EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals

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The Endocrine Society's first Scientific Statement in 2009 provided a wake-up call to the scientific community about how environmental endocrine-disrupting chemicals (EDCs) affect health and disease. Five years later, a substantially larger body of literature has solidified our understanding of plausible mechanisms underlying EDC actions and how exposures in animals and humans—especially during development—may lay the foundations for disease later in life. At this point in history, we have much stronger knowledge about how EDCs alter gene-environment interactions via physiological, cellular, molecular, and epigenetic changes, thereby producing effects in exposed individuals as well as their descendants. Causal links between exposure and manifestation of disease are substantiated by experimental animal models and are consistent with correlative epidemiological data in humans. There are several caveats because differences in how experimental animal work is conducted can lead to difficulties in drawing broad conclusions, and we must continue to be cautious about inferring causality in humans. In this second Scientific Statement, we reviewed the literature on a subset of topics for which the translational evidence is strongest: 1) obesity and diabetes; 2) female reproduction; 3) male reproduction; 4) hormone-sensitive cancers in females; 5) prostate; 6) thyroid; and 7) neurodevelopment and neuroendocrine systems. Our inclusion criteria for studies were those conducted predominantly in the past 5 years deemed to be of high quality based on appropriate negative and positive control groups or populations, adequate sample size and experimental design, and mammalian animal studies with exposure levels in a range that was relevant to humans. We also focused on studies using the developmental origins of health and disease model. No report was excluded based on a positive or negative effect of the EDC exposure. The bulk of the results across the board strengthen the evidence for endocrine health-related actions of EDCs. Based on this much more complete understanding of the endocrine principles by which EDCs act, including nonmonotonic dose-responses, low-dose effects, and developmental vulnerability, these findings can be much better translated to human health. Armed with this information, researchers, physicians, and other healthcare providers can guide regulators and policymakers as they make responsible decisions. **(*Endocrine Reviews* 36: E1–E150, 2015)**

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Abbreviations: AGD, anogenital distance; AhR, aryl hydrocarbon receptor; AHS, Agricultural Health Study; AR, androgen receptor; ARC, arcuate nucleus; ATR, atrazine; AVP, arginine vasopressin; AVPV, anteroventral periventricular nucleus; BBP, butyl benzyl phthalate; BMI, body mass index; BPA, bisphenol A; BPH, benign prostatic hyperplasia; CI, confidence interval; CPP, Collaborative Perinatal Project; CVD, cardiovascular disease; D1 (Dio1), type 1 deiodinase; DBP, di-n-butyl phthalate; DCP, dichlorophenol; DDD, dichlorodiphenyldichloroethane; DDE, dichlorodiphenyldichloroethylene; DDT, p,p′-dichlorodiphenyltrichloroethane; DEHP, di(2-ethylhexyl)phthalate; DES, diethylstilbestrol; DINP, diisononyl phthalate; DNMT, DNA methyltransferase (enzyme); DOHaD, developmental origins of health and disease; E, embryonic day; EDC, endocrine-disrupting chemical; (continued p.2)

(continued) EPA, Environmental Protection Agency; EPM, elevated plus maze; ER, estrogen receptor; EZH2, enhancer of Zeste homolog 2; G, gestational day; GPER, G protein-coupled ER; GR, glucocorticoid receptor; GST, glutathione transferase; HCB, hexachlorobenzene; HCH, hexachlorocyclohexane; HDAC, histone deacetylase; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic-pituitary-gonadal; HPT, hypothalamic-pituitary-thyroid; HPTE, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane; HSD, hydroxysteroid dehydrogenase; iAs, inorganic arsenic; IVF, in vitro fertilization; KCC2, potassium chloride cotransporter 2; LQ, lordosis quotient; MBP, monobutyl phthalate; MBzP, mono-n-benzyl phthalate; MEHHP, mono-(2-ethyl-5-hydroxylhexyl) phthalate; MEHP, mono-(2-ethylhexyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MEP, monoethyl phthalate; MPOA, medial POA; MWM, Morris water maze; MXC, methoxychlor; NCD, noncommunicable disease; NHANES, National Health and Nutrition Examination Survey; NIEHS, National Institute of Environmental Health Sciences; NIS, sodium/iodide symporter; NOAEL, no observed adverse effect level; NTP, National Toxicology Program; OVX, ovariectomized; P, postnatal day; PBB, polybrominated biphenyl; PBDE, polybrominated diphenyl ether; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzodioxin; PCDF, polychlorinated dibenzofuran; PCOS, polycystic ovarian syndrome; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PIN, prostatic intraepithelial neoplasia; POA, preoptic area; POP, persistent organic pollutant; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PRL, prolactin; PSA, prostate-specific antigen; P450scc, P450 side-chain cleavage; PTU, propylthiouracil; PVN, paraventricular nucleus; RXR, retinoid X receptor; SNP, single nucleotide polymorphism; StAR, steroidogenic acute regulatory (protein); TBBPA, tetrabromobisphenol A; TBT, tributyltin; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; T1D, type 1 diabetes mellitus; T2D, type 2 diabetes mellitus; TDS, testicular dysgenesis syndrome; TEB, terminal end bud; TGCC, testicular germ cell cancer; ThR, thyroid hormone receptor; TPO, thyroperoxidase; VDR, vitamin D receptor; VMH, ventromedial nucleus of the hypothalamus; WAT, white adipose tissue.

I. Introduction to EDC-2

A. Five years after the Endocrine Society's first Scientific Statement

It has been 5 years since the Endocrine Society convened a group of experts to review the state of the science on endocrinological effects of environmental contaminants that perturb hormonal systems, termed endocrine-disrupting chemicals (EDCs). That team conducted a thorough review of the extant literature up to that time (2008), and wrote an initial white paper that was then developed into the landmark Scientific Statement on EDCs published in 2009, herein referred to as "EDC-1" (1). Since that time, numerous publications have emerged. What has influenced the field most deeply since 2008 has been four types of studies: 1) those describing the consequences of EDC exposures on development and physiology (mainly conducted in rodent models); 2) those investigating the mechanistic underpinnings of these disorders (gene expression and epigenetic changes induced in cell and tissue culture, together with molecular and cellular work conducted in endocrine tissues of EDC-exposed animals); 3) work seeking to document associations between body burdens of certain EDCs to disease propensity in humans (mainly epidemiological work); and 4) those reports of humans with known occupational or acute exposures to a particular chemical or group of chemicals with EDC activity (eg, pesticide applicators, or families residing near the Seveso, Italy, factory, site of a large dioxin leak). In 2014–2015 when this second Scientific Statement, EDC-2, was written, there was far more conclusive evidence for whether, when, and how EDCs perturb endocrine systems, including in humans. Thus, it is more necessary than ever to minimize further exposures, to identify new EDCs as they

emerge, and to understand underlying mechanisms in order to develop interventions.

Through the years there has been controversy around endocrine disruptors, in part because different stakeholders, some with financial incentives, may review the literature from a very different perspective. The chemical industry and environmental nongovernmental organizations have often been in conflict, and the lay press sometimes oversimplifies the research results. It is also notable that the goals of industry-funded studies and federal granting agency-funded studies can differ both in design and in desired outcomes. The former (industry) are often done to prove safety, and negative results are considered a favorable outcome and are published. By contrast, government-funded research is usually hypothesis-driven, seeking underlying mechanisms, and not necessarily intended to prove or disprove safety. As a result, such studies may be omitted from the risk assessment process, something that ought to be overcome by better integration of the different types of studies.

Here, our goal was to present relevant research independent of the results, and we considered both industry- and government-funded work. As we discuss later, there is always a potential bias toward positive study findings, ie, finding of adverse effects rather than reporting no effects. However, there is also, unfortunately, a possibility of the opposite kind of bias that might be in the best interests of a producer to show that its product is safe, and therefore a negative test result is desirable. Thus, readers of papers on EDC effects should consider whether articles are peer-reviewed and whether there might be a conflict of interest of reviewers, editors, or publishers with industry connections, and readers should use their scientific judgment in evaluating the strength of the scientific work. Consideration of the quality of research should also include whether or not experiments were done in a blind or double-blind fashion to avoid inadvertent experimental bias.

An Executive Summary of this statement has been published separately (2), summarizing the key points of the full Scientific Statement.

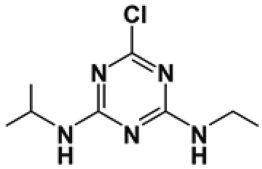
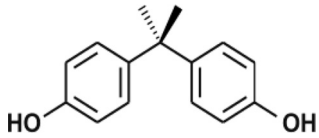
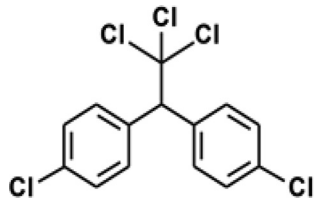
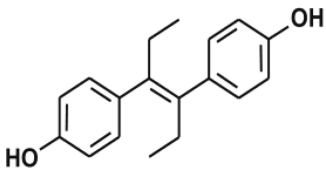
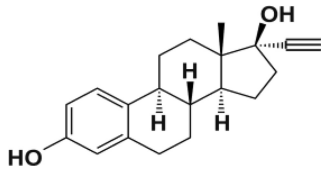
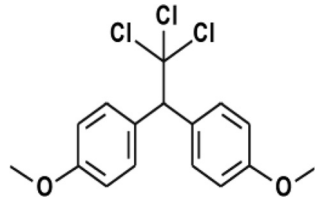
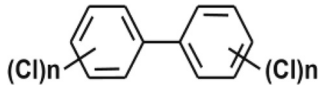
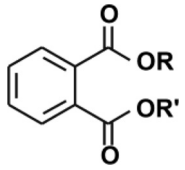
1. Definition of EDCs and prototypical examples

For the purposes of EDC-2, we provide an operational working definition of an EDC as: "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (3). Although there may be hundreds or more environmental chemicals with EDC activity, several classes are most commonly studied and will be introduced briefly here; these and several others are summarized in Table 1.

a. Bisphenol A. Bisphenol A (BPA) was first synthesized in 1891 and was discovered to be estrogenic in 1936 (4). More BPA is produced annually than any other chemical, with 15 billion pounds produced in 2013 (5). It is used in a very wide array of manufacturing, food packaging, toys, and other applications, and BPA resins are found in the lining of many canned foods and beverages such that virtually everyone is exposed continuously (6). In food contact materials, BPA may leach into food or water under high heat, physical manipulation, or repetitive use. Due to its ubiquitous nature and continuous exposure, 93% of Americans have a measurable amount of BPA in their urine (7, 8). It is also detected in breast milk of some women (9). BPA is so prevalent in our daily environment that elimination of BPA contamination during carefully controlled quantitative procedures has proven difficult (10, 11). BPA is rapidly metabolized to nonbioactive forms and has a short half-life of approximately 4–5 hours in adult humans, with lower metabolic rates in the fetus and infants (12, 13). Measurements of bioactive or free BPA in human serum is controversial at present, with some documenting nanograms per milliliter quantities in samples using contamination-free conditions (13–15), whereas others report that ordinary exposures result in picograms per milliliter levels or lower (16). Although relevant internal exposure remains a critical issue that is still unresolved, it is noteworthy that industrial exposures, vulnerable populations, and individual variations in metabolism and susceptibility must be taken into consideration (17). Currently, the US Environmental Protection Agency (EPA) safety level of BPA is set at 50 μg/kg/d, whereas the European Food Safety Authority's temporary tolerable daily intake was recently lowered to 4 μg/kg/d. Several studies in the present report will document BPA effects in mammalian systems at or below these current safety levels.

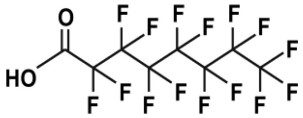
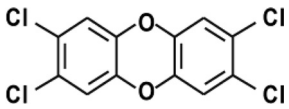
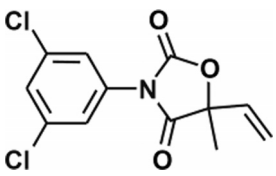
b. Phthalates. Phthalates and phthalate esters are a large group of compounds used as liquid plasticizers found in a wide range of products including plastics, coatings, cosmetics, and medical tubing. These compounds were first introduced as additives in the production of plastic in the 1920s and resulted in the rapid widespread use of polyvinyl chloride plastic in the 1930s and later. Because they are not chemically bound to the plastic, phthalates can leach into the environment. Moreover, a variety of consumer products use various phthalates, including personal care products, medical tubing, vinyl flooring materials, and toys. In one case, food companies in Taiwan began deliberately using a variety of phthalates as emulsifiers at very high concentrations (18). When this was discovered, the government notified the population that certain kinds of foods were contaminated, including sports drinks, fruit beverages, tea drinks, fruit jam or jelly, and health foods or supplements in powder or tablet form. But it was later

Table 1. Classifications, Histories, Chemical Properties, and Physiological Effects of Common EDCs

EDC	General Chemical Structure	Group	Introduction date	Restricted/ Ban Date	Route of Exposure	Sources	Half-Life	Effects/Body Burden
ATR		Chlorotriazine herbicide	1959	European Union ban 2004	Ingestion, inhalation	Pesticide/ herbicide, contaminated water and soil	10–12 h	Endocrine, respiratory and nervous system targets, liver damage
BPA		Bisphenols	1960s	Restricted 2012	Ingestion, inhalation, dermal absorption	Polycarbonate plastics, epoxy resins, plastic toys and bottles, lining of food cans	4–5 h	Estrogenic, obesogenic, neurological effects, adverse thyroid hormone action, reproductive and developmental effects
DDT		Organochloride	1940s	Banned 1972	Ingestion, inhalation, dermal absorption	Contaminated water, soil crops, fish	6–10 yr	Carcinogen, central nervous system, kidney, liver and peripheral nervous system effects
DES		Non-steroidal synthetic estrogen	1941–1947	Restricted 1971–1975	Ingestion, injection, vaginal suppository	Pharmaceutical	2–3 days	Transplacental carcinogen, teratogen
EE2		Synthetic derivative of 17β-estradiol	1943		Oral	Oral contraceptives, contaminated water	13–27 h	Cardiovascular disease, cerebrovascular disease, thromboembolic disease, gallbladder disease, carcinogenic
MXC		Organochlorine insecticide	1948	United States 2003 banned use as pesticide	Ingestion, inhalation, dermal absorption	Contaminated soil, water, and food	Aerobic soil >100 days	Central nervous system depression, damage to liver and kidney, developmental and reproductive effects in animals, transgenerational kidney and ovary disease, obesogen
PCBs		Organochloride	1927	Banned 1979	Ingestion, inhalation, dermal absorption	Contaminated air and food, skin contact with old electrical equipment	12 days to 16 yr	Carcinogen, chloracne, stomach and liver damage, reproductive and nervous system effects and thyroid injury
Phthalates		Plasticizers	1920s	Restricted 2009	Ingestion, inhalation, dermal absorption	Contaminated food, PVC plastics and flooring, personal care products, medical devices and tubing	~12 h	Carcinogen, liver damage, reproductive and developmental effects, asthma, obesogen

(*Continued*)

Table 1. Continued

EDC	General Chemical Structure	Group	Introduction Date	Restricted/ Ban Date	Route of Exposure	Sources	Half-Life	Effects/Body Burden
PFOA		Fluorosurfactant	1940s	United States 2015 voluntary production restiction	Ingestion, inhalation	Contaminated food and water, dust, floor waxes, fire fighting foam, electrical wiring, lining of food wrappers, stain resistant carpeting	2–4 yr	Liver, and mammary gland developmental, and immune system toxicant, carcinogen
TCDD		Polychlorinated dibenzo-p-dioxin	Synthesized 1872		Ingestion, inhalation	By-product of chlorinated herbicide production, smelting, chlorine bleaching of paper	7–11 yr	Liver damage, weight loss, atrophy of thymus gland, immunosuppression, reproductive effects and cancer
Vinclozolin		Dicarboximide fungicide	1981		Ingestion, inhalation, dermal absorption	Diet and occupational	Aerobic soil 28 days, plasma 20 h	Antiandrogenic activity, male reproductive and neurological effects, transgenerational reproductive effects, potential carcinogen

Abbreviations: EE2, ethinyl estradiol; PVC, polyvinyl chloride.

shown that the contamination was more widespread, including ice cream, frozen food, and cake mixes (19). In fact, phthalates are detectable in human urine, serum, and milk samples (20–22), and the estimated daily exposure to one major phthalate, di(2-ethylhexyl)phthalate (DEHP), ranges from 3–30 μg/kg/d (23).

c. Atrazine. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) (ATR) is a widely used chlorotriazine herbicide used to control broadleaf and grass weed growth on crops such as commercial corn, sorghum, and sugar cane. Christmas tree farms, parks, and golf courses also use ATR. ATR has been the major herbicide used worldwide since its registration in 1959 because of its ability to remain active for extended periods of time, its economical price, and its broad spectrum of weed control (24). ATR and its metabolites are commonly reported groundwater contaminants and the most commonly detected pesticide in US surface waters, including drinking water. For this reason, the EPA is particularly concerned about the potential of ATR and related chlorotriazines to affect aquatic organisms and the carcinogenic risk from exposure (25, 26).

d. Polychlorinated biphenyls and polybrominated diphenyl ethers. Polychlorinated biphenyls (PCBs) are a class of industrial chemicals with paired phenolic rings and variable degrees of chlorination. They were synthesized by exposing the biphenyl molecule to chlorine gas in the presence of a catalyst, resulting in complex mixtures of 209 possible congeners (27–29). PCBs were mass-produced globally from the late 1920s until they were banned in 1979. A wide variety of applications used these mixtures, including plasticizers in rubber and resins, carbonless copy paper, adhesives, and paints and inks. The varied nature of their use resulted in widespread environmental contamination, including buildings and schools (30). These persistent organic pollutants (POPs) bioaccumulate in the environment and are stored in body fat, and they therefore have continued potential for adverse health effects (31). Some PCBs are classified as EDCs because they have thyroidogenic, estrogenic, and antiandrogenic actions (32, 33). The commercial production of polybrominated diphenyl ethers (PBDEs) began in the late 1970s (34), just about the time that PCB production was banned. They were used as flame retardants in upholstered products, mattresses, and clothing. In 2001, approximately 33 000 metric tons of PBDEs were produced, most of which was used in North America (34). As a result, North Americans have the highest blood levels of PBDEs, compared to those living in other regions of the world (35, 36). PBDEs consist of predominantly three congener mixtures, pentaBDE, octaBDE, and decaBDE. The first two have been banned in Europe and Asia, but decaBDE mixtures continue to be widely used globally (37). Of the 209 possible congeners, the five that account for over 90% of body burden in human tissues are tetraBDE47; pentaBDE99, -100, and -153; and decaBDE209.

e. DDT and DDE. p,p'-Dichlorodiphenyltrichloroethane (DDT) is a synthetic industrial and household insecticide with a long half-life, extensive use, and lipophilic nature that have made it a prominent environmental contami-

nant. The United States banned DDT in 1972 due to its effects on the environment and potential human health effects, despite the benefit of decreased incidence of malaria and typhoid (38, 39). DDT and its metabolites, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD), have been associated with endocrine-related diseases such as testicular tumors (40), endometrial cancer (41), pancreatic cancer (42), type 2 diabetes mellitus (T2D) (43), and breast cancer (44, 45).

We must note that in the list of chemicals described above, there are EDCs, such as BPA and phthalates, that are commonly detected in most of the population because of their widespread use. These latter EDCs have relatively low accumulation in body fat tissue; therefore, serum or urine levels of these chemicals, their metabolites, or specific reaction products likely reflect the so-called "body burden," defined as the total amounts of these chemicals that are present in the human body at a given point in time. On the contrary, PCBs, PBDEs, DDT, and DDE are POPs. These are highly lipophilic and accumulate in the food chain and in white adipose tissue (WAT) with important consequences. This storage depot can contain a large reservoir of POPs that are liberated into blood, especially during weight loss periods. At the same time, it is a mechanism of protection as well because it limits the availability of chemicals to enter the blood and access other tissues, thereby eliciting detrimental effects. In fact, retention of POPs in WAT may limit the ability to estimate the real burden of these chemicals from measurements in serum or urine levels, as occurs in most animal and human studies.

B. Endocrine systems are a physiological interface with the environment, and gene-by-environment interactions are perturbed by EDCs

The endocrine glands are distributed throughout the body and produce the hormones that act as signaling molecules after release into the circulatory system (Figure 1). Development, physiological processes, and homeostatic functions are regulated and maintained by hormones. Several functions of natural hormones are critical for both health and disease and are relevant to EDCs. First, many hormones bind to receptors with remarkable affinity, having dissociation constants between 10^{-12} and 10^{-9} M, which approximates their very low concentrations in the

Figure 1.

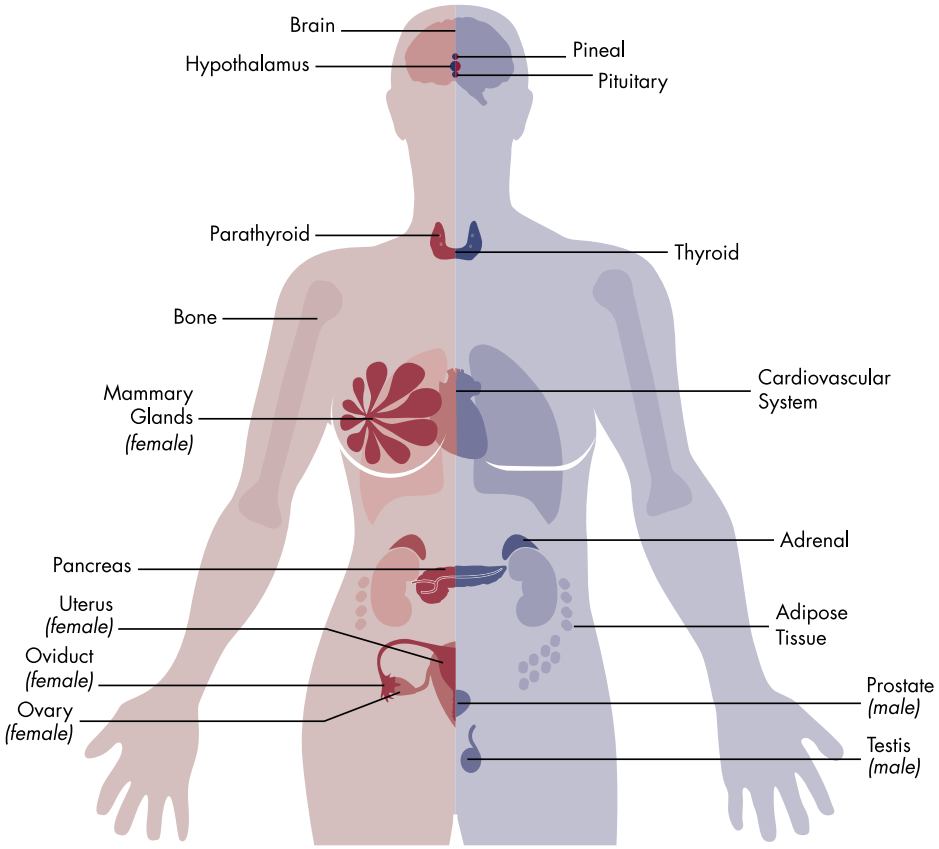


Figure 1. Diagram of many of the body's endocrine glands in females (left) and males (right).

circulation. The maximal response of a cell is achieved at ligand concentrations at which most of the receptors are still not occupied. Second, the amount of a hormone that is synthesized and released is determined by the interplay of numerous molecular and physiological processes, including regulation of gene and protein expression, exocytosis of vesicles containing peptide or protein hormones, metabolism and steroidogenesis of lipophilic hormones, transport through circulation (often in association with binding partners), actions at target receptors, metabolism, degradation, excretion, and many others. Third, a fundamental property determining hormone levels involves the ability of the gland and its targets to interact, most often by negative feedback of a hormone on its receptor in the original target gland. Fourth, levels of any hormone must be within a physiologically relevant range to be most effective. Excursions outside of that range to superphysiological (elevated) or subphysiological (depressed) levels for any extended period nearly always result in dysfunction or disease. This latter concept is exemplified by the thyroid system: normal (euthyroid) levels of the thyroid hormones are needed for appropriate metabolic health. Hyperthyroidism is associated with a range of symptoms due to elevated metabolism, and hypothyroidism, with a very different disease phenotype, results from depressed hormone levels.

EDCs interfere with the action of hormones, disrupt homeostasis, and may alter physiology during the whole life span of an individual, from fetal development to adulthood (1). Understanding how EDCs affect physiological processes and initiate pathophysiology is essential in understanding the etiology of hormone-related diseases. Some EDCs, at environmentally relevant doses, bind to hormone receptors and act either as agonists or antagonists, thus enhancing, dampening, or blocking the action of hormones. They also alter the number of hormone receptors in different cell types and the concentration of circulating hormones (46, 47). These effects, depending on timing and exposure dose, give rise to alternative phenotypes, which may lead to increased disease susceptibility. If exposure alters hormone actions during ontogenesis, the effects are often permanent and can affect organ development and function. Furthermore, these effects could have lifetime consequences that are both complex and difficult to predict. The extent and nature of long-term consequences depend on the interaction of genes and environment and involve many variables, including the developmental window of exposure, the individual's metabolism, and his or her genetic background.

Long-term permanent physiological changes that result from early EDC exposure alter one's susceptibility to common noncommunicable diseases (NCDs), which have markedly increased during the last few decades (48). The four principal NCDs are cancers, cardiovascular diseases (CVDs), chronic respiratory diseases, and diabetes mellitus. Cumulatively, they kill 36 million people each year, 80% in low- and middle-income countries (49). Both genetics and environment play a role in the incidence of these NCDs, and increasing interest is turning toward finding how chronic, low-dose lifelong exposures to EDCs contribute to these statistics.

An individual's genes play important roles in determining health and physiological parameters, such as insulin sensitivity and blood pressure. Single nucleotide polymorphisms (SNPs) in the genome are related to incidence and severity of CVDs, diabetes mellitus, obesity, and abnormalities in metabolism, reproduction, and other endocrine systems. Such polymorphisms may also contribute to variability in responses to chemical exposures. It is important to note that a SNP is rarely responsible for a NCD; rather, there are a large number of SNPs, each with a small effect that accumulates and is associated with these diseases (50, 51). For example, there are 65 SNPs known to be associated with the risk of one of the most common NCDs, T2D (52). The interactions between EDC exposure and SNPs associated with NCDs are still greatly unknown, and further studies are necessary. The rise in common NCDs, therefore, is thought to be attributable to environmental and social factors, including EDCs (53), more than any common gene variant. The mechanisms for these changes, involving molecular epigenetic processes, are discussed in *Section I.D*.

Although much of the focus of EDC-2 is on developmental exposure, EDCs also interact with receptors during adulthood. As will be discussed later, this may elicit effects such as weight gain ("obesogens") and/or insulin resistance and hyperinsulinemia ("diabetogens"). Although actions in adults may be reversible if the insult is removed, exposure in the real world is more often continuous and to a mixture of chemicals. In such circumstances as chronic lifelong exposure, EDCs may predispose individuals to pathologies such as T2D (54) or thyroid dysfunction.

C. The developmental origins of health and disease

During embryonic development, organogenesis and tissue differentiation proceed through a series of tightly regulated and temporally coordinated events at the cellular, biochemical, and molecular levels, ultimately resulting in a functional, mature structure. Development is an *Einbahnstrasse* (one-way street), and thus natural substances such as hormones as well as environmental changes, including exposures to exogenous environmental chemicals, alter this unidirectional process. These latter perturbations may impart structural and functional changes that can profoundly deflect the developmental trajectory, often

leading to lifelong phenotypic changes such as increased endocrine disease propensity. Overt toxicant exposures during gestation have been recognized for decades to cause adverse outcomes in exposed children, with examples that include links between thalidomide and limb malformations, folate deficiency and spina bifida, methyl mercury and Minamata disease, as well as fetal alcohol syndrome, to name a few (55). Childhood and puberty are also periods of rapid change in endocrine-dependent organ systems and are beginning to be recognized as additional sensitive periods (56–59).

The targets of endocrine glands typically exhibit heightened sensitivity to hormones during specific developmentally critical windows. During these periods, hormonal signals cause changes to cells at the molecular (often gene expression and/or epigenetic) level (see *Section I.D.*) and dictate or modify structural and functional organization of the tissues. Such early-life programming events are best exemplified in studies on brain development where androgens and estrogens play early, essential roles in imprinting sexual dimorphisms in structure, gene expression, and signaling that determine behaviors throughout life (60). Likewise, there is growing appreciation that development during the critical period is particularly vulnerable to the effects of exogenous EDCs that can reprogram essential signaling/differentiation pathways and lead to lifelong consequences (61). In fact, molecular changes in response to EDCs often precede morphological consequences, sometimes by weeks, years, or decades (depending upon life span), and experimental studies showing gene or protein expression changes in response to EDCs may be sentinels for disease propensity later in life.

This reprogramming process is best appreciated within the framework of the developmental origins of health and disease (DOHaD) hypothesis, which posits that an adverse environment experienced by a developing individual can increase the risk of disease later in life (62, 63). As originally formulated, the DOHaD paradigm initially focused on multiple studies that documented links between poor nutrition in utero and increased risk in offspring of obesity, CVD, and diabetes mellitus over a life span (62). The most notable example is the Dutch "hunger" winter during World War II when maternal starvation—in a trimester-specific manner—correlated with increased cardiovascular and metabolic diseases of the offspring in adulthood (64, 65). Importantly, the DOHaD hypothesis readily expands to accommodate perturbations in the endocrine system during early development, including aberrations in endogenous hormones (in timing, sequence, and levels), maternal intake of synthetic hormones, and inadvertent exposures to environmental chemicals including EDCs.

1. Diethylstilbestrol and beyond

Perhaps the best-studied endocrine-based example is in utero exposure to diethylstilbestrol (DES), a potent synthetic nonsteroidal estrogen taken by pregnant women from the 1940s to 1975 to prevent miscarriage and other complications. DES was prescribed at doses from less than 100 mg (in most cases) upward to 47 000 mg, with a median dose of 3650 to 4000 mg in the United States (IARC 2012). Most women received low doses (ie, 5 mg) and increased their intake (up to 125 mg) as symptoms or pregnancy progressed, translating to doses of about 100 μg/kg to 2 mg/kg DES per day (66). In 1953, a study proved DES was ineffective (67). Its use was discontinued when a subset of exposed daughters presented with early-onset vaginal clear-cell adenocarcinoma (68), with a 40-fold increase in risk compared to unexposed individuals (69) (Table 1). A highly significant incidence ratio for clear-cell adenocarcinoma was also found in the Dutch DES cohort, a population that may have had lower exposures than US women (70). It was subsequently determined that exposed offspring of both sexes had increased risk for multiple reproductive disorders, certain cancers, cryptorchidism (boys), and other diseases (71–73), although the risk for sons is more controversial (74). New data are emerging to implicate increased disease risk in grandchildren (75). Not surprisingly, a plethora of examples is emerging for increased disease susceptibility later in life as a function of developmental exposures to EDCs that include BPA, phthalates, PCBs, pesticides, dioxins, and tributyltin (TBT), among others.

D. Epigenetics and transgenerational effects of EDCs

The mechanisms of action of EDCs are varied (Table 2) and not entirely understood, but recent evidence suggests that some EDCs may cause epigenetic changes, which in turn may lead to transgenerational effects of EDCs on numerous organ systems (76–80). Epigenetic changes are described as heritable changes in gene expression that are not due to changes in DNA sequence (ie, not due to mutation). Several possible mechanisms of epigenetic change exist, including methylation of cytosine residues on DNA, post-translational modification of histones, and altered microRNA expression. To date, most studies on the effects of EDCs on epigenetic changes have focused on DNA methylation, but recent studies have also addressed the effects of EDCs on histone modifications and microRNA expression (77, 78, 81).

DNA methylation is a process in which methyl groups are attached to cytosine residues by DNA methyltransferase enzymes (DNMTs), usually in cytosine-guanosine dinucleotide pairs (CpG sites), although DNA methylation can occur on non-CpG residues (82, 83). DNA meth-

Table 2. Mode of Action for EDCs

EDC	Mechanism	Mode of Action
BPA	Nuclear receptor	ER agonist (859, 1246); strong affinity for ERRγ (860, 1247); antiandrogen (1248); increased PR expression (477, 1249); hPXR agonist (1250)
	ER-mediated nongenomic pathway	Activates membrane-associated ERα, ERβ signaling cascades through PI3K-pAkt and MAPK-pErk and GPER-pErk pathways (960, 1251–1255)
	Nonsteroidal receptor	Antagonist of ThR (1095); binds to GPR30 (861)
	Ion channels	Activates membrane ERβ-Ca^{2+} pathway; activates ERβ-KATP and Ca^{2+} mobilization (293); up-regulation of Ca^{2+} ion channel gene and protein, Orai1 (966, 326, 1256, 1257)
	Uninhibited growth	Alters MaSC gene expression and induces early neoplastic lesions (348); induces beaded ducts and increases hyperplasia (362, 1258, 1259)
	Inflammation	Induces proinflammatory cytokines and chemokines (1260)
DDT and metabolites	Nuclear receptor	Binds and transactivates ERα and ERβ (1246, 1261); DDE binds AR and represses transcription (1262)
	Microenvironment/stroma	Induced estrogenic microenvironment in breast adipose tissue (865)
DES	Nuclear receptor	ERα agonist (1246, 1263); AR binding (1264); suppresses activation of ERR α, β, and γ (1265)
	ER-mediated non-genomic pathway	Activates MAPK and PI3K and induces phosphorylation of ERK (1266, 1267)
	Epigenetic	Hypermethylation of HOXA10 (1268); DNA methylation (1269)
Dioxins	Nonsteroidal receptor	Binds to AhR (1270)
	Coactivator recruitment	Recruitment of coactivator p300 (1270)
PCBs	Steroid hormone biosynthesis	Inhibits sulfotransferase (1271), inhibits aromatase (1272); increases T_4 glucuronidation, competes with thyroid hormone binding proteins (1273)
	Nuclear receptor	Weak binding to ER (1246), weak binding to AR (1264)
PFOA	Nuclear receptor	Binds to ER and EREs (845, 846)
	Nonsteroidal receptor	PPARα agonist (157, 1274)
	Uninhibited growth	Increased hyperplasia and stromal density (853)
Phthalates	Nuclear receptor	DBP weak affinity for ER (874)
	Microenvironment/stroma	MEHP induced PPARβ in adipose (1274)

Abbreviations: EREs, estrogen response elements; ERR, estrogen-related receptor; PI3K, phosphatidylinositol-3-kinase.

ylation is important for several normal developmental and reproductive processes such as gametogenesis and embryogenesis. Hypermethylation in a promoter region is thought to repress gene transcription because the methylated promoter region has a decreased affinity for transcription factors and an increased affinity for methylated DNA-binding proteins, methyltransferases, histone deacetylases (HDACs), and/or corepressors (84, 85).

Histone modification is a process in which specific amino acids in the N-terminal ends of histones undergo post-translational modification, including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination by enzymes such as histone acetyltransferases, deacetylases, methyltransferases, and demethylases (86, 87). These modifications determine whether the DNA wrapped around histones is available for transcription and play roles in determining the rate of transcription. Histone modifications also help regulate replication, recombination, and higher-order organization of the chromosomes (88). Changes to these modifications are often found in diseases such as cancer and are best studied for those diseases (89, 90).

The molecular mechanisms by which microRNAs and other noncoding RNAs affect gene expression are not entirely understood, but it is likely that microRNAs play a role in gene regulation and chromatin organization (91, 92). MicroRNAs often bind to the 3′ end of gene transcripts and initiate mRNA degradation or suppression of protein translation (93). Studies also suggest that microRNAs can affect the expression of other epigenetic regulators such as DNMTs and histone-modification enzymes (94).

Both hormones and EDCs cause DNA methylation, histone modifications, and altered microRNA expression (95). These epigenetic changes often cause phenotypic changes in organisms, which may appear immediately or long after EDC exposure. These properties are dictated by the timing of exposure. When EDCs introduce epigenetic changes during early development, they permanently alter the epigenome in the germline, and the changes can be transmitted to subsequent generations. When an EDC introduces epigenetic changes during adulthood, the changes within an individual occur in somatic cells and are not permanent or transmitted to subsequent generations (76, 77, 81, 96). For an EDC to have truly transgenerational effects, exposure must occur during development, and the effects need to be observed in the F3 generation. This is because when a pregnant F0 female is exposed to an EDC, germ line cells in her F1 fetus are directly exposed to the EDC. These exposed F1 germ line cells are then used to produce the F2 generation, and thus, the F2 generation was directly exposed to the EDC via the germ cells. This

exposure scenario makes the F3 generation the first generation that was not directly exposed to the EDC (97–99).

EDC-induced epigenetic changes are also influenced by dose of exposure, and they are tissue specific (77, 78, 81). Thus, it is important to consider both dose of EDC and the tissue before making firm conclusions about the epigenetic effects of EDCs. DNA methylation changes are the best-studied mechanism in this regard. For example, prenatal exposure to DES caused hypermethylation of the *Hoxa10* gene in the uterus of mice and was linked to uterine hyperplasia and neoplasia later in life (100). Beyond the effects of prenatal exposure to DES on the daughters exposed in utero are suggestions that this leads to transgenerational effects of the chemical on the reproductive system (101–103), although whether this is linked to DNA methylation changes in humans is unknown.

The two EDCs that are best studied for transgenerational epigenetic effects, especially DNA methylation, are BPA and vinclozolin (Table 1). Prenatal exposure to BPA decreased CpG methylation in agouti mice (104), altered the methylation status of the *Hoxa10* gene in the rodent uterus (100), and changed DNA methylation of key genes associated with prostate cancer in rats (105, 106). Prenatal BPA exposure at physiologically relevant levels altered DNA methylation of imprinted genes in the mouse embryo and placenta (107). Furthermore, urinary concentrations of BPA were associated with less genomic methylation of genes involved in immune function, transport activity, metabolism, and caspase activity in bisulfite-converted saliva DNA from girls aged 10–13 years, and BPA levels (50 ng/kg to 50 mg/kg) were linked with hypermethylation in tail tissue in mice (108, 109). Additional studies, however, are required to directly determine whether the BPA-induced changes in DNA methylation result in abnormal phenotypes in subsequent generations. Furthermore, whereas a few studies showed that BPA induced testicular abnormalities in the F1–F3 generations of rats (110) and caused changes in social interaction tasks in the F2 and F4 generations (111), it is not clear whether these effects are due to changes in DNA methylation patterns, meriting further work.

Prenatal exposure to vinclozolin in rodents has also been shown to induce transgenerational effects on physiological and behavioral phenotypes in the F3 generation (112–114), but whether these outcomes are due to altered DNA methylation changes occurring in the germline, as shown in the sperm (115), is not known. In males, prenatal exposure to vinclozolin caused germ cell death and the appearance of disease-like phenotypes in the prostate through the F3 generation (116, 117). In females, vinclozolin caused more ovarian cysts and a reduced number of oocytes and primary follicles through the F3 generation (117, 118). Again, the mechanism is proposed to involve hypo- or hypermethylation of DNA (116, 117, 119). Importantly, work using this model relied on high-dose exposures to the pregnant dams (50–100 mg/kg/d) and must be replicated using environmentally relevant doses.

Although there is less research on other EDCs, evidence suggests that they can cause DNA methylation changes (80, 120, 121). Exposure to methoxychlor (MXC) altered DNA methylation in multiple CpGs in the ERβ promoter (80). DEHP exposure caused global increases in cytosine methylation in the testes (122) and modified DNA methylation of imprinted genes in F1 and F2 oocytes in mice (120). Furthermore, whereas prenatal exposure to DEHP delayed puberty in the F1 and F3 generations and decreased sperm counts, testicular germ cell function, and the number of normal seminiferous tubules in the F3 generation (121), studies are required to determine whether DEHP-induced DNA methylation changes are responsible for these adverse transgenerational outcomes.

Little is known about the ability of EDCs to cause histone modifications and whether this leads to transgenerational effects in animals or humans. The herbicides paraquat and dieldrin caused histone modifications in immortalized rat mesencephalic dopaminergic cells (123, 124), and the insecticide propoxur causes histone modifications in gastric cells in vitro (125). DES caused histone deacetylation in the promoter region of the cytochrome P450 side chain cleavage (P450scc) gene (126). Further studies, however, need to be conducted to identify other EDCs causing histone modifications in animals and humans and to determine whether such modifications lead to transgenerational effects.

There is also little knowledge about the ability of EDCs to alter microRNA expression. Studies show that BPA exposure induced expression of the microRNA miR-146a in human placental cell lines (127), down-regulated several microRNAs in ovaries of ewes (128), and altered expression of microRNAs in the rat penis (129). Another study showed that both BPA and DDT altered the expression profile of microRNA in MCF-7 breast cancer cells (130). Future studies are needed to identify the specific effects of EDCs on microRNA expression and to determine whether EDC-induced changes in microRNA expression lead to adverse phenotypes in the exposed and subsequent generations.

In summary, a prominent mechanism for increased disease risk in adulthood as a function of early-life EDC exposure is attributed to epigenomic reprogramming, a result of high plasticity as the epigenetic code is installed during development (131, 132). Furthermore, the environment-gene interface must be considered as a basis for individual disease susceptibility whereby EDC-induced modifications of the epigenetic code early in life permit

Figure 2.

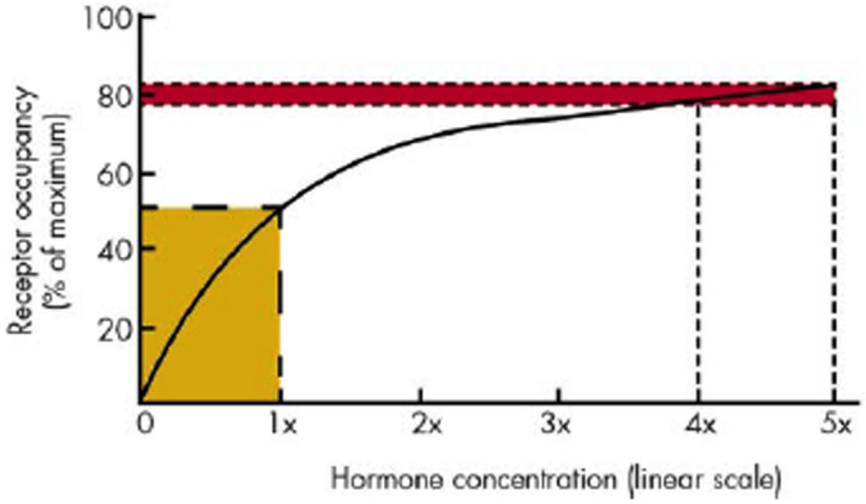


Figure 2. Schematic example of the relationship between receptor occupancy and hormone concentration. In this theoretical example, at low concentrations, an increase in hormone concentration from 0 to 1× causes an increase in receptor occupancy of approximately 50% (from 0 to 50%; see yellow box). Yet the same increment in hormone concentration at higher doses (from 4× to 5×) causes an increase in receptor occupancy of only approximately 4% (from 78 to 82%; see red box). However, it is important to recognize that receptor occupancy is not linearly related to hormone effect, and low receptor occupancy (1 to 10%) can be associated with maximal effects. [Reprinted from L. N. Vandenberg et al: Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev*. 2012;33: 378–455 (133), with permission. © Endocrine Society.]

cryptic genetic variants or low-penetrant mutations to emerge and to manifest a phenotype at later life stages, long after the initial EDC exposure.

E. Dose-response characteristics of EDCs

Hormones have complicated concentration-response patterns, and these lay the foundation for dose-response characteristics exhibited by EDCs. The mechanisms that are important to consider when discussing dose-response characteristics of EDCs include, but are not limited to: 1) receptor characteristics, such as abundance, isoforms, and signal transduction requirements; and 2) cognate ligand characteristics including background level, mechanisms of synthesis and secretion regulation, as well as delivery to the site of action. These fundamental endocrine principles demonstrate why it is difficult or impossible to define the "potency" of an EDC, why "thresholds" of action are not possible to identify, and why nonlinear dose-responses cannot be ignored despite some continued controversy on this point. These and other concepts relevant to dose-responses, thresholds, and receptor kinetics are thoroughly reviewed in Ref. 133.

As mentioned, hormones act at very low concentrations, in part because hormone receptors are high-affinity receptors; that is, very low concentrations of hormone can bind to the receptor population and initiate important biological effects. In the study of EDCs, the term "low dose" is used in different ways, typically to distinguish studies that examine effects that are: 1) below the doses used in traditional toxicology studies, ie, doses below the no observed adverse effect level (NOAEL) or the low observed adverse effect level; 2) at doses in the range of typical human exposures; or 3) at doses in animals that are in the range of circulating concentrations of a substance in humans (133). The classic hormone concentration-response pattern is that of a sigmoidal curve (Figure 2). In this case, small changes in hormone concentration at the low end of the dose-response curve produce substantially greater differences in effect than similar changes in hormone concentration at the high end of the dose-response curve. This is important because very low concentrations of EDCs could add to the endogenous hormone concentration, producing an effect that is much greater than would be predicted based on its ability to bind to the receptor in isolated systems. In addition, hormone receptors expressed at different concentrations will affect the various characteristics of the dose-response curve. In this case, as receptor concentrations increase, the dose-response curve is shifted to the left; ie, it requires less hormone to produce the same biological effect. This can explain both why some endpoints of hormone action are more sensitive to the hormone than others, and why some are more sensitive to endocrine disruptors than others.

A feature of hormone-response relationships that is often overlooked is that of "spare receptors." This concept was originally named because it was observed that a maximum response to hormone could be achieved with receptor occupancy that was far below 100% (134). In fact, in some systems, the maximum hormone response can be achieved with as little as 1% receptor occupancy. An important insight, however, is that all the receptors are necessary to produce the maximum response, but the receptors are only occupied for some proportion of time that is less than 100% (134). Viewed this way, receptor concentration and the dynamic equilibrium between receptor and ligand become even more important to consider when EDCs enter into the mix.

Because hormones interact with and activate their receptors in a nonlinear fashion, dose-responses are at least sigmoidal, but they can also be more complex, including being nonmonotonic (Figure 2). These dose-response curves are often referred to as "U-shaped" (with maximal responses observed at low and high doses) or "inverted U-shaped" (with maximal responses observed at intermediate doses). In vitro studies have been instrumental in understanding the complex mechanisms behind nonmonotonic dose-responses. One mechanism involves integrating two (or more) monotonic responses that affect a common endpoint. For example, studies of prostate cell lines have shown that these cells proliferate to the highest degree when provided intermediate concentrations of androgen (135, 136). The reason for this inverted U-shaped response is that the cell line actually contains two populations of cells: one population proliferates in response to testosterone, whereas testosterone inhibits cell proliferation in the other population. At low doses, the first population has minimal proliferation, and at high doses the second population has a low level of proliferation because it is being inhibited. When looking only at cell number, intermediate doses have the maximal effect because at these concentrations the first population is somewhat proliferative and the second population is only somewhat inhibited. Ultimately, these two cell populations were isolated from each other, and when observed individually, each one had a monotonic response to androgen.

Nonmonotonic dose-responses also occur because of receptor down-regulation when hormones are present in high concentrations, bind to their receptors, and decrease receptor number. The degradation of receptors is increased when the hormone is abundant, and the ability of the cell to replace these receptors is slower than the rate at which they are removed from the system. Thus, high concentrations of hormone eventually lead to fewer available receptors and a natural shift in the receptor-mediated response. In addition to this mechanism, nonmonotonic responses can be caused by the increased toxicity of a hormone (cytotoxicity) at high doses. For example, the MCF7 breast cancer cell line proliferates in response to estrogen until high doses are reached (10^{-5} to 10^{-4} M) where it is cytotoxic, resulting in cell death (137). The same toxicity has been observed in a subpopulation of MCF7 cells that no longer express the estrogen receptor (ER), suggesting that estradiol (the natural estrogen) is not having endocrine effects at these high doses, but is generally toxic.

The concept of nonmonotonicity is also seen in vivo, underscoring the point that the above models are not simply an artifact of cell line work. We provide examples from several disciplines to illustrate this point. In the realm of metabolic effects of EDCs, multiple-dose studies have described a nonmonotonic relationship between prenatal or perinatal BPA exposure and weight increase as well as other metabolic alterations such as insulin resistance, glucose tolerance, and hyperinsulinemia (138–140). Similar results have been obtained with the synthetic surfactant perfluorooctanoic acid (PFOA), a putative obesogen in female mice (141), and the phthalate, mono-(2-ethylhexyl) phthalate (MEHP) (142). In adult male mice, BPA exposure regulates pancreatic insulin content at low doses (100 μg/kg/d), but it has no effect at high doses (1 mg/kg/d) (143). In the prostate, nonmonotonic dose-response curves for prostatic growth responses to estradiol, DES, and more recently BPA have been reported by several laboratories, showing that low- and high-dose exposures have no effect, whereas middle range exposures have growth stimulatory responses in rodent models (144). For female reproduction, in utero BPA exerted nonmonotonic effects on the age at vaginal opening (a marker of puberty) in female mice. Specifically, exposure (from embryonic day [E] 10.5 to birth) to 0.5 or 50 μg/kg/d, but not 20 μg/kg/d, delayed vaginal opening in the F3 generation (145).

Considering these features of hormones, it is important to view concepts such as *potency* of EDCs and *threshold* of EDC actions within this context. For EDCs that can interact directly with a hormone receptor, issues of receptor isoform, abundance, and signal transduction characteristics will be important characteristics defining potency (or efficacy). However, this will be endpoint-specific, and hormones often have many targets of action that change over the course of the life span of an individual. This is another essential feature in that hormone action during development produces effects that persist throughout life, and disruption during development is not likely to be reversible (see *Section I.F*). Because of this, potency is not simply defined for an EDC, and it is most certainly not the same as "affinity" (for the receptor). Moreover, some EDCs (eg, BPA) can bind to multiple hormone receptors.

Likewise, the concept of threshold as it applies to EDCs is equally complicated and must be considered with caution. Put simply, the concept of threshold for an EDC means that there is an exposure level below which no effects occur. In its simplest form, this implies that one can measure the difference between "no effect" and "some effect," something that the limits of measurement tools (assays) as well as the intrinsic variability in the biological system will always blur. This becomes more complicated because EDC effects during development may not be manifested until later in life, so operationalizing the definition of a threshold will be much more complicated. Finally, the choice of endpoint around which to build evidence for a threshold will also limit the definition; for example, the

threshold for BPA action on uterine weight in a rat study may be very different from the threshold on brain development, which may be very different from the increased risk of prostate or breast cancer.

F. Identifying effects of EDCs on human health: where to start?

There are numerous lines of evidence that environmental factors play a substantive role in disease causation or progression, or may alter the susceptibility to disease over a lifetime. A cohort analysis of nearly 45 000 pairs of twins from Sweden, Denmark, and Finland (146) concluded that the environment, and not genetics, played a principal role in sporadic cancers of the prostate, breast, and female reproductive system. Accidental exposures to EDCs vary by individual and are usually at higher levels than exposures experienced by the average citizen. Environmental accidents, such as a 1976 pesticide plant explosion in Seveso, Italy (147), have demonstrated a relationship between dioxin exposure and significantly increased cancer rates in women (148), increased metabolic disease in women who were 12 or younger at the time of the explosion (149), permanently reduced sperm quality in men who were breastfed as infants just after the explosion (150), and a dose-related association between serum dioxin levels and time to pregnancy and infertility in women (151). Pollution events have also been correlated with health effects in large residential cohorts. Specific examples are Agent Orange exposure to servicemen in South Vietnam (152), contamination of drinking water sources with volatile organic chemicals at the Camp Lejeune Marine base, Jacksonville, North Carolina, from the 1950s to mid 1980s (153), and PFOA contamination of the Little Hocking River and surrounding areas of northern Kentucky and Ohio from potentially the 1950s to the present (154)—all leading to numerous documented health effects, including endocrine-sensitive disease endpoints.

When correlations are made between health outcomes in humans and a particular chemical, the confirmation of cause and effect and the elucidation of a mechanism or mode of action must be derived from studies in animals, usually rodent models. One way in which individual compounds or mixtures of compounds are tested for their long-term health effects is the 2-year bioassay in rats and/or mice, as described by the National Toxicology Program (NTP) (155) or through similar test guidelines, such as those developed and used within the Organisation for Economic Co-operation and Development (http://www.oecd.org/env/chemicalsafetyandbiosafety/testingofchemicals/oecdguidelinesforthetestingofchemicals.htm) and the European Union's 2007 REACH (Registration, Evaluation, Authorisation and Restriction of Chemical Substances) regulation. In these assays, the test compound is usually fed to or inhaled by the animals from about 8 weeks to 2 years of age, and cancer formation is traditionally the endpoint of interest. Typically, a dose range of the highest tolerable concentrations is used in these studies, and endocrine-disrupting activity may be missed or masked by the more toxic effects (156). The Report on Carcinogens, produced by the NTP, documents data on nominated chemicals that may potentially put people in the United States at increased risk for cancer (http://ntp.niehs.nih.gov/pubhealth/roc/index.html). Of the tens of thousands of chemicals in the marketplace, about 2500 chemicals have been evaluated for any health effect (156). Of those, 2-year rodent cancer studies have been conducted on just over 600 chemicals (156). About 250 of the chemicals have shown carcinogenic potential in female rodents, and as an example of how they were further evaluated, about 60 of those showed evidence as mammary gland carcinogens (157). Furthermore, many of those mammary gland carcinogens caused cancer in other parts of the body. Since the time of the EDC-1 (1), the NTP has reinstituted an experimental design that exposes the fetus and developing offspring to the test chemical, in addition to exposure as an adult (http://ntp.niehs.nih.gov/testing/types/cartox/index.html). However, even with the neonatal exposure, the high-dose designs are still meant to identify cancer-causing agents and may not identify EDCs.

Recently, the Toxic Substances Control Act (TSCA) Chemical Substances Inventory (US EPA, TSCA Inventory) has been audited for accuracy, and duplicate listings were removed. At the time of this writing, there are nearly 85 000 existing chemical substances on the updated TSCA Chemical Substance Inventory (http://www2.epa.gov/tsca-inventory/) that were manufactured or processed in the United States. This does not include chemicals deemed as "new," naturally occurring materials, exempted polymers, or branded materials of confidential composition. This list may also not include the ingredients of a chemical solution or mixture that are considered "inert." Because only a few chemicals might be tested in the 2-year bioassay-like studies each year, other means of identifying possible EDCs are needed.

The US EPA's Endocrine Disruptor Screening Program (EDSP) has developed a multitiered set of animal and cell-based assays to test a handful of EDCs at a time; the pubertal protocol, for example, exposes rats to test compounds before, during, and after the expected time of puberty, assessing preputial separation in the male and vaginal opening in the female. There are several other endocrine-based endpoints in these assays, but it falls short in that it does not require collection or evaluation of mammary tissue, an important pubertal marker in humans; proper thyroid characterization; or other histopathological endpoints of importance (848). There are also other assays that measure specific effects on the male and female reproductive tract, and may indicate (anti)estrogenic and androgenic activities in vivo. The limitation of the tiered testing is that the assays were designed to test one compound at a few doses at a time.

Because compounds that have been suggested or defined as EDCs vary greatly in their structural makeup, the time it takes to be metabolized and/or excreted from the body (the EDC half-life), and their exposure routes and abundance in the environment, an entirely new direction of investigation was undertaken in about 2004 to test the thousands of untested chemicals for endocrine-perturbing activity. These testing programs have gained traction in the last decade. The US EPA Toxicity Forecaster (ToxCast) and the Toxicity Testing in the 21st Century (Tox21) (158, 159) are two federally funded efforts that have the potential to prioritize chemicals for potential human health effects. Tox21 is a collaborative program between the US EPA's National Center for Computational Toxicology, National Institute of Environmental Health Sciences (NIEHS)/NTP, the National Institutes of Health's National Center for Advancement of Translational Sciences, and the US Food and Drug Administration, and it uses primarily high throughput screening methods to characterize toxicity pathways (158, 159). ToxCast and Tox21 have some overlap in their assays but differ in their assay numbers and the number of specific chemicals tested. Furthermore, all of the assays used in Tox21 are part of the ToxCast battery, and all of the compounds in ToxCast are part of the large Tox21 library. It is likely that information from Tox21 and ToxCast can be used in basic science studies to investigate mechanisms of action of toxic chemicals identified by the programs and/or to investigate the effects of identified chemicals in vivo.

Since the last EDC Scientific Statement, ToxCast has evaluated about 1800 chemicals from a broad range of sources, in more than 700 assays that evaluate approximately 300 signaling pathways. Tox21 has generated information on approximately 10 000 chemicals using nearly 75 cell-based assays. These testing schemes incorporate assays to determine the potential effects of chemicals on nuclear receptor-based signaling (ie, estrogen, androgen, thyroid, peroxisome proliferator-activated receptor [PPAR]), stress responses, cellular proliferation regulators, mitochondrial toxicity, and drug metabolism, among others. The use of these types of assays allows for comparison of activity across a number of structurally related compounds, in a wide number of areas of endocrine disruption. Because these tests do not cover all of the potential routes of endocrine disruption, others have suggested a set of in vitro and in vivo tests that may be designed to enhance detection of more specific EDCs such as breast toxicants (160). Of course, there are also limitations to the use of these systems; the cell-based systems often lack the ability to metabolize the test compound or in other ways be extrapolated to humans; the accuracy of outcome is dependent on test chemical purity; individual responses may not always predict population responses to an exposure (and single cell response may not predict whole system responses); and finally, a good understanding of the relationships between pathways and disease outcomes in humans and animal models from the perspective of how hormones act in physiological systems is needed for successful prediction of effect. Moreover, species differences may lead to large differences in responses, so interpretation of data from rodent models should be done with this in mind. The use of transgenic animals, especially humanized mice, may help fill the gap, and large animal (eg, sheep) and nonhuman primate models ought to fill in this missing link in translatable research. In addition, efforts are currently under way at the US EPA, NIEHS and numerous other laboratories to determine the accuracy of the Tox21 and ToxCast data in predicting "valid" endocrine disruptors. Those efforts will compare accumulated data from the high throughput assays with animal data from, for example, the EDSP assays, to assess the reliability or predictability of a compound's potential to cause endocrine disruption.

