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Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses

Laura N. Vandenberg, Theo Colborn, Tyrone B. Hayes, Jerrold J. Heindel, David R. Jacobs, Jr., Duk-Hee Lee, Toshi Shioda, Ana M. Soto, Frederick S. vom Saal, Wade V. Welshons, R. Thomas Zoeller, and John Peterson Myers

Center for Regenerative and Developmental Biology and Department of Biology (L.N.V.), Tufts University, Medford, Massachusetts 02155; The Endocrine Disruption Exchange (T.C.), Paonia, Colorado 81428; Laboratory for Integrative Studies in Amphibian Biology (T.B.H.), Molecular Toxicology, Group in Endocrinology, Energy and Resources Group, Museum of Vertebrate Zoology, and Department of Integrative Biology, University of California, Berkeley, California 94720; Division of Extramural Research and Training (J.J.H.), National Institute of Environmental Health Sciences, National Institutes of Health, U.S. Department of Health and Human Services, Research Triangle Park, North Carolina 27709; Division of Epidemiology and Community Health (D.R.J.), School of Public Health, University of Minnesota, Minneapolis, Minnesota 55455; Department of Preventive Medicine (D.-H.L.), School of Medicine, Kyungpook National University, Daegu 702-701, Korea; Molecular Profiling Laboratory (T.S.), Massachusetts General Hospital Center for Cancer Research, Charlestown, Massachusetts 02129; Department of Anatomy and Cellular Biology (A.M.S.), Tufts University School of Medicine, Boston, Massachusetts 02111; Division of Biological Sciences (F.S.v.S.) and Department of Biomedical Sciences (W.V.W.), University of Missouri-Columbia, Columbia, Missouri 65211; Biology Department (T.Z.), University of Massachusetts-Amherst, Amherst, Massachusetts 01003; and Environmental Health Sciences (J.P.M.), Charlottesville, Virginia 22902

For decades, studies of endocrine-disrupting chemicals (EDCs) have challenged traditional concepts in toxicology, in particular the dogma of "the dose makes the poison," because EDCs can have effects at low doses that are not predicted by effects at higher doses. Here, we review two major concepts in EDC studies: low dose and nonmonotonicity. Low-dose effects were defined by the National Toxicology Program as those that occur in the range of human exposures or effects observed at doses below those used for traditional toxicological studies. We review the mechanistic data for low-dose effects and use a weight-of-evidence approach to analyze five examples from the EDC literature. Additionally, we explore nonmonotonic dose-response curves, defined as a nonlinear relationship between dose and effect where the slope of the curve changes sign somewhere within the range of doses examined. We provide a detailed discussion of the mechanisms responsible for generating these phenomena, plus hundreds of examples from the cell culture, animal, and epidemiology literature. We illustrate that nonmonotonic responses and low-dose effects are remarkably common in studies of natural hormones and EDCs. Whether low doses of EDCs influence certain human disorders is no longer conjecture, because epidemiological studies show that environmental exposures to EDCs are associated with human diseases and disabilities. We conclude that when nonmonotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. Thus, fundamental changes in chemical testing and safety determination are needed to protect human health. (***Endocrine Reviews*** **33: 0000–0000, 2012)**

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Abbreviations: A4, Androstenedione; AhR, aryl hydrocarbon receptor; BPA, bisphenol A; CDC, Centers for Disease Control and Prevention; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane;DES, diethylstilbestrol; EDC, endocrine-disrupting chemicals; EPA, Environmental Protection Agency; ER, estrogen receptor; FDA, Food and Drug Administration; GLP, good laboratory practices; LOAEL, lowest observed adverse effect level; mER, membrane-associated ER; NHANES, National Health and Nutrition Examination Survey; NIS, sodium/iodide symporter; NMDRC, nonmonotonic dose-response curve; NOEL, no observed effect level; NOAEL, no observed adverse effect level; NTP, National Toxicology Program; PIN, prostatic intraepithelial neoplasias; POP, persistent organic pollutants; ppb, parts per billion; SERM, selective ER modulator; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WoE, weight of evidence.

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I. Introduction

This review focuses on two major issues in the study of endocrine-disrupting chemicals (EDCs): low-dose exposures and nonmonotonic dose-response curves (NMDRCs). These concepts are interrelated, and NMDRCs are especially problematic for assessing potential impacts of exposure when nonmonotonicity is evident at levels of exposure below those that are typically used in toxicological assessments. For clarity of presentation, however, we will first examine each of the concepts separately.

A. Background: low-dose exposure

It is well established in the endocrine literature that natural hormones act at extremely low serum concentrations, typically in the picomolar to nanomolar range. Many studies published in the peer-reviewed literature document that EDCs can act in the nanomolar to micromolar range, and some show activity at picomolar levels.

1. What is meant by low dose?

In 2001, at the request of the U.S. Environmental Protection Agency (EPA), the National Toxicology Program (NTP) assembled a group of scientists to perform a review of the low-dose EDC literature (1). At that time, the NTP panel defined low-dose effects as any biological changes 1) occurring in the range of typical human exposures or 2) occurring at doses lower than those typically used in standard testing protocols, *i.e.* doses below those tested in traditional toxicology assessments (2). Other definitions of low dose include 3) a dose below the lowest dose at which a biological change (or damage) for a specific chemical has been measured in the past, *i.e.* any dose below the lowest observed effect level or lowest observed adverse effect level (LOAEL) (3), or 4) a dose administered to an animal that produces blood concentrations of that chemical in the range of what has been measured in the general human population (*i.e.* not exposed occupationally, and often referred to as an environmentally relevant dose because it creates an internal dose relevant to concentrations of the chemical measured in humans) (4, 5). This last definition takes into account differences in chemical metabolism and pharmacokinetics (*i.e.* absorption, distribution, and excretion of the chemical) across species and reduces the importance of route of exposure by directly comparing similar blood or other tissue concentrations across model systems and experimental paradigms. Although these different definitions may seem quite similar, using just a single well-studied chemical like bisphenol A (BPA) shows how these definitions produce different cutoffs for exposure concentrations that are considered low dose (Table 1). For many chemicals, including EDCs, a large number of studies meet the criteria for low-dose studies regardless of whether the cutoff point for a low dose was based on the range of typical human exposures, doses used in traditional toxicology, or doses that use an internal measure of body burden.

Whether low doses of EDCs influence disease is a question that now extends beyond the laboratory bench, because epidemiological studies show that environmental exposures to these chemicals are associated with disorders in humans as well (see for examples Refs. 6–16). Although disease associations have historically been observed in individuals exposed to large concentrations of EDCs after

TABLE 1. Low-dose definitions and cutoff doses: BPA and DEHP as examples

Chemical	Estimated range of human exposures	Doses below the NOAEL	Doses below the LOAEL	Administered doses (to animals) that produce blood levels in typical humans
BPA	0.4–5 μg/kg · d (679)	No NOAEL was ever established in toxicological studies (38)	<50 mg/kg · d (38)	~400 μg/kg · d to rodents and nonhuman primates (4, 253)
DEHP	0.5–25 μg/kg · d (680)	<5.8 mg/kg · d (681, 682)	<29 mg/kg · d (681, 682)	Unknown

Estimates of human exposure are made from consumer product consumption data but do not take into account that there are unknown sources of these chemicals. DEHP, Bis(2-ethylhexyl) phthalate.

industrial accidents (17–19) or via occupational applications (20–22), recent epidemiological studies reveal links between environmentally relevant low concentrations and disease prevalence. With the extensive biomonitoring studies performed by the U.S. Centers for Disease Control and Prevention (CDC) (23, 24) and similar environmental surveys performed in Europe (25) and elsewhere (www.statcan.gc.ca/concepts/hs-es/measures-mesures-eng.htm), knowledge about environmental exposures to EDCs and their associations with human health disorders has increased substantially.

Low-dose effects have received considerable attention from the scientific and regulatory communities, especially when examined for single well-studied chemicals like BPA (4, 27–32). The low-dose literature as a whole, however, has not been carefully examined for more than a decade. Furthermore, this body of literature has been disregarded or considered insignificant by many (33, 34). Since the NTP's review of the low-dose literature in 2001 (2), a very large body of data has been published including 1) additional striking examples of low-dose effects from exposures to well-characterized EDCs as well as other chemicals, 2) an understanding of the mechanisms responsible for these low-dose effects, 3) exploration of nonmonotonicity in *in vivo* and *in vitro* systems, and 4) epidemiological support for both low-dose effects and NMDRCs.

2. Is the term low dose a misnomer?

Endogenous hormones are active at extremely low doses, within and below the picomolar range for endogenous estrogens and estrogenic drugs, whereas environmental estrogen mimics are typically active in the nanomolar to micromolar range (for examples, see Refs. 35–38), although some show effects at even lower concentrations (39–41). Importantly, the definitions above do not take into account the potency or efficacy of the chemical in question, a topic that will be discussed in greater detail below. Instead, low dose provides an operational definition, in which doses that are in the range of human exposure, or doses below those traditionally tested in toxicological studies, are considered low. To be clear, none of these definitions suggest that a single concentration can be set as a low dose cutoff for all chemicals. Using the above definitions, for some chemicals, low doses could potentially be in the nanogram per kilogram range, but for most chemicals, doses in the traditional micro- and milligram per kilogram range could be considered low doses because traditional approaches to testing chemicals typically did not examine doses below the milligram per kilogram dose range.

B. Background: NMDRCs

We have defined low-dose studies according to the definitions established by the NTP panel of experts (2). However, because the types of endpoints that are typically examined at high doses in toxicological studies are often different from the types of endpoints examined in low-dose studies, one cannot assume that an effect reported in the low-dose range is necessarily different from what would be observed at higher doses. For example, low doses of a chemical could affect expression of a hormone receptor in the hypothalamus, an endpoint not examined in high-dose toxicology testing, and high doses could similarly affect this same endpoint (but are likely to be unreported because high doses are rarely tested for these types of endpoints). Thus, the presence of low-dose effects makes no assumptions about what has been observed at higher concentrations. (As discussed elsewhere, for the majority of chemicals in commerce, there are no data on health effects and thus no established high- or low-dose range.) Therefore, low-dose effects could be observed at the lower end of a monotonic or linear dose-response curve.

In contrast, the definition of a NMDRC is based upon the mathematical definition of nonmonotonicity: that the slope of the dose-response curve changes sign from positive to negative or vice versa at some point along the range of doses examined (42). Often NMDRCs have a U- or inverted U-shape (43); these NMDRCs are thus also often referred to as biphasic dose-response curves because responses show ascending and descending phases in relation to dose. Complex, multiphasic curves have also been observed (41, 44, 45). NMDRCs need not span from true low doses to high (pharmacologically relevant) doses, although experiments with such a broad dose range have been performed for several EDCs; the observation of nonmonotonicity makes no assumptions about the range of doses tested. Examples of NMDRCs from *in vitro* cell culture and *in vivo* animal experiments, as well as epidemiological examples, are presented in detail later in this review (see *Sections III.C.1–3*). Additional examples of NMDRCs are available in studies examining the effects of vitamins and other essential elements on various endpoints (see for example (46); these will not be examined in detail in this review due to space constraints.

NMDRCs present an important challenge to traditional approaches in regulatory toxicology, which assume that the dose-response curve is monotonic. For all monotonic responses, the observed effects may be linear or nonlinear, but the slope does not change sign. This assumption justifies using high-dose testing as the standard for assessing chemical safety. When it is violated, high-dose testing regimes cannot be used to assess the safety of low doses.

It should be noted that both low dose and nonmonotonicity are distinguished from the concept of hormesis, which is defined as a specific type of response whereby "the various points along [the dose response] curve can be interpreted as beneficial or detrimental, depending on the biological or ecological context in which they occur" (47). Estimations of beneficial or adverse effects cannot be ascertained from the direction of the slope of a dose-response curve (48–50). In their 2001 Low Dose Peer Review, the NTP expert panel declined to consider whether any effect was adverse because "in many cases, the long-term health consequences of altered endocrine function during development have not been fully characterized" (2). There are still debates over how to define adverse effects (51–53), so for the purposes of this review, we consider any biological change to be an effect. Importantly, most epidemiological studies are by definition examining low doses (unless they are focusing on occupationally exposed individuals), and these studies typically focus on endpoints that are accepted to be adverse for human health, although some important exceptions exist (54–56).

Finally, it is worth noting that any biological effect, whether it is observed to follow linear relationships with administered dose or not, provides conclusive evidence that an EDC has biological activity. Thus, other biological effects are likely to be present but may remain undetected or unexamined. Many EDCs, including those used as pesticides, were designed to have biological effects (for example, insecticides designed to mimic molting hormone). Thus, the question of whether these chemicals have biological effects is answered unequivocally in their design; the question is what other effects are induced by these biologically active agents, not whether they exist.

C. Low-dose studies: a decade after the NTP panel's assessment

In 2000, the EPA requested that the NTP assemble a panel of experts to evaluate the scientific evidence for low-dose effects and dose-response relationships in the field of endocrine disruption. The EPA proposed that an independent and open peer review of the available evidence would allow for a sound foundation on which the EPA could "determine what aspects, if any, of its standard guidelines for reproductive and developmental toxicity testing [would] need to be modified to detect and characterize low-dose effects" (2). The NTP panel verified that low-dose effects were observed for a multitude of endpoints for specific EDCs including diethylstilbestrol (DES), genistein, methoxychlor, and nonylphenol. The panel identified uncertainties around low-dose effects after exposure to BPA; although BPA had low-dose effects on some endpoints in some laboratories, others were not found to be consistent, leading the panel to conclude that it was "not persuaded that a low-dose effect of BPA has been conclusively established as a general or reproducible finding" (2).

Since the NTP's review of low-dose endocrine disruptor studies, only a few published analyses have reexamined the low-dose hypothesis from a broad perspective. In 2002, R. J. Witorsch (57) analyzed low doses of xenoestrogens and their relevance to human health, considering the different physiologies associated with pregnancy in the mouse and human. He proposed that low doses of endocrine disruptors would not likely affect humans because, although low-dose effects had been observed in rodents, the hormonal milieu, organs controlling hormonal release, and blood levels of estrogen achieved are quite different in humans. There are, of course, differences in hormones and hormone targets between rodents and humans (58), but the view that these differences negate all knowledge gained from animal studies is not supported by evolutionary theory (59–61). This human-centered stance argues against the use of animals for any regulatory testing (62) and runs counter to the similarities in effects of EDCs on humans and animals; rodents proved to be highly predictive of the effects of DES on humans (63, 64). In a striking example, studies from mice and rats predicted that gestational exposure to DES would increase mammary cancer incidence decades before women exposed *in utero* reached the age where this increase in risk was actually observed (65–67).

In 2007, M. A. Kamrin (68) examined the low-dose literature, focusing on BPA as a test case. He suggested that three criteria were required to support the low-dose hypothesis. First is reproducibility, which he defined as "the same results are seen from the same causes each time a study is conducted." Furthermore, he proposed that the dose response for the effects must be the same from study to study. Second is consistency, which he defined as the results all fitting into a pattern, whereby the results collected from multiple species and under variable conditions all show the same effect. And third is proper conduct of studies, which he defined as including the appropriate controls and performance under suitable experimental conditions as well as the inclusion of multiple doses such that a dose-response curve can be obtained.

Although we and others (69–72) agree with the use of these criteria (reproducibility, consistency, and proper experimental design), there are significant weaknesses in the logic Kamrin employed to define these factors. First, suggesting that reproducibility is equivalent to the same results obtained each time a study is conducted is unrealistic and not a true representation of what is required of replication. As has been discussed in other fields, "there is no

end to the ways in which any two experiments can be counted as the same — or different . . . All experiments are the same in respect of their being experiments; they are all different by virtue of being done at different places, at different times, by different people, with different strains of rat, training regime, and so on" (73).

Furthermore, according to the Bradford-Hill criteria, a set of requirements accepted in the field of epidemiology to provide adequate evidence of a causal relationship between two factors, a single negative result (or even several studies showing negative results) cannot negate other studies that show adverse effects (74). Essentially, all scientists know that it is very easy for an experiment to find no significant effects due to a myriad of reasons; it is more difficult to actually find effects, particularly when using highly sophisticated techniques (69).

Second, the concept of consistency as a pattern that can be derived from all results is one we will use below, using a weight-of-evidence (WoE) approach and several specific examples. However, Kamrin's proposed idea that every study must show the same effect has the same weaknesses as discussed for the proposed definition of reproducibility and does not acknowledge the obvious differences in many species and strains. It also suggests that the identification of a single insensitive strain could negate any number of positive studies conducted with appropriate animal models (75).

And finally, Kamrin suggested that only studies with appropriate controls should be used for analyses, a criterion we agree should be followed. However, his own scrutiny of the low-dose animal literature fails to do so (68). He also suggested that studies use multiple doses so that a dose-response curve can be obtained. Although studies using a single dose can be informative, we agree that dose-response relationships provide important information to researchers and risk assessors alike. However, this requirement is not helpful if there is an insistence on observing a linear response; as we discuss in depth in this review, there are hundreds of examples of nonmonotonic and other nonlinear relationships between dose and endpoint. These should not be ignored.

In 2004, Hayes (76) reviewed the available literature concerning the effects of atrazine on amphibian development, with a specific focus on the effect of ecologically relevant doses of this EDC on malformations of the gonads and other sexually dimorphic structures; in the case of aquatic exposures, it can be difficult to determine what a cutoff for a low dose would be; thus, Hayes focused on studies examining the effects of atrazine at levels that had been measured in the environment. He reviewed the results produced by several labs, in which it was independently demonstrated that low concentrations of atrazine produced gonadal abnormalities including hermaphroditism, males with extra testes, discontinuous gonads, and other defects. Hayes' work also clearly addressed the so-called irreproducibility of these findings by analyzing the studies that were unable to find effects of the pesticide; he noted that the negative studies had multiple experimental flaws, including contamination of the controls with atrazine, overcrowding (and therefore underdosing) of experimental animals, and other problems with animal husbandry that led to mortality rates above 80%.

In 2006, vom Saal and Welshons (77) examined the low-dose BPA literature, identifying more than 100 studies published as of July 2005 that reported significant effects of BPA below the established LOAEL, of which 40 studies reported adverse effects below the 50 μg/kg · d safe dose set by the EPA and U.S. Food and Drug Administration (FDA); all of these studies would be considered low dose according to the NTP's definition (2). The authors proposed that these examples should be used as evidence to support the low-dose hypothesis. Furthermore, this publication detailed the similarities among the studies that were unable to detect any effects of low doses of BPA and established a set of criteria required to accept negative studies. We have adapted the criteria detailed by Hayes (76) and vom Saal and Welshons (77) to produce a set of requirements for low-dose studies; these criteria are described in some detail below.

D. Why examine low-dose studies now?

The developmental origins of health and disease hypothesis originated from studies showing that fetal DES exposure could cause severe malformations and cancers of the reproductive tract, and other studies demonstrating that fetal malnutrition could lead to adult diseases including metabolic syndrome, diabetes, and increased stroke incidence (78–81). Since that time, the developmental origins of health and disease hypothesis has been extended to address whether diseases that are increasing in prevalence in human populations could be caused by developmental exposures to EDCs (67, 82–85). Evidence from the animal literature has been tremendously informative about the effects of EDC exposures early in development and has driven new hypotheses to be tested in epidemiology studies (86). Studies including several discussed in this review provide supportive evidence that the fetal and neonatal periods are specifically sensitive to chemicals that alter endocrine signaling and that EDCs could be contributing to a range of diseases.

Strong, reliable, and reproducible evidence documents the presence of low concentrations of EDCs and other chemicals in human tissues and fluids, as well as in environmental samples (28, 87–89). These studies indicate

that samples collected from humans and the environment typically contain hundreds of contaminants, usually in the parts-per-billion (ppb) range (90, 91). The obvious question with potentially large public health implications is whether these concentrations are so low as to be irrelevant to human health. The fact that epidemiological analyses (reviewed in *Section III.C.3*) repeatedly find associations between the measured concentrations in human samples and disease endpoints suggests it is inappropriate to assume the exposures are too low to matter. That is especially the case given the empirical data (reviewed in *Section II.A*) from animal and cell culture experiments showing effects can be caused by concentrations comparable (and sometimes below) what is measured in humans and also the detection of NMDRCs in some of those same experiments.

In the human biomonitoring field, large databases such as the CDC's National Health and Nutrition Examination Survey (NHANES) have allowed researchers to make comparisons between groups of individuals with various exposure criteria; some of these studies will be addressed in detail in subsequent sections of this review. Although by definition these databases examine low-dose exposures, their use has been the subject of significant debate. Because of the large number of chemicals that have been measured (>300 in the most recent NHANES by the CDC) and the large number of health outcomes and other disease-related data collected from the individuals that donated biological samples, it has been argued that the number of possible associations that could be made would lead to a significant number of false positives (92); thus, associations could be found simply because of extensive data dredging. This has led some to suggest that these studies as a whole should be rejected (93, 94).

In response to these criticisms, epidemiologist Jan Vandenbroucke (95) notes, "researchers do not mindlessly grind out one analysis after another"; the examination of these databases for associations between chemical exposures and health effects does not entail the statistical comparison between all possible factors, calculated as some 8800 comparisons in the CDC's NHANES database (92). Instead, epidemiologists typically focus on a select number of comparisons that address relationships between chemicals and diseases identified *a priori* (96, 97), often because of mechanistic data obtained in laboratory animals or *in vitro* work with human and animal cells and tissues. Repeated findings of links between EDC exposures and diseases in epidemiological analyses of biomonitoring data based on *a priori* hypotheses suggests these relationships should not be rejected as a statistical artifact and, instead, should be the basis for significant concern that low-dose effects can be detected in the general population (85, 98).

E. Mechanisms for low-dose effects

The endocrine system is particularly tuned to respond to very low concentrations of hormone, which allows an enormous number of hormonally active molecules to coexist in circulation (38). As a ligand-receptor system, hormones act by binding to receptors in the cell membrane, cytosol, or the nucleus. The classical effects of nuclear hormone receptors influence gene expression directly, although rapid nongenomic actions at membrane-associated receptors are now well documented and accepted. Membrane receptors are linked to different proteins in the cell, and binding to these receptors typically changes cellular responses in a rapid fashion (99), although the consequence of a rapid signaling event could be the activation of a nuclear transcription factor, leading to responses that take longer to detect. Peptide hormones can also influence gene expression directly (see Refs. 100 and 101 for examples).

There are several means by which the endocrine system displays specificity of responses to natural hormones. Many hormone receptors are expressed specifically in a single or a few cell types (for example, receptors for TSH are localized to the thyroid), whereas some (like thyroid hormone receptors) are found throughout the body (102). For receptors that are found in multiple cell types, different effects are produced in part due to the presence of different coregulators that influence behaviors of the target genes (103–105). And finally, some hormones have multiple receptors [for example estrogen receptor (ER)α and ERβ], which are expressed in different quantities in different cell types and organs and can produce variable effects on gene expression or cellular phenomena (cell proliferation *vs.* apoptosis) (102, 106).