EDCs migrate into the air, food, and water of humans and wildlife because of accidental release, pollution (and drift in air or water), leaching from the product they are contained in, volatilization (aerosolization), as food residues after agricultural application, lipophilic distribution into milk from body stores, etc. Lotions, sunscreens, soaps, and other mixtures containing endocrine disruptors may be applied to the skin, leading to dermal absorption and activity (161). Occupational exposure to these chemicals may encompass multiple routes of exposure to higher concentrations than the average resident. This review will highlight several EDCs, some of which are also known carcinogens, for which evidence has accumulated over the last 5 years. These compounds are summarized in Table 1, and some of their known mechanisms of action are discussed in Table 2.

G. Review criteria for EDC-2

The authors of this article agreed upon several criteria in reviewing the literature and determining what to include or exclude. From the "big-picture" perspective, we focused primarily on those EDCs for which the evidence spans several levels of analysis, particularly when there was epidemiological information in humans for which there was also a body of work on experimental animal data, especially during development, and mechanistic and molecular information from cells and animal tissues after exposures. Some EDCs, including BPA, POPs such as PCBs, phthalates, and pesticides, cut across virtually every area of endocrinology studied for their effects and were included in all of the sections. We also attempted to identify what we think are the critical re-

search gaps for each topic covered in this Statement. Each original research paper cited was critically evaluated for appropriateness of the model and the use of adequate controls (negative and positive) and was considered for the range of dosages tested. Sample size needed to be high enough to have adequate power to draw accurate statistical conclusions using appropriate statistical methods. Other methodological confounds were considered as they arose, and if deemed problematic, the paper was excluded. Excluded papers included those that did not meet these requirements or have other methodological problems that confounded the work. Whether a study found an effect of an EDC, or no effect, was not a determinant in including or excluding that study.

It is important to note that there may be publication bias, in that studies with positive findings (ie, an effect of an EDC is identified) are more likely to be published than those with negative findings (no effect of an EDC). There is simply no way for us to know about that work. Of importance, there are now more journals that make a point of publishing any study that is reviewed and considered well performed, even if results are negative, and over time more negative studies may emerge. Methods to limit the risk of bias (eg, distributing animals within a litter to different treatment groups) are routine procedures in endocrinology labs but are seldom described formally. Therefore, we could not use the description of these methods as a criterion for inclusion or exclusion. However, we emphasize the need for researchers in this field to fully describe these methods. Another point, whether in human, animal, or in vitro work, is whether work is done with the investigator blind to treatment. This is often not specified, and whereas authors may state that work was done in a blinded fashion, it is virtually never stated that work is done in an *unblinded* fashion, making it impossible to exclude such studies.

An example of a prototypical literature search, and inclusion criteria, is provided here for female reproductive health (see *Section III*). A PubMed search was conducted using search terms, their variants, and their combinations, such as BPA, DES, MXC, pesticide, phthalate, plasticizer, PCB, dioxin, ovary, oocyte, oviduct, fallopian tube, follicle, vagina, uterus, anterior pituitary, steroid, hormone, female, girl, women, cyclicity, estrus, menses, pregnancy, endometriosis, fibroid, leiomyoma, fertility, infertility, puberty, polycystic ovarian syndrome (PCOS), premature ovarian failure, birth, preterm, birth outcome, and menopause. We considered all journal articles that our search generated within the given time frame. We included all human studies, experimental mammalian in vivo studies, and in vitro studies using mammalian cells. We did not omit any journal articles based on whether they contained positive or negative findings. We omitted journal articles that focused on nonmammalian systems (eg, fish) because the focus of this article is on EDCs mammals. Thus, we conclude by reiterating that it was the rigor of the science that led to an article's inclusion or exclusion. Because EDC-2 picks up where EDC-1 left off in 2008, most of the literature reviewed herein is from the years 2008 to the present (2014).

II. Obesity, Diabetes Mellitus, and Cardiovascular Diseases

A. Introduction

T2D and obesity are two of the world's greatest health care issues. Globally, diabetes mellitus affects 347 million people, and in 2004, 3.4 million people died from the disease. By the year 2030, the World Health Organization estimates that diabetes will be the seventh leading cause of death. The World Health Organization also estimates that obesity has almost doubled since 1980. In 2008, 10% of the men and 14% of the women in the world were obese, compared with 5% for men and 8% for women in 1980, and at least 2.8 million adults die each year as a result of being overweight or obese (162). Notably, prevalence has increased in children and adolescents in developed countries; 23.8% of boys and 22.6% of girls were overweight or obese in 2013 (9). Given that obesity is directly associated with roughly 44% of diabetes mellitus cases (162), understanding the processes involved in the etiology of obesity and diabetes mellitus and how these diseases interact will help us prevent their development, reduce incidence, and develop new treatments. It will also help reduce the enormous social and financial burden of these disorders. The estimated annual medical cost of obesity in the United States was US $147 billion in 2008, and individual medical costs for obese people were US $1,429 higher than for normal-weight people (163).

Most of the research on the effects of EDCs on obesity, diabetes mellitus, and associated metabolic disorders is relatively new, appearing over the last decade. Before 2000, several EDC studies examining other endpoints reported increased weight as a collateral effect but did not focus on obesity or metabolic disorders as the primary outcome. In 2002, Baillie-Hamilton (164) reviewed the literature and hypothesized the obesogenic action of toxic chemicals, based on the parallel increase of pollutants and the incidence of obesity. This wake-up call led to a new hypothesis: that exposure to environmental chemicals during development may play a role in predisposing to obesity later in life (165, 166). These chemicals were named "obesogens" (167). Since 2008, multiple cellular,

animal, and epidemiological studies have emerged regarding the links between EDCs and obesity.

Diabetes mellitus is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Any toxic chemical that kills β-cells or disrupts their function has been termed a "diabetogen." Beginning more than 30 years ago, researchers described how environmental chemicals (eg, dioxins, DDT, BPA) altered β-cell function (168–171). Recently, numerous articles have been published demonstrating the link between EDC exposure and alterations in glucose homeostasis and/or diabetes mellitus in animals as well as epidemiological studies in humans (138, 172–175).

Here we will review the evidence published since 2008 implicating EDC exposure in the etiology of obesity and T2D. It is difficult to know which EDCs are risk factors for obesity, diabetes, or both, because they have interlinked pathologies. However, there is enough evidence to suggest that some EDCs act as obesogens and others act as diabetogens (Table 3). Therefore, we will review EDC action on obesity and diabetes separately. Additionally, the role of EDCs acting as cardiovascular disruptors is an important emerging area, which will be briefly reviewed.

B. Definition and etiology of obesity

1. Definition of obesity

The World Health Organization defines overweight and obesity as "abnormal or excessive fat accumulation that presents a risk to health" and categorizes normal-weight, overweight, and obese people by their body mass index (BMI). BMI is calculated as the ratio of an individual's body mass divided by the square of their height (BMI = kg/m^2). For adult men and women, BMI between 18.5 and 24.9 is considered normal, between 25 and 29.9 is overweight, and 30 or higher is obese. It is of note that BMI cutoff points differ between Western and non-Western countries (176). In some special cases, BMI does not correlate accurately with the percentage of body fat. Body fat modifications associated with changes in height over time (during childhood development) or large amounts of muscle tissue may produce higher BMI values and result in misclassification as overweight or obese. Nevertheless, BMI is a measure used to determine childhood overweight and obesity. The Centers for Disease Control and Prevention (CDC) defines overweight in children and teens as "a BMI at or above the 85th percentile and below the 95th percentile for children and teens of the same age and sex." Obesity is defined as "a BMI at or above the 95th percentile for children and teens of the same age and sex" (http://www.cdc.gov/obesity/childhood/defining.html). Waist circumference is another index used to screen for possible health risks that come with overweight and obesity. The risk goes up with a waist size that is greater than 88 cm for adult women or greater than 102 cm for adult men (https://www.nhlbi.nih.gov/health/educational/lose_wt/risk.htm). It is of note that cutoffs for increased metabolic risk may differ based on race/ethnicity; this places some populations at a heightened risk at lower waist circumferences.

2. Etiology of obesity

Obesity is caused by an energy imbalance when intake of energy is higher than expenditure. The origin of obesity is multifactorial and is influenced by both genetic and environmental factors. Studies of the heritability of obesity in monozygotic and dizygotic twins indicate a gene-by-environment interaction, associated with obesity (177, 178). How genes and environment interact to regulate body adiposity is complex. It is a difficult task to accommodate genetics as well as social and environmental influences in order to understand how body fat is controlled (179). In any case, environmental factors such as changes in lifestyle (eg, increase in stress or sedentary habits), changes in diet, and increased exposure to EDCs seem to play varying roles in the propensity to develop obesity.

The historical global increase in the production of synthetic organic and inorganic chemicals (many of which are EDCs) parallels the worldwide increase in obesity prevalence, lending support to the obesogen hypothesis (164). The likelihood that there are common environmental factors leading to the increase in the prevalence of obesity is supported by evidence in research animals, feral rats, and domestic dogs and cats (180). Interestingly, one study reported an increase in T2D in domestic cats and showed that this is associated with plasma levels of organohalogenated contaminants (181). Whether EDCs are one of the common factors driving mammals to obesity is still a matter of conjecture, yet it is one that we should continue to explore through experimental research and epidemiology.

a. The obesogen hypothesis. A positive balance between food energy intake and expenditure generates obesity. When there is an excess of lipids to store, the adipose tissue expands to accommodate it through the hypertrophy of existing adipocytes and by differentiation of preadipocytes (182). The inability of WAT to expand as much as necessary and the development of a dysfunctional adipose tissue contributes to obesity and obesity-associated metabolic complications (183).

How do EDCs make us fat? The 2006 study by Grun and Blumberg (167) that coined the term "obesogens" defined them as "xenobiotic chemicals that can disrupt the normal developmental and homeostatic controls over adipogenesis and/or energy balance." This and other related

Table 3. Effects of EDCs on Diabetes Mellitus and Obesity

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Obesogenic/Diabetogenic	Endpoints	Ref
BPA	Wistar rats	M/F	E0, P21	50, 250, 1250 μg/kg/d, oral, gavage	13–26 wk	Obesogenic; diabetogenic; exacerbates obesogenic and diabetogenic action of HFD; nonmonotonic	Body weight, glucose tolerance, insulin resistance, plasma insulin, β-cell mass, β-cell mRNA expression, lipid homeostasis	139
BPA	S-D rats	M/F	E6–P21	1 mg/L in drinking water	P21	Obesogenic	Body weight, fat weight, mRNA expression	241
BPA	CF-1 mice	M/F	E11–17	2.4 μg/kg/d, oral, food	P0, P22, and P26	Obesogenic	Body weight at weaning, age at vaginal opening, and interval between vaginal opening and first vaginal estrus	237
BPA	S-D rats	M/F	10-wk prebreeding exposure, mating, and E0–P21	50 and 500 mg/kg/d, dietary concentrations	F0 to F3 generations	Weight reduction in all generations	Body weight	250
BPA	Long-Evans rats	M	E12–P21, P21–P90	2.4 μg/kg/d, oral, dissolved in corn oil	90 d	Obesogenic in offspring, no increase in weight in adults	Body weight	236
BPA	ICR/Jcl mice	M/F	E0–P21	20 μg/kg/d, sc injection	P21, 10 wk	No change in weight	Body weight	248
BPA	Wistar rats	M	Gestation and lactation	50 μg/kg/d, oral gavage	3 wk	Diabetogen	Weight, serum insulin, insulin resistance, DNA methylation, hepatic glucokinase expression	310
BPA	C57BL/6 mice	M	Acute administration, adulthood	50 μg/kg, oral, water	6 mo	Possible diabetogenic	Hepatic glucokinase activity	307
BPA	C57BL/6J mice	M/F	E1 lactation	0, 3, 10, 30, 100, 300, 3000 μg/kg/d, oral, food	23 wk	Decreased weight in females, sex-dependent small alterations in energy homeostasis	Body weight, organ weight, hormones in plasma, glucose tolerance, locomotor activity, histopathology	249
BPA	CD-1 mice	M	E8–P16	0.025, 0.25, 25 μg/kg/d, osmotic pumps	P2 and P21	Possible diabetogen	Global metabolism by metabolomics	309
BPA	CD-1 mice	M	28 d, adulthood	0, 5, 50, 500, 5000 μg/kg/d, oral, food contamination	10 wk	Possible diabetogen and obesogen	Hepatic transcriptome, liver triglyceride accumulation	306
BPA	OF-1 mice	M/F	E9–E16	10, 100 μg/kg/d, sc injection	Mothers at E16–E18 and 4 mo after delivery, offspring at 3 and 6 mo old	Diabetogenic in mothers and offspring; obesogenic in mothers	Glucose tolerance; insulin sensitivity; insulin, leptin, triglycerides and glycerol plasma levels; pancreatic β-cell function; changes in body weight; measurement of η-cell mass and proliferation	138
BPA	OF-1 mice	M	4 d, adulthood	10, 100 μg/kg/d, sc injection	8–12 wk, adulthood	Diabetogenic	Insulin sensitivity, glucose tolerance, plasma insulin and glucose levels, pancreatic β-cell function	172
BPA	OF1 mice	M	8 d, adulthood	100 μg/kg/d, sc injection	8–12 wk	Diabetogenic	Global metabolism, glucose tolerance, insulin resistance, plasma insulin, insulin secretion, Akt phosphorylation in skeletal muscle and liver	244
BPA, DEHP, DBP	Harlan S-D rats	M/F	E8–E14	Mix of 50 BPA, 750 DEHP, and 66 DBP mg/kg/d, ip injection	F1 generation, F3 generation	Obesogenic in F3 generation	Body weight, abdominal adiposity	262
BPA, DES	CD-1 mice	M/F	E9–E18	5, 50, 500, 5000, 50 000 μg/kg/d BPA, or 0.1 μg/kg/d DES, oral	3–19 wk	Obesogenic, diabetogenic, nonmonotonic	Body weight, fat weight, glucose tolerance, insulin resistance, plasma insulin, plasma leptin, plasma adiponectin	140
BPA, DES	CD-1 mice	M/F	E0 to weaning	1 μg/kg/d DES, 0.25 μg/kg/d BPA, oral, feeding	P21–P15 wk	No increase in weight	Body weight, length, and fat and lean composition; food intake; glucose tolerance test	246
BPA, EE	C57/Bl-6 mice	M/F	E3–P21	2 and 200 μg/kg/d BPA, 5 μg/kg/d EE, oral, gavage	P21	No increase in weight	Body weight, anxiety	247
BPA, estrone	S-D rats	M/F	E6 throughout lactation	0.1 mg/kg/d BPA, 1.2 mg/kg/d estrone, oral, drinking water	4 d to 4–6 mo	Obesogenic	Analysis of body weight, pattern of estrous cyclicity, and uterotrophic assay	240
BPA, EQ, EB, DPN, PPT	Long-Evans rats	M	P0–P3	50 μg EB, 1 mg/kg; PPT, 1 mg/kg; DPN, 10 mg/kg; EQ, 50 μg/kg; BPA, sc injection	P68	Obesogenic	Body weight	239

(*Continued*)

Table 3. Continued

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Obesogenic/Diabetogenic	Endpoints	Ref
BPA HFD	ICR mice	M/F	E13–E16	0.2 and 3 mg/kg/d, oral, drinking water	1 mo	Obesogenic	Body weight, fat weight, serum leptin and lipids	238
BPA HFD	OF-1 mice	M	E9–E16	10 μg/kg/d, sc injection	17–28 wk	Obesogenic, diabetogenic	Body weight, fat weight, glucose tolerance, insulin resistance, plasma insulin, insulin secretion, mRNA expression	242
DDT	C57BL/6J mice	F	E11.5–P5	1.7 mg/kg body weight	Up to 6 mo	Obesogenic, diabetogenic	Body composition, energy homeostasis, metabolic parameters, glucose tolerance. Energy balance, metabolic consequences of HFD	259
BPA, TCDD, PCB-153, DEHP	C57BL/6J mice	M/F	Before gestation, during gestation, lactation, combined with standard diet and HFD	Tolerable daily intake of each component of the mixture, oral in pellets	10–12 wk	Diabetogen	Weight, hepatic gene expression, plasma leptin, insulin and cholesterol, glucose and insulin tolerance	313
DEHP	Wistar rats	M/F	Gestation and lactation	1.25, 6.25 mg/kg/d, oral gavage	3, 15, 26 wk	Diabetogenic	Weight, glucose and insulin tolerance, β-cell mass and function	312
DES	CD-1 mice	M/F	P1–P5	1 μg/kg/d, 1 mg/kg/d, sc injections	2, 6 mo	Obesogenic	Analysis of body weight and fat pads; serum levels of leptin, adiponectin, IL-6, insulin, and triglycerides	1277
DES	C57BL/6J	M/F	E12–P7	0.01, 0.025, 0.05, 0.1 mg/kg/d, oral, gavage	8 wk	Obesogenic	Body weight, fat weight, cholesterol, triglycerols, glucose	1278
DDT	Harlan S-D rats	M/F	E8–E14	50, 25 mg/kg/d, ip injection	10 mo, F1 generation, F3 generation	Obesogenic in F3 generation	Body weight, abdominal adiposity	263
FM 550	Wistar rats	F	E8–P21	100, 1000 μg/d, oral, in pellets	21 d, 17 wk	Obesogenic, cardiovascular disruptor	Serum T_4, hepatic carboxylesterase activity, body weight, glucose tolerance, heart wall thickness	565
MEHP	C57BL/6J mice	M/F	E12–P7	0.5, 0.25, 0.05 mg/kg/d, oral, gavage	60 d	Obesogenic, diabetogenic, nonmonotonic	Weight, fat weight, blood glucose, cholesterol, triacylglycerols, mRNA expression adipogenic genes	142
PCB-126	Wistar rats	M	E7–P21	100 mg/kg/d, oral, food	20 mo	Obesogenic	Neurobehavior, body weight	256
PCB-153	C57BL/6J mice	M	12 wk, adulthood	50 mg/kg × 4, ip injection	12 wk	Exacerbates obesogenic action of HFD	Body weight	1279
PFOA	CD-1 mice	F	E1–E17	0, 0.01, 0.1, 0.3, 1, 3, 5 mg/kg/d, oral, gavage	15–16 wk, 42, wk, 70–74 wk	Obesogenic; nonmonotonic	Body weight, serum, insulin, leptin, fat weight	141
PFOA	CD-1 mice	F	17 d, adulthood	1 mg/kg/d, oral, gavage	10 wk	No change in weight	Body weight	141
PFOS	CD-1 mice	M/F	Gestation and lactation	0, 0.3, 3 mg/kg, oral gavage	P21 and P63	Diabetogenic	Fasting glucose and insulin levels, glucose tolerance	304
PFOS	CD-1 mice	M	3, 7, 14, 21 d, adulthood	0, 1, 5, 10 mg/kg/d, oral gavage	8–12 wk	Hepatic steatosis, possible diabetogenic	Histology of liver, hepatic liver metabolism	304
POPs	CB57BL/6J	M	Adulthood	Different diets with farmed salmon fillets, with different POP concentrations, oral, food	Adulthood	Diabetogenic; obesogenic	Insulin and glucose tolerance, fat distribution, mRNA levels of target genes	303
POPs, PCDDs, PCDFs, non-ortho-PCBs, mono-ortho- substituted PCBs, organochlorine pesticides	S-D rats	M	28 d, adulthood	Organochlorine pesticides, 0.36–2.63 ng/g diet; DDTs, 0.13–3.57 ng/g diet; PCDDs, 0.11–1 pg/g diet; PCDFs, 0.32–0.99 pg/g diet; non-ortho-substituted PCBs, 0.83–23.7 pg/g diet; mono-ortho-substituted PCBs, 4.30–1314.35 pg/g diet; PCBs, 0.07–2.35 ng/g diet, oral, food	Adulthood	Diabetogenic; obesogenic	Insulin sensitivity, hepatic lipid homeostasis and mRNA levels of target genes, glucose uptake, daily energy intake, quantification of body weight and visceral fat	285
TBT	KM mice	M	From puberty once every 3 d over 45 d	0.5, 5, 50 μg/kg/d, oral, gavage	45 d after treatment, adulthood	Obesogenic	Body weight; fat mass; plasma insulin, leptin, adiponectin, and resistin levels; histological liver changes; levels of hepatic adiponectin and resistin	1280

(*Continued*)

Table 3. Continued

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Obesogenic/Diabetogenic	Endpoints	Ref
TBT	C57BL/6J mice	M/F	E12–E18	0.05–0.5 mg/kg/d, ip injection	P1–P10 wk	Obesogenic	Changes of body weight, changes of adiposity in liver, testis and inguinal adipose and mammary adipose depots, quantitative analysis RXR:PPARγ target genes	184
TBT TBBPA	Swiss mice	NS	Single dose	40 mg/kg, 2.1 g/kg, oral gavage, sc injection	1 d	Possible metabolic disruptor	T_3-independent transcription of Mc4r and Trh transcription	252
TBT TBBPA	Swiss mice	NS	E6 to delivery, for 7 d from E12	0.5 mg/kg/d 150 mg/kg/d, oral gavage	2 d	Possible metabolic disruptor	T_3-independent transcription of Mc4r and Trh transcription	252
TBT, ROSI	C57BL/6J mice	M/F	E16	0.1 mg/kg TBT, 1 mg/kg ROSI, oral, gavage	8 wk	Obesogenic	Effects of TBT on stem cell proliferation, adipose, bone and cartilage differentiation	251
TCDD	C57BL/6J mice	M	Single dose, 4 wk old	100 μg/kg/d, oral, gavage	4–5 wk	Possible metabolic disruptor	Changes in body weight, analysis of intestinal disaccharide activity, gene and protein expression of intestinal glucose transporters, glucose tolerance test	1281
TCDD	Short-hair guinea pig	M	Single injection, 4–6 wk old	0.01, 0.03, 0.1, 0.3, 1.0 μg/kg/d, ip injection	0.25, 0.5, 1, 5, 10 and 28 d after single injection	Possible diabetogenic	Measurement of glucose uptake in pancreas, adipose tissue, liver and brain	168
TCDD	S-D rats	M	Single injection	25, 125 μg/kg/d, ip injection	1, 2, 4, 8, 16, 28 d after 125 μg/kg dose, 32 after 25 μg/kg dose	Possible metabolic disruptor	Changes in body weight, measurement of liver PEPCK and E-6-Pase activity, mean time to death	1282
TCDD	S-D rats	M	Single injection	1 μg/kg/d, ip injection	24 h after single injection	Diabetogenic	Pancreatic insulin content and release, pancreatic glucose uptake and protein levels of GLUT-2, plasma insulin, triglycerides, free fatty acids, and leptin levels	286
TCDD	C57BL/6J mice	M	Single injection, 8 wk old	10 μg/kg/d, ip injection	8 wk, adulthood	Diabetogenic	Glucose tolerance test, pancreatic insulin release	287

Abbreviations: DPN, diarylpropionitrile; EB, estradiol benzoate; EE, ethinyl estradiol; EQ, equol; F, female; FM, Firemaster; HFD, high-fat diet; ip, intraperitoneal; M, male; PEPCK, phosphoenolpyruvate carboxykinase; PPT, propyl pyrazole; ROSI, rosiglitazone; sc, subcutaneous; S-D, Sprague-Dawley.

studies gave birth to the "obesogen hypothesis," which suggests that prenatal or early-life exposure to certain EDCs predisposes some individuals to gain fat mass and become obese (184–186). Evidence published during the last few years indicates that EDCs promote adipogenesis in cellular models and promote adipogenesis and obesity in animal models as well as in humans, indicating that EDCs must alter energy balance. A recent comprehensive review by Casals-Casas and Desvergne (187) further explores the action of EDCs and obesity.

b. Epidemiology and evidence in humans. In January 2011, the NIEHS division of the NTP organized a workshop to evaluate the role of environmental chemicals in diabetes mellitus and obesity. The workshop reviewed epidemiological data for several EDCs, including maternal smoking, arsenic, BPA, phthalates, and POPs, and subsequently published a consensus report (188). Readers are also referred to several reviews (189–191) for a more detailed description on epidemiological studies about EDC exposure and obesity and T2D association.

i) Bisphenol A. There are a number of epidemiological studies that associate EDCs, especially BPA, with obesity in humans. Most studies are cross-sectional analyses of the US National Health and Nutrition Examination Survey (NHANES) data in adults and children that show that the higher the urinary BPA concentration, the higher the odds of obesity and larger waist circumference (192–195). Another study using a cohort in China reported an association between urinary BPA concentrations and overweight, obesity, insulin resistance, and diabetes mellitus (196). To our knowledge, only one prospective study has examined the association between prenatal and early-life exposure to BPA and children's body mass in 9-year-old girls (197). That work concluded that girls with the highest exposure to BPA in utero had lower weight for the same height than girls with the lowest exposure, a result that contradicts the previous studies; these inconsistencies require further studies on larger sample sizes.

ii) Phthalates. Elevated levels of phthalates have also been associated with increased BMI and waist circumference in adults and children. A cross-sectional analysis of the NHANES data showed that mono-n-benzyl phthalate (MBzP), mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl)

phthalate (MEOHP), and monoethyl phthalate (MEP) were associated with increased waist circumference and BMI in males (198, 199). BMI and waist circumference increased with MEP exposure in adolescent girls, as well as in 20- to 59-year-old women, although the study did not find any association in children (199). Results from a Swedish cohort of elderly people showed that circulating concentrations of mono-isobutyl phthalate were positively associated with waist circumference, total fat mass, and trunk fat mass, as well as the subcutaneous abdominal region in women, but no statistically significant relationship was found in men (200). In children, results are varied. In Chinese school children, exposure to MEHP and MEP was positively associated with BMI and waist circumference (201). Other studies have not found a correlation between these EDCs and body weight in normal-weight children, but in overweight children, MEP and the sum of low-molecular-weight phthalates were associated with higher BMI and waist circumference (202). Low-molecular-weight phthalates were associated with childhood obesity in non-Hispanic blacks, showing that race/ethnicity may play a role in vulnerability. No significant association was found for any race/ethnicity subgroup for high-molecular-weight phthalates or DEHP (203).

It is of note that BPA and phthalates have a short half-life in the organism. Most epidemiological studies, including those cited here, are based on a single measurement of exposure that may not accurately reflect true exposure.

iii) Persistent organic pollutants. Other EDCs, especially POPs, have been identified as obesogens, with BMI and waist circumference associations reported independently of gender (204). A prospective cohort study associated low-dose developmental exposure to PFOA with overweight in female offspring at 20 years of age (205). The directionality of this finding is consistent with animal studies discussed previously (141). A very comprehensive review about the link between POPs, obesity, and diabetes has been published recently (206). It concluded that human evidence on POPs and obesity remains insufficient, yet evidence is much stronger on POPs and T2D, as will be discussed later.

In summary, there is variability between cohorts, gender specificity, and roles of race/ethnicity that must be considered in interpreting these epidemiological data. Because the nature of cross-sectional studies does not enable the establishment of a cause-effect link, we can only infer causality from cellular and animal studies. Therefore, more prospective studies are needed to establish causation between EDCs and obesity in humans.

c. Cellular models

i) Chemicals that induce adipogenesis. One of the first EDCs identified as an obesogen was the organotin TBT, widely used as an antifouling pesticide in marine paints or as an antifungal for textiles. TBT acts as an agonist of PPARγ and retinoid X receptor (RXR) α at nanomolar levels and promotes adipogenesis in the preadipocyte cell line 3T3-L1 (184, 207). Because PPARγ is a pivotal molecule in the regulation of adipogenesis (208), any EDC acting as an agonist on this receptor will cause adipocyte expansion after increasing the number of fat cells (1, 209). Most of the cellular studies published to date with EDCs are centered on their adipogenic action, yet adipogenesis per se does not explain obesity. An excess of adipocytes, however, can be filled up with fat in the presence of a positive energy balance state. This can be accomplished by direct effects in adipose tissue, changing the balance of adipokines and leading to increased food intake or decreased energy expenditure.

Studies have shown that a number of EDCs activate PPARγ and thus increase adipogenesis in preadipocyte cell lines. For example, TBT and triphenyltin produced adipocyte differentiation (184, 207, 210). Phthalates (including mono-n-butyl phthalate, MBzP, and MEHP) (211, 212), parabens (212, 213), 4-nonylphenol (214), and BPA (215) at micromolar levels resulted in adipogenesis. DEHP and TBT enhanced adipogenesis after differentiation of mesenchymal stem cells (216), and the fungicide triflumizole promoted adipogenesis in 3T3-L1 preadipocytes and human multipotent mesenchymal stromal stem cells at low nanomolar concentrations (217).

In addition to binding to PPARγ, some EDCs promote adipogenesis through other mechanisms [ie, DES via ERs (218) and BPA through ERs (219–221)].

Both BPA diglycidyl ether and BPA induced adipogenesis in 3T3-L1 preadipocytes, but only BPA diglycidyl ether was able to induce adipogenesis in human and mouse mesenchymal stem cells (222). Interestingly, BPA at the environmentally relevant concentration of 10 nM increased the mRNA expression as well as the enzymatic activity of 11β-hydroxysteroid dehydrogenase type 1 (11-βHSD type 1), which transforms cortisone to cortisol in human adipose tissue and induces adipogenesis. This action may involve a glucocorticoid receptor (GR) and thereby lead to the acceleration of adipogenesis, but this was not tested (223). The binding of EDCs to GRs has revealed a potentially novel pathway for EDC modifications of energy metabolism. The pesticide tolylfluanid, widely used in Europe, bound to GR and promoted preadipocyte differentiation in vitro (224). It also increased insulin-stimulated lipogenesis (225). The aryl hydrocar-

bon receptor (AhR) was also shown to mediate PCB-induced adipogenesis (226). Finally, exposure to the brominated flame retardant BDE47 resulted in adipocyte differentiation in vitro (227).

In summary, cellular models indicate that the biocides TBT and triphenyltin, the fungicide triflumizole, the pesticide tolylfluanid, the POP PCB, phthalates and BPA used in plastic and many other products, and the detergent 4-nonylphenol make new adipocytes in vitro and may increase obesity susceptibility in animal models and/or humans in the presence of energy imbalance. Other comprehensive reviews by Regnier and Sargis (228) and La Merrill et al (229) provide further molecular details about EDCs and adipocytes.

d. Animal models. Obesity requires eating more food and/or consuming less energy. To date, most of the obesity studies in animals are based in the observation that EDC exposures induce weight increases and changes in adiposity, as well as affecting hormones and adipokines involved in the regulation of food intake and energy expenditure. Figure 3 and Table 3 show a summary of EDCs with obesogenic effect. There are fewer studies related to how EDCs disrupt energy balance. Therefore, more studies are necessary to gain mechanistic insights into the role that EDCs play in the etiology of obesity.

i) Environmental estrogens: DES and BPA. Studies of rodents that were prenatally, neonatally, or perinatally exposed to EDCs support the obesogen hypothesis. For example, DES exposure effects in mouse models replicate human findings (102, 230). DES is an estrogenic chemical that binds with high affinity to the ERs, ERα and ERβ, which play an important role in adiposity regulation as well as central

Figure 3.

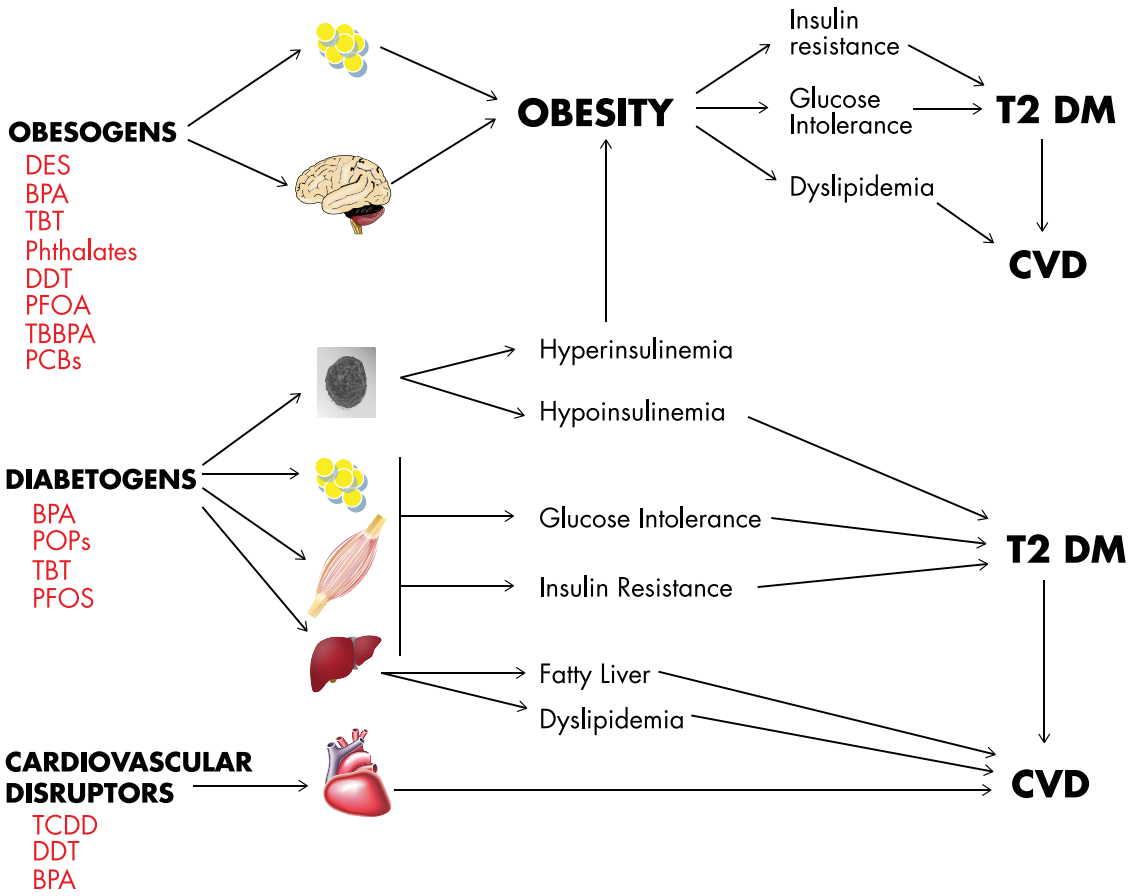


Figure 3. EDCs can act as obesogens, diabetogens, and/or cardiovascular disruptors. For obesogenic effects, EDCs act upon adipocytes and the brain to induce obesity, which generates insulin resistance, glucose intolerance, and dyslipidemia and greatly increases the susceptibility to T2D and CVDs. Additionally, EDCs work as diabetogens that directly affect the islet of Langerhans and increase or decrease normal insulin biosynthesis and release, generating hyper- or hypoglycemia. An excess of insulin signaling, as well as insulin resistance, can result in metabolic syndrome. In animal models, EDCs induce insulin resistance, glucose intolerance, fatty liver, and dyslipidemia on WAT, liver, and skeletal muscle. This generates T2D and CVDs. The EDC BPA has been shown to act directly on the heart, increasing the probability of CVDs in animal models.

and peripheral energy balance (231–234). Developmental exposure to DES in mice induced adipogenesis and caused mice to become obese or overweight (235).

Other chemicals classified as environmental estrogens, particularly BPA, produced similar effects. Perinatal exposure to low doses of BPA caused increased body weight; adiposity; alterations in blood levels of insulin, leptin, and adiponectin; as well as a decrease in glucose tolerance and insulin sensitivity in an age-dependent manner (139, 140, 236–243).

The central control of food intake and energy expenditure requires the precise coordination of several hypothalamic and extrahypothalamic nuclei. One study suggested that BPA decreased locomotor activity, a measure of energy expenditure, after 8 days of exposure in adult male mice, suggesting that it alters central energy regulatory pathways; however, that study did not examine the mechanisms and the involvement of the central nervous system (244). Perinatal exposure of CD-1 mice to environmentally relevant doses of BPA via specially prepared diets induced sexually dimorphic alterations in the structure of the hypothalamic energy balance circuitry as well as causing other metabolic alterations, such as glucose intolerance (245).

It is plausible that a concerted action of estrogenic EDCs on adipocytes, the endocrine pancreas, and the central nervous system may explain the increase in adipogenesis together with energy imbalance driving to weight increase. We must consider, however, that there is a great variability in results among the different reports summarized below, particularly when weight increase was measured as the endpoint. This can be explained by dissimilar BPA dosing, timing of exposure, and the sex and age of the animals. Perinatal exposure to lower levels of BPA (2.5 ng/kg/d) produced an increase in size but no change in adiposity and had no effect on glucose homeostasis at 15 weeks of age (246). Other studies of perinatal exposure to 2, 20, and 200 μg/kg/d of BPA reported no increase in weight in adult mouse offspring (247, 248). Prenatal exposures to low doses (10 and 100 μg/kg/d) of BPA in mice produced no changes in weight in adult males, and decreased weight in adult females. Males, however, had hyperinsulinemia, glucose intolerance, and insulin resistance at 6 months but not at 3 months of age (138). Another mouse study also reported no increase in weight in males and a decrease in weight in female offspring (249). This study reported sex-dependent alterations in parameters related to glucose and energy homeostasis, such as plasma glucagon levels, decreased energy expenditure in males, and increased expression of uncoupling protein 1 in female brown adipose tissue (249). In another study, high toxicological doses of BPA produced a decrease in weight (250).

In recent years, a number of studies have stated that several EDCs exhibited nonmonotonic dose-response curves. As a consequence, more recent studies have begun to test a range doses (133). Examination of BPA at doses from 5 to 5000 μg/kg/d demonstrated a nonmonotonic relationship between prenatal exposure and weight increase. Only one BPA concentration (500 μg/kg/d) increased weight at 18 weeks of age. Of note, low doses promoted glucose intolerance and insulin resistance despite no change in weight (138, 140). These multiple-dose studies also reported a nonmonotonic relationship between BPA exposure, weight increase, and other metabolic alterations such as insulin resistance, glucose tolerance, and hyperinsulinemia (138–140). Therefore, negative results obtained in studies conducted using only one dose must be interpreted with caution.

We can conclude that the obesogenic effect of BPA in animals still needs further characterization because it depends on dosing, timing of exposure, and age at analysis. We must, nevertheless, emphasize that in the great majority of the reports summarized above, BPA caused metabolic alterations. Those include: insulin resistance and hyperinsulinemia possibly as a consequence, glucose intolerance, hyperleptinemia, hypoglucagonemia, and alterations in energy expenditure. Therefore, BPA can be considered as a metabolic disruptor, and as discussed below, it is diabetogenic and alters cardiovascular function.

ii) PPARγ agonists: TBT and phthalates. Mice treated prenatally with TBT showed elevated lipid accumulation at birth and increased fat depots at 10 weeks of age (184). TBT altered the fate of multipotent mesenchymal stem cells, which differentiate to adipocytes rather than to bone cells (251). Notably, the effect was imitated by the PPARγ agonist rosiglitazone, indicating the likely involvement of this receptor. Another PPARγ agonist, MEHP, exerted adipogenic actions in adult mice as well as after in utero exposure (142). In that study, 6-week-old mice injected with a single dose of MEHP (0.5 mg/kg/d) had increased adipogenic markers after 24 hours, such as PPARγ, adipocyte-specific fatty acid binding protein, and lipoprotein lipase in adipocytes, as well as Fas in the liver, actions that were mimicked by troglitazone. The same researchers dosed pregnant mice (via gavage) with MEHP at 0.05, 0.25, or 0.5 mg/kg/d from E12 to lactational day 7. At 8 weeks of age, mice offspring exposed in utero to the lowest MEHP had increased weight compared to controls. Those groups exposed to higher concentrations did not have increased weight, constituting another example of a nonmonotonic dose-response. The MEHP-induced weight in-

crease was associated with accumulation of epididymal and perirenal fat. Moreover, total cholesterol, triacylglycerol, and blood glucose levels were all increased, although glucose levels remained within the physiological range. Interestingly, these changes were sex dependent, with males being more sensitive than females.

iii) Tetrabromobisphenol A. The flame retardant tetrabromobisphenol A (TBBPA) as well as TBT affected TRH expression and type 4 melanocortin receptors in the paraventricular nucleus (PVN) of the hypothalamus. The thyroid hormone T_3 regulates the expression of these two important proteins, both of which are involved in metabolic control (252). Acute exposure to TBT (40 mg/kg) increased TRH translation in the PVN of newborn offspring. TBBPA (150 mg/kg) exposure of gestating dams for 7 days decreased T_3-independent TRH and type-4 melanocortin receptors expression; this in turn should decrease catabolism, thermogenesis, as well as hypothalamic sensitivity to melanocortin, thus stimulating orexigenic pathways. Both actions are predicted to produce a positive energy balance state (253). Recently, TBBPA and tetrachlorobisphenol A were shown to function as obesogens in zebrafish (254). The more we know about how EDCs act on central energy regulation, the better we will understand how EDCs may trigger obesity. However, this is a largely understudied area of research that should be prioritized for future study.

iv) PFOA and other POPs. Further studies have tested the synthetic surfactant PFOA as a putative obesogen in female mice (141). Exposure to a range of doses (from 0.01 to 5 mg/kg/d) during 17 days of pregnancy revealed that the lowest doses (0.01 and 0.1) produced higher weight in adults from 20 to 40 weeks, together with higher serum levels of insulin and leptin. Increased levels of these two hormones may change energy balance. Food intake, basal blood glucose levels, and glucose tolerance remained unaltered; energy expenditure was not measured. Although PFOA binds to PPARs at high concentrations (255), its role in the effects described above is presently unknown.

Other EDCs led to a weight and adiposity increase, including PCB126 (256), PCB77 (226), and the organophosphate insecticides chlorpyrifos, diazinon, and parathion, especially when combined with a high-fat diet (see Ref. 257 for a review). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) administration at high doses accelerated weight gain in mice fed a high-fat diet (258).

Recent work has demonstrated that perinatal exposure to environmentally relevant doses of DDT reduced energy expenditure and impaired thermogenesis. This is associated with a decrease in the expression of mRNA involved in thermogenesis and substrate utilization in the brown adipose tissue of adult female mice (259). Because we now know that human brown adipose tissue plays an essential role in energy balance (260), its alteration by EDC exposure represents a new area of interest to study.

3. Epigenetics: gene-by-environment connection

To understand how EDCs affect obesity, diabetes mellitus, and other metabolic disorders, we must look to genes and their regulatory factors. The mechanism of the phenotypic changes in F1 and F2 generations described in the above paragraphs likely involves epigenetic modifications—specifically, mitotically and/or meiotically heritable changes in gene function without changes in DNA sequence (98, 261). Most of the current epigenetic evidence for EDC effects on the obesity phenotype is from F1 and/or F2 generations, suggesting a direct effect of EDCs on the gestating mother or the fetus (104).

Interestingly, a mix of plastic derivatives used in consumer and industrial applications (BPA, DEHP, and di-n-butyl phthalate [DBP]) promotes the epigenetic transgenerational inheritance of adult-onset obesity in rats (262). That study characterized the F1 and F3 phenotypes, with direct exposure of the fetus and somatic cells to EDCs in the F1 generation and epigenetic transgenerational inheritance through the germline in the F3 generation. An obesity phenotype was observed in 1-year-old F3 female rats whose weight, fat deposition, and adiposity was increased in most of the organs examined (262). Interestingly, these obese female rats had PCOS, a pathology seen in many obese women and that may, in part, be a result of EDC exposure (1). Surprisingly, the F1 female rats had normal weight, a strong indication that the molecular mechanisms involved in F1 generation and F3 generation are different. In any case, this work demonstrates an epigenetic transgenerational inheritance of obesity, which is not due to the direct effect of EDCs. Whether this can happen with environmentally relevant doses of EDCs is still unknown because the levels of plastic compound mixture used in that study (262) were high compared to current levels of human exposure. This same research group also studied transgenerational actions of DDT in rats and demonstrated a transgenerational obesity phenotype in F3 (but not F1) animals, along with other associated diseases (263). The study also identified sperm DNA methylation regions in a number of genes involved in obesity and polycystic ovarian disease in DDT-exposed rats. This important body of work, although it needs to be replicated and extended to lower dosages, has shown that ancestral environmental exposures may influence the predisposition to obesity even without direct exposure to the environmental compound.

C. Definition and etiology of type 2 diabetes mellitus

According to the American Diabetes Association, "diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels" (264).

The etiology of T2D is multifactorial and not completely understood. T2D development is normally associated with a strong genetic predisposition as well as with environmental factors such as obesity, aging, and lack of exercise. Obesity triggers insulin resistance via signaling molecules circulating in blood that are either released from adipocytes or are not taken up by adipocytes (265, 266). Some EDCs have been proven to induce insulin resistance in cellular and animal models, leading to the "diabetogen hypothesis" (very much in parallel with the "obesogen hypothesis"). The diabetogen hypothesis proposes that "every EDC circulating in plasma able to produce insulin resistance, independently of its obesogenic potential and its accumulation in adipocytes, may be considered a risk factor for metabolic syndrome and type 2 diabetes" (267). The International Diabetes Federation defines the metabolic syndrome as "a cluster of the most dangerous myocardial infarction risk factors: diabetes and prediabetes, abdominal obesity, high cholesterol and high blood pressure" (http://www.idf.org/metabolic-syndrome).

EDCs acting as obesogens are a risk factor for T2D and can potentially induce "diabesity," a form of diabetes that typically develops in later life and is associated with obesity. However, it is important to note that many EDCs produce insulin resistance and alter insulin production and secretion by directly acting on adipocytes, liver, and β-cells in the absence of overweight or obesity. Therefore, these EDCs are diabetogens and constitute a risk factor for T2D on their own.

The diabetogenic action of EDCs could explain, at least in part, the concept of being metabolically obese despite a normal weight (268). This phenotype is particularly frequent in Asia, where there is a divergence between the rates of obesity and T2D (269, 270). Here we will review the evidence that EDCs can act as diabetogens.

1. The diabetogen hypothesis

a. Epidemiology and evidence in humans. As previously mentioned in the Obesity section, readers are referred to reviews (188–191) written after the NTP Workshop: Role of Environmental Chemicals in the Development of Diabetes and Obesity organized in 2011. Since the NTP workshop, a comprehensive review of epidemiological research about environmental EDCs and T2D including seven prospective studies has been published, and it is highly recommended (271).

i) Persistent organic pollutants. There is substantial evidence linking some POP exposure to T2D in humans, including trans-nonachlor, DDE, PCBs, and dioxin-like chemicals. The role of POPs, particularly organochloride pesticides and PCBs, in the incidence of T2D currently represents the most solid association between a class of EDCs and the increase in the prevalence of T2D (206). Although there are some discrepancies, results with occupational cohorts, nonoccupational populations, as well as the general population indicate an independent association of POP exposure and diabetes mellitus (272). Notably, nonmonotonic relationships and low-dose effects appear in humans (206).

Several prospective studies have linked POPs to T2D in the general population. Those include DDE (273), PCBs, organochlorine pesticides, hexachlorobenzene (HCB), and dioxins (274–278). None of these prospective studies demonstrate that a single POP significantly predicted T2D; however, they all concluded that some POPs or combination of POPs predicted the future risk of T2D.

ii) BPA, arsenic, and phthalates. Several cross-sectional studies have associated BPA levels in urine with the incidence of T2D. The first study appeared in 2008 when the NHANES released the first large-scale data on urinary BPA concentrations (175). This study associated BPA urinary levels with a higher incidence of self-reported T2D as well as CVDs. Subsequent studies linked urinary BPA levels to diabetes in normal-weight, overweight, and obese people (279), and also to insulin resistance in normal-weight and obese people (196). Interestingly, a recent study established a relationship between urinary BPA and hyperinsulinemia and insulin resistance, particularly in men (280). This prediabetic state in men matches the data discussed above in adult mice (172, 244). Other studies using data from NHANES (2003–2006 and 2003–2008) found an association in the pooled analysis but not during the cycle 2003–2006 or cycle 2003–2008 (281, 282). A new prospective study established an association between BPA and phthalate exposures with risk of T2D in middle-aged women (283). One study found no such association (284), although the authors declared strong conflict of interest. Arsenic and phthalates are also important EDCs that have been associated with T2D in cross-sectional studies (see Ref. 271 for a review).

b. Cellular models

i) Persistent organic pollutants. Adipocytes are a target of numerous widespread EDCs and may represent a reservoir for EDCs, especially POPs (229). Nanomolar concentrations of POPs induced insulin resistance and decreased expression of insulin-dependent genes involved in lipid homeostasis in the differentiated adipocyte cell line, 3T3-L1, demonstrating direct actions of POPs on insulin signaling (285). It is of note that not all POPs contributed equally to the alteration of glucose uptake by adipocytes. Those that impaired insulin action were non-*ortho*-substituted and mono-*ortho*-substituted PCB mixtures, organochlorine pesticide mixtures, and DDTs. Polychlorinated dibenzodioxin (PCDD) and polychlorinated dibenzofuran (PCDF) mixtures presented with normal insulin action (285). Exposure to TCDD decreased glucose uptake in adipocytes and pancreas (168) and impaired insulin secretion (286, 287). Dioxins and dioxin-like PCBs interact with the AhR, which activates signaling pathways that regulate other nuclear receptors such as PPARγ or ERs involved in adipogenesis (187, 229). Moreover, AhR−/− mice presented with increased insulin sensitivity and improved glucose tolerance (288). It is, therefore, plausible that activation of AhR by POPs with dioxin activity elicits an opposite action.

Interestingly, in addition to an adipogenic effect, TCDD and PCB77 also triggered a proinflammatory action on mouse 3T3-L1 cells, likely through AhR activation (226). TCDD activates genes related to the inflammatory pathway in a human model of adipocytes (289). Because inflammation is linked to dysfunction of adipocytes and insulin resistance (290), it is possible that in addition to their obesogenic action, POPs may contribute to adipose tissue inflammation and thereby increase insulin resistance and T2D (229).

ii) Bisphenol A. In cultured adipose cells derived from human subcutaneous tissue and 3T3-L1 adipocytes, exposure to 1 nM BPA decreased insulin sensitivity and glucose utilization and potentiated the release of proinflammatory molecules such as IL-6 and IFNγ (220). BPA at environmentally relevant picomolar and nanomolar levels decreased the release of adiponectin from human adipocytes (291). In the rat hepatoma cell line FaO, BPA at concentrations of 100 nM and above induced lipid accumulation by interfering with lipid oxidation and secretion (292).

The pancreatic β-cell is one of the main targets for environmentally relevant concentrations of BPA (54, 267). BPA can affect pancreatic β-cell function in two ways. The first is a very rapid closure of ATP-sensitive K^+ channels, potentiation of glucose-stimulated Ca^{2+} signals, and release of insulin via binding at extranuclear ERβ (169, 170, 293). This rapid action has also been demonstrated in human islets of Langerhans (293) and in glucagon-releasing pancreatic α-cells (294). The second way BPA can affect pancreatic β-cell function is by increasing glucose-induced insulin biosynthesis after binding to extranuclear ERα (143). Concentrations of BPA as low as 1 nM can trigger these actions, and the extranuclear ERα and ERβ sites of action described in the above studies were demonstrated using knockout mice lacking these receptors (293, 295). The direct effect of BPA in β-cells is thought to cause hyperinsulinemia in vivo (244). This hyperinsulinemia may be elicited to counteract the BPA-induced insulin resistance, or may be a direct action of BPA on β-cells causing hyperinsulinemia prior to insulin resistance, or both (293).

iii) Heavy metals. Arsenic is considered an EDC; at low dosages it can act upon hormone receptors, and at submicromolar and micromolar concentrations, arsenic blocked glucose-stimulated insulin secretion in isolated rat β-cells (296) and in murine islets of Langerhans, without affecting pancreatic insulin content (297). Other heavy metals such as inorganic mercury induced β-cell death (298), and cadmium impaired glucose-stimulated insulin secretion from pancreatic β-cells (299).

These and other actions of EDCs on β-cells and insulin sensitivity have been reviewed, and we refer readers to these additional papers (54, 267, 300–302). Further studies are still necessary in order to understand the direct effect of EDCs and the molecular mechanisms in key cell types involved in metabolism. Most studies have been centered in adipocytes and the endocrine pancreas, yet the roles of the liver, skeletal muscle, and brain, which are key tissues involved in metabolism, are still greatly unknown and deserve attention.

c. Animal models

i) Persistent organic pollutants. Evidence in adult animals shows that EDCs alter plasma insulin levels, insulin sensitivity, and glucose tolerance. Exposure of adult rats to POPs during 28 days worsened high-fat diet-induced insulin resistance and produced abdominal obesity as well as hepatic steatosis as already discussed (285). This same group reported that the presence of POPs in farmed salmon fillets together with a high-fat diet induced obesity and insulin resistance, glucose intolerance, and hyperinsulinemia in mice (303).

Perinatal exposure to perfluorooctane sulfonate (PFOS; 0.3 and 3 mg/kg/d) affected glucose metabolism in adult offspring, both at weaning and at 2 months of age

(304). Weanling males presented higher insulin levels at both doses, and at 2 months of age fasting serum glucose and insulin were elevated in animals on a normal diet or high-fat diet. Animals on a high-fat diet that were exposed to PFOS had diminished insulin sensitivity compared to the high-fat diet group. Perinatal exposure to environmentally relevant doses of DDT reduced energy expenditure and impaired thermogenesis, as already discussed in the Obesity section. This work also demonstrated an impaired glucose tolerance, particularly in animals exposed to DDT in utero and treated with a high-fat diet during adulthood (259).

ii) BPA and TBT. Much of the work conducted in this area has focused on BPA. BPA exerted both acute and long-term effects in male mice. Injections of BPA at 10 μg/kg/d rapidly (in minutes) increased plasma insulin levels and decreased glycemia (172). Longer exposures of mice to BPA at 100 μg/kg/d over 4–8 days resulted in reduced food intake, body temperature, and locomotor activity, together with hyperinsulinemia, insulin resistance, and glucose intolerance, without weight increase (172, 244). In Long Evans Tokushima Fatty rats (a breed that naturally develops metabolic syndrome at about 25 weeks of age), BPA exposure at 100 μg/kg/d by oral gavage throughout puberty accelerated the onset of glucose intolerance and hyperinsulinemia (305).

In the liver of adult male mice treated orally with a whole range of BPA doses (5, 50, 500, 5000 μg/kg/d) (306), no increase in weight was reported, and there was an increase of adiposity only at a concentration of 50 μg/kg/d. However, lower doses of BPA (5, 50, and 500 μg/kg/d) increased plasma insulin levels, hepatic mRNA, and protein expression related to lipid biosynthesis and increased lipid deposition. The study reported no effects at BPA levels at the NOAEL of 5 mg/kg/d. In a different study, BPA (50 μg/kg/d, orally) suppressed the activity of hepatic glucokinase (the glucose sensor of the liver) both acutely after 2 hours of exposure and after 2 weeks of chronic exposure (307). Because impaired hepatic glucose sensing is a risk factor for diabetes mellitus, this supports the diabetogen hypothesis. When administered chronically over 60 days, TBT decreased β-cell mass contributing to the disruption of glucose homeostasis (308). Thus, TBT is also a putative diabetogen.

Numerous studies have consistently reported that prenatal or perinatal exposure to EDCs results in alterations in glucose homeostasis. Prenatal exposure to BPA (10 μg/kg/d) provoked insulin resistance, hyperinsulinemia that likely developed to counteract the decrease in insulin sensitivity, and glucose intolerance without any change in weight in male offspring at 6 months of age. Neither females nor younger males (3 mo old) were affected (138). BPA (5 to 50 000 μg/kg/d) administered orally to pregnant mice on gestational days (G) 9–18 resulted in an altered glucose homeostasis phenotype in 3-month-old offspring (140). That study reported insulin resistance and decreased serum adiponectin at all doses tested, and glucose intolerance appeared at 5 and 5000 μg/kg/d. Hyperinsulinemia, likely a consequence of insulin resistance, was seen at the lowest dose of 5 μg/kg/d. An increase in weight was observed only at 500 μg/kg/d (140). Perinatal exposure to BPA (3.5 μg/kg/d) induced glucose intolerance in male offspring at 3 months of age, but not in female offspring (245). Metabolomics research has demonstrated that low doses of BPA disrupted global metabolism, including energy metabolism and brain function, in CD-1 mouse pups (309). Molecular epigenetic changes may provide a mechanistic explanation of how perinatal EDCs lead to insulin resistance observed in adulthood. The insulin resistance in offspring after perinatal exposure to BPA may be induced by abnormal DNA methylation in hepatic cells, which decreased glucokinase expression and increased PPARγ expression (310). Interestingly, multigenerational effects in glucose homeostasis have been observed in the F2 generation after exposure of F0 animals to BPA (40 μg/kg/d). These alterations may result from epigenetic methylation of the CpG island in the promoter of the glucokinase gene in the liver (311).

iii) Phthalates. Perinatal exposure to DEHP at doses of 1.25 and 6.25 mg/kg/d in rats produced important alterations in glucose homeostasis, which are gender- and age-dependent. By contrast, adipocyte size and body fat percentages were comparable to controls (312). That study also reported that 15-week-old female rats had increased fasting serum insulin, whereas glucose and insulin tolerance remained unchanged. Later in life, at 27 weeks of age, the females' fasting blood glucose levels were elevated, and insulin was decreased. The rats presented with impaired glucose tolerance and reduced insulin release in vivo. In males at 15 weeks, insulin levels were normal and glucose tolerance was improved. At 27 weeks, serum insulin was higher and glucose tolerance was unaltered. The study also reported disruptions of β-cell mass and function in both sexes at weaning and a strong decrease in insulin content, loss in β-cell mass, and abnormal β-cell ultrastructure at 27 weeks. This indicates that the endocrine pancreas may be a primary target for phthalates during development.

iv) EDC mixtures. Eating a high-fat diet together with a mixture of pollutants including DEHP, BPA, PCB153, and TCDD induced sex-specific metabolic alterations in offspring, but no weight gain (313). The study administered

each EDC at the tolerable daily intake reference dose, starting 5 weeks before pregnancy and continuing through gestation and lactation. This mixture of diet and chemicals in males produced an increase in the expression of hepatic genes related to cholesterol biosynthesis and a decreased hepatic total cholesterol level, whereas glucose tolerance was normal. By contrast, in females, glucose tolerance was greatly impaired together with a decrease in ERα expression and estrogen target genes. Notably, cholesterol metabolism in females remained unaltered. In addition to the alterations in metabolic endpoints, this study is interesting because it used a mixture of chemicals found in food at low doses, resembling nonexperimental conditions of exposure to a whole cocktail of chemicals rather than exposure to one EDC at a time.