The typical physiological levels of the endogenous hormones are extremely low, in the range of 10–900 pg/ml for estradiol, 300–10,000 pg/ml for testosterone, and 8–27 pg/ml for T_4 (see Table 2). Importantly, steroid hormones in the blood are distributed into three phases: free, representing the unconjugated, unbound form; bioavailable, representing hormones bound to low-affinity carrier proteins such as albumin; and inactive, representing the form that is bound to high-affinity binding proteins such as SHBG or α-fetoprotein (38) (Fig. 1A). When the circulating levels in blood are corrected for the low fraction of the hormones that are not bound to serum binding proteins, the free concentrations that actually bring about effects in cells are even lower, for example 0.1–9 pg/ml for estradiol. Concentrations of active hormones will vary based on the age and physiological status of the individual (*i.e.* plasma testosterone levels are less than 1 ng/ml in male children but increase to approximately 5–7 ng/ml in adulthood; during menses, estradiol levels are typically less than 100

TABLE 2. Ranges of endogenous hormones in humans (from Ref. 108)

Hormone	Free concentration (females)	Total concentration (females)	Free concentration (males)	Total concentration (males)
Cortisol	20–300 ng/ml		20–300 ng/ml	
Estradiol	0.5–9 pg/ml (adult female)	<20 pg/ml (prepubertal) 20–800 pg/ml (premenopausal) <30 pg/ml (postmenopausal)		10–60 pg/ml (adult)
Progesterone		0.2–0.55 ng/ml (prepubertal) 0.02–0.80 ng/ml (follicular phase) 0.90–4 ng/ml (luteal phase) <0.5 ng/ml (postmenopausal)		0.1–0.4 ng/ml (prepubertal) 0.2–2 ng/ml (adult)
Insulin		0–250 pmol/liter		0–250 pmol/liter
GH		2–6 ng/ml		2–6 ng/ml
Prolactin		0–15 ng/ml		0–10 ng/ml
Testosterone	9–150 pg/ml (adult)		0.3–250 ng/ml	
Thyroid hormone	8–30 pg/ml (10–35 pM)		8–30 pg/ml (10–35 pM)	
TSH	0.5–5 μU/ml		0.5–5 μU/ml	

pg/ml, but just before ovulation, they spike to 800 pg/ml; *etc.*) (107, 108). Of course, it should be noted that active concentrations of natural hormones vary somewhat from species to species and can even vary between strains of the same species (109).

There are several reasons why endogenous hormones are able to act at such low circulating concentrations: 1) the receptors specific for the hormone have such high affinity that they can bind sufficient molecules of the hormone to trigger a response, 2) there is a nonlinear relationship between hormone concentration and the number of bound receptors, and 3) there is also a nonlinear relationship between the number of bound receptors and the strongest observable biological effect. Welshons and colleagues (38) describe how hormone concentration influences receptor occupancy: "receptor occupancy is never determined to be linear in relation to hormone concentration . . . At concentrations above the K_d [the dissociation constant for receptor-ligand binding kinetics], saturation of the response occurs first, and then at higher concentrations, saturation of receptors is observed." What this means is that at low doses of hormone, a 10-fold increase in hormone concentration can have a 9-fold increase in receptor occupancy, whereas at high doses of hormone, a 10-fold increase in hormone concentration produces a less than 1.1-fold increase in receptor occupancy (38) (Fig. 1B). Thus, even moderate changes in hormone concentration in the low-dose range can produce substantial changes in receptor occupancy and therefore generate significant changes in biological effects. Welshons *et al.* (38) also note that a near-maximum biological response can be observed without a high rate of receptor occupancy, a situation that was previously termed the spare receptor hypothesis (110, 111); that is, the response mechanism saturates before all of the receptors are saturated. The presence of spare receptors is the basis for saying that these receptor systems are tuned to detect low concentrations that lead to occupancy of 0.1–10% of total receptors. Within this range of low receptor occupancy, there is high proportionality between changes in the free hormone concentration and changes in receptor occupancy, and a change in receptor occupancy by a ligand for the receptor is required to initiate changes in receptor-mediated responses (38).

There are additional reasons why natural hormones are active at low doses: 4) hormones have a strong affinity for their receptors (relative to affinity for other receptors) because many hormones are secreted from a single gland or site in the body but must have effects throughout the body in multiple tissues and 5) blood concentrations of hormones are normally pulsatile in nature, with the release of one hormone often controlled by the pulsatile release of another hormone (112, 113), and both the frequency and the amplitude of pulses modulate the biological response; hormones are also influenced by circadian rhythms, with dramatic differences in hormone secretion depending on the time of day (114, 115).

For many years, the mechanisms by which some environmental chemicals acted at low doses were not well understood. In 1995, the National Research Council appointed the Committee on Hormonally Active Agents in the Environment to address public concerns about the potential for adverse effects of EDCs on human health (116). At the time, work on understanding the mechanisms by which EDCs exert their effects was in its infancy, and in the executive summary, the committee stated, "Lack of knowledge about a mechanism does not mean that a reported effect is unconfirmed or unimportant, nor does demonstration of a mechanism document that the resulting effects are unique to that mechanism or are pervasive

Figure 1.

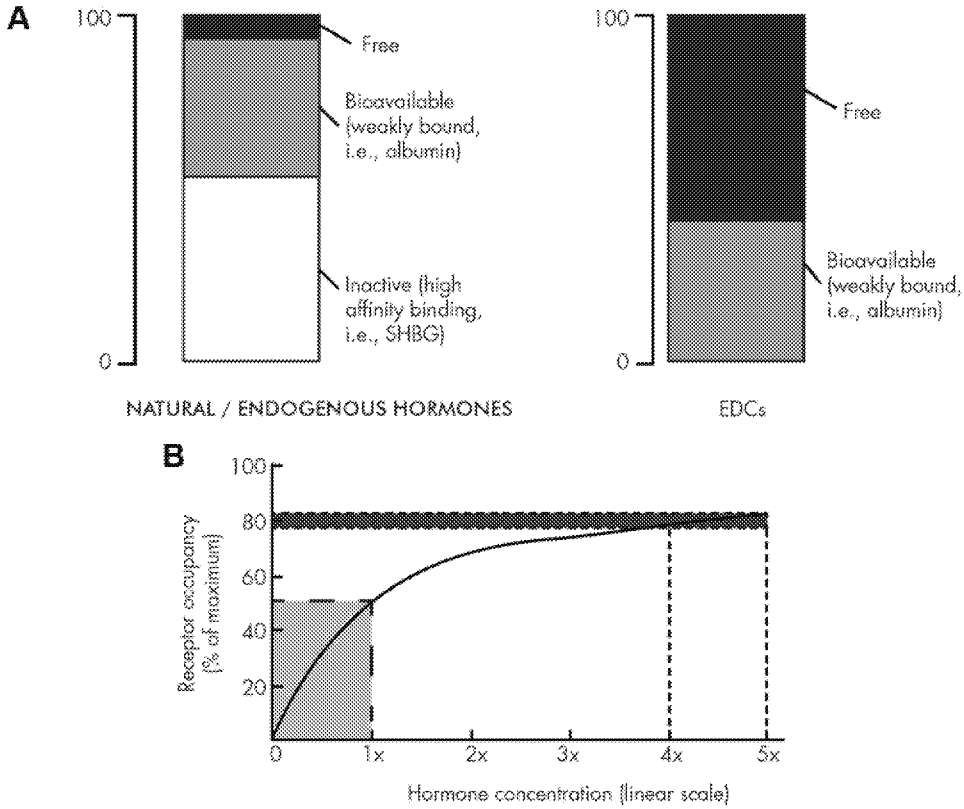

Figure 1. Characteristics and activities of natural hormones. A, This schematic depicts a typical relationship of three phases of circulating hormones: free (the active form of the hormone), bioavailable (bound weakly to proteins such as albumin), and inactive (bound with high affinity to proteins such as SHBG). These three phases act as a buffering system, allowing hormone to be accessible in the blood, but preventing large doses of physiologically active hormone from circulating. With EDCs, there may be little or no portion maintained in the inactive phase. Thus, the entirety or majority of a circulating EDC can be physiologically active; the natural buffering system is not present, and even a low concentration of an EDC can disrupt the natural balance of endogenous hormones in circulation. B, Schematic example of the relationship between receptor occupancy and hormone concentration. In this theoretical example, at low concentrations, an increase in hormone concentration of x (from 0 to 1x) causes an increase in receptor occupancy of approximately 50% (from 0 to 50%, see *yellow box*.) Yet the same increase in hormone concentration at higher doses (from 4x to 5x) causes an increase in receptor occupancy of only approximately 4% (from 78 to 82%, see *red box*).

in natural systems." Since that time, a tremendous amount of work has been dedicated to understanding the molecular mechanisms of action of EDCs, and in particular the mechanisms responsible for low-dose effects.

1. General mechanisms for EDC action

As discussed above, the endocrine system evolved to function when unbound physiologically active ligands (hormones) are present at extremely low doses (117). Because of shared receptor-mediated mechanisms, EDCs that mimic natural hormones have been proposed to follow the same rules and therefore have biological effects at low doses (38, 118). Similarly, EDCs that influence in any way the production, metabolism, uptake, or release of hormones also have effects at low doses, because even small changes in hormone concentration can have biologically important consequences (38, 119).

The estrogen-response mechanisms have been extensively studied with regard to the effects of endogenous estrogens and estrogenic drugs. In classical, genomic estrogen action, when endogenous estrogens bind to ER, those receptors bind to estrogen response element sequences or to a number of other response element sites adjacent to the genes directly responsive to estrogens; this binding influences transcription of estrogen-sensitive genes (120). Xenoestrogens produce the same reactions; these chemicals bind to ERs, which then initiate a cascade of molecular effects that ultimately modify gene expression. Therefore, for the actions of estrogenic EDCs, molecular mechanisms and targets are already known in some detail. Similar mechanisms are induced by the binding of androgens to the androgen receptor, or thyroid hormone agonists to the thyroid hormone receptor, among others.

Additionally, there are EDCs that act as antagonists of these hormone systems, binding to a receptor, but not activating the receptor's typical response, and preventing the binding or activity of the endogenous ligand. Finally, many EDCs bind to the receptor and trigger a response that is not necessarily the same as that triggered by the endogenous estrogens; these are termed selective ER modulators (SERMs). Ultimately, all of these actions occur at the level of the receptor.

Many studies have been dedicated to the understanding of which EDCs bind to which nuclear hormone receptors and how the binding affinities compare to the natural steroid. Thus, many of these chemicals have been classified as weak hormones. Yet studies have shown that, for example, the so-called weak estrogens like BPA can be equally potent as endogenous hormones in some systems, causing biological effects at picomolar levels (30, 38, 41, 121). Both endogenous estrogens and EDCs can bind to ER associated with the cell membrane [membrane-associated ER (mER)α and mERβ] that are identical to the nuclear ER (122–124), and a transmembrane ER called G-protein coupled receptor 30 that is structurally dissimilar to the nuclear ER and encoded by a distinct gene (125, 126). In many cells, 5–10% of total ERα and ERβ are localized to the plasma membrane (124); these membrane-associated receptors are capable of nongenomic steroid action in various cell types (30, 121, 127); thus, rapid and potent effects are well documented for many EDCs including BPA, DES, endosulfan, dichlorodiphenyldichloroethylene (DDE), dieldrin, and nonylphenol, among others (41, 128–130).

Finally, EDCs have other effects that are not dependent on binding to either classical or membrane-bound steroid hormone receptors. EDCs can influence the metabolism of natural hormones, thus producing differences in the amount of hormone that is available for binding either because more (or less) hormone is produced than in a typical system or because the hormone is degraded faster (or slower) than is normal. Other EDCs influence transport of hormone, which can also change the amount of hormone that is available for receptor binding. And EDCs can also have effects that are independent from known endocrine actions. One example is the effect of endogenous hormones and EDCs on ion channel activity. BPA, dichlorodiphenyltrichloroethane (DDT), DES, nonylphenol, and octylphenol have all been shown to disrupt Ca^{2+} channel activity and/or Ca^{2+} signaling in some cell types (131–134). This example illustrates how both natural hormones and EDCs can have hormonal activity via binding to nuclear hormone receptors but may also have unexpected effects via receptor-mediated actions outside of the classical endocrine system.

2. Mechanisms of EDC-induced low-dose actions

The various mechanisms by which EDCs act *in vitro* and *in vivo* provide evidence to explain how these chemicals induce effects that range from altered cellular function, to abnormal organ development, to atypical behaviors. Just as natural hormones display nonlinear relationships between hormone concentration and the number of bound receptors, as well as between the number of bound receptors and the maximal observable biological effect, EDCs obey these rules of binding kinetics (38). Thus, in a way, EDCs exploit the highly sensitive endocrine system and produce significant effects at relatively low doses.

To gain insight into the effects of natural hormones and EDCs on gene expression profiles, it is possible to calculate doses that produce the same effect on proliferation of cultured cells, *i.e.* the quantitative cellular response doses, and determine the effect of those doses on transcriptomal signature profiles. When this is done for estradiol and EDCs with estrogenic properties, the affected estrogen-sensitive genes are clearly different (135). However, an interesting pattern emerges: comparing profiles among only the phytoestrogens shows striking similarities in the genes up- and down-regulated by these compounds; profile comparisons between only the plastic-based estrogens also show similarities within this group. Yet even more remarkable is what occurs when the doses are selected not based on cell proliferation assays but instead on the ability of estradiol and estrogen-mimics to induce a single estrogen-sensitive marker gene. When doses were standardized based on marker gene expression, the transcriptomal signature profiles were very similar between estradiol and estrogen mimics (135). Taken together, these results suggest that the outcomes of these experiments are contextual to the normalization parameter and that marker gene expression and cell proliferation are not superimposable. This indicates that the biological level at which the effects of chemicals are examined (*i.e.* gene expression, cellular, tissue, organ, or organismal) can greatly impact whether low-dose effects are observed and how these effects are interpreted.

There are several other mechanisms by which low-dose activities have been proposed. One such possibility is that low doses of EDCs can influence the response of individuals or organs/systems within the body to natural hormones; thus, the exposed individual has an increased sensitivity to small changes in endogenous steroids, similar to the effects of intrauterine position (see Ref. 136 and *Section I.F*). In fact, several studies have shown that exposure to EDCs such as BPA during perinatal development can influence the response of the mammary gland to estrogen (137, 138) and the prostate to an estrogen-testosterone

mixture similar to the concentrations produced in aging men (139–142). There is also evidence that EDCs work additively or even synergistically with other chemicals and natural hormones in the body (143–145). Thus, it is plausible that some of the low-dose effects of an EDC are actually effects of that exogenous chemical plus the effects of endogenous hormone.

Finally, it should be noted that during early development, the rodent fetus is largely, but not completely (146), protected from estrogen via the binding activity of α-fetoprotein, a plasma protein produced in high levels by the fetal liver (147). Some estrogen-like EDCs, however, bind very weakly to α-fetoprotein, and therefore, it is likely that this protein does not provide protection to the fetus during these sensitive developmental periods (36, 148). Furthermore, because EDCs may not bind to α-fetoprotein or other high-affinity proteins in the blood (148–150) and can have a higher binding affinity to proteins like albumin (compared with natural estrogens) (36, 149), the balanced buffer system in place for endogenous hormones may be disturbed (Fig. 1A). Thus, whereas only a portion of endogenous hormones are bioavailable, the entirety of a circulating EDC could be physiologically active.

The effects of hormones and EDCs are dependent on dose, and importantly, low (physiological) doses can be more effective at altering some endpoints compared with high (toxicological) doses. There are many well-characterized mechanisms for these dose-specific effects including signaling via single *vs.* multiple steroid receptors due to nonselectivity at higher doses (30), receptor down-regulation at high doses *vs.* up-regulation at low doses (151, 152), differences in the receptors present in various tissues (153, 154), cytotoxicity at high doses (155), and tissue-specific components of the endocrine-relevant transcriptional apparatus (104, 105). Some of these factors will be addressed in *Section III.B* in the section dedicated to NMDRCs.

F. Intrauterine position and human twins: examples of natural low-dose effects

Hormones have drastically different effects at different periods of development. In a now classical *Endocrinology* paper, Phoenix and colleagues (156) showed that hormone exposures during early development, and in particular fetal development, had organizational effects on the individual, whereby the developing organs were permanently reorganized by exposure to steroids. Permanent, nonreversible masculinization of the developing body plan by androgen exposure *in utero* is an example. These organizational effects are in contrast to the effects of the same hormones, at similar or even higher doses, on adults. The effects of steroids on individuals after puberty have been termed activational, because the effects on target organs are typically transient; withdrawal of the hormone returns the phenotype of the individual to the preexposed state (157), although this is not always the case (158).

One of the most striking examples of the ability of low doses of hormones to influence a large repertoire of phenotypes is provided by the study of intrauterine positioning effects in rodents and other animals. The rodent uterus in particular, where each fetus is fixed in position along a bicornate uterus with respect to its neighbors, is an excellent model to study how hormones released from neighboring fetuses (159) can influence the development of endocrine-sensitive endpoints (31). Importantly, differences in hormonal exposures by intrauterine position are relatively small (see Fig. 2) (160). Thus, even a small magnitude in differences of hormonal exposures is sufficient to generate effects on behavior, physiology, and development.

The earliest studies of intrauterine position compared behavioral characteristics of females relative to their position in the uterus (161–164); male behavior was also affected by intrauterine position (161, 165–167). Subsequent studies of intrauterine position showed that position in the uterus influenced physiological endpoints (157, 160–162, 168–174) as well as morphological endpoints in female rodents (160, 161, 163, 164, 175–177). Male physiology and morphological endpoints were similarly affected by intrauterine position (165, 167, 177–179).

The endocrine milieu of the uterine environment has been implicated in these effects because differences in hormonal exposure have been observed based on intrauterine position (Fig. 2). The production of testosterone in male mice starting at approximately d 12 of gestation allows for passive transfer of this hormone to neighboring fetuses (159, 160, 180). Thus, fetuses positioned between two male neighbors have slightly higher testosterone exposures compared with fetuses positioned between one male and one female or two female neighbors (168, 181–183). These data indicate that very small differences in hormone exposures during fetal development are capable of influencing a variety of endpoints, many of which become apparent only during or after puberty. Furthermore, small differences in hormone exposures may be compounded by other genetic variations such as those normally seen in human populations.

Intrauterine effects have been observed in animals with both large litters and singleton or twin births including ferrets, pigs, hamsters, voles, sheep, cows, and goats (136, 184, 185). But perhaps the most compelling evidence for intrauterine effects comes from human twin studies. Many

Figure 2.

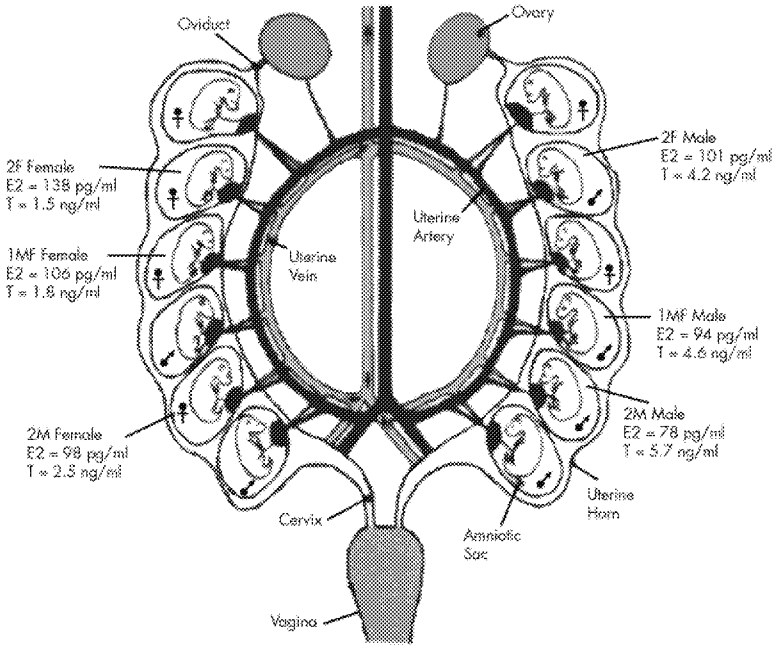

Figure 2. Intrauterine position produces offspring with variable circulating hormone levels. Fetuses are fixed in position in the bicornate rodent uterus, thus delivery via cesarean section has allowed for study of the influence of intrauterine position on behaviors, physiology, and organ morphology. Illustrated here are the differences in estradiol (E2) and testosterone (T) concentrations measured in male and female fetuses positioned between two male neighbors (2M), two female neighbors (2F), or neighbors of each sex (1MF). Direction of blood flow in the uterine artery (dark vessel) and vein (light vessel) is indicated by an *arrow* (159).

studies have found that the sex of the fetuses impacts the phenotype of one or more of the twins, with significant evidence suggesting that male twins strongly influence a female co-twin; endpoints including sensation seeking (186), ear superiority (187, 188), brain and cerebellum volume (189), masculine/feminine behaviors and aggression levels (190–192), handedness (193, 194), reproductive fitness (192, 195), finger length ratios (196), risk for developing eating disorders (197), and birth weight (198) were all affected in females with a male twin. From these studies, many authors have concluded that testosterone from male fetuses influences developmental parameters in female twins; typically, male same-sex twins do not display altered phenotypes for these endpoints. Yet importantly, limited studies indicate that female twins can influence their uterine pairs, with some behaviors affected in male co-twins (191); breast cancer incidence in women and testicular cancer in men have also been shown to be influenced by having a female co-twin (83, 199, 200).

Although the mechanisms for these intrauterine effects are not completely understood, very small differences in hormone exposures have been implicated, making the effects of twin gestations a natural example of low-dose phenomena. In the human fetus, the adrenals produce androgens that are converted to estrogen by the enzyme aromatase, specifically in the placenta. In a human study designed to compare hormone levels in the amniotic fluid, maternal serum, and umbilical cord blood of singleton male and female fetuses, significant differences were observed in the concentrations of testosterone, androstenedione (A4), and estradiol (201). Specifically, amniotic fluid concentrations of testosterone and A4 were approximately twice as high in male fetuses, whereas estradiol concentrations were slightly, but significantly, higher in female fetuses. Yet, interestingly, there were no differences for any of the hormones in maternal serum, similar to findings in mice that litters with a high proportion of males or females did not impact testosterone, estradiol, or progesterone serum levels in mothers (180). In umbilical cord serum, concentrations of A4 and estradiol were higher in males compared with females (201), although it must be noted that these samples were collected at parturition, long after the fetal period of sexual differentiation of the reproductive organs.

Several studies have specifically compared steroid hormone levels in maternal and umbilical cord blood samples collected from same-sex and opposite-sex twins. Male twins, whether their co-twin was a male or a female, had higher blood concentrations of progesterone and testosterone compared with female twins (202). Furthermore, for both sexes, dizygotic twins had higher levels of these hormones, as well as estradiol, compared with monozygotic twins. Fetal sex had no effect on maternal concentrations of testosterone, progesterone, or estrogen, suggesting that any differences observed in fetal samples are due to contributions from the fetuses' own endocrine systems and the placental tissue (203). Yet an additional study conducted in women carrying multiple fetuses (more than three) indicates that both estradiol and progesterone concentrations in maternal plasma increase with the number of fetuses, and when fetal reduction occurs, these hormone levels remain elevated (204).

It has been proposed that low-dose effects seen in different intrauterine positions in litter-bearing animals could be an evolutionary adaptation, whereby the genotypes of the fetuses are relatively similar but a range of phenotypes can be produced via differential hormone exposures (136, 168). For example, female mice positioned between two females are more docile and thus have better

reproductive success when resources are plentiful, but females positioned between two males are more aggressive and therefore are more successful breeders under stressful conditions (161, 171, 175). In this way, a mother produces offspring with variable responses to environmental conditions, increasing the chances that her own genetic material will continue to be passed on. Yet although there is evidence to suggest that a variable intrauterine environment is essential for normal development (171), intrauterine positional effects appear to have little effect on offspring phenotypes in inbred rodent strains (168, 205). This result may be related to the link between genetic diversity and hormone sensitivity (206, 207), suggesting that outbred strains are the most appropriate for studying endocrine endpoints and are also most similar to the effects of low doses of hormones on human fetuses.

Finally, it has been proposed that similar mechanisms are used by the developing fetus in response to natural hormones via intrauterine position and EDCs with hormonal activity (136). To this end, several studies have examined the effects of both exposure to an EDC and intrauterine position or have considered the effect of intrauterine position on the response of animals to these chemicals (174, 176, 181, 208, 209). For example, one study found that intrauterine position affected the morphology of the fetal mammary gland, yet position-specific differences were obliterated by BPA exposure (176). Additional studies suggest that prostate morphology is disrupted by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure in males positioned between two females, but this chemical does not affect prostate morphology in males positioned between two males (181). Finally, male rodents positioned between two males have higher glucose intolerance than males positioned between two females, yet when these males are given a diet high in phytoestrogens, glucose tolerance is dramatically improved in the males positioned between two males, whereas their siblings positioned between two females do not benefit (209). What is clear from these studies is that low doses of natural hormones are capable of altering organ morphology, physiology, and reproductive development, similar to the effects of EDCs.

It has been suggested that the endocrine system allows for homeostatic control and that the aim of the endocrine system is to "maintain normal functions and development in the face of a constantly changing environment" (210). Yet studies from intrauterine position, together with studies of EDCs (see *Sections II.C–F*), clearly indicate that the fetal endocrine system cannot maintain a so-called homeostasis and is instead permanently affected by exposures to low doses of hormones.

II. Demonstrating Low-Dose Effects Using a WoE Approach

A. Use of a WoE approach in low-dose EDC studies

In 2001, the NTP acknowledged that there was evidence to support low-dose effects of DES, genistein, methoxychlor, and nonylphenol (2). Specifically, the NTP expert panel found that there was sufficient evidence for low-dose effects of DES on prostate size; genistein on brain sexual dimorphisms, male mammary gland development, and immune responses; methoxychlor on the immune system; and nonylphenol on brain sexual dimorphisms, thymus weight, estrous cyclicity, and immune responses. Using the NTP's definitions of low dose (*i.e.* effects occurring in the range of typical human exposures or occurring at doses lower than those typically used in standard testing protocols), we propose that most if not all EDCs are likely to have low-dose effects. Yet an important caveat of that statement is that low-dose effects are expected for particular endpoints depending on the endocrine activity of the EDC, and not for any/all endocrine-related endpoints. For example, if a chemical blocks the synthesis of a hormone, blood levels of the hormone are expected to decline, and the downstream effects should then be predicted from what is known about the health effects of low hormone levels. In contrast, if a chemical binds a hormone receptor, the effects are expected to be very complex and to be both tissue specific and dose specific. Finally, most EDCs interact with multiple hormone pathways, or even multiple hormone receptors, making the expected effects even more complex and context specific (211–213).

Table 3 summarizes a limited selection of chemicals that have evidence for low-dose effects, with a focus on *in vivo* animal studies. As seen by the results presented in this table, low-dose effects have been observed in chemicals from a number of classes with a wide range of uses including natural and synthetic hormones, insecticides, fungicides, herbicides, plastics, UV protection, and other industrial processes. Furthermore, low-dose effects have been observed in chemicals that target a number of endocrine endpoints including many that act as estrogens and antiandrogens as well as others that affect the metabolism, secretion, or synthesis of a number of hormones. It is also clear from this table that the cutoff for low-dose effects is not only chemical specific but also can be effect dependent. And finally, although this table is by no means comprehensive for all EDCs or even the low-dose effects of any particular chemical, the affected endpoints cover a large range of endocrine targets.

Several EDCs have been well studied, and the number of publications focusing on low-dose effects on a particular developmental endpoint is high; however, other

TABLE 3. EDCs with reported low-dose effects in animals (or humans, where stated)

Chemical	Use	EDC action	Low-dose cutoff	Affected endpoint	Refs.
Aroclor 1221 (PCB mixture)	Coolants, lubricants, paints, plastics	Mimics estrogens, antiestrogenic activity, *etc.*	0.1–1 mg/kg (produces human blood levels)	Brain sexual dimorphisms	683, 684
Atrazine	Herbicide	Increases aromatase expression	200 μg/liter (334, 335)	Male sexual differentiation/development	See this review
BPA	Plastics, thermal papers, epoxy resins	Binds ER, mER, ERRγ, PPARγ, may weakly bind TH receptor and AR	400 μg/kg · d (produces human blood concentrations)	Prostate, mammary gland, brain development and behavior, reproduction, immune system, metabolism	See this review
Chlordane	Insecticide	Binds ER	100 ng/g (produces human blood levels)	Sexually dimorphic behavior	685
Chlorothalonil	Fungicide, wood protectant	Aromatase inhibitor	164 μg/liter (environmental concentrations, EPA)	Corticosterone levels (amphibians)	686
Chlorpyrifos	Insecticide	Antiandrogenic	1 mg/kg · d (EPA)	Acetylcholine receptor binding (brain)	687
DDT	Insecticide	Binds ER	0.05 mg/kg (EPA)	Neurobehavior	688
DES	Synthetic hormone	Binds ER	0.3–1.3 mg/kg · d (dose typically administered to pregnant women)	Prostate weight	689
Dioxin (TCDD)	Industrial byproduct	Binds AhR	1 μg/kg · d (397)	Spermatogenesis, immune function and oxidative stress, tooth and bone development, female reproduction, mammary gland, behavior	See this review
Genistein	Phytoestrogen	Binds ER	50 mg/kg (EPA)	Brain sexual dimorphisms	690
Heptachlor	Insecticide	Induces testosterone hydroxylases	0.15 mg/kg · d (EPA)	Immune responses	691
Hexachlorobenzene	Fungicide	Modulates binding of ligand to TRE, weakly binds AhR	0.08 mg/kg · d (EPA)	Anxiety and aggressive behaviors	692
Maneb	Fungicide	Inhibits TSH release, may bind PPARγ	5 mg/kg · d (EU Commission)	Testosterone release	693
Methoxychlor	Insecticide	Binds ER	5 mg/kg · d (WHO)	Immune system	694, 695
4-Methylbenzylidine camphor	UV screen	Weakly estrogenic	10 mg/kg · d (Europa)	Sexual behavior	696
Methyl paraben	Preservative	Estrogenic	1000 mg/kg · d (EFSA)	Uterine tissue organization	697
Nicotine	Natural alkaloid in tobacco	Binds acetylcholine receptors, stimulates epinephrine	Human use of nicotine substitutes	Incidence of cryptorchidism (humans)	698
Nonylphenol	Detergents	Weakly estrogenic	15 mg/kg · d (EPA)	Testosterone metabolism	699
Octylphenol	Rubber bonding, surfactant	Weakly binds ER, RXR, PRGR	10 mg/kg · d (700)	Testes endpoints	701
Parathion	Insecticide		0.2 mg/kg · d (WHO)	Cognitive and emotional behaviors	702
PBDE-99	Flame retardant	Alters TH synthesis	0.3 mg/kg · d (EPA)	TH levels in blood	703
PCB180	Industrial lubricant, coolant	Impairs glutamate pathways, mimics estrogen	Examined normal human populations	Diabetes (humans)	704
PCB mixtures	Coolants, lubricants, paints, plastics	Binds AhR, mimic estrogens, antiestrogenic activity, etc.	Each at environmentally relevant levels	TH levels	705
Perchlorate	Fuel, fireworks	Blocks iodide uptake, alters TH	0.4 mg/kg · d (436)	TSH levels (humans)	See this review
Sodium fluoride	Water additive (to prevent dental caries), cleaning agent	Inhibits insulin secretion, PTH, TH	4 mg/liter water (EPA standard)	Bone mass and strength	706
Tributyltin oxide	Pesticide, wood preservation	Binds PPARγ	0.19 mg/kg · d (EPA)	Obesity	707
Triclosan	Antibacterial agent	Antithyroid effects, androgenic and estrogenic activity	12 mg/kg · d (Europe SCCP)	Altered uterine responses to ethinyl estradiol	708
Vinclozolin	Fungicide	Antiandrogenic	1.2 mg/kg · d (EPA)	Male fertility	709

EDC action indicates that for some chemicals, an effect is observed (*i.e.* estrogenic, androgenic), but for many EDCs, complete details of receptor binding are unavailable or incomplete. Low-dose cutoff means the lowest dose tested in traditional toxicology studies, or doses in the range of human exposure, depending on the data available. Affected endpoint means at least one example of an endpoint that shows significant effects below the low-dose cutoff dose. This list is not comprehensive, and the lack of an endpoint on this table does not suggest that low doses do or do not affect any other endpoints. AR, Androgen receptor; EFSA, European Food Safety Authority; ERR, estrogen related receptor; PCB, polychlorinated biphenyl; PPARγ, peroxisome proliferator-activated receptor-γ; PRGR, progesterone receptor; RXR, retinoid X receptor; SCCP, Scientific Committee on Consumer Products; TH, thyroid hormone; TRE, thyroid response element; WHO, World Health Organization.

chemicals are less well studied with fewer studies pointing to definitive low-dose effects on a given endpoint. In fact, there are a significant number of EDCs for which high-dose toxicology testing has been performed and the no observed adverse effect level (NOAEL) has been derived, but no animal studies in the low-dose range have been conducted, and several hundred additional EDCs where no significant high- or low-dose testing has been performed (see Table 4 for examples). Balancing the large amount of data collected from some well-studied chemicals like BPA and atrazine with the relative paucity of data about other chemicals is a difficult task.

TABLE 4. Select examples of EDCs whose potential low-dose effects on animals remain to be studied

Chemical	Use	EDC action	Low-dose cutoff
Antiseptics and preservatives			
Butyl paraben	Preservative (cosmetics)	Estrogenic, antiandrogenic	2 mg/kg · d (EPA)
Propyl paraben	Antimicrobial preservative found in pharmaceuticals, foods, cosmetics, and shampoos	Estrogenic activity	LOAEL 10 mg/kg · d, NOEL 6.5 mg/kg · d (Europa)
Cosmetics and personal care products			
2,4-Dihydroxybenzophenone	UV absorber in polymers, sunscreen agent	Estrogenic activity	Not identified
3-Benzylidene camphor	UV blocker used in personal care products	Estrogenic activity	0.07 mg/kg · d (710)
4,4′-Dihydroxybenzophenone	UV light stabilizer used in plastics, cosmetics, adhesives, and optical fiber	Estrogenic activity	Not identified
Benzophenone-2	Used in personal care products such as aftershave and fragrances	Estrogenic activity, changes in T_4, T_3, and TSH levels, alterations in cholesterol profile	NOEL 10–333 mg/kg · d (711)
Benzophenone-3	UV filter	Estrogenic, PPARγ activator	200 mg/kg · d (Europa)
Multiple use (other)			
Melamine	Flame-retardant additive and rust remover; used to make laminate, textile, and paper resins; metabolite of cyromazine	Affects voltage-gated K^+ and Na^+ channels and Ca^{2+} concentrations in hippocampal neurons	63.0 mg/kg · d (FDA)
Resorcinol	Used in the manufacturing of cosmetics, dyes, flame retardants, hair dye formulations, pharmaceuticals, skin creams, and tires	Alters T_4 and TSH levels	80.00 mg/kg · d (Europa)
Pesticides			
Aldrin[a]	Insecticide	Estrogenic activity	0.025 mg/kg · d (Health Canada)
Alachlor	Herbicide	Decreases serum T_4, binds PR, weakly binds ER	1 mg/kg · d (EPA)
Amitrole	Herbicide	Decreases thyroid hormone	0.12 mg/kg · d (FAO)
Bitertanol	Fungicide	Alters aromatase	30 mg/kg · d (EPA)
Carbendazim	Fungicide	Affects FSH, LH, and testosterone levels; alters spermatogenesis and Sertoli cell morphology	8 mg/kg · d (712)
Diazinon	Insecticide	Alters glucocorticoids	0.065 mg/kg · d (CDC)
Endrin[a]	Insecticide	Stimulates glucocorticoid receptor	0.025 mg/kg · d (CDC)
Fenoxycarb	Insecticide	Alters acetylcholinesterase	260 mg/kg · d (CDC)
Mirex[a]	Insecticide	Decreases testosterone levels	0.075 mg/kg · d (CDC)
Zineb	Fungicide	Alters T_4 and dopamine levels	LOAEL 25 mg/kg · d (EPA)
Ziram	Fungicide	Alters norepinephrine levels	1.6 mg/kg · d (EPA)
Resins			
Bisphenol F	Used in polycarbonates	Alters T_4, T_3, and adiponectin levels, has estrogenic activity	LOAEL 20 mg/kg · d (713)
Styrene	Precursor to polystyrene	Alters dopamine	200 mg/kg · d (EPA)

PPARγ, peroxisome proliferator-activated receptor-γ; PR, progesterone receptor.

[a] These chemicals were identified in the 1990s as part of the dirty dozen, 12 chemicals that were acknowledged to be the worst chemical offenders because of their persistence in the environment, their ability to accumulate through the food chain, and concerns about adverse effects of exposures to wildlife and humans. These chemicals were banned by the Stockholm convention and slated for virtual elimination. Yet there is still very little known about the low-dose effects of these chemicals, likely in the range of past and current human and/or wildlife exposures.

WoE approaches have been used in a large number of fields to determine whether the strength of many publications viewed as a whole can provide stronger conclusions than any single study examined alone. Although the term, weight of evidence, is used in public policy and the scientific literature, there is surprisingly little consensus about what this term means or how to characterize the concept (214). Historically, risk assessors have used qualitative

approaches (*i.e.* professional judgment to rank the value of different cases) and quantitative approaches (*i.e.* scoring methods to produce statistical and mathematical determinations of chemical safety), but it has been argued that these methods lack transparency and may produce findings that are unrepeatable from one risk assessor to another (215, 216). Whatever the method used, when EDCs are being assessed, it is important to use the principles of endocrinology to establish the criteria for a WoE approach. We do this in *Section II.B*, identifying three key criteria for determining whether a study reporting no effect should be incorporated into a WoE approach. It also should be noted that in epidemiology, the term, weight of evidence, is typically not used, but the concept is actuated by meta-analysis, formally and quantitatively combining data across studies, including a plot of individual and pooled study findings and also a measure of heterogeneity of findings between studies.

For some well-studied chemicals, there are large numbers of studies showing both significant effects, and additional studies showing no effects, from low-dose exposures. In these cases, extensive work is needed to deal with discordant data collected from various sources; studies showing no effect of low-dose exposures must be balanced in some way with those studies that do show effects. As stated by Basketter and colleagues (217), "it is unwise to make a definitive assessment from any single piece of information as no individual assay or other assessment . . . is 100% accurate on every occasion . . . This means that from time to time, one piece of conflicting data has to be set aside." WoE approaches in EDC research have typically dealt with datasets that have some conflicting studies, and these conflicts are even more difficult to sort out when studies have attempted to directly replicate published findings of adverse effects (see for example Refs. 218–221).

Most previously published WoE analyses have examined chemicals broadly (asking questions such as, "Does BPA produce consistent adverse effects on any endpoint?") (see Ref. 222). This can lead to problems including those encountered by the NTP expert panel, which found that there was some evidence for low-dose effects of BPA on certain endpoints but mixed findings for other endpoints. For example, the panel noted that some studies found low-dose effects of BPA on the prostate, but other studies could not replicate these findings. In *Section II.B*, we address criteria that are needed to accept those studies that are unable to detect low-dose effects of chemicals; these criteria were not used by the NTP in 2001, but they are essential to address controversies of this sort and perform WoE analyses using the best available data. In the sections that follow, we employed a WoE approach to examine the evidence for low-dose effects of single chemicals on selected endpoints or tissues, also paying attention to when in development the EDC in question were administered.

B. Refuting low-dose studies: criteria required for acceptance of studies that find no effect

Over the past decade, a variety of factors have been identified as features that influence the acceptance of low-dose studies (69, 71, 76, 77, 90, 205, 223, 224). In fact, the NTP low-dose panel itself suggested that factors such as strain differences, diet, caging and housing conditions, and seasonal variation can affect the ability to detect low-dose effects in controlled studies (2). In particular, three factors have been identified; when studies are unable to detect low-dose effects, these factors must be considered before coming to the conclusion that no such effects exist.

1. Negative controls confirm that the experimental system is free from contamination

Although all scientific experiments should include negative (untreated) controls, this treatment category is particularly important for EDC research. When a study fails to detect low-dose effects, the observed response in control animals should be compared with historical untreated controls; if the controls deviate significantly from typical controls in other studies, it may indicate that these animals were, in fact, treated or contaminated in some way or that the endpoint was not appropriately assessed (77, 205, 225). For example, if an experiment was designed to measure the effect of a chemical on uterine weight, and the control uteri have weights that are significantly higher than is normally observed in the same species and strain, these animals may have been inadvertently exposed to an estrogen source, or the uteri may not have been dissected properly by the experimenters. In either case, the study should be examined carefully and likely cannot be used to assess low-dose effects; of course, untreated controls should be monitored constantly because genetic drift and changes in diet and housing conditions can also influence these data, thus explaining changes from historical controls. Importantly, several types of contamination have been identified in studies of EDCs including the leaching of chemicals from caging or other environmental sources (226, 227), the use of pesticide-contaminated control sites for wildlife studies and contaminated controls in laboratory studies (76), and even the use of food that interferes with the effects of EDCs (224, 228). It is also important to note that experiments must consider the solvent used in the administration of their test chemical, and thus good negative controls should test for effects of the solvent itself. Using solvent negative controls helps prevent false posi-

tives as well as the possibility that the vehicle could mask the effects of the chemical being studied.

2. Positive controls indicate that the experimental system is capable of responding to low doses of a chemical acting on the same pathway

Many studies do not include a positive control, either because of the size and cost of the experiment when including an additional treatment or because an appropriate positive control has not been identified for the endpoint being examined. If the experiment detects an effect of the chemical in question, the exclusion of a positive control does not necessarily affect the interpretation of the results; instead, it can be appropriately concluded that the test chemical is significantly different from unexposed (but similarly handled/treated) negative controls. However, if the study fails to detect low-dose effects of a test chemical, no convincing conclusion can be made; in this case, a positive control is required to demonstrate that the experimental system was capable of detecting such effects (71, 75, 77, 205).

Several issues must be considered when addressing whether the positive control confirms the sensitivity of the assay. First, an appropriate chemical must be selected, and it must be administered via the appropriate route, *i.e.* if the test chemical is administered orally, a positive control that is orally active, such as ethinyl estradiol, should be used; if the test chemical is administered sc, a positive control that is active via this route, such as 17β-estradiol, is most appropriate. The use of 17β-estradiol in studies that use oral exposures is particularly inappropriate (see Ref. 229) for example) because this hormone, like most natural steroids, has very low oral activity (77). Second, the positive control chemical must be examined, and effective, at appropriately low doses. Thus, if the test chemical is 100 times less potent than the positive control, a dose of the positive control 100 times lower than the test compound must produce effects (69, 71, 205). For example, studies that report effects of ethinyl estradiol only at doses that are hundreds of times higher than the dose that is effective in contraceptives (230) are not capable of detecting low-dose effects of test chemicals. Without appropriate and concurrent positive and negative controls, studies that fail to detect low-dose effects of test chemicals should be rejected.

3. Species and animal strains that are responsive to EDCs must be used

The NTP expert panel specifically noted that "because of clear species and strain differences in sensitivity, animal-model selection should be based on responsiveness to endocrine-active agents of concern (*i.e.* responsive to positive controls), not on convenience and familiarity" (2). An analysis of the BPA literature clearly showed that many of the studies that failed to detect effects of low doses used the Charles River Sprague-Dawley rat (75); this strain was specifically bred to have large litters (231), and many generations of inbreeding have rendered the animal relatively insensitive to estrogens (205). The NTP expert panel noted the lack of effects of BPA on Sprague-Dawley rats and concluded that there were clear differences in strain sensitivity to this chemical (2). Importantly, this may not be true for Sprague-Dawley rats that originate from other vendors, indicating that animal origin can also influence EDC testing.

Many studies in mice (138, 206, 207, 232–234) and rats (232, 235–239) have described differences displayed between two (or more) animal strains to a natural hormone or EDC. Often these differences can be traced to whether a strain is inbred or outbred. Genetically diverse strains are generally found to be more sensitive to estrogens (206). Importantly, well-controlled studies demonstrate that strain differences in response to estrogen treatment may be organ dependent or may even differ between levels of tissue organization within the same organ. For example, the Sprague-Dawley rat is more sensitive to ethinyl estradiol than other strains when measured by uterine wet weight. However, when other endpoints were measured, *i.e.* height of cells in the uterine epithelium, the Sprague-Dawley rat was indistinguishable from the DA/Han rat; instead, the Wistar rat had the most heightened response (237). Additionally, there are data to indicate that strain differences for one estrogen may not be applicable for all estrogenic chemicals. In comparing the responses of DA/Han, Sprague-Dawley, and Wistar rats to other xenoestrogens, additional differences were observed including a greater increase in uterine wet weight of DA/Han and Sprague-Dawley rats but not Wistar rats after exposure to 200 mg/kg BPA; increased uterine epithelium thickness was observed in Wistar and Sprague-Dawley rats but not DA/Han rats after exposure to 200 mg/kg octylphenol (237). Attempts have been made, at times successfully, to map the differences in strain response to genetic loci (240). However, it appears that strains with differences in response that manifest in some organs do not have divergent responses in other organs, a phenomenon that is not explained by genetic differences alone. For these reasons, the NTP's recommendation that scientists use animals that are proven responsive to EDCs (2) must be observed.

4. Additional factors?

Additional factors have also been identified as influential in the ability (or inability) to detect low-dose effects in

EDC studies. Although these factors must be considered when interpreting studies and using a WoE approach, some issues that were previously identified as essential factors in the design of studies (*i.e.* route of administration) have more recently been disputed (241).

The first factor is the use of good laboratory practices (GLP) in the collection of data. When assessing the EDC literature for risk assessment purposes, the FDA and European Food Safety Authority (EFSA) have given special prominence to studies that complied with GLP guidelines, essentially giving scientific priority to industry-funded studies because that group typically conducts GLP guideline studies (33, 242). Because GLP guidelines are designed only to control data collection, standards for animal care, equipment, and facility maintenance, and they do not ensure that studies were designed properly with the appropriate controls, it has been argued that the use of GLP methods is not appropriate or required for EDC studies (69).

GLP studies are typically large, with dozens of animals studied for each endpoint and at each time point. Thus, it has been concluded that these studies are better simply because they are larger. Yet small studies designed with the use of power analysis, statistical tools that allow researchers to determine *a priori* the number of animals needed to determine significant differences based on effect size, are equally capable of detecting effects while reducing the number of animals used (69). GLP studies also typically (but not necessarily) rely upon standardized assays, which are not generally considered contemporary tools and are often shown to be incapable of detecting adverse effects on endpoints that employ modern tools from molecular genetics and related disciplines. Furthermore, some fields of EDC research have no GLP studies (243). Finally, there is no published evaluation of whether studies performed under GLP are more capable of providing accurate results. The priority given to GLP studies therefore does not appear to have been justified based on any comparative analysis. Thus, as long as studies include appropriate measures of quality assurance, they need not be performed under GLP standards to provide reliable and valuable information, and many GLP studies are inadequate to assess important and relevant endpoints. Instead, the most valuable studies consider the factors presented above, along with appropriate dose selections and choice of endpoint.

The second factor worth considering is the source of funding for studies. In several fields, significant controversy has been produced based on the results obtained from independent scientists compared with results obtained from scientists affiliated with the chemical industry (75, 76). Funding source *per se* should not dictate the outcome of a research study, but that does not mean that researchers are not subject to underlying biases. In our own WoE analyses, presented in *Sections II.C–G*, we do not discount studies merely because they were conducted with industry funds, nor do we lend higher weight to studies conducted in independent or government laboratories; if a study, regardless of funding, finds no effect of a chemical, it is given weight only if the three criteria described in *Sections II.B.1–3* (successful and appropriate negative and positive controls and appropriate choice of animal model) were met.