D. EDCs and type 1 diabetes mellitus

The etiology of type 1 diabetes mellitus (T1D) is very different from T2D. It usually occurs at early ages, triggered by an autoimmune reaction accompanied with a profound inflammatory process that kills pancreatic β-cells (314). There is some commonality, however, between both types of diabetes. For instance, insulin resistance accelerates the progression to T1D in people with islet autoimmunity (315, 316). The incidence of T1D in children is growing inexplicably. Studies relating EDCs and other contaminants to T1D are beginning to emerge, although they are still very preliminary (317, 318).

In animals, BPA in drinking water increased insulitis (inflammation of the islet of Langerhans caused by the infiltration of lymphocytes) in mice and accelerated the onset of T1D in adult female nonobese diabetic mice (319). Transmaternal exposure to BPA during pregnancy also accelerated the incidence of T1D in female offspring (319). The relevance of these nonobese diabetic animals to humans is questionable because the action of other contaminants in nonobese diabetic mice does not translate well to humans (318). In conclusion, this is an important area that deserves further research and more studies in humans.

E. EDCs and cardiovascular diseases

The previous EDC Statement by the Endocrine Society mentioned an association between EDCs and CVD (1). The Statement discussed the possibility that EDCs acting as obesogens may increase the incidence of CVD because EDCs are linked to obesity. Likewise, any EDC acting as a diabetogen, inducing insulin resistance and/or dyslipidemia, enhances the risk of CVD. Nevertheless, since 2009 the scenario has changed. There are now new studies suggesting a direct link between EDCs and CVD, independently of those EDCs acting as obesogens or diabetogens.

1. Classes of EDCs associated with CVD

a. Persistent organic pollutants. Before 2008, epidemiological studies indicated an association between dioxin exposure and cardiovascular mortality, including ischemic heart disease. These studies are synthesized in a systematic review (320). It is important to note that in most of these results there was an absence of adjustment for other major risk factors for CVD, including smoking, lack of exercise, or alcohol consumption, which represents a major limitation. Organochlorine pesticides were associated with peripheral artery disease, particularly in obese people (321). Animal studies support a possible role for dioxins after binding AhR and inducing inflammation and atherosclerosis (322) and are indicative of TCDD-induced hypertension (323). There exists a clear association between prenatal p,p'-DDT and self-reported hypertension, as recently demonstrated in a longitudinal birth cohort study (324). This confirms previous associations between DDT exposure and hypertension (325).

b. BPA. There is evidence that BPA acts directly as a cardiovascular disruptor in rodents, independently of any obesogenic or diabetogenic effect. One study showed that low concentrations of BPA (1 nM) promoted arrhythmias in female rat hearts through a rapid alteration of Ca^{2+} handling in myocytes in an ERβ-dependent manner, verified in ERβ null mice (326). The authors also showed rapid action (in minutes) in isolated ventricular myocytes. Chronic exposure to BPA accelerated atherosclerotic lesions in the aorta and increased high-density lipoprotein cholesterol levels in apolipoprotein-E knockout mice, with no change in weight (327). Oral administration of BPA elicited hypertension in adult mice (328). In excised female rat hearts, BPA exposure (0.1–100 μM) slowed electrical contraction (329).

The first study that reported an epidemiological association between urinary BPA and T2D and CVD came out in 2008 (discussed above) (175). Two years later, using a similar approach with a larger NHANES data set, a study reported an association between urinary BPA and CVD, but not diabetes mellitus (281). Other recent studies have claimed a link between BPA exposure and the incidence of coronary heart disease (330) and high blood pressure (331). A new crossover intervention trial in humans demonstrated a rapid increase of blood pressure of about 4.5 mm Hg (332). Although the associations of repeated or chronic BPA exposure with CVDs still require further evaluation, this study shows the potential cardiovascular risk associated with BPA (332).

These studies are currently insufficient to establish a firm causality between EDC exposure and CVD. How-

ever, they should call attention to the need for more studies assessing whether EDCs act as cardiovascular disruptors.

F. Conclusions

The obesogen and diabetogen hypotheses propose that exposure to EDCs promotes the development of obesity, T2D, or both. Both cellular and animal models demonstrate a role for EDCs in the etiology of these two pathologies. For obesogens, animal studies show that EDC-induced weight gain depends on the timing of exposure and the age of the animals. Exposure during the perinatal period seems to trigger obesity later in life (after 3 to 6 mo of age). New results covering a whole range of EDC doses have pointed to the importance of nonmonotonic dose-response relationships; some doses induced weight increase, whereas others did not. Obesity is an important risk factor for T2D and CVDs, and therefore obesogens represent an indirect risk for these pathologies (Figure 3).

EDCs can also act as diabetogens, directly targeting β- and α-cells in the endocrine pancreas, adipocytes, and liver cells, and provoking insulin resistance together with hyperinsulinemia. These changes can also be associated with altered levels of adiponectin and leptin. Therefore, we can classify these EDCs as diabetogens that constitute a risk factor for T2D. This diabetogenic action is also a risk factor for CVDs and for obesity. Increased levels of insulin and leptin in plasma and decreased levels of adiponectin among other metabolic changes may disrupt energy balance and can lead to diet-induced obesity (333) (Figure 3). Although there are fewer articles on EDCs and cardiovascular disruption, evidence in humans and in animal models suggests that dioxins, DDTs, and plastic components such as BPA may directly target the cardiovascular system and cause atherosclerosis and/or hypertension.

PCBs, dioxins, brominated flame retardants, and organochlorine pesticides such as DDT are POPs. They are highly lipophilic and accumulate in the food chain and in body tissues. Phthalates and BPA are common chemicals, and although lipophilic, they accumulate less in body fat tissues. Nevertheless, because of their widespread use, they are found in the circulation of almost all individuals in the industrialized world. Because we are exposed to a changing mix of these EDCs throughout our lives, it is important to consider additive or even synergistic effects of mixtures. In real life, a mixture of EDCs will target different organs, exerting an integrated action that drives to metabolic alterations, which will increase susceptibility to obesity, T2D, and CVDs (Figure 3).

Epidemiological studies in humans also point to an association between EDC exposure and obesity and/or T2D. There are strong prospective studies supporting a serious link between chlorinated POPs and T2D. However, with regard to other EDCs, causality is based, for the moment, only on animal data because most epidemiological studies are cross-sectional, with diet as an important confounding factor (334). Therefore, although the weight of evidence in humans is adequate to suggest a possible link between EDCs and metabolic disorders, to establish a solid causality we need new studies, particularly with EDCs other than POPs, and including prospective intervention trials in controlled populations.

Box 1. Key Points: Obesity and Diabetes Mellitus

- **Disruption of glucose and lipid homeostasis is a risk factor for metabolic disorders including obesity and diabetes mellitus.**
- **BPA, phthalates, TBT, arsenic, PBDEs, PFOA, TCDD, PCBs, and DDTs are known to have effects on cellular and animal models.**
- **In animal models, prenatal and perinatal exposures to some EDCs disrupt the homeostatic control of adipogenesis and/or energy balance and induce obesity. A growing number of EDCs alter insulin production, secretion, and/or function, increasing the susceptibility for T2D. Some animal models suggest that EDCs have direct adverse effects on the cardiovascular system.**
- **A number of cross-sectional epidemiological studies associate EDC levels with obesity, diabetes mellitus, and CVDs in humans. There are important prospective studies associating exposure to POPs and T2D.**
- **Obesogenic and diabetogenic effects are induced in a nonmonotonic dose-dependent manner. Exposures to different levels produce diverse phenotypes.**
- **The molecular mechanisms involved are still largely unknown, but alteration of gene expression after binding to the AhR, PPARγ, and ERs seems to play a role.**
- **The interaction between EDC exposure and SNPs associated with obesity, T2D, and CVDs is a key issue for future studies.**

III. Female Reproductive Health

A. Introduction to EDCs and female reproduction

The female reproductive organs include the ovaries, oviducts/fallopian tubes, uterus, and vagina, and they are hormonally regulated by the hypothalamus and pituitary. The hypothalamic-pituitary-ovarian axis organs interact to fulfill the main

roles of the female reproductive system: to produce female sex hormones, produce female gametes, transport gametes to a site where sperm can fertilize them, and provide a favorable environment for the development and delivery of the fetus. The proper function of every organ in the female reproductive system is critical to the operation of these complex processes. Several studies indicate that EDCs can adversely affect the ovary, uterus, vagina, anterior pituitary, and/or steroid production, which can lead to reproductive disorders such as early puberty, infertility, abnormal cyclicity, premature ovarian failure/menopause, endometriosis, fibroids, and adverse pregnancy outcomes (59, 80, 335–345). Sections IIIB to IIIH below provide an update on studies published over the last 5 years that examine the impact of EDCs on female reproductive organs and reproductive function. A brief summary of the most common reproductive outcomes is provided in Table 4.

B. Effects of EDCs on the ovary

In the past 5 years, numerous studies have examined the effects of EDCs on ovarian development, primarily using animal models or in vitro systems. Collectively, these studies show that some EDC exposures adversely affect the developing ovary by interfering with germ cell nest breakdown, meiosis, and follicle formation and viability (Figure

Table 4. Summary of the Main Effects of EDCs on the Female Reproductive System

Organ or Condition	Category	BPA	Phthalates	Pesticides	Environmental Contaminants	DES
Ovary	Ovarian development			Decreased ovarian weight	Delayed ovarian development	
	Germ cell nests	Decreased germ cell nest breakdown				Decreased germ cell nest breakdown
	Atresia	Increased atresia	Increased atresia		Increased atresia	Increased atresia
	Oocytes	Increased number of multioocyte follicles, interference with meiosis	Decreased no. of viable oocytes		Decreased oocyte quality	
	Primordial follicles	Decreased number of primordial follicles	Increased primordial follicle recruitment	Increased activation of primordial follicles		
	Follicle growth	Decreased antral follicle growth	Decreased antral follicle growth	Decreased antral follicle growth	Decreased follicle growth	
	Steroidogenesis	Altered steroidogenesis	Altered steroidogenesis	Altered steroidogenesis	Decreased steroidogenesis	
	Gene expression	Altered gene expression	Altered gene expression	Altered gene expression	Altered gene expression	
Uterus	Structure	Development of endometrial-like structures		Altered uterine weight	Shorter fundi and uterine lengths, fewer uterine glands	
	Proliferation/hyperplasia/ carcinoma	Impaired proliferation				Endometrial hyperplasia, uterine adenocarcinoma
	Immune function	Increased immune responsiveness			Chronic active inflammation	
	Receptivity		Compromised uterine receptivity, decreased implantation sites	Decreased implantation sites		
	Gene expression	Altered gene expression			Altered gene expression	Altered gene expression
Vagina	Carcinoma					Carcinoma Altered gene expression
Anterior pituitary	Gonadotropins	Increased gonadotropin mRNA	Increased ability to produce gonadotropins	Altered gonadotropin release	Altered gonadotropin levels	Decreased LH-secreting gonadotropes
Reproductive cycles	Puberty			Altered vaginal opening	Altered onset of puberty	
Pathophysiological reproductive conditions	Fertility	Reduced fertility	Reduced fertility	Reduced fertility	Reduced fertility	
	Early menopause/premature reproductive failure	Early menopause/ premature ovarian failure	Early menopause	Early menopause	Early menopause	Early menopause
	Fibroids	Increased risk of fibroids	Increased risk of fibroids		Increased risk of fibroids	Increased risk of fibroids
	Endometriosis		Increased risk of endometriosis	Increased risk of endometriosis	Increased risk of endometriosis	
Pregnancy and birth outcomes	Adverse birth outcomes	Increased risk of adverse birth outcomes	Increased risk of adverse birth outcomes	Increased risk of adverse birth outcomes	Increased risk of adverse birth outcomes	

4). Information on the effects of specific EDCs on ovarian development follows.

1. Ovarian development

a. Bisphenol A. Currently, no information is available on the effects of BPA on ovarian development in humans. However, low-dose BPA increased the incidence of multioocyte follicles and altered the fetal ovarian steroidogenic gene and microRNA expression that mediate gonadal differentiation and folliculogenesis in sheep (128, 346). BPA also increased the incidence of unenclosed oocytes in macaques (347), inhibited germ cell nest breakdown in mice (348), and decreased the number of primordial follicles in CD-1 mice (218, 349; reviewed in Ref. 344).

BPA also interfered with ovarian development by affecting the onset of meiosis in the fetal ovary. For example, daily low-dose BPA exposure (<1 ng/mL in maternal serum) significantly disrupted synapsis and recombination between homologous chromosomes at the onset of meiosis in macaques (347). Similarly, gestational exposure to low-dose BPA altered the expression of genes that control meiosis in mice (*Stra8*, *Dazl*, *Nobox*) (350, 351). Furthermore, BPA exposure after the onset of meiosis induced meiotic delay at G17.5 in CD-1 mice (218).

Consistent with the results from in vivo studies, in vitro studies indicate that BPA (1–30 μM) impaired meiotic progression in cultured human fetal oocytes, increased levels of recombination (MLH1 foci), and induced epigenetic changes that may contribute to chromosome congression failure (352–354). Given the limited information on the effects of BPA on the developing ovary in humans, future studies should be designed to determine whether BPA adversely affects human ovarian development.

b. Phthalates. Currently, little information is available on the effects of phthalates on ovarian development in humans. One study showed that DEHP increased the expres-

Figure 4.

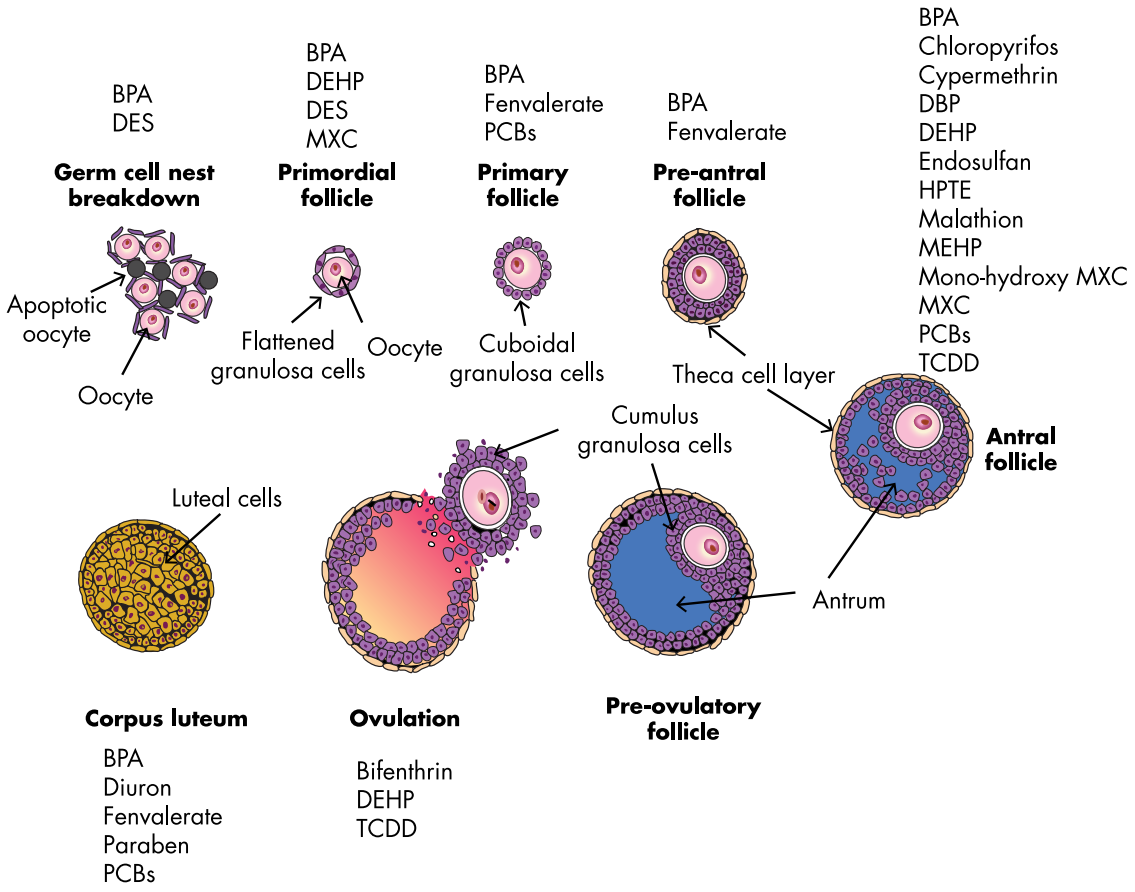


Figure 4. The effects of EDCs on the ovary. This schematic shows the normal developmental stages of ovarian follicles beginning with germ cell nest breakdown around birth, formation of primordial follicles, and their growth to primary follicles, preantral follicles, antral follicles, and finally, preovulatory follicles. This schematic also shows ovulation and the formation of the corpus luteum. Examples of EDCs that adversely affect the ovary are listed in red font above or below their likely site of action.

sion of liver X receptor α and sterol regulatory element binding protein members in the fetal human ovary (355). A few studies examined the effects of phthalates on the mouse ovary and showed that MEHP decreased the number of viable oocytes in fetal mouse ovaries, likely by dysregulating the expression of Cu-Zn superoxide dismutase (*Sod1*) and mitochondrial respiratory chain protein (*Nd1*) (356). Moreover, DEHP impaired primordial follicle assembly in cultured newborn mouse ovaries (357). Given the limited information on the effects of phthalates on the developing ovary in humans, future studies should be designed to determine whether phthalates adversely affect ovarian development in humans as well as animal models. Furthermore, considering that limited information is available on many phthalates, experimental studies should examine the effects of phthalates other than just DEHP and MEHP on the developing ovary.

c. Pesticides. As with BPA, no information is available on the effects of pesticides on the developing human ovary. However, animal studies showed that MXC increased the expression of a number of genes associated with cell death and primordial follicle activation in mice (358). It also activated primordial follicles and increased lipid peroxidation in neonatal mouse ovaries (358). Future studies should be conducted to determine the effects of other pesticides and their mechanisms of action on the developing ovary using both experimental and epidemiological approaches.

d. Environmental contaminants. Similar to the EDCs mentioned above, no information exists on the impact of environmental contaminants on the developing ovary in humans. In Wistar rats, however, exposure to TCDD during pregnancy delayed the development of the ovaries in the offspring (359). It also reduced the expression of steroidogenic acute regulatory (StAR) protein, a key steroidogenic enzyme, in fetal ovaries (359). Collectively, these few studies provide evidence that TCDD adversely affects the developing ovary in animals. Additional studies are required, however, to determine whether other environmental contaminants have similar effects on the developing ovary.

2. Effects of EDCs on the postnatal ovary

Many studies also show that EDCs affect the structure and/or function of the postnatal ovary (Figure 4). Information on the effects of specific EDCs on the structure/function of the postnatal ovary follows.

a. Bisphenol A. Although information on the effects of BPA on the postnatal human ovary is limited, animal studies consistently show that BPA adversely affected the postnatal ovary (344). Gestational BPA exposure accelerated follicle transition, decreasing primordial and increasing primary follicle numbers in lambs and rats (346, 360). Low-dose neonatal BPA exposure decreased the numbers of all follicle types and increased atretic follicles in rats during adulthood (361). High doses (100 000 and 300 000 μg/kg/d), but not low doses (2.5–2700 μg/kg/d), of BPA during prenatal life increased cystic follicles, depleted corpora lutea, and reduced antral follicle numbers in rats (362).

BPA exposure also adversely affected the postnatal ovary in vitro. BPA inhibited the growth of cultured mouse antral follicles (363–365), aberrantly up-regulated the expression of cell cycle regulators and pro- and antiatretic factors (364), and caused atresia (364). These effects of BPA on antral follicles were not mouse strain-specific because BPA inhibited follicle growth in antral follicles isolated from FVB, C57BL/6, and CD-1 mice, with CD-1 follicles being slightly more sensitive to BPA at early time points compared to the other strains (366).

The effects of BPA on the ovary may depend on which other chemicals are present in the ovary. Studies indicated that triclosan interacted with BPA, magnifying its presence in reproductive tissues in mice (367), and that the extract *Genista tinctoria L* ameliorated the toxic effects caused by BPA on rat ovaries (368).

Collectively, these previous studies provide strong evidence that BPA adversely affects the postnatal ovary by inhibiting follicle growth and/or increasing atresia/apoptosis. Further studies are required to determine the exact mechanisms by which BPA adversely affects the postnatal ovary. Studies are also needed to determine whether other plasticizers and their metabolites have similar effects to BPA on the postnatal ovary.

b. Phthalates. Like BPA, information on the effects of phthalates on the postnatal human ovary is limited. One study showed that benzyl butyl phthalate induced cell death in human granulosa cells via a mechanism that was AhR and CYP1B1 dependent (369). In in vivo experimental studies, DEHP induced apoptosis, a hallmark of atresia, in granulosa cells in adult ICR mice (370). Furthermore, DEHP accelerated primordial follicle recruitment in adult mice, likely through alterations in the phosphatidylinositol 3-kinase signaling pathway (371). DEHP, in combination with benzo[a]pyrene, increased atresia and decreased follicle numbers in rats (372).

In in vitro studies, both DEHP and MEHP inhibited follicle growth in mouse antral follicles (373), and MEHP inhibited follicular growth in cultured rat ovarian follicles (374). Furthermore, both DEHP and MEHP induced atre-

sia in cultured mouse antral follicles by mechanisms that included decreased expression of cell-cycle regulators and antiapoptotic regulators, as well as increased expression of proapoptotic factors and oxidative stress (375, 376). MEHP caused atresia in cultured mouse follicles and increased the expression of the proapoptotic gene *Aifm1*, but decreased the proapoptotic gene *Bok* and the antiapoptotic gene *Bcl2l10*. In addition, exogenous estradiol interfered with MEHP-induced changes in *Aifm1* and *Bcl2l10* in cultured mouse follicles, suggesting that MEHP-induced follicle atresia and changes in gene expression required a decrease in estradiol levels (377). Consistent with these results, MEHP caused apoptosis of rat granulosa cells (374), and DEHP caused apoptosis in mare cumulus cells by causing oxidative stress (378).

Another phthalate, DBP, inhibited follicle growth and induced atresia in isolated mouse antral follicles via mechanisms that included cell cycle arrest, changes in the expression of regulators of apoptosis, and the inhibition of estradiol production (379). Collectively, these previous studies provide evidence that phthalates adversely affect the postnatal ovary, but further studies are required to determine the exact mechanisms. Studies are also needed to determine whether other plasticizers and their metabolites have effects similar to phthalates on the postnatal ovary.

c. Pesticides. Although limited information is available on the effects of pesticides on the human postnatal ovary, animal studies clearly showed that MXC decreased ovarian weights and increased the incidence of cystic ovaries in rats (380, 381). Furthermore, MXC inhibited growth and induced atresia in mouse (382–387) and baboon antral follicles (388). The mechanism by which MXC inhibited antral follicle growth was mediated, in part, by the AhR pathway (382) and the ER pathway (389, 390). The mechanism by which MXC induced atresia involved alterations in factors that regulate apoptosis (eg, Bcl-2 and caspase) (385, 387, 391) and oxidative stress (383, 392, 393).

The major metabolite of MXC, 1,1,1-trichloro-2, 2-bis(4-hydroxyphenyl)ethane (HPTE), also inhibited growth and induced atresia in antral follicles from adult mouse ovaries (394). It is likely that this process involved ER pathways (394), as well as the altered expression of genes regulating signal transduction, transport, cell cycle, adhesion, differentiation, motility growth, apoptosis, development, and metabolism (395). Similarly, another metabolite of MXC, monohydroxy MXC, inhibited follicle growth and caused antral follicle atresia in mice (396).

Other pesticides also adversely affected the postnatal ovary in animal models. Both endosulfan and malathion decreased the number of healthy follicles and increased the number of atretic follicles in Wistar rats (397). Chlorpyrifos and endosulfan decreased viability and developmental competence in oocytes from buffalos (398). Cypermethrin increased atresia in rats (399), and daily carbamate pesticide exposure for 90 days decreased the number of small follicles and decreased fertility in albino mice (400). Imidacloprid decreased ovarian weight and increased atresia in female rats (401). Fenvalerate decreased ovarian weight and reduced the numbers of preantral follicles and corpora lutea in rats (402), and it inhibited follicle diameters in primary cultures of rat preantral follicles (403). Trifluralin exposure slightly increased cytoplasmic degeneration in oocytes (404). Bifenthrin inhibited LH-responsive ovulatory gene expression in rat granulosa cells, suggesting that it may increase the risk of ovulatory dysfunction (405). Long-term exposure to diuron reduced ovarian weight and corpora lutea numbers in Sprague-Dawley rats (406). 2,4,-Dichlorophenoxyacetic acid altered the activity of antioxidant enzymes and increased lipid peroxide concentrations in the ovaries of pre- and postnatal exposed rats (407).

Taken together, studies conducted during the past 5 years confirm previous studies that pesticides alter gene expression, impair follicle growth, increase atresia, and reduce oocyte quality in the postnatal ovary. Further studies are needed, however, to examine additional pesticides and to fully understand the impact of pesticide exposure on the human postnatal ovary.

d. Diethylstilbestrol. In the past 5 years, no new information became available on the effects of DES on the postnatal human ovary. Recent animal studies indicate that DES adversely affected the postnatal ovary. Neonatal exposure to DES inhibited germ cell nest breakdown (408) and caused the formation of polyovular follicles in mice, likely by interfering with the ERβ pathway and inhibiting programmed oocyte death and germ cell loss (409). It also reduced the primordial follicle pool and increased atresia in prepubertal lambs (346), and it caused polyovular ovaries in hamsters (410). Although these previous studies provide solid evidence that DES adversely affects ovarian structure in a variety of species, studies are needed to determine whether other synthetic estrogens adversely affect the ovary.

e. Environmental contaminants. In the previous 5 years, limited information was published on environmental contaminants and the human ovary. One study reported that chlorinated biphenyl levels in follicular fluid samples were associated with decreased fertilization rates and a lower chance of oocytes developing into high-quality embryos in women (411).

Several studies, however, show that environmental contaminants affect the postnatal ovary in animal models. For example, TCDD altered expression of the canonical clock genes, *Bmal1* and *Per2*, in adult mouse ovaries (412). It also reduced the number of cells in S-phase and inhibited the levels of *Cdk2* and *Cdk2* after pregnant mare serum gonadotropin treatment in immature rats, suggesting that TCDD inhibited ovulation by blocking cell cycle progression (413). Oral TCDD administration once a week for 29 weeks up-regulated the expression of selenium binding protein 2, glutathione S-transferase mu type 3, Lrpap1 protein, reduced nicotinamide adenine dinucleotide phosphate, and peptidylprolyl isomerase D, but inhibited the expression of prohibitin and N-ethylmaleide-sensitive factor in Sprague-Dawley rat ovaries (414). Oral TCDD treatment for 2 years caused chronic inflammation in rat ovaries (415). Furthermore, in utero low-dose oral exposure to TCDD induced the expression of inflammatory genes in rats (416). PCB congeners 77, 126, and 153 increased the expression of genes involved in oxytocin synthesis in bovine granulosa and luteal cells (ie, precursor-neurophysin-oxytocin in granulosa cells and peptidylglycine α-amidating mono-oxygenase) (417). A mixture of PCB congeners 101 and 118 reduced ovarian weight, reduced oocyte developmental capacity, and increased follicular atresia in CD-1 mice (418). Prenatal exposure to PCBs 118 and 153 increased the sum of secondary, early antral, and antral follicles, whereas prenatal exposure to PCB 153 increased primary follicles in lambs (419). PCBs 126, 77, and 153 interfered with the mobilization of intracellular calcium in bovine granulosa and luteal cells (420).

Taken together, studies conducted during the past 5 years confirm previous studies that environmental contaminants such as TCDD and PCBs alter gene expression, impair follicle growth, increase atresia, and reduce oocyte quality in the postnatal ovary. Additional studies are required to understand the mechanisms by which these EDCs affect the postnatal ovary. Additional studies are also required to determine whether other environmental contaminants affect the postnatal ovary in humans and animal models.

f. Other EDCs. A limited number of studies show that a variety of other EDCs adversely affect the postnatal ovary. The fungicide mancozeb inhibited proliferation but not migration in human and mouse granulosa cells in vitro (421). Paraben exposure during the juvenile-prepubertal period decreased the number of corpora lutea, increased the number of cystic follicles, and thinned the follicular epithelium in rats (422). Tributyltin induced the expression of apoptotic factors (TNFα and TNFR1), which may in turn induce apoptosis and result in a loss of ovarian function in rats (423). Finally, high levels of phytoestrogens interfered with adult ovarian function by inhibiting follicle growth and inducing atresia (424). Given that only a limited number of studies have examined the effects of EDCs such as mancozeb, parabens, tributyltin, and phytoestrogens on the ovary, future studies are needed to fully determine the impact of these chemicals on reproductive health.

g. Transgenerational effects of EDCs on the postnatal ovary. Interestingly, the effects of EDCs on the ovary may be transgenerational in nature because studies indicate that both fetal and neonatal exposure to MXC caused epigenetic alterations in ovarian genes in adults (80, 425, 426). Furthermore, developmental exposure to a pesticide mixture containing permethrin and N,N-diethyl-m-toluamide caused primordial follicle loss in the F3 generation of rats (427). Similarly, developmental exposure to TCDD caused primordial follicle loss in the F3 generation of rats (428). Although these studies provide some evidence that the effects of EDCs on the ovary may be transgenerational in nature, they are limited in scope because they have only been conducted in rodents and have focused on a limited number of chemicals. Thus, future studies should fully examine the transgenerational impact of EDCs on the ovary, taking into consideration various EDCs as well as species (ie, humans and rodents).

3. Effects of EDCs on ovarian steroidogenesis

The ovaries are the main producers of sex steroid hormones through a combined effort of the theca and granulosa cells in preantral and antral follicles. During steroidogenesis, cytochrome P450scc enzyme (also called CYP11A1) converts cholesterol (present in the mitochondria of theca cells) into pregnenolone. Steroidogenic enzymes (such as 3β-HSD, cytochrome P450 17α, and aromatase) convert pregnenolone into progesterone, androgens, and finally estrogens. Disruptions in hormone production could be due to direct effects of EDCs on either the granulosa or theca cells, or indirectly through the effects of EDCs on factors required to produce hormones, such as the steroidogenic enzymes. EDCs that interfere with enzymes or hormone intermediates in the steroidogenic pathway may cause abnormal levels of sex steroid hormones (Figure 5). This in turn may lead to reduced fertility/infertility, abnormal cyclicity, or a variety of other health problems because normal levels of sex steroids are required for normal bone, brain, and cardiovascular function. Sections a through d below provide information on the effects of specific EDCs on ovarian steroidogenesis.

Figure 5.

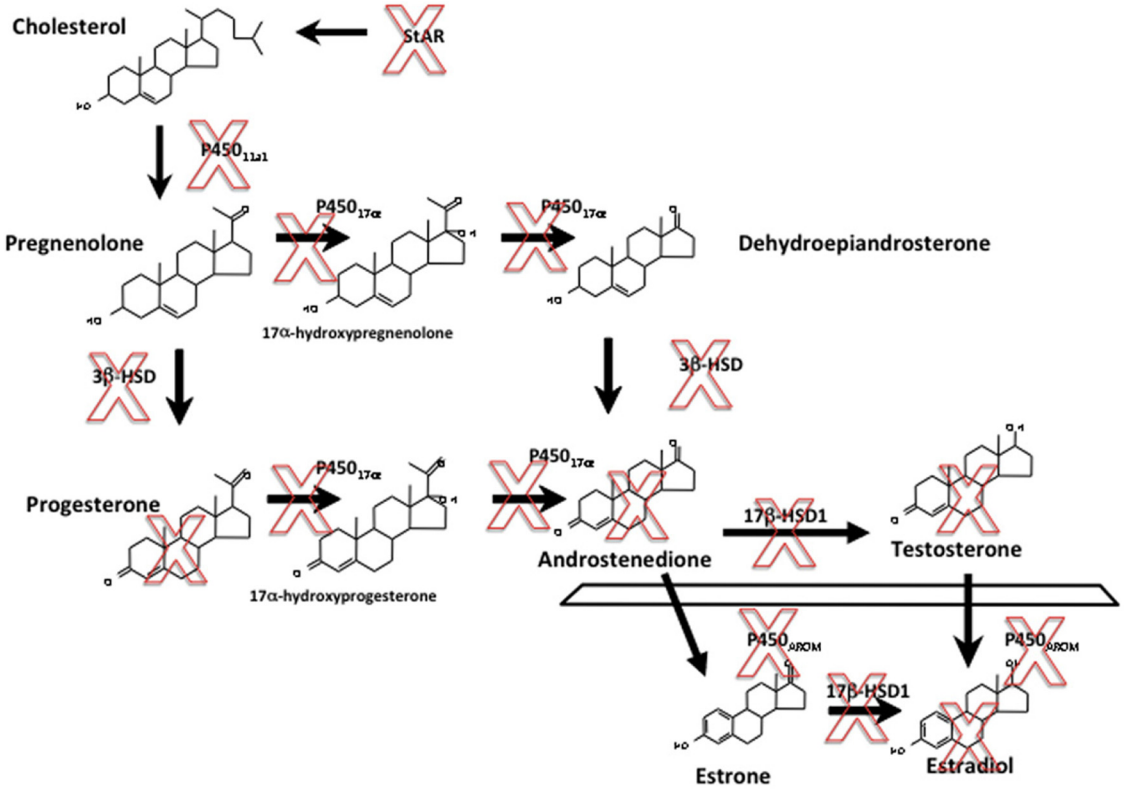


Figure 5. Steroidogenic pathways leading to estradiol biosynthesis. The red Xs indicate the hormones or enzymes that have been shown to be affected by EDCs. Each hormone in the pathway is derived from cholesterol. Reactions are catalyzed by steroidogenic enzymes as they metabolize each hormone to a different hormone down the pathway. Estradiol is the major hormone produced by antral follicles.

a. Bisphenol A. Human studies demonstrate relationships between BPA and in vitro fertilization (IVF) hormone levels. In one report, higher levels of BPA were associated with lower peak serum estradiol levels before oocyte retrieval in women undergoing IVF (429–431). Another study by Ehrlich et al (432) reported an association between urinary concentrations of BPA and CYP19 gene expression in a nonmonotonic manner in women undergoing IVF. Furthermore, BPA exposure was associated with increased levels of testosterone, estradiol, and pregnenolone in girls with precocious puberty (433).

In experimental studies, BPA also altered ovarian steroidogenesis, but the directionality of effects (increased or decreased) depended upon the species, age of exposure, and doses used in the studies (344, 345). In rats, prenatal exposure to BPA at high doses (100 000 and 300 000 μg/kg/d) increased serum estradiol levels and decreased serum progesterone levels (362). Perinatal (434) and postnatal (435, 436) low-dose BPA exposure increased serum estradiol levels as well as testosterone and progesterone levels in rodents. Low-dose BPA (below 0.1 mg/kg/d) decreased estradiol, testosterone, Cyp19 (aromatase), and StAR protein levels in adult rats (437).

Several in vitro studies confirmed the results of in vivo studies on BPA (344). BPA inhibited estradiol, testosterone, androstenedione, estrone, dehydroepiandrosterone, and progesterone production, and decreased *Star* and *Cyp11a1* expression in cultured intact antral follicles in murine models (363, 365, 438). The ability of BPA to inhibit steroidogenesis was seen in multiple strains of mice (CD-1, FVB, and C57BL/6) (366). Another study showed opposite effects of BPA (100 nM to 100 μM), increasing testosterone synthesis and *Cyp17a* (cytochrome P450 17α hydroxylase/lyase), *Cyp11a1*, and *Star* expression in isolated rat theca-interstitial cells (439). In addition, a study using porcine granulosa cells showed that 0.1 μM of BPA increased estradiol levels, whereas 1 and 10 μM BPA decreased estradiol levels, and that 0.1, 1, and 10 μM decreased progesterone levels (440). Collectively, studies on BPA using in vitro models, animal models, and epidemiological approaches consistently show that BPA exposure impaired steroidogenesis. Future studies should determine

the mechanisms by which BPA alters steroidogenesis and determine the reasons for differences between studies in the directionality of the effects of BPA.

b. Phthalates. Although studies in the past 5 years on phthalate exposure and steroidogenesis in women have been limited in number, they suggest that phthalate exposure may be associated with altered steroidogenesis in women. The Western Australian Pregnancy Cohort Study reported a negative association between phthalate metabolites and human maternal SHBG, but the association between phthalate metabolites and maternal androgens was inconsistent (441). Another human study showed that maternal urinary levels of MEHP and another phthalate, MEHHP, were negatively associated with both free testosterone and the ratio of free testosterone to estradiol measured in the cord serum from female infants (173).

Several in vivo studies in animals also indicated that phthalate exposure interfered with normal steroidogenesis. DEHP and benzo[a]pyrene exposure inhibited estradiol production in rats, likely due to reduced aromatase (372). Additional DEHP studies in rats showed that it lowered serum levels of progesterone and estradiol in 20-day-old females (442) and lowered sex hormone levels in adults (443), and that prenatal exposure inhibited estradiol levels in adult offspring (444). DEHP and MEHP inhibited the secretion of serum progesterone levels in adult mice, but the MEHP-induced decrease in progesterone was not due to the inhibition of aromatase (370). Moderate to high doses of MEHP during late gestation in mice caused high levels of estradiol and altered the expression of aromatase and StAR (445). A study in cycling ewes showed that DEHP increases plasma concentrations of progesterone (446).

Consistent with this in vivo work, in vitro studies confirm that phthalates can alter steroidogenesis, with most (but not all) studies showing phthalate-induced decreases in steroidogenesis. DEHP inhibited estradiol biosynthesis in cultured mouse antral follicles (373, 447). Similarly, MEHP inhibited estradiol levels in cultured mouse antral follicles by decreasing steroidogenic enzyme levels (447). In addition, DEHP significantly changed the production of progesterone by oocyte cumulus complexes in porcine ovaries (448). MEHP increased the inactivation of estradiol to estrone in cultured follicles in mice (449), and it inhibited the production of estradiol in human granulosa-lutein cells by reducing the expression of aromatase (450, 451). Similarly, MEHP inhibited androstenedione, testosterone, and estradiol levels in cultured rat follicles (374). Another phthalate, DBP, decreased the levels of estradiol produced by isolated mouse antral follicles (379). By contrast to the aforementioned studies showing inhibitory effects of phthalates on steroidogenesis, MEHP stimulated basal steroidogenesis in KK-1 granulosa tumor cells, likely by increasing the amount of cholesterol available for steroidogenesis (452).

Although these previous studies provide consistent evidence that DEHP and MEHP impair steroidogenesis in animal models, future studies are needed to fully understand the mechanisms by which this occurs. In addition, given that the effects of many phthalates on steroidogenesis have not been examined, future studies should focus on whether other phthalates impair steroidogenesis.

c. Pesticides. Limited recent information is available on the association between pesticide exposure and steroidogenesis in women. One study of 457 participants in Hawaii reported an association between heptachlor exposure and a longer luteal phase length and a drop in estradiol/progesterone metabolites after ovulation (453). Given the few studies on the association between pesticide exposure and steroidogenesis in women, future studies are needed to confirm these results.

Multiple studies consistently show that a variety of pesticides alter ovarian steroidogenesis in laboratory animals. For example, compared to vehicle, MXC inhibited the production of estradiol, testosterone, androstenedione, and progesterone in isolated mouse antral follicles (454). The effects of MXC on steroid levels likely stem from its ability to inhibit the expression of key factors in the estradiol biosynthesis pathway, including aromatase, 17β-HSD, 17α-hydroxylase/17,20-lyase, 3β-HSD, P450scc, and StAR protein, together with the ability of MXC to induce expression of Cyp1b1, an enzyme that metabolizes estradiol (454). The MXC metabolite, HPTE, inhibited CYP450-cholesterol side-chain cleavage activity, leading to decreased progesterone production by cultured rat ovarian follicular cells (455). The MXC metabolite, monohydroxy MXC, inhibited steroidogenesis by both reducing the availability of pregnenolone (396) and inhibiting the expression of Cyp11a1, Cyp17α1, and Cyp19 mRNA in mouse antral follicles in vitro (456).

Studies also show that pesticides other than MXC and its metabolites alter ovarian steroidogenesis. For example, cypermethrin inhibited the activity of 3β-HSD in rats (399) and inhibited luteal cell viability and progesterone secretion in the bovine corpus luteum cells (457). Fenvalerate inhibited StAR and P450scc gene expression, resulting in decreases in progesterone, testosterone, and estradiol production in primary cultures of rat preantral follicles (403). Furthermore, HCB inhibited testosterone and estradiol secretion, and pentachlorobenzene stimulated estradiol and testosterone secretion in porcine ovarian follicles (458). These disparate effects may be due to

differential actions on steroidogenic enzymes because HCB inhibited Cyp17α, 17β-HSD, and CYP16 expression, whereas pentachlorobenzene stimulated Cyp17α and Cyp19, but did not affect 17β-HSD expression (458). Bifenthrin decreased progesterone expression, likely by reducing the expression of key steroidogenic enzymes (eg, P450scc, StAR) in rat ovarian granulosa cells (459). ATR increased progesterone secretion from newly formed corpora lutea by increasing steroidogenic factors (eg, StAR, Cyp450ssc, 3β-HSD) and down-regulating the luteolytic gene, 20α-HSD, in rat ovarian granulosa cells (460). ATR also increased the estrogen-to-androgen ratio in rats by increasing levels of aromatase (461), and it increased progesterone and estradiol production and aromatase activity in primary rat granulosa cells (462). Furthermore, ATR disrupted steroidogenesis in swine granulosa cells (463).

The effects of ATR on steroidogenesis, however, appear to differ with age, dose, and experimental model. Fa et al (464) showed that ATR decreased steroidogenic enzyme expression and estradiol levels in immature rat granulosa cells in vitro. This contrasts with data from in vivo studies in adult animals showing that various doses of ATR increased steroidogenic enzymes and sex steroid hormone levels (460–463). Taken together, studies in the previous 5 years consistently show that pesticides impair steroidogenesis in a variety of animal models, although outcomes may differ depending on species, dose, and timing of exposure. Future studies should determine whether pesticide exposures are also associated with altered steroidogenesis in women.

d. Environmental contaminants. A human study showed that high concentrations of PCDDs/PCDFs and PCBs are linked to lowered estradiol levels in a cohort of 33 girls (465). To our knowledge, this is the only such study conducted in humans. Experimental studies consistently show that environmental contaminants alter ovarian steroidogenesis in a variety of systems. For example, TCDD exposure decreased estradiol in both virgin and multiparous mice (466). Oral administration of TCDD weekly for 29 weeks reduced estradiol levels in Sprague-Dawley rats (414), inhibited estradiol production in female mice (466), and inhibited estradiol biosynthesis in isolated mouse antral follicles (467), likely by reducing the expression of key enzymes (eg, 17β-HSD) in the estrogen biosynthesis pathway (468). TCDD reduced serum testosterone levels and the ratio of testosterone to estradiol in juvenile mice (469), and TCDD decreased estradiol levels in chicken ovarian follicles (470) as well as chicken ovaries (471). Taken together, studies on environmental contaminants in the past 5 years confirm previous work indicating that environmental contaminants such as TCDD and PCBs impair steroidogenesis in a variety of animal models. Given the limited information on the association between environmental contaminants and steroid levels in humans, further studies are needed to fully understand the association between environmental contaminant exposure and steroid hormone levels in girls and women.

C. Effects of EDCs on uterine structure and function

The uterus is a muscular organ comprised of a fundus, a corpus, and a narrow caudal portion called the cervix. The functional portion of the uterus is endometrial tissue — complex glandular tissue and stroma responsible for creating a healthy environment for embryo implantation and sustained pregnancy. EDCs that affect uterine responses to hormones and/or uterine gross morphology could impair normal uterine functions (such as embryo implantation) and lead to adverse birth outcomes. Sections 1a through 1e describe the effects of specific EDCs on the uterus.

1. EDCs that affect the uterus

a. Bisphenol A. Although the effect of BPA on the structure/function in the human uterus is unknown, several studies indicate that BPA affects uterine structure/function in animal models (Figure 6) (344, 345). Gestational and neonatal exposure to BPA caused development of endometrial-like structures with glands and stroma in adipose tissue surrounding the genital tract in adult female Balb-c mice (472, 473). Parental dietary exposure to BPA increased uterine weight in the F1 offspring in CD-1 mice (474), and lactational exposure to BPA increased uterine weight in neonatal rats (475). Furthermore, gestational exposure to BPA did not affect the morphological appearance of the fetal uterus, but it altered the expression of genes (eg, HOXA13, WNT4, and WNT5A) that may regulate uterine function in later life in rhesus macaques (476). Gestational BPA exposure also increased expression of Hoxa10 in adult CD-1 and ICR mice (100), and postnatal exposure to BPA decreased estradiol-induced progesterone receptor (PR) expression in African green monkeys (477). BPA exposure also impaired proliferation of uterine cells both in vivo and in vitro (100, 478–483), increased the immune responsiveness of the uterus in C57BL/6 mice in vivo (484), and exerted estrogenic gene expression profiles in ovariectomized (OVX) mice (485). In cultured primary heterogeneous populations of uterine cells, BPA significantly inhibited cell contractions, increased oxytocin-related pathways, and decreased prostaglandin-related signaling (486).

Some of the effects of BPA on the uterus may be transgenerational in nature. Hiyama et al (487) showed that prenatal exposure to BPA altered the methylation

Figure 6.

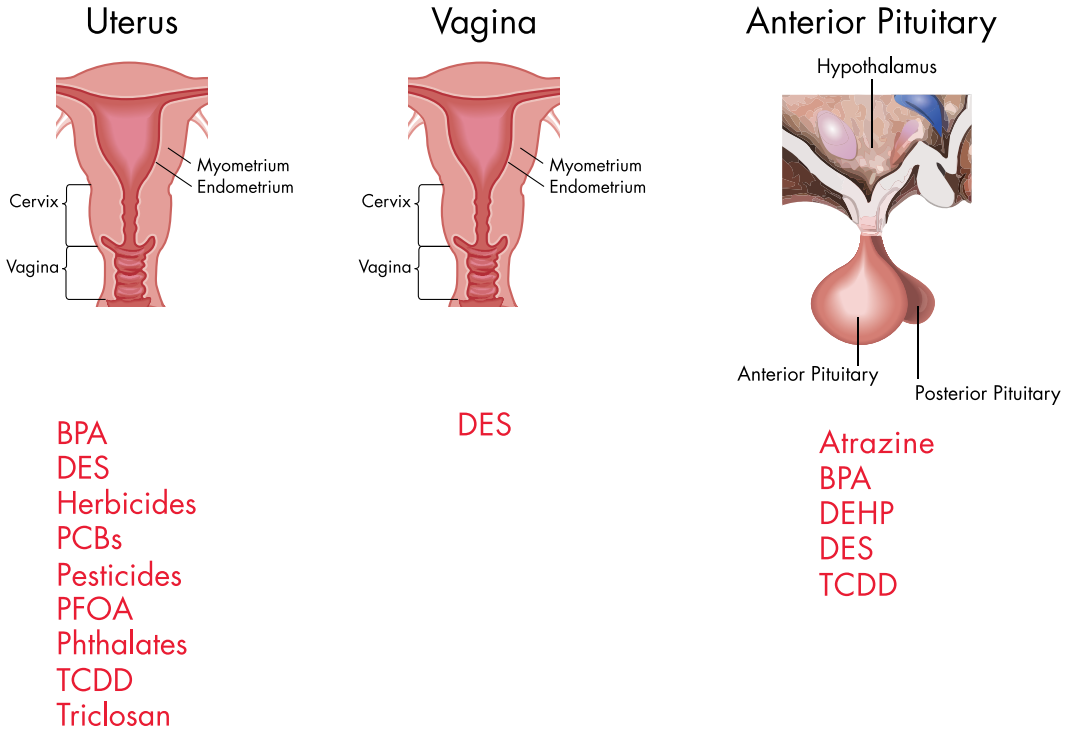


Figure 6. The effects of EDCs on the uterus, vagina, and anterior pituitary gland. This schematic shows the normal structure of the uterus, vagina, and anterior pituitary, with a list of EDCs (in red text) that have been shown to perturb the development and function of these structures.

pattern of Hoxa10 in the F2 generation of mice. However, future studies need to determine whether the altered methylation pattern persists into the F3 generation and how the altered methylation pattern affects the function of the uterus.

Some of the effects of BPA on the uterus may be mediated by BPA metabolites and not BPA itself. Okuda et al (488) showed the BPA metabolite, 4-methyl-2,4,bis(4-hydroxyphenyl)pent-1-ene (mono-BPA), has potent estrogenic activity in terms of effects on uterine weight, myometrial thickness, and luminal epithelial cell height in OVX rats, but that BPA does not elicit the same estrogenic responses. Furthermore, they estimated that mono-BPA was about 500-fold more potent than BPA in the OVX rat uterus (488). Future studies are required to determine whether BPA and its metabolites alter uterine function in women as well as other species.

b. Phthalates. Phthalate exposure has been associated with uterine structure/function in one human study (Figure 6). In the Western Australian Pregnancy Cohort Study, concentrations of mono-(carboxy-iso-octyl) phthalate were positively associated with uterine volume (441).

In pregnant mice, DEHP compromised endometrial receptivity and decreased implantation sites by disrupting the MAPK and nuclear factor-κB signaling pathways (489). Dibenzyl phthalate inhibited estradiol-induced uterine growth in immature mouse uterotrophic assays (490). DEHP and MEHP stimulated the secretion of prostaglandin F2α and inhibited the secretion of prostaglandin E2 in cultured bovine endometrial cells (491).

It is important to note that not all studies on phthalate exposure showed an impact on the uterus. Polypropylene and polyethylene terephthalate did not increase uterine weight in Sprague-Dawley rats (492). Similarly, exposure to plastic samples eluted from plastic food containers did not result in differences in uterine weight between controls and treated groups of immature Sprague-Dawley rats (492). Future studies should be conducted to examine in detail the association between phthalate exposure and uterine outcomes in women and to determine the mechanisms by which phthalates impair uterine structure/function in animal models.

c. Pesticides. Although recent studies have not examined the effect of pesticides on the structure/function of the human uterus, several studies indicated that pesticides disrupted uterine structure and/or function in animal models (Figure 6). MXC increased uterine weight in nonpregnant rats and decreased the number of implantation sites in pregnant

rats (380). The MXC metabolite HPTE induced estrogen gene expression profiles in OVX mice (485). Furthermore, a mixture of organophosphorus pesticides (dichlorvos, dimethoate, and malathion) increased uterine weight in Sprague-Dawley rats (493). In immature rats, mirex reduced uterine weight, whereas Aroclor 1221 and endosulfan increased uterine weight, suggesting that Aroclor 1221 and endosulfan are estrogenic in the immature uterotrophic assay (494). Fenvalerate increased resorption sites in rats (402). DDT and hexachlorocyclohexane (HCH) stimulated the contraction of myometrial uterine strips; this may enhance the risk of abortions in pregnant females (495). Benomyl and carbendazim inhibited cell proliferation in human endometrial cells (496). Expression profiling studies have shown that ethinyl estradiol and o,p-DDT exhibit similar temporal profiles, suggesting that o,p-DDT elicits estrogenic responses in uteri from immature OVX mice and rats (497). Low doses of endosulfan disrupted the expression of proteins that regulate uterine development and differentiation (ERα, Hoxa10, and α-SMA) in rats (498). Furthermore, the herbicide pendimethalin increased uterine weight and up-regulated expression of ERβ in rats (499). It is important to note, however, that not all studies showed that pesticides, insecticides, or herbicides affected uterine structure and function. For example, pyrethroid metabolites did not affect uterine weight in Sprague-Dawley rats (500). Further studies are required to fully determine which pesticides adversely affect uterine structure and function.

d. Diethylstilbestrol. Synthetic estrogens are well known disruptors of uterine structure and function in humans and animals (Figure 6). Consistent with previous studies (102, 103), recent data indicate that neonatal DES exposure caused endometrial hyperplasia/dysplasia in hamsters (410) and increased uterine adenocarcinoma and uterine abnormalities in Donryu rats (501). Neonatal DES exposure also caused the differential expression of 900 genes in one or both layers of the uterus (502). Specifically, DES altered multiple factors in the PPARγ pathway that regulate adipogenesis and lipid metabolism, and it perturbed glucose homeostasis, suggesting that DES affects energy metabolism in the uterus (502). In the mouse uterus, DES altered the expression of chromatin-modifying proteins (503) and Wnt signaling pathway members (504), caused epigenetic changes in the sine oculis homeobox 1 gene (503), and decreased the expression of angiogenic factors (505). DES also altered the expression of genes commonly involved in metabolism (506) or endometrial cancer in mice (507), and it activated nongenomic signaling in uterine myometrial cells (508) and increased the incidence of cystic glands in rats (509).

e. Environmental contaminants. Only one study has been conducted in humans on environmental contaminants and uterine structure/function. This report indicated that high levels of PCBs were associated with shorter fundi and uteri lengths in a study of 33 girls (465).

Several experimental studies are consistent with the human study in that they show that industrial environmental contaminants affect uterine structure and function (Figure 6). Polychlorinated dibenzo-p-dioxins and furans and PCB-153 caused chronic active inflammation of the uterus, and 92 polychlorinated dibenzo-p-dioxins and furans increased endometrial cystic hyperplasia in Sprague-Dawley rats (415). PCB77 stimulated the force of myometrial contractions by increasing myometrial synthesis of PGF2α in cattle (510). Exposure to TCDD at G15 caused fewer uterine glands, morphological anomalies, and decreased expression of genes involved in fluid transport (Aqp3 and Aqp5), cytoarchitecture (Dsc2 and Sprr2A), and immune regulation (Lcn2 and Ltf) in mice at 9 weeks in response to hormone treatment (511). These findings persisted, although the half-life of TCDD in mice is 7–11 days, and the mice were cross-fostered at birth to avoid TCDD exposure through lactation (511). Furthermore, TCDD exposure induced Cyp1A1 expression and inhibited β-catenin and E-cadherin in trophoblastic endometrial epithelial cells in vitro, suggesting that TCDD modulates the Wnt-signaling pathway (512). Acute TCDD exposure disrupted cannabinoid signaling in human endometrial biopsy samples (513). Future studies should further examine the mechanisms by which environmental contaminants adversely affect the uterus. In addition, future studies are required to fully understand which environmental contaminants pose a risk to uterine health in women.

f. Other EDCs. A variety of other EDCs may affect uterine structure and function (Figure 6). The antibacterial agent triclosan enhanced uterine responses to ethinyl estradiol and increased the ethinyl estradiol-induced stimulation of epithelial cell height in rats (514). PFOA at low doses increased absolute and relative uterine weights, but did not antagonize the histopathological effects of estradiol in CD-1 mice (515). In contrast, paraben exposure did not alter the number of implantation sites or uterine mass in CF-1 mice (516). Given the relatively few studies on the effects of triclosan, PFOA, and parabens on uterine structure and function, future studies should be conducted to examine the effects of triclosan, PFOA, and parabens on the uterus. Such studies should examine a variety of species and obtain detailed dose-response and time-course information.

D. Effects of EDCs on the vagina

Only a limited number of studies assessed the effects of EDCs on the vagina, and of these, all but one on phthalates focused on DES (Figure 6). A recent study of women showed an association between in utero exposure to DES and clear cell carcinoma of the vagina (517), confirming previous findings. Furthermore, DES disrupted the expression of transformation-related protein 63, which makes cell fate decisions of Müllerian duct epithelium and induces adenosis lesions in the cervix and vagina in women (518).

Studies in mice showed that DES induced vaginal adenosis by down-regulating RUNX1, which inhibits the BMP4/activin A-regulated vaginal cell fate decision (519); induced epithelial cell proliferation and inhibited stromal cell proliferation (520); and caused persistent down-regulation of basic-helix-loop-helix transcription factor expression (Hes1, Hey1, Heyl) in the vagina, leading to estrogen-independent epithelial cell proliferation (521, 522). Neonatal exposure to DES caused persistent changes in expression of IGF-1 and its downstream signaling factors in mouse vaginas (521). It also up-regulated Wnt4, a factor correlated with the stratification of epithelial cells, in mouse vaginas (521). Interestingly, the simultaneous administration of vitamin D attenuated the ability of DES to cause hyperplasia of the vagina in neonatal mice (523).

In the one study in the previous 5 years that did not focus on DES, polypropylene and polyethylene terephthalate did not increase vaginal weight in Sprague-Dawley rats (492). Although a few studies have been conducted during the previous 5 years on the effects of EDCs on the vagina, such studies are very few in number, small in scope, and focused on DES. Thus, future studies are needed in this largely understudied area before we fully appreciate whether other EDCs impair the vagina.

E. Effects of EDCs on the anterior pituitary gland

Female reproductive function is driven by signals, many of which initiate from the hypothalamus, are propagated by the gonadotropes of the anterior pituitary gland, and ultimately play out as actions on the ovary, uterus, and other parts of the reproductive tract. In response to the hypothalamic neuropeptide GnRH, as well as to proteins and steroids produced by the ovary, the anterior pituitary synthesizes and secretes the gonadotropins LH and FSH, which control follicle growth, ovulation, steroidogenesis, and cyclicity. The effects of EDCs on hypothalamic-pituitary neuroendocrine function are covered extensively in *Section VIII*, and thus only brief coverage of the anterior pituitary gland will be given here. Sections 1a through 1e provide information on the effects of specific EDCs on the anterior pituitary.