To perform a WoE evaluation, we identified some basic information about the chemical in question, the dose that would be considered a low-dose cutoff, and the studies in support of and against low-dose effects. We then considered whether the majority of studies found effects of low doses of a chemical on a single endpoint in question. If studies did not find low-dose effects, we considered whether they adhered to the criteria discussed above for proper design of an EDC low-dose study. In particular, we considered whether appropriate animal strains as well as positive and negative controls were used. With regard to animal strain, as discussed briefly in *Section II.B.3*, there is variability between animal strains that can significantly influence the ability to detect effects of EDCs; using insensitive strains to produce negative data cannot refute positive data in a sensitive strain. In several cases, it was easy to conclude that there was a strong case for low-dose effects because there were no studies finding no effects at low doses or because all of the negative studies were inappropriately designed. For other chemicals, a significant number of studies found effects on the endpoint being considered, but other (adequately designed) studies refuted those findings. Under those circumstances, we determined whether the findings of harmful effects came from multiple laboratories; when they did, we cautiously concluded that there was evidence for low-dose effects. Below (*Sections II.C–G*), we present five examples where a significant number of studies were available examining low-dose effects of an EDC on a single particular endpoint.

C. BPA and the prostate: contested effects at low doses?

As discussed briefly above, BPA is one of the best-studied EDCs, with more than 200 published animal studies, many of which focused on low doses (29, 31). The effects of this chemical on wildlife species have also been described in detail (28). BPA is found in a myriad of consumer products, and it leaches from these items under normal conditions of use (4). It has also been regularly detected in air, water, and dust samples. The majority of individuals in industrialized countries have BPA metabolites in their urine, and trends indicate increasing expo-

sures in developing nations like China (87, 244). Although it was long suspected that most human exposures originate from BPA contamination of food and beverages, a study comparing the excretion of BPA metabolites with the length of time spent fasting suggests that there are also likely to be significant exposures from sources other than food and beverages (245). BPA has recently been shown to be used in large quantities in thermal and recycled papers and can enter the skin easily via dermal absorption (246–248). Thus, despite the large amount of information available on BPA sources, our understanding of how these sources contribute to total human exposures remains poor; these studies also point to significant gaps in current knowledge about BPA metabolism in humans (243).

BPA binds to the nuclear and membrane ER, and thus most of the effects of this chemical have been attributed to its estrogenic activity (27). However, there is evidence that it can activate a number of additional pathways, including thyroid hormone receptor, androgen receptor, as well as peroxisome proliferator-activated receptor-γ signaling pathways (249–252). The cutoff for a low dose has been set at several different concentrations depending on which studies and definitions are used (see Table 1). The EPA calculated a reference dose for BPA of 50 μg/kg · d based on a LOAEL of 50 mg/kg · d (38). More recent pharmacokinetic scaling experiments have estimated that exposures to approximately 400 μg/kg · d produce blood concentrations of unconjugated BPA in the range of human blood concentrations (4). Thus, for the two WoE analyses of the BPA literature we conducted, doses of 400 μg/kg · d or lower were considered low dose; pharmacokinetic studies from nonhuman primates support the appropriateness of this dose for approximating human exposure levels (253). Furthermore, because this dose is below the toxicological LOAEL, it is a conservative cutoff for low-dose studies (see Refs. 3 and 38 and Table 1).

One of the most well studied and hotly debated examples of a low-dose effect comes from the BPA literature; regulatory agencies and scientists have addressed several times whether low doses of BPA during fetal and perinatal development affect the rodent prostate (118, 205, 254, 255). In 1997, the first study on BPA and the prostate determined that fetal exposure to low doses (2 and 20 μg/kg · d administered orally to pregnant mice) increased the weight of the adult prostate compared with unexposed male offspring (256). Since that time, several additional studies have verified that prostate weight is affected by fetal exposure to similar low doses (257–259). Studies have also shown that low doses of BPA affect androgen receptor binding activity in the prostate (257), tissue organization, and cytokeratin expression in the gland (260–262) as well as the volume of the prostate and the number and size of dorsolateral prostate ducts (208). Several recent studies have also examined whether low doses of BPA (10 μg/kg · d) influence the incidence of adult-onset prostatic intraepithelial neoplasia (PIN) lesions. Perinatal BPA exposure, whether administered orally or sc to pups, increases the incidence of PIN lesions in response to a mixture of testosterone and estradiol in adulthood (139, 141, 263); this hormonal cocktail was designed to mimic the endocrine changes associated with aging in men that also typically accompany the onset of prostate cancer. In addition to the effects of BPA on PIN lesions, these low doses also produced permanent alterations in the epigenome of exposed males, with prostates displaying completely unmethylated sequences in genes that are hypermethylated in unexposed controls (140, 263). In examining these studies, although the same effects of BPA on the prostate were not observed in all studies, there is an obvious trend demonstrating that low doses of BPA during early development significantly affect several aspects of prostate development.

Since the initial report showing effects of low doses on the prostate, approximately nine studies, including several designed specifically to replicate the original positive study, have shown no effects of low doses on the prostate (264–272); every one of these studies examined the prostate weight, and Ichihara *et al.* (264) also examined the effects of BPA on PIN lesions (without hormonal treatment) and the response of the prostate to a chemical carcinogen. Three of these studies failed to include a positive control of any kind (264, 268, 270); three studies used DES as a positive control but found no effect from exposure to this potent xenoestrogen (265–267) (*i.e.* the positive control failed); another study used 17β-estradiol as a positive control, inappropriately administered orally, and found no effects of this hormone on the prostate (271); and two studies used an estrogenic positive control (ethinyl estradiol) and found effects from its exposure, but only at inappropriately high doses (269, 272). These two studies clearly showed that the positive control dose was too high, because rather than increase the weight of the prostate (as seen after low doses of estrogens in other studies), the positive control decreased the weight of the adult prostate (269, 272).

Although this topic was once considered controversial, using a WoE approach, it is clear that there is strong evidence in support of low-dose effects of BPA on the development of the prostate. The evidence clearly shows that several endpoints, including prostate weight, were affected in similar ways in multiple studies from several different labs at doses below 400 μg/kg · d; most effects were seen at doses below 50 μg/kg · d. Furthermore, PIN lesions were reported after neonatal exposure to 10 μg/kg · d with

hormonal treatment in adulthood. No appropriately conducted studies contest this evidence. Therefore, the WoE analysis demonstrates that low doses of BPA significantly alter development of the rodent prostate. The NTP's review of the BPA literature in 2008 indicated that this agency agrees that there is now significant evidence that low-dose BPA adversely affects development of the prostate (273).

D. BPA and the mammary gland: undisputed evidence for low-dose effects

The mammary gland is a conspicuous choice to examine the effects of estrogenic compounds because this organ depends on estrogen for proper development at several critical periods in life (274). The fetal gland expresses ER in the mesenchymal compartment, and just before birth, the epithelium becomes ER positive as well (275). At puberty, estrogen is responsible for ductal elongation and overall development of the gland, allowing the epithelium to fill the stromal compartment in preparation for pregnancy and lactation. Although BPA is an example of a chemical that has been classified as a weak estrogen because it binds with a much lower affinity to ERα compared with 17β-estradiol, even weak estrogens are known to affect the development of the mammary gland during early development (276).

In the first study to examine the effects of BPA on the mammary gland, prepubertal rats were exposed to relatively high doses (100 μg/kg · d or 54 mg/kg · d) for 11 d. After even this short exposure, mammary gland architecture was affected in both dose groups, with increased numbers of epithelial structures and, in particular, structures that suggest advanced development (277). BPA exposure also altered proliferation rates of mammary epithelium and cell cycle kinetics, with an increased number of cells in S-phase and a decreased number of cells in G1. Although relatively high doses of BPA were examined, this initial study indicated that the prepubertal and pubertal gland could be sensitive to BPA.

Many additional studies have examined another critical period, the fetal and neonatal periods, which are sensitive to environmental estrogens (78, 276, 278). Mice exposed prenatally to low doses of BPA via maternal treatment (0.25 μg/kg · d) displayed altered development of both the stromal and epithelial compartments at embryonic d 18, suggesting that exposures affect tissue organization during the period of exposure (176). In addition, similar low doses produced alterations in tissue organization observed in puberty and throughout adulthood, long after exposures ended, and even induced pregnancy-like phenotypes in virgin females (137, 279–282). Female mice exposed to BPA *in utero* displayed heightened responses to estradiol at puberty, with altered morphology of their glands compared with animals exposed to vehicle *in utero* (138). Another study demonstrated that perinatal BPA exposure altered the mammary gland's response to progesterone (283). Remarkably, all of these effects were observed after maternal exposures to low doses (0.025–250 μg/kg), suggesting that the gland is extremely sensitive to xenoestrogen exposures. These studies are in contrast to one that examined the effects of higher doses (0.5 and 10 mg/kg · d) when BPA was administered for 4 d to the dam, which reported advanced development of BPA-exposed glands before puberty but no effects in adulthood (284).

Adult exposure to BPA is only now being examined in the mouse mammary gland model. A recent study examined the effects of BPA on mice with mutations in the *BRCA1* gene. This study reported that 4 wks of exposure to a low dose of BPA altered the tissue organization of the mammary gland in ways that are similar to the effects observed after perinatal exposure (285). This study focused on altered development of the gland during exposure; additional studies are needed to determine whether these effects are permanent or whether normal mammary morphology could be achieved by cessation of BPA exposure.

Another obvious endpoint is the effect of BPA exposure on mammary cancer incidence. Several studies indicate that exposure to BPA *in utero* produces preneoplastic (281, 286, 287) and neoplastic lesions (286) in the gland in the absence of any other treatment. Additionally, other studies show that females exposed to BPA during the perinatal period are more sensitive to mammary carcinogens, decreasing tumor latency and increasing tumor incidence (287–290). These studies are also supported by subsequent studies examining gene and protein expression, which show that low-dose BPA specifically up-regulates expression of genes related to immune function, cell proliferation, cytoskeletal function, and estrogen signaling and down-regulates apoptotic genes (282, 288, 289, 291).

Postnatal BPA exposures also influence mammary cancer incidence; animals exposed lactationally to BPA from postnatal d 2 until weaning displayed decreased tumor latency and increased tumor multiplicity after treatment with DMBA [7,12-dimethylbenz(a)anthracene], a carcinogen (292). This study suggested that BPA exposure led to increased cell proliferation and decreased apoptosis in the gland and shifted the period where the gland is most susceptible to mammary carcinogens, a result that has important implications for human breast cancer. Finally, an additional study examined the effects of adult BPA exposure on mammary cancer; this study demonstrated that low doses of BPA accelerate the appearance of mammary tumors in a tumor-prone mouse strain (293). Interestingly,

high doses did not have this effect; thus, this study is also an excellent example of a NMDRC.

Two studies of BPA and the mammary gland seem to contradict this body of literature, but both examined extremely high doses. In the first study, Nikaido *et al.* (294) exposed female mice to 10 mg/kg BPA from postnatal d 15–18. Mammary glands from these animals were examined at 4, 8, and 24 wk of age, and no differences were observed in the exposed animals relative to controls. Although the lack of effects reported in this study could be due to the high dose employed, they could also be related to the relatively short exposure period during the preweaning phase. In the second study, Yin and colleagues (295) examined the effects of BPA during the first few days after birth (0.1 or 10 mg BPA, equivalent to approximately 10 and 1000 mg/kg) on the incidence of mammary tumors after exposure to a mammary carcinogen at puberty. Similar to the study described above, this one also examined the effects of BPA after a relatively short period of exposure (only three injections administered between postnatal d 2 and 6). Although the study showed that BPA affected tissue organization, there was no change in the incidence of tumors in BPA-exposed females. Because both of these studies examined both high doses and relatively short periods of exposure, it is difficult to compare them directly to the studies finding effects of BPA on the mammary gland after longer exposures to lower doses; at the very least, they cannot refute studies suggesting that BPA alters development of this gland.

In summary, the WoE clearly shows that low-dose BPA exposure affects development of the mammary gland, mammary histogenesis, gene and protein expression in the gland, and the development of mammary cancers. In fact, this example of low-dose effects produced remarkably similar effects across more than a dozen studies conducted in several different labs. These results are also consistent with the effects of low-dose BPA exposure on mammary epithelial cells in culture (reviewed in Ref. 30). Although epidemiology studies examining the influence of BPA on breast cancer rates have proven to be inconclusive at best (296), to replicate the animal studies discussed above, epidemiologists must collect information about prenatal and neonatal exposures and relate them to adult breast cancer incidence. These types of studies would take decades to conduct (67) and should take into consideration the effects of other estrogens, because their effects can be additive or even synergistic (143, 144, 297).

Although our analyses of BPA have focused on its effects on the mammary gland and prostate (see *Sections II.C–D*), it is worth noting that several other endpoints have strong data to support the hypothesis that BPA has low-dose effects. In a recent review using similar WoE approaches, Hunt and colleagues (298) focused on those studies that examined the effects of BPA on the oocyte, specifically scrutinizing studies that reported effects, or no effects, on meiotic aneuploidy and other alterations in the intracellular organization and chromosome abnormalities. Similar to what has been observed with the prostate and mammary gland, the effects observed in the oocyte are variable from study to study, but overall consistent, and suggest that BPA exposure produces defects in these cells.

A large number of studies have also focused on the effects of BPA on the brain and behavior, with the most significant effects on sexually dimorphic regions of the brain and behaviors (299–307). Other affected behaviors include social behaviors, learning and anxiety, and maternal-neonate interactions (reviewed in Refs. 29 and 308). The NTP expert panel statement concluded that there were significant trends in these behavioral data and wrote that there was some concern that BPA could have similar effects in humans (273). Low-dose effects have also been reported for BPA in the female reproductive tract (309, 310), immune system (311, 312), maintenance of body weight and metabolism (313, 314), fertility (315–317), and the male reproductive tract (259, 318) (see Refs. 29 and 319 for comprehensive reviews).

E. Another controversial low-dose example: atrazine and amphibian sexual development

Atrazine is an herbicide that is applied in large volumes to crops, and there is concern that agricultural runoff of this chemical can affect nontarget animal species, especially amphibians that live and reproduce in small ponds and streams where significant amounts of atrazine have been regularly measured (320–322). It is the most commonly detected pesticide in ground and drinking water. Atrazine induces aromatase expression in cells and animals after exposure (323); this ultimately causes an increase in the conversion of testosterone to estrogen (324, 325). This effect has been reported in all vertebrate classes examined: fish, amphibians, reptiles, birds, and mammals, including human cell lines (see Ref. 326 for review). Another well-documented effect of atrazine is that it decreases androgen synthesis and activity, again, in every vertebrate class examined (326). In addition, endocrine-disrupting effects of atrazine occur through a number of other mechanisms, including antiestrogenic activity (327), altered prolactin release (328), and increased glucocorticoid release from the adrenal glands (329, 330), among others (327).

Because of atrazine's indirect effect on estrogen levels, one relevant endpoint that has been given attention is the effect of this chemical on gonad differentiation in various amphibian species. The early gonad is bipotential, and in

mammals, the expression of genes on the Y-chromosome is needed to masculinize the undifferentiated gonad; when this does not occur, the gonad develops into ovarian tissue. In *Xenopus laevis* frogs (and some other animals like birds), the opposite is true: females are heterogametic (*i.e.* ZW-chromosomes) and males have two of the same chromosomes (*i.e.* ZZ). In *X. laevis*, the W-chromosome is the dominant one, containing a gene, DM-W, which induces aromatase expression (331). Thus, having a W-chromosome is needed to produce estrogen; without the conversion of testosterone to estrogen, the frog develops as a male (332). Changes in sex ratio and gonadal morphology are therefore good indicators that an estrogen, or a chemical that up-regulates aromatase and indirectly increases estrogen levels, is present (76).

Determining a low-dose cutoff for atrazine is not a simple task. Although the safe limit of 3 μg/liter in drinking water was set by the EPA, actual levels in the environment often exceed this concentration (333), and levels in ponds and streams can reach 100 μg/liter (322) or more. In traditional toxicology studies examining several amphibian species, the LOAEL was set at 1.1 mg/liter, and the no observed effect level (NOEL) was 200 μg/liter (334, 335). Thus, using the definitions of low dose established by the NTP (2), we consider any treatment at or below 200 μg/liter to be a low dose.

In 2002, one of the first published studies to connect atrazine exposures to altered gonadal morphology examined *X. laevis* frogs exposed to 0.01–200 μg/liter throughout larval development (336). All doses from 0.1–200 μg/liter produced gonadal malformations including the presence of multiple gonads and hermaphroditism. Several other reports showed similar effects of low doses on gonadal phenotypes including studies that report the production of hermaphrodites and intersex frogs, males with ovotestes, and males with testicular oocytes (337–343). Additional studies showed that low-dose atrazine exposure (0.1–200 μg/liter in the water) during sexual differentiation caused testicular dysgenesis, testicular resorption, and testicular aplasia in male frogs (343, 344), and others indicated effects on sex ratios (339, 342, 345, 346). Importantly, these effects were not all observed at the same atrazine concentration, and the studies were conducted in several different species, with some reporting effects at low doses but no effects at higher doses (341) and others reporting effects in some but not all species (339). Examining these studies as a whole, there is clearly a pattern of effects that are reproducible from study to study, and they collectively support the hypothesis that atrazine disrupts sex hormone concentrations.

To date, five peer-reviewed studies have reported no effects of atrazine on sex ratios, gonadal morphology, the incidence of testicular abnormalities or testicular oocytes, gonad size, or the incidence of intersex phenotypes (347–351). Little can be ascertained from these negative studies, however, because four did not include any positive control, suggesting that the frogs used in those studies may have been incapable of responding to atrazine or any other hormonal treatment (347–350). Additionally, one of those studies reported testicular oocytes in the control frogs, suggesting either that the negative control population was contaminated with atrazine (or another EDC or hormone), or that an inappropriate strain of *X. laevis* was selected for the experiments (347). Only one study remains that did not find any effects of atrazine; this study used an appropriate positive control (17β-estradiol) and found effects of that hormone on sex ratios and the incidence of intersex gonads (351). An EPA expert panel noted, however, that this study used a strain of *X. laevis* that was obtained from a new, unexamined population of frogs from Chile and suggested that this strain may be insensitive to environmental chemicals. Furthermore, the panel called for additional analysis of the data in this study, including the statistical approaches; they suggested that an independent laboratory should evaluate the histopathological results; and they requested that atrazine metabolites be measured (352). The panel also proposed that these experiments should be repeated with an established *X. laevis* strain. Taking together the results of those studies that found effects of atrazine on sexual differentiation, and this one negative study, the WoE for the case of low-dose atrazine on sexual differentiation is clearly in support of adverse effects of this chemical.

Just as epidemiological studies have found links between EDCs and human diseases, ecological field studies have examined whether exposure to atrazine in natural environments affects the development of wild amphibians (343, 353–358). These studies have many of the same constraints as those observed in epidemiology: a paucity of data on early life exposures (including exposure levels of controls), limitations on the total number of EDCs that can be measured in environmental and biological samples, and a lack of causative relationships that can be established between exposures and effects. For these reasons, studies that found relationships between atrazine exposure (or concentrations in environmental samples) and effects on one or more aspect of sexual differentiation (343, 353–355) are considered weak, but significant, evidence for low-dose effects. The presence of several studies suggesting a relationship between low-dose exposure to atrazine in the wild and altered sexual differentiation indicates a plausible causal relationship. Because the ecological and laboratory data show similar effects of atrazine on go-

nadal development, this strengthens the conclusions of our WoE that low doses of atrazine cause harm to amphibians.

Feminization of males after atrazine exposure is not restricted to amphibians; exposure of zebrafish to low doses increased the ratio of female to male fish and increased expression of aromatase (359). Close to a dozen additional studies also report that environmentally relevant doses of atrazine can up-regulate aromatase, decrease testosterone, and/or increase estrogen levels in a large number of species (reviewed in Ref. 119), suggesting that low-dose effects of atrazine may be more widespread than their effects on the gonads of amphibians. Other studies indicate that low-dose atrazine affects the immune system and stress responses of salamanders (360–362), survivorship patterns of several frog species (363), and thyroid hormone and plasma ion concentrations in salmon (364).

An important factor to consider when examining the effects of atrazine on different animal models is the difficulty in identifying an appropriate low, environmentally relevant dose for all species. Aquatic animals can be housed in water containing levels of atrazine found in wild habitats, yet no toxicokinetic studies are available to determine what administered dose produces the levels of atrazine metabolites, typically in the parts-per-million or ppb range (365, 366), measured in human samples. There are also no blood or urine measurements in exposed rodents to compare with human levels; thus, extrapolations across species are estimates at best.

Keeping this qualification in mind, exposures in the range of 25–100 mg/kg · d during development have been shown to alter mammary gland development (367, 368), estrous cyclicity (369), serum and intratesticular testosterone concentrations (370), timing of puberty in males and prostate weight (371), and immune function (372) in rodents. Lower doses of atrazine metabolites (0.09–8.73 mg/kg · d) altered development of the mammary gland (373), male pubertal timing and prostate development (374). Identifying the range of doses administered to animals that produce the levels of atrazine and its metabolites measured in human blood and urine is an essential research need to pursue low-dose studies in rodents and other mammals.

F. Dioxin and spermatogenesis: low-dose effects from the most potent endocrine disruptor?

Dioxin, or TCDD, is formed as a byproduct of industrial processes as well as during waste incineration. Because TCDD is extremely toxic to some animals, with 1 μg/kg capable of killing 50% of guinea pigs, it has been labeled the most toxic chemical on earth (375). But interestingly, other animals are less sensitive to lethal effects of TCDD, with an LD_{50} of approximately 1000 μg/kg in hamsters, and studies also suggest that humans are not a hypersensitive species for lethality (376). Additionally, there are differences in the half-life of TCDD in different animals; in rodents, the half-life is 2–4 wks, but in humans, the half-life is approximately 10 yrs, and additional factors influence TCDD pharmacokinetics including the exposure level and the amount of body fat present (377–379). In cell cultures, doses as low as 10^{-11} M are toxic, with decreased viability observed even in cells maintained in nonproliferative states (380).

TCDD binds to the aryl hydrocarbon receptor (AhR), and differences in the affinity for the receptor may be responsible for differences in sensitivity between species (381). The K_d (dissociation constant for receptor-ligand binding kinetics) in human samples typically ranges from 3–15 nM, but in samples from rodents, the K_d is less than 1 nM (382). Importantly, there are also nongenomic pathways affected by TCDD that are mediated by AhR that are typically altered within minutes of TCDD exposure and therefore without changes in transcription (383). Yet many studies suggest that important differences exist between species regarding binding affinity of TCDD for AhR and the toxicity of this chemical, but that other adverse effects, including those related to the endocrine-disrupting activities of TCDD, occur at similar doses (or body burdens) across animal species (384, 385). Thus, it is plausible that AhR affinity alone can predict some, but not all, effects of TCDD and related chemicals.

The mechanisms responsible for many of the endocrine-disrupting activities of TCDD are currently not well understood. Knocking out AhR disrupts morphogenesis of several organ systems even in the absence of a ligand like TCDD, suggesting that this receptor plays important roles in early development (386). AhR is translocated to the nucleus after loss of cell-cell contacts and is often localized to the nucleus in embryonic cells, suggesting that it could have ligand-independent effects on development and/or that endogenous ligands could be present during early development (387). When TCDD is present, AhR translocates to the nucleus and dimerizes with ARNT, the aromatic hydrocarbon receptor nuclear translocator (388). Although the (currently unidentified) physiological activators of AhR are likely to induce rapid on/off signaling via AhR, TCDD and related compounds appear to maintain activation of AhR, and the presence of TCDD prevents the normal action of the AhR signaling pathway in the maintenance of homeostasis (389). This induces changes in the expression of genes and promotes the production of toxic metabolites. These effects may be responsible for some of the endocrine-related endpoints affected by TCDD exposure. Additionally, recent studies have shown complex and intricate interactions between the

AhR and ER signaling pathways (390), suggesting that dioxin may also have indirect effects on some ER-mediated endpoints via AhR signaling.

Teratogenic effects of TCDD have been well documented after high-dose (391, 392) and low-dose exposures (393). These studies show that almost every organ and system in the body is affected by this chemical. High doses that did not produce lethality caused severe weight loss, intestinal hemorrhaging, alopecia, chloracne, edemas, and severe liver damage. Sadly, there are now several examples in humans of accidental exposures after the industrial release of TCDD where a number of individuals have been exposed to large doses (389, 394) as well as a few documented intentional poisonings (395). The tolerated daily intake level was set at 1–4 pg/kg · d, although the doses consumed by nursing infants are likely to exceed these levels by a factor of 10 (375). Adult exposures usually result from the consumption of contaminated foods, and because TCDD is lipophilic, it is concentrated in the fat component of breast milk and therefore passed in large quantities from a nursing mother to her infant.

Using classical toxicology methods, the effects of single TCDD doses were examined in adult male rats, specifically focusing on the effects of this chemical on the number of spermatids per testis and the integrity of the testicular germinal epithelium (396). In one of the earliest studies, Chahoud and colleagues (397) determined a LOAEL of 3 μg/kg · d and set the NOAEL at 1 μg/kg · d for effects on the testes. Because there are significant differences in the toxicity of TCDD between animal models, and different endpoints have different identified NOAELs, we have selected the 1 μg/kg · d identified by Chahoud *et al.* as the cutoff for low-dose studies of this compound. This cutoff is based on the NTP's definition of low dose as occurring at doses lower than those tested in traditional toxicology assessments (2). However, it is important to acknowledge that body burdens that mimic those observed in human populations are likely the best indicators of low doses for TCDD (384), and thus we recommend that future studies determine body burdens after administration of TCDD for the specific strain, origin, and species of animal being tested to ensure that truly low doses, relevant to human populations, are being tested.

Several recent epidemiological studies have indicated that relatively high exposures to TCDD during early life (due to industrial release of high amounts of the chemical) can permanently affect semen quality and sperm count in men (398). Yet epidemiology studies also clearly show that the timing of TCDD exposure can vastly influence the effect of this chemical on spermatogenesis; exposures during perinatal life significantly reduced sperm parameters, but exposures during puberty increased sperm counts; exposures in adulthood had no effect on sperm parameters (399). Thus, it is also important for animal studies to focus on exposures during critical periods for development of the male reproductive tract and spermatogenesis in particular.

We are aware of 18 studies that have examined the effects of low doses ($\leq$1 μg/kg · d) of TCDD during perinatal development on male fertility endpoints in adulthood. The endpoints assessed vary, including epididymal sperm counts, ejaculated sperm number, daily sperm production, sperm transit rate, and percent abnormal sperm, and the sensitivity of these endpoints appears to impact the ability to detect low-dose effects in different studies (400, 401) (Table 5). In total, 16 rodent studies examined the effect of low-dose TCDD on epididymal sperm count; 12 showed significant effects on this endpoint (402–413), whereas the other four did not (414–417). Of the five studies that examined ejaculated sperm counts, four studies (404, 405, 408), including one examining rhesus monkeys (418), showed effects of low-dose TCDD, *i.e.* a significant decrease in sperm counts; one study found no effect (417). Daily sperm production was a less-sensitive endpoint, with four studies showing significant decreases after prenatal exposure to low doses (402, 403, 407, 409) and four studies showing no effects (406, 412, 413, 416); sperm transit rate was examined in only two studies, although both showed significant decreases in sperm tranfer rates (403, 410); and finally, three studies determined that low-dose TCDD produced abnormalities in sperm appearance or motility (414, 415, 419), but one study was not able to replicate these findings (417).

When examining the TCDD literature as a whole, the WoE strongly suggests that prenatal exposure to low doses of TCDD affects sperm-related endpoints in adulthood (Table 5). In all, only two studies were unable to detect any effect of TCDD on the sperm endpoints assessed, although both studies found effects of TCDD on other endpoints including the weight of the adult prostate (416) and the timing of puberty (417). No study on TCDD used a positive control, likely due to a paucity of information on the mechanisms of dioxin action, but this raises obvious questions about the ability of these experimental systems to detect effects on spermatogenesis. Finally, some of the inability to detect effects of TCDD could be due to the use of insensitive strains, because 1000-fold differences in sensitivity have been reported for different rodent strains (420).

Even though we have focused the majority of our attention on the effects of low-dose TCDD exposure on spermatogenesis, it should be noted that low doses of this chemical affect a multitude of endpoints in animals, altering immune function (421, 422), indicators of oxidative

TABLE 5. Summary of low-dose animal studies examining the effects of TCDD on spermatogenesis endpoints

Study	Administered dose (time of administration)	Animal	Epididymal sperm count	Ejaculated sperm no.	Daily sperm production	Sperm transit rate	% abnormal sperm
Mably *et al.* (409)	0.064–1 μg/kg (gestational d 15)	Rat	Decreased	NA	Decreased	NA	NA
Bjerke and Peterson (402)	1 μg/kg (gestational d 15)	Rat	Decreased	NA	Decreased	NA	NA
Gray *et al.* (404)	1 μg/kg (gestational d 8)	Rat	Not significant	Decreased	NA	NA	NA
	1 μg/kg (gestational d 15)	Rat	Decreased	Decreased	NA	NA	NA
	1 μg/kg (gestational d 11)	Hamster	Decreased	Decreased	NA	NA	NA
Sommer *et al.* (408)	1 μg/kg (gestational d 15)	Rat	Decreased	Decreased	Decreased	Not significant	Not significant
Wilker *et al.* (410)	0.5, 1 or 2 μg/kg (gestational d 15)	Rat	Decreased	NA	Unaffected	Increased	NA
Gray *et al.* (405)	0.05–1 μg/kg (gestational d 15)	Rat	Decreased	Decreased	Decreased	NA	NA
Faqi *et al.* (403)	0.025–0.3 μg/kg (before mating, then 0.005–0.06 μg/kg weekly [to dams])	Rat	Decreased	NA	Decreased	Increased	Increased
Loeffler and Peterson (412)	0.25 μg/kg (gestational d 15)	Rat	Decreased	NA	Unaffected	NA	NA
Ohsako *et al.* (416)	0.0125–0.8 μg/kg (gestational d 15)	Rat	Not significant	NA	Unaffected	NA	NA
Ohsako *et al.* (406)	1 μg/kg (gestational d 15)	Rat	Decreased	NA	Unaffected	NA	NA
	1 μg/kg/gestational d 18	Rat	Unaffected	NA	Unaffected	NA	NA
	1 μg/kg/postnatal d 2 (to pups)	Rat	Unaffected	NA	Unaffected	NA	NA
Simanainen *et al.* (407)	0.03–1 μg/kg (gestational d 15)	Rat	Decreased	NA	Decreased	NA	NA
Yonemoto *et al.* (417)	0.0125–0.8 μg/kg (gestational d 15)	Rat	Unaffected	Unaffected	NA	NA	Unaffected
Yamano *et al.* (714)	0.3 or 1 μg/kg (postnatal d 1 and then every week [to dams])	Rat	Not significant	NA	NA	NA	NA
Ikeda *et al.* (715)	0.4 μg/kg (before mating, then 0.08 μg/kg weekly [to dams])	Rat	Unaffected	NA	NA	NA	NA
Bell *et al.* (414)	0.05–1 μg/kg (gestational d 15)	Rat	Increased (at certain ages)	NA	NA	NA	Increased
Bell *et al.* (415)	0.0024–0.046 μg/kg (d 12 weeks before pregnancy through parturition)	Rat	Unaffected	NA	NA	NA	Increased
Arima *et al.* (418)	0.03 or 0.3 μg/kg (gestational d 20, then 5% of dose monthly [to dams])	Rhesus monkey	Decreased	Not significant	NA	NA	Not significant
Yamano *et al.* (419)	0.3 or 1 μg/kg (weekly to dams then pups [all postnatal])	Rat	NA	NA	NA	NA	Increased
Jin *et al.* (411)	1 μg/kg · d (postnatal days 1–4 [to dams])	Mouse	Decreased	NA	NA	NA	NA
Rebourcet *et al.* (413)	0.01–0.2 μg/kg (gestational d 15)	Rat	Decreased (at some ages)	NA	Not significant	NA	NA

Not significant indicates trend for effect but did not reach statistical significance. Unaffected means assessed, but no differences were observed relative to controls. Here, low doses were considered any at or below 1 μg/kg · d (see text for discussion of how this cutoff was established for rodent studies). NA, Not assessed.

stress (423–425), bone and tooth development (426, 427), female reproduction and timing of puberty (428–430), mammary gland development and suceptibility to cancers (431), behaviors (432, 433), and others. In several cases, lower doses were more effective at altering these endpoints than higher ones (423, 424, 426, 433). Epidemiology studies of nonoccupationally exposed individuals also indicate that serum TCDD levels may be linked to diseases in humans as well (434). Mean serum TCDD levels have decreased by a factor of 7 over a 25-yr period (1972–97) in several industrial nations (435), but results from both animal and epidemiological studies suggest that even the low levels detected now could have adverse effects on health-related endpoints.

G. Perchlorate and thyroid: low-dose effects in humans?

A significant challenge with observing low-dose effects of EDCs in the human population is that human chemical exposures are multivariate along the vectors of time, space, and sensitivities. In addition, chemicals can exert effects on several systems simultaneously. Therefore, associations in human studies between exposures and disease are difficult to reconcile with experimental studies in animal model systems. For this reason, the literature describing the potential impacts of perchlorate contamination on the human population is potentially clarifying because to the best of our knowledge, perchlorate exerts only a single effect, and the pharmacology of perchlorate exposures has been studied in human volunteers (436). This

literature offers a unique perspective into the issue of low-dose effects, perhaps providing important hypotheses to explain mechanistically why high-dose, short-term experiments can fail to predict the outcome of low-dose, lifetime exposures.

In the 2001–2002 NHANES dataset, perchlorate was detected in the urine of each of the 2820 samples tested (437). This widespread exposure means that the human population is being continuously exposed because perchlorate has a half-life in the human body of about 8 h (438). Human exposures to perchlorate are likely attributed to both contaminated drinking water and food (439); in fact, a recent analysis concludes that the majority of human exposure to perchlorate comes from food (440).

The predominant theory proposed to explain the source of perchlorate contamination in the United States is that it has been employed for many decades as the principal oxidant in explosives and solid rocket fuels (441). Perchlorate is chemically stable when wet and persists for long periods in geological systems and in ground water. Because of disposal practices during the 1960s through 1990s, perchlorate became a common contaminant of ground water in the United States (441, 442). Perchlorate is also formed under certain kinds of natural conditions (443), although the relative contributions to human exposure of these different sources is not completely understood. As a result of perchlorate contamination of natural waters, the food supply has become contaminated through irrigation in part because both aquatic and terrestrial plants can concentrate perchlorate more than 100-fold over water levels (444).

This exposure profile in the human population is important because high doses of perchlorate are known to reduce functioning of the thyroid gland, and poor thyroid function is an important cause of developmental deficits and adult disease (445). The primary question is: at what dose does perchlorate inhibit thyroid function sufficiently to cause disease? The current literature, reviewed below, supports the view that background exposure may affect thyroid function in adult women. These exposure levels, however, are considerably lower than predicted by early toxicology experiments in humans.

Perchlorate reduces thyroid function by inhibiting iodide uptake by the sodium/iodide symporter (NIS) (446), which is the only known effect of perchlorate on human physiology (438). NIS is responsible for transporting iodide into the thyroid gland, which is required for the production of thyroid hormone (447). However, NIS is also expressed in the gut (448, 449), in lactating breast (448, 450, 451), and in placenta (452), presumably all as a delivery mechanism for iodide to the developing and adult thyroid gland. Because the NIS transports perchlorate (450), the pathway by which humans take up and concentrate perchlorate is the same as the pathway by which humans take up and concentrate iodide. Interestingly, NIS expression in the human fetal thyroid gland is the rate-limiting step in production of thyroid hormone (453). Moreover, NIS transport of perchlorate explains why high levels of perchlorate are found in human amniotic fluid (454, 455) and breast milk (456–459).

This effect of perchlorate on thyroid function is important because thyroid hormone is essential for normal brain development, body growth as well as for adult physiology (445, 460). Moreover, it has become clear that even small deficits in circulating thyroid hormone in pregnant women (461, 462) or neonates (463) have permanent adverse outcomes. In fact, recent work indicates that very subtle thyroid hormone insufficiency in pregnant women is associated with cognitive deficits in their children (461). Because of the importance of thyroid hormone in development and adult physiology, and because perchlorate is a potent inhibitor of iodide uptake and thyroid hormone synthesis, identifying the dose at which these events occur is critical.

Perchlorate was used medically to reduce circulating levels of thyroid hormone in patients with an overactive thyroid gland in the 1950s and 1960s (reviewed in Ref. 446); therefore, it was reasonable to examine the dose-response characteristics of perchlorate on the human thyroid gland. Because perchlorate inhibits iodide uptake, several studies were performed to evaluate the effect of perchlorate exposure on iodide uptake inhibition in human volunteers (438, 464–466). In one study, 0.5 or 3 mg/d (approximately 0.007 and 0.04 mg/kg · d) perchlorate was administered to healthy volunteers (n = 9 females and 5 males, age 25–65 yr), and no effects were observed (466). Of course, it is important to note that the 2 wk of administration tested in this study is not sufficient to see any effect on serum concentrations of T_4 or TSH; the healthy thyroid can store several months' worth of thyroid hormone in the gland (467). Another small study also found no effects of administering 3 mg/d (approximately 0.04 mg/kg · d) on any thyroid endpoint assessed (n = 8 adult males) (464).

In contrast, two studies examining adult volunteers administered perchlorate found effects of this chemical on at least one endpoint. The first found that radioactive iodide uptake was affected by 2 wk of exposure to 10 mg/d (0.13 mg/kg · d), but other measures of thyroid function were not altered (n = 10 males) (465). The second examined adults (n = 37) given doses ranging from 0.007–0.5 mg/kg · d; all but the lowest dose altered radioactive iodide uptake, and only the highest dose altered TSH levels (438). These studies were interpreted to suggest that adults would have to consume 2 liters of drinking water daily that

was contaminated with at least 200 ppb (200 μg/liter) perchlorate to reach a level in which iodide uptake would begin to be inhibited. Yet, these administered doses are high and relatively acute, so the derivation of a safe dose from these studies, applied to vulnerable populations such as those with low iodide intake, has been strongly disputed (471).

Studies of occupational exposures have also been used to examine the effects of exposure to relatively high levels of perchlorate. In the first such study, more than 130 employees were separated into eight groups based on exposure estimates from airborne perchlorate in the workplace (472). The authors found that individuals with longer daily exposures to perchlorate, due to longer work shifts, had significant decreases in TSH levels compared with individuals with shorter exposures. But this study was hampered because actual exposure levels were not measured via urine or blood samples. A second study examined 37 employees exposed to perchlorate and 21 control employees from an azide factory; actual exposure measures were not conducted, but estimates were calculated based on exposures to perchlorate dust and air samples (473). This study found no effects of perchlorate exposures on any thyroid endpoint, although the sample size examined was small. In the final occupational exposure study, serum perchlorate levels were measured and compared with several measures of thyroid function in workers (n = 29) who had spent several years as employees in a perchlorate production plant (474). In this study, the most complete because of the biomonitoring aspect of the exposure measures, higher perchlorate levels were associated with lower radioactive iodide uptake, higher urinary iodide excretion, and higher thyroid hormone concentrations.

Although iodide uptake was often inhibited in these studies, serum thyroid hormones were typically not altered, perhaps because of sufficient stored hormone. Based on these observations, the National Academy Committee to Assess the Health Implications of Perchlorate Ingestion (467) estimated that perchlorate would have to inhibit thyroid iodide uptake by about 75% for several months to cause a reduction in serum thyroid hormones. Moreover, the drinking water concentration of perchlorate required for this kind of inhibition was estimated to be over 1,000 ppb (438). Therefore, the National Academy of Sciences committee recommended a reference dose of 0.0007 mg/kg · d (467), based on the dose at which perchlorate could inhibit iodide uptake, and the EPA used this value to set a provisional drinking water standard of 15 ppb.

Considering these data and general knowledge about the thyroid system, it was unexpected that Blount *et al.* (475) would identify a positive association between urinary iodide and serum TSH in adult women in the NHANES 2001–2002 dataset. Yet several features of this dataset were consistent with a causal action of perchlorate on thyroid function. First, in the general population of adult women, urinary perchlorate was positively associated with serum TSH. In the population of adult women who also had low urinary iodide, however, urinary perchlorate was more strongly associated with serum TSH and was negatively associated with serum T_4. The strength of this association was such that the authors calculated that women at the 50th percentile of perchlorate exposure experienced a 1 μg/dl T_4 reduction (reference range = 5–12 μg/dl). Should this magnitude of reduction in serum T_4 occur in a neonate, measurable cognitive deficits would also be present (476). Finally, Steinmaus *et al.* (477), using the same NHANES dataset, showed that women with low urinary iodide who smoke had an even stronger association between urinary perchlorate and measures of thyroid function. Tobacco smoke delivers thiocyanates, which also inhibit NIS-mediated iodide uptake (446).

The NHANES dataset suggests that perchlorate exposures of 0.2–0.4 μg/kg · d (440) are associated with depressed thyroid function, even when urinary iodide is not reduced. This is a considerably lower dose than the 7 μg/kg · d dose required to suppress iodide uptake in the Greer *et al.* (438) study or the 500 μg/kg · d the NAS estimated would be required for several months to actually cause a decline in serum T_4. Therefore, it is reasonable to question whether these associations represent a causative relationship between perchlorate and thyroid function.

A number of epidemiological studies have been published to test for a relationship between perchlorate exposure and thyroid function. Early work used neonatal screening data for T_4 as a measure of thyroid function, and the city of birth (Las Vegas, NV, compared with Reno, NV) as a proxy measure of exposure (478, 479). The reported findings were negative, but we now know that all Americans are exposed to perchlorate, so there was considerable misclassification of exposure, and no relationship should have been observed. Several additional studies using similar flawed designs also found no relationship between proxy measures of perchlorate exposures and clinical outcomes (480–484).

A recent study of the neonatal screening data from 1998 in California identified a strong association between neonatal TSH and whether or not the mother resided in a contaminated area (485). This study included over 497,000 TSH measurements and 800 perchlorate measurements. In addition, they used as a cutoff a variety of TSH levels (as opposed to the 99.9th percentile used for the diagnosis of congenital hypothy-

roidism), indicating that perchlorate exposure is not associated with congenital hypothyroidism. Two additional studies have shown similar relationships between perchlorate and TSH levels, particularly in families with a history of thyroid disease (486, 487).

Several studies in pregnant women have failed to identify a relationship between perchlorate exposure and measures of thyroid function (488–490). Although these are important studies that need to be carefully scrutinized, they do not replicate or refute the NHANES dataset. It thus remains important to conduct additional studies exploring the relationship between background exposure to perchlorate and thyroid function in adults, pregnant women, neonates, and infants. This effort will be challenging because of the different characteristics of thyroid function and hormone action at different life stages (460). In addition, it will be important to obtain individual measurements of exposures to perchlorate and other NIS inhibitors (thiocyanate and nitrate), and iodide itself as well as individual measures of thyroid function (free and total T_4 and TSH).

If background levels of perchlorate affect thyroid function in any segment of the population, it will be challenging to explain how the high-dose, short-term experiments of Greer *et al.* (438) completely underestimated the sensitivity of the human thyroid gland to perchlorate exposure. One possibility is that physiological systems respond to short durations of robust stress with compensatory mechanisms that reset during periods of long-term stress.

When these data are examined together, several important issues are raised. First, this example illustrates the difficulties inherent in studying human populations; epidemiology yields associations, not cause-effect relationships, in many cases using surrogate markers for perchlorate, and is not able to distinguish short- *vs.* long-term exposure duration. Second, our WoE analysis suggests that there is weak evidence for low-dose effects of perchlorate; further research is needed. The relationship between low-dose perchlorate exposures and thyroid endpoints would be strengthened by the addition of studies that measure biological concentrations of perchlorate and compare them with thyroid endpoints in neonates and other vulnerable populations. Third, the published studies that reported low-dose effects of perchlorate typically examined very specific populations, with several focusing on women with low iodine intake. This observation suggests that some groups may be more vulnerable to low doses of perchlorate than others (491).

H. Low-dose summary

These examples, and the examples of low-dose effects in less well-studied chemicals (Table 3), provide evidence that low-dose effects are common in EDC research and may be the default expectation for all chemicals with endocrine activity. Many known EDCs have not been examined for low-dose effects, but we predict that these chemicals will have effects at low doses if studied appropriately. Although studies unable to detect effects at low doses have received attention, including some studies designed to replicate others that reported low-dose effects, the majority of these studies contain at least one major design flaw. Thus, a WoE approach clearly indicates that low-dose effects are present across a wide span of chemical classes and activities.

III. Nonmonotonicity in EDC Studies

A concept related to low dose is that of nonmonotonicity. As noted in *Section I.B*, in a monotonic response, the observed effects may be linear or nonlinear, but the slope does not change sign (Fig. 3, A and B). In contrast, a dose-response curve is nonmonotonic when the slope of the curve changes sign somewhere within the range of doses examined (Fig. 3C). NMDRCs are often U-shaped (with maximal responses of the measured endpoint observed at low and high doses) or inverted U-shaped (with maximal responses observed at intermediate doses) (Fig. 3C, *top panels*). Some cases are more complicated, with multiple points along the curve at which the slope of the curve reverses sign (Fig. 3C, *bottom left*). Nonmonotonicity is not synomymous with low dose, because there are low-dose effects that follow monotonic dose-response curves. Thus, it is not required that a study include doses that span from the true low-dose range to the high toxicological range to detect nonmonotonicity. The consequence of NMDRCs for toxicity testing is that a safe dose determined from high doses does not guarantee safety at lower, untested doses that may be closer to current human exposures.

Examples of NMDRCs from the cell culture, animal, and epidemiological literature will be discussed in detail in *Section III.C*. Importantly, our review of the literature finds that NMDRCs are common in the endocrine and EDC literature. In fact, it is plausible that, considering the mechanisms discussed below, NMDRCs are not the exception but should be expected and perhaps even common.

A. Why is nonmonotonicity important?

NMDRCs in toxicology and in the regulatory process for EDCs are considered controversial. In addition to discussions of whether NMDRCs exist, there is also discussion of whether those that do exist have relevance to

Figure 3.