1. EDCs that affect the pituitary

a. Bisphenol A. To our knowledge, no information is available on the effects of BPA on the human anterior pituitary gland. Animal studies, however, show that gestational exposure to BPA increased mRNA levels of the gonadotropins, but not other pituitary hormones in mice (524), and BPA exposure during the perinatal and postnatal periods increased FSH mRNA levels in mice (434). Neonatal exposure to BPA lowered basal and GnRH-induced LH levels and increased GnRH pulsatility in adult rats, suggesting that neonatal exposure to BPA permanently affects GnRH pulsatility and pituitary GnRH signaling (525). Furthermore, BPA reduced the percentage of cells that responded to estradiol via calcium channel opening and inhibited the activation of ERKs in a nonmonotonic dose-response manner in cultured GH3/B6/F10 rat pituitary cells (526). Taken together, studies in the previous 5 years indicate that BPA adversely affects the anterior pituitary in animal models. However, limited information is available on its mechanisms of action in animal models and its effects in humans. Future studies should focus on the mechanisms by which BPA affects the pituitary and explore whether other EDCs have similar effects.

b. Phthalates. To our knowledge, no information is available on the effects of phthalates on the human anterior pituitary. A few experimental studies, however, indicate that phthalates alter anterior pituitary function (Figure 6). DEHP increased the ability of primary cultures of pituitary cells to produce and secrete LH in response to GnRH, suggesting that DEHP stimulates hormonal function (442). DEHP also increased gonadotropin levels in prepubertal rats (527). Given the limited number of studies, future studies should examine the effects of phthalates on the anterior pituitary using both in vitro and in vivo models.

c. Pesticides. As with BPA and phthalates, no information is available on the effects of pesticides on the anterior pituitary in humans. Studies in animals, however, showed that the pesticide ATR adversely affected the function of the anterior pituitary (Figure 6). ATR both activated the pituitary release of hormones (528) and inhibited LH release from the pituitary via a mechanism that includes effects on the LH pulse generator through alterations in adrenal hormone secretion (529, 530). Given the limited number of studies, future studies should examine the effects of pesticides on the anterior pituitary using both in vitro and in vivo models.

d. Diethylstilbestrol. Synthetic estrogens affect the anterior pituitary, although research in this arena is largely focused on DES in animal models (Figure 6). Neonatal DES exposure decreased the percentage of LH-secreting gonadotropes in neonatal mice (531), and it also decreased LHβ in 3-month-old mice (532). Interestingly, whereas DES reduced the gene expression of LH-secreting gonadotropes in vitro, it did not affect their numbers (501). Future research is needed to determine whether DES, as well as other synthetic estrogens, affects the anterior pituitary in humans and animal models.

e. Environmental contaminants. Evidence for the effects of the environmental contaminant TCDD on the anterior pituitary in humans is lacking, and evidence for the effects of TCDD in animal models is equivocal. Gestational TCDD exposures reduced the expression of pituitary gonadotropins in rodents (533–536), increased LH secretion by pituitary cells during the follicular phase in pigs (537), and decreased LH and FSH levels in virgin female C57BL/6J mice (466). By contrast, in cultured pituitary cells, TCDD did not affect the expression of gonadotropin mRNAs in the absence of GnRH, but it inhibited the synthesis of gonadotropin β-subunit mRNAs in the presence of GnRH (359). TCDD also up-regulated known AhR target genes (Cyp1a1 and Cyp1b1) in the rat pituitary gland, but the physiological consequences of this up-regulation are unclear (538). Given the equivocal nature of the findings on the effects of TCDD on the anterior pituitary, studies should be conducted to elucidate the reasons for discrepancies.

f. Other EDCs. To date, only one study has examined the impact of other EDCs on the anterior pituitary. This study showed that a mixture of endocrine disruptors (13 chemicals including phthalates, pesticides, UV filters, BPA, parabens, and paracetamol) given from G7 to postnatal day (P) 22 increased the incidence of pituitary adenoma in rats (539). However, further studies are required to determine the mechanisms by which the mixture causes pituitary adenoma and whether other mixtures adversely affect the anterior pituitary.

F. Female reproductive cycles

1. Effects on puberty

a. Bisphenol A. Research on BPA and puberty has produced inconsistent results in epidemiological studies (reviewed in Refs. 344 and 345). In one epidemiological study, BPA levels were associated with idiopathic central precocious puberty in Turkish girls (540), but in another study BPA levels were not associated with precocious puberty in girls (433). In a study of 1151 girls ages 6 to 8 years and a study of 192 girls age 9, BPA exposure was not associated with accelerated breast or pubic hair development (541, 542). Similarly, a study of 82 patients with precocious puberty and 32 patients without precocious puberty did not show an association between BPA and precocious puberty (543).

The data on BPA and puberty in animal models are also equivocal. Some studies show that BPA did not affect vaginal opening in gestationally, neonatally, and orally exposed Long Evans rats (544) or in lactationally exposed rats (475), and BPA did not affect the onset of puberty in CD-1 mice (474). However, other studies in neonatally exposed ICR mice, Sprague-Dawley rats, and Long Evans rats showed that BPA accelerated vaginal opening (525, 545, 546). Given the disparate findings in both epidemiological and experimental studies, future studies should be designed to determine whether and how BPA exposure impacts puberty.

b. Phthalates. Studies on phthalate exposure and puberty in humans are also equivocal (547). The Western Australian Pregnancy Cohort Study showed an association between the sum of all DEHP metabolites and a nonsignificant tendency toward an early age at menarche ($P = .069$) (441). Furthermore, a review by Jurewicz and Hanke (548) reported an association between urinary levels of phthalates and pubertal gynecomastia, and between serum levels of phthalates and premature thelarche and precocious puberty in girls. Similarly, in a case-control study of 104 girls in Taiwan, urinary phthalate metabolites were significantly higher in girls with precocious puberty (185). In a multiethnic longitudinal study of 1151 girls in the United States (New York City, Cincinnati, northern California), high-molecular-weight phthalate metabolites were borderline associated with pubic hair development, and high-molecular-weight phthalates were inversely associated with hair stage (541). Furthermore, girls with precocious puberty had higher levels of kisspeptin, suggesting that phthalates may promote female puberty by increasing kisspeptin activity (549). However, other studies suggested that phthalate exposure may be associated with delayed puberty. In a multiethnic study of 1239 girls from New York City, Cincinnati, and San Francisco, urinary concentrations of DEHP metabolites were associated with later pubic hair development, and monobutyl phthalate (MBP) levels were associated with older age at first breast development (550). A Danish study reported that the highest levels of MBP, MBzP, DEHP metabolites, and diisononyl phthalate (DINP) metabolites were associated with delayed age at puberty (551).

In contrast, a few studies did not find an association between plasticizer exposure and puberty in humans. In a multicenter cross-sectional study of 28 girls with central precocious puberty and 28 prepubertal controls, levels of nine phthalate metabolites were similar (552). A longitudinal study of 168 healthy girls examined every 6 months for 5 years did not show an association between phthalate exposure and age at pubertal milestones (553).

Recent animal studies on the potential impact of phthalate exposure on puberty provide interesting but equivocal data. Some studies showed that DBP and butyl benzyl phthalate (BBP) exposure did not affect vaginal opening in rats (554), whereas others show that DBP exposure induced earlier pubertal timing in female Sprague-Dawley rats (555), or that it delayed vaginal opening and completely blocked vaginal opening at high doses (750 and 1000 mg/kg/d) in Wistar rats (556). The differences in the results could be due to differences in dose, timing of dose, and strain. Given the equivocal nature of the findings in both epidemiological and experimental studies, future studies should be designed to determine whether and how phthalate exposure impacts puberty.

c. Pesticides. The existing human data on pesticide exposure and puberty are limited (557). A small study of 45 girls living in the Menderes region in Turkey did not show an association between pesticide levels and precocious puberty, but the authors suggested that pesticide exposure may be associated with obesity and that obesity may be the underlying cause for precocious puberty (558). The study was limited because most pesticides, except 4,4′ DDE, were undetectable in the samples. Interestingly, the authors reported that the long axis of the uterus and ovaries was different in girls with detectable levels of 4,4-DDE compared to those without detectable levels of DDE (558). In a small study of 78 children with idiopathic precocious puberty and 100 control children, the levels of p,p,-DDE did not differ between children with precocious puberty and normal age at puberty (559).

The animal studies on pesticides and puberty are inconsistent with the human studies in that the animal studies showed that several herbicides/pesticides altered puberty. A high dose of ATR during prenatal life delayed vaginal opening in Sprague-Dawley rats (560). Similarly, exposure to simazine for 21 days delayed vaginal opening, decreased the number of estrous cycles, and delayed the first day of estrus in Wistar rats (561). Interestingly, exposure to simazine for a longer period of time (41 d) delayed vaginal opening and the time of the first estrus but did not alter the number of normal estrous cycles (561). By contrast to these effects of ATR and simazine on the retardation of the pubertal process, neonatal exposure to the herbicide acetochlor accelerated vaginal opening and caused irregular cyclicity in female rats (562). In contrast, exposure to pyrethroid metabolites did not affect the onset of puberty in rats (500). Given the limited data in humans, future studies are needed to determine whether pesticide exposure is associated with age at puberty in girls. Furthermore, experimental studies are required to determine whether and how other pesticides adversely affect puberty.

d. Environmental contaminants. Although reports on environmental contaminants and puberty in humans are lacking, environmental contaminants may influence puberty in animals. For example, gestational exposure to TCDD promoted early-onset puberty in the F3 generation of rats (563). Tributyltin exposure from G6 through lactation advanced the onset of vaginal opening and the first vaginal estrus in mice (564). Firemaster 550, a fire-retardant mixture used in foam-based products and a common containment in house dust, advanced puberty in female rats (565). In one study, exposure to PCBs during pregnancy and lactation caused early onset of puberty but did not cause cycle irregularity in rats (566). By contrast, another study showed that prenatal exposure of rats to an estrogenic mixture of PCBs, Aroclor 1221, advanced the timing of vaginal opening and increased the percentage of animals with irregular estrous cycles (567). Given the limited data in humans, future studies are needed to determine whether environmental contaminant exposure is associated with age at puberty in girls. Furthermore, experimental studies are required to determine whether and how other environmental contaminants affect puberty.

e. Perfluorooctanoic acid. Studies in humans indicate that PFOA may be associated with the timing of puberty (568, 569). PFOA was measured in blood collected from Danish women during their pregnancies (568). Their daughters (n = 343) then completed questionnaires at age 20 that assessed the timing of menses. PFOA is known to cross the placental barrier; therefore, the study used maternal levels of the chemicals as a proxy for prenatal exposure. Daughters with the highest levels of prenatal PFOA exposure reported having their first menstrual period 5.3 months later than daughters exposed prenatally to lower levels of PFOA (568). Similarly, in a 2005–2006 survey of residents with PFOA water contamination from the mid-Ohio Valley in the United States, higher serum concentrations of PFOA or a related compound, PFOS, were associated with later age of sexual maturation in 2931 girls aged 9–18 years (130-d delay for PFOA and 138-d delay for PFOS exposure) (569).

In contrast, experimental studies suggest that PFOA may not affect puberty in animal models. For example, in CD-1 mice, prenatal PFOA exposure (at doses that recapitulate serum concentrations reported in females) did not affect vaginal opening or time to first estrus, but it altered mammary gland development (570). The reasons for the discrepancies between the human and animal studies are unclear. It is possible that humans and mice have different sensitivities to PFOA, and this should be examined in future studies.

f. Other EDCs. A few studies indicate that other EDCs are associated with pubertal outcomes. In 12- to 16-year-old females in the NHANES study, the levels of 2,5-dichlorophenol (2,5-DCP) and summed environmental phenols (2,5-DCP and 2,4-DCP) were inversely associated with age at menarche after adjustment for BMI and race/ethnicity (571). However, parabens, BPA, triclosan, benzophenone 3, total phthalates, and 2,4-DCP were not significantly associated with age at menarche (571). In a multiethnic longitudinal study of 1151 girls in the United States (New York City, Cincinnati, northern California), daidzein was inversely associated with breast stage, and triclosan was inversely associated with hair stage (541). Future studies should confirm these findings and determine whether other EDCs are associated with pubertal outcomes in humans as well as animal models.

2. Effects on estrous and menstrual cyclicity

a. Bisphenol A. Recent studies suggest that plasticizer exposure may alter estrous cyclicity in rodents, but the data are inconsistent. For BPA exposure, some studies found that it decreased the time spent in estrus in rats and mice (348, 525, 546), whereas others reported that it increased the time spent in proestrus and estrus in rats (572). Yet in other studies, BPA had no effect on estrous cyclicity in gestationally and neonatally exposed CD-1 mice and Long-Evans rats (545, 573).

b. Phthalates. Little information is available on the effects of phthalates on menstrual/estrous cyclicity. One study on DEHP showed that exposure during adult life prolonged the duration of estrus in mice (371).

c. Pesticides. Relatively few studies have examined pesticide exposure and menstrual cyclicity in women. One study showed a significant reduction of bleeding (1 d) in women in the highest tertile of aromatic fungicide exposure (574). A cross-sectional study of women residing in agricultural communities showed an association between ATR exposure and increased menstrual irregularity (575).

d. Environmental contaminants. In the past 5 years, few studies have focused on the association between environmental contaminant exposure and menstrual characteristics. A longitudinal study of women trying to become pregnant showed a significant 3-day increase in cycle length for women in the highest tertile relative to the lowest tertile of estrogenic PCB congeners (574). Given the overall lack of information on EDCs and estrous/menstrual cyclicity, future studies should be designed to determine whether and how EDCs impact estrous and menstrual cyclicity.

3. Effects of EDCs on fertility

a. Bisphenol A. Limited information exists on BPA exposure and fertility in women (reviewed in Refs. 344 and 345). In the Longitudinal Investigation of Fertility and the Environment study, women's concentrations of BPA were not associated with time to pregnancy (576). In a study of 137 women undergoing IVF, an association between higher quartiles of urinary BPA concentrations and increased odds of implantation failure was found (577). Furthermore, a retrospective cohort study of 115 women undergoing early pregnancy monitoring showed an association between maternal conjugated BPA levels and a higher risk of miscarriage (578). Similarly, BPA levels (but not PFOS and PFOA, MEHP, and DEHP levels) were higher in infertile women compared to fertile women (579). By contrast, BPA levels were undetectable in follicular fluid from women undergoing infertility treatments (580), but this study did not assess fertility outcomes.

Data from experimental studies indicated that BPA reduced fertility. For example, BPA exposure during early gestation completely ablated embryo implantation (581, 582), and BPA exposure during neonatal life decreased implantation sites in pregnant rats (110, 583–585). BPA exposure during gestation reduced fertility with age in mice (348), and neonatal BPA exposure also decreased pregnancy maintenance (583). Furthermore, prenatal BPA exposure may have transgenerational effects on female fertility (145). Specifically, prenatal BPA exposure significantly reduced fertility index and the ability of mice to maintain pregnancies in the F3 generation (145). Future studies are required to determine how prenatal BPA exposure impacts fertility in the F3 generation. Along the same lines, future studies should be conducted to clearly determine whether BPA exposure is associated with infertility in women.

b. Phthalates. Limited information exists on phthalate exposure and fertility outcomes in women. In a cohort of women planning their first pregnancy, urinary levels of MEHP were associated with pregnancy loss (489), but given the small sample size (48 women), the findings need

to be confirmed. Furthermore, urinary levels of phthalates were significantly higher in 56 couples recruited from assisted reproduction clinics, compared with 56 couples that were parents of at least one child (586). By contrast, phthalate levels were lower than 15 ng/mL in follicular fluid from women undergoing infertility treatments (580). This study, however, did not assess fertility outcomes, so it is unclear how the low levels of phthalate in the follicular fluid are associated with fertility outcomes.

Experimental studies show an association between phthalate exposure and reduced fertility. DEHP exposure for 8 weeks caused a 100% pregnancy loss in C3H/N mice (587). Prenatal exposure to DEHP increased the time to pregnancy in CD-1 mice (588). Given the limited information on phthalates and fertility, future studies in both humans and animals should examine the effects of a wide variety of phthalates on fertility.

c. Pesticides. The data on pesticide exposure and infertility in humans are equivocal. Several studies indicated that pesticide exposure was associated with reduced fertility or infertility, whereas others showed no such association. For example, levels of HCB, but not DDT/DDE, significantly increased the odds of implantation failure in 720 women undergoing IVF (589). In a cross-sectional study of 264 Mexican women chronically exposed to organophosphate pesticides, mothers with PON1192RR (paraoxonase) genotypes had a 2-fold increased risk of miscarriage compared to mothers with PON1192QR/PON119QQ genotype. These latter mothers also had a 4-fold increased risk of miscarriage compared to mothers with the PON155LL genotype (590). A study of pregnant women from Poland, Ukraine, and Greenland showed an association between higher levels of 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) and p,p'-DDE and an increased risk of fetal loss (591). In a cross-sectional study of 911 women working in agriculture in Africa, spraying pesticides during the first 3 months of pregnancy was associated with spontaneous miscarriage (592), and the pesticide DDT was detected in 100% of infertile women at higher levels than in pregnant women from Rio de Janeiro, Brazil (593).

In contrast, p,p-DDE levels in serum or follicular fluid were not associated with fertilization rate or pregnancy outcome in a case-control study of 619 Saudi women (19–50 y old) seeking IVF treatment (594). Similarly, using data obtained through the Danish Occupational Hospitalization Register, women working in horticulture did not have increased odds of infertility compared with the standard population (595). Furthermore, in a study of 99 infertile women undergoing IVF treatment in the Czech Republic, the levels of DDT were correlated with the number of diploid oocytes, but the findings were not statistically significant (596).

Prior to the past 5 years, several studies indicated that pesticide exposures reduce fertility or cause infertility in animal models (337, 343, 597). These previous findings have been confirmed in more recent studies. Specifically, prenatal exposure to MXC reduced fertility in rodents (380), likely by altering key molecules in the phosphatase and tensin homolog, IGF-1, and estrogen signaling pathways in the ovary (425). Furthermore, a mixture of organophosphorus pesticides made of dichlorvos, dimethoate, and malathion decreased pregnancy and live birth rates in Sprague-Dawley rats (493). Future studies should expand these findings by determining whether other pesticide mixtures affect fertility in animal models and whether they are associated with subfertility or infertility in women.

d. Environmental contaminants. In a study of 99 infertile women undergoing IVF treatment in the Czech Republic, PCB 158 levels were not significantly correlated with fertilization of oocytes, but the authors noted that if the level of PCB 158 doubled in their statistical model, the ratio of fertilized to nonfertilized oocytes dropped by 15% (596). The authors also noted that PCB 47 levels were borderline negatively associated with successful pregnancy (596). Given the lack of studies on environmental contaminants and fertility, future studies examining environmental contaminants and fertility are warranted in both animal models and human populations.

4. Premature ovarian failure/early menopause

a. Bisphenol A. Studies in women showed that BPA exposure is associated with premature ovarian failure and early menopause in women. In one study, urinary concentrations of BPA were positively associated with markers of oxidative stress and inflammation in women, suggesting that BPA exposure promotes oxidative stress and inflammation and may make postmenopausal women susceptible to other BPA-induced health effects related to aging (598). A prospective cohort study of women undergoing infertility treatments showed that higher BPA levels were associated with lower antral follicle counts, suggesting that BPA exposure accelerated ovarian failure (599). To date, studies have not determined whether BPA causes premature ovarian failure in animal models. Thus, future studies are required to determine whether this is the case.

b. Phthalates. In the past 5 years, limited information became available on the impact of phthalate exposure on premature ovarian failure/early menopause in women. In a cross-sectional survey using the NHANES, women with

high levels of phthalate metabolites had an earlier mean age at menopause compared to women with low levels of phthalate metabolites (600). To date, animal studies have not determined whether phthalate exposure causes premature ovarian failure. Thus, future studies are required to determine whether this is the case. In addition, epidemiological studies are required to confirm the one study that has examined phthalate exposure and early menopause in women.

c. Pesticides. In the past 5 years, limited information became available on the impact of pesticide exposure on premature ovarian failure/early menopause in women. In a cross-sectional survey using the NHANES, women with high levels of β-HCH and mirex had an earlier mean age at menopause compared to women with low levels of β-HCH and mirex (600).

Animal studies are consistent with this epidemiological study because they indicated that exposure to pesticides may cause premature ovarian failure. Perinatal exposure to MXC or estradiol benzoate advanced the onset of reproductive senescence in Fischer rats (601). Future studies should expand these findings by determining whether other pesticides are associated with premature ovarian failure/early menopause, and if so, future studies should determine the underlying mechanisms.

d. Diethylstilbestrol. Combined data from three studies on DES indicated that in utero exposure was associated with an increased lifetime risk of early menopause in women (602). However, animal studies have not determined whether DES exposure causes premature ovarian failure. Thus, future studies should focus on this issue.

e. Environmental contaminants. One epidemiological study showed that women with higher levels of p,p'-DDE had an earlier age at menopause compared with women with lower levels (600). This finding is consistent with those from animal studies indicating that environmental contaminants caused premature reproductive senescence. For example, TCDD exposure accelerated the onset of acyclicity and causes early loss of reproductive function with age in Lewis rats (603). Similarly, prenatal exposure to low levels of a PCB mixture resulted in rats with significantly longer estrous cycles over time as they aged, compared to control rats (604). Given the lack of existing studies, there is a need for additional studies on environmental contaminants and premature ovarian failure and early menopause.

f. Other EDCs. A few studies suggested that EDC exposures may be associated with premature ovarian failure in women. A prospective cohort study of women seeking fertility treatment at Massachusetts General Hospital showed that urinary levels of propyl paraben (a preservative in personal care products) were associated with a trend toward lower antral follicle counts as well as higher day-3 FSH levels (indicators of ovarian aging) (605). Furthermore, exposure to a mixture of 13 chemicals including phthalates, pesticides, UV filters, BPA, parabens, and paracetamol from G7 until P21 caused early reproductive senescence in rats (539). Future studies, however, are needed to determine whether other EDCs are associated with premature ovarian failure/early menopause and to determine the mechanisms by which EDCs cause premature ovarian failure/early menopause.

g. Transgenerational effects. The potential effects of EDCs on premature ovarian failure may be transgenerational in nature because developmental exposure to a pesticide mixture (permethrin and N,N-diethyl-m-toluamide) increased ovarian insufficiency in the F3 generation of rats (427). Similarly, TCDD increased the incidence of ovarian insufficiency in the F3 generation of rats (428). Future studies should examine the mechanisms by which EDCs cause transgenerational effects.

G. Pathophysiological reproductive conditions

1. Polycystic ovarian syndrome

PCOS is an endocrinopathy characterized by oligo/anovulation and hyperandrogenemia, and often by metabolic dysfunction such as insulin resistance. Very few studies examined whether EDC exposure is associated with PCOS, and those that have are limited in sample size and scope (606). Sections 1a and 1b below provide information on the association between specific EDCs and PCOS.

a. Bisphenol A. The data on BPA exposure and PCOS in women are limited and equivocal. One case-control study (71 women with PCOS and 100 women without PCOS) reported an association between serum BPA levels and increased testosterone, androstenedione, and insulin resistance in women with PCOS (607). In contrast, a study of 60 patients with PCOS (23 with insulin resistance and 37 without insulin resistance) and 29 women without PCOS showed that serum levels of octylphenol, but not phthalic acid esters or BPA, were higher in PCOS patients with insulin resistance compared to PCOS patients without insulin resistance (608).

In rodents, prenatal and neonatal low-dose BPA exposures led to the disruption of ovarian cyclicity (545), increased testosterone production (435), and ovarian cysts (609); and high-dose BPA exposure led to ovarian cysts (435) and an accumulation of large antral follicles (545).

However, one study indicated that developmental BPA exposure (G6 through P40) did not induce key features of PCOS in rats (610).

It is difficult to extrapolate studies on PCOS in animal models to humans because the ovarian phenotype in rodents (cystic-appearing follicles) often differs from the ovarian phenotype of women with PCOS (accumulation of small antral follicles). Therefore, work in monkeys and sheep has helped bridge this gap by enabling experimental analyses of effects of prenatal androgens and/or EDCs. In rhesus monkeys, exposure to testosterone in early to midgestation caused metabolic dysfunctions that are similar to those observed in women with PCOS, and late gestational exposure to dihydrotestosterone or DES caused ovarian hyperandrogenism and menstrual abnormalities similar to those in women with PCOS (611). Similarly, in ewes, testosterone propionate injections during pregnancy caused female lambs to develop hyperinsulinemia (612, 613). However, EDC effects in these latter species are lacking and provide a basis for future studies.

b. Phthalates. To date, limited information is available on phthalates and PCOS. In the Western Australian Pregnancy Cohort study, MEP and the sum of all phthalate metabolites measured were protective against PCOS, suggesting that antenatal exposure to phthalates may be associated with estrogenic and/or antiandrogenic outcomes in adolescent girls (441). Future studies should confirm these findings and examine whether other phthalate metabolites are associated with PCOS.

2. Endometriosis

Endometriosis is a gynecological condition in which uterine endometrial cells grow outside the uterine cavity. This condition often causes pain and infertility. Sections 2a through 2d describe the relations between EDC exposure and endometriosis.

a. Bisphenol A. To date, not many studies have focused on whether BPA exposure is associated with risk of endometriosis in women. One case-control study of 69 women suggested that serum BPA concentrations may be associated with the occurrence of endometriosis (614), but these findings need to be confirmed by other studies before definitive conclusions can be drawn about the association between BPA exposure and endometriosis.

b. Phthalates. Several studies have focused on whether phthalate exposure is associated with an increased risk of endometriosis and suggest that there is an association. In a prospective case-control study in Korea, women with advanced stages of endometriosis had significantly higher plasma levels of MEHP and DEHP than women without advanced stages of endometriosis (615). In another case-control study, women with endometriosis had significantly higher levels of total urinary mono-n-butyl phthalate than controls (616). A cross-sectional study of urinary phthalates (NHANES) showed a positive association between MBP and an increased risk of endometriosis (617). Similarly, in the Natural History, Diagnosis, and Outcomes Study, six phthalate metabolites were significantly associated with 2-fold increased odds of endometriosis (618).

It is important to note that not all epidemiological studies have shown that phthalate exposure increased the risk of endometriosis. For example, a population-based, case-control study of women in a large healthcare system in the United States Pacific Northwest showed a strong inverse association between MEHP concentration and the risk of endometriosis and suggested a nonsignificant inverse association between DEHP, MEHHP, MEOHP, and the risk of endometriosis (619). Similarly, a study of infertile Japanese women did not show an increased risk of endometriosis in association with urinary concentrations of phthalate metabolites (620). However, the sample size in the Japanese study was small and only included 57 cases (women with endometriosis) and 80 controls (women without endometriosis) (620). Furthermore, a cross-sectional study of 1227 women in NHANES did not show an association between most phthalate metabolites and endometriosis, although MEHP was inversely related to the odds of having endometriosis combined with leiomyoma (617).

If phthalates are associated with endometriosis, the mechanism of action is not clear. One study indicated that in vitro treatment with DEHP increased the viability of Ishikawa cells and endometrial stromal cells in serum free conditions after exposure to hydrogen peroxide (621). These data suggest that the increased viability may have led to some aspects of endometriosis (621). Thus, it is possible that phthalate exposure increased the viability of cells, a condition that predisposes women to endometriosis. Future studies should determine whether this is the case.

c. Pesticides. In the past 5 years, only a few studies focused on the association between pesticide exposure and endometriosis. A case-control study of women enrolled in a large healthcare system in the United States Pacific Northwest showed that serum concentrations of β-HCH and mirex were positively associated with endometriosis (622). A study of women aged 18–40 years undergoing laparoscopy showed a significant association between aromatic fungicides, t-nonachlor, and HCB and an in-

creased risk of endometriosis (623). Future studies are required to confirm these findings and to determine the mechanisms by which pesticides may increase the risk of endometriosis.

d. Environmental contaminants. Several earlier studies showed that the environmental contaminant TCDD was associated with an increased risk of endometriosis in nonhuman primates and women (624). Recent studies have expanded on this work by examining the potential mechanisms behind this association. These studies indicated that TCDD exposure disrupted cannabinoid signaling in the human endometrium, leading to increased inflammation in the endometrium (513) and that it inhibited progesterone responsiveness in humans and animal models (624). Interestingly, exposing mice to TCDD caused a progesterone-resistant phenotype in adults that persisted over multiple generations, suggesting that TCDD exposure had transgenerational effects on endometriosis (625). Furthermore, recent studies showed that TCDD increased the expression of thymus-expressed chemokine and promoted the invasiveness of endometrial stromal cells by increasing the expression of matrix metalloproteinase-2 and -9, which in turn may have contributed to the onset and progression of endometriosis (626). In addition, recent studies showed that TCDD reduced the expression of CD82 (a wide-spectrum tumor metastasis suppressor that inhibits the mobility and invasiveness of cells), and increased the expression of CCL2-CCR2, which recruited macrophages and further down-regulated CD82 (627). TCDD, in combination with estradiol, also increased RANTES (C-C chemokine, regulated on activation, normal T cell expressed and secreted) in endometriosis-associated coculture, with the resulting effect of recruiting macrophages, inhibiting apoptosis, thereby facilitating the progression of endometriosis (628).

A few additional recent studies indicated that other environmental contaminants were associated with an increased risk of endometriosis. A case-control study in Rome showed a positive association between dioxin-like PCBs and an increased risk of endometriosis (629). Interestingly, a case-control study in Italian women showed that genetic polymorphisms in glutathione transferase (GST) modified the association between PCB exposure and endometriosis (630). Specifically, GSTP1 wild-type genotype in the presence of medium-high blood levels of PCB-153 significantly increased the risk of endometriosis. Furthermore, a recent study showed that total equivalence and concentrations of dioxins and PCBs were higher in patients with deep infiltrating endometriosis compared to controls without endometriosis (631). In contrast, among patients at a reproductive medicine clinic in Atlanta, Georgia, levels of serum dioxins and PCBs were similar in women with and without endometriosis (632). In the ENDO study (633), the POP β-HCH was positively associated with endometriosis. In a case-control study of group health enrollees in western Washington (251 cases and 538 controls), summed and estrogenic PCB concentrations were not associated with endometriosis, but PCB170, PCB201, and PCB196 were all significantly below the null (634). Further studies are required to confirm these results and to determine why certain environmental contaminants, but not others, are associated with endometriosis.

3. Fibroids

Fibroids are benign smooth muscle tumors that develop in the uterus. They often cause pain and heavy menstruation. In the past 5 years, a few studies examined the link between EDC exposure and fibroids. Sections 3a through 3e below provide specific examples of EDCs that are associated with fibroids.

a. Bisphenol A. A few studies examined the associations between known EDCs and fibroids in women. A case-control study in China showed that the mean concentrations and ranges of distribution of BPA, nonylphenol, and octylphenol were higher in women with fibroids compared to women without fibroids (635, 636). A case-control study of 128 patients showed a borderline significant increase in levels of BPA in women with no or mild fibroids vs women with moderate or severe fibroids (637).

Data from in vitro studies are consistent with those from human studies. One study showed that BPA promoted growth of primary cultures of human uterine fibroid cells (638). Another study showed that BPA enhanced cell proliferation and colony-forming efficiency in human uterine myoma mesenchymal stem cells (639). Although the current epidemiological and in vitro studies are consistent, future studies need to confirm these results.

b. Phthalates. Several recent studies focused on whether phthalate exposures were associated with fibroids in women. A cross-sectional study of 1227 women in NHANES showed a positive association between MBP and an increased risk of fibroids, but MEHP, MEHHP, and MEOHP were inversely associated with fibroids (617). Similarly, women with fibroids had significantly higher levels of total urinary MEHP; and women with the GSTM1 null genotype who have been exposed to MEHP had an even higher risk of fibroids, indicating that both GSTM1 null genotype and phthalate exposure are associated with fibroids (616). Although these studies provide important information, the findings need to be confirmed

and expanded to include assessment of the association between other phthalates and endometriosis.

c. Pesticides. Limited information is available on pesticide exposure and uterine fibroids. In one study, fenvalerate stimulated the growth of uterine fibroid cells by enhancing cell cycle progression and inhibiting apoptosis through an ER-independent pathway (640). Furthermore, fenvalerate-induced cell proliferation required the down-regulation of p27 (641). Given the limited amount of information on pesticides and fibroids, future studies should be designed to examine pesticides and fibroids in epidemiological and experimental studies.

d. Diethylstilbestrol. A few recent studies confirmed the known association between DES exposure and fibroids. In the Sister Study, in utero exposure to DES was positively associated with early-onset fibroids (642, 643). Similarly, in the Nurses' Health Study II, prenatal DES exposure was associated with uterine fibroids, with the strongest risk being for women exposed to DES in the first trimester (644). Given the consistency in findings, future studies should be focused on determining the mechanism by which DES exposure increases the risk of fibroids.

e. Environmental contaminants. Only a few epidemiological studies examined the association between environmental contaminants and fibroids. In a cohort study of women undergoing laparoscopy or laparotomy at 14 hospital centers, PCB levels were positively associated with fibroids in the absence of other gynecological disorders (645). In the Great Lakes Fish Consumption Study, years of sport fishing were associated with an increased risk of fibroids (646). Future studies should confirm these findings as well as examine the mechanisms underlying the association between environmental contaminant exposure and fibroids.

H. Pregnancy and birth

1. Preterm birth

a. Bisphenol A. Studies on the link between BPA exposure and preterm birth produced equivocal results (344). A nested case-control study of 60 pregnant women showed a positive association between urinary BPA concentration and preterm birth (647). In contrast, several experimental studies showed that BPA did not alter gestation length in gestationally and neonatally exposed mice (648), adult exposed mice (573), or gestationally and neonatally exposed Sprague-Dawley rats (649). Given the equivocal nature of the findings, future studies are needed to determine whether BPA exposure is associated with premature birth.

b. Phthalates. The data on phthalate exposure and preterm birth are also equivocal. In a pregnancy cohort study of 283 women from California, Iowa, Minnesota, and Missouri, women at the 75th percentile of DEHP metabolite concentrations had a 2-day longer mean length of gestation than women at the 25th percentile (650). Furthermore, MEHP and MEOHP concentrations were associated with increased odds of delivering at 41 weeks or later and reduced odds of preterm delivery, suggesting that DEHP exposure may interfere with the timing of parturition (650). In contrast, in a large Mexican birth cohort study, urinary levels of MBP and MBzP were associated with preterm birth (651). Similarly, in a study of 311 African American or Dominican women from New York City, urinary MEHP levels were associated with shorter gestational age, on average 5 days less among women with the highest vs lowest quartile concentrations of MEHP (652). Interestingly, the results were similar for other DEHP metabolites (652). Furthermore, in a nested case-control study in Massachusetts, MEHP, mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), and total DEHP metabolites were associated with increased odds of preterm birth (653). MEHP, MECPP, MBP, mono-93-carboxypropyl phthalate, and total DEHP metabolites were associated with increased odds of prematurity (653).

The potential mechanisms by which phthalates may affect preterm birth are unknown. It is possible that phthalate exposure might affect placental function, and altered placental function may increase the risk of preterm birth. This possibility is supported by studies that indicate that gestational phthalate exposures in humans are associated with decreased expression of genes required for trophoblast differentiation and placenta steroidogenesis (654). Given the equivocal epidemiological findings, future studies should examine the reasons for discrepancies among studies. If phthalate exposure is associated with preterm birth, future studies should examine the underlying mechanism.

c. Pesticides. Several previous studies indicate that pesticide exposure is associated with an increased risk of preterm birth in humans (655, 656), and studies conducted in the past 5 years confirm these previous findings. In a case-control study of 156 women with preterm birth and 151 women without preterm birth, women with preterm delivery had significantly higher levels of HCH, γ-HCH, and p'p'-DDE (657). Furthermore, the presence of polymorphisms in CYP1A1m2 and GSTM1 null genotypes magnified the risk of preterm birth, suggesting a gene-environment interaction in pregnant women (657). Similarly, a review of Kentucky birth certificate data for 2004–2006 and ATR levels in public drinking water for 2000–2008

showed increased odds of preterm birth for women living in the counties with the highest ATR exposure (658). In addition, chlordecone concentration was associated with decreased risk of length of gestation and an increased risk of preterm birth in a study of 818 women in Guadeloupe (659), and pesticide consumption per capita was associated with premature birth in Brazil (660).

In contrast, not all studies indicate that pesticide exposure is associated with preterm birth. A study of 1568 mother-child pairs in three Spanish areas did not show an association between prenatal exposure to hexachlorobenzene and preterm birth (661). Similarly, a retrospective cohort study in Indiana did not find an association between ATR exposure and preterm birth, although ATR is associated with a small-for-gestational age condition (662). The mechanisms by which pesticides may increase the risk of preterm birth are unclear. However, chlorpyrifos modifies genes relevant for placenta function (eg, ABCG2 [ATP-binding cassette, subfamily G], GCM1 [glial cells missing homolog 1], and βhCG [human chorionic gonadotropin]) in JEG-3 cells (663), and thus, it is possible that the gene modifications affect placental function. Future studies should determine the reasons for discrepancies among existing studies and determine whether and how unexamined pesticides and pesticide mixtures are associated with preterm birth.

d. Environmental contaminants. Limited recent information has been published on environmental contaminants and preterm birth in humans. Experimental studies, however, indicate that TCDD exposure caused premature birth in F1 mice as well as in three subsequent generations (664). It is thought that TCDD exposure increased sensitivity to inflammation, which further negatively impacted gestation length (664). This is supported by studies using second trimester placental explants, in which TCDD exposure shifted immunity to a proinflammatory phenotype at the maternal-fetal interface that could increase the risk of infection-mediated preterm birth (665). It is also supported by studies showing that developmental TCDD exposure was associated with preterm birth in a subsequent adult pregnancy and that this was due to altered PR expression and inflammation in the placenta (666). It will be important for future studies to determine whether environmental contaminants have similar effects in humans and whether other unexamined environmental contaminants are associated with preterm birth.

2. Pregnancy and birth outcomes

a. Bisphenol A. Studies show that BPA exposure increased the risk of adverse birth outcomes in humans (667). In a study of 72 pregnant women, urinary BPA levels were associated with a 1.1-day decrease in gestation length in males, but not females (349). In a multicenter birth cohort study of mothers and children in Korea, prenatal exposure to BPA was associated with increased birth weight and length, particularly in male neonates (543). In the Generation R study in The Netherlands, however, prenatal BPA exposure was associated with reduced fetal weight and head circumference (668). Future studies are required to understand the mechanism underlying the association between BPA exposure and adverse birth outcomes.

b. Phthalates. In some studies, phthalate exposure was associated with an increased risk of adverse birth outcomes in humans. In the Generation R study of pregnant women participating in a prospective cohort study in The Netherlands, maternal phthalate exposure was associated with an increased time-to-pregnancy (669) as well as impaired fetal growth during pregnancy and decreased placental weight (670). In a study of 72 pregnant women, urinary MEHHP was associated with a 4.2-day decrease in gestation (349). Interestingly, the decreases in gestation time were only observed in males, suggesting sex-specific alterations during the initiation of labor (349). In 111 pregnant Japanese women, maternal urinary MEHP levels were negatively associated with anogenital distance (AGD) in male offspring (671). In contrast, amniotic fluid levels of MBP were negatively associated with AGD in female offspring (672).

Not all studies, however, indicated an association between phthalate exposure and adverse birth outcomes in humans. In a case-control study nested in a French mother-child cohort study, phthalates were not associated with birth weight (673) although urinary concentrations of DCP and 2,5-DCP were associated with decreased birth weight in males, and urinary levels of benzophenone were positively associated with birth weight and head circumference (673). Similarly, in a study of 149 pregnant Japanese women, urinary levels of phthalate esters were not associated with birth outcomes; however, urinary levels of 1-hydroxypyrene, a polyaromatic hydrocarbon metabolite, were negatively correlated with birth weight, birth length, and head circumference (674).

Data from animal studies indicate that phthalate exposure caused adverse birth outcomes. Gestational exposure to DEHP (500 mg/kg/d) caused complete pregnancy failure in mice (675) and decreased fetal quality and weight in rats (676). DEHP exposure at 3000 mg/kg before mating until G7 decreased pregnancy rate, but lower doses did not impact fertility or early embryonic development in rats (677). Furthermore, exposure to DEHP from G12 to P21 restricted growth and delayed lung maturation in newborn rats (678). Similarly, gestational exposure to

DBP significantly increased fetal weight in female rats (679). It is important to point out that these were high-dose studies. Future studies should determine the reasons for discrepancies among epidemiological studies and determine the mechanism by which phthalates cause adverse birth outcomes in animal models.

c. Pesticides. Pesticide exposure has been associated with adverse effects on pregnancy/birth outcomes in epidemiological studies. In a search on occupational exposure to chemicals and time to pregnancy, pesticide exposure was associated with an increased time to pregnancy (670). In 442 participants in the Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS), high methyl bromide use within 5 km of the home during the second trimester of pregnancy was negatively associated with birth weight, birth length, and head circumference; similar associations exist between methyl bromide use during the second trimester (680). Interestingly, infants with the paraoxonase 1 (PON1) genotype (-108TT), a low enzyme activity phenotype, were more susceptible to organochlorine pesticide exposure effects on fetal growth and length of gestation compared to infants without the low enzyme activity phenotype (681). In a cohort study of 11 446 women-neonate pairs in France, levels of ATR metabolites and nitrates were significantly associated with infants being small for gestational age (682). In the Agricultural Health Study, a large study of 2246 farm women, "ever use" of the pesticide carbaryl was associated with decreased birth weight (683). In a case-cohort study nested in a prospective birth cohort of 579 pregnant women in France, the presence of ATR was associated with fetal growth restriction and small head circumference for sex and gestational age, but not with any congenital abnormalities. Furthermore, head circumference was inversely associated with presence of urinary metolachlor (684). In a study of 150 women who underwent an elective cesarean delivery at term in New Jersey, high metolachlor concentrations in cord blood were significantly associated with birth weight, and increasing cord dichloran concentrations were associated with an increase in abdominal circumference (685). In a cross-sectional pilot study in women from Mexico, pesticide exposure was associated with higher placental maturity, which may potentially affect nutrient transport from the mother to the fetus (686).

Some of these associations between pesticide exposure and birth outcomes may be sex-dependent. In 187 healthy pregnant women in Shanghai, China, urinary diethyl phosphate levels were associated with a decrease in gestational duration in girls, but not boys (687). Furthermore, some of the associations between pesticide exposure and preterm birth may be due to oxidative stress and inflammation. Organochlorine pesticide levels were associated with preterm delivery and increased oxidative stress in a small study of 30 women with preterm delivery and 30 women without preterm delivery (688), and environmental pesticide exposure increased the frequency of the anti-inflammatory cytokine IL-13 in the placenta, which may lead to up-regulation of enzymes implicated in tissue repair (689). In addition, in a study of 50 women delivering babies with low birth weight and 50 women delivering babies with normal birth weight, levels of γHCH and total HCH and oxidative stress markers were observed in women with low birth weight babies compared to controls (690).

Data from animal studies confirm the association between pesticide exposure and numerous adverse pregnancy outcomes in women. Collectively, these studies showed that p,'p-DDT levels in adipose tissue were significantly related to birth length (691), and that p,p'-DDE concentrations were also significantly associated with decreased birth weight and shortened gestational age (692). An in utero study showed that low exposure to DDT was negatively correlated with head circumference, crown-heel length, and birth weight, (independent of gestational age and/or preterm birth) (693). Furthermore, synthetic insecticides (XenTari and deltamethrin) reduced implantation sites in pregnant rats (694). In sheep, MXC exposure from G30 to G90 caused low birth weight offspring that were hypergonadotropic during early postnatal life and had severely dampened preovulatory surges (695). Additional studies are needed to determine whether other pesticides cause adverse birth outcomes and to fully understand the underlying mechanism by which pesticides cause adverse birth outcomes.

d. Perfluorooctanoic acid. A few studies indicate that PFOA exposure may be associated with adverse pregnancy outcomes in humans. Data collected in 2005–2006 demonstrated that serum PFOA and the related compound PFOS were associated with pregnancy-induced hypertension in a group of over 1300 women. Furthermore, a recent meta-analysis of both human and rodent data demonstrated that PFOA exposure was associated with a decrease in mean birth weight of −0.023 g (95% confidence interval [CI], −0.029, −0.016) per 1-unit increase in dose (mg/kg body weight/d) in rodents and was associated with a −18.9 g (95% CI, −29.8, −7.9) difference in birth weight in humans (696–698). These findings should be confirmed in future studies. Furthermore, experimental studies should be designed to determine the mechanisms by which PFOA causes adverse pregnancy outcomes.

e. Environmental contaminants. Epidemiological studies indicate that environmental contaminant exposure is associated with adverse birth outcomes. In a prospective cohort study of 514 pregnant women, significant associations between PCBs and birth weight were found for male, but not female infants (699). A study of 3421 pregnant women reported that time to pregnancy increased with increasing levels of PCBs (700). Furthermore, reduced fecundity was associated with levels of p,p'-DDE, total PCBs, and PCB153 and PCB187 (700). In the Seveso Women's Health Study, every 10-fold increase in serum levels of TCDD was associated with a 25% increase in time to pregnancy and almost a doubling in the odds of infertility (151). In a study of Japanese mothers living in the coastal areas of Japan, concentrations of PCDDs and dibenzofurans in breast milk were inversely correlated with newborn length, and TCDD was negatively correlated with newborn head circumference, even after adjustment for gestational age, infant birth weight, and other confounders (701). A mother-child cohort study in Greece (1117 mothers and newborns) showed that birth weight was negatively associated with increasing levels of HCB and PCBs and reported small negative associations between POPs and head circumference, but not gestational age (702). In the Hokkaido Study on Environment and Children's Health, an ongoing cohort study in Japan (large-scale cohort = 20 940; Sapporo cohort = 514), PCDF and PCDD exposures were negatively associated with birth weight and infant development, with males being more susceptible than females (308). In a study of 248 pregnant Inuit women, cord blood concentrations of PCB153, HCB, and mercury were significantly associated with shorter duration pregnancy (703). Finally, a meta-analysis of 12 European birth cohorts reported that increasing concentrations of PCB153 were associated with decreased birth weight (704).

There are exceptions to those studies demonstrating an association between environmental contaminant exposure and adverse birth outcomes. For example, in the Seveso Women's Health Study, a retrospective cohort study of TCDD exposure and reproductive health, serum TCDD was not significantly associated with spontaneous abortion fetal growth, gestation length, or birth weight (705).

Animal studies are consistent with epidemiological studies indicating that environmental contaminant exposure is associated with adverse birth outcomes. Several such studies indicated that exposure to environmental contaminants adversely affects birth outcomes. In rats, TCDD exposure caused aberrations in cytokinesis in two-cell embryos and morulas (706). In a three-generation experiment in mice, TCDD exposure significantly reduced pup survival in the F2 and F3 generation (466). Further studies are required to determine how TCDD affects the survival of pups in the F2 and F3 generations.

I. Conclusions

During the past 5 years, several studies examined the effects of EDCs on the female reproductive system. Collectively, these studies consistently show that: 1) plasticizers, the pesticide MXC, and TCDD impair ovarian development by interfering with germ cell nest breakdown and follicle formation; 2) pesticides and plasticizers affect postnatal ovarian structure and function in animal models by decreasing ovarian weight, inhibiting follicle growth, and/or increasing atresia/apoptosis; and 3) pesticides, phthalates, BPA, and environmental contaminants impair ovarian steroidogenesis in animal models and women. Although the data are not always consistent between experimental and epidemiological studies, they suggest that EDCs may adversely affect the structure and/or function of the uterus, vagina, and anterior pituitary, and that they may be associated with abnormal puberty, irregular cyclicity, reduced fertility, infertility, PCOS, endometriosis, fibroids, preterm birth, and adverse birth outcomes. It is important to note, however, that limited data are available on the effects and associations of EDCs and human reproductive outcomes. Furthermore, limited information is available to explain the inconsistencies in results among studies and to understand the mechanisms by which EDCs adversely affect female reproduction. In addition, many potential EDCs have not been studied at all in experimental or epidemiological studies. Thus, there is a real need for future studies to focus on the effects and mechanisms by which EDCs affect female reproductive outcomes in both experimental and epidemiological studies.

IV. Male Reproductive Health

A. Introduction

The EDC field has a decades-long history in the area of male reproductive health due to long-standing concerns regarding how chemicals affect reproduction in wildlife and humans (707, 708). Furthermore, the DES experience pointed to the possibility that pharmaceutical estrogens, and potentially environmental estrogens, can affect not only female but also male reproductive health (709). This discovery spurred a comprehensive assessment on how hormone exposure affected human health (710), eventually leading to an understanding and more mechanistic evidence that EDCs, especially those that are antiandrogenic, play key roles in the development and maintenance of male health (711). This section focuses primarily on the

literature in humans, with briefer summaries of animal studies. Therefore, in providing background on male reproductive development, the life stages and ages refer to humans.

B. Male sexual development, and Nature's experiments

Normal development of the male gonads begins early in embryogenesis, with the differentiation of the testis from a bipotential gonad. The internal sex organs arise as a consequence of the maintenance of the mesonephric ducts (or Wolffian ducts) and the regression of the paramesonephric (or Müllerian ducts) due to the testicular hormones testosterone and anti-Müllerian hormone, respectively. The development of external genitalia, scrotum, and testicular descent are under the control of several genetic and hormonal pathways. Testosterone and its metabolite dihydrotestosterone drive the masculinization of the external genitalia. Testicular insulin-like peptide 3 is also necessary for testicular descent. Timing of the hormone action is important. In humans, normal androgen action during gestational weeks 8–15 is critical for normal development of the genitalia; penile malformations arise at this time window, whereas development of sperm production capacity occurs in a much broader time window that continues to puberty. In rodents, the early androgen-dependent developmental phase is called the male programming window, which occurs at E13–E17 (712). Another sex-specific indicator of androgen action in fetal life is AGD, which is larger in males than females beginning in fetal life through adulthood (713). In males, the onset of puberty is androgen-driven, with regulation occurring at the hypothalamic level via neurons secreting kisspeptin that starts the regulatory cascade via GnRH neurons to the pituitary gland and from there to the testis. Testicular growth is the hallmark of the onset of puberty, but it is difficult to monitor without physical measurements; therefore studies on the timing of male puberty in humans are less frequent than those in females. Furthermore, findings on the effects of endocrine disruptors on the timing of male puberty have been rather limited, and consistent findings have only been reported for lead exposure, which is associated with delayed puberty (for review, see Ref. 714).

Natural mutations or hormonal abnormalities have been extremely informative in helping us better understand the consequences of disturbances in the developmental processes described above, including their consequences on male reproductive organs. Genetic mutations affecting androgen production or action cause testicular dysgenesis syndrome (TDS), including cryptorchidism, hypospadias, impaired semen quality, and markedly increased risk of testicular cancer (715). The mutations encompass a large number of genes, including those that encode ARs, key steroidogenic enzymes, and regulatory transcription factors. Although defects in these genes cause TDS, there is another group of genes associated with an increased risk for TDS, such as Kit-ligand, BMP7, and TGFbR3 (716). Despite this knowledge, attempts to find a genetic basis of TDS outcomes have largely failed, and we can only give molecular genetic diagnoses for a small minority of cases. It seems likely that all components of TDS have a multifactorial etiology, and environmental factors play an important role.

Considering the importance of normal androgen action in male reproductive development and function, it is not surprising that chemical compounds that disrupt

Box 2. Key Points: Female Reproduction

- **The female reproductive system is complex and requires proper structure and function of many organs, including the ovary, uterus, vagina, and anterior pituitary. EDCs have the potential to interfere with female reproduction by adversely affecting the structure and/or function of female reproductive organs.**
- **Studies consistently show that some EDCs impair key processes in ovarian development (germ cell nest breakdown and follicle formation) in animal models; adversely affect the structure and function of the postnatal ovary by inhibiting follicle growth and/or increasing atresia/apoptosis in animal models; and disrupt steroid hormone levels in animals and women.**
- **Although the data are not always consistent between experimental and epidemiological studies, they suggest that some EDCs may adversely affect the structure and/or function of the uterus, vagina, and anterior pituitary. Some EDCs are also associated with abnormal puberty, irregular cyclicity, reduced fertility, infertility, PCOS, endometriosis, fibroids, preterm birth, and adverse birth outcomes.**
- **Limited information is available to explain the inconsistencies in findings among studies and to explain the mechanisms by which EDCs adversely affect female reproduction. Thus, more research is needed in this area.**
- **Many potential EDCs have not been studied at all in experimental or epidemiological studies. Thus, there is a real need for future studies to focus on the effects and mechanisms by which EDCs affect female reproductive outcomes in both experimental and epidemiological studies.**

androgen production or action can cause TDS symptoms such as hypospadias, cryptorchidism, and impaired spermatogenesis in experimental animals. Although there are no animal models for human testicular germ cell cancer (TGCC), antiandrogenic compounds do cause structural alterations in the testis resembling the abnormalities seen in human testicular cancer (711). Animal models show that antiandrogens can act in a dose-additive manner, which has challenged the current no adverse effect levels because the adverse outcomes have appeared when the animals have been exposed to a combination of chemicals far below their individual NOAELs (717–719). In addition to antiandrogens, estrogens and dioxins cause similar effects, via their cognate ERs and AhRs, respectively. The balance between these hormone-receptor pathways is also important, beyond the individual roles of each hormonal system. Because the bulk of studies have focused on antiandrogens, these are currently the main focus of EDC research in male reproduction.

C. Hypospadias

Hypospadias is a condition in which the urethral folds do not fuse properly, and the urethra is exteriorized on the ventral side rather than the tip of the penis. The incidence of hypospadias varies regionally, and temporal increases in the number of cases have been reported in several countries over different time periods (720). These epidemiological variations may reflect environmental influences, but surprisingly few studies have evaluated the role of environmental chemicals in hypospadias. Case-control studies of hypospadias are challenging because the malformation is rare and its severity is variable. Therefore, the number of subjects is small in most of the studies, many of which are not adequately statistically powered to enable the detection of significant associations.

1. Pesticides, PCBs, phthalates, and other industrial exposures

a. Humans. One meta-analysis suggested an association between an increased risk of hypospadias in sons of parents exposed to pesticides (721). Exposure was assessed by an evaluation of medical charts, parental interviews, occupation, job exposure matrix, or linkage of agricultural census and birth records. Pooled risk ratios were 1.36 (95% CI, 1.04–1.77) and 1.19 (95% CI, 1.00–1.41) for maternal and paternal exposures, respectively (721). Because these pesticides represented a number of chemicals, it is not clear which ones may be linked to hypospadias.

Later studies using the job exposure matrix (a list of levels of exposure to a variety of harmful [or potentially harmful] agents for selected occupational titles) as a proxy for pesticide exposure did not show significant associations with hypospadias (722, 723), but they suggested a link to heavy metals or maternal exposure to any EDC (724). A similar relationship was also found in a small Italian study (725). However, there are caveats to this work because exposure assessments in the absence of measurements of chemical exposure leave a lot of uncertainty and can cause inconsistencies (726).

In the Collaborative Perinatal Project (CPP) conducted in the United States in the 1950s and 1960s, maternal serum samples were collected during pregnancy and analyzed for several chemicals. The children of these mothers were examined several times during childhood until the age of 7 years (727–730). Results indicated an increased odds ratio for hypospadias for the sum of some PCBs, but no linear association with PCB levels was reported (730). Results also did not indicate any statistically significant associations between hypospadias and chlordane-related contaminants, DDE, β-HCH, or other pesticides (727–730). A similar study using maternal serum samples from the 1950s and 1960s and assessing hypospadias from medical records did not find an association between hypospadias and levels of DDT or DDE during pregnancy (731). A more recent study that recruited families between 2005 and 2007 reported an association between an increased risk of hypospadias and above-median levels of HCB in primiparous women several weeks after delivery (724). A small questionnaire-based study found no association between the rate of hypospadias in offspring and levels of polybrominated biphenyl (PBB)-153 in mothers' serum at the time of conception (732). Similarly, another study reported no significant associations between hypospadias and midpregnancy serum levels of PCBs, PBDEs, HCB, DDT, or DDE (733). A common problem in the studies of hypospadias is the small number of cases, as indicated earlier, and therefore the statistical power to detect any significant associations is very limited. This is less of an issue in cryptorchidism studies, discussed next, because undescended testicles are fairly common.

b. Animal studies. In animal studies, hypospadias is a common outcome in male pups that have been exposed to antiandrogens in utero. Table 5 lists some of the chemicals causing hypospadias in experimental animals. Some of the chemicals inhibit testosterone production (eg, phthalate esters [BBP, DBP, DEHP, DINP]), whereas others block the androgen receptor (AR) (eg, the pesticide DDE, and fungicides vinclozolin and procymidone). Despite their dissimilar mechanism of action, these chemicals act in a dose-additive manner, with increased likelihood of adverse effects of low doses of individual chemicals in the mixture (734, 735).