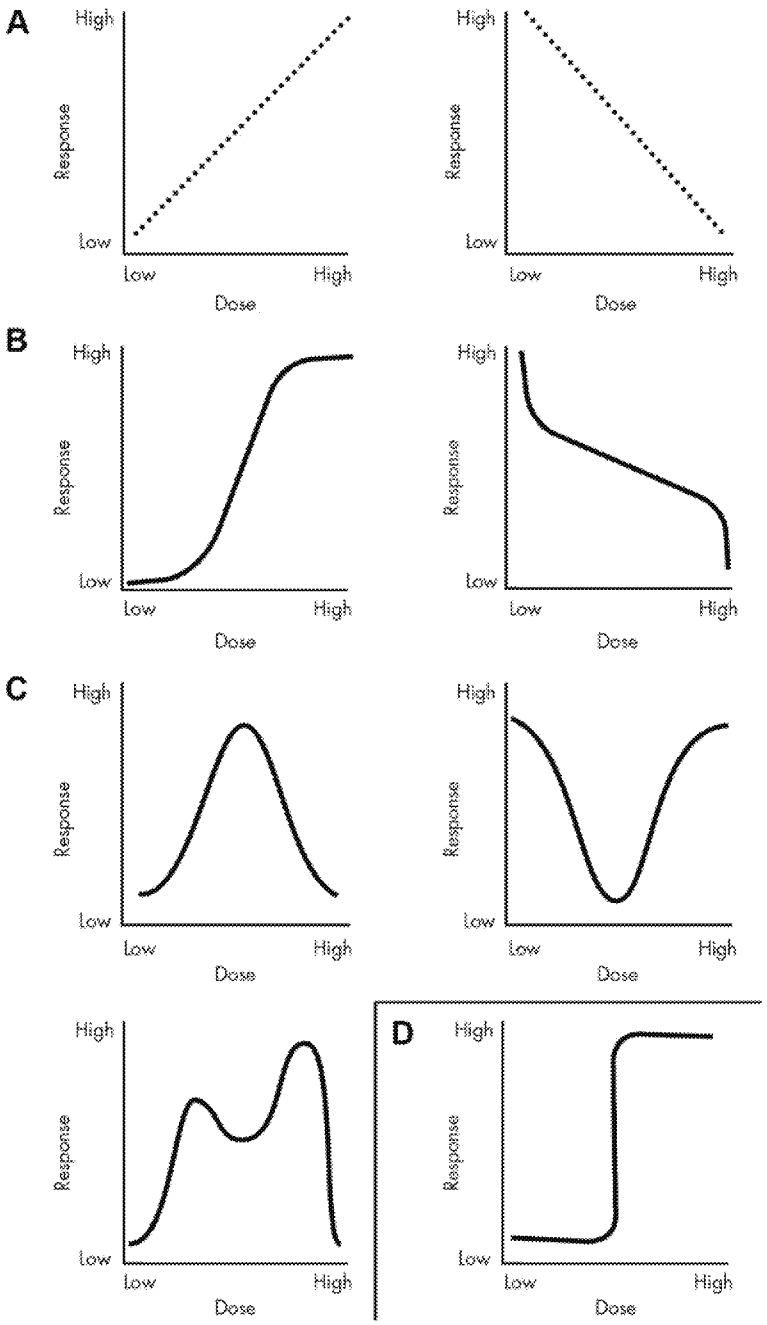

Figure 3. Examples of dose-response curves. A, Linear responses, whether there are positive or inverse associations between dose and effect, allow for extrapolations from one dose to another. Therefore, knowing the effects of a high dose permits accurate predictions of the effects at low doses. B, Examples of monotonic, nonlinear responses. In these examples, the slope of the curve never changes sign, but it does change in value. Thus, knowing what happens at very high or very low doses is not helpful to predict the effect of exposures at moderate doses. These types of responses often have a linear component within them, and predictions can be made within the linear range, as with other linear responses. C, Displayed are three different types of NMDRCs including an inverted U-shaped curve, a U-shaped curve, and a multiphasic curve. All of these are considered NMDRCs because the slope of the curve changes sign one or more times. It is clear from these curves that knowing the effect of a dose, or multiple doses, does not allow for assumptions to be made about the effects of other doses. D, A binary response is shown, where one range of doses has no effect, and then a threshold is met, and all higher doses have the same effect.

toxicological determination of putative safe exposures. In the standard practice of regulatory toxicology, the calculated safe dose, also called a reference dose, is rarely tested. In a system that is responding nonmonotonically, it is not appropriate to use a high-dose test to predict low-dose effects. Unfortunately, all regulatory testing for the effects of chemical exposures assume that this is possible. All current exposure standards employed by government agencies around the world, including the FDA and EPA, have been developed using an assumption of monotonicity (492, 493). The low-dose range, which presumably is what the general public normally experiences, is rarely, if ever, tested directly.

The standard procedure for regulatory testing typically involves a series of tests to establish the lowest dose at which an effect is observable (the LOAEL), then a dose beneath that at which no effect is observable (the NOAEL). Then a series of calculations are used to acknowledge uncertainty in the data, species differences, age differences, *etc.*, and those calculations, beginning with the LOAEL or the NOAEL, produce a reference dose that is presumed to be a safe exposure for humans (Fig. 4). Typically, the reference dose is 3- to 1000-fold lower than the NOAEL. That reference dose then becomes the allowable exposure and is deemed safe, even when it is never examined directly. For chemicals with monotonic linear dose-response curves (Fig. 3A), this may be appropriate. But for any chemicals that display nonmonotonic patterns, it is likely to lead to false negatives, *i.e.* concluding that exposure to the reference dose is safe when in fact it is not.

As described above, there are other nonlinear dose-response curves that are monotonic (Fig. 3B). These curves may also present problems for extrapolating from high doses to low doses because there is no linear relationship that can be used to predict the effects of low doses. Equally troubling for regulatory purposes are responses that have a binary response rather than a classical dose-response curve (Fig. 3D). In these types of responses, one range of doses has no effect on an endpoint, and then a threshold is met, and all higher doses have the same effect. An example is seen in the atrazine literature, where doses below 1 ppb had no effect on the size of the male larynx but doses

at or above 1 ppb produced a significant decrease in size of approximately 10–15% (336). Even doses of 200 ppb, the toxicological NOEL, produce the same effect. Thus, this all-or-none effect is observed because atrazine does not shrink the larynx; instead, it removes the stimulatory agent (*i.e.* androgens). In the absence of some threshold dose of androgen, the larynx simply remains at the unstimulated (female) size. The EPA's assessment of this study and others was that the lack of a dose-dependent response negates the importance of this effect (352). The lack of a dose response for a threshold effect like larynx size does not mean that the effects are not dose dependent; thus, understanding these types of effects and their implications for risk assessments is essential for determining the safe levels of chemicals.

It is important to mention here that the appropriateness of determining NOAEL concentrations, and therefore calculating reference doses, from exposures to endogenous hormones or EDCs has been challenged by several studies (Fig. 4A) (494–496). These studies show that hormonally active agents may still induce significant biological effects even at extremely low concentrations and that presently available analytical methods or technologies might be unable to detect relatively small magnitudes of effects. Previous discussions of this topic have shown that as the dose gets lower (and approaches zero) and the effect size decreases, the number of animals needed to achieve the power to detect a significant effect would have to increase substantially (497). Even more importantly, the assumption of a threshold does not take into account situations where an endogenous hormone is already above the dose that causes detectable effects and that an exogenous chemical (whether an agonist or antagonist) will modulate the effect of the endogenous hormone at any dose above zero (Fig. 4B). There can thus be no threshold or safe dose for an exogenous chemical in this situation. Forced identification of NOAEL or threshold doses based on the assumption that dose-response curves are always monotonic without considering the background activity of endogenous hormones and the limitations of analytical techniques supports the misconception that hormonally active agents do not have any significant biological effects at low doses. Thus, the concept that a toxic agent has a safe dose that can be readily estimated from the NOAEL derived from testing high, acutely toxic doses is overly simplistic and contradicted by data when applied to EDC (5, 497, 498).

B. Mechanisms for NMDRCs

Previously, the lack of mechanisms to explain the appearance of NMDRCs was used as a rationale for ignoring these phenomena (492, 493). This is no longer acceptable because there are several mechanisms that have been identified and studied that demonstrate how hormones and EDCs produce nonmonotonic responses in cells, tissues, and animals. These mechanisms include cytotoxicity, cell- and tissue-specific receptors and cofactors, receptor selectivity, receptor down-regulation and desensitization, receptor competition, and endocrine negative FEEDBACK loops. These mechanisms are well understood, and by providing detailed biological insights at the molecular level into the etiology of NMDRCs, they strongly negate the presumption that has been central to regulatory toxicology that dose-response curves are by default monotonic.

1. Cytotoxicity

The simplest mechanism for NMDRCs derives from the observation that hormones can be acutely toxic at high doses yet alter biological endpoints at low, physiologically relevant doses. Experiments working at concentrations that are cytotoxic are incapable of detecting responses that are mediated by ligand-binding interactions. For example, the MCF7 breast cancer cell line proliferates in response to estradiol in the low-dose range (10^{-12} to 10^{-11} M) and in the pharmacological and toxicological range (10^{-11} to 10^{-6} M), but toxic responses are observed at higher doses (38). Thus, when total cell number is graphed, it displays an inverted U-shaped response to estrogen. But cells that do not contain ER, and therefore cannot be affected by the hormonal action of estradiol, also display cytotoxic responses when treated with high doses of hormone. These results clearly indicate that the effects of estradiol at high doses are toxic via non-ER-mediated mechanisms.

2. Cell- and tissue-specific receptors and cofactors

Some NMDRCs are generated by the combination of two or more monotonic responses that overlap, affecting a common endpoint in opposite ways via different pathways. For example, *in vitro* cultured prostate cell lines demonstrate a nonmonotonic response to increasing doses of androgen where low doses increase cell number and higher doses decrease cell number, thus producing an inverted U-shaped curve (499, 500). Although the parental cell expressed an inverted U-shaped dose-response curve, after a long period of inhibition, the effects on cell number could be segregated by selecting two populations of cells: one that proliferated in the absence of androgens and other cells that proliferated in the presence of high androgen levels (501). Thus, the observed inverted U-shaped response is due to actions via two independent pathways that can be separated from each other in an experimental setting (502). Similarly, estrogens have been shown to induce cell proliferation and inhibit apoptosis in several cell populations, but inhibit proliferation and induce apopto-

Figure 4.

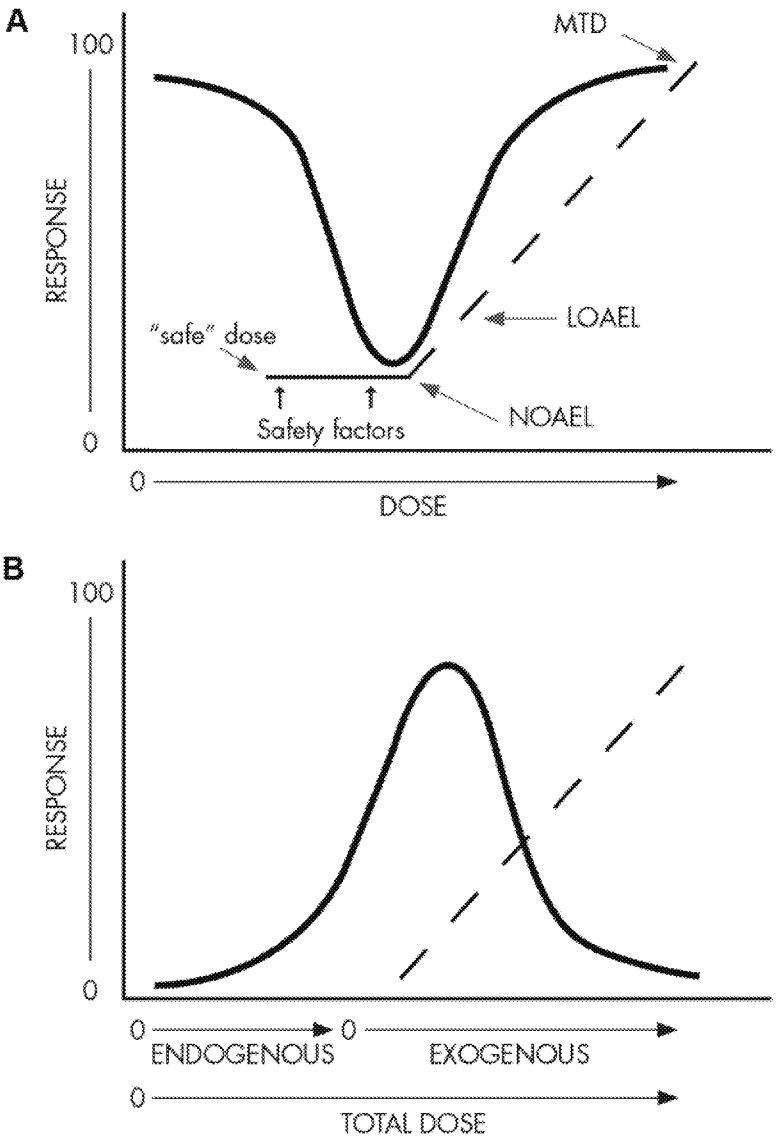

Figure 4. NOAEL, LOAEL, and calculation of a safe reference dose. A, In traditional toxicology testing, high doses are tested to obtain the maximum tolerated dose (MTD), the LOAEL, and the NOAEL. Several safety factors are then applied to derive the reference dose, *i.e.* the dose at which exposures are presumed safe. This reference dose is rarely tested directly. Yet when chemicals or hormones produce NMDRCs, adverse effects may be observed at or below the reference dose. Here, the doses that would be tested are shown by a *dotted line*, and the calculated safe dose is indicated by a *thick solid line*. The actual response, an inverted U-shaped NMDRC, is shown by a *thin solid line*. B, Experimental data indicate that EDCs and hormones do not have NOAELs or threshold doses, and therefore no dose can ever be considered safe. This is because an exogenous hormone (or EDC) could have a linear response in the tested range (*dotted line*), but because endogenous hormones are present (*thin solid line*), the effects of the exogenous hormone are always observed in the context of a hormone-containing system.

sis in others (503, 504), with the combined effect being an inverted U-shaped curve for cell number (505).

Why does one single cell type have different responses to different doses of the same hormone? The case of the prostate cell line described above is reminiscent of the results described from the transcriptome of MCF7 cells, whereby a discrete global response like cell proliferation manifests at significantly lower estrogen doses than the induction of a single marker gene (135). That a response like cell proliferation requires a significantly lower dose of hormone than the dose needed to induce a given target gene is counterintuitive but factual; it may be interpreted as consistent with the notion that metazoan cells, like cells in unicellular organisms, are intrinsically poised to divide (503, 506, 507) and that quiescence is an induced state (508, 509). The biochemical details underlying these different responses are largely unknown; however, recent studies showed that steroid receptors control only a portion of their target genes directly via promoter binding. The majority of the changes are indirect, through chromatin rearrangements (510, 511).

Why do different cell types (*in vitro* and *in vivo*) have different responses to the same hormone? One answer is that they may express different receptors, and these receptors have different responses to the same hormone. For example, some tissues express only one of the two major ER (ERα and ERβ), and actions via these receptors are important not just for responsiveness to hormone but also for cellular differentiation and cross talk between tissue compartments (512). Yet other tissues express both ERα and ERβ, and the effects of signaling via these two receptors often oppose each other; *i.e.* estrogen action via ERα induces proliferation in the uterus, but ERβ induces apoptosis (154). Complicating the situation further, different responses to a hormone can also be obtained due to the presence of different cofactors in different cell and tissue types (513, 514); these coregulators influence which genes are transcriptionally activated or repressed in response to the presence of hormone. They can also influence ligand selectivity of the receptor and DNA-binding capacity, having tremendous impact on the ability of a hormone to have effects in different cell types (105, 515, 516).

Although much of these activities occur on a biochemical level, *i.e.* at the receptor, there is also evidence that nonmonotonicity can originate at the level of tissue organization. The mammary gland has been used as a model to study inter- and intracompartmental effects of hormone treatment: within the ductal epithelium, estro-

gen has distinct effects during puberty, both inducing proliferation, which causes growth of the ductal tree, and inducing apoptosis, which is required for lumen formation (517, 518); in cell culture, the presence of stromal cells can also enhance the effects of estrogen on epithelial cells (519, 520), suggesting that stromal-epithelial compartmental interactions can mediate the effects of estrogen.

3. Receptor selectivity

NMDRCs can occur because of differences in receptor affinity, and thus the selectivity of the response, at low *vs.* high doses. For example, at low doses, BPA almost exclusively binds to the ER (including mER), but at high doses it can also bind weakly to other hormone receptors, like androgen receptor and thyroid hormone receptor (249, 521). This type of receptor nonselectivity is quite common for EDCs, and it has been proposed that binding to different receptors may be an explanation for the diverse patterns of disease observed after EDC exposures (522). In fact, several of the chemicals shown to have low-dose effects are known to act via multiple receptors and pathways (Table 3). Thus, the effects seen at high doses can be due to action via the binding of multiple receptors, compared with the effects of low doses, which may be caused by action via only a single receptor or receptor family.

4. Receptor down-regulation and desensitization

When hormones bind to nuclear receptors, the ultimate outcome is a change in the transcription of target genes. When the receptor is bound by ligand, an increase in response is observed; as discussed previously in this review, the relationship between hormone concentration and the number of bound receptors, as well as the relationship between the number of bound receptors and the biological effect, is nonlinear (38). After the nuclear receptor is bound by hormone and transcription of target genes has occurred (either due to binding of the receptor at a DNA response element or the relief of a repressive event on the DNA), the reaction eventually must cease; *i.e.* the bound receptor must eventually be inactivated in some way. Thus, nuclear hormone receptors are ubiquitinated and degraded, usually via the proteasome (523). Importantly, the role of the hormone in receptor degradation differs depending on the hormone; binding of estrogen, progesterone, and glucocorticoid mediates the degradation of their receptors (524–526), whereas the presence of hormone may actually stabilize some receptors and prevent degradation (527), and other receptors are degraded without ligand (528). As hormone levels rise, the number of receptors being inactivated and degraded also rises, and eventually the number of receptors being produced cannot maintain the pace of this degradation pathway (523). Furthermore, the internalization and degradation of receptors can also influence receptor production, leading to an even stronger down-regulation of receptor (529). In the animal, the role of receptor down-regulation is actually quite complex, because signaling from one hormone receptor can influence protein levels of another receptor; *i.e.* ER signaling can promote degradation of the glucocorticoid receptor by increasing the expression of enzymes in the proteasome pathway that degrade it (530).

There is also the issue of receptor desensitization, a process whereby a decrease in response to a hormone is not due to a decrease in the number of available receptors but instead due to the biochemical inactivation of a receptor (531). Desensitization typically occurs when repeated or continuous exposure to ligand occurs. Normally seen with membrane-bound G protein-coupled receptors, the activation of a receptor due to ligand binding is quickly followed by the uncoupling of the activated receptor from its G proteins due to phosphorylation of these binding partners (532). Receptor desensitization has been observed for a range of hormones including glucagon, FSH, human chorionic gonadotropin, and prostaglandins (533). Importantly, desensitization and down-regulation can occur in the same cells for the same receptor (534), and therefore, both can play a role in the production of NMDRCs.

5. Receptor competition

Mathematical modeling studies suggest that the mixture of endogenous hormones and EDCs establishes a natural environment to foster NMDRCs. Using mathematical models, Kohn and Melnick (42) proposed that when EDC exposures occur in the presence of endogenous hormone and unoccupied hormone receptors, some unoccupied receptors become bound with the EDC, leading to an increase in biological response (*i.e.* increased expression of a responsive gene, increased weight of an organ, *etc.*). At low concentrations, both the endogenous hormone and the EDC bind to receptors and activate this response, but at high doses, the EDC can outcompete the natural ligand. The model predicts that inverted U-shaped curves would occur regardless of the binding affinity of the EDC for the receptor and would be abolished only if the concentration of natural hormone were raised such that all receptors were bound.

6. Endocrine negative FEEDBACK loops

In several cases, the control of hormone synthesis is regulated by a series of positive- and negative feedback loops. Several hormones are known to control or influence their own secretion using these feedback systems. In one example, levels of insulin are known to regulate glucose uptake by cells. Blood glucose levels stimulate insulin pro-

duction, and as insulin removes glucose from circulation, insulin levels decline. Thus, NMDRCs can occur as the free/available ligand and receptor concentrations are influenced by one another. In another example, thyroid hormone secretion is stimulated by TSH, and thyroid hormone suppresses TSH; thus, feedback between these two hormones allows thyroid hormone to be maintained in a narrow dose range.

Several studies indicate that these negative feedback loops could produce NMDRCs when the duration of hormone administration is changed (535). For example, short exposures of estrogen induce proliferation in the uterus and pituitary, but longer hormone regimens inhibit cell proliferation (236, 536). Thus, the outcome is one where exposure to a single hormone concentration stimulates an endpoint until negative feedback loops are induced and stimulation ends (537).

7. Other downstream mechanisms

Removing the variability that can come from examining different cell types, or even single cell types in the context of a tissue, studies of cultured cells indicate that different gene profiles are affected by low doses of hormone compared with higher doses. In a study of the genes affected by low *vs.* higher doses of estrogen, researchers found that there were a small number of genes in MCF7 breast cancer cells with very high sensitivity to low doses of estradiol (10 pM) compared with the total number of genes that were affected by higher (30 or 100 pM) exposures (538). But the surprising finding was the pattern of estradiol-induced *vs.* estradiol-suppressed gene expression at high and low doses; when 10 pM was administered, the number of estradiol-suppressible genes was approximately three times higher than the number of estradiol-inducible genes. However, the overall profile of the number of estradiol-suppressible genes was approximately half the total number of estradiol-inducible genes. This observation suggests that low doses of estrogen selectively target a small subset of the total number of estrogen-sensitive genes and that the genes affected by low doses are most likely to be suppressed by that treatment. The mechanisms describing how low doses of estrogen differently affect the expression of genes compared with higher doses have yet to be elucidated, but low doses of estradiol inhibit expression of apoptotic genes (539), indicating that which genes are affected by hormone exposure is relevant to understand how low doses influence cellular activities.

C. Examples of nonmonotonicity

1. Examples of NMDRCs from cell culture

A tremendous amount of theoretical and mathematical modeling has been conducted to understand the production of nonlinear and nonmonotonic responses (42, 540). These studies and others suggest that the total number of theoretical response curves is infinite. Yet this does not mean that the occurrence of NMDRCs is speculative; these types of responses are reported for a wide variety of chemicals. Cell culture experiments alone provide hundreds of examples of nonmonotonic responses (see Table 6 for examples). In the natural hormone category, many different hormones produce NMDRCs; this is clearly not a phenomenon that is solely attributable to estrogen and androgen, the hormones that have been afforded the most attention in the dose-response literature. Instead, NMDRCs are observed after cells are treated with a range of hormones, suggesting that this is a fundamental and general feature of hormones.

Chemicals from a large number of categories with variable effects on the endocrine system also produce NMDRCs in cultured cells. These chemicals range from components of plastics to pesticides to industrial chemicals and even heavy metals. The mechanisms for nonmonotonicity discussed in *Section III.B* are likely explanations for the NMDRCs reported in a range of cell types after exposure to hormones and EDCs. Table 6 provides only a small number of examples from the literature, and it should be noted that because these are studies of cells in culture, most of these studies typically examined only a few types of outcomes: cell number (which could capture the effects of a chemical on cell proliferation, apoptosis, or both), stimulation or release of another hormone, and regulation of target protein function, often examined by measuring the phosphorylation status of a target.

2. Examples of NMDRCs in animal studies

Some scientists suggest that nonmonotonicity is an artifact of cell culture, however, a large number of NMDRCs have been observed in animals after administration of natural hormones and EDCs, refuting the hypothesis that this is a cell-based phenomenon only. Similar to what has been observed in cultured cells, the NMDRCs observed in animals also span a large range of chemicals, model organisms, and affected endpoints (Table 7). These results underscore the biological importance of the mechanisms of nonmonotonicity that have been largely worked out *in vitro*.

Although NMDRCs attributable to estrogen treatment are well documented, the induction of NMDRCs is again observed to be a general feature of hormone treatment; a wide range of hormones produce these types of responses in exposed animals. Importantly, a number of pharmaceutical compounds with hormone-mimicking or endocrine-disrupting activities also produce NMDRCs. Finally, as expected from the results of cell culture

TABLE 6. Examples of NMDRCs in cell culture experiments

Chemicals by chemical class	Nonmonotonic effect	Cell type	Refs.
Natural hormones			
17β-Estradiol	Cell number	MCF7 breast cancer cells	135, 716
	Dopamine uptake	Fetal hypothalamic cells (primary)	717
	pERK levels, prolactin release	GH3/B6/F10 pituitary cells	41, 718, 719
	β-Hexosaminidase release	HMC-1 mast cells	720
	Cell number	Vascular smooth muscle cells	721
	Production of L-PGDS, a sleep-promoting substance	U251 glioma cells	722
5α-Dihydrotestosterone	Cell number	LNCaP-FGC prostate cancer cells	499
	Cell number, kinase activity	Vascular smooth muscle cells	721
5α-Androstenedione	Cell number	LNCaP-FGC prostate cancer cells	499
Corticosterone	Mitochrondrial oxidation, calcium flux	Cortical neurons (primary)	723
Insulin	Markers of apoptosis (in absence of glucose)	Pancreatic β-cells (primary)	724
Progesterone	Cell number	LNCaP-FGC prostate cancer cells	499
Prolactin	Testosterone release	Adult rat testicular cells (primary)	725
hCG	Testosterone release	Adult rat testicular cells (primary)	725
T_3	Rate of protein phosphorylation	Cerebral cortex cells (primary, synaptosomes)	726
	LPL mRNA expression	White adipocytes (rat primary)	727
GH	*IGF-I* expression	Hepatocytes (primary cultures from silver sea bream)	728
Pharmaceutical hormones			
DES	Cell number	MCF7 breast cancer cells	716
	Prolactin release	GH3/B6/F10 pituitary cells	41
Ethinyl estradiol	CXCL12 secretion	MCF7 breast cancer cells, T47D breast cancer cells	729
R1881 (synthetic androgen)	Cell number	LNCaP-FGC cells	499
Trenbolone	Induction of micronuclei	RTL-W1 fish liver cells	730
Plastics			
BPA	Cell number	MCF7 breast cancer cells	135, 716
	Dopamine efflux	PC12 rat tumor cells	40
	pERK levels, intracellular Ca^{2+} changes, prolactin release	GH3/B6/F10 pituitary cells	41, 718
	Cell number	LNCaP prostate cancer cells	731
DEHP	Number of colonies	*Escherichia coli* and *B. subtilis* bacteria	732
Di-*n*-octyl phthalate	Number of colonies	*E. coli* and *B. subtilis* bacteria	732
Detergents, surfactants			
Octylphenol	Cell number	MCF7 breast cancer cells	716
	Dopamine uptake	Fetal hypothalamic cells (primary)	717
	pERK levels	GH3/B6/F10 pituitary cells	718
	hCG-stimulated testosterone levels	Leydig cells (primary)	733
Propylphenol	pERK levels	GH3/B6/F10 pituitary cells	718
Nonylphenol	pERK levels, prolactin release	GH3/B6/F10 pituitary cells	41, 718
	β-Hexosaminidase release	HMC-1 mast cells	720
	Cell number	MCF7 breast cancer cells	135
PAH			
Phenanthrene	All-trans retinoic acid activity	P19 embryonic carcinoma cells	734, 735
Benz(a)acridine	All-trans retinoic acid activity	P19 embryonic carcinoma cells	734
Naphthalene	hCG-stimulated testosterone	Pieces of goldfish testes	736
B-naphthoflavone	hCG-stimulated testosterone	Pieces of goldfish testes	736
Retene	hCG-stimulated testosterone	Pieces of goldfish testes	736
Heavy metals			
Lead	Estrogen, testosterone, and cortisol levels	Postvitellogenic follicles (isolated from catfish)	737
Cadmium	Expression of angiogenesis genes	Human endometrial endothelial cells	738

(Continued)

TABLE 6. Continued

Chemicals by chemical class	Nonmonotonic effect	Cell type	Refs.
Phytoestrogens and natural antioxidants			
Genistein	Cell number	Caco-2BBe colon adenocarcinoma cells	739
	CXCL12 secretion, cell number	T47D breast cancer cells	729
	Cell number, cell invasion, MMP-9 activity	PC3 prostate cancer cells	740
	pJNK levels, Ca^{2+} flux	GH3/B6/F10 pituitary cells	719
Coumesterol	Prolactin release, pERK levels	GH3/B6/F10 pituitary cells	719
Daidezin	Prolactin release, pERK levels	GH3/B6/F10 pituitary cells	719
	Cell number	MCF7 breast cancer cells	135
	Cell number	LoVo colon cancer cells	741
Resveratrol	Expression of angiogenesis genes	Human umbilical vein endothelial cells	742
Trans-resveratrol	pERK levels, Ca^{2+} flux	GH3/B6/F10 pituitary cells	719
Artelastochromene	Cell number	MCF7 breast cancer cells	743
Carpelastofuran	Cell number	MCF7 breast cancer cells	743
Biochanin A	Induction of estrogen-sensitive genes in the presence of testosterone	MCF7 breast cancer cells	744
Licoflavone C	Induction of estrogen-sensitive genes	Yeast bioassay	745
Quercetin	Aromatase activity	H295R adrenocortical carcinoma cells	746
	Cell number	SCC-25 oral squamous carcinoma cells	747
Dioxin			
TCDD	Cell number, gene expression	M13SV1 breast cells	748
PCB			
PCB-74	Cell viability, GnRH peptide levels	GT1-7 hypothalamic cells	749
PCB-118	Cell viability, GnRH peptide levels	GT1-7 hypothalamic cells	749
Aroclor 1242 (PCB mixture)	β-Hexosaminidase release	HMC-1 mast cells	720
POP mixture	Apoptosis of cumulus cells	Oocyte-cumulus complexes (primary, isolated from pigs)	750
Herbicides			
Glyphosphate-based herbicide (Round-Up)	Cell death, aromatase activity, ERβ activity	HepG2 liver cells	751
Atrazine	Cell number	IEC-6 intestinal cells	752
Insecticides			
Endosulfan	Cell number	IEC-6 intestinal cells	752
	β-Hexosaminidase release	HMC-1 mast cells	720
	ATPase activity of P-glycoprotein	CHO cell extracts	753
Diazinon	Cell number	IEC-6 intestinal cells	752
Dieldrin	β-Hexosaminidase release	HMC-1 mast cells	720
DDT	Cell number	MCF7 breast cancer cells	144
DDE	β-Hexosaminidase release	HMC-1 mast cells	720
	Prolactin release	GH3/B6/F10 pituitary cells	41
3-Methylsulfonyl-DDE	Cortisol and aldosterone release, expression of steroidogenic genes	H295R adrenocortical carcinoma cells	754
Fungicides			
Hexachlorobenzene	Transcriptional activity in the presence of DHT	PC3 prostate cancer cells	755
Prochloraz	Aldosterone, progesterone, and corticosterone levels; expression of steroidogenic genes	H295R adrenocortical cells	756
Ketoconazole	Aldosterone secretion	H295R adrenocortical cells	757
Fungicide mixtures	Aldosterone secretion	H295R adrenocortical cells	757
PBDE			
PBDE-49	Activation of ryanodine receptor 1	HEK293 cell (membranes)	758
PBDE-99	Expression of *GAP43*	Cerebral cortex cells (primary)	759

Due to space concerns, we have not elaborated on the shape of the curve (U, inverted U, or other nonmonotonic shape) or the magnitude of observed effects in this table. CXCL12, Chemokine (C-X-C motif) ligand 12; DEHP, bis(2-ethylhexyl) phthalate; DHT, dihydrotestosterone; hCG, human chorionic gonadotropin, MMP, matrix metalloproteinase; PAH, polyaromatic hydrocarbons; PBDE, polybrominated diphenyl ethers; PCB, polychlorinated biphenyl; pERK, phospho-ERK; PGDS, prostaglandin-D synthase; pJNK, phospho-c-Jun N-terminal kinase.

TABLE 7. Examples of NMDRCs in animal studies

Chemicals by chemical class	Nonmonotonic effect	Organ/sex/animal	Refs.
Natural hormones			
17β-Estradiol	Morphological parameters	Mammary gland/female/mice	138, 541
	Accumulation of cAMP	Pineal/female/rats	760
	Prostate weight	male/mice	689
	Uterine weight	female/mice	761
	Antidepressant effects, measured by immobility assay	Behavior/male/mice	762
	Nocturnal activity, gene expression in preoptic area	Brain and behavior/female/mice	763
Corticosterone	Spatial memory errors	Behavior/male/rats	764
	Cholinergic fiber loss in cortex after treatment with neurodegenerative drugs	Brain/male/rats	765
	Mitochondrial metabolism	Muscle/male/rats: strain differences	766
	Contextual fear conditioning	Behavior/male/rats	767
	Locomotor activity	Behavior/male/captive Adelie penguins	768
Glucocorticoid	Na^+/K^+-ATPase activity	Brain/tilapia (fish)	769
Testosterone	Na^+/K^+-ATPase activity	Brain/tilapia (fish)	769
	Gonadotropin subunit gene expression	Pituitary/sexually immature goldfish	770
11β-Hydroxyandrosterone	Gonadotropin subunit gene expression	Pituitary/sexually immature goldfish	770
T_4	Bone growth	Tibia/male/rats with induced hypothyroidism	771
Leptin	Insulin production (in the presence of glucose)	Pancreas/male/rats	560
Oxytocin	Infarct size, plasma LDH levels, creatine kinase activity after ischemia/ reperfusion injury	Brain and blood/male/rats	772
	Memory retention	Behavior/male/mice	773
Melatonin	Brain infarction and surviving neuron number after injury	Brain/female/rats	774
Dopamine	Memory	Brain/both/rhesus monkey	775
	Neuronal firing rate	Brain/male/rhesus monkey	776
Pharmaceutical			
DES	Sex ratio, neonatal body weight, other neonatal development	Mice	777
	Adult prostate weight	Male/mice	689
	Uterine weight	Female/mice	761
	Expression of PDGF receptor	Testes/male/rats	778
	Morphological parameters	Mammary gland/male and female/ mice	779
Estradiol benzoate	Dorsal prostate weight, body weight	Male/rats	780
	Sexual behaviors, testes morphology	Male/zebra finches (birds)	781
Ethinyl estradiol	GnRH neurons	Brain/zebrafish	782
Tamoxifen	Uterine weight	Female/mice	761
Fluoxetine (antidepressant)	Embryo number	*Potamopyrgus antipodarum* (snails)	783
Fadrozole (aromatase inhibitor)	Aromatase activity	Ovary/female/fathead minnows	784
Plastics			
BPA	Fertility	Reproductive axis /female/mice	316
	Reproductive behaviors	Behavior/male/rats	785
	Protein expression	Hepatopancreas/male/*Porcellio scaber* (isopod)	786
	Timing of vaginal opening, tissue organization of uterus	Reproductive axis/female/mice	577
	Expression of receptors in embryos	Brain and gonad/both/ mice	787
DEHP	Aromatase activity	Hypothalamus/male/rats	788
	Cholesterol levels	Serum/male/rats	569
	Timing of puberty	Reproductive axis /male/rats	789
	Body weight at birth, vaginal opening, and first estrous	Female/rats	790
	Seminal vesicle weight, epididymal weight, testicular expression of steroidogenesis genes	Male/rats	791
	Responses to allergens, chemokine expression	Skin/male/mice	792

(*Continued*)

TABLE 7. Continued

Chemicals by chemical class	Nonmonotonic effect	Organ/sex/animal	Refs.
Detergents, surfactants			
Nonylphenol ethoxylate	Fecundity	*Biomphalaria tenagophila* (snails)	793
Octylphenol	Embryo production	*P. antipodarum* (snails)	794
	Spawning mass and egg numbers	*Marisa cornuarietis* (snails)	795
Semicarbazide	Timing of preputial separation, serum DHT	Male/rats	796
Antimicrobial			
Triclocarban	Fecundity	*P. antipodarum* (snails)	797
PCB			
Mixture of PCB	Corticosterone levels	Male/kestrels (birds)	798
Environmental PCB mixture	Corticosterone levels	Female/tree swallows (birds)	799
UV filters			
Octyl methoxycinnamate	Activity, memory	Behavior/both/rats	800
Aromatic hydrocarbons			
B-naphthoflavone	Testosterone	Plasma/male/goldfish	736
Toluene	Locomotor activity	Behavior/male/rats	801
Dioxins			
TCDD	Cell-mediated immunity	Immune system/male/ rats	802
	Proliferation after treatment with chemical carcinogen	Liver/female/rats	803
Heavy metals			
Cadmium	Expression of metallothionein, *p52/TFF1*	Intestine and kidney/ female/rats	804
	Activity of antioxidant enzymes	Earthworms	805
	Size parameters, metamorphic parameters	*Xenopus laevis*	806
Lead	Growth, gene expression	*Vicia faba* seedlings (plant)	807
	Retinal neurogenesis	Eye and brain/female/rats	808
Selenium	DNA damage, apoptotic index	Prostate/male/dogs	809
	Hatching failure	Eggs/red-winged blackbirds (wild population)	810
Phytoestrogens			
Genistein	Aggressive, defensive behaviors	Behavior/male/mice	811
	Retention of cancellous bone after ovariectomy	Tibia bones/female/rat	812
	Expression of *OPN*, activation of Akt	Prostate/male/mice	740
Resveratrol	Angiogenesis	Chorioallantoic membrane/chicken embryos	742
	Ulcer index after chemical treatment, expression of gastroprotective genes	Stomach/male/mice	813
Phytochemicals			
Phlorizin	Memory retention	Behavior/male/mice	814
Herbicides			
Atrazine	Time to metamorphosis	Thyroid axis/*Rhinella arenarum* (South American toad)	815
	Survivorship patterns	Four species of frogs	363
	Growth parameters	*Bufo americanus*	816
Pendimethalin	Expression of *AR*, *IGF-I*	Uterus/female/mice	817
Commercial mixture with mecoprop, 2,4-dichlorophenoxyacetic acid and dicamba	Number of implantation sites, number of live births	Female/mice	818
Simazine	Estrous cyclicity	Reproductive axis/female/rat	819
Insecticides			
Permethrin	Dopamine transport	Brain/male/mice	820
Heptachlor	Dopamine transport	Brain/male/mice	820
DDT	Number of pups, sex ratios, neonatal body weight, male anogenital distance	Mice	777
Methoxychlor	Number of pups, anogenital distance (males and females), neurobehaviors (males and females)	Mice	777
Chlorpyrifos	Body weight	Male/rats	821
	Antioxidant enzyme activity	*Oxya chinensis* (locusts)	822
Malathion	Antioxidant enzyme activity	*O. chinensis* (locusts)	822

(*Continued*)

TABLE 7. Continued

Chemicals by chemical class	Nonmonotonic effect	Organ/sex/animal	Refs.
Fungicides			
Carbendazim	Liver enzymes, hematology parameters	Blood and liver/male/rats	823
Chlorothalonil	Survival, immune response, corticosterone levels	Several amphibian species	686
Vinclozolin	Protein expression	Testes/male/*P. scaber* (isopod)	786

Due to space concerns, we have not elaborated on the shape of the curve (U, inverted U, or other nonmonotonic shape) or the magnitude of observed effects in this table. DEHP, Bis(2-ethylhexyl) phthalate; DHT, dihydrotestosterone; LDH, lactate dehydrogenase; PCB, polychlorinated biphenyl; PDGF, platelet-derived growth factor.

experiments, chemicals with many different modes of action generate NMDRCs in treated animals.

Perhaps most striking is the range of endpoints affected, from higher-order events such as the number of viable offspring (which could be due to alterations in the reproductive tissues themselves or the reproductive axis), to behavioral effects, to altered organ weights, and to lower-order events such as gene expression. The mechanisms responsible for these nonmonotonic phenomena may be similar to those studied in cell culture systems, although additional mechanisms are likely to be operating *in vivo* such as alterations in tissue organization (541) and the interactions of various players in the positive and negative feedback loops of the endocrine system.

3. Examples of NMDRCs in the epidemiology literature

Perhaps not surprisingly, natural hormones produce NMDRCs in human populations as well (Table 8). Although the methods needed to detect NMDRCs in humans are specific to the field of epidemiology, these results sup-

TABLE 8. NMDRCs for natural hormones identified in the epidemiology literature

Hormone	Affected endpoint	NMDRC	Study subjects	Refs.
Testosterone (free)	Incidence of coronary events	Incidence of 25% at extremes of exposure, 16% at moderate exposure	Rancho Bernardo Study participants, women aged 40+ (n = 639)	824
	Depression	Hypo- and hypergonadal had higher depression scores than those with intermediate free testosterone	Androx Vienna Municipality Study participants, manual workers, men aged 43–67 (n = 689)	825
PTH	Mortality	~50% excess risk for individuals with low or high iPTH	Hemodialysis patients (n = 3946)	826
	Risk of vertebral or hip fractures	~33% higher for low or high iPTH compared to normal levels	Elderly dialysis patients (n = 9007)	827
TSH	Incidence of Alzheimer's disease	About double the incidence in lowest and highest tertile in women (no effects observed in men)	Framingham Study participants (elderly) (n = 1864, 59% women)	828
Leptin	Mortality	Mortality ~10% higher for lowest and highest leptin levels	Framingham Heart Study participants (elderly) (n = 818, 62% women)	563
Insulin	Coronary artery calcification	Higher for low and high insulin area under the curve measures.	Nondiabetic patients with suspected coronary heart disease, cross-sectional (n = 582)	829
	Mortality (noncardiovascular only)	Relative risk ~1.5 for highest and lowest fasting insulin levels	Helsinki Policemen Study participants, men aged 34–64 (n = 970)	830
Cortisol	BMI, waist circumference	Low cortisol secretion per hour for individuals with highest and lowest BMI, waist circumference	Whitehall II participants, adults, cross-sectional (n = 2915 men; n = 1041 women)	831
	Major depression (by diagnostic interview)	Slight increases at extremes of cortisol	Longitudinal Aging Study Amsterdam participants, aged 65+, cross-sectional (n = 1185)	832

BMI, Body mass index; iPTH, intact PTH; PTH, parathyroid hormone.

port the idea that NMDRCs are a fundamental feature of hormones. Importantly, it should be noted that most of the individuals surveyed in studies examining the effects of natural hormones have a disease status or are elderly. This of course does not mean that natural hormones induce NMDRCs in only these select populations but may instead be a reflection of the types of individuals available for these studies (for example, there are very few clinical events in younger people).

NMDRCs observed in the epidemiology literature from human populations exposed to EDCs are now starting to receive attention (Table 9). Here, most reports of NMDRCs come from studies of healthy individuals exposed to persistent organic pollutants POPs, chemicals that do not easily degrade and consequently bioaccumulate in human and animal tissues (542). These POPs do encompass a range of chemical classes including components of plastics, pesticides, and industrial pollutants. A large number of these studies have focused on endpoints that are relevant to metabolic disease, and together, these studies show that there is a recurring pattern of NMDRCs related to POPs and disease. Of course, not every study of POPs shows NMDRCs, and this is probably due to the distribution of EDCs in the populations examined.

In addition to the studies that show strong evidence for NMDRCs in human populations, there is also a subset of studies that provide suggestive evidence for nonmonotonic relationships between EDCs and human health endpoints (Table 9). In fact, the authors of many of these papers clearly identify U- or inverted U-shaped dose-response curves. However, when authors do not perform the appropriate statistical tests to verify the presence of a NMDRC, there is some ambiguity in their conclusions. The usual cross-sectional *vs.* prospective design dichotomy in epidemiology also is a factor that can influence the strength of a NMDRC, or prevent the detection of one at all. This disjunction in design is often incongruous with EDC exposure studies because we often know very little about clearance rates of the chemical, interactions with adiposity, and changes to these factors with age and gender. Yet regardless of any possible weaknesses in these studies, they provide supportive evidence that NMDRCs are observed in human populations.

Because these reports of NMDRCs in human populations are relatively new, few mechanisms have been proposed for these phenomena. Why would risk curves be nonmonotonic over the dose distribution observed in human populations? Why would individuals with the highest exposures have less severe health outcomes compared with individuals with more moderate exposures? One plausible explanation is that the same mechanisms for NMDRCs in animals and cell cultures operate in human populations: chronic exposures to high doses can activate negative feedback loops, activate receptors that promote changes in different pathways that diverge on the same endpoint with opposing effects, or produce some measure of toxicity. Accidental exposures of very large doses may not behave the same as background doses for a variety of reasons, including the toxicity of high doses; these large doses tend to occur over a short time (and therefore more faithfully replicate what is observed in animal studies after controlled administration).

Another explanation is that epidemiology studies, unlike controlled animal studies, examine truly complex mixtures of EDCs and other environmental chemicals. Some chemical exposures are likely to be correlated due to their sources and their dynamics in air, water, soil, and living organisms that are subsequently eaten. Therefore, intake of these chemicals may produce unpredicted, likely nonlinear outcomes whether the two chemicals act via similar or different pathways.

The design of observational epidemiological studies is fundamentally different from studies of cells or animals, in that the EDC exposure distributions are given, rather than set by the investigator. In particular, as shown in Fig. 5, different epidemiological populations will have different ranges of exposure, with the schematic example showing increasing risk in a population with the lowest exposures (labeled group A), an inverted U-shaped risk in a moderate dose population (labeled group B), and an inverse risk in a population with the highest exposures (labeled group C). An additional example is provided (labeled group D) in which an industrial spill shows high risk, but the comparison with the entire unaffected population with a wide variety of risk levels due to differential background exposure could lead to a high- or a low-risk reference group and a wide variety of possible findings.

It is reasonable to suggest that even though epidemiological studies are an assessment of exposures at a single time point, many of these pollutants are persistent, and therefore a single measure of their concentration in blood may be a suitable surrogate for long-term exposures. The movement of people from relatively low- to higher-exposure groups over time depend on refreshed exposures, clearance rates, and individual differences in ability to handle exposures (*i.e.* due to genetic susceptibilities, amount of adipose tissue where POPs can be stored, *etc.*).