Table 5. Effects of EDCs Observed in Reproductive System of Male Animals

EDC	Observation	Ref.
Alkyl phenol ethoxylates (*p*-tert-octylphenol, *p*-nonylphenol)	Reduction in testis weight, decreased seminiferous tubule diameter	1283
	Increased testis weight, reduction in epithelial height of the efferent ducts	1284
	Decreased testis weight	1283, 1285
	Decreased epididymal weight, decreased total cauda epididymal sperm count	1285
BPA	Reduced sperm count	110
DDE	Nipple retention	1262, 1286, 1287
	Hypospadias	1286
	Reduced accessory sex organ weights	1262, 1286, 1288
	Reduced AGD	1262, 1287
	Delayed preputial separation	1262
	Abnormally small penis, poorly organized testis, decreased plasma testosterone levels	1289
DES	Sterility, epididymal cysts, cryptorchidism, testicular lesions, inflammatory disease of the accessory sex glands, reduction in the number of spermatogonia with multinucleate cells in lumina of testis, nodular enlargements of the seminal vesicles and/or prostate	1290
	Reduction in testis weight	1284, 1291, 1292
	Distension and overgrowth of the rete testis	1284, 1292, 1293
	Distension and reduction in epithelial height of the efferent ducts	1284
	Underdevelopment of the epididymal duct epithelium, convolution of the extra-epididymal vas; decreased AR expression in testis, epithelium of the rete testis, caput and cauda epididymis, and vas deferens	1292
	Reduction in epithelial height in the vas deferens	1292, 1293
	Decreased testosterone levels	1293, 1294
	Increased gonadotropin levels, increased pubertal FSH levels	1294
	Decreased pituitary response to GnRH	1295
Dicarboximide fungicides (vinclozolin, procymidone, prochloraz)	Hypospadias	1286, 1288, 1296, 1297
	Cleft phallus	1288, 1296
	Reduced AGD	734, 981, 1286, 1296–1299
	Decreased testis weight	1297, 1298
	Cryptorchidism	1296, 1297, 1300
	Increased number of apoptotic germ cells in testis, reduced elongated spermatid content per testis	981
	Nipple retention	1286, 1296, 1299, 1300
	Reduced accessory sex organ weights	981, 1286, 1296, 1298, 1300
	Glandular atrophy and chronic inflammation of prostate	981, 1297, 1300
	Reduced secretion and chronic inflammation of seminal vesicles, chronic inflammation of epididymis, spermatogenic granuloma	1297
	Epididymal granulomas	1296, 1300
	Agenesis of prostate, decreased fertility, decreased testosterone levels	1296
	Decreased sperm number and daily sperm production	1286, 1296, 1298
	Increased sperm head abnormalities	1298
	Low ejaculated sperm count	1286
	Abnormal morphology of seminiferous tubules	1296, 1298
	Reduction of erections during the ex copula penile reflex test, increase in seminal emissions during the ex copula penile reflex tests	1301
Dioxins	Reduced accessory sex organ weights	808, 1302–1304
	Decreased testis weight, altered sex behavior, decreased daily sperm production, dose-related tendency for decreased plasma testosterone and DHT	1302
	Reduced AGD	808, 1302–1304
	Epididymal malformations	808, 1304
	Decreased sperm numbers	808, 1302, 1304
Herbicides (linuron)	Nipple retention	1160, 1286, 1305
	Reduced accessory sex organ weights, delayed preputial separation, decreased testis weight, reduced spermatid number, decreased AGD	1286
	Decreased AGD	1160, 1286, 1305
	Epispadias	1286
	Testicular and epididymal malformations	1286
	Delayed preputial separation	1306
	Decreased weight of ventral prostate	1306
	Decreased testosterone level	735
	Reduced sperm count	735
Paracetamol	Decreased AGD	744
PCBs 77, 118, 126, 132	Reduced accessory sex organ weights	1286, 1307, 1308
	Decreased testis weight	1286, 1308
	Increased testis weight, increased epididymis weight, reduced AGD, increased daily sperm production, altered sex behavior	1307
	Increased AGD, increased number of abnormal sperm	1308
	Delay in onset of spermatogenesis, preputial separation, and sex accessory growth	1286
Decreased sperm number and total motile sperm count	1286, 1308	
Phthalate esters (DEHP, BBP, DINP, DBP)	Nipple retention	1286, 1309–1313
	Decreased testis weight	718, 1300, 1311–1315
	Reduced AGD	718, 1286, 1309–1313
	Cryptorchidism	718, 1286, 1311–1313, 1315
	Reduced accessory sex organ weights	1286, 1309, 1311–1313, 1315
	Lesion of the rete testis	1309
	Hemorrhagic testis	1286, 1311
	Cleft phallus and hypospadias	1286, 1309, 1311–1313
	Multinucleated gonocytes, agenesis of bulbourethral glands and gubernacular cords	1311

(*Continued*)

Table 5. Continued

EDC	Observation	Ref.
	Agenesis of the seminal vesicles and coagulating glands	1311, 1313
	Agenesis of epididymis and vas deferens	1286, 1312, 1313
	Histopathological changes of testis	718, 1309, 1312–1315
	Delayed preputial separation	1286, 1312
	Reduced fertility and fecundity	1286
	Reduced daily sperm production, increased serum testosterone levels	1315
	Reduced testicular testosterone levels	1310, 1314
	Reduced serum inhibin B levels, increased plasma LH levels	1310
Tributyltin	Increased AGD	1316
	Reduced number of Sertoli cells and gonocytes in fetal testis	1317

Abbreviation: DHT, dihydrotestosterone. [Updated from J. Toppari et al: *Endocrine Disrupters and Child Health. Possible Developmental Early Effects of Endocrine Disrupters on Child Health*. World Health Organization, Geneva, Switzerland, 2012 (1318), with permission.]

D. Cryptorchidism

Cryptorchidism is the failure of the testis to fully descend to the bottom of the scrotum. The incidence of congenital cryptorchidism in full-term boys varies between 1 and 9% in different countries, whereas the frequency is consistently higher in preterm boys and correlates negatively to gestational age (736). In countries where prospective clinical studies with well-documented diagnostic criteria have been performed, the incidence of cryptorchidism has increased from 1–3% in the late 1950s to 7–9% in the 2000s (720).

During the last 20 years, it has become evident that in addition to congenital cryptorchidism, many boys acquire cryptorchidism during childhood, when one or both testes ascend to an abnormal position. The frequency of this condition varies greatly. In a clinical follow-up study in England, the cumulative incidence at 2 years of age was 7% (737). In epidemiological studies, particularly those based on registry data, congenital and acquired cryptorchidism are not separated, although their etiology may be very different.

Cryptorchidism shows familial clustering, which may be the result of either genetic or environmental factors. Studies have also reported similar concordance of cryptorchidism in heterozygotic and monozygotic twins, suggesting that the shared fetal milieu might play a role (738). There are several known risk factors for cryptorchidism, including small for gestational age; gestational diabetes in the mother; and maternal alcohol, acetaminophen, tobacco, or nicotine substitute use during pregnancy (739–744). The roles of alcohol, smoking, and painkillers have remained somewhat controversial because of conflicting results. It is also difficult to discern individual effects, because these substances are often used together.

1. Pesticides

a. Humans. Several studies have explored the association between cryptorchidism and pesticide exposure. Adipose tissue from cryptorchid boys undergoing orchiopexy had higher levels of heptachloroepoxide and HCB than controls, whereas there were no differences for PCBs (745). A number of case-control studies have reported on EDCs in peripheral serum, cord blood serum, placenta, and mother's milk. A Finnish-Danish study reported higher levels of eight chlorinated pesticides in breast milk of cryptorchid compared to noncryptorchid boys (746). The US CPP study (described above) reported negative associations between cryptorchidism and p,p'DDE, p,p'DDT, heptachloroepoxide, HCB, and chlordane-related pesticides, whereas β-HCH (in concentration percentiles between 50 and 90) showed a positive association (727–730). California children in the two higher quartiles of DDT exposure (measured from maternal pregnancy serum) had a higher incidence of cryptorchidism, whereas the study found no significant association with DDE (731). A Spanish study that assessed total estrogenic burden in placenta samples linked cryptorchidism with xenoestrogen exposure and associated detectable levels of lindane and mirex with higher cryptorchidism risk (747).

2. Flame retardants, PCBs, DDT, and dioxin

a. Humans. Flame retardants, PCBs, DDT, and dioxin have been assessed for their relationships with cryptorchidism. The US CPP found no association between PCBs and cryptorchidism (730). In a Finnish-Danish study, PCBs and PBDEs were measured in mother's milk and placenta (56, 57). The concentration of these chemicals in placenta did not show any positive association with cryptorchidism, but the Danish cryptorchid boys were exposed to significantly lower levels of PCBs in breast milk compared to controls (748), whereas the level of PBDEs in breast milk was higher in the cryptorchid group vs controls (749). A small study (nine cases, 450 controls) reported no association between maternal serum levels of PBB-153 at the time of conception and cryptorchidism (732).

Dioxin levels in breast milk were associated with an increased risk of cryptorchidism in Danish boys (750), whereas placental levels had no such association (748). Dibutyltin concentrations in placenta were associated

with an increased incidence of cryptorchidism in Danish boys, whereas there was an opposite association with dibutyltin in Finnish boys (751). Cord blood levels of perfluorinated compounds were not associated with cryptorchidism in the Finnish-Danish study when the Finnish and Danish children were combined into one group, but in Finnish children the level of PFOA was negatively correlated to the risk of cryptorchidism (752).

A French case-control study on cryptorchidism measured concentrations of DDE, PCBs, DBP, and MBP in colostrum and cord blood (753). The boys in the high-exposure group (based on combined exposure to DDE and PCBs) had a higher risk of cryptorchidism than boys in the lower exposure group. A similar tendency was found for MBP. A US study that examined testicular position at the mean age of 12.8 months reported an association between levels of maternal urinary MEHP and incomplete testes descent (754). Maternal urinary levels of five phthalate metabolites were also associated negatively with AGD, suggesting an antiandrogenic effect (754). The Finnish-Danish study reported an association between phthalate levels in mother's milk and serum testosterone and LH concentrations at 3 months of age in boys, although there was no direct association between cryptorchidism and milk phthalate levels (755). High phthalate exposure was associated with a decline in free testosterone and an increase in LH concentration, again suggesting an antiandrogenic effect (755).

b. Animal studies. Experimental animal studies (Table 5) show that exposures to antiandrogenic EDCs lead to cryptorchidism, similar to the effect of these compounds on hypospadias (719). Moreover, estrogenic compounds can also lead to cryptorchidism (756). Thus, mixture effects of EDCs may be due to their various estrogenic and antiandrogenic properties. There is no reason why the same mechanisms do not apply to humans, who share the same biological mechanisms of testicular descent as rodents, but it remains to be proven whether human exposure to EDC mixtures may be responsible for the outcome of cryptorchidism (757).

E. Testicular cancer

The incidence of TGCC, which represents 95% of all testicular cancer cases, has increased in recent decades. Basic research suggests that TGCC has a fetal origin, and hormonal stimulation from puberty onward stimulates tumor development (758), with a peak incidence in young men. There are large incidence differences between regions and populations (759). The highest incidence is among Caucasians, with African Americans having much lower incidence than Caucasians living in the same geographic areas of the United States (759, 760). However, incidence has increased in both races. Genome-wide association studies have identified genetic polymorphisms that increase susceptibility to TGCC, but it has become apparent that environmental factors play an important role. Swedish immigration studies show that TGCC risk in the first generation of immigrants follows the pattern of their home country, whereas the second generation (born in Sweden) adopts the Swedish risk profile (761). All these findings point to environmental effects. Cryptorchidism and hypospadias are well-characterized risk factors of TGCC, and they have risk factors in common, such as small for gestational age. Because these diseases and birth defects are linked together and are also associated with poor semen quality, Skakkebaek et al (715) hypothesized that they could be signs of a common entity, TDS. Experimental animal studies show that exposure to EDCs with antiandrogenic properties (such as DBP, DEHP, vinclozolin, and procymidone) cause typical TDS defects and support the possibility that the same is the case in humans (720). However, TGCC does not develop in rats and mice as it does in humans, and animal models of human testicular cancer are lacking. Therefore, research on EDCs and TGCC are limited to human epidemiological studies.

1. PCBs and PBDEs

a. Humans. Although the roots of the disease may originate in utero, associations between EDC exposures and TGCC are based primarily on assessments in adults. Not surprisingly, these studies reported mostly negative results, ie, no association with occupational or environmental exposure (762). Hardell et al (763–765) studied both prenatal and postnatal exposures and found that maternal serum concentrations of PBDEs and PCB congeners (collected when the men were adults) were higher in the TGCC group than in controls, whereas no association was found between TGCC and PCB levels in the adult men themselves. One US study reported an inverse association between TGCC and PCB (730). Clearly, we need more studies assessing the association of fetal exposure to EDCs with TGCC, something that is extremely difficult to do due to the long lag between exposure in utero and adult outcome.

2. DDT and DDE

a. Humans. Five studies analyzed the association between TGCC and DDT and DDE (40, 764, 766–768). None of the studies found an association with DDT; however, four of the studies found a positive association with DDE (reviewed in 769, 770). A separate study reported an association between chlordane exposure (cis-nonachlor and trans-nonachlor) and TGCC; however, it did not establish any associations between TGCC and exposure to oxy-

chlordane, MC6, heptachlor, dieldrin, mirex, HCB, or HCH (729).

b. Animal studies. Although there are no good animal models for TGCC, ultrastructural changes in testicular histology have been used as a proxy for cancer risk (771). For example, phthalate esters produced structural abnormalities in rat testes after fetal exposure (711). Table 5 lists several other chemicals that have been shown to cause structural abnormalities in the developing testis and are therefore considered potential testicular germ cell carcinogens.

F. Semen quality

Insults to spermatogenic cells in adult men typically have a temporary effect on sperm production. Many cancer treatments and low-dose irradiation are examples of such insults. If the spermatogonial stem cells are not destroyed, sperm production can reappear after months or years. A good example comes from maternal smoking during pregnancy, which is associated with reduced sperm concentration and total sperm counts in sons, whereas an adult male's own smoking has less of an effect, if any (750). Moreover, combining exposure in utero with adult exposure impairs testicular function even more than either of these alone (772). Interestingly, maternal smoking does not seem to affect all male reproductive health problems because there is no association with testicular cancer and very weak or no association with cryptorchidism (773).

1. Humans

By contrast to smoking, chemical exposures to the developing testes, particularly those that influence spermatogonial stem cells or Sertoli cells, can cause irreversible changes that result in permanently low adult sperm numbers. Several studies have examined the association between adult PCB exposure and semen quality (774). The results varied considerably and included positive (775, 776), inverse (777, 778), or no associations (775–782). There are also conflicting results in studies that assessed sperm morphology and DNA integrity. Sperm morphology did not differ between exposure groups in three studies (776, 779, 780), was positively correlated in one study (777), and was inversely associated in another study (783). Sperm DNA integrity was impaired in association with PCB exposure in four studies (779, 784–786), whereas one study reported no association (778). Sperm motility was negatively associated in most of these studies. Thus, PCB exposure may affect sperm DNA integrity and motility, but the underlying mechanism is unknown.

Two studies from endemic malaria areas with high DDT use and levels (South Africa and Mexico) reported that DDE exposure was inversely associated with sperm motility (787) and total sperm count (787), and positively correlated with defects in sperm chromatin condensation and morphology (788). Inverse associations with sperm motility were also found in regions where DDE levels were not as high as in malaria areas (776, 781). Two other studies found no association (775, 778). Sperm concentration was positively but weakly associated with DDE exposure in two Nordic studies (775, 776). There are many studies showing no association between DDE and sperm morphology (776, 778, 780) or sperm DNA integrity (780, 784, 786). Follow-up studies on children born after in utero exposure during the Taiwanese food accident (high PCB and dibenzofuran exposure) showed that they had impaired sperm morphology and motility as adults (789).

Studies assessing men exposed in the Seveso accident have revealed no association between adult exposure to dioxin and any long-term effects on semen quality (150). In contrast, fetal and perinatal exposure via breast-feeding was associated with reductions in sperm concentration, number of motile sperm, and total sperm number. The critical exposure window appears to have been infancy because the effect was seen only in breast-fed sons and not in formula-fed sons (150). This exposure window occurs at the time of rapid Sertoli cell proliferation, which may explain the association.

Two small studies have assessed the association between semen quality and PBDEs used as flame retardants (790, 791). A Japanese study of 10 men found a negative association with sperm concentration and testicular size (790), whereas a Canadian study of 52 men found that only sperm motility was inversely associated with PBDE exposure (791).

Five studies examined the association between exposure to perfluorinated compounds and semen quality (792–796). Two of these studies found an inverse association with sperm morphology (793, 794), but otherwise there were no consistent findings. Men from Greenland had compromised sperm DNA integrity associated with PFOA, but not men from Poland or Ukraine (796). Fetal exposure to PFOA was inversely associated with sperm concentration and total sperm number in young adulthood, whereas there was no association with PFOS exposure (797).

Studies on semen quality suffer from the same problem as those on testicular cancer: long time lags between critical exposure and the manifestation of adverse effects. Although it is possible to conduct studies on POPs years after exposure due to the persistence of these chemicals, correlations with more rapidly metabolized compounds must be made much closer to the time of exposure. Our knowledge is therefore limited mostly to the POPs reviewed above. The studies clearly agree with the hypothesis that early exposure windows are key to determining the outcome because the adverse associations are most often

found in groups with early-life exposure (eg, to dioxin and PFOA). Forthcoming prospective studies of cohorts with early exposure to other POPs will advance our knowledge of a wider spectrum of chemicals. However, we will have to wait until the existing cohorts grow into adulthood. In addition, animal research shows that fetal exposure to dioxins, antiandrogens, and estrogenic chemicals affects semen quality as summarized in Table 5.

G. Conclusions

As a whole, research on EDCs and male reproductive function is suggestive of links between exposures and a range of disorders that include developmental abnormalities such as cryptorchidism and hypospadias, poor semen quality, and increased risk of TGCC (Box 3). However, drawing direct links and determining which EDCs may play causal roles in these aspects of TDS has not been possible in humans. The animal literature (Table 5) is beginning to identify potential candidates, especially antiandrogens. Much more research is needed on these and other EDCs to which humans are commonly exposed, including during prenatal and early-life development.

V. Hormone-Sensitive Cancers in Females

A. Introduction

The incidence of hormone-sensitive cancers of the breast, endometrium, ovary, testis, prostate, and thyroid has risen at a quicker pace than genetic drift over the last few decades (798, 799). Although some of the increase in diagnosis may be from enhanced detection and screening, it has been hypothesized that increases in these types of cancers are, in part, due to lifestyle choices (to include nutritional intake, age at which the first child is conceived, pharmaceutical use, and food/drink storage containers) and EDCs in the environment. Much of the research investigating the effects of EDCs and cancer has focused on the breast, prostate, and testes, whereas other less common hormone-related diseases such as endometrial and ovarian cancer have received little attention. Additionally, much of the attention in the area of environmental chemicals has been on trying to identify carcinogens in exposed adult humans and rodents. EDCs may have a different role in reproductive tissues than carcinogens; altered hormone receptor patterns, competition for endogenous hormone, inadequate feedback loops, and improper methylation patterns are just some results of EDC exposure that may disrupt development and disease risk for life.

Box 3. Key Points: Male Reproduction

- **Development of the male reproductive system is hormonally regulated, with androgens providing the driving force in masculinization of genitalia.**
- **Cryptorchidism and hypospadias are the most common birth defects of male reproductive organs, and their incidence has increased in many countries over the same period when the incidence of testicular cancer has multiplied.**
- **Although results are variable, a large proportion of men have decreased semen quality, resulting in delayed time to conception.**
- **Animal experiments demonstrate clearly that disruption of hormones regulating development of the reproductive tract cause cryptorchidism, hypospadias, poor semen quality, and ultrastructural testicular abnormalities that are similar to changes associated with human testicular cancer. These outcomes can therefore be considered signs of a common problem, TDS.**
- **Antiandrogens, xenoestrogens, and dioxins are the best-characterized endocrine disruptors of the male reproductive system. Antiandrogenic chemicals act additively, irrespective of their mechanism of action (ie, whether they are receptor antagonists or inhibitors of hormone synthesis).**
- **The number of known antiandrogenic and xenoestrogenic EDCs is increasing, and it remains to be established which of them might contribute most to human reproductive problems.**

B. Critical periods of mammary gland development

The mammary gland, a reproductive organ required for lactation in all mammals, is particularly sensitive to EDCs because its complex development involves growth, differentiation, secretory activity, and regression, all orchestrated by hormones, growth factors, and stromal factors. The multicellular components producing the signals for tissue development in humans and rodents are shown in Figure 7. Several cell types must coordinate their actions for normal mammary gland development, and they are all targets for endocrine disruption. Three stages of mammary gland development are particularly vulnerable to exogenous perturbation: the prenatal or gestational period, puberty, and pregnancy (800, 801). Other female reproductive organs, such as the ovaries and uterus, are more vulnerable to EDCs during the in utero, neonatal, and pubertal phases of development. This section focuses on mammary development.

Gestation, puberty, and pregnancy are known critical periods during which EDC exposure may affect mammary gland development (800, 801) (Figure 8). Endocrine disruption during

Figure 7.

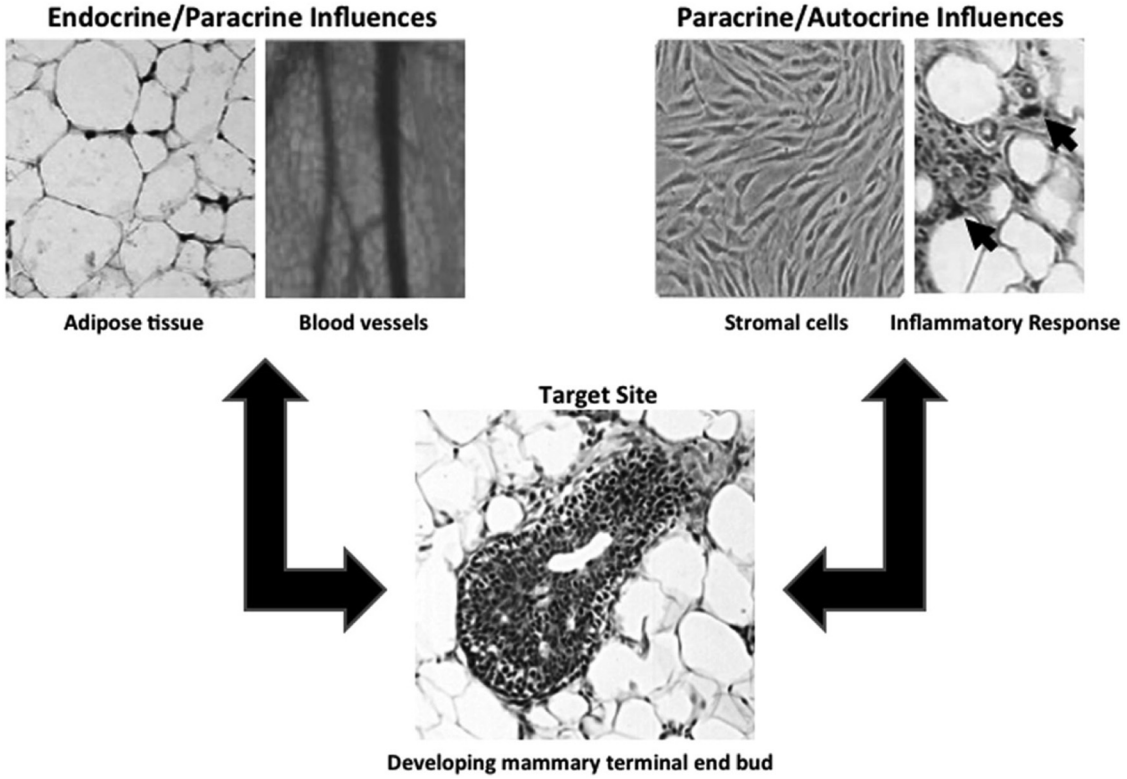


Figure 7. The multiple influences of the environment on mammary development. Environmental factors may affect breast development and later-life risk for disease or dysfunction via indirect and direct mechanisms. Environmental exposures may change endogenous signals (hormones and growth factors) that affect endocrine organs, as well as tissues near the mammary gland (ie, fat). Those nearby tissues can send atypical messages through the vascular system, culminating in perturbed mammary development. Mammary tissue may also be a direct target of environmental exposures; epithelia, fibroblasts, fat cells, and inflammatory cells express unique and shared receptors that are targets for environmental chemicals. The tight cellular junctions signal across cell types, affecting neighboring cells as well as the target cells (ie, epithelial cells in the terminal end bud). These various endocrine/paracrine/autocrine signaling mechanisms may affect the status of mammary epithelia over the lifetime of the individual. [Reprinted from Figure 2 in S. E. Fenton et al: Perinatal environmental exposures affect mammary development, function, and cancer risk in adulthood. *Annu Rev Pharmacol Toxicol*. 2012;52:455–479 (1276), with permission. © Annual Reviews.]

gestation, when the mammary epithelial bud is receiving signals to form ducts and extend into the fat pad, could lead to altered developmental timing and/or unusual formation of glandular structures. Epigenetic modifications during early life may also lead to altered programming of the gland and altered structure or function in later life. Additionally, stem cell nests may be modified during this time; their role in normal development has been rarely studied. During the pubertal period, mammary growth is exponential due to the proliferation and rapid division of terminal end buds (TEBs); the highly proliferative nature of TEBs is what makes these structures particularly vulnerable to EDCs. TEBs are the least mature undifferentiated terminal structures and the most susceptible, whereas lobules are more mature and less susceptible to chemical carcinogens (802). The TEBs eventually differentiate, and the mature gland settles into its normal adult cycle. Prolonging the window of time that TEBs are present could lead to increased risk of deleterious effects from a chemical carcinogen exposure. In fact, several studies have determined that the presence of TEBs at the time of exposure to a carcinogen is positively associated with tumor multiplicity (803, 804). This is likely due to the presence of stem cells, the hypothesized site of eventual tumor initiation, in the TEBs during puberty. The microenvironment of the gland at this time may also play a role in eventual risk for tumor formation. Modified stromal makeup (increased breast density) may lead to a more permissive environment and enable a switch from epithelial cell dormancy to metastatic growth (805). Finally, the gland undergoes a third critical period of development during pregnancy as it prepares itself for functional lactation. Interruption of lactation can lead to mortality or malnutrition of the offspring and is particularly important in wildlife and domestic species that rely solely on maternal nourishment for repro-

Figure 8.

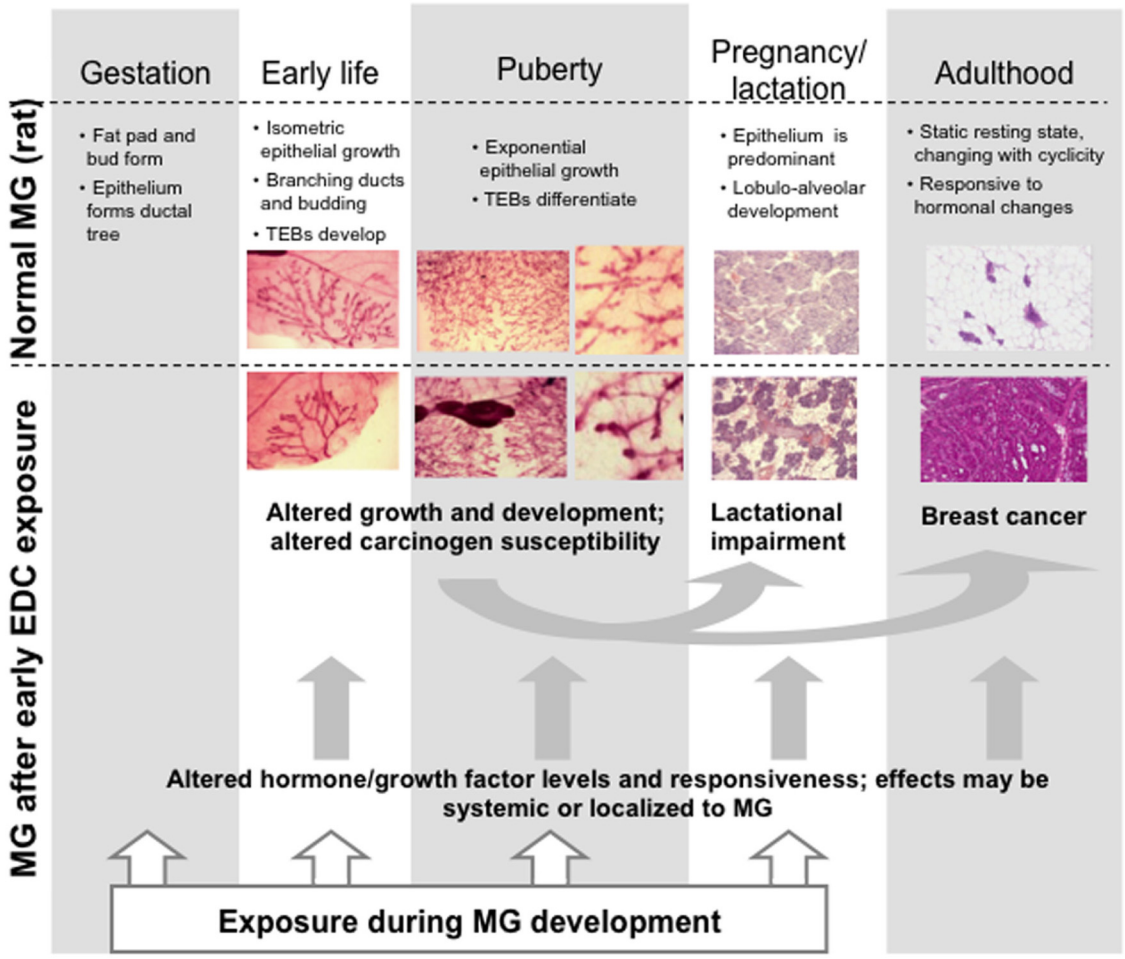


Figure 8. Stages of normal rat mammary gland (MG) development and effects of environment on subsequent events. Different effects and outcomes after EDC exposure are strongly dependent upon the age of exposure (neonatal period, puberty, pregnancy) and time of analysis. Early-life effects such as altered thelarche or gynecomastia present themselves in adolescents, whereas effects on lactation or mammary tumorigenesis become evident during adulthood. Arrows indicate plausible (gray) or more certain (white) mechanistic pathways. Photomicrographs for early life and puberty were all taken at 16× magnification on a macroscope. [Adapted from R. R. Enoch et al: Mammary gland development as a sensitive end point after acute prenatal exposure to an atrazine metabolite mixture in female Long-Evans rats. *Environ Health Perspect*. 2007;115:541–547 (884), with permission.] Photomicrographs for pregnancy/lactation and adulthood were taken at 10× magnification on a standard microscope (from S.E.F.). [Reprinted from Figure 1 in R. A. Rudel et al: Environmental exposures and mammary gland development: state of the science, public health implications, and research recommendations. *Environ Health Perspect*. 2011;119:1053–1061 (801), with permission.]

duction. Pregnancy has been shown to be protective of breast tissue to later-life disease (cancer) in humans (800).

C. Effects of EDCs on the mammary gland: rodent models and epidemiological studies

Research on EDCs and mammary tissue has been informed by the history of certain estrogenic pharmaceuticals used over the last several decades. These compounds, especially DES, tamoxifen, and hormones given to perimenopausal women, have helped experts to define and further understand the critical windows of susceptibility in regard to cancer development in reproductive organs. Some of these drugs given for the purpose of having endocrine "correcting" effects resulted in endocrine "disruption" instead, along with long-term adverse effects (95).

There are numerous environmental chemicals that are linked to mammary gland and breast development and an enhanced risk for cancer development. Some of this evidence is from epidemiological studies, but the majority is from rodent models. Because a comprehensive literature review was recently completed on these chemicals (801), we used this as a starting platform. We performed another PubMed search looking for the keywords breast, environment, exposure, mammary gland, cancer, tumor, devel-

opment, dioxin, TCDD, PCB, BPA, phthalate, DDT, DDE, xenoestrogen, perfluorochemical, PFOA, ATR, chlorotriazine, DES, ethinyl estradiol, and estrogen to update the knowledge base in this document. Of these EDCs, we have summarized the modes of action for many of those most commonly studied in the mammary gland (Table 2). Notably, these mechanisms apply to EDC actions in other systems discussed throughout this review.

Data that were strictly single cell type-based (not representative of the breast cellular makeup) were excluded, unless there was in vivo confirmation of the effect. We included rat, mouse, nonhuman primate, and human data, regardless of whether the data reported a positive or negative outcome. The data discussed here do not represent all chemicals known to affect the breast, but highlight several examples for which both positive and negative data have accumulated.

1. Dioxins

There are more than 400 types of dioxin-related compounds, about 30 of which are significantly toxic to human health, with TCDD being the most toxic. TCDD is an organochlorine that is a product of industrial processes, such as paper bleaching, smelting, and the manufacture of herbicides and pesticides. TCDD is lipophilic and has a long half-life in humans and large mammals (eg, whales, polar bears) of 7–11 years, which causes it to bioaccumulate in animals and the environment (806). TCDD is a ligand for the AhR and functions as a signal transducer with antiestrogenic actions (807). TCDD alters development in a wide range of estrogen-responsive female tissues including the vagina (808, 809) and the mammary gland (810). TCDD can also reduce estradiol production and elevate ER mRNA expression in exposed rat uteri and ovaries (811).

a. Humans. Studies of adolescent Belgian children living in a dioxin-polluted suburb revealed a correlation between high serum levels of dioxin and an increased chance of delayed breast development (812). Likewise, high prenatal and/or lactational exposure to dioxin-like compounds was significantly correlated with delayed initiation of breast development in a longitudinal study of Amsterdam-born adolescents (813). In that study, dioxin measurements at the time of puberty were not correlated with pubertal development, demonstrating a critical window (fetal/infancy) for the effects of dioxin on breast development.

In Seveso, Italy, an industrial accident in 1976 resulted in the highest contamination of TCDD known in human residential populations. Data collected after this accident have helped researchers evaluate the potential long-term effects of TCDD exposure on cancer incidence. Ten- and 15-year follow-up studies found no increase in incidence or mortality (814, 815), and a 20-year follow-up study found that women who resided in the most heavily contaminated zones had a slight increase of cancer risk (816). However, these results were based on only five cases. The Seveso cohort was reinvestigated in the Women's Health Study to explore a possible link between breast cancer incidence and early-life exposure. Serum from women who were infants at the time of the accident had a 10-fold increase in serum TCDD, which was associated with a 2-fold increase in breast cancer incidence (817). These women are not yet between the ages of 40 and 55 years, the age of highest breast cancer risk; therefore, it is important to continue to follow and assess breast cancer incidence in these women as they age. A second epidemiological study in Chapayevsk, Russia, a region contaminated by dioxin, reported elevated breast cancer mortality among women living near the chemical plant (818).

b. Animal studies. Studies conducted in rodents exposed in utero to dioxin have reported a range of mammary gland developmental effects that are consistent with the delayed developmental effects in children. These include an increased TEB number in exposed rats at a time when duct ends in control rats are differentiated (810, 819), undersized mammary glands with decreased branching (819), and deficient lobule development (819, 820). Rat studies also confirmed that prenatal exposure to dioxin was the critical window for persistent developmental effects (819). Prenatal exposure to dioxin also increased the number of chemically induced mammary adenocarcinomas in female rats (810, 821).

2. DDT and DDE

a. Humans. Human studies have revealed that DDT and DDE may affect a woman's ability to lactate. Mothers with high levels of DDE (milk and serum levels measured) tended to discontinue breast-feeding sooner than those with lower levels of exposure (822, 823). Furthermore, a second study found that women who previously breast-fed demonstrated shortened lactation duration associated with DDE exposure (824); these effects were proposed to be due to lactation failure. In contradiction to this, other studies have found no association between DDT or any of its metabolites and lactation duration (825, 826). Research has shown that DDE has estrogen-like or antiandrogenic activity (39), and this may interfere with normal hormonal control during lactation.

Exposure to DDT or its metabolites has been inconsistently associated with an increase of breast cancer incidence. Little evidence for increased risk was reported in studies measuring exposure levels at the time of diagnosis

(827). In a recent meta-analysis that found no link between DDT or DDE levels and breast cancer (828), there was large variability in data sets regarding study designs, method of reporting exposures, and consideration of variables such as age and menopausal status. A case-control study by Cohn et al (829) that took age at exposure into consideration revealed that high levels of serum *p*,*p'*-DDT correlated with a statistically significant 5-fold increased risk among women who were born after 1931. These women were exposed to *p*,*p'*-DDT before 14 years of age because DDT was in peak use in 1945. These findings substantiate the importance of evaluating exposures at younger ages when assessing breast cancer risk and highlight the sensitivity of the mammary gland around puberty. With that in mind, another study examined associations between self-reported acute exposure to a DDT fogger truck and ER-positive/PR-positive breast cancer in the Long Island Breast Cancer Study project. An increased risk of breast cancer for women who were 20 years of age or less that reported seeing a fogger truck, compared to women in the same birth cohort that did not see a fogger truck, was reported (830).

In an effort to sort out the conflicting association between breast cancer risk and DDT and its metabolites, Boada et al (831) hypothesized that most studies did not take into account that human beings are exposed to multiple organochlorine pesticides simultaneously and that these mixtures could exert different effects than those exerted by one chemical alone. That study evaluated serum profile of mixtures of organochlorines in healthy women and women diagnosed with breast cancer from Gran Canaria Island. Although the serum levels of DDE and DDD were higher in breast cancer patients than in healthy women, after a multivariate analysis, only DDD showed a moderate increase in the risk of developing breast cancer. Breast cancer patients presented more frequently with a combination of aldrin, DDE, and DDD, and this mixture was not found in the healthy women (831).

b. Animal studies. Studies in animals have reported several developmental effects due to exposure to DDT at different critical periods. Pubertal acute exposure to DDT in rats acted as a morphogen causing increased mammary cell proliferation, which promotes the progression of TEBs to more differentiated lobular terminal ductal structures (832). However, rats dosed with DDE 5 weeks before mating and through gestation and lactation exhibited normal lactation (833).

Most of these studies centered on the estrogenic congeners *o*,*p'*-DDT and *o*,*p'*-DDE, but one study explored the antiandrogenic *p*,*p'*-DDE and its effects on mammary tumor multiplicity in a tumor-forming strain of mouse. After implanting *p*,*p'*-DDE pellets into MMTV-Neu transgenic mice, Johnson et al (834) found that *p*,*p'*-DDE did not increase mammary tumor multiplicity but did act as a tumor promoter. Tumors were detected at 3 months of age in mice treated with *p*,*p'*-DDE pellets, nearly 2 months before the first tumor appeared in the control group, suggesting that early-life exposure to *p*,*p'*-DDE may predispose females to early-onset breast cancer (834).

3. Diethylstilbestrol

a. Animal studies. In addition to the better-known effects of early-life DES exposure on reproductive tract development and vaginocervical cancer in humans, animal studies have also shown that DES exposure affects mammary glands. In utero and lactational exposure to high doses of DES increased mammary gland growth and decreased the number of TEBs (835). Neonatal high-dose exposure to DES triggered extensive ductal dilation at P33 and promoted precocious lactogenesis in postpubertal, nulliparous 12-week-old female mice (836). In contrast, low-dose DES reduced ductal branching at P6 and P33 (837). Low-dose exposure to DES during pregnancy caused impaired lactation in rats (838).

Gestational exposure of DES in rats resulted in an increase in spontaneous mammary gland tumors (839). Furthermore, multiple studies in mice showed that prenatal exposure to DES increased the risk of mammary tumorigenesis in females exposed to a known carcinogen, as well as the numbers of TEBs (840, 841). In mice treated prenatally with DES there was a significant increase in enhancer of Zeste homolog 2 (EZH2) protein and EZH2 activity (measured by increased mammary histone H3 trimethylation)—a histone methyltransferase that may be linked to breast cancer risk and epigenetic regulation of tumorigenesis, as well as an increase in adult mammary gland EZH2 (842). However, two studies found that relatively high-dose exposure during neonatal development reduced TEB numbers and prevented spontaneous mammary gland tumors (841, 843). The mechanism for effects of DES on reproductive tissues likely varies by tissue. Taken together, this highlights the likelihood that DES daughters may have increased risk of adverse breast outcomes, cancer, and developmental abnormalities of the vagina.

4. Perfluorooctanoic acid

PFOA, also referred to as C8, is a well-studied synthetic surfactant that has proven to be a developmental toxicant. Industrial uses for PFOA include grease proofing, waterproofing, stain repellents, fire-fighting foams, and friction reducers. Consumer products that contain PFOA include dental products and food container linings. PFOA is an

environmentally ubiquitous, persistent, and stable compound that has a half-life of 16–22 days in mice and 2–4 years in humans (844). It does not share any structural similarities with other EDCs, and although it is capable of binding to ERs and causing binding of a compound-ER interaction with the response elements, it has not traditionally demonstrated estrogenic activity in mammals (845, 846), although this type of activity has been reported in fish (845). In vitro studies have shown that PFOA in combination with estradiol is both estrogenic and antiestrogenic (845), but it does not interfere with or enhance estrogenic signaling in uterotrophic assays (515).

a. Humans. Epidemiological studies have suggested a positive association between PFOA and delayed puberty, early menopause, and pre-eclampsia (569, 847). Delayed pubertal timing (self-reported) was correlated with high PFOA concentrations in young girls living near a chemical plant in the Mid-Ohio Valley (569). Due to the positive association of PFOA with factors that reduce breast cancer risk, it has been suggested that PFOA may also reduce breast cancer risk. However, recent data from the highly exposed population of Greenlandic Inuit women revealed a positive association between serum concentrations of perfluorinated compounds and increased breast cancer risk (849).

There are, to our knowledge, no reports on the relationship between breast developmental timing and early-life perfluorinated exposure in girls. However, the Breast Cancer and the Environment Research Program reported two important findings that may link the two endpoints. First, researchers found a linear relationship between the amount of breast-feeding time and the serum PFOA levels measured in girls at 6–8 years of age (850). In the same study, there was a highly significant relationship between the sources of the drinking water in an area in northern Kentucky called the Cincinnati Center and girls' serum PFOA concentrations. This suggests that the child is exposed to PFOA through breast milk. Second, a study that compared formula- and breast-fed girls found that girls that were predominantly breast-fed underwent later-onset breast development (850). Breast-feeding duration was directly associated with delayed age at onset of breast development ($P = .008$). This effect was evident in the Cincinnati Center girls, and the entire three-center cohort. These studies suggest that early-life exposure to PFOA via drinking water or breast milk may result in later-onset breast developmental timing, as has been shown in mice. Overall nutritional status is certainly an important consideration in these types of studies.

b. Animal studies. The link between PFOA and altered breast development timing and function was first uncovered by studies in animal models. PFOA delivered to pregnant mice caused a lack of normal lactational competency in the exposed dam (851) and led to increased offspring mortality (851, 852). Gestational PFOA exposure resulted in delays in mammary gland epithelial cell development and ductal elongation, as well as reductions in ductal branching and TEB appearance at weaning in CD-1 female mouse offspring (851, 853). Abnormal mammary gland maturation persisted at 18 months in these animals and included increased epithelial hyperplasia and increased stromal density—factors associated with increased breast cancer risk (851, 853–855). In addition, White et al (855) demonstrated that chronic low-dose PFOA exposure, similar to the concentrations found in highly contaminated human water supplies, slowed mammary gland differentiation in F1 and F2 female CD-1 mice. In subsequent studies, using doses that were 10- and 30-fold lower than in the previous studies, exposures for both full gestation (1–17 d) and half gestation (10–17 d) focused around the time of mammary bud development were sufficient to stunt mouse mammary gland development (856) and alter gene expression required for normal gland development. Another study found similar developmental deficits after low-dose prenatal exposures in C57Bl/6 mice (570), with no change in the timing of vaginal opening or first estrus. Studies that reported on PFOA exposure in the peripubertal period found no effects at 1 mg PFOA/kg body weight or less and mixed effects at higher doses, including altered estrous cyclicity, decreased ovarian steroid hormonal synthetic enzymes, and reduced gene expression of steroid-induced mammary growth factors. Results varied significantly depending on the strain of mouse tested (570, 857). A recent paper highlighting the strain differences in metabolism or excretion of PFOA reported that when Sv/129 mice received doses three to 300 times higher than those that affected CD-1 mice, Sv/129 mice had no mammary effects and lower than expected PFOA blood concentrations (858).

5. Bisphenol A

BPA shares some structural similarity to estradiol and binds to ERα with weak affinity (859). BPA has a strong binding affinity to the estrogen-related receptor γ and the G protein-coupled ER (GPER) (860, 861). In vitro BPA induced the proliferation of an ERα-positive breast epithelial carcinoma cell line (MCF-7) and increased the expression of the PR (862).

a. Animal studies. Many in vitro and rodent-based studies have shown that BPA is an estrogenic chemical that can act

as an endocrine disruptor, even at low doses (863). However, the evidence linking elevated levels of BPA in humans and increased breast cancer risk is both lacking and unconvincing (863). This may be due to the short half-life of the chemical, the difficulty in assessing exposure during susceptible life stages, and the growing list of chemicals (analogs) on the market that may replace it.

On the other hand, many animal studies have demonstrated that BPA alters mammary development, growth, and the risk for forming tumors. Fetal exposure to low, human-relevant doses of BPA altered the development of mouse mammary glands by increasing the percentage of ducts, TEBs, and alveolar buds at 6 months of age (864). In addition, perinatal low-dose exposure resulted in enhanced lateral branching and increased TEBs at puberty in mice (865). Of special concern is the ability of BPA to increase TEB density because these structures contain the cells that are the proposed targets in which cancer arises in humans and rodents. Gestational exposure to BPA in rats was associated with preneoplastic lesions by P50 and mammary gland adenocarcinomas by P90 (866). More recently, BPA given orally during gestation and through 90 days of age induced a significant increase in minimal grade ductal hyperplasia in female rats at 21 days in three dose groups and significant hyperplasia at 90 days in the highest dose group. When lesion severity was taken into consideration, significant hyperplasia at 90 days was evident in two of the three dose groups that demonstrated that effect at 21 days (362). In the 90-day-old group, the National Center for Toxicological Research study reported a mammary gland ductal adenocarcinoma at 2.5 μg BPA/kg body weight per day. BPA administration was also associated with ductal hyperplasia in 90-day-old male rats when severity was taken into consideration (362). Rats and mice exposed to BPA during early life were susceptible to carcinogen-induced mammary tumors and showed a decrease in tumor latency (867, 868). Recently, prenatal BPA exposure was shown to induce growth and branching of the male CD-1 mouse rudimentary gland (869) and the male rat mammary epithelium (870). Nonhuman primate studies reported similar growth-promoting effects of the immature female breast linked to BPA (871).

6. Phthalates

a. Humans. In a study of nearly 1200 peripubertal girls (550), urinary concentrations of high molecular weight (including DEHP) phthalates, known to have antiandrogenic effects in other studies, were associated with delayed pubic hair acquisition. Age at breast development in that same study was older in girls in the fifth quintile of urinary MBzP concentrations compared to those in the first quintile. However, in a small cohort of Puerto Rican girls, significantly higher levels of high molecular weight phthalates [dimethyl, diethyl, dibutyl, and di-(2-ethylhexyl)] and its major metabolite, MEHP, were identified in 68% of samples from precocious thelarche patients (872).

A recent case study examined the association between urinary concentrations of phthalate metabolites and breast cancer in women of northern Mexico. Phthalates were detected in 82% of women, and the concentrations of MEP were higher in breast cancer patients than in controls. Conversely, MBP, MEOHP, and mono (3-carboxypropyl) phthalates were inversely associated with breast cancer risk because they were more abundant in control women. The authors concluded that exposure to diethyl phthalate, the parent compound of MEP, may be associated with a 2-fold increase in breast cancer risk (873).

b. Animal studies. DBP induced reproductive toxicity in rodents and has the potential to bind weakly to ERs (874, 875). In rats, DBP exposure from late gestation through lactation resulted in poor mammary alveolar branching and hypoplasia in the adult female offspring (876). By contrast, in utero exposure to BBP resulted in a marginal acceleration of mammary gland growth by increasing the proliferative index of TEBs, but delaying and in delayed pubertal onset (877, 878).

7. Atrazine

a. Humans. Epidemiological studies have revealed little or no association between occupational or agricultural exposures to ATR and breast cancer. In 1997, an ecology study showed a statistically significant increase in breast cancer risk with triazine exposure from contaminated surface water in the years 1991–1992 in Kentucky. Additionally, this paper reports an association between groundwater contamination and increased breast cancer, but no such association was found for surface water contamination in the years 1993–1994 (879). The authors noted that the inconsistent associations seen between the two measurements may have been due to surface water data that did not measure actual triazine levels but instead was given a positive or negative detection score. However, Muir et al (880) used a population census and pesticide application data from 1989–1991 in urban and rural regions of Lincolnshire and Leicestershire counties in the United Kingdom and observed a positive association between ATR application and breast cancer for the rural regions of Leicestershire County, but not for Lincolnshire County. In a second Kentucky study using data from 1993–1997, investigators found no association between ATR-contaminated water and breast cancer risk (881). Similar results have been seen by McElroy et al (882) in a case-control study of ATR-contaminated well water in

Wisconsin. Overall, these studies, however limited, do not suggest an increased risk of breast cancer from adult exposure to ATR. Considering the rodent data on early-life exposure to ATR and its connection to mammary gland tumorigenesis (see *Section V.C.7.b*), more studies are needed to investigate the relationship between levels and timing (ie, prenatal, adult) of ATR exposure and breast cancer in humans.

b. Animal studies. Several rat studies have examined the impact of ATR on the developing mammary gland during gestation. ATR exposures caused delayed mammary gland development (small gland size, sparse branching, and abundant TEBs) in female offspring compared to controls (883). Low-dose prenatal exposure to ATR and its metabolites produced similar effects in offspring (884). Later studies carried out in less sensitive rat strains (560) or in rats with an abundance of pup death (885) found that ATR had little effect on mammary epithelial development. This underscores the importance of the species and strain in evaluating endocrine-disrupting actions of environmental chemicals.

Although ATR is not classified as a direct carcinogen, chronic dietary ATR exposure increased mammary adenocarcinomas in female Sprague-Dawley rats and increased hyperplasia in high-dosed males (886). In contrast, ATR did not increase tumorigenesis in female F334, male Sprague-Dawley, or male F334 rats (887, 888). In addition, chronic high-dose dietary ATR caused lengthened estrous cycles, increased days in estrus, and increased the incidence or earlier onset of mammary tumor formation in female Sprague-Dawley rats (888). Moreover, ATR increased the incidence of mammary adenomas and adenocarcinomas in human c-HA-*ras* proto-oncogene transgenic rats that were pre-exposed to a chemical carcinogen (889).

D. Uterine cancer, ovarian cancer, and EDCs

Endometrial adenocarcinoma, commonly referred to as uterine cancer, is the most common malignancy of the female genital tract, and incidence rates are rising as life expectancy increases. Type I endometrial cancer is associated with endometrial hyperplasia in premenopausal and perimenopausal women with a history of elevated estrogen levels (890). Type II endometrial cancer is associated with endometrial atrophy in older women and is estrogen-independent. Numerous studies have shown that elevated levels of unopposed endogenous estrogens are associated with an increased risk of endometrial cancer (891). Despite the role hormones play in disease progression, only a handful of studies have examined the risks of EDC exposure and endometrial cancers.

1. Humans

In 1995, a study by Sturgeon et al (892) did not find a significant association between serum DDT levels and endometrial cancer in women. A second study confirmed these results (84), showing that neither DDT nor PCB exposure increased risk. A third study in women found a weak but nonsignificant association between serum DDE levels and an increased risk of endometrial cancer (41). All of these studies investigated DDE concentrations of endometrial cancer patients compared to hospital-based controls, but none explored the possibility that fetal exposure to EDCs may be of importance. Thus, we need future studies to clarify potential links between fetal exposure to DDT and endometrial cancer.

Several epidemiological studies have revealed an increased risk of endometrial cancer in association with EDC exposure. Occupational exposure to dioxins increased endometrial cancer risk in female workers (893). No difference in serum BPA levels was found in patients with simple hyperplasia compared to healthy controls. However, BPA levels were lower in women suffering from hyperplasia that had malignant potential than in patients with endometrial cancer (894). Also, phthalate metabolites are associated with a 2-fold increase in magnetic resonance imaging diagnoses of endometriosis (618), and another study revealed that as the concentration of phthalates in urine increases, so does the risk of endometrial effects (619). Other combinations of EDCs have been implicated in uterine cancer, but limited evidence is available for the link between any individual EDC and uterine cancer (895).

In Western countries, ovarian cancer is the leading cause of death from a gynecological malignancy. A majority of ovarian tumors overexpress ERα, and this facilitates tumor growth through estrogen signaling (896). High serum levels of estrogens and hormone replacement treatments are potential risk factors for ovarian cancer, but the signaling pathways are not completely clear (897, 898).

EDCs might also increase ovarian cancer risk, but studies are limited in number. In a population-based case-control study, women previously exposed to chlorotriazine herbicides showed a significant 2.7-fold increased risk for ovarian neoplasms (899). In contrast, an epidemiological study showed a weak but nonsignificant association between ovarian cancer and occupational exposure to chlorotriazines (900). In the Agricultural Health Study (a large cohort of private and commercial pesticide applicators from Iowa and North Carolina), investigators found increased risk of ovarian cancer among women employed as private applicators (901). All of these studies demonstrating consistent effects of herbicide exposure and ovarian cancer risk are limited by the number of women that work as pesticide applicators, so combining and analyzing data

sets collected with similar variables in the future would be recommended. Research conducted in 2013 revealed that higher PFOA levels were associated with ovarian cancer among residents living near the DuPont Teflon plant in West Virginia (902).

2. Animal studies

In rats, neonatal exposure to DES induced transcription of estrogen-responsive genes and caused an increase in susceptibility to develop hyperproliferative lesions, thought to be precursors of endometrial cancers (904, 905). In mice, neonatal exposure to DES caused more than 90% of female offspring to develop endometrial cancer by 18 months of age (906). A recent study revealed that an in utero exposure to BPA could elicit an endometriosis-like phenotype in female mice offspring (472). TBBPA, a commonly used flame-retardant replacement, was recently reported by the NTP to induce aggressive uterine cancer in rats (2014 NTP Technical Report, CAS no. 79-94-7), potentially by altering steroid enzymatic activity. Finally, animal studies have shown that chronic exposure to TCDD promotes the development of ovarian tumors in female Sprague-Dawley rats (903). These examples underscore the need to further investigate female reproductive cancer endpoints in rodent models and its relationship with environmental exposures that demonstrate structural or mechanistic similarity to DES, BPA, or TBBPA.

E. Cellular and molecular mechanisms of EDCs in mammary, ovary, and uterus

Most studies examining the mechanism by which EDCs exert their effects have focused on nuclear hormone receptors. This large superfamily includes ERs, ARs, and PRs that play diverse roles in development, proliferation, and metabolism. Beyond these traditional mechanisms, EDCs can also affect transcriptional coactivators, enzymes of steroid hormone biosynthesis, nonsteroid receptors (thyroid, retinoid, glucocorticoid, PPAR-related signaling, and AhR), DNA-mediated mechanisms (mutagenic and epigenetic), and microenvironment signaling. Table 1 lists a number of examples by which EDCs disrupt female reproductive organ development and function and increase the risk for cancer. Outcomes for various target organs are listed separately under each EDC because the effect of an EDC on the breast may be entirely different from the effect on the uterus; ie, estrogens are "protective" in the breast and may have "hyperproliferative" effects in the uterus, and progestins may enhance breast cancer risk but act to protect the uterus from cancer. However, we need additional studies to better understand these mechanisms, as well as yet undiscovered pathways for current and potentially new EDCs.

F. Conclusions

Over the years, evidence has accumulated for the potential adverse effects that EDCs have on breast development and risk for cancer susceptibility. EDCs may act at many levels to disrupt female reproductive tissue development and to make tissue more vulnerable to a subsequent hit of an environmental chemical or hormonal insult. For decades, chemicals were tested in rodents at high doses for carcinogenicity, but today we understand that effects of chemicals at low levels may show endocrine-disrupting activity that is not present at high exposure levels. Therefore, it is important to consider the modifying effects of EDCs on female reproductive tissues. Several chemicals, such as PFOA and BPA, are known to modify the stromal compartment of the mammary gland (907, 908) or uterus (515) in rodents, and others have clear effects on reproductive function in the female (see *Section III*) and brain (see *Section VIII*). Many of these outcomes are known risk factors for breast cancer (timing of puberty and menopause, age at first child, breast density, etc) and should be considered as mediators/modulators of the latent effects of EDCs in the breast, uterus, and ovary.

Adding to this complexity is the fact that EDCs also act as obesogens (see Section II). Obesity plays a significant role in pubertal timing; fat makes up the primary portion of the developing breast tissue and may house lipophilic chemicals for extended periods of time. Furthermore, obesity plays a role in breast cancer risk. These multifactorial effects of EDCs may enhance obesity, alter normal hormonal milieu, and change the developmental patterns of reproductive tissues all at once, leading to a snowball effect for the EDC or mixture of EDCs in question. Furthermore, although EDCs are often investigated as single chemicals for effects on disease in rodent and epidemiological studies, evidence is accumulating that EDCs sharing structural homology, the ability to bind and activate certain receptors, or similar outcomes in high-throughput tests designed to indicate endocrine-disrupting activity may act in like ways and should be considered in groups or mixtures.

VI. Prostate Gland Disruption

A. Prostate Development and Hormone Sensitivity

The prostate gland is a male accessory gland that produces seminal fluids that transport sperm and enhance their fertilizing capacity. A critical feature of the prostate gland, particularly with regard to EDCs, is its absolute dependence on hormones, especially androgens, for embryological development, growth, function, and homeostasis throughout life. Heightened androgen action in the

prostate gland is made possible by expression of 5α-reductase (SRD5A1 and SRD5A2), which converts testosterone to dihydrotestosterone, an androgen with 5-fold higher affinity for AR relative to testosterone. Several additional steroidogenic enzymes expressed in the prostate are critical for balanced steroidal regulation of the gland and prostate health. Prostate cells and tissues express AR, ERα, ERβ, other nuclear hormone receptors (retinoic acid receptor, RXR, AhR), membrane receptors (GPER), and the vitamin D receptor (VDR) in a cell-specific and developmentally dependent manner (909). Protein hormones such as prolactin (PRL), GH, and IGF-1 also have specific actions in the prostate through receptors expressed in prostate epithelial cells (910–912).

In addition, there is a strong influence of androgens, estrogens, and other steroid and protein hormones in both prostate cancer and benign prostatic hyperplasia (BPH) regarding etiology, progression, and therapeutic modalities. Initially, all prostate cancer is dependent on androgens, whereas aberrant AR signaling and altered steroid metabolism play essential roles in androgen-dependent progression to castration-resistant prostate cancer (for reviews, see Refs. 913–915). Importantly, there is clear evidence from both humans and rodent models that estrogens can initiate prostate cancer and drive progression (916–918). Similarly, androgens combined with elevated estrogens are essential for the development of BPH (919). Thus, EDCs that augment or interfere with these hormone signaling pathways have marked potential to influence prostate disease incidence and progression in the human population. Prostate stem cells also play a role in these processes because there is an equilibrium between self-renewal and differentiation in prostate epithelial stem cell populations (920) that may be altered by exposure to EDCs.

The importance of developmental exposure and latent disease in the prostate is exemplified in sons of mothers who used DES during pregnancy. These individuals had structural abnormalities in the prostatic utricle with persistent ectasia (dilation) (921), similar to reports in rodent studies of developmental estrogenization. Studies conducted to date on prostatic disease incidence in DES-exposed sons followed through 25–60 years of age have not identified an increased risk of BPH or prostatic inflammation (922); however, they did not assess prostate cancer. Because these cohorts are only now entering the age for elevated BPH rates and prostate cancer onset, a thorough follow-up over the next 25 years is needed to accurately assess whether fetal DES exposure influences prostate disease risk and outcomes. The animal findings, together with the literature showing the importance of appropriate endogenous hormonal exposures for normal prostate development and function support a developmental basis of adult prostate diseases as a function of early-life hormonal imbalances and, potentially, EDC exposures that modulate these pathways.

Box 4. Key Points: Hormone-Sensitive Cancers in Females

- **Incidences of breast (especially in men and women under 40 years of age), endometrial, and ovarian cancers are increasing, and it is suspected that EDCs and other environmental factors are contributing to this increase.**
- **Critical periods of mammary gland development enhance susceptibility to lifelong or persistent adverse effects of EDC exposure.**
- **Rodent studies have identified EDCs altering mammary development, susceptibility to tumors, and lactation after critical period exposures. Examples include industrial chemicals, pollutants, herbicides, and pharmaceuticals.**
- **Dioxin is an EDC demonstrating DOHaD principles; early-life exposure leads to delayed pubertal breast tissue development in female rodents and in girls. Dioxin has also demonstrated effects on later life lactation and breast and ovarian cancer risk.**
- **Recent epidemiological studies focused on adult breast cancer risk indicate the importance of evaluating EDC exposures at a young age when assessing hormone-sensitive cancers in women.**
- **Mechanisms of action for female reproductive cancers are not well understood; the roadblocks result from the innate complexity of tissue-specific multicell communication, lack of appropriate in vitro test systems, variables such as obesity and cyclicity, and latency (often 2–3 y in rodents and 40–50 y in women) from the time of exposure to adverse outcomes.**
- **Because many targets of EDCs are conserved across species, future epidemiological studies focused on mechanisms of action for cancer of the breast, ovary, or uterus should test reported rodent biomarkers of effect and vice versa to expand translation of effects.**
- **There is a critical need for testing mixtures of EDCs based on their structural or activity homology and for integrating adequate precancerous cell lines representing female reproductive tissues in high-throughput chemical screening and testing.**

B. EDC actions in the prostate gland

The previous EDC review (1) highlighted research studies available on EDCs and prostate cancer, using both epidemiology data and work from animal models that either supported these findings or, in the absence of any human data, predicted that effects may occur. This update will summarize highlights of the previous review, expand the discussions with new data, and evaluate agents not previously discussed for which prostate-specific evidence is now available (Table 6). The focus will be on human epidemiology or other human-related studies with additions, where available, of in vitro and in vivo animal models that provide controlled conditions and mechanistic insight. It should be noted that spontaneous prostate cancer in rats and mice occurs at very low incidence and thus does not effectively recapitulate the high incidence of prostate cancer in men. Use of rodents as prostate cancer models requires the use of chemical carcinogens and hormonal or genetic manipulation with >1 year for tumorigenesis (923). Thus, negative tumorigenic findings in rodents must be interpreted with caution.

1. Farming and pesticides

a. Human exposures. The most compelling data for a link between prostate disease and environmental factors apart from diet comes from extensive epidemiology studies on occupational exposure to pesticides, largely in the farming communities, and increased prostate cancer. The primary

Table 6. Effects of EDCs on Prostate Cancer Risk and Mortality

EDC	Exposure Period	Effect	Ref.
Human studies			
Agent Orange/dioxin	Adult	Increased PCa risk, increased recurrence, progression	944–948
Aldrin (pesticide)	Adult	Increased aggressive PCa, increased PCa risk with family history	927, 928
Arsenic (heavy metal)	Adult	Increased PCa incidence, mortality	986, 990
ATR (pesticide)	Adult	Increased PCa risk with family history	937
BPA	Development	Increased carcinogenic susceptibility	960
	Adult	Increased PCa incidence	958
Cadmium (heavy metal)	Adult	Increased or decreased PCa risk	999–1001
Coumaphos (pesticide)	Adult	Increased PCa risk with family history	927, 928
DES	In utero	Persistent ectasia	921
DDT/DDE	Adult	No change in PCa risk	976, 977
Dieldrin, lindane, toxaphene, MXC, heptaphor, dicofol (pesticides)	Adult	Increased PCa risk	934, 1213
Endosulfan (pesticide)	Adult	Increased PCa risk	933, 934
Fonofos (pesticide)	Adult	Increased aggressive PCa, increased PCa risk with family history	926, 936, 937, 939
Malathion (pesticide)	Adult	Increased PCa risk, Increased aggressive PCa	926
PCBs	Adult	No change in PCa risk	976, 977
	Adult occupational	Increased PCa risk, mortality	975
Phorate (pesticide)	Adult	Increased PCa risk with family history	939, 943
Simazine (pesticide)	Adult	Increased PCa risk	933
TCDD	Adult	Increased PCa risk	949
Terbufos (pesticide)	Adult	Increased aggressive PCa, increased PCa risk with family history	926, 933, 939–941
Animal studies			
Arsenic (heavy metal)	Adult	Increased stem cell transformation, human cells	992, 993
BPA	Neonatal	Increased carcinogenic susceptibility in rats	105, 106, 959, 969, 1319
	Embryonic to adult	Increased stem/progenitor cell proliferation, human cells	960, 1320
	Adult	Increased aromatase activity, increased estradiol:testosterone ratio in rats	974
	Adult	Increased PCa cell proliferation, xenografts growth, human cell line	1236
	Adult	Increased PCa cell migration, invasion, human cell lines	966
	Adult	Increased centrosome amplification, benign and PCa human cell lines	958
Cadmium (heavy metal)	Adult	Increased stem cell transformation, human cells	1005
	Adult	Epithelial hyperplasia in rats, increased AR, ER	1003, 1004
DES	Perinatal	PIN, tumors with aging	970, 1275, 1321
PCBs	Adult	Altered AR, ER, decreased secretions in rats	973
	Adult	Increased proliferation, PSA, human cell line	979
TCDD	Perinatal	Increased epithelial hyperplasia in mice	956
	Adult	Decreased PCa progression in TRAMP mice	956
Vinclozolin	In utero	Chronic prostatitis as adults in rats	981, 983, 984

Abbreviation: PCa, prostate cancer.

source for these studies is derived from the prospective Agricultural Health Study (AHS) in the United States, conducted by governmental health agencies (www.aghealth.org), that has followed approximately 57 000 private (farmer) and commercial pesticide applicators since 1993. Since the previous EDC review (1), many additional studies have been published that interrogate this and other pesticide-exposed populations in greater detail, identifying more chemicals associated with increased prostate cancer rates and providing further insight into the interaction between pesticides and prostate cancer risk. Of relevance to this review, a subset of these compounds are linked to hormonal activities suggesting their potential as endocrine disruptors, and these are highlighted below. It should be noted that the individual pesticides were not measured in the AHS participants, as is the case for most other epidemiology studies on pesticides. Rather, exposures were linked to disease outcomes based on subject recall of exposure through questionnaires or documented occupational exposures. Thus, direct links with each agent are difficult to assess and remain to be addressed in future studies.

Although initial studies had implicated methyl bromide exposure as having the strongest risk with prostate cancer (924), this has not persisted with longer follow-up in the AHS (925). However, a subsequent analysis of the AHS cohort has identified specific organophosphate insecticides (fonofos, malathion, terbufos, and aldrin) with increased risk of aggressive prostate cancer (926). Additionally, multiple analyses have again confirmed an association between certain organophosphate (eg, coumaphos) and organochlorine (eg, aldrin) pesticides and increased prostate cancer risk in men with a familial history of the disease (927, 928). Although exposure to these compounds can induce oxidative stress, reactive oxygen species buildup, and DNA damage, certain organophosphate and organochlorine chemicals may influence sex hormone homeostasis and act as EDCs. Specifically, studies show that chlorpyrifos, coumaphos, fonofos, and phorate strongly inhibited the hepatic CYP1A2 and CYP3A4 enzymes that metabolize testosterone, estradiol, and estrone (929, 930). Malathion reduced serum FSH, LH, and testosterone (931), and aldrin increased aromatase activity (932). The AHS findings are supported by other population studies, including a large case-control Canadian analysis that observed a significant increase in prostate cancer among farmers in British Columbia (933). Interrogation of this group in relationship to specific pesticides determined that increased prostate cancer risk was associated with exposure to several chemicals known to exhibit EDC activity, including simazine (an aromatase inducer), endosulfan (an estrogen agonist/androgen antagonist and aromatase inducer), DDT (xenoestrogen that binds ERs and antagonizes AR), diazinon (estrogenic activity), and malathion (antiandrogenic properties). Of further note is a recent case-controlled study that examined prostate cancer risk in the general population (ie, nonoccupational exposures) living in an agriculturally intensive area in California (934). A significant association was found between increased prostate cancer rates and ambient pesticide exposure (residential and soil/dust drift) to a group of organochlorines with known EDC actions: dieldrin, endosulfan, MXC, lindane, toxaphene, dicofol, and heptaphor. Thus, in addition to farmers and other groups with occupational exposures to pesticides, prostate cancer risk may be applicable to individuals living in agricultural regions of the country. This is supported by data from the cross-sectional NHANES study that examined prostate and breast cancer rates as a function of serum organochlorine pesticides measured in the participants (935). Although breast cancer did not associate with the examined organochlorines, prostate cancer risk was significantly associated with background exposures to β-HCH, transnonachlor, and dieldrin, all with known EDC activities. Thus, the prostate cancer link with several pesticides that have EDC activity is not restricted to applicators and farmers alone but to the general population, at least in the limited studies conducted to date.

To understand specific genes that may be involved in the gene-environment interactions with prostate cancer, detailed gene SNP analysis was undertaken in nested case-control substudies within the AHS cohort and was examined against exposures for specific chemicals. Associations were found between several specific pesticide chemicals and interactions with SNPs in base or nucleotide excision repair pathway genes, suggesting a role of these chemicals in oxidative stress and DNA damage/repair (936, 937). Interestingly, those pesticides interacting with nucleotide excision repair genes included fonofos, and those interacting with base excision repair genes included fonofos and ATR, the latter having actions that include alterations in estrogen metabolism that elevate estradiol levels (929, 938). Specific analysis of polymorphisms in xenobiotic metabolizing genes (phases I and II) identified interactions with a handful of SNP variants, petroleum oil distillate chemicals (terbufos, fonofos, phorate, methyl bromide), and increased prostate cancer risk (939), again supporting potential action of a subset of pesticide chemicals as EDCs. One gene associated with increased prostate cancer risk in the pesticide applicator population was TXNRD2, a redox enzyme (939). Of particular interest, this gene partially overlaps on chromosome 22 with catechol-O-methyl transferase, a phase II enzyme important in androgen and estrogen metabolism. Furthermore, SNPs in

several phase I/phase II enzyme genes, including the CYP2C family, CYP2C18, and CYP2C19 that directly metabolize pesticides and steroids, have positive associations with prostate cancer, suggesting that alterations in the levels of steroidal and toxic pesticide intermediates may influence prostate cancer risk (939). Although fonofos usage has been discontinued due to its potential carcinogenic activities, terbufos is the fourth most common organophosphate insecticide in the United States, and it is associated with elevated prostate cancer risk in the AHS cohort (940). An analysis of vitamin D pathway gene variants in relation to prostate cancer in the AHS cohort identified five significant, monotonic interactions with increasing cancer risk involving terbufos with vitamin D binding protein variants, and parathion with VDR and RXRβ gene variants (941). These gene variants lower circulating 25-hydroxyvitamin D levels and reduce VDR and RXRβ activity, abrogating the chemoprotective actions of vitamin D against prostate cancer (941, 942). Aldrin use and variants in TET2 and PP2A genes, the latter that dephosphorylates AR, were associated with increased prostate cancer risk (928). Finally, interactions were identified between coumaphos, terbufos, and phorate with gene variants of chromosome 8q24, a hotspot previously associated with prostate cancer risk (943). Of note, one 8q24 variant that statistically associates with these insecticides resides within an AR enhancer site, suggesting a potential hormone-related mechanism of action.

b. Cell and animal models. Animal models and in vitro experiments support these data and are available for some of the individual chemicals mentioned above as well as for the organophosphates and organochlorines as a group. Due to the large number of chemicals in the separate studies, they are not individually discussed herein, and readers are directed to Table 6 for details. In summary, a significant amount of new data has accumulated in the past 5 years that confirms and expands on exposure to EDC-active pesticides, particularly in the organophosphate and organochlorine class, and its direct association with elevated prostate cancer risk in humans.

2. Agent Orange and dioxin

a. Human exposures. The first EDC Scientific Statement (1) did not detail the known associations between Agent Orange and dioxin exposures and increased prostate cancer risk, so we briefly review the data. For greater details, we refer the reader to the most recent Veterans and Agent Orange Update 2012, released by the National Academy of Sciences (944). During the Vietnam conflict (1962–1971), the herbicide Agent Orange, a 50:50 mixture of 2,4-di-chlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid with picloram and cacodylic acid, was sprayed to defoliate the jungle. TCDD, the most toxic form of dioxin, was an unintentional contaminant generated during the production of 2,4,5-trichlorophenoxyacetic acid by the Dow Chemical Company. The subsequent health effects of Agent Orange are largely attributed to this chemical. In response to a Congressional law in 1991, the National Academy of Sciences convened a committee to evaluate potential health effects from this exposure in the Vietnam veterans. Published epidemiological data, mechanistic studies, and animal research results are compiled and reanalyzed, and health risks are identified in reports that are released biannually. Increased prostate cancer rates in Agent Orange-exposed veterans have been identified, with a relative increased risk of 2.3–6.0 in the highest exposure group (Air Force Ranch Hand sprayers) compared to veterans who served in Southeast Asia but did not spray (944, 945). Furthermore, this highly exposed group developed the disease earlier and had more aggressive forms of prostate cancer at diagnosis (946). Based on these and other studies, the Veterans and Agent Orange Committee concludes that there is suggestive evidence of an association between exposure to Agent Orange chemicals of interest and prostate cancer (944). In addition, a more recent study of prostate cancer diagnosis at biopsy in Agent Orange-exposed veterans, compared to nonexposed veterans, found a 1.75-fold increased risk of high-grade prostate cancer (Gleason score $\geq$7) and a 2.1-fold increase in detecting prostate cancer with a Gleason score $\geq$8, including both aggressive and potentially lethal forms of the disease (947). Furthermore, a follow-up analysis (up to 60 mo) of veterans with prostate cancer found that those exposed to Agent Orange were more likely to have recurrence and progression (948). That TCDD is the toxic agent in these studies is supported by a recent meta-analysis of TCDD-exposed cohorts representing 40 286 participants, which reported a positive association between TCDD exposure and prostate cancer (meta-standardized mortality ratio = 1.26) (949).

b. Cell lines/mechanistic studies. The biological effects of Agent Orange chemicals and TCDD in particular are mediated through the binding to the transcription factor AhR that is expressed in nearly every cell, leading to dimerization with aryl hydrocarbon receptor nuclear translocator and binding of aryl hydrocarbon response elements upstream of TCDD-regulated genes (950–952). Many AhR-regulated genes encode phase I metabolizing enzymes such as CYP1A1, CYP1A2, CYP1B1, and several phase II conjugating enzymes, which serve as biomarkers of activation. Recent studies have also identified nongenomic signaling of TCDD through the AhR that contributes to its

toxic effects (953). Of note, AhR and aryl hydrocarbon receptor nuclear translocator are expressed in adult human prostate epithelial progenitor cells, and dioxin exposure results in a dose-dependent expansion of prostate stem and progenitor cell numbers (954), which could permit increased opportunities for carcinogenic hits, thus driving increased cancer incidence. Interestingly, studies on TCDD effects on prostate cancer cells in vitro have produced variable results, with some showing proliferative effects and others showing no stimulation or repression (955).

c. Animal studies. Although animal studies are limited, the murine model does not support TCDD as a carcinogenic in the adult prostate. In fact, selective AhR activators inhibited prostate cancer cell proliferation and delayed prostate cancer progression in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of cancer (956). This indicates that the rodent prostate may not be an effective model for TCDD-driven prostate cancers in humans. However, developmental exposure to TCDD increased prostate hyperplastic lesions with aging in a wild-type mouse model, suggesting that timing of TCDD exposure may be a critical variable in determining prostatic effects, with the developing prostate being more sensitive than the adult prostate (956).

3. Bisphenol A

There have been a considerable number of studies over the past several years investigating the potential for adverse health effects from BPA, including its role as a potential carcinogen (957). Of relevance to prostate cancer, some new studies have been conducted in human populations, tissues, and cells, although most work has focused on animal models, the latter providing valuable mechanistic information.

a. Human studies. The first direct clinical evidence that BPA exposure may be associated with prostate cancer comes from a recent prospective study of 60 urological patients evaluated for potential prostate cancer due to elevated serum prostate-specific antigen (PSA) (958). Urinary BPA-glucuronide levels before prostate biopsy were significantly higher in the biopsy-confirmed prostate cancer patients than those with no diagnosis of cancer. When the data were analyzed by patient age, cancer-positive patients <65 years old had higher urine BPA-glucuronide levels than noncancer patients, whereas there was no difference in BPA levels in cancer vs noncancer patients in men >65 years old. One possible interpretation is that BPA exposure is associated with earlier-onset prostate cancer. Alternatively, the presence of high BPA concentrations may suggest a lifestyle that sustains higher BPA exposures, perhaps compounded with other factors associated with increased risk of prostate cancer (958). Nonetheless, these positive associations between BPA levels and prostate cancer diagnosis are provocative, and we need larger case-controlled studies to confirm potential correlations.

b. Human stem cells. Recent studies have shown that human prostate stem and progenitor cells are targets of BPA that can reprogram the cells after brief, low-dose exposures. Adult prostate stem-progenitor cells were isolated from the prostates of young disease-free men (960), and treatment with BPA increased stem cell self-renewal, progenitor cell proliferation, and stemness gene expression in a dose-dependent manner. Both 10 nM BPA and 17β-estradiol induced equimolar rapid membrane-initiated signaling in these ER-positive cells, identifying the human prostate stem and progenitor cells as direct BPA targets. Genome-wide profiling of BPA-exposed progenitor cells found that several genes were consistently altered across organ donors, including multiple SNORDs, a class of noncoding RNAs (961). Furthermore, BPA-regulated SNORDs were associated with specific histone modifications (H3K4me3, H3K9me3, H3K27me3), thus identifying an epigenetic basis for prostate progenitor cell reprogramming. These findings have been corroborated by studies using human embryonic stem cells for directed differentiation to prostatic organoids in vitro (962). Exposure to BPA modified branching morphogenesis, with 1 nM accelerating and 10 nM restricting early organoid branching. Furthermore, low-dose BPA stimulated focal aggregates of resident stem cells in the mature prostate organoids, suggesting that BPA increased stem cell symmetric self-renewal. Together, these studies indicate that embryonic and adult prostate stem cells are influenced by low-level BPA exposures that amplify their numbers and modify gene expression. This is particularly relevant because lifetime cancer risk is strongly correlated with the total number of divisions of the stem cells that maintain tissue homeostasis (963). Thus, chronic low-level stimulation of stem-progenitor cell numbers may stochastically increase the potential for carcinogenic hits to cells that can populate tumors.

Earlier work with rodent models determined that developmental exposure to low-dose BPA increased prostatic susceptibility to estrogen-driven carcinogenesis with aging (959). To examine whether the human prostate is similarly sensitive to low-dose BPA exposures, prostate stem-progenitor cells from disease-free men were grown in nude mice, forming an in vivo humanized prostate-like gland producing PSA+ epithelium (960). Developmental BPA exposure was modeled by feeding the murine host

low doses of BPA as the prostates formed (peak free-BPA, 0.39 or 1.35 ng/mL serum). At maturation, mice were given estradiol implants to model rising estradiol levels in aging men. After 2–4 months, the incidence of prostatic intraepithelial neoplasia (PIN) and adenocarcinoma in the human prostate epithelium significantly increased from 13% in oil-fed controls to 33–36% in grafts exposed to either BPA dose in vivo. Together, the above models indicate that developmental stage BPA exposures can increase pathological lesions and carcinogenic susceptibility in the human prostate epithelium.

c. Cell lines/mechanistic studies. The previous EDC report (1) reviewed in vitro and in vivo studies in human prostate cancer cell lines. Low-dose BPA exposure stimulated cell proliferation and xenograft growth of LNCaP cells, a line that contains a gain-of-function mutated AR resulting in promiscuous ligand binding. BPA did not have this effect in AR-negative cells (PC-3, DU-145) or a prostate cancer cell line containing wild-type AR, suggesting that it is activation of the gain-of-function AR that mediated the effects of BPA. The gain-of-function point mutation in the AR ligand binding domain of LNCaP cells is found in approximately 5% of advanced prostate cancers in patients who relapse after androgen deprivation therapy. Because many other AR-ligand binding domain mutations are also observed in advanced disease, the findings suggest that BPA exposure may be a risk factor for progression and therapeutic relapse in a subpopulation of men with prostate cancer (964, 965). Since that time, new studies with LNCaP cells showed that BPA (0.1–10 nM) markedly stimulated cell migration and invasion, an effect mediated through the up-regulation of the Ca^{2+} ion channel gene and protein, Orai1 (966). Because the AR-negative PC3 cells exhibited a similar response, these studies indicate that BPA can also influence human prostate cancer cell migration and invasion in an AR-independent manner, most likely through ERβ, which is expressed at high levels in both LNCaP and PC3 cells. That BPA exposure may provide a procarcinogenic environment is implicated by studies with nontumorigenic human prostate cells (PNT1a) and PC3 cancer cells exposed to 200 nM BPA short term (24 h) or 1 nM BPA long term (2 mo), demonstrating significantly increased levels of DNA bulky adducts (967). These data propose a plausible mechanism involving BPA oxidation to semiquinone or quinone intermediates that form DNA adducts, leading to direct DNA damage within prostate epithelial cells. Another recent study reported that low-dose BPA exposures (0.01–0.1 nM) potentiated centrosome amplification in nontransformed and transformed human prostate epithelial cell lines, with a nonmonotonic dose-response observed in four of six lines (958). Furthermore, in that same study, BPA exposure promoted anchorage-independent growth in the androgen-independent C4–2 cell line. Together, these studies support the concept that BPA may contribute to neoplastic transformation and modulate the aggressiveness of human prostate cancer cells.

d. Animal models. In the past 5 years, data derived from animal models have provided additional evidence to support a consequence of BPA exposure in relation to prostate diseases, including prostate cancer. Previous studies in rats determined that a brief perinatal exposure to low-dose BPA (10 μg/kg body weight) epigenetically reprogrammed the prostate tissues and markedly increased the incidence and severity of prostatic precancerous lesions upon treatment with estradiol as adults (105). A subsequent study by this group directly addressed the issue of BPA exposure route, finding a nearly identical heightened susceptibility to prostate neoplasia and tumors after either oral or subcutaneous administration of 10 μg/kg body weight BPA during the postnatal period (959). This was recently confirmed in an independent study using rat pups neonatally exposed to either 50 μg/kg body weight orally or 10 μg/kg body weight subcutaneously, which produced comparable serum free BPA levels. As compared to neonatal oil-control rats, BPA exposures led to an elevated incidence of severe PIN in the lateral prostates given elevated estradiol levels as adults (968). However, rates of prostate cancer were not influenced by developmental BPA treatments. In another new study, pregnant rat dams were treated orally with low-dose (25 μg/kg body weight) or 10-fold higher levels of BPA from day 10 to 21 of gestation. A significant increase in total prostatic lesions (inflammation, hyperplasia, dysplasia) was observed at 6 months, with the greatest responses seen at the lower dose (969). This suggests that fetal BPA exposures (during prostate determination and initial budding) may produce heightened prostate pathology with aging as compared to postnatal exposures (during branching morphogenesis and cell differentiation) when BPA exposures alone were insufficient to induce prostatic pathology (105, 959). It is noteworthy that concurrent treatment with the phytochemical indole-3-carbinol during gestational BPA exposure was able to attenuate the deleterious effects of BPA on prostatic lesions, largely by reducing the inflammatory response and proliferative index in the ventral prostate tissues, providing promise for chemoprotective approaches using natural products (969).

To evaluate the potential for transgenerational effects of BPA on prostate pathology in future generations, a recent study exposed pregnant rats to high doses of BPA mixed with phthalates. Although prostatic epithelial at-

rophy or hyperplasia was observed in the male offspring at 1 year, this was not transmitted to subsequent generations (262). However, an epigenetic basis for the lifelong memory of developmental BPA exposure was elaborated further using prostates collected over the life span of rats treated neonatally with low-dose BPA and given adult estrogens that drove prostatic neoplasia (106). Using global DNA methylation analysis, three distinct patterns of functional DNA methylation changes were identified in response to early-life BPA: those that appeared early and persisted throughout life, those that appeared after later-life hormonal events such as puberty and rising adult estrogens, and those that increased and decreased over the life span of the animal (106). Specific genes with aberrant expression were characterized in detail (*Pde4D4*, *Hpcal1*, *Nsbp1*), each previously implicated in various cancers, including prostate. Furthermore, upon BPA exposure, early and persistent overexpression of prostate DNA methyltransferases (*Dmnt*3a/b) and methyl-CpG binding domain proteins with demethylase activity (*Mbd*2/4) were noted, which may mechanistically underlie early-life reprogramming and permit dynamic changes in response to secondary estrogenic exposures throughout life (106). Together, these results highlight the complexity of developmental reprogramming events initiated by BPA exposures that may predispose to carcinogenesis with aging.

Earlier studies using primary cultures of fetal mouse prostate mesenchymal cells established that, similar to estradiol, BPA stimulated AR and ERα gene expression in a dose-dependent manner (971), providing a molecular mode of action for BPA effects in the developing prostate. This was recently confirmed in studies with in vivo low-dose BPA exposures of mice during gestation, demonstrating increased Cyp19A1 gene expression and aromatase activity, and resultant elevations in estradiol synthesis in the embryonic urogenital sinus tissue that together amplify estrogenic actions during this critical developmental window (972, 973). New studies on prostates from rats gestationally exposed to low-dose BPA reported elevated AR and ERα protein in prostate tissues prepubertally as well as at 6 months, suggesting that altered steroid receptor levels throughout life may underlie the aberrant prostatic growth responses over the life span (969). In this regard, it is noteworthy that brief exposure of adult rats to BPA increased aromatase levels, resulting in a higher plasma estradiol:testosterone ratio in a dose-dependent manner (974), suggesting that the adult prostate gland may remain susceptible to BPA effects throughout the course of life.

4. Polychlorinated biphenyls

a. Human studies. The previous EDC review (1) highlighted a few reports showing significant associations between PCB153 and PCB180 and elevated prostate cancer risk and increased prostate cancer mortality in men occupationally exposed to PCBs. A recent updated analysis of this later cohort of 24 865 capacitor manufacturing workers in the United States exposed to PCBs with 35 years of follow-up data confirmed the elevation in mortality from prostate cancer, indicating that the disease was significantly associated with the estimated 20-year lagged cumulative PCB exposure among long-term workers (975). Furthermore, using a sophisticated job exposure matrix, prostate cancer deaths showed an increased trend in association with increasing levels of cumulative PCB exposure (975). On the other hand, a new prospective, nested case-control study in 14 203 Japanese men with a mean follow-up of 12.8 years found no association between prostate cancer incidence and any organochlorine measured in plasma, including o,p'-DDT, p,p'-DDT, p,p'-DDE, nonachlor, and $>$ 40 PCBs, suggesting that the general population may not be at increased risk as compared to occupationally exposed workers (976). Due to the aging component of prostate cancer risk, however, a longer-term follow-up is necessary to validate these results. Given that Asian men have the lowest worldwide incidence of prostate cancer (as reflected in the small number of 201 prostate cancer cases in this large cohort), race-based factors may have contributed to these negative findings. However, a small case-control study of urology patients in Canada similarly found no correlation between prostate cancers in the general population and plasma levels of nine PCB congeners including PCB153 and PCB180, as well as other organochlorines (977). The studies referenced above all relate to adult exposures to PCBs, so whether developmental exposures to these agents may differentially influence adult prostate cancer risk is a possibility that has not been addressed. Together, the above studies strongly support the hypothesis that higher-dose exposures occurring in men occupationally exposed to PCBs result in increased prostate cancer rates later in life. Whether this increased risk occurs in the general population will require continued analysis in larger cohorts and in populations at higher risk for prostate cancer onset, eg, African American and US Caucasian men.

b. Cell lines and animal studies. The number of in vitro prostate models or in vivo animal studies on PCBs is limited. An in vitro analysis of the effects of selected PCBs on the human prostate cancer cell line LNCaP found several that reduced cell proliferation, PSA secretion, and 5α-reductase activity, whereas others (including PCB153 and 118) exhibited biphasic effects, inducing proliferation and PSA secretion at low concentrations (978). A recent examina-

tion of PCB11, a ubiquitous PCB found in air samples, reported a buildup of oxidation injury and growth inhibition in the normal human prostate cell line RWPE-1 (979). Of potential clinical relevance, a study examining the effect of Aroclor 1254 or PCB153 on oxidative stress and growth inhibition in RWPE-1 cells, reported that adverse effects were mitigated by antioxidant treatment after exposure (980). Similarly, Aroclor 1254 treatment of adult rats altered prostate AR and ER expression and compromised prostate secretory activity; interestingly, α-tocopherol treatment prevented this effect, suggesting that this antioxidant may be protective against PCB toxicity (973). Clearly, more mechanistic studies are required before modes of action can be derived.

5. Vinclozolin

The fungicide vinclozolin has antiandrogenic properties through antagonism of the AR (982). After in utero exposure, chronic prostatitis was observed in adult male rats, an effect transmitted transgenerationally through the male gamete over four generations (983). A new study examining the effects of direct in utero vinclozolin exposure on ventral lobe prostates in rats reported that vinclozolin-induced prostatitis was associated with reduced AR levels and increased nuclear factor κB activation in prostatic epithelium, an effect that was fully reversible with high-dose testosterone treatments during puberty (981, 984). This supports the hypothesis that vinclozolin actions in the prostate are induced through its AR antagonistic actions. At present, there are no reports on the effects of adult exposure to vinclozolin on subsequent prostate disease, nor are there studies reporting adverse effects in humans. In light of the potential association between vinclozolin and chronic prostatitis, we need more research on both of these issues.

6. Trace metals

Exposure to several types of heavy metals has been directly linked to increased risk of multiple cancers, including prostate cancer (985). Among these molecules are two trace metals, arsenic and cadmium, which are classified as EDCs due to their ability to act as a ligand and/or interact with members of the steroid receptor superfamily.

a. Inorganic arsenic. Inorganic arsenic (iAs) is found naturally in soils and in ground and surface water. Depending on locale and diet, human exposure to harmful levels can be widespread (985). Although toxicity is attributable to multiple mechanisms including oxidative stress and DNA damage, evidence also exists for iAs as an EDC due to its interference with steroid receptor activity, perhaps due to common zinc-finger interactions (986–989). As previously reviewed, epidemiology studies have shown an association between iAs exposure and prostate cancer mortality in Taiwan and the United States in hotspot areas of high exposure (1). A recent evaluation of low-moderate iAs exposure with urinary measures in a prospective cohort of approximately 4000 Native Americans identified a significant increase in mortality from prostate cancer over 20 years of follow-up (990). However, rodent models of iAs carcinogenicity did not support these human results; this may be attributable to marked species differences in arsenical biokinetics between rodents (991). In vitro studies using benign human prostate cells reported that iAs induced malignant transformation of prostate epithelial cells and drove them to an androgen-independent state (992). Interestingly, compared to the individual compounds, combined exposures of RWPE-1 cells to both iAs and estradiol resulted in increased transformation, suggesting synergistic pathways (993). The Waalkes laboratory reported that prostate epithelial stem cells are a direct target for iAs transformation, which may underpin the increased prostate cancer risk and mortality (994–996). Of note is the activation of the RAS oncogene and increased ERK activity in the prostate stem cells transformed by iAs (994) because these are important mediators of hormone actions in human prostate stem cells (960). That stem cells are targets of iAs also raises the issue of a developmental exposure to arsenicals because the prostate is enriched in stem cells during this time (997). Future research into these areas, including in utero exposure in human populations with prostate cancer as adults, is critical for delineating this possibility.

b. Cadmium. Cadmium exposures come from smoking, diet, and a variety of occupations. Because cadmium acts as a ligand and activates ERs, among other modes of action, it is considered an estrogen mimic (998). There is conflicting epidemiological evidence for an association between cadmium exposure and prostate cancer risk or mortality, with some data showing no interactions and others showing significant correlations (999–1002), meriting further work to resolve these discrepancies. Two recent studies in rodent models found that brief exposure to low-dose cadmium increased epithelial hyperplasia and upregulated AR and ERα levels, suggesting endocrine perturbations leading to abnormal proliferation (1003, 1004). Similar to iAs, cadmium exposure transforms normal human prostate epithelial cells in vitro, enabling them to recruit nearby stem cells into an oncogenic phenotype (1005). These provocative findings highlight novel pathways whereby cadmium exposure may influence the carcinogenic potential of prostate epithelial cells. Continued

research in this area may help to elucidate mechanisms that might be useful for future disease prevention.

C. Conclusions

Data continue to emerge that identify the prostate gland as a direct EDC target tissue, whereby exposures especially during development result in lifelong changes that influence disease susceptibility and contribute to prostate cancer incidence, progression, and mortality. Undoubtedly, the sensitivity of the prostate to EDC exposure is due to the absolute dependence of this gland on androgens and multiple other hormones that regulate its development, secretory activity, and homeostasis throughout life, with hormone imbalances contributing to disease ontogeny in this structure. Accumulating data confirm that the adult human prostate is directly affected by EDCs, with increased cancer rates and mortality occurring in men exposed to pesticides, Agent Orange chemicals, alkylphenols, and trace metals, all with known hormonal actions as a common factor. It is important to note that not all findings are in agreement, and for several, negative findings suggest that previously suspected compounds (eg, methyl bromide) most likely have limited or no prostatic effect. Furthermore, several chemicals have very limited evidence for adverse effects and well controlled studies are required to assess their potential for harm to the prostate gland. New findings in human and animal models also support previous data showing a developmental window of heightened sensitivity to EDCs resulting in increased susceptibility to aging-associated pathologies including carcinogenesis. Modes of action include altered steroid receptor levels and actions, changes in steroidogenic enzyme activity, and epigenomic reprogramming that may underpin an increased disease propensity throughout life. Additionally, important new data identify the stem and progenitor cells within the developing and adult prostate gland as direct targets of EDC actions, further contributing to long-term memory of prior exposures within the gland. Because these cells populate the prostate during embryonic development, maintain adult homeostasis, and repopulate the prostate after acute or chronic injury, and because stem-like cancer cells play a fundamental role in tumor progression, reprogramming of prostate stem and progenitor cells by EDCs have marked potential for transmitting lifelong changes within the gland, including a predisposition to diseases with aging.

Continued studies by researchers and governmental agencies examining the role of EDCs in prostate disease are essential at several levels. First, it is necessary to investigate more individual EDCs and classes of compounds to identify all chemicals that may adversely affect prostate health so that we can undertake needed steps to reduce human exposures. Next, it is important to delineate the critical periods of exposure vulnerability for the various EDCs, from early development through puberty, as culprits for increasing disease risk in adult men. Third, it is fundamental that we understand EDC modes of action and the molecular underpinnings of lifelong prostatic perturbations because this knowledge will be critical for effective prevention and treatment strategies. Fourth, we must discover biomarkers of exposure that will best identify at-risk populations. Finally, we need to continue efforts to understand the stem cell as a target of prostate reprogramming by EDCs and develop prostate stem cell models as effective, functional screening tools to rapidly identify chemicals that are potentially harmful to prostate health.

VII. Thyroid Disruption

A. Characteristics of the hypothalamic-pituitary-thyroid (HPT) axis

Thyroid hormone is essential for normal development and for the control of many aspects of adult physiology in vertebrates. Thus, it is important that thyroid function and thyroid hormone action be maintained within normal physiological limits both during development and in adulthood. Environmental factors such as the micronutrients iodine, selenium, and iron are important for the proper control of thyroid function. In addition, a number of foods contain goitrogenic (goiter-inducing) agents such as thiocyanates and isoflavones can interfere with thyroid function. Nitrates, chlorates, and perchlorates are anions found in water and foods, and these also can inhibit thyroid function. Finally, manufactured chemicals can interfere with thyroid function or thyroid hormone action. The goal of this section is to review recent studies concerning the impact of EDC exposures on the thyroid and to place these studies within the context of new information informing us about the basic biology of thyroid hormone.

Blood levels of thyroid hormones are maintained within a somewhat narrow range in an individual, but individuals differ in their "set point" such that the population variance (the reference range) can be 10-fold greater than individual variance (1006, 1007). This fact is important in considering EDC effects within individuals and populations.

The control of thyroid function involves a dynamic interaction among the hypothalamic releasing hormone TRH, the pituitary hormone TSH, and the thyroid hormones that exist in two major forms: T_4 and T_3 (1008) (Figure 9). The HPT axis is one of the body's neuroendo-

crine systems (Figure 9) (see *Section VIII* for more on neuroendocrine disruption). T_4 is the predominant hormone secreted from the thyroid gland, and it is converted to T_3 by the action of a deiodinase (type 1 [referred to as Dio1 or D1] or type 2 [Dio2, or D2]) in various tissues (1009). Thyroid disruption by EDCs can occur at any level of the HPT axis including thyroid hormone synthesis, release, transport, and metabolism, or thyroid hormone actions on target tissues. This occurs, in part, because of structural similarities between some EDCs and thyroid hormones (Figure 10).

There are two important implications of the complexity of HPT regulation. First, some tissues can regulate their own sensitivity to thyroid hormone by changes in the expression of various enzymes and transporters (eg, Ref. 1010). This creates a situation in which changes in thyroid hormone action in specific tissues and cells would not reflect changes in circulating levels of thyroid hormones. Second, EDCs can interfere with thyroid hormone action in a complex manner in the thyroid gland, the hypothalamus, or pituitary, or in thyroid hormone-regulated tissues and cells. These characteristics of the thyroid axis represent a major challenge for interpreting results of EDC studies (1011) because it means that to fully evaluate the ability of an EDC to interfere with thyroid hormone action, we cannot rely solely on thyroid hormone levels in blood for our measurements. In humans, genetic defects in HPT function have informed our basic understanding of the thyroid system, and they provide evidence that EDCs may interfere with thyroid hormone action in tissues in a manner that is independent of circulating levels of thyroid hormone (1012). The clinical manifestation of these genetic defects is important to consider for studies of EDCs because they point to adverse effects that are not traditionally viewed as being manifestations of thyroid disease.

Box 5. Key Points: Prostate

- **The prostate gland is a hormone-dependent structure, and dysregulation of hormonal signaling is a known contributor to the high rates of prostate disease with aging. A number of EDCs have been associated with aberrant prostate growth, making it a likely EDC target.**
- **Disruption of multiple hormonal pathways by EDCs have been identified in the prostate, including ERs, AR, VDR, retinoic acid receptor/RXRs, PRL, and steroid-metabolizing enzymes. This may underlie increased prostate cancer risk by certain EDCs.**
- **Gene-environment interactions have been identified for several pesticides in population studies and suggest that certain genetic alterations such as SNP variants may predispose subpopulations of men to heightened prostate cancer susceptibility from exposure.**
- **EDC classes with known prostatic effects include pesticides, insecticides, herbicides, Agent Orange chemicals, PCBs, alkylphenols, BPA, and some heavy metals.**
- **Epidemiological evidence indicates increased prostate cancer rates and mortality in men exposed to specific pesticides, Agent Orange, alkylphenols, PCBs, and inorganic arsenic.**
- **Animal models and human cell-based studies provide evidence for elevated prostate cancer risk from BPA exposures, with increased sensitivity to BPA reprogramming during early-life developmental.**
- **Cell-based and animal studies support the human data, extend to additional EDCs of concern, and identify cellular (eg, stem cells) and molecular pathways (eg, epigenetics) that underpin increased prostate disease risk.**
- **Occupational pesticide/herbicide exposure levels provide greater prostate cancer risk although emerging evidence is suggestive of general population risk in areas of pesticide/herbicide utilization. PCB risk appears limited to occupational exposure levels, and arsenic risk occurs in hotspot areas of high iAs in drinking water. BPA effects in animal models have been noted at low-dose equivalents to general population exposures.**

Future studies are needed to identify prostate disease risk with low-dose EDC exposures, identify critical life periods of exposure vulnerability, investigate adverse effects of several unstudied EDC classes that interfere with steroid actions, and elucidate modes of action for EDC prostatic effects and discovery of biomarkers of EDC exposure to identify at-risk populations.

B. Role of the micronutritional environment in thyroid hormone action

Several micronutrients are essential for thyroid hormone production (1013), and this may be an important variable contributing to differential effects of EDCs on thyroid hormone action. Thyroid hormones are iodine-containing thyronines: modified tyrosyl residues connected by an ether link. Two atoms of iodine are bound to each of the inner and outer rings to form T_4 (1014), explaining the dietary requirement for iodine (1015). Considering the importance of iodine to thyroid hormone

Figure 9.

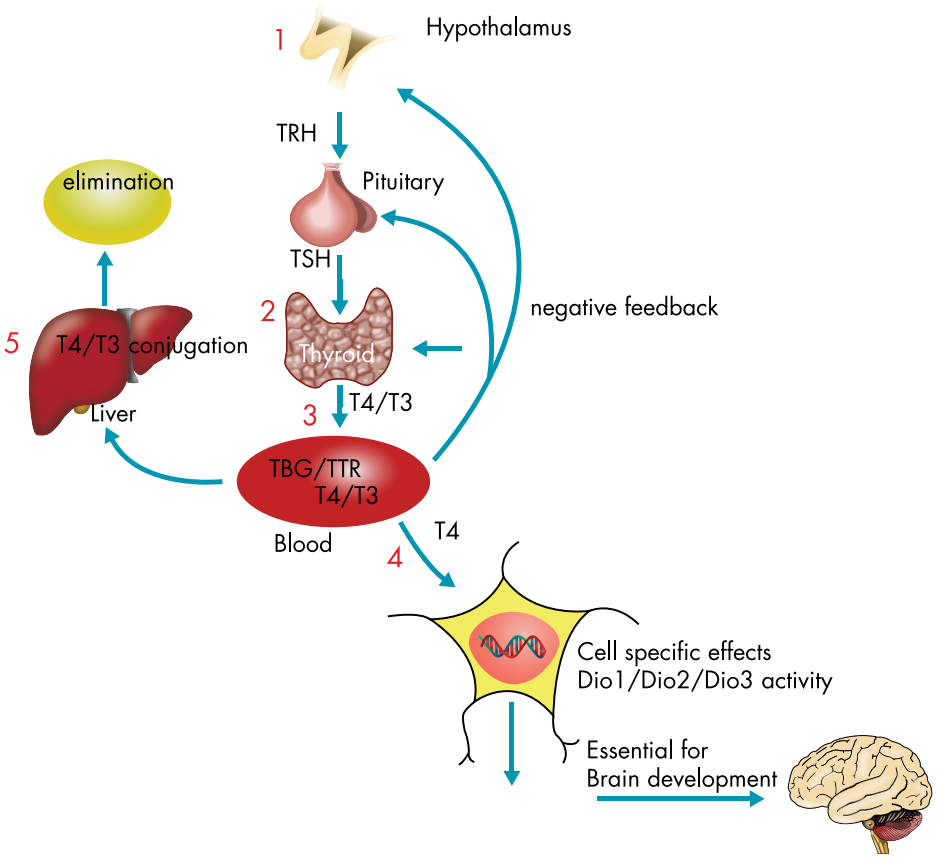


Figure 9. The HPT axis can be disrupted at several points of regulation. The control of thyroid hormone delivery to the site of action in tissues is a highly complex process that includes synthesis in the thyroid gland, transport through blood, selective uptake into tissues and cells, and metabolism at the site of action. These elements are represented as follows. 1) TRH is produced in parvocellular neurons of the hypothalamic PVN. Only a subset of these neurons appears to control pituitary TSH release (ie, are hypophysiotropic). These neurons are controlled by a combination of neuronal afferents and thyroid hormone negative feedback. This feedback is mediated principally by T_4 from serum, which is converted to T_3 by tanycytes and delivered to TRH neurons through the cellular transporter MCT8. Negative feedback itself is mediated selectively by the ThRB2 receptor. TRH stimulates the synthesis and release of TSH in pituitary by the action of membrane receptors that signal through protein kinase C. 2) Thyroid hormone synthesis requires the active uptake of iodide through the NIS, the production of thyroglobulin, and its iodination by the TPO enzyme. TSH stimulates the thyroid cell through cAMP, which increases thyroid hormone synthesis and release simultaneously. Thyroid hormone release requires the endocytosis of iodinated thyroglobulin in the colloid, vesicular transport through the thyrocyte, during which time iodotyrosyl residues are coupled and excised from the protein backbone before exocytotic release. Once in blood, 3) thyroid hormones are carried on binding proteins (so-called "distributor proteins"). Most T_4 (75%) is carried on T_4 binding protein (TBG), about 25% on transthyretin (TTR), and a small proportion on albumin. This leaves about 0.01% "free" (unbound). 4) T_4 in serum gains entry into tissues and cells through selective transporters such as the organic anion transport protein 1C1 (OATP1c1) or the monocarboxylate transporter 8 (MCT8). The delivery of biologically active T_3 is complex especially in the nervous system. T_4 is taken up by glial cells and converted to T_3 by the action of Dio2. Then, T_3 is transported actively to neurons and acts on the ThR α or β. 5) The half-life of T_4 in serum is 7–10 days in humans and 24 hours in rodents. This is controlled by the liver, which expresses enzymes (glucuronidases or sulfotransferases) that modify T_4 and T_3 such that they are eliminated in bile.

synthesis and the singularity of the sodium/iodide symporter (NIS) that transports iodide, it is potentially important that several environmental chemicals can interfere with iodide uptake through the NIS.

The enzymes Dio1 and Dio2 that convert T_4 to T_3 through removal of an iodine atom are selenoproteins (1013), and therefore dietary selenium is required for this function. In fact, patients with genetic defects in selenoprotein synthesis have impaired thyroid function (1016, 1017). In rats, PBDE exposure reduced Dio1 activity in the liver (1018). This is a potentially important mechanism of thyroid hormone vulnerability to disruption by EDCs, but this requires further study.

C. Chemicals with direct actions on the thyroid gland: perchlorate, chlorate, nitrate, thiocyanate

1. Iodide uptake

A number of environmental chemicals, mostly complex anions, can interfere with NIS function and iodide uptake

Figure 10.

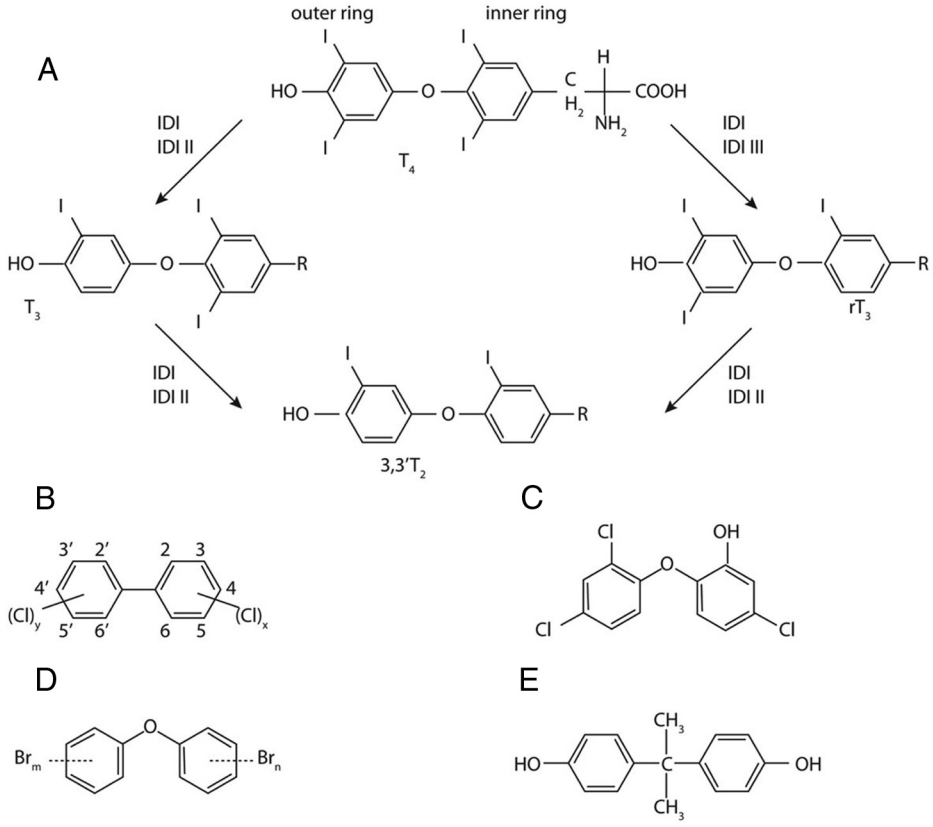


Figure 10. Representative environmental chemicals with structures similar to that of thyroid hormone. A) Thyroid hormone (T_4) is metabolized by a series of enzymes to form more a potent (outer ring deiodination by Dio1 and Dio2) or less potent (inner ring deiodination by Dio3) hormone. These metabolic events are important steps in the control of thyroid hormone action in tissues and take place in specific cells as described in the text. It is important to recognize that some environmental chemicals may interfere with specific enzymes, but little is known of this pathway. Some of these chemicals are: B, PCBs; C, triclosan; D, PBDEs; and E, BPA. [Modified from M. E. Gilbert et al: Developmental thyroid hormone disruption: prevalence, environmental contaminants and neurodevelopmental consequences. *Neurotoxicology*. 2012;33:842–852 (1322), with permission. © Elsevier.]

and thereby interfere with thyroid function. Perhaps the most important among these are perchlorate, chlorate, nitrate, and thiocyanate. Chlorate and nitrate can be found in high levels in some water supplies; high levels of thiocyanate are derived from a variety of sources including cigarette smoke. Although there are natural sources of perchlorate, it is also made in large quantities in the production of explosives (solid rocket fuel, automobile air bags, fireworks). All of these ions inhibit iodide intake, and this can be particularly important when iodide intake is low to insufficient (1019). Perhaps because all of these factors interact (including iodide intake), the effect of perchlorates on human thyroid function is complex (1020). A recent study showed that perchlorate levels in pregnant women with borderline thyroid function were inversely related to measures of cognitive function in the offspring (1021). Therefore, it is possible that environmental perchlorate affects thyroid function when other risk factors are present, including low dietary iodide intake, the presence of other complex anions, underlying subclinical thyroid disease, and perhaps the presence of other environmental antithyroid agents.

2. Iodide organification

The thyroperoxidase (TPO) enzyme is essential in thyroid hormone synthesis and is the target of goitrogenic agents such as isoflavones and thiocyanates (1022, 1023). In a recent study, Paul et al (1024) developed a high-throughput assay to detect chemicals that affect TPO enzyme activity and tested several chemicals, some of which showed TPO inhibition. The Tox21 set of assays will include this tool and it will likely be an important indicator of such chemicals. Likewise, Song et al (1025) developed an in vitro assay for interaction of various chemicals with human TPO and found that some chemicals could inhibit, and some could increase TPO activity in vitro. Thus, a variety of natural and synthetic chemicals can interfere with this important enzyme.

3. Chemicals that increase thyroid hormone clearance

A large number of chemicals that reduce circulating levels of thyroid hormone do so by activating enzymes in the liver that increase T_4 and/or T_3 clearance from blood (1026). However, some chemicals that reduce serum total and free T_4 do not increase serum TSH and do not produce effects on thyroid hormone action in tissue that are consistent with their effects on serum T_4 concentrations (1012). The mechanisms that account for this observation are not known.

4. Chemicals that bind to T_4 binding globulin or transthyretin

A significant number of chemicals bind to thyroid hormone binding proteins in blood, sometimes with greater affinity than with thyroid hormone itself (1027–1032). The functional significance of this binding is not clear. On one hand, this might reduce the amount of thyroid hormone normally distributed across tissues. It may also interfere with analog measures of free hormone. Most of these chemicals are phenolic compounds, such as PCBs, PBDEs, and others.

5. Chemicals that interfere with thyroid hormone metabolism

T_3 and T_4 are metabolized by deiodinase enzymes, as well as hepatic enzymes including glucuronidases and sulfatases. The outer ring deiodinases (Dio1 and Dio2) convert T_4 to T_3. In contrast, the type 3 deiodinase (Dio3) is an inner ring deiodinase, which converts T_4 to rT_3 and T_3 to T_2. These enzymes are critical for health, as evidenced by severe goitrous hypothyroidism in patients with genetic defects in the Dio2 (DEHAL1) gene (1033). Considering this, it is possible that environmental chemicals that have structures similar to that of thyroid hormone (eg, halogenated biphenyls and biphenyl ethers) could affect thyroid hormone metabolism in ways that would be difficult to detect. However, this issue has not been intensely studied. Recently, Shimizu et al (1034, 1035) began to evaluate the ability of chemicals to interfere with deiodinases involved in iodine salvage in the thyroid gland. They reported that several chemicals significantly inhibited this activity, although the 20% inhibitory concentration was in the micromolar range.

6. Chemicals that interfere with thyroid hormone transport

There are very few studies of this important issue. One recent study showed that the insecticide fipronil altered hepatic expression of the thyroid hormone transporter Oatp1c1 (1036). However, few studies have evaluated the ability of environmental chemicals to interfere directly with either T_4 or T_3 transmembrane transport.

7. Conclusions

These studies show that a number of manufactured and natural chemicals can interact with thyroid hormone production, transport through serum, metabolism, and clearance, all of which—singly or in combination—could explain the ability of various chemicals to cause a reduction in circulating levels of thyroid hormone. A significant area of uncertainty is that it is not clear to what the degree various adaptive responses within the thyroid system can compensate for these effects or when during development these adaptive responses become mature.

D. EDCs and the thyroid

An increasing number of studies are reporting on the relationship between EDC exposures and measures of thyroid function in the human population, most focused on PCBs, PBDEs, phthalates, BPA, and perchlorate. Studies in humans do not consistently associate chemical exposures with circulating levels of hormones, possibly because an association between chemical exposures and circulating levels of hormones is difficult to test directly in humans. However, animal studies have identified a large number of environmental chemicals that cause a decrease in serum thyroid hormone (reviewed in Refs. 1026 and 1037). These, as well as in vitro studies, do show a consistent association between EDC exposures and thyroid hormones. One of these reviews also provided a timeline for the development of the rodent and human thyroid gland in relation to neural development and categorized antithyroid chemicals in terms of their site of action within the thyroid system, enabling a head-to-head comparison of species (1026).

Here, we focus on the mechanisms by which some manufactured chemicals are known to interfere with thyroid hormone action. The strength of this approach is that it provides a simple way for readers to approach this issue and emphasizes the mechanistic aspect of thyroid disruption. We realize that thyroid cancer is increasing in incidence, but little is known about the role of environmental chemicals. This issue has been well reviewed recently (799) and will not be discussed further in the current article.

1. Perchlorate

a. Human studies. Experimental studies in humans indicate that the serum half-life of perchlorate is about 8 hours and that an exposure level of about 5.2 μg/kg/d is sufficient to begin to reduce iodide uptake into the thyroid gland (1038). The adult thyroid gland stores a great deal of hormone in the form of iodinated thyroglobulin, and human toxicology studies indicated that only very high exposures to perchlorate would inhibit thyroid hormone synthesis (1038), so it was surprising that one study found that

background exposure to perchlorate was associated with serum TSH in the general population of women (not in men) (1039). It is less surprising that this association was greater in women with urinary iodine below 100 μg/L, and stronger still among these women who smoked (1040), because cigarettes contain thiocyanates that also inhibit iodine uptake. These observations were repeated in a later NHANES survey (1041). Because infants are particularly vulnerable to thyroid hormone insufficiency (1042) and because perchlorate levels are particularly high in breast milk (1043), perchlorate may be affecting thyroid hormone signaling in early infant development in some proportion of the US population (1044).

Studies showing an association of low-dose long-term perchlorate exposure and thyroid disruption (1039, 1040) were not consistent with other studies of the high-dose short-term human exposure (1038, 1045, 1046), although there was a link between perchlorate exposure and thyroid disruption in the 2007–2008 NHANES sample (1041). In addition, a recent European study reported a relationship between perchlorate exposure in pregnant women and measures of reduced cognitive function in the offspring (1021).

b. Animal studies. Because the mechanism of perchlorate action is well known, and because both the toxicology and epidemiology have been performed on humans, there are fewer animal studies investigating the mechanism of perchlorate action. Perhaps the most important was studied by Gilbert and Sui (1047), who showed that perchlorate exposure to pregnant rats affected synaptic function in the adult offspring.

2. Polychlorinated biphenyls

a. Human studies. A considerable literature shows that developmental PCB exposure (both prenatal and postnatal) is associated with a variety of cognitive deficits in children (1048). The most recent reports have begun to evaluate the specific cognitive domains affected by PCB exposures and to distinguish them from those affected by lead and methylmercury (27). There is very little consistency in the literature describing the relationship between PCB exposure and measures of thyroid function in humans, despite the established relationship between PCB exposure and adverse cognitive measures (1049, 1050).

b. Animal and in vitro studies. Research in rodents consistently shows that PCB exposure leads to a reduction in serum total and free T_4 (eg, Ref. 1051). Importantly, in vitro and animal research indicates that at least some PCBs may interfere with thyroid hormone action at the tissue or cell level. Variations between effects of the 209 PCB congeners exist due to the number and placement of bromine atoms on the diphenyl oxide ring (1052). A commercial mixture of PCB congeners, Aroclor 1254, exerted thyroid hormone-like effects on thyroid hormone-regulated genes in the developing rat brain (1053). In addition, a number of studies demonstrated that very low concentrations of some hydroxylated PCBs (10^{-10} M) interfered with the ability of T_3 to activate the thyroid hormone receptor (ThR), which affected neuronal growth in primary cortical neurons (1054, 1055). Hydroxylated PCB metabolites also interfered with ThR activation in GH3 cells (1056), in rodents (1057), and in humans (1058). All of these studies show that the ability of PCBs to interfere with ThR activation was not reflected by changes in circulating levels of thyroid hormone. These are important observations, but it should not be taken to mean that a large number of chemicals interact directly with the ThRs. In fact, Hofmann et al (1059) identified very few chemicals that directly interacted with the ThR.