Figure 5 therefore further illustrates that observational epidemiological studies yield the composite effect of varying mixtures of EDCs at various exposure levels for various durations, combining acute and chronic effects. These studies are important, however, in that they are the only way to study EDC effects in the long term in intact humans, as opposed to studying signaling pathways, cells,

TABLE 9. NMDRCs for EDCs identified in the epidemiology literature

Chemicals by chemical class	Affected endpoint	NMDRC	Study subjects	Refs.
Insecticides				
Trans-nonachlor	Diabetes incidence	Highest risk in groups with intermediate exposures (quartile 2)	CARDIA participants, case-control study (n = 90 cases and n = 90 controls)	833
	Telomere length in peripheral leukocytes	Increased length in intermediate exposures (quintile 4)	Adults aged 40+ (Korea, n = 84)	591
p,p'-DDE	BMI, triglyceride levels, HDL cholesterol	Highest risk in groups with intermediate exposures (quartile 3)	CARDIA participants (n = 90 controls from nested case control study)	590
	Risk of rapid infant weight gain	For infants born to women of normal weight prepregnancy, risk is highest with intermediate exposures.	Infants from Childhood and the Environment project, Spain (n = 374 from normal prepregnancy weight mothers; n = 144 from overweight mothers)	834
	Telomere length in peripheral leukocytes	Increased length with intermediate exposures (quintile 4)	Adults aged 40+ (Korea, n = 84)	591
Oxychlordane	Bone mineral density of arm bones	With low exposures, fat mass had inverse associations with bone mineral density; with high exposures, fat mass had positive associations with bone mineral density	NHANES 1999–2004 participants, aged 50+ (n = 679 women, n = 612 men)	835
Plastics				
Mono-methyl phthalate (MMP)	Atherosclerotic plaques	Increased risk in intermediate exposure groups (quintiles 2–4)	Adults aged 70, living in Sweden (n = 1016)	836
Perfluorinated compounds				
PFOA	Arthritis (self-reported)	Increased risk in intermediate exposure groups (quartile 2)	NHANES participants, aged 20+ (both sexes, n = 1006)	837
Fire retardants				
PBB-153	Blood triglyceride levels	Increased risk in intermediate exposure groups (quartile 2)	NHANES participants, aged 12+ (n = 637)	604
PBDE-153	Prevalence of diabetes,	Prevalence of diabetes highest in intermediate groups (quartiles 2–3 relative to individuals with undetectable levels)	NHANES participants, aged 12+ (n = 1367)	604
	Prevalence of metabolic syndrome, levels of blood triglycerides	Prevalence of metabolic syndrome highest in intermediate exposure groups (quartile 2 relative to individuals with undetectable levels); blood triglycerides highest in low exposure groups (quartile 1 relative to individuals with undetectable levels)	NHANES participants, aged 12+ (n = 637)	604
PCB				
PCB-74	Triglyceride levels	Lowest levels are observed in intermediate groups (quartile 2)	CARDIA participants (n = 90 controls from nested case-control study)	590
PCB-126	Bone mineral density in right arm	With low exposures, fat mass had inverse associations with bone mineral density; with high exposures, fat mass had positive associations with bone mineral density	NHANES participants, aged <50 (n = 710 women, n = 768 men)	835
PCB-138	Bone mineral density in right arm	With low exposures, fat mass had inverse associations with bone mineral density; with high exposures, fat mass had positive associations with bone mineral density	NHANES participants, women aged 50+ (n = 679 women, n = 612 men)	835
PCB-153	Telomere length in peripheral leukocytes	Increased length with intermediate exposure groups (quintile 4)	Adults aged 40+ (Korea, n = 84)	591
PCB-170	Diabetes incidence	Highest risk in groups with intermediate exposures (quartile 2)	CARDIA participants, case-control study (n = 90 cases and n = 90 controls)	833
	Endometriosis	Decreased risk in groups with intermediate exposures (quartile 3)	Participants from the Women at Risk of Endometriosis (WREN) study, 18–49 yr old, case-control study (n = 251 cases; n = 538 controls)	838
PCB-172	DNA hypomethylation (by Alu assay)	Highest levels of hypomethylation in groups with lowest and highest exposures	Adults aged 40+ (Korea, n = 86)	839
PCB-180[a]	BMI	Highest BMI with intermediate exposures (quartile 2)	CARDIA participants (n = 90 controls from nested case control study)	590
PCB-187[a]	HDL cholesterol levels	Lowest levels with intermediate exposures (quartile 2)	CARDIA participants (n = 90 controls from nested case control study)	590
PCB 196–203	Diabetes incidence	Highest risk in groups with intermediate exposures (quartile 2)	CARDIA participants, case-control study (n = 90 cases and n = 90 controls)	833
PCB-196	Endometriosis	Decreased risk in groups with intermediate exposures (quartile 3)	Participants from the Women at Risk of Endometriosis (WREN) study, 18–49 yr old, case-control study (n = 251 cases; n = 538 controls)	838

(Continued)

TABLE 9. Continued

Chemicals by chemical class	Affected endpoint	NMDRC	Study subjects	Refs.
PCB-199[a]	Triglyceride levels	Highest risk in groups with intermediate exposures (quartiles 2–3)	CARDIA participants (n = 90 controls from nested case control study)	590
PCB-201	Endometriosis	Decreased risk in groups with intermediate exposures (quartiles 2–3)	Participants from the Women at Risk of Endometriosis (WREN) study, 18–49 yr old, case-control study (n = 251 cases, n = 538 controls)	838
Heavy metals				
Selenium	Fasting glucose levels (by modeled exposure)	Intermediate exposures have highest fasting glucose levels	NHANES 2003–2004 participants, aged 40+ (n = 917)	840
	Glycosylated hemoglobin (by modeled exposure)	Intermediate exposures have highest % glycosylated hemoglobin	NHANES 2003–2004 participants, aged 40+ (n = 917)	840
	Diabetes incidence (by modeled exposure)	Intermediate exposures have highest risk for diabetes	NHANES 2003–2004 participants, aged 40+ (n = 917)	840
	Blood triglyceride levels	Intermediate exposures have highest triglyceride levels	NHANES participants, aged 40+ (n = 1159)	841
Arsenic	Cytokines in umbilical cord blood	Lower inflammatory markers at intermediate exposures (quartile 2)	Pregnant women in Bangladesh (n = 130)	842
Manganese	Mental development scores in infants and toddlers	Intermediate exposures had highest mental development scores at 12 months of age; association lost in older toddlers	12-month-old infants, Mexico (n = 301)	843
	Sperm count, motility and morphology	Intermediate doses had lowest sperm counts and motility; intermediate doses also had the worst sperm morphologies	Men aged 18–55 (infertility clinic patients, n = 200)	844
Mixtures				
31 POP	Diabetes incidence	Highest incidence in intermediate groups (sextiles 2–3)	CARDIA participants, case-control study (n = 90 cases and n = 90 controls)	833
16 POP	Diabetes incidence	Highest incidence in intermediate groups (sextiles 2–3)	CARDIA participants, case-control study (n = 90 cases and n = 90 controls)	833
Non-dioxin-like PCB (mix)	Metabolic syndrome	Highest incidence in intermediate groups (quartile 3)	NHANES 1999–2002 participants, aged 20+ (n = 721)	845
Dioxin-like PCB (mix)	Triacylglycerol levels by quartile of exposure	Highest levels in intermediate groups (quartile 3)	NHANES 1999–2002 participants, aged 20+ (n = 721)	845
Additional supportive evidence for NMDRC in the epidemiology literature				
Insecticides				
Heptachlor epoxide	Prevalence of newly diagnosed hypertension	Highest risk in intermediate groups (quartile 2); other endpoints do not have NMDRC	NHANES participants, women aged 40+, cross-sectional (n = 51 cases, n = 278 total)	826
β-Hexachloro-cyclohexane	Triacylglycerol levels by quartile of exposure	Highest risk in intermediate group (quartile 2)	NHANES participants, aged 20+ (n = 896 men, 175 with metabolic syndrome)	845
Plastics				
Mono-*N*-butyl phthalate (MBP)	BMI, age-specific effects	Effects seen only in elderly participants (age 60–80); risk is lowest in quartile 3	NHANES male participants (n = 365; age 60–80)	470
Mono-benzyl phthalate (MBzP)	BMI, age-specific effects	Effects seen only in young participants (age 6–11); risk is highest in quartiles 2–3	NHANES participants (both sexes, n = 329 males; n = 327 females)	470
Flame retardants				
PFOA	Thyroid disease (self-reported)	Lowest risk in intermediate groups (quartile 3)	NHANES 1999–2000, 2003–2006 participants, males aged 20+ (n = 3974)	837
Dioxin and related compounds				
TCDD	Age at natural menopause	Highest for intermediate exposure group (quintile 4)	Highly exposed women; Seveso Women's Health Study participants (n = 616)	468
HCDD	Bone mineral density in right arm by quintile of fat mass	With low exposures, fat mass had inverse associations with bone mineral density; with high exposures, fat mass had positive associations with bone mineral density	NHANES participants, women aged 50+ (n = 679 women, n = 612 men)	835
Heavy metals				
Selenium	Prevalence of peripheral artery disease	Disease prevalence decreased in intermediate doses, then increased gradually with higher doses	NHANES participants, aged 40+ (n = 2062)	469

BMI, Body mass index; HCDD, hexachloro-dibenzo-p-dioxin; HDL, high-density lipoprotein. PCB, polychlorinated biphenyls; PFOA, perfluorooctanoic acid; PBB, polybrominated biphenyl; PBDE, polybrominated diphenyl ethers; POP, persistent organic pollutants

[a] In many cases, multiple chemicals in the same class had similar effects. A few chemicals were selected to illustrate the observed effect. This list is not comprehensive.

organs, or animal models over limited periods of time. Causal inference is not done directly from the epidemiological study results; instead, it is done via combining information from the epidemiological observations with findings from the detailed studies of pathways and animals.

We have suggested that NMDRCs are a fundamental and general feature of hormone action in cells and animals.

Figure 5.

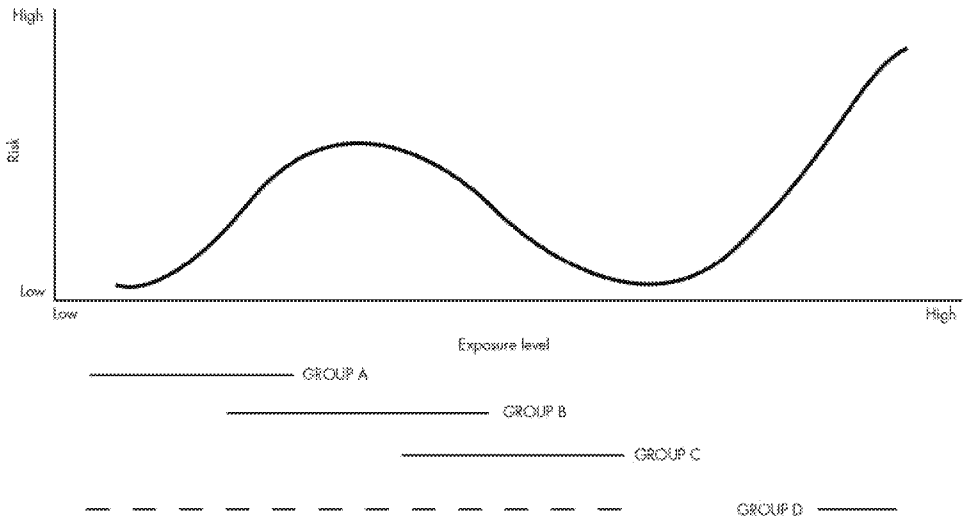

Figure 5. Example of a NMDRC in humans and the sampling populations that could be examined in epidemiology studies. This schematic illustrates a theoretical NMDRC in a human population. If a study were to sample only group A, the conclusion would be that with increasing exposures, risk increases monotonically. Sampling group B would allow researchers to conclude that there is a nonmonotonic relationship between exposure level and risk. If a study included only group C, the conclusion would be that with increasing exposures, there is decreased risk of disease. Group D represents a population that was highly exposed, *i.e.* due to an industrial accident. This group has the highest risk, and there is a monotonic relationship between exposures and risk, although risk is high for all individuals. In the group D situation, there is generally a background population with which high-dose exposure is compared (*dotted line*); relative risk for group D would depend on whether that background population resembles group A, B, or C. From this example, it is clear that the population sampled could strongly influence the shape of the dose-response curve produced as well as the conclusions reached by the study.

It is therefore worth asking whether NMDRCs are expected in the epidemiology literature. The endpoints assessed in epidemiology studies are typically integrated effects, rather than short-term effects; therefore, the various cell- or organ-specific effects may cancel each other, particularly if they are NMDRCs (because they are unlikely to all have nonmonotonicity at the same dose and direction). Thus, NMDRCs are likely to be rarer in the epidemiology literature compared with studies examining the effects of a wide range of doses of an EDC on animals and cultured cells. Yet it is also important to ask what can be concluded if a NMDRC is detected in one epidemiology study but not in others examining the same chemical and outcome. There are several factors that must be considered. The first is that differences in the populations examined between the two studies could explain why a monotonic relationship is observed in one group and a nonmonotonic relationship in another (see Fig. 5). The second is that one or more studies may not be statistically designed to detect NMDRCs. Finally, it is plausible that the NMDRC is an artifact due to residual confounding or some other factor that was not considered in the experimental design. As more becomes known about the mechanisms operating in cells, tissues, and organs to generate NMDRCs, our ability to apply this information to epidemiology studies will increase as well.

4. Tamoxifen flare, a NMDRC observed in cells, animals, and human patients

Although there is controversy in toxicology and risk assessment for endocrine disruptors, NMDRCs are recognized and used in current human clinical practice, although under a different specific term, flare. Flare is often reported in the therapy of hormone-dependent cancers such as breast and prostate cancer. Clinically, failure to recognize the NMDRC that is termed a flare would be considered malpractice in human medicine.

Tamoxifen flare was described and named as a transient worsening of the symptoms of advanced breast cancer, particularly metastases to bone associated with increased pain, seen shortly after the initiation of therapy in some patients (543). If the therapy could be continued, the patients showing tamoxifen flare demonstrated a very high likelihood of subsequent response to tamoxifen, including arrest of tumor growth and progression of symptoms for some time.

The subsequent mechanism of the flare was described in basic lab studies in athymic mouse models of human hormone-dependent breast cancer xenografts (544) and in tissue culture of hormone-dependent human breast cancer cells (545–547). In these models, it was observed that although high, therapeutic concentrations of tamoxifen inhibited estrogen-stimulated proliferation of breast cancer cells, lower concentrations of tamoxifen actually stimulated breast can-

Figure 6.

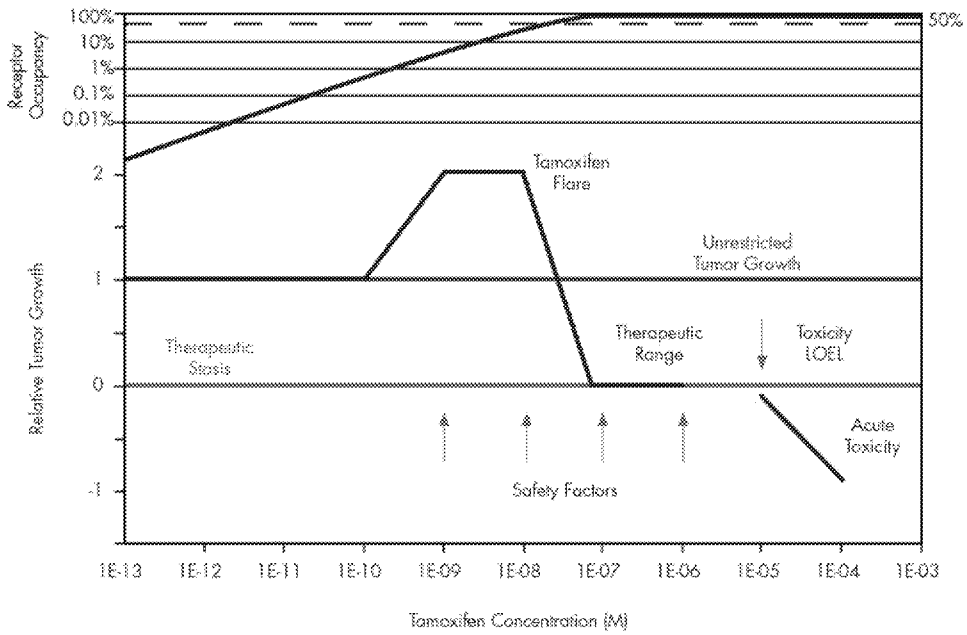

Figure 6. Dose-response ranges for tamoxifen in breast cancer therapy. This figure demonstrates the NMDRC, also called flare, in tamoxifen treatments. As the circulating dose of tamoxifen increases when treatment starts, patients initially experience flare, *i.e.* growth of the tumor (546), followed by a decrease in tumor size as the circulating levels of tamoxifen rise into the therapeutic range (676, 677). High doses of tamoxifen are acutely toxic (546). Starting from the highest concentrations, where acute toxicity is observed, and going to lower concentrations on the X-axis, the acute toxicity diminishes towards zero growth, *i.e.* therapeutic stasis (*green* baseline). This occurs at approximately 1E-05 m, the lowest observed effect level (LOEL) for toxicity. The vertical arrows show the results of applying three or four 10-fold safety factors to the LOEL for the high-dose toxicity of tamoxifen, and would calculate a safe or reference dose for tamoxifen in the region of flare, the least safe region of exposure in actual practice. Above the diagram of dose response ranges is estimated ER occupancy by tamoxifen. This was calculated from the affinity constant of tamoxifen for ERs determined in human breast cancer cells (Ki = 29.1 nM; Ref 678); flare appears to correspond to low receptor occupancy (*blue axis*), therapeutic range with mid and upper-range receptor occupancy, and acute toxicity well above 99% receptor occupancy. (678).

cer cell growth as long as the cells were estrogen dependent (548). Tamoxifen was also shown to disrupt tissue organization of the mammary gland, with specific effects on the stroma that may contribute to the observed effects on proliferation of epithelial cells (549, 550).

Tamoxifen therapy is administered as 10 mg twice per day (20 mg/d; approx 0.3 mg/kg body weight per day), but the target circulating levels are in the near submicromolar range (0.2–0.6 μM); these levels are reached slowly, after approximately 2 wks of therapy (551). In the initial period, where tamoxifen flare is observed, the circulating concentrations are ascending through lower concentrations, in the range below therapeutic suppression of growth, where breast cancer cell proliferation is actually stimulated by the drug, both in tissue culture, in animal xenograft studies, and in human patients (reviewed in Ref. 548). The recognition of this dual dose-response range for tamoxifen (low-dose, low-concentration estrogenic growth-stimulatory and higher-dose, higher-concentration estrogenic growth-inhibitory responses) led to the definition of the term selective estrogen response modulator, or SERM, activity (552–554). This SERM activity has since been observed for many or even most estrogenic EDCs, including BPA (3, 555–557).

These observations defined three separate dose-response ranges for the SERM tamoxifen in human clinical use. The lowest dose-response range, the range of flare, stimulated breast cancer growth and symptoms in some patients with hormone-dependent cancer. The next higher dose-response range is the therapeutic range where tamoxifen inhibits estrogen-dependent tumor growth. The highest dose range causes acute toxicity by the SERM (see Fig. 6).

Tamoxifen provides an excellent example for how high-dose testing cannot be used to predict the effects of low doses. For tamoxifen (as for other drugs), the range of acute human toxicity for tamoxifen was determined in phase I clinical trials. Phase I trials also defined an initial therapeutic range, the second dose-response range, as a dose below which acute toxicity was not observed. The therapeutic dose range was tested and further defined in phase II and later clinical trials to determine efficacy (see for example Ref. 558). Standard toxicological testing from

Figure 7.

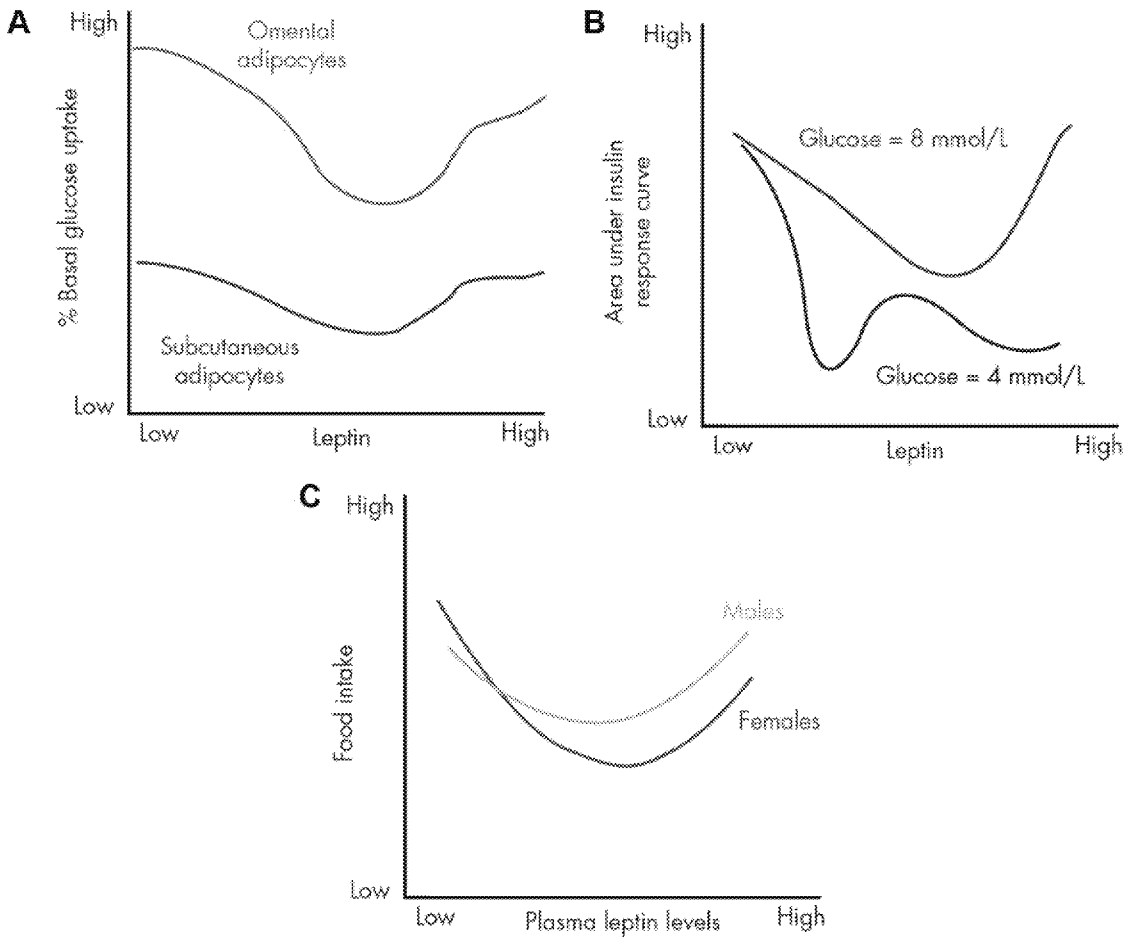

Figure 7. Leptin as an example of a NMDRC. Several studies report NMDRCs in response to leptin treatments. A, NMDRCs are observed in cultured primary adipocytes after leptin exposure. This graph illustrates the relationship between administered leptin dose and glucose uptake in two types of adipocytes, those isolated from omental tissue (*green*) and others from sc fat (*purple*) (schematic was made from data in Ref. 559). These data are on a log-linear plot. B, *Ex vivo* rat pancreas was treated with leptin and various doses of glucose, and the insulin response curves were examined. Area under the curve is a measure of the ability of the pancreas to bring glucose levels under control. Different dose-response curves were observed depending on the amount of glucose administered: a U-shaped curve when 8 mmol/liter was included (*pink*) or a multiphasic curve with 4 mmol/liter (*blue*) (schematic made from data in Ref. 560). These data are on a linear-linear plot. C, U-shaped NMDRCs were also observed when food intake was compared with leptin levels in the blood of rats administered the hormone. This response was similar in males (*orange*) and females (*cyan*) (schematic made from data in Ref. 562). These data are on a linear-linear plot.

high doses to define a LOAEL or NOAEL are equivalent to the phase I clinical testing, and in risk assessment, a safe dose or reference dose is calculated from these tests. However, the lowest dose range, with the highly adverse effects termed flare, was not detected in the phase I trials and was determined only for tamoxifen in breast cancer therapy at the therapeutic doses (543). The implication for risk assessment is that NMDRCs for EDCs, particularly those already identified as SERMs, would likely not be detected by standard toxicological testing at high doses. That is, the consequence of high-dose testing is the calculation of a defined but otherwise untested safe dose that is well within the range equivalent to flare, *i.e.* a manifestly unsafe dose of the EDC (Fig. 6).

5. Similarities in endpoints across cell culture, animal, and epidemiology studies: evidence for common mechanisms?

There are common trends in some findings of NMDRCs in cell, animal, and human studies and therefore evidence for related mechanisms for NMDRCs at various levels of biological complexity. Tamoxifen flare, discussed in *Section III.C.4*, is an informative example. Another illustrative example is that of the effect of the hormone leptin (Fig. 7). In cultured primary adipocytes, NMDRCs are observed after leptin exposure; moderate doses of leptin significantly reduce insulin-mediated glucose intake, whereas low and high doses maintain higher glucose intake in response to insulin (559). The rat pancreas shows a similar response to leptin; the amount of

secreted insulin has an inverted U-shaped response to leptin (560, 561). Even more striking is the relationship between leptin and food intake. Rats administered moderate doses of leptin consume less food compared to rats dosed with low or high levels of leptin (562); mechanistically, this lower food intake could be due to higher circulating glucose levels in these animals due to ineffective insulin action. And finally, in a human study, leptin levels were found to correlate with body mass index but have a U-shaped relationship with mortality (563). These results suggest that hormones can produce similar responses at several levels of biological complexity (cell, organ, animal, and population).

A large number of epidemiology studies with NMDRCs have found relationships between EDC exposures like POPs and metabolic diseases including obesity and diabetes (Table 9) (see also Ref. 564 for a review), and the mechanisms for these relationships have begun to be explored. Human and animal cells treated with EDCs in culture display NMDRCs that are relevant to these diseases: BPA has nonmonotonic effects on the expression of adipocyte proteins in preadipocytes and the release of adiponectin from mature adipocytes (565–567). Similarly, in female rodents, low doses but not high doses of BPA increased adipose tissue