3. Polybrominated diphenyl ethers

a. Human studies. PBDEs are found in the blood of pregnant women (35), in cord blood, and in breast milk (1060, 1061). Studies show a significant negative correlation between cord blood PBDE levels and cognitive function, including full scale, verbal, and performance IQ (eg, Ref. 1062). These findings are consistent with animal studies, which documented a number of neurobehavioral deficits in rodents caused by various PBDEs, and in some cases their interactions with PCBs (1063–1066). Moreover, others have reported that PBDE exposure is negatively correlated with cognitive function in humans (700, 1067, 1068).

b. Animal and in vitro studies. The relationship between PBDE exposure and neurodevelopment in humans likely involves multiple mechanisms. In addition to affecting thyroid hormone action, some PBDEs affect intracellular calcium regulation (1069) and other signaling or molecular mechanisms that could affect brain development (1061). However, because of the critical role of thyroid hormone in brain development, small deficits in thyroid hormone levels during developmental stages can produce cognitive deficits (1009). By inference, if PBDE exposure alters thyroid hormone levels in pregnant women, fetuses, or neonates, this may result in cognitive deficits due at least in part to thyroid disruption. Moreover, if some PBDE congeners or their metabolites can interfere with thyroid hormone action at

the cellular level, then PBDE exposure may also be linked to cognitive deficits by this mechanism.

Findings in animal, in vitro, and biochemical studies have supported epidemiological studies examining the association between PBDE exposure neurotoxicity and thyroid disruption. Several early rodent studies indicated that PBDE exposure reduced circulating levels of total and free T_4 (1070–1072). In some cases, TSH levels were elevated, and in others the TSH levels were not elevated. A recent animal study compared PBDE exposure with propylthiouracil (PTU) exposure in their effects on serum total and free T_4, TSH, and "downstream" measures of thyroid hormone action in tissues (1012). Results showed that PBDE exposure reduced serum free and total T_4, but that serum TSH levels were not increased. Moreover, PBDE exposure reduced serum T_4 to a level that was similar to that produced by PTU, but the effects on thyroid hormone action in liver and heart were not similar to those of PTU. Thus, PBDE exposure produced enigmatic effects on serum thyroid hormone levels and thyroid hormone action in tissues, suggesting that caution should be applied to the underlying assumption that the effects of PBDE on thyroid hormone action are predicted by changes in serum hormone levels.

These findings and others suggest that some PBDE congeners may act directly on the ThR. This interpretation is supported by the observation of Ren et al (1073) who found that several hydroxylated PBDEs can displace T_3 from ThRβ and ThRα with IC_{50} values in the 1–10 μM range. Likewise, using a yeast-based assay containing a human ThRβ full-length protein, Li et al (1074) identified several hydroxylated PBDEs that interacted with the ThR. Importantly, Koibuchi's group (1075) reported that BDE209 could interfere directly with ThR binding to DNA at concentrations in the picomolar–nanomolar range and that this was dependent upon the DNA motif to which the ThR was bound. These are a few of the studies demonstrating a direct physical interaction between some PBDEs or their hydroxylated metabolites and the ThR. Because there are 209 different PBDE congeners and multiple kinds of metabolites, it is likely that additional PBDE species could be identified that interact with the ThRα and/or ThRβ. Moreover, several groups have identified PBDEs that can activate luciferase expression in a "transactivation" assay or in cell-based proliferation assays (1076). These kinds of studies may identify chemicals that interact directly with the ThR or may affect deiodinase enzymes or transporters in a manner that produces similar effects. A review of the literature in this field within the context of neurotoxicity was published several years ago (1077).

4. Phthalates

a. Human studies. Several epidemiological studies have reported relationships between urinary phthalate levels and thyroid function measures including T_4, T_3, and TSH. One study reported a negative association between urinary phthalate monoesters and serum free and total T_4 (1078). A single blind study found that a 1-week topical application of diethyl phthalate or DBP did not affect serum total or free T_4 or serum TSH (1079). However, a third study reported that urinary DEHP was negatively correlated with serum free T_4 in men (1080). In addition, using data from NHANES, urinary MEHP was significantly negatively associated with serum total and free T_4 and positively associated with serum TSH (6). Interestingly, a negative association was reported in nonobese subjects between phthalate metabolites in urine and serum T_4, but not in obese subjects (1081). Finally, another study reported a positive association between intake of phthalate-tainted foods and serum TSH in Taiwanese children (1082).

Overall, there seems to be surprising agreement among human studies showing that specific phthalates are associated with reduced serum T_4, and in some cases increased serum TSH, and that there appear to be several mechanisms in play.

b. Animal and in vitro studies. Among the first studies evaluating effects of phthalates on the thyroid was a 2-year carcinogenesis study of diallyl phthalate in mice, which noted limited effects on thyroid histology, but did not measure hormone levels (1083). A second study evaluated the effect of exposure to bis-DEHP, di-n-hexyl phthalate, or di-n-oxyl phthalate on various health measures in rats and observed that all caused a decrease in serum T_4 but not T_3 (1084). In contrast, a third study reported that high doses of DBP administered for 15 days via oral gavage reduced both T_3 and T_4 (1085). Studies have shown that phthalates enhanced the expression of NIS in a rat thyroidal cell line (PC C13), as well as perchlorate-sensitive iodide uptake into FRTL5 cells (1086). In addition, a study showed that several phthalates decreased T_3 uptake in a *Rana catesbeiana* erythrocyte assay (1087). Finally, several phthalates were evaluated for their ability to interfere with ThRs. One study reported that a low dose (10^{-7} M) of DEHP suppressed T_3-mediated receptor activation and that this was not related to its ability to cause receptor dissociation from thyroid hormone-response elements or to the altered ability of the ThR to interact with cofactors (1088). Using the rat pituitary cell line GH3 in a T-SCREEN, another study identified several phthalates that affected T_3-stimulated cell proliferation (1089). These experimental studies point to the need for more

research into the mechanisms by which various phthalates interfere with the thyroid system because these chemicals are ubiquitous and have the potential to interfere with the healthy function of the human thyroid gland.

5. Bisphenol A

a. Human studies. Several recent epidemiological studies indicate a link between BPA exposure and thyroid hormone levels. A weak positive association between halogenated BPA and serum T_4 was reported in one study (1090), similar to a previous study (1091). In contrast, two other reports indicated a negative association between serum free T_4 and BPA exposure (6, 1092). Given the individual and population variability of serum thyroid hormone levels (1006, 1007) and the rapid clearance of BPA (1093, 1094), any observed relationships between the two measures should raise some interest. However, there has been little research in this area.

b. Animal and in vitro studies. In 2002, two studies showed that BPA is a weak ligand for ThRs, therefore acting as an indirect antagonist (1095, 1096). BPA inhibited the transcription of genes activated by TRs in cell culture (1097, 1098). A more recent study using CV-1 kidney cells suggests that BPA can interfere with thyroid hormone action on the TRβ receptor by a nongenomic mechanism (1099).

A study of dietary exposure to BPA during pregnancy and lactation reported an increase in serum total T_4 levels without affecting TSH, a pattern similar to that found in thyroid resistance syndrome, as would be expected for a ThR antagonist (1100). Seemingly paradoxical, that study also found that the thyroid hormone-responsive gene *RC3* was elevated in the dentate gyrus of the hippocampus where ThRα is predominantly expressed. Thus, BPA may act as an antagonist selectively on the ThRβ isoform, disrupting the negative feedback mechanism but increasing the transcription of thyroid hormone-activated genes where TRα is present as a response to elevated T_4 levels. If BPA creates a pattern of disruption that mimics thyroid resistance syndrome, it may affect the development of the cortex. This would also be consistent with the observed relationship between thyroid resistance and attention deficit in both mice (1101, 1102) and humans (1103, 1104).

E. Conclusions

There are several important conclusions to be drawn from the recent data on EDCs and thyroid disruption. First, there is good evidence from animal, biochemical, and human studies that specific chemicals can interfere with thyroid hormone action and cause adverse effects at the population level. Second, the current biomarker of thyroid "disruption"—circulating levels of thyroid hormone—may not be faithfully reflecting EDC effects on thyroid hormone action in tissues. Therefore, new biomarkers of thyroid hormone action must be developed for use in epidemiological studies. Third, there are a large number of EDCs that can interact with and potentially disrupt the thyroid system at different nodes of thyroid hormone action regulation, and very few studies have evaluated this. Fourth, given the importance of thyroid hormone in development, especially of the brain, we need more studies on this topic.

VIII. Neurodevelopmental and Neuroendocrine Effects of EDCs

A. Introduction to EDCs and the developing brain

Parts of the brain function as an endocrine gland. Certain groups of neurons release their neurohormones by the classical endocrine mechanism into a portal capillary vasculature that vascularizes the anterior pituitary. This neuroendocrine region at the base of the brain, the hypothalamus, is integrally involved in controlling how the body adapts to the environment and in the regulation of several peripheral endocrine systems. Different groups of hypothalamic neurons control reproduction, growth, metabolism, lactation, stress responsiveness, uterine contractions at parturition, energy balance, circadian rhythms, temperature regulation, and electrolyte balance, among other functions. For the purposes of EDC-2, we will focus primarily on the hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) neuroendocrine axes controlling reproduction and stress, respectively (Figure 11). Importantly, the steroid hormones produced by the gonads and adrenals, beyond their classical endocrine actions, act upon the brain via steroid hormone receptors that are not only expressed in the hypothalamus but are also widely and heterogeneously expressed throughout the nervous system.

Thus, the brain is a target of EDCs in two principal ways: through perturbations of neuroendocrine processes that originate in the hypothalamus, and via actions of EDCs on steroid hormone receptors and other signaling pathways (eg, steroidogenesis) that occur much more widely throughout the brain. Here, we discuss the literature on neurodevelopment and neuroendocrine actions of EDCs.

Readers will note that there is little discussion of the human literature. Assays of neurodevelopment require postmortem tissues, on which no studies are available. Neuroendocrine neurons cannot be directly evaluated because hypothalamic releasing hormones cannot be mea-

sured in peripheral blood samples, urine, or other tissues. Therefore, the human data are limited to behavioral outcomes, which are discussed toward the end of *Section VIII*.

B. EDC effects on steroid hormone receptors and steroidogenic enzymes

The brain's dense and widespread distribution of hormone receptors, its high hormone sensitivity, and its ability to synthesize steroids through the expression of steroidogenic enzymes, among other characteristics, make it particularly vulnerable to hormonal perturbations. This concept is particularly important when put into the context of development because there are critical periods during which even minute changes in hormone exposures can affect a neurobiological outcome. This issue of sensitive life periods is central to understanding EDC effects on brain and behavior (1105).

1. EDC effects on steroid hormone receptors in the brain

There is robust evidence that EDCs change the expression, abundance, and distribution of steroid hormone receptors in the developing central nervous system. Research on many classes of EDCs consistently shows effects on mRNA levels, protein expression, and neuroanatomical changes to nuclear hormone receptors studied to date, as well as functional consequences of altered receptor action. Although beyond the scope of this review article, there is a very robust and rich literature on EDC effects in fish brains and behavior, and we refer readers to some reviews on the subject (1106–1109). This section will focus on BPA and PCBs for which there is the most evidence for EDC actions on hormone receptors in the brain.

Box 6. Key Points: Thyroid

- **A large number of chemicals and chemical classes are known to affect the thyroid system.**
- **Animal studies have also demonstrated that a number of chemicals can reduce circulating levels of thyroid hormone, including but not limited to PCBs, PBDEs, some phthalates, and perchlorate. Interestingly, not all chemicals also cause an increase in serum TSH.**
- **Some chemicals that affect the thyroid system in animals have been shown to be associated with cognitive deficits in humans. However, exposure is not always correlated with reductions in thyroid hormone in humans.**
- **Thyroid hormone produces different effects at different developmental stages—in humans as well as in animals—and the consequences of disruption are stage-specific.**
- **Some chemicals clearly exert actions on the thyroid system in humans and animals at environmentally relevant concentrations. The mechanism(s) by which chemicals can produce this effect varies.**
- **Three key areas of research are urgently needed: 1) identify biomarkers of thyroid hormone action in tissues to test the ability of chemicals to interfere with hormone action in the absence of effects on serum hormone concentrations; 2) determine whether chemicals with different mechanisms of action on the thyroid system can synergize to cause adverse effects; and 3) identify high throughput assays that predict thyroid "disruption."**

a. Bisphenol A

i) Hypothalamus. In the hypothalamus of rodents, developmental BPA exposures affect expression of the nuclear ER genes and proteins, but the directionality of results is mixed. For example, several studies have shown that hypothalamic ERα protein and mRNA expression was increased by prenatal and/or early postnatal BPA in some studies. Although the vast majority of work comes from rodent models, this outcome was reported for the female sheep medial preoptic area (MPOA) after prenatal (d 30–90 of the 147-d gestation; 5 mg/kg/d to the ewe dam) exposure of the females. In mice, numbers of ERα immunoreactive cells in the MPOA were increased by BPA (50 μg/kg/d or 5 mg/kg/d, from E15 to P21) in both sexes at P56 (1110). A similar outcome for ERα cells in the anteroventral periventricular nucleus (AVPV) was reported for postnatal (P1 to P7) BPA treatment of female rats (0.05 or 20 mg/kg/d) when analyzed at P100 (572). Another rat study administered BPA (2 μg/kg/d to dams) from E10 to P7 in rats and reported *Esr1* up-regulation in both sexes at P80 (1111). In this same species, BPA (E6 to E21, 2.5 or 25 μg/kg/d) increased *Esr1* in the medial basal hypothalamus shortly after birth in males and females on P1 (1112).

By contrast, other experiments showed decreased ERα protein and mRNA expression after BPA, or mixed effects. Prenatal BPA treatment (E6 through the day of birth; 2.5 or 25 μg/kg/d) decreased *Esr1* expression on P21 in the AVPV of female rats (1113). In the study of Monje et al (572), whereas postnatal BPA increased ERα cells in AVPV (discussed above), it decreased this endpoint in the arcuate nucleus (ARC) at P100. In

Figure 11.

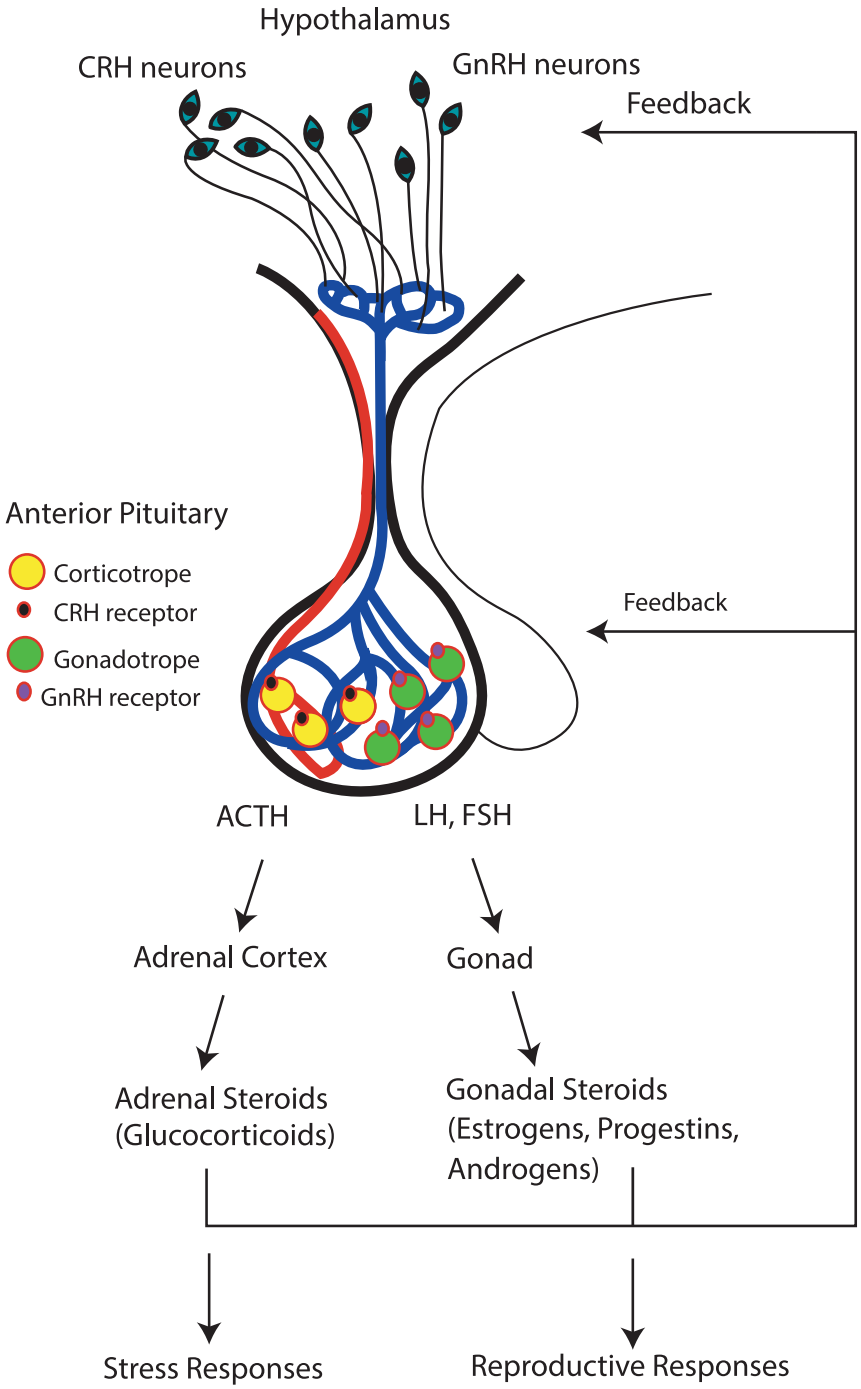


Figure 11. The HPG and HPA neuroendocrine axes are shown. For each system, a group of hypothalamic neurons synthesizes and secretes a neuropeptide, CRH (HPA axis) or GnRH (HPG axis). These hormones travel through the portal capillary vasculature between the base of the hypothalamus and the anterior pituitary. There, CRH activates the synthesis and secretion of ACTH from corticotropes. Similarly, GnRH stimulates LH and FSH release. These pituitary hormones, when they reach their target glands (adrenal or gonad), regulate steroidogenic processes involved in biosynthesis of the glucocorticoids and sex steroid hormones. These hormones activate stress and reproductive responses, respectively, in the body. They also exert feedback actions on the hypothalamus and pituitary to enable adaptation of the body and to modulate homeostasis of these neuroendocrine systems.

mice, prenatal BPA (2, 20, 200 μg/kg/d from E0 to E19) resulted in inverted U-shaped dose-response curves for *Esr1* in whole hypothalamus of male and female offspring on P28 (1114).

Finally, ERα protein and mRNA were also unaffected by several BPA treatment regimes. Such a finding was made in female rats for exposure from E15 to P21 (5 mg/kg/d) on ERα protein in the MPOA, ventromedial nucleus of the hypothalamus (VMH), and ARC at P56 (1115); for male rats (E8–E23; 25 or 250 μg/kg/d) for *Esr1* mRNA in the preoptic area (POA) and medial basal hypothalamus at P30 or P120 (1116); and for male and female rats exposed (E6–E21) to 2.5 or 25 μg/kg/d BPA and analyzed at P1 (1112). Another rat study reported no change to *Esr1* in the MPOA, VMH, or ARC of female rats at P21 or P90 exposed to BPA (2.5 or 25 μg/kg/d) from E6 through birth (1113). It is notable that some of these results with different directionality come from the same study but with different brain regions or age at analysis.

The literature on BPA actions on the ERβ is similarly complex, with increases (475, 1112), decreases (475, 1113, 1117), mixed results—inverted U-shaped dose-response curve (1114), or no changes (1112, 1113, 1115) reported. Many of these studies also measured ERα, so details of exposure period, dose, and age of analysis will be limited. Stimulatory effects of BPA were seen for rats exposed prenatally and assessed for *Esr2* in the VMH at P1 (1112). Those studies showing inhibitory effects of BPA on ERβ included the sheep model (1117) and several rodent studies. In rats, BPA decreased *Esr2* in the male but not in the female hypothalamus (1111). Another rat study showed that prenatal BPA de-

creased *Esr2* in the AVPV and MPOA of female rats at P90 (1113). It is clear that there are marked differences in how studies were conducted that make it difficult to reconcile results.

There are few studies on the AR, PR, and GR. For the AR, BPA exposure increased numbers of AR-immunoreactive cells in the POA of mice exposed to BPA from E15 to P21 (50 μg/kg/d or 5 mg/kg/d) and evaluated at 8 weeks of age (1110). Monje et al (572) reported that PR protein was slightly decreased by BPA (P1–P7, 0.05 or 20 mg/kg/d) in the AVPV, but not the ARC, two structurally and functionally distinct subregions of the hypothalamus. GR protein was decreased by BPA in the hypothalamus of female rats, but not male rats, exposed during gestation and through lactation (40 μg/kg/d) and assessed at P46 (1118).

ii) Nonhypothalamic brain regions. In the hippocampus, developmental exposure of male rats to BPA changed expression of the ERα gene and protein, an effect that was dependent upon the age at which ERα was measured, and this effect was blocked by the ER antagonist ICI 182 780 (46). A study from this same group reported that BPA decreased ERβ protein in hippocampus (475). In the amygdala of rats of both sexes, BPA treatment increased both ERα and ERβ gene expression at P1 in several subregions (1112). In prefrontal cortex, BPA caused little effect on ERα mRNA, and a decrease in ERβ gene expression, but only in females (1114). BPA (40 μg/kg/d) had no effect on GR protein in the hippocampus of male or female rats (1119).

b. Polychlorinated biphenyls. Effects of developmental PCB exposures have been studied for effects on ERs, AR, and PR. Prenatal PCB treatment (Aroclor 1221, or a mix of three PCBs; 1 mg/kg at E16 and E18) decreased ERα-immunoreactive cell numbers in the AVPV of female but not male rats when assessed at P60 (567). In that same study, gene expression of *Esr1*, *Esr2*, and *Pgr* was unaffected. *Ar* gene expression was decreased in female but not male POA of these rats (567). Developmental PCB and PBDE exposures abolished sex differences in expression of PRs (1120), but this work was conducted at higher dosages (10 or 30 mg/kg/d of A1254, a PCB mixture, or 1 or 10 mg/kg/d of PBDE99). In addition, *Esr1* expression was increased, and *Esr2* decreased in male and female rats, whereas *Pgr* did not change (1120).

2. Developmental EDC effects on steroidogenic and steroid-metabolizing enzymes in the brain

Nearly all of the steroidogenic enzymes are detectable in the brain, with each enzyme having unique developmental and brain region-specific profiles. Beginning with those enzymes necessary for cholesterol transport and side chain cleavage, and continuing through the pathways leadings to glucocorticoids, mineralocorticoids, estrogens, progestins, and androgens, these enzymes are capable of interconverting, metabolizing, and inactivating steroid hormones in the brain (1121–1131). This is a relatively underexplored area for EDC research, but available publications provide evidence that early-life EDC exposures can modulate expression and activity of these enzymes. Detailed experimental information is found in Table 7.

a. Bisphenol A. Gestational and early postnatal BPA exposures increased expression of the p450 aromatase protein in the hippocampus (475). Although we have focused this article on developmental exposure, treatment of adult male and female rats for 4 days with low-dose BPA (50 μg/kg/d) had significant effects on mRNAs for aromatase (increased in both sexes) and 5α-reductase 1 (decreased in females) in the prefrontal cortex (974).

b. Polychlorinated biphenyls. Exposure of rat pups to a reconstituted mixture of PCBs had sex- and age-specific effects on mRNA of 5α-reductase 1 and 2 and aromatase in the hypothalamus (566). Embryonic treatment of fetuses with Aroclor 1254, a PCB mixture, affected 5α-reductase 2 mRNA in females but not males when assessed at P20; it did not affect 5α-reductase 1 or aromatase mRNA, or aromatase activity in either sex (1132). Other enzymes (catechol-O-methyltransferase, Cyp1A1) were affected (mostly decreased) by mixtures of PCBs, PCDDs and PCDFs given to rats during postnatal development (1133).

3. Conclusions

The literature shows that the brain's expression of genes and proteins for steroid hormone receptors is highly sensitive to developmental EDC exposure. However, it is difficult to draw any generalized conclusions because the way in which the hypothalami were dissected, the way that endpoints were measured, the exposure period, the doses, and the ages at analysis differed from lab to lab. It is important for future work to include replication of past methodologies to allow for better extrapolation of results.

Nonreceptor-mediated mechanisms of action of EDCs are also important points of consideration for the brain, particularly steroidogenic enzymes. The widespread localization and developmental changes in expression of these enzymes makes them potential targets of EDCs. This mechanism has been studied better in the ovary (see *Section III*), and more research on the steroid-synthesizing organs of the body is needed.

Table 7. Developmental EDC Effects on Steroidogenesis in the Brain

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis and Brain Region	Effect	Endpoint(s)	Ref.
BPA	Rat (S-D)	M/F	E7 to P21	0.01, 0.5, 5, 50, 200 mg/kg/d by gavage to dams	P4, P7, P14, P21, P56; Hippocampus	Aromatase protein was increased in a dose-dependent manner by BPA at ages prior to P56	Aromatase protein	475
PCBs: reconstituted mixture of PCBs 126, 138, 153, 180	Rat (S-D)	M/F	E15–E19 to dam, then 2 × weekly to dam until pups were P21	10 mg/kg/d given to dams sc	E20, P12, P21, P60; Hypothalamus	5α-reductase 1 mRNA was decreased in females at P21 and in both sexes at P60. 5α-reductase 2 mRNA was increased in females at P60. Aromatase mRNA was increased in males at P21	mRNA of 5α-reductase1, 5α-reductase 2, aromatase	566
PCBs: Aroclor 1254	Rat (S-D)	M/F	E15–E19 to dam; neuronal cultures also performed	25 mg/kg/d given to dams by gavage for in vivo work. In neuronal cultures, 10, 100, 500 μg/mL	E20 hypothalamus (in vivo) or hypothalamic neuronal cultures	In vivo, there were no effects of A1254 on aromatase activity or mRNA, or on 5α-reductase 1 mRNA. 5α-reductase 2 mRNA was increased in females, and AR mRNA was decreased by A1254. In vitro, the two higher doses of A1254 decreased aromatase activity	mRNA of 5α-reductase 1, 5α-reductase 2; aromatase activity	1132
PCB, PCDD, PCDF reconstituted mixture	Rat (S-D)	M/F	P1, P5, P10, P15, and P20	Gavaged at 1×, 10×, 100×, 100 × concentrations estimated based on human infant exposures from breast milk	P21; hypothalamus, hippocampus, cortex	The 1000 × mixture had significant effects on *Comt* (reduced in hypothalamus, hippocampus, cortex of males), *Cyp1A1* (elevated in males, all regions), *Esr1* (decreased in males in hypothalamus and hippocampus) and *Dnmt1* (decreased in hypothalamus of males). 100 × mixture increased *Comt* in cortex of females. Other data were not shown	mRNA of *Cyp1A1*, *Cyp1B1*, *Cyp2B1*, *Comt*, *Esr1*, *Dnmt1*	1133

Abbreviations: M, male; F, female; sc, subcutaneous; S-D, Sprague-Dawley.

C. Molecular epigenetic mechanisms for EDC effects in the brain

The molecular mechanisms underlying the effects of EDC exposures during development are beginning to be better understood for the nervous system and include DNA methylation and histone modifications. DNA methylation in the brain is sexually dimorphic (in a brain region-specific manner) and highly sensitive to the perinatal hormonal environment (1134–1136). Histone methylation and acetylation are also sexually dimorphic, and can be modified by early-life hormonal manipulations, as shown in the hypothalamus, cortex, and hippocampus, and this can change adult behaviors (1137–1141). These molecular mechanisms are therefore vulnerable targets for developmental EDC exposures.

1. DNA methylation

a. Bisphenol A. Evidence that BPA induces changes in DNA methylation, primarily hypomethylation, was first shown for the agouti viable (A^{vy}) mouse (104) and in prostate cancer cells (105). In one of the first studies investigating the effects of BPA on global DNA methylation in embryonic forebrain (1142), mice were exposed to BPA beginning on E0 (20 μg/kg/d), and CpG methylation was assayed by restriction landmark genomic scanning. The results showed that BPA induced methylation and demethylation at roughly similar levels and supported the ability of BPA to affect methylation in the fetal brain. In another study, pregnant mice were fed BPA through gestation (~20 μg/kg/d), and their embryos were collected at E18.5 for analysis of *Dnmt1*, *Dnmt3a*, and *Dnmt3b* in whole brain (1143). That study did not find any differences caused by BPA, but it is notable that the use of whole brains may have masked region-specific effects. In fact, Kundakovic et al (1114) reported sex- and brain region-specific effects of BPA (2, 20, or 200 μg/kg/d from E0 to E19) on *Dnmt1* and *Dnmt3a* gene expression, and differential effects on CpG methylation of *Esr1*. Additional differences between these studies may be due to the different ages at analysis, which were P28 (1114) and E18.5 (1143). Finally, *Dnmt1* mRNA was significantly increased in the basolateral amygdala of female rats at P45 that had been exposed to BPA through gestation and lactation (1144).

b. Methoxychlor. In one study, the effects of perinatal MXC treatment did not affect CpG methylation of the gene for ERα, *Esr1*, in the aged female POA, although estradiol benzoate treatment in the positive control group resulted in hypermethylation at three CpG sites (601). When prenatal PCBs were given in late gestation (A1221 at 1 mg/kg

on E16 and E18) and DNA methylation of the genes for the androgen receptor (*Ar*) and Period 2 (*Per2*) were measured in the AVPV of male and female rats during postnatal development, only very small effects were found (1145). This latter study also quantified mRNA levels of *Dnmt1* and *Dnmt3a* and found a significant effect of PCBs on *Dnmt1* but not *Dnmt3a* mRNA in the AVPV of both females and males. In the ARC of the hypothalamus, *Dnmt3a*, but not *Dnmt1*, was affected in both sexes (1145). Thus, there are brain region-specific differences in effects of EDCs on expression of these genes.

2. Histone modifications

Although less is known about EDC effects on histone acetylation, methylation, and other characteristics of histones, the existing literature suggests some effects.

a. Bisphenol A. A recent important study looked at BPA treatment of cultured cortical neurons from rats, mice, and humans, focusing on outcomes related to the potassium chloride cotransporter 2 (KCC2), which is essential for neuronal development and the chloride shift that occurs as the brain develops (1146). Results of that study showed that BPA treatment of primary cultures attenuated the chloride shift, something that was rescued by blockade of HDAC1. BPA also increased MECP2 expression and binding of the MECP2 protein to CpG shores in the KCC2 gene, together with decreased H3K9ac binding. That group also went on to study cortical cultures from newborn mouse pups exposed to BPA during gestation (as opposed to application of BPA to cultures in the former studies). Those results were consistent in showing diminished KCC2 expression and in effects on CpG methylation of the KCC2 promoter (1146). Thus, this study is important for proving biologically relevant epigenetic mechanisms of DNA methylation and histone deacetylation in models of the developing nervous system. Because of the widespread importance of the chloride shift in neuronal development throughout the entire brain, beyond the cortex, this finding is likely to be applicable to other brain regions.

b. Phthalates. In a human neuroblastoma cell line (SH-SY5Y), phthalate treatment affected expression of the HDAC genes HDAC4 (increased) and HDAC5 (decreased), and HDAC inhibitors blocked the effects of phthalates on cellular viability (1147).

3. Conclusions

Environmental epigenetics is an emerging field that has its roots in the DOHaD model. In fact, the organizational-activational hypothesis of brain sexual differentiation underscores the exquisite sensitivity of the brain to the changing hormonal milieu during development. Now that evidence is stronger for which epigenetic molecular mechanisms may underlie these processes, the corresponding EDC work is likely, over the next few years, to provide more mechanistic information about how these chemicals affect brain development in a sexually dimorphic manner.

D. Developmental EDC effects on neuroendocrine systems

In EDC-1, we comprehensively reviewed the evidence for EDC effects on the HPG axis and the neuroendocrine control of reproduction (1). For EDC-2, we will provide an update and add discussion of recent information about the HPA control of stress. The HPT system is covered in *Section VII*. There is still only limited information on the effects of EDCs on the neuroendocrine control of growth and lactation, which will not be covered here. The emerging field of EDC actions on energy balance is discussed in *Section II*, but it is notable that studies linking EDCs to perturbations in metabolism were conducted primarily in the realm of peripheral energy balance, with limited information on the central control of energy balance. Although there are some suggestions of links between EDCs and neurotransmitters involved in energy balance control (1148), this area of research is in its infancy. One example is a study of exposure to BPA followed by feeding a high-fat diet in adulthood that showed effects on glucose tolerance and on expression and activation of neuropeptides involved in feeding behaviors (245). However, this should be a research priority for the future.

1. HPG systems

The hypothalamic neurons that synthesize and release the neuropeptide GnRH (encoded by the *Gnrh1* gene) are vulnerable to several classes of EDCs. This section will review the literature on EDCs and GnRH function, together with the emerging evidence for effects of EDCs on hypothalamic kisspeptin neurons (Table 8). First identified about a decade ago as an important neuropeptide involved in the control of reproduction through its actions on the GPR54 receptor (encoded by the *Kiss1r* gene), kisspeptin has emerged as a critical player in the activation of GnRH neurons during puberty and in mediating negative feedback (both sexes) and positive feedback (females) of steroid hormones (1149–1151). Since then, it has also become clear that kisspeptin is integrally involved in energy balance and may be involved in the coordination of energy demands and reproductive function (1152). For this section, additional details are provided in Table 8.

a. Bisphenol A. A number of articles reported stimulatory actions of BPA on GnRH and kisspeptin systems (ie, increased gene and/or protein expression). For example, developmental BPA exposure increased *Gnrh1* and *Kiss1* mRNA in the hypothalamus of P50 males and females (434). Perinatal BPA exposure of male rats slightly increased GnRH neuron numbers, significantly increased AVPV kisspeptin neuron numbers, and enabled gonadectomized, estradiol-primed males to have an LH surge (1153). In female mice, gestational plus lactational exposure to BPA increased the numbers of kisspeptin-immunoreactive neurons only in the rostral periventricular nucleus, but not in the caudal periventricular nucleus or AVPV (1115). No changes in the numbers of GnRH neurons were detected in that study (1115). In lambs gestationally exposed to PCB153 but not PCB118, GnRH-induced LH release, and to a lesser extent FSH release, was amplified (419).

Other articles have reported inhibitory actions of BPA. In female rats exposed neonatally to BPA, then OVX and hormone primed, effects of hormone priming on kisspeptin fiber density in the AVPV and ARC that normally occur in OVX rats were abolished in BPA-treated rats (1154). No effects of BPA were found in intact adult males in that same study (1154). This same laboratory also reported that in the ARC but not AVPV of peripubertal female rats, kisspeptin fiber density was decreased by BPA, that the neuropeptide RFRP3 fiber density was decreased, and that appositions between RFRP3 and GnRH neurons were decreased by BPA (1155). Another group exposed neonatal female rats to BPA and investigated GnRH-induced LH release at P13 or P120, reporting diminished LH responses at both ages (525).

Finally, some studies reported mixed actions or no effects of BPA, and one of these studies also investigated the action of MXC. Early postnatal BPA exposure to female rats that were subsequently OVX in adulthood and given hormone priming increased *Gnrh1* mRNA (low-dose BPA) and decreased GnRH primary transcript (low and high doses of BPA) and the steroid-induced GnRH/LH surge (572). Female rats exposed to BPA from P0 to P3 were OVX in adulthood and treated with estradiol plus progesterone. There was no effect on the percentage of activated GnRH neurons (coexpressing Fos) during the anticipated GnRH/LH surge (545). In mice, the numbers of kisspeptin-immunoreactive cells in the POA/AVPV of males perinatally exposed to BPA were unchanged in adulthood (1110). In sheep, the effects of gestational BPA or MXC were tested on steroid-induced negative and positive feedback on gonadotropins in adulthood or GnRH-stimulated LH secretion (1156). None of these parameters were affected in the sheep.

b. Polychlorinated biphenyls. Published studies of developmental PCB actions have demonstrated either inhibitory or mixed effects on GnRH and kisspeptin systems. In a model of prenatal PCB exposure to rats, kisspeptin immunoreactivity and coexpression of Fos in GnRH neurons (a marker of GnRH activation) were suppressed in the PCB-treated females (567). mRNAs for *Gnrh1*, *Kiss1*, and *Kiss1r* were unaffected in both males and females in the latter study (567).

Prenatal exposure of rats to PCBs (Aroclor 1221 or a reconstituted PCB mix) for 2 days of gestation had no significant effects on *Gnrh1* or *Kiss1* mRNA in the POA in male and female pups at P1 (1157). However, mixed effects were found for *Kiss1r* mRNA, levels of which were significantly increased in the males and decreased in the females by the PCB treatment. In two studies using this same paradigm in A1221-treated rats, postnatal developmental expression of *Gnrh1* and *Kiss1r* in the AVPV was masculinized in the females, and *Kiss1* was increased in the ARC (1145). When rats were aged to 9 months, these relationships were no longer apparent (604), underscoring the point that the age and physiological status are important variables to consider when evaluating outcomes.

c. Phthalates. For phthalates, when DBP was given to neonatal (P1–P5) or peripubertal (P26–P30) female rats, there were mixed effects on gene expression of *Gnrh1* and *Kiss1r* in the AVPV and ARC, dependent upon dosage and age of administration (555). Kisspeptin immunoreactivity was also measured and had similar outcomes, with increases primarily due to neonatal treatment in the low-to-intermediate dosages and decreases (ARC) due to peripubertal treatment (555).

d. Sewage sludge. In sheep fetuses of ewes feeding on pastures treated with sewage sludge, decreased hypothalamic *Kiss1* mRNA (but not *Kiss1r* or *Esr1*) and decreased numbers of pituitary cells immunoreactive for kisspeptin, double-labeled for kisspeptin/LHβ, kisspeptin/ERα, and LHβ/ERα, were found (1158).

e. Conclusions. It is clear that effects of developmental EDC exposures on GnRH and kisspeptin systems are not consistent. In fact, even within a study, the outcomes of early-life treatments with EDCs can be an up-regulation, down-regulation, or no change, depending upon the endpoint and the age at which an effect was determined and how that measurement was made (eg, gene expression vs protein and methodology, such as immunohistochemistry, Western blotting, PCR, etc). Therefore, whereas we cannot provide a generalizable conclusion that EDCs up- or down-regulate the GnRH and kisspeptin system, we have indicated the directionality of effects in Table 8.

Table 8. Developmental EDC Effects on GnRH-Gonadotropin and Kisspeptin Systems in Vivo

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Endpoint(s)	GnRH/LH Effect	Kisspeptin Effect	Ref.
BPA, MXC	Sheep	F	E30–E90 (full term is 147 d)	5 mg/kg/d via daily sc injections to ewe	Adulthood, ~21 mo	GnRH mRNA in anterior hypothalamus and POA	GnRH mRNA was decreased by both MXC and BPA ↓		1117
BPA, MXC	Sheep	F	E30–E90 (full term is 147 d)	5 mg/kg/d via daily sc injections to ewe	OVX at 21 mo of age	LH response to steroid feedback and to GnRH challenge	LH and FSH response to steroid negative feedback and LH response to positive feedback were unaffected by the EDCs. GnRH-induced LH release was similarly unaffected ✖		1156
EDC mix from sewage sludge	Sheep	Both sexes combined	E0–E110	Sewage sludge was applied to pastures; control pastures received conventional inorganic fertilizer	Ewes and fetuses were euthanized at E110.	*Kiss1*, *Kiss1r*, *Esr1* mRNA in rostral, medial and caudal hypothalamus; colocalization of kisspeptin and LHβ and ERα in the pituitary gland by dual IHC	Numbers of LHβ cells, and those co-expressing ERα in pituitary, were decreased in the sewage sludge group ↓.	*Kiss1* was significantly decreased ↓ through the hypothalamus and in the pituitary of sewage-sludge exposed fetuses. *Kiss1r* (GPR54), and *Esr1* mRNA were unaffected in hypothalamus and pituitary ✖. Numbers of kisspeptin cells, double-labeled kisspeptin/LHβ cells, and double-labeled kisspeptin/ERα cells, were decreased in the sewage sludge fetuses ↓	1158
PCBs	Sheep	F	E0 to birth	Ewes were gavaged 3 × weekly with PCB153 (98 μg/kg/d or PCB118 (49 μg/kg/d	Lambs	GnRH-induced LH and FSH release	Basal LH and FSH concentrations did not differ among groups ✖. The PCB153 group had significantly greater GnRH-induced LH release, and slightly but not significantly higher FSH release ↑. No effect of PCB 118 was found for this endpoint ✖		419
BPA	Mouse (C57BL/6J)	M	E15–P21	Gavage of dams 50 μg or 5 mg/kg/d	8 wk	Kisspeptin immunolabeling in the MPOA and AVPV		Kisspeptin immunoreactive neuron numbers were unaffected by BPA ✖	1110
BPA	Mouse (C57bl/6J)	F	E15–P21	0.05 or 5 mg/kg/d by gavage to dam	8 wk (young adult)	GnRH, kisspeptin, and ERα immunolabeling	Numbers of GnRH immunoreactive neurons and the percentage of those with kisspeptin apposition were unchanged by BPA ✖	Numbers of kisspeptin neurons were unchanged in AVPV and caudal periventricular nucleus ✖, and increased in rostral periventricular nucleus ↑	1115
BPA	Rat (Wistar)	F	P1–P7	0.05 or 20 mg/kg, sc	P100, OVX + estradiol to induce the GnRH/LH surge	*Gnrh1* mRNA and primary transcript; serum LH; ERα protein by IHC in AVPV and ARC	*Gnrh1* mRNA was increased by the low-dose BPA ↑. GnRH primary transcript (presence of intron A) was decreased by both BPA dosages ↓. Serum LH was decreased by high-dose BPA ↓		572

(*Continued*)

Table 8. Continued

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Endpoint(s)	GnRH/LH Effect	Kisspeptin Effect	Ref.
BPA	Rat (Long-Evans)	F/M	P0–P3	50 μg or 50 mg/kg/d	Adults (P148 or older), females OVX and hormone primed with estradiol + progesterone. Males were intact	Kisspeptin fiber density in the AVPV and ARC		Hormone priming in females reduced kisspeptin fiber density in AVPV, but not in BPA females ↓. In the ARC, hormones reduced kisspeptin immunoreactivity, but not in the low-dose BPA group ↓. No effect in males ✖	1154
BPA	Rat (Long-Evans)	F/M	P0–P3	50 μg or 50 mg/kg/d	Adults (P148 or older). Females were OVX and hormone primed with estradiol + progesterone	GnRH-Fos double labeling by IHC, and number of GnRH neurons	Number of GnRH neurons was unaffected; % of GnRH neurons that co-expressed Fos was unaffected ✖		545
BPA	Rat (GnRH-GFP transgenic Wistar)	F/M	P0–P3	50 μg or 50 mg/kg/d	Female (P17, P21, P24, P28, P33); male (P21, P33)	GnRH was detectable by GFP labeling. Kisspeptin and RFRP3 were detected by IHC	Kisspeptin appositions on GnRH neurons were unaffected by BPA ✖. Appositions of RFRP3 on GnRH neurons were decreased in the low-BPA females ↓	In AVPV, kisspeptin fiber density was unaffected by BPA ✖. In ARC, in P33 females, the high-BPA exposure group had decreased kisspeptin ↓, similar to that in males. RFRP3 fiber density was decreased by low-dose BPA in the females at P28 and P33 ↓	1155
BPA	Mouse (CD-1)	M/F	E1 to P20 to dams. Some pups continued gavage from P21 to 49	12, 25, or 50 mg/kg/d to dames via gavage	P50 (females on proestrus)	GnRH, Kisspeptin, GPR54 (hypothalamus); and pituitary mRNAs (including LHβ, FSHβ, and others)	*Gnrh1*: increased in both sexes ↑. Pituitary LH was unaffected ✖; FSH was increased in both sexes ↑. TSH, GH, PRL were unaffected ✖	*Kiss1* was increased in both sexes ↑. *Kiss1r* was unaffected ✖	434
BPA	Rat (S-D)	M/F	E10 to P7	2 μg/kg/d sc to dam	Male at P30, P50, P90. Males from BPA dams, and males and females from vehicle dams, were also gonadectomized and given estradiol for up to 7 d. Intact rats were also used	Number of kisspeptin immunoreactive cells in the AVPV. Number of GnRH cells in the POA	BPA slightly but significantly increased GnRH+ cells in the male POA ↑. When the LH surge was measured, females and BPA males, but not control males, exhibited an LH surge ↑	In gonadectomized, estradiol-treated rats, BPA increased AVPV kisspeptin neuron numbers at all 3 ages ↑, making them closer to the female (higher) levels. In intact rats, BPA kisspeptin cell numbers were also higher in both estradiol-treated and untreated males ↑. The LH surge in females and in BPA males was attenuated by the GPR54 inhibitor P234 ↓	1153

(*Continued*)

Table 8. Continued

EDC	Animal	Sex	Exposure Period	Dosage, Route	Age at Analysis	Endpoint(s)	GnRH/LH Effect	Kisspeptin Effect	Ref.
BPA	Rat (S-D)	F	P1–P10	BPA 50 (2.5–6.2) or BPA 500 (25–62 mg/kg/d) given sc to pups	P13 or P120 (in estrus)	GnRH pulsatility, GnRH-induced LH release	At P13, in vivo GnRH-induced LH release was decreased by BPA 500 ↓, the number of GnRH peaks/hour in an in vitro perifusion increased ↑, and the interpulse interval decreased ↓. At P120, GnRH-induced LH release was decreased at 15 and 50 min post-BPA ↓		525
DBP	Rat (S-D)	F	P1–P5 (neonatal) or P26–P30 (peripubertal)	0.5, 5, or 50 mg/kg/d given sc to pups	Adulthood (on proestrus)	mRNA of *Gnrh1* and *Kiss1r* in the AVPV and ARC	*GnRH1* mRNA in the AVPV and ARC was increased by DBP (5 mg/kg group only) in the neonatal group ↑. For the pubertal group, *Gnrh1* was increased (0.5 mg/kg) ↑ or decreased (5 mg/kg) ↓ in AVPV, and increased (0.5 mg/kg) ↑ or decreased (5, 50 mg/kg) ↓ in the ARC	*Kiss1r* in AVPV was increased by DBP (5 mg/kg) in the neonatal group ↑, and increased (0.5 mg/kg) ↑ or decreased (50 mg/kg) ↓ in the peripubertal group. In the ARC, *Kiss1r* was decreased (all doses) ↓ in the neonatal group, and increased (0.5, 50 mg/kg) ↑ or decreased (5 mg/kg) ↓ in the peripubertal group. Kisspeptin immunoreactivity in AVPV was increased (0.5 mg/kg, neonatal group) ↑. In the ARC, kisspeptin immunoreactivity was increased (5 mg/kg, neonatal) ↑ or decreased (all dosages, peripubertal) ↓	555
PCBs	Rat (S-D)	M/F	E16, E18	1 mg/kg of A1221 or a mix of PCBs 138, 153, 180, given ip to dam on E16 and E18	P1	Gene expression in the POA	*GnRH1* mRNA was unaffected by PCB treatment ✖	*Kiss1* mRNA was unaffected ✖. *Kiss1r* mRNA was significantly decreased by PCBs in males ↓ and increased in females ↑	1157
PCBs	Rat (S-D)	M/F	E16, E18	1 mg/kg of A1221 or a mix of PCBs 138, 153, 180, ip to dam on E16 and E18	P60 (females on proestrus)	Gene expression in the POA, and IHC for kisspeptin, GnRH-Fos, and ERα in the AVPV	GnRH-Fos double labeling was significantly decreased by PCBs in females on proestrus ↓. mRNA for *Gnrh1* was unaffected in both sexes ✖	Kisspeptin immunofluorescence density was significantly decreased by PCBs in the female ↓, but not the male ✖, AVPV. mRNA for *Kiss1* and *Kiss1r* were unaffected, both sexes ✖	567
PCBs	Rat (S-D)	M/F	E16, E18	1 mg/kg of A1221, ip to dam on E16 and E18	P15, P30, P45, P90	mRNA of 48 genes in the AVPV and ARC	In females, A1221 masculinized *Gnrh1* in the AVPV ↓	In females, A1221 masculinized expression of *Kiss1r* in the AVPV ↓, and *Kiss1* was increased by A1221 in the ARC ↑	1145

Abbreviations: F, female; IHC, immunohistochemistry; M, male; S-D, Sprague-Dawley. Directionality of responses: ↓, down-regulated; ↑, up-regulated; ✖, no change.

2. HPA systems

In EDC-1, we cited this quotation from Harvey et al (1159): "The adrenal is arguably *the* neglected organ in endocrine toxicology, and the lack of recognition of the importance of adrenal function in a regulatory endocrine disruption context, and the need for an adrenal toxicology assessment strategy has been pointed out." There have been some advances in research; we will discuss all of the papers we were able to locate that focused on hypothalamic-pituitary control of stress, focusing on developmental exposure periods, although we note that there is a small literature on adult exposures, especially to ATR (528, 1160, 1161).

a. Bisphenol A. Two papers have been published recently on the effects of BPA on the HPA axis using gestational/lactational exposure. The first study reported higher corticosterone levels in BPA-treated female rats than control females, but no effect of BPA on GR protein in the hippocampus (1119). In the second study, BPA effects in rats were investigated for adrenal morphology, corticosterone levels, hypothalamic expression of GR protein by Western blot, and mRNA of *Crhr1* and *Pomc* in the pituitary (1118). Results showed that: 1) BPA females had heavier adrenals compared to control females; 2) corticosterone levels, both basal and in response to a stressor (forced swim), were attenuated in BPA females compared to control females; 3) GR protein was lower in BPA females than control females under basal but not stress conditions; and 4) *Crhr1* mRNA in the pituitary was slightly elevated and *Pomc* mRNA was substantially elevated in stressed BPA males (1118). Another group tested effects of a slightly different timing/duration/dosage of BPA and found elevated corticosterone, ACTH, and *Crh1* mRNA in the PVN of males but not females (1111). In addition, the corticosterone response to stress (forced swim) was attenuated in BPA females, but not males. GR mRNA was differentially affected by BPA in the hippocampus (increased in BPA females, decreased in BPA males) and in PVN (increased in BPA females, unaffected in males), and mineralocorticoid mRNA and proteins for neuronal nitric oxide synthase and CREB were also changed in the BPA groups (1111). Finally, in the deer mouse, exposure of the dam to BPA before and throughout pregnancy had no effect on adult corticosterone levels, although behaviors were significantly affected (1162).

b. PCBs and PBBs. Pregnant rat dams were fed PBBs or Aroclor 1254 (a PCB mix) throughout pregnancy, and HPA function was determined in pups at P15 (1163). Basal corticosterone, as well as CRH- or ACTH-induced corticosterone release, was significantly suppressed in the PCB treatment group but not the PBB group. A recent study showed that prenatal PCBs (A1221, 0.5 mg/kg given on E16 and E18) significantly elevated basal serum corticosterone in adulthood in female but not male rats (1164). In goats, pregnant does were exposed to PCB126 or PCB153 from day 60 of gestation to delivery at 13 weeks, after which the kids were monitored and tested for effects on the HPA axis hormones (1165). Basal cortisol was decreased in females exposed to PCB126 at specific time points from weeks 35–51 of monitoring; in males, both PCBs were associated with significant effects at specific time points, and there was an overall decrease in mean cortisol in the PCB153 males. When goats were catheterized and subjected to serial blood drawing at 9 months of age, the PCB animals had higher plasma cortisol concentrations compared to control goats (1165).

c. Conclusions. More studies on the mechanistic basis for EDC actions on the developing HPA axis are much needed, especially considering that this system is well known for being susceptible to prenatal and postnatal maternal stress. Not only do offspring of stressed mothers behave differently and have aberrant responses to stressors; they also show molecular epigenetic changes to their brains consistent with reprogramming of the nervous system of these individuals (1166, 1167). Because developmental EDC exposures in some cases cause similar types of physiological HPA responses, it is important to investigate the potential underlying epigenetic mechanisms.

3. Posterior pituitary hormones: oxytocin and vasopressin

A small but important area of research that has emerged since EDC-1 is that of effects on the central vasopressin (also called arginine vasopressin [AVP]) and oxytocin systems. These neuropeptides are synthesized in large (magnocellular) neurons in the PVN and supraoptic nucleus of the hypothalamus and project axon terminals into the posterior pituitary gland from which they are released into general circulation. The vasopressin and oxytocin nonapeptides are highly conserved and structurally similar; they also overlap in their behavioral functions such as social and affiliative behaviors (1168). The literature on EDC effects on the neuroanatomy and neurochemistry of the oxytocin and/or vasopressin systems overlaps extensively with literature on the effects of EDCs on social behaviors because many studies investigate a behavioral outcome together with an underlying change in gene or protein expression in the brain. Thus, we will summarize the literature for neuroanatomical or molecular changes in these systems and return to some of these studies in the section on behavioral effects of EDCs (*Section VIII.E*). Experimental details are provided in Table 9. Nearly all

work has been conducted in animal models, but we do provide one example of a human study for PCBs and DDT below.

a. Bisphenol A. Effects of prenatal BPA treatment of mice were evaluated for oxytocin receptor (*Oxtr*) gene expression in whole embryo brains at E18.5. Expression was lower in the BPA males than in other control males or in the females (1169). In this same study, some behaviors (social interaction) but not others (social preference, social anxiety) were changed in pups tested as juveniles. More recently, Sullivan et al (1170) treated prairie voles in early postnatal life with BPA, and the animals were run through a battery of social behavior tests. Results were dependent upon sex and dosage of BPA, with the greatest effect occurring in response to a higher dosage in females, with increased vasopressin neurons and decreased oxytocin neurons in the PVN. Behavioral testing of these animals also revealed complex outcomes and more effects in females than males (see Table 9). In rats, exposure to BPA through gestation and postnatally until puberty affected anxiogenic behaviors but did not significantly change gene expression of *Oxt*, *Oxtr*, or *Avpr1a*, although there was a trend for an effect on the latter (1171).

b. PCBs, PBDEs, and DDT. Organohalogens such as PCBs and PBDEs also influence AVP function; as reviewed in Ref. 1172, they affect peripheral and central osmoregulatory function. In humans, there are positive associations between serum PCB or DDT concentrations and measures of blood pressure and hypertension (324, 1173, 1174) (see *Section II* for additional information on cardiovascular effects of EDCs). In rats, PBDEs resulted in exaggerated systolic blood pressure responses in association with slightly (but not significantly) elevated AVP levels in serum (1175).

c. MXC and chlorpyrifos. In pine voles, a monogamous species often used in social behavioral testing, gestational and lactational exposure to MXC decreased oxytocin receptor binding in the cortex but had no effect in the lateral septum of the females as adults (1176). There were also few effects on mate preference in that study. Another pesticide, chlorpyrifos, was tested in mice (1177), with effects found on hypothalamic oxytocin protein (increased by prenatal chlorpyrifos, especially in males) and on AVP protein (decreased by prenatal chlorpyrifos in males). This same group has recently reviewed the literature on links between chlorpyrifos and sexually dimorphic social behaviors (1178).

4. Conclusions

The past 5 years represent a period of important advances in the effects of developmental EDC exposures on hormones, brain, and behavior. In the animal literature, more papers relate these endpoints within the same individuals, allowing for a more direct inference between exposure and a complex phenotypic outcome that includes a behavioral change and underlying differences in hormones and gene expression in the brain. As discussed in the next section (VIII.E), this area is of high relevance to humans because of suspected links between environmental chemicals and neurodevelopmental outcomes.

E. Neurobehavioral effects of developmental EDCs

1. Human evidence from epidemiological studies

The epidemiological data on potential links between EDCs and neurodevelopmental disorders have grown in the past 5 years; although we will focus on recent studies, we will also present results (briefly) from older studies for an historical perspective. The nature of this work generally involves measurements of body burden from maternal media (urine, blood, milk), umbilical cord blood, infant urine, and ultimately, a correlation with some neurodevelopmental measure in the child such as tests of cognitive function. What has changed since EDC-1 (1) is the number of studies conducted around the world, increased sample sizes, more EDCs measured with better methodologies such as liquid chromatography-tandem mass spectrometry, better storage of samples and avoidance of contaminants, and the variety of neurobehavioral tests conducted. What is still very controversial is whether there are direct links among environmental EDCs and specific disorders such as autism spectrum disorders, attention deficit hyperactivity disorders, and others, although the hypothesis has been postulated that EDCs may contribute to the increasing prevalence of these disorders (1179, 1180). A recent review has covered the subject of how aberrant prenatal steroid hormone levels produced by the mother, placenta, or fetal adrenal or gonad, along with pharmaceuticals and to a lesser extent EDCs, could affect the developing brain of the fetus in humans (95). It must be emphasized, however, that by definition such work is always correlational. Furthermore, the ability to extrapolate from experimental animal work to humans on effects of these substances on behavior is probably the most difficult compared to EDC effects on physiological processes or hormone levels, the latter which are more easily quantified and much less subjective. Behavior in humans, including social and affective behavior, neurodevelopmental disorders, and sexual behavior and orientation, is unique in the animal kingdom. Although most people

Table 9. EDC Effects on Neural Vasopressin and Oxytocin Systems and on Related Social/Affiliative Behaviors

EDC	Animal	Exposure Period	Dosage, Route	Sex, Age at Analysis	Brain Effect on AVP and OXT Systems	Behavioral/Physiological Effect	Endpoint(s)	Ref.
ATR	Mouse	E14 to P21	1 or 100 μg/kg/d given to pregnant dam in drinking solution	Males and females tested at P31		Significant effects of ATR treatment on investigative-affiliative behaviors that were increased in frequency in males ↑ but not females ✖.	Social interaction test with a same-sex, same-treatment mouse.	1219
BPA	Cynomolgus monkey	E20 to 132	10 μg/kg/d given to pregnant mother via sc osmotic pump	Male and female juveniles, aged 1–2 y		Frequencies of exploring the environment and presenting were decreased in males ↓ but unaffected in females ✖. Visual exploration was robustly increased in the males ↑.	Exploratory behavior and presenting	1218
BPA	California mice	Dams were exposed beginning 2 wk before mating and continued through lactation	50 mg/kg feed weight	Males and females tested as adults		Territorial marking by the males was decreased by BPA exposure ↓. There was no effect in either sex on spatial learning. BPA abolished the sex difference in exploratory behavior.	Territorial marking	1212
BPA	Prairie vole	P8 to P14	5 or 50 μg/kg/d or 50 mg/kg/d given orally to pups	P28–P30 and P60–P75	In females, BPA (high dose) increased AVP neurons (anterior PVN) ↑ and decreased OXT neurons (posterior PVN) ↓. Effects on tyrosine hydroxylase neurons (elimination of sex difference) also occurred in response to BPA ↓.	Open field: BPA had mixed effects dependent on dosage in females ↓ ↑; no effect in females. Novel social test: high-dose BPA increased sniff time in females ↑ and decreased it in males ↓. Partner preference: whereas female controls showed a preference, this was lost in BPA females ↓. Males had no preference (control or BPA).	Behavior in the open field, novel social test, partner preference test; IHC of OXT, AVP in the PVN, and tyrosine hydroxylase in the BNST.	1170
BPA	Rat	E1 through puberty	1 mg/L in drinking water	Males, females, tested as juveniles and/or adults	No significant effects on mRNAs ✖, although there was a trend for an effect on *Avpr1a*.	Juveniles given BPA showed increased anxiogenic behaviors ↑ but only when fed a soy-free diet.	Anxiogenic behaviors, and gene expression in amygdala of *Oxt*, *Oxtr*, *Avpr1a* (among others).	1171
BPA	Mouse	E1 through E18.5	5 μg BPA daily in chow to dam	Males, females (E18.5)	*Oxtr* mRNA was lower in BPA males than other groups ↓. In addition, the glutamate transporter (*Slc1a1*) was higher in BPA females ↑.	BPA had greater effects in females than males in the social interaction tests ↓ ↑, but no effect in the social preference or EPM ✖.	OXT receptor (*Oxtr*) and other mRNAs in whole embryo brain; juvenile social interactions, EPM, social preference.	1169
Chlorpyrifos	Mouse	E15 to E18 and P11 to P14	3 or 10 mg/kg. Dams were given oral chlorpyrifos on E15–E18; offspring were given chlorpyrifos sc on P11–P14.	Males, females at 5 mo	OXT (whole hypothalamus) was increased by prenatal chlorpyrifos ↑, particularly in males. AVP was decreased by chlorpyrifos (prenatal) ↓, again in males.		OXT, AVP protein (Western blot, ELISA).	1177
MXC	Pine vole	E1 and weaning	2 mg/kg/d to dam (orally)	Females as adults	OXT receptor binding was decreased in the cortex of MXC females ↓; no change in the lateral septum ✖.	Partner preference behaviors were minimally affected ✖.	Behaviors: preference test for a female 2 d after co-habitating with a male, choosing the known partner or a stranger. Maternal behavior toward pups. OXT receptor binding assays.	1176
PBDE	Rat	E6 to P21	1.7 or 30.6 mg/kg/d by gavage to dam	Males at 12 mo	Plasma AVP was slightly but not significantly higher in the high PBDE group ✖.	Basal cardiovascular parameters were unaffected by PBDEs ✖. In response to a hyperosmotic stimulus, PBDE rats had higher systolic blood pressure after 3 h ↑. There were no differences in diastolic blood pressure ✖. A small increase in heart rate was found in the lower-dose PBDE group ↑.	Blood pressure; AVP in plasma.	1175
PCBs (A1221)	Rat	E16 and E18	0.5 or 1 mg/kg, ip to dam	Males and females at P60–P90		No effects of PCBs on sociability behavior (choice between empty cage or a cage containing a same-sex conspecific) ✖. In the social novelty test, prenatal A1221 (1 mg/kg in females, 0.5 mg/kg in males) disrupted novelty preference. Males (0.5 mg/kg A1221) spent less time nose-touching ↓.	Sociality and social novelty behavior in adulthood; serum corticosterone (increased in females by 0.5 mg/kg A1221).	1164

Abbreviations: BNST, bed nucleus of the stria terminalis; F, female; IHC, immunohistochemistry; M, male; OXT, oxytocin; S-D, Sprague-Dawley. Directionality of responses: ↓, down-regulated; ↑, up-regulated; ↓ ↑, mixed effect depending on dose; ✖, no change.

agree that genetics, the social and family environment, natural hormones, and hormone disruptors affect the endpoints we have discussed in this review, we are truly limited in evaluating how much each factor plays into a highly complex neurobiological outcome.

Historically, some of the earliest evidence for a connection between EDC exposures and human outcomes was in the realm of cognitive functions (Table 10). Studies of pregnant women who lived near Lake Michigan, where concentrations of PCBs were relatively high, revealed that children of mothers with the highest exposure levels were much more likely to have lower average IQ levels and poorer performance on reading comprehension (1181). Since then, studies on relationships between PCB concentrations in mother and/or child and neurobehavioral and cognitive deficits have found similar effects (1182–1184). Although a decrease of 5 IQ points may not appear on the surface to be clinically relevant to an individual, the potential population effects of a downward shift of IQ curve has enormous impact in terms of increasing numbers of people categorized as having developmental disabilities, together with the economic impact of care for these individuals (1185).

a. BPA and phthalates. For BPA, higher levels in maternal urine were associated with behavioral problems (1186) and increases in anxiety and depressive behaviors (1187) in their children. Another BPA study reported that higher maternal BPA, as measured in urine, was associated with more internalizing problems in boys when assessed at age 7 years (1186).

In a Korean study, an inverse associations were reported between several classes of phthalates (MEHHP, MEOHP, MBP) in urine from pregnant women in the third trimester of pregnancy and poorer performance on the mental developmental index and psychomotor index in male (but not female) infants at 6 months of age (1188). In a study of associations between phthalates and sexually dimorphic play behavior, Swan et al (1189) reported that masculine play behaviors were reduced in boys whose mothers had higher urinary phthalate levels during pregnancy. The Mount Sinai Children's Environmental Health study showed that increased concentrations of low molecular weight phthalate metabolites were associated with poorer performance on a battery of behavioral tests (1190) (Table 10).

b. PBDEs and PCBs. For PBDEs, maternal concentrations of BDE47 were not associated with the child's performance on the Bayley or the Psychomotor developmental indices, but higher BDE47 levels were associated with a 4.5-point decline in IQ and an increase in the hyperactivity score (1191). In an adult population from upstate New York near the General Electric capacitor facility or a reference site, PBDE and PCB concentrations were measured in serum, and a neuropsychological assessment of intellectual functioning, attention, and verbal memory and learning was performed (1192). Although the sum of PBDE congeners did not have an association with performance, there were significant interactions (inverse association) between PBDEs and PCBs for some of the measures of verbal learning and memory. When Inuit children were tested on a visual go/no-go task related to impulsivity and error monitoring, and results related to plasma PCB153 concentrations, higher PCBs were associated with diminished error monitoring (1193). Another study from this group tested performance of Inuit infants on several tasks and found that PCB concentrations were inversely associated with performance on the visual recognition memory tasks (27).

c. Pesticides. Several classes of pesticides are consistently associated with declines in measures of cognitive function, although whether this is due to effects on neurotransmitter synthesis, release, metabolism, etc, or to hormone actions (eg, via steroid hormone receptors localized in neurons that signal via acetylcholine, dopamine, glutamate, and others) cannot be distinguished, nor are they mutually exclusive. One report on chlorpyrifos exposure showed that increasing levels measured in umbilical cord blood plasma were associated with declines in full-scale IQ and working memory in children at 7 years of age (1194). A birth cohort study of children primarily from Latino families residing in an agricultural area of California where organophosphate pesticides were used showed that children in the highest quintile of maternal concentrations of dialkyl phosphate metabolites (measured in urine during pregnancy) had an average 7-point deficit in full-scale IQ (1195). Another study on organophosphates showed an association between total dialkyl phosphate metabolites, genotype with respect to the paraoxonase 1 gene, and performance on tests of mental development (1196). Pesticide use near the home of pregnant women was associated with increased likelihood for children to have autism spectrum disorder or developmental delay (60 and 150% increase, respectively) (1197). By contrast, studies on EDC mixtures were more difficult to interpret because a report looking at associations between 52 EDCs and autistic symptoms found some positive and some negative associations, and effect sizes were very small (1198).

d. Conclusions. In summary, most epidemiological studies find small but significant associations between early-life markers of EDC exposures and subsequent performance

Table 10. Associations Between EDCs and Neurodevelopmental and Cognitive Outcomes in Humans

EDC	Population Studied	Outcome	Ref.
BPA	Mothers and 3-y-old children in Cincinnati, Ohio	Higher BPA in maternal urine was associated with increases in anxiety and depressive behaviors on the behavior assessment system for children-2 test, and poorer emotional control on the behavior rating inventory of executive function-preschool test, particularly in girls	1187
BPA	Mother-child pairs	Higher maternal BPA (urine) was associated with increased internalizing problems in boys at age 7 y	1186
Chlorpyrifos	Inner city mothers and children in the Columbia Center for Children's Environmental Health Study	Each SD increase in chlorpyrifos exposure (umbilical cord plasma) was associated with a decline in full-scale IQ (1.4%) and working memory (2.8%)	1194
Organophosphates	Children in the Mount Sinai Children's Environmental Health Study at 1–2 and 6–9 y of age	Prenatal total dialkyl phosphate metabolites (cord blood plasma) were associated with decreased mental development at 1 y in blacks and Hispanics, and were associated with a polymorphism of the paraoxonase 1 gene	1196
Organophosphate pesticides	Children from predominantly Latino farmworker families in an agricultural area of California	Children of mothers with the highest quintile of dialkyl phosphate metabolites (urine) had a 7-point deficit in IQ score	1195
PBDEs	Children 1–5 y of age from Cincinnati, Ohio	Maternal serum PBDE47 was not associated with the child's performance on the Bayley Scale or the Psychomotor Developmental Index, but higher prenatal BDE47 was associated with a 4.5-point decrease in overall IQ and an increase in hyperactivity score	1191
PBDEs, PCBs	Adults aged 55–74 y who resided for the past 25 y in sites in upstate New York near the General Electric capacitor facility	Although the sum of serum PBDEs did not correlate with performance, there was an interaction between PBDEs and PCBs such that there was an inverse association with performance on the verbal learning and memory task. Other neuropsychological measures were not correlated	1192
PCB153	Inuit children (~11 y of age)	Performance on a visual go/no-go task, specifically error monitoring, was diminished in children with higher PCB153 concentrations in plasma	1193
PCB153	Inuit infants from Arctic Quebec at 6.5 and 11 mo of age	Some aspects of performance on the Fagan Test of Infant Intelligence and the A-not-B test, but not the Bayley Scales of Infant Development-2nd edition, were affected by PCBs in umbilical cord plasma, especially visual recognition memory	27
Pesticides	Children whose families lived in close proximity to agricultural pesticide use	Children with autism spectrum disorder or developmental delay were 60% and 150% more likely (respectively) to have lived near sites with organophosphate pesticide application	1197
Phthalates	Children in the Mount Sinai Children's Environmental Health Study at 4–9 y of age	Increased concentrations of low molecular weight phthalate metabolites (maternal urine) were associated with poorer performance on tests for aggression, conduct problems, and depression clinical scales, the externalizing problems and behavioral symptom index, and the global executive composite index	1190
Phthalates	Mother-infant pairs recruited in 2006–2009 in three Korean cities	High maternal phthalates (MEHHP, MEOHP, MBP) in maternal urine were associated with poorer performance on the mental developmental index and the psychomotor developmental index in 6-mo-old male (but not female) infants	1188
Phthalates	Children in the Study for Future Families whose mothers had previously had phthalate metabolites measured during mid pregnancy (urine)	Certain phthalates were associated with a decreased masculine score in boys on play behaviors	1189
52 commonly detectable chemicals, including BPA, phthalates, PCBs, brominated flame retardants, perfluoroalkyl substances	Mothers and 4- to 5-y-old children from the HOME study, Cincinnati, Ohio	Tested relationships between the social responsiveness scale (SRS) as a measure of autistic behavior, and concentrations of EDCs in maternal blood or urine. Overall, SRS scores were negligibly associated with most EDCs. PBDE28 was associated with more autistic behaviors, but fewer autistic behaviors were seen in children with detectable PCB178, β-HCH, or PBDE85, compared to those with undetectable levels. Increased PFOA concentrations were associated with fewer autistic behaviors	1198

Studies are organized chronologically from top to bottom for each EDC class. [Modified from A. C. Gore et al: Implications of prenatal steroid perturbations for neurodevelopment, behavior, and autism. *Endocr Rev*. 2014;35:961–991 (95), with permission. © Endocrine Society.]

on cognitive and neurobehavioral tests. Further information is needed to confirm and extend these studies. Many were based on relatively small sample sizes, and the magnitude of changes was often quite small. Therefore, it is important to be cautious in interpreting results and, of course, in inferring causal relationships. It should be noted that research published in recent years largely confirms previous work, giving more confidence to outcomes of these studies over time.

2. Animal models

Developmental exposures to EDCs have long been associated with a number of behavioral changes in animals studied at various postnatal stages, especially in adulthood, but also in infancy and adolescence. Studies have primarily focused on the areas of EDCs and: 1) reproductive behaviors; 2) cognition, learning, and memory; 3) affective behaviors (eg, depressive-like and anxiety-related behaviors); 4) maternal behaviors; and 5) social and affiliative behaviors. We will review the literature from the past 5 years in these arenas. A description of the common behavioral tests used in the animal literature is provided in Table 11.

a. Reproductive behaviors. Considering the widespread effects of EDCs on female and male reproductive physiology (see *Sections III and IV*, respectively), the recent experimental literature on EDC effects on reproductive behaviors is surprisingly mixed in outcomes. Although many studies show behavioral outcomes, an equal number do not. We discuss this literature below.

i) Bisphenol A. In prairie voles, a species that is more prosocial than rats or mice, BPA exposure abolished partner preference behaviors in females, but not males (1170). In female rats, several reports show no effects of BPA on lordosis quotient (LQ) (544), on proceptive or receptive behaviors (1199), or on both proceptive and receptive behaviors (1200). In female mice, developmental BPA exposure at one but not all dosages increased LQ (in that study, mice were OVX and hormone primed as adults) (1115). Another BPA study conducted in female rats found that early postnatal BPA treatment significantly decreased some types of proceptive behaviors (hops and darts) but not others (ear wiggling), and had no effects on LQ (1201).

Male mice exposed to BPA developmentally showed few behavioral changes in one study (1110), whereas male rats had impairments in sexual behaviors in another (1199). In this latter study, males exposed to BPA had fewer numbers of intromissions, decreased copulatory efficiency, and other behavioral changes that were dose-dependent when observed at lower or intermediate dosages, but not the highest dosage (1199). Another group reported that BPA exposure of male mice via the maternal diet caused decreases in the numbers of intromissions and ejaculations, especially at low/intermediate dosages, but had no effect on latencies to mount, intromit, or ejaculate

Table 11. Neurobehavioral Tests Used in the Experimental Animal Developmental EDC Literature

A. Tests of affective states such as anxiety-like and depressive-like behaviors
1. Elevated plus maze (EPM): considered a test of anxiety, this is an X-shaped apparatus that is elevated above the ground. One arm of the X is walled, and the other arm is open. Animals can explore the apparatus, and the time spent is used as an index of an animal's willingness to spend more time in the "safe" (enclosed) vs the "unsafe" (unenclosed) set of arms
2. Light-dark box: another test of anxiety-like behaviors, consisting of a large two-chamber rectangular apparatus. The partition contains a door allowing the animal free access from one chamber to the other. Typically, one compartment is darkly lit and the other side is brightly lit. Time spent and distance travelled in the two compartments is used as an index of anxiety, based on rodents' preference for dark over bright environments
3. Open-field test: a large (usually square) arena is used. An animal is placed in the center of the arena, and the time spent and distance traveled in different components (edges, corners, center) are measured. Time spent in the center is considered to represent willingness to engage in risky behavior, compared to time along the edges or in corners that provide a safer environment

B. Tests of cognition, learning, and memory
1. Morris water maze (MWM): a water-filled arena with a hidden platform is used to determine how long it takes an animal to swim to the platform and remember its position based on spatial cues. The water is made opaque so that the platform cannot be visualized
2. Other maze tests (eg, Y-maze, Biel maze, radial arm maze) are also commonly used in the behavioral neurosciences and are briefly described in the text

C. Tests or indices of reproductive behaviors and status
1. Lordosis behavior: a prototypical arched-back posture assumed by a receptive female rodent in response to reproductive stimuli (eg, a male mount or intromissions). The lordosis quotient (LQ) is defined slightly differently by researchers but typically refers to the number of lordoses divided by the number of mounts, or the number of mounts plus intromissions, $\times$ 100
2. Paced mating behavior: a test of female-typical reproductive behaviors in which the female is allowed to set the pace of mating. It typically uses a male that is restrained or confined to a chamber, whereas the female has free access. The numbers, latencies, frequencies, and timings of behaviors are scored during the trial

(1202). Thus, it is clear that more experimentation is needed to resolve many of the disparities.

ii) PCBs and PBDEs. The effects of PBDEs and PCBs were evaluated for outcomes on feminine sexual behaviors (1120). Proceptive behaviors were decreased, and LQ and other markers of female sexual activity were reduced (1120). This latter result is consistent with an earlier study showing that PCBs perturbed paced mating behavior in female rats (1203). However, Cummings et al (1204) found no effect of PCB77 on feminine mating behaviors in rats, although partner preference (choice for a male vs a female) was significantly impaired by PCBs and varied by timing (prenatal, postnatal) of exposure.

iii) UV filters. Feminine sexual behaviors were tested in rats exposed to chemicals in UV filters that are putative EDCs. Results showed that prior 4-methylbenzylidene camphor (7 or 24 mg/kg/d fed to sire and dam before mating, and to dam and subsequently offspring throughout life) and 3-benzylidene camphor (0.24, 0.7, 2.4, or 7 mg/kg, similar treatment regime) exposures reduced proceptive behaviors and receptive behaviors and increased rejection behaviors toward a male rat (1205).

b. Cognitive, affective, social, and anxiety-like behaviors. A burgeoning literature has emerged over the past 5 years linking EDCs to a range of nonreproductive behavioral outcomes, primarily those involved in learning/memory, affective state (eg, depressive- or anxiety-like behaviors), and social interactions. These behaviors are quite diverse and involve different neural pathways, but we will discuss them together because of the overlapping literature (eg, a single study might test four or more types of behaviors). More information on the behavioral tests is found in Table 11.

i) Bisphenol A. BPA has been extensively tested for cognitive and affective outcomes and has the largest literature. Most studies on low-dose early life exposures have revealed some behavioral effects, with differences between the sexes. In addition, while many of the reported effects are significant they are usually relatively small. Thus, the conclusion we draw from this literature is that prenatal EDCs such as BPA cause subtle behavioral effects that are dependent upon aspects of the experimental model such as dosage, route, age at treatment, species, sex, and the behavioral test. We will briefly summarize some examples.

Using the Morris water maze (MWM), Xu et al (46) found that rats took longer to find the target platform, consistent with impaired performance (46). Using a Y-maze test, which allows exploration in a Y-shaped chamber where rats distinguish between a previously explored arm (familiar) vs an unexplored arm (novel), Poimenova et al (1119) reported that BPA-exposed rats, especially females, showed less exploration, and there was impaired discrimination between the novel and familiar arms in both sexes, together with changes in the apparent strategies used by rats to negotiate the maze (1119). Another study using a Y-maze (1206) reported that alternation behavior was decreased in BPA-exposed animals, and in the novel object recognition task, BPA impaired object recognition. In the radial arm maze, BPA-exposed rats at two doses (0.05 or 5 mg/kg), but not at other doses (0.5 or 50 mg/kg), made more working memory errors and, to a lesser extent, reference memory errors (1207). Using a food reward-motivated maze test, male rats exposed earlier to BPA needed more time to reach the reward and made more errors (1208). Last, a study testing the effects of *paternal* exposure to BPA (as opposed to the *maternal* exposure of all the other studies reviewed in this section) showed that performance on the MWM was impaired, with exposed offspring of both sexes swimming longer distances and taking more time to find the platform (1209).

By contrast to these results showing the effects of BPA on learning and memory, several others did not. Stump et al (1210) used a Biel water maze (a variant of the MWM) and found some small differences in performance on P22 and P62 that they attributed to natural variability in the test. Sadowski et al (1211) found no effect of BPA on performance in the radial arm maze. Another study on adult mice found no effect of BPA on performance on the MWM (248). In California mice, BPA had no effect on spatial navigational skills tested in the Barnes maze (1212). Thus, whereas most studies we reviewed showed some impairment, there were differences in experimental results.

Many studies have assessed anxiety-like behavior using tests such as the open field test, the elevated plus maze (EPM), and the light-dark box (Table 11). Because results are mixed, we will begin with those studies that showed anxiogenic (ie, increased anxiety) effects of BPA. For the open field test, BPA-exposed rats spent less time in the center area and traveled a longer distance, compared to control rats (1213). This anxiogenic effect of BPA was also seen in the work of Patisaul et al (1171) in which fewer animals entered the light chamber of the light-dark box, spent less time there, and took longer to enter (both sexes were tested as juveniles). That lab also used the EPM to show that a smaller percentage of BPA-exposed males entered the open arm and had fewer open arm entries. Although some of these anxiogenic effects were maintained

into adulthood, the magnitude of effects, in general, was diminished compared to the juvenile period (1171). Another mouse study found that BPA decreased center time in the open field in males and to a lesser extent females when tested as juveniles, and in adulthood, this effect was again seen in the males (1214). Anxiogenic effects of BPA were also reported by Xu et al (1215), who detected greater effects in females than males in the light-dark transition, open field, and EPM tests. In deer mice (1216), BPA exposure affected strategies used in the Barnes maze (feminized males) and increased anxiety-like behaviors in the EPM. In California mice, BPA-exposed females spent less time in the open arms compared to control females, something that was not seen in the males (1212). In the open field test, female (but not male) rats exposed to developmental BPA showed decreased time in the center (1144), again consistent with anxiogenic actions of BPA.

By contrast, Tian et al (1206) showed anxiolytic effects of BPA in mice using both the open field test and the EPM. In the former, BPA animals traveled a greater distance in the center, and in the latter, they spent more time in the open compared to the enclosed arm of the EPM. Chen et al (1111) reported anxiolytic effects of BPA in rats, especially in females. Other studies (eg, Refs. 248 and 1208) reported no differences in one or both of these tests. Yet another study comparing males and females reported that low-dose BPA eliminated some of the sex differences in performance on the EPM (increased in males and decreased in females by BPA), although overall the magnitude of the effects was small (1217).

A considerable literature has been published in the last 5 years on social behaviors, again showing mixed results (Table 9). Most studies suggest some change in these behaviors, but whether these have "positive" or "negative" valence is more difficult to interpret because social behaviors are complex and changes to these behaviors need to be interpreted from the perspective of a conspecific, something that we cannot do easily as humans. Thus, our interpretation of changes caused by EDC exposures as "disruption" should be interpreted by readers to mean that the animals differ from a normal conspecific. We will begin this discussion with the one paper that, to our knowledge, addressed this question using male cynomolgus monkeys born to pregnant monkeys that were implanted with a subcutaneous pump that administered BPA (10 μg/kg/d) or vehicle from G20 to 132 (1218). Monkeys were tested for social interactions as juveniles, aged 1–2 years. Frequencies of exploring the environment and presenting were decreased in males but unaffected in females. Visual exploration was robustly increased in the males (similar to levels in females, which perform this behavior at much higher rates than males) (1218).

The remainder of the literature has (to our knowledge) been conducted in rodents. In California mice, territorial marking by the males was decreased by BPA exposure (1212). In juvenile lab mice exposed to BPA, social interactions with a same-sex, same-age conspecific, and social preference for an adult male compared to an empty cell, were measured (1169). The nature of social interactions in juveniles was changed in the social interaction test, especially in females, but was unchanged in the social preference test. Low-dose BPA exposure to prairie voles changed social behaviors in a social novelty test in females, but not in males (1170). Similar studies have been conducted for ATR using a social interaction test with a same-sex, same-treatment mouse at P31 (1219). A principal component analysis was used, identifying significant effects of ATR treatment on investigative-affiliative behaviors in males (1219).

ii) Polychlorinated biphenyls. There are several published studies on the effects of PCBs on cognitive and social behaviors, and all demonstrate at least some change in these behaviors. Exposure of rats to PCB153 or PCB126 increased the number of trials needed to learn the Y-maze task in both males and females (1220). However, mice exposed to a mixture of six nondioxin-like PCBs showed no impairment on the MWM (1221). A mix of PCBs was tested for its effects on a variety of anxiety-related behaviors (1221). Mice exhibited an increase in anxiety-like behaviors as demonstrated through testing on the EPM (longer latency to enter the open arm and decreased percentage of time in the open arm) and the light-dark box (more time to leave the dark box and less time spent in the light box). Exposures to PCB47 and PCB77 in rats also affected aspects of social behaviors tested in juveniles and adults (1222). Recently, a study using exposure of rats to the PCB mixture A1221 showed that males decreased their interactions with another conspecific male in a test of social novelty, although overall the behavioral phenotype for both sexes was relatively modestly affected by exposure (1164).

iii) Phthalates. Effects of phthalates on the EPM were measured in rats tested through adolescence into adulthood (1223). A significant decrease in entries into the open arm and in time spent in the open arm was seen in males at P45 and P60, but not on P30, and there were no effects in females.

iv) Atrazine. Mice exposed to ATR were tested on a battery of behavioral tests as juveniles and adults (1224). In a novel object recognition test, the ability to discriminate the novel object was abolished in the ATR-treated animals as juveniles, and to a lesser extent as adults. Effects of ATR were also tested on the open field test in mice, with only

one significant but small effect (increased frequency of exploration in the lower-dose group) (1219).

Therefore, the literature as a whole is mixed in results depending upon EDC used, exposure period, and the behavioral test, with some effects greater in females and others in males.

c. Transgenerational effects of EDCs on brain and behavior. The original observation in 2005 that ancestral exposure to relatively high doses of the EDC vinclozolin resulted in reproductive and other diseases in the F3 generational descendant male rats (116) called attention to the possibility that heritable germline epigenetic changes caused by environmental toxicants can be transmitted across generations. That laboratory has since gone on to show a number of phenotypic disease changes to F3 male and female descendants of pregnant rats exposed to vinclozolin (118, 1225–1227). Other EDCs were tested as well and showed significant adverse outcomes in the F3 generation (262, 1228). This is still a controversial area because the vinclozolin work has not been replicated by other laboratories (1229, 1230), and the high dosages are not environmentally relevant. Nevertheless, links between other EDC exposures (eg, BPA at much lower dosages) and transgenerational effects have been reported, with compounds utilized at much lower dosages (1143).

i) Vinclozolin. Behavioral studies conducted on F3 descendants of endocrine-disrupted animals have revealed considerable changes to the behavioral phenotype, but again it must be emphasized that these animals descended from high-dose-treated progenitors. Using F3 male and female rats that descended from the vinclozolin- or vehicle-treated great-grandmothers, a series of behavioral tests were conducted. First, a mate preference test was conducted in which F3 animals were given a choice between a vehicle- or a vinclozolin-F3 lineage opposite-sex partner (113). Although males showed no preference between the two lineages of females, the females showed a significant preference for the vehicle descendants over the vinclozolin descendants. In addition, tests of anxiety-like behaviors in the F3-vinclozolin rats revealed significant differences from the F3-vehicle lineage animals (1231). Crews et al (112) went on to show that F3 vinclozolin-lineage rats that experienced stress during adolescence were behaviorally very different from their vehicle-lineage counterparts. Moreover, the interactions of ancestral EDC exposure and stress in an individual's own adolescence were substantially different between the sexes, consistent with natural behavioral sexual dimorphisms, as well as differential transgenerational responses to EDCs (114).

ii) Bisphenol A. Two studies have been published from the Rissman laboratory that demonstrated transgenerational effects of BPA on social behaviors in descendant mice (111, 1143). This latter body of work is important because the dosages of BPA used were relevant to those in humans, and studies were performed by an independent group of investigators using a mouse model. One of the most important gaps, therefore, is a more robust literature confirming transgenerational EDC actions on brain and behavior.

F. Conclusions

A summary of key points regarding EDC effects in the brain is provided in Box 7.

From animal work, there is consensus that there are critical periods of brain development. However, an important gap in knowledge in humans is whether and when such sensitive periods exist, comparable to what is known from the experimental animal literature. Even basic knowledge about the roles of hormones on brain sexual differentiation in humans is mainly inferred from known perturbations such as congenital adrenal hyperplasia and genetic disorders (reviewed in Ref. 95). When it comes to EDC effects, it is obvious that exposures are not controlled, and humans are exposed throughout their lives to very individualized mixtures of chemicals, some of which may never have been tested for endocrine-disrupting actions. An additional point that is rarely considered in both basic and clinical research is just how important the age at analysis is in determining an outcome. Developmental studies evaluating EDC actions in animals of different ages within a laboratory show that the response—the phenotypic outcome—can evolve across the life cycle. This point must be extended to human research in considering when, and how, to evaluate neurobiological and other effects of known exposures, or to relate the body burden of a chemical or mixture.

IX. Conclusions and Recommendations

The past 5 years represent a leap forward in our understanding of EDC actions on endocrine health and disease. The scientific literature published during this period has provided much deeper insights into the underlying molecular and cellular mechanisms of action, the importance of critical developmental exposure periods, and stronger epidemiological studies in humans from around the world. Despite limitations due to differences in experimental design in cell and animal studies and the need for caution in inferring causality from epidemiological work in humans, most studies support links between exposure and adverse outcomes. We conclude this review article by discussing the key areas of research for

the next 5 years (Box 8) and make recommendations for policy makers, physicians, and the general public (Box 9).

A. Research gaps

1. Mechanisms of action of EDCs

It is well-established that many actions of EDCs are mediated by the classical nuclear hormone receptors, especially ERs, AR, ThR, and the orphan receptor AhR. There is increasing evidence, both in vitro and in vivo, for EDC effects on PR, GR, and PPARs, although this latter area merits more work. Much of the functional assay data on steroid receptor actions has been undertaken in vitro, at times utilizing steroid hormone receptor transfection assays, and the relationship of these findings to in vivo and in utero events has not been well delineated. Intracellular signaling by receptors and their coregulatory elements can be co-opted by EDCs, resulting in outcomes that are not necessarily predicted by effects of the natural hormone. Other members of the nuclear hormone superfamily also ought to be investigated for EDC actions, and beyond the nucleus are the membrane steroid hormone receptors including membrane ERs, GPER- and STX-sensitive ERs, membrane progestin receptors (PGRMC1 and PGRMC2), and membrane ARs. These are burgeoning fields of research that not only need systematic study by researchers in the EDC field, but also require much more study for physiological roles played by these receptors. We have also reviewed the evidence that steroidogenesis and metabolism of hormones are affected by EDCs; however, most of this research has been conducted in the brain and gonads, whereas steroidogenic enzymes are distributed throughout numerous organs and tissues including the adrenal glands, adipose, and other parts of the reproductive system. This area requires investigation throughout the body in a developmental context with more mechanistic insight.

EDCs also affect the biosynthesis, release, and metabolism of nonsteroid hormones such as peptides, proteins, and monoamines. In the brain (see *Section VIII*), EDCs affect neuroendocrine peptide hormones, and they act upon neurotransmitter receptor systems such as dopamine, serotonin, glutamate, γ-aminobutyric acid, and others. The biosynthesis, release, and plasma levels of protein hormones such as insulin are also directly affected by EDCs and are indirectly regulated via nuclear and extranuclear steroid hormone receptors. Therefore, further work is needed to determine whether and how EDCs bind to the membrane receptors that mediate effects of hydrophilic hormones and neurotransmitters; the actions of EDCs on the biosynthesis and processing of these hormones, many of which are synthesized as prohormones and undergo subsequent post-translational processing; and the release and metabolism of these hormones. In addition, the types of tissues in which these studies have been conducted need to be expanded. This would include the liver, which is involved in the metabolism of steroids and several EDCs and is a steroid-responsive organ itself. Understanding how the liver responds to EDCs and the fundamental differences between hepatic actions in humans and rodent models is necessary for a clearer appreciation of EDC actions across species. It would also enhance the

Box 7. Key Points: Brain

- **The experimental animal literature consistently shows that the structure and function of the brain's neuroendocrine systems can be altered by developmental exposures to EDCs.**
- **The adult neurobiological consequences of developmental exposures include alterations in peripheral hormones and changes in behaviors.**
- **Underlying mechanisms of EDC actions in the brain include molecular and cellular changes in expression of particular genes and proteins involved in neuroendocrine and other behaviors, including those involved in cognitive and affective functions.**
- **The brain is highly vulnerable to EDC exposures because of the widespread distribution of nuclear hormone receptors, steroidogenic enzymes, and neurotransmitter systems on which EDCs can act.**
- **Strong experimental evidence in animals shows that there are sex differences in EDC effects on the brain. Moreover, epidemiological work also shows that relationships between body burdens of chemicals and particular behaviors often differ between the sexes in human studies.**
- **In humans, epidemiological data support associations between higher exposures to EDCs with decreased IQ, increased neurodevelopmental problems, and other neurocognitive outcomes.**
- **Future research needs to focus on sex differences in endocrine disruption of the brain and to consider both age of exposure and age at assessment in interpreting results.**

ability to make predictions of chemical actions for humans from rodent study results.

A point that should be mentioned is that this Statement, although clearly focused on the actions of EDCs on the body's endocrine systems, also provides evidence for neurobiological and, to a lesser extent, immune functions that are acted upon by these same chemicals. Furthermore, cross talk among these three systems, representing the body's major way of communicating with the environment, is a basic part of physiology and homeostasis, and research should better integrate these levels. Moreover, exciting new discoveries on the role of the microbiome in health and disease (1232) should be considered for future research.

2. Translating research from animals to humans

Most of the experimental animal work on EDCs has been conducted in rodent models. Endocrine systems and hormones are highly conserved across mammalian species, making rodents an excellent model in many ways, but there are a few important differences. A specific example is that of female reproductive cycles. Rats and mice have short estrous cycles, with ovulation occurring every 4–5 days. Humans and many nonhuman primates have 28-day menstrual cycles. Although the hormonal cycles of estrogens, progestins, GnRH, and gonadotropins across these cycles are similar between rodents and primates, the much shorter follicular stage in rats and mice and the lack of a prolonged luteal phase in these latter species result in substantial differences in reproductive organs and tissues because they respond to different durations of hormone exposures compared to primates. Another major difference is pregnancy. Rodents have bifurcated uterine horns and give birth to multiple offspring; each fetus has its own placenta, and the maternal-fetal interface is substantially different from that of humans. In addition, the position of a rodent fetus in the uterine horn with respect to its male and female siblings can affect hormones and development (1233, 1234), something that is not relevant to species bearing singleton offspring. It should be noted that some rodent species, especially the guinea pig, have longer reproductive cycles (~14 d) with true luteal phases, longer pregnancies, and fewer offspring, making them a prime candidate for EDC research in reproductive biology. Importantly, transgenic animals have been very underutilized in this realm, and humanized mice and other models may greatly contribute to the field.

Box 8: Recommendations for Research Over the Next 5 Years

- **Mechanistic studies of EDC actions on nuclear hormone receptors need to be extended beyond ERs, AR, PR, GR, ThR, and PPARs to other nuclear hormone superfamily members and to membrane steroid hormone receptors.**
- **Investigate EDC effects on enzymes involved in steroidogenesis, hormone metabolism, and protein processing in humans and animal models.**
- **Consider tissue-specific effects of EDCs.**
- **Translate research from rodents into nonhuman primates, sheep, and other species; and take advantage of transgenic (especially humanized) animals, keeping in mind the need for a better understanding of hormones and early-life development in humans.**
- **Test additional critical periods beyond prenatal and early postnatal— eg, adolescence as an additional sensitive developmental window.**
- **Evaluate EDC outcomes at different life stages–not just adulthood.**
- **Design studies to consider sex and gender differences in response to EDCs.**
- **Perform longitudinal and multigenerational analyses in animals and humans.**
- **Evaluate and implement emerging and sensitive testing systems, including high-throughput systems, for hazard assessment, screening, and prioritization.**
- **In humans, consider genetic diversity and population differences in exposures and outcomes. This should include racial, ethnic, socioeconomic, and geographic variables.**
- **Expand research to emerging "EDCs of interest" and to mixtures of low-dose EDCs.**
- **The team science approach, including teams of basic, translational, and clinical scientists; epidemiologists; health care providers; and public health professionals, needs to be a priority for future research and funding.**

The sheep is more similar physiologically to humans in terms of extended gestation and reproductive cycles. This model is beginning to be exploited in the DOHaD field, in which pregnant ewes are exposed to EDCs and effects on physiological outcomes in the offspring are explored. Research in this arena to date has primarily focused on reproductive and metabolic outcomes (128, 1117, 1156), and future studies should extend to other latent endpoints.

Nonhuman primates, especially macaque species, are widely considered to be the most translationally relevant animal models. Monkeys have been underutilized for EDC research, largely due to the high expense, long life

span, and ethical considerations for primate research. Thus, what little research has been conducted in monkey models has, for the most part, utilized adult exposures. Performing research using the DOHaD model is much more difficult because the long period of gestation and postnatal development in monkeys has both benefits and shortcomings. Most macaques have an extended life period, sometimes up to 5 years, before the onset of puberty and the acquisition of adult reproductive function. Although this better approximates the prolonged childhood and adolescence of humans than rodents (in which adult reproduction is attained within 3 mo), it limits the ability to test the effects of prenatal exposures on adult outcomes because maintaining and paying for a monkey colony for 5 years or longer may not be logistically possible. Nevertheless, funding agencies ought to recognize the need to draw definitive conclusions about EDC effects in the monkey to resolve concerns about translating from the rodent literature. In the past few years, a series of studies has focused on pharmacokinetics and pharmacodynamics of EDCs (especially BPA) and the effects of prenatal exposures on reproductive outcomes (347, 476, 871, 1235), and these studies should set the stage for future research. Due to obvious limitations in human work, there are still gaps in knowledge in the basic biology of hormones and their actions during fetal development, underscoring the need to capitalize upon appropriate animal models.

Another approach that may provide useful information is the use of human fetal specimens, surgical specimens, and cells from organ donors collected from adults. Once obtained for research purposes using approved Institutional Review Board guidelines, these tissues and cells can be cultured and/or transplanted into immunodeficient murine hosts for in vivo studies to assess whether EDC results from animal models translate to humans. Advantages of this approach include direct human application and the use of cells and tissues that are not immortalized or diseased. Caveats of these approaches are the limited duration of primary cell and tissue viability, prior uncontrolled exposures of human specimens to various chemicals, and specimen variability. The latter is particularly true for fetal specimens that are collected at different developmental time points, which can create significant variability in organs that develop in utero. A few examples of these approaches have been recently published in prostate studies, using fetal prostate tissues (1236) and adult prostate primary cells (960) to assess the ability of estrogens and BPA exposures to influence prostate carcinogenesis, respectively.

We suggest that future translational research focus on collaborations between experimental, clinical, and epidemiological studies. It is important to determine whether the effects of EDCs observed in animal models occur in humans and vice versa. Furthermore, it is important to develop methods to assess whether known mechanisms underlying EDC effects in animal models and in vitro systems also occur in humans. Transient actions of EDCs in humans ought to be studied by randomized intervention trials, such as those published recently (332). Moreover, long-term prospective intervention studies should be conducted in which EDC exposures are reduced via lifestyle changes. By better understanding the mechanisms underlying EDC effects, we will be able to develop better preventative and treatment strategies for EDC-related disorders and diseases.

3. The importance of life stage at evaluation and the need for longitudinal and multigenerational studies

It is now well established that exposures to EDCs during critical life stages affect health and disease in the offspring. What is far less appreciated is the age at which an endocrine outcome must be assessed. The vast majority of work with DOHaD models has evaluated the adult animal, often because endocrine systems are not fully mature until adulthood and a disease state is not manifested until this life period. The reproductive systems of females and males provide excellent examples because a reproductive dysfunction such as infertility cannot be evaluated in a prereproductive individual. In addition, certain hormone-dependent cancers become much more prevalent in adulthood, after pubertal hormone levels have increased and act upon hormone-sensitive cells to induce carcinogenesis.

However, the evaluation of a developmental EDC exposure effect at a single life stage can be very problematic. Endocrine and nervous tissues undergo substantial developmental changes, and the release of hormones as an individual develops from infancy to childhood to adolescence to adulthood is a highly dynamic process. Furthermore, each hormone has a unique postnatal developmental profile. When added together, the expectation that a hormone or EDC effect at one cross-section of life would reflect the effect at another stage of life is likely incorrect. This is predicated upon the idea that tissues and organs do not change their functions over time and that a molecular, cellular, or physiological property of a cell is static throughout life. This is clearly not the case.

In fact, research across disciplines shows that EDCs have differential effects at different life stages, even within a single tissue. An excellent example is the developing neuroendocrine system. Profiling studies of expression of neuroendocrine genes in the developing hypothalamus show that each gene has a specific developmental pattern across life, that there are sex differences in these profiles, and that

EDCs affect these patterns in a sex-specific manner (131, 1145). In addition, the relationship among patterns of gene expression (ie, the interconnected network of genes and proteins that function together to maintain a biological function) undergoes dynamic change as an animal develops. This latter point underscores the necessity of assessing multiple endpoints when determining effects of EDC exposures, together with considering developmental or life stage in interpreting data.

This concept must be extended across the entire life cycle. Although associations between EDC exposures and the onset of puberty have been shown in animals and humans, much less is known about premature reproductive senescence and other aging-related changes. Considering that many, if not most, endocrine disorders have increasing prevalence with age, this must be factored into future studies conducted in a longitudinal study design.

EDC exposures to pregnant animals have been shown to cause multigenerational or transgenerational effects on a number of disease endpoints, particularly reproduction, neurobehavior, and adiposity (111–113, 116, 209). This work needs much more follow-up to better determine the underlying mechanisms, which are likely to include epigenetic molecular programming changes. Moreover, research is needed in human populations. Some work has been conducted in grandchildren of DES-exposed women who took this estrogenic pharmaceutical during pregnancy. The consequences on the offspring (F1 generation) are well-studied, and research is beginning to be published on the grandchildren (F2 generation) (1237). For environmental chemicals, several ongoing projects need continued funding. The AHS in the United States has followed pesticide applicators for potential health outcomes related to neurobehavior, respiratory health, reproductive health, and cancers. The Child Health and Development Studies at the University of California at Berkeley is another example of a longitudinal and multigenerational project designed to study relationships between the environment and health outcomes. The consequences of widespread dioxin exposure that followed the industrial accident in Seveso, Italy, in 1976 have been followed up in the Seveso Women's Health Study and other research endeavors. It is important to follow these cohorts to further determine disease outcomes, as well as to ascertain the mechanisms that may be underlying them.

4. Tissue- and organ-specific effects of EDCs

The literature reviewed in *Sections II to VIII* of this Statement demonstrates that the most commonly studied EDCs—BPA, dioxins, PCBs, pesticides, and others—have effects on multiple physiological systems. However, the nature of these effects and the underlying mechanisms can differ considerably. Even within an organ, there may be differing outcomes due to the heterogeneity of tissues. For example, studies on developmental EDC effects on the hypothalamus discussed in *Section VIII* show that each subregion has unique responses to EDCs that differ from responses in a neighboring region (eg, Ref. 1145). This tissue specificity, and even cellular specificity, in responses to EDCs means that one cannot infer the action of an EDC from one tissue to another or between two different cell types within a tissue. As researchers, we tend to focus on a specific body part in our investigations; we recommend that more collaborations be developed to capitalize upon tissues that are not normally saved in one laboratory for investigation by experts in another complementary disciplines.

5. New EDCs and mixtures

Much EDC research over the past 5 years has focused on relatively few EDC classes such as pesticides, industrial chemicals, and plastics/plasticizers such as BPA and phthalates. We have far

Box 9: Recommendations Beyond Research for the Next 5 Years

- **Educate the public, the media, politicians, and governmental agencies about ways to keep EDCs out of food, water, and air and to protect developing children, in particular.**
- **Develop industrial partners such as "green chemists" and others who can create products that test and eliminate potential EDCs.**
- **Recognize that EDCs are an international problem and develop international collaborations.**
- **Cultivate the next generation of EDC researchers, green chemists, physicians, and public health experts with expertise in endocrine systems.**
- **Funding agencies need to go beyond the "one scientist, one project" and "one clinician, one patient" perspective to fund team science and healthcare.**
- **Funding agencies need to prioritize EDC research in the basic, clinical, and epidemiological realms, especially considering that the cost of research and prevention will result in substantial cost savings in treatment and mitigation.**
- **Emphasize the need for precaution and prevention.**
- **Determine how much evidence is enough, based on rigorous, peer-reviewed science— keeping in mind that absolute proof of harm, or proof of safety, is not possible.**

more limited information on the impact of "emerging EDCs of interest" such as triclosan, PFOA, parabens, and replacements for BPA and other chemicals. A good example of the latter is bisphenol S, now in use as a BPA replacement but recently identified as a putative EDC (1238, 1239). Often, new chemicals are introduced, but in the absence of policies requiring careful testing and disclosure, researchers cannot determine whether or not these chemicals are of concern as EDCs.

An important area that requires extensive and careful research is that of EDC mixtures. Every person is exposed to some combination of chemicals throughout his or her life; the compounds we are exposed to, and their consequences on our bodies, are highly individualized due to differences in genetics, metabolism, and lifestyle. Therefore, the days of studying individual chemicals are coming to a close, and we should transition into research on mixtures (1240). Studies are beginning to be published in the realm of both basic research and epidemiological work relating body burdens of multiple EDCs to endocrine or reproductive outcomes.

However, research on EDC mixtures has several caveats, as researchers try to devise a "representative mixture" that is somehow comparable to the representative human exposure. Considering that each of us has a unique exposome, there is no such thing as representative. Furthermore, reviewers of journal articles and grant proposals need to be educated about reasonable expectations for what can and cannot be done in mixture research. For example, in testing a mixture of five, 10, or more chemicals, it is not possible to test each and every individual compound at a range of dosages, nor is it possible to look at combinations of every subset of chemicals before arriving at a mixture. If the experimental mixture has relevance in terms of widespread use and range of dosages, if work is carefully conducted, and if experiments include appropriate controls and endpoints, then such research should be supported by the scientific community and funding agencies.

A similar argument can be made for the concept of "ecologically relevant dosages" (1240). With the exception of exposures to a single pure chemical, usually due to a toxic spill, contaminated food, or industrial accident, the definition of "ecological relevance" is purely subjective. We often rely on measures of specific chemicals in a bodily fluid to determine "human relevant" levels, but this must always be interpreted with caution. In fact, the epidemiological data as a whole show that a range of exposures can be detected in individuals even within a geographically limited population. Furthermore, individual and population exposures are not only different—they are unique. As people relocate, as they change their diet and lifestyle, and as their physiological and life status change, potential exposures and ways the body processes them will also change. Developing an animal model to approximate human exposures begs the question: Which human? What is relevant ecologically, environmentally, or humanly? Reviewers and readers of the literature must keep this in mind in interpreting data, and mixture research must be prioritized for the future.

B. Recommendations beyond research

1. Educating the public, the media, and governments

The public and the media have played a very important role over the last decade in calling attention to concerns about EDCs. Although some press coverage has been alarmist, responsible journalism has also increased public knowledge about substances such as BPA and triclosan, leading to bans in some states in the United States and the European Union.

Clinicians need to play a greater role in talking to their patients about EDCs. They should ask questions to determine potential exposures of patients through diet, work, and lifestyle behaviors and educate them about minimizing exposures to EDCs (1241). It is important to develop tools and teaching materials (eg, brochures) that healthcare professionals can use to explain EDCs at a level that can be understood by people from diverse educational, cultural, and ethnic backgrounds. The Hormone Health Network of the Endocrine Society has developed materials to help patients understand EDCs, sources of exposure, and health effects, and it provides links to other resources (http://www.hormone.org/hormones-and-health/scientific-statements/edcs). Furthermore, environmental health scientists and green chemists should communicate with one another about the biological effects of EDCs and how to design the next generation of chemicals to be free of EDC activity. Such activities are already under way (http://www.tipedinfo.com/), and a protocol for determining hormonal activity in chemicals was recently published (1242).

Scientific and international organizations are already playing a key role in communicating the science of EDCs to the general public. The Endocrine Society (1) (https://www.endocrine.org/advocacy-and-outreach/position-statements), the American Public Health Association (http://www.apha.org/policies-and-advocacy/public-health-policy-statements/policy-database/2014/07/09/09/03/a-precautionary-approach-to-reducing-american-exposure-to-endocrine-disrupting-chemicals), the American Chemical Society (http://www.acs.org/content/acs/en/policy/publicpolicies/promote/endocrinedisruptors.html), the American College of Obstetricians and Gynecologists jointly with the American Society of Reproductive Medicine (http://www.acog.org/~/

media/Committee%20Opinions/Committee%20on%20Health%20Care%20for%20Underserved%20Women/co575.pdf?dmc=1&ts=20140912T1804036966), and the Royal College of Obstetricians and Gynaecologists (https://www.rcog.org.uk/en/guidelines-research-services/guidelines/sip37/) have issued scientific and policy statements about the consequences of EDCs on human health and the need to minimize exposures during development. International organizations such as the Secretariat of the Strategic Approach to International Chemicals Management and the World Health Organization-United Nations Environment Programme have also weighed in on the importance of assessing exposures. Although there are differences of opinion both within and between these organizations and critiques from both sides of the table (1243, 1244), it is crucial to initiate and maintain these conversations and to work on areas of common ground with the goal of improving quality of life and health across the globe. Attempts to bring these issues to the attention of politicians who write and pass regulatory laws, as well as to regulatory agencies that evaluate EDCs, have been made by many individual scientists, scientific organizations, environmental organizations, and public health policy boards.

2. EDCs as a worldwide problem and the need for international population cohorts

There is a common misperception that the problem of EDCs is limited to developed countries. The spread of environmental chemicals by air and water currents, through consumption of migratory species that spend parts of their lives in contaminated regions, and through consumption of imported processed foods means that EDCs are a global problem and that all humans are exposed. Furthermore, developing countries are sometimes dumping grounds for chemicals used in developed countries, and this contributes enormously to their growing burden. A new international guide published in collaboration between the Endocrine Society and IPEN (http://www.endocrine.org/edcguide) addresses the impact of EDCs in developing countries. The World Health Organization's recent report also tackles this topic (799). The fact that breast cancer rates are climbing quickly in Arctic areas of the world—regions where there has not been heavy industry or chemical use (1245)—further supports this contention. Given that humans are at the top of the food chain, consuming a chemical-laden diet, this could potentially explain the increases in obesity, diabetes, and cancers, which are rising at rates inconsistent with genetic drift. Thus, EDCs are a global problem that requires regulation based on precaution and solid science.

Biological monitoring, data compilation, and data analysis in human populations must be taken to a higher order of collaboration at the global level. We recommend the establishment of a worldwide consortium of existing cohorts and the formation of new partnerships across the globe. The research community can substantially increase the power of studies by examining the relationships between exposures to multiple natural and man-made chemicals and their relationships to a multitude of biological endpoints and disease outcomes. Efforts such as these are under way in the European Union in consortia such as the ENRIECO Project (Environmental Health Risks in European Birth Cohorts; http://www.enrieco.org/) with more than 30 European birth cohorts aimed to examine environment and health relationships. HEALS (Health and Environment-wide Associations Based on Large Population Surveys; www.heals-eu.eu) is another such effort aimed at integrating technology to support exposome studies. Further expansion to include countries in North and South America, Asia, and Africa should be inclusive of region-specific exposures and provide racial diversity that may influence data outcomes. By interrogating and integrating large longitudinal epidemiology studies at the international level, the human data can aid in best identifying the most critical and relevant EDCs and mixtures that adversely affect human health. This information in turn should be used to prioritize chemicals for subsequent in vitro, in vivo, and mechanistic research studies. Careful data mining has the potential to aid in developing early biomarkers of EDC exposures and associated diseases, identifying at-risk individuals, and formulating viable intervention strategies. In parallel with ongoing high-throughput screening strategies (eg, Tox21 and ToxCast), global epidemiology studies on EDCs have the potential to guide chemical policies and regulatory agencies toward well-informed decisions that improve human health.

3. Cultivating the next generation of researchers and physicians with expertise in endocrine systems

We suggest that emphasis should be placed on training new researchers in the field and enhancing career development opportunities for early-stage investigators. Although much work is needed to fully understand the impact of EDCs on a variety of systems, the number of scientists and clinicians who have been trained in the field is relatively limited. Funding agencies around the world should support the research itself and the individuals conducting the research to ensure an ample pipeline of appropriately trained basic and clinical scientists, epidemiologists, and public health professionals. In this time of diminishing funding for research in many countries, the need to cultivate early-stage investigators and to provide them with a fundamental understanding of hormone actions and consequences of perturbations, confounding

variables, and ubiquitous exposures is paramount. Basic theories including low-dose actions of hormones and EDCs, nonmonotonic dose-response curves, and whether there is a threshold of exposure need to be incorporated into the decision-making processes involved in regulating potential EDCs.

4. The need for precaution, and how much evidence is enough?

In the first EDC Scientific Statement, we advocated for prevention and the precautionary principle (1). Here, we will discuss the terminology and philosophy utilized by some organizations such as the US NTP, which uses the phrase "evidence integration" (http://ntp.niehs.nih.gov/pubhealth/hat/noms/index-2.html) in determining whether a chemical may have an impact on human health. The NTP has recommended this terminology to the National Research Council and the EPA (http://www.nap.edu/catalog/18764/review-of-epas-integrated-risk-information-system-iris-process). When high-quality endocrinological studies demonstrate that a chemical interferes with hormone action in vivo and in vitro at environmentally (human) relevant concentrations, and when we have a high degree of evidence that these hormone systems are essential for normal development, it is reasonable to infer that these chemicals will produce adverse effects in humans. This inference is scientifically based but is often considered to be "precautionary" because overt adverse effects may not have been fully characterized. Where to set the bar for evidence of hazard and risk represents a balance of "precautionary principles" designed to protect industry and protect public health.

There are other points that several of the authors of this Statement would like to raise with respect to precaution. It simply is not reasonable to assume a chemical is safe until proven otherwise. Clearly, not all chemicals are EDCs, but substantial information needs to be provided before inclusion of a new compound in a food storage product, a water bottle, or a household product. Replacement chemicals provide excellent examples of why precaution is merited. The BPA substitute, bisphenol S, is now shown to have endocrine-disrupting activity on par with BPA in experimental studies discussed in EDC-2. A further need for precaution is based on evidence that individuals exposed to EDCs may carry that body burden for their entire lives in the case of long-lived chemicals; that even short-lived chemicals may induce changes that are permanent; and that some actions of EDCs are observed in an individual's offspring. Transgenerational effects of EDCs mean that even if a chemical is removed from use, its imprints on the exposed individual's DNA may persist for generations and possibly forever. These observations, which have cut across all areas of EDC research reviewed in EDC-2, make it paramount to evaluate any new chemical before inclusion on the open market in any form to avoid any further contribution to the problem. Although one may argue that precaution may stifle innovation, there are real opportunities for using the scientific evidence about EDCs to stimulate innovation in a profitable manner. A case in point is the initiative called TIPED (Tiered Protocol for Endocrine Disruption), a meeting of the minds of environmental health scientists and green chemists who are working together to communicate to one another what are the biological effects of EDCs and how the next generation of chemicals can be designed to be free of EDC activity (http://www.tipedinfo.com/). This team has produced a protocol for determining hormonal activity in chemicals (1242), and testing of the first chemicals is under way.

The body of literature identifies EDCs as contributing to outcomes related to impaired reproduction, neurodevelopment, thyroid function, and metabolism and increased propensity for hormone-sensitive cancers. This information needs to be used to limit exposures and/or develop interventions. Unfortunately, what continues to be problematic is the difficulty in directly relating chronic disease burden to exposures in humans. The increased prevalence of such diseases underscores the need to invoke precaution in introducing new (and usually untested) chemicals into the environment.

The field of EDCs has become far more established over the last 5 years based on the body of literature reviewed in this Statement. However, trying to interpret diverse and sometimes contradictory results can lead to confusion and the impression that there is still controversy about whether or not EDCs have biological effects in humans. We contend that there is irrefutable evidence across multiple chemicals and organ systems for which EDCs cause adverse effects in humans, although the doses at which this occurs (occupational vs routine life exposures) remain unresolved for several compounds. Differences between results are usually attributable to variability in how experiments are conducted, differences in laboratory environments, and differences in species/sex/age at exposure/age at analysis/treatment models. In interpreting the literature, one needs to recognize when a biological effect is seen again and again across studies. Such is certainly the case for the systems discussed in *Sections II–VIII*.

The scientific conclusions drawn in this document need to be used for risk or human health assessments. Methods need to be developed to consider nonlinear dose-responses, and benchmark dosages should be arrived upon using sensitive endpoints with a biological health outcome of interest in humans. As we move forward, we believe that the evidence is sufficient to recommend greater regulation,

more precaution, better communication between healthcare professionals and patients, and efforts to avoid introducing new EDCs in a misguided effort to replace previous chemicals in the absence of proper testing.

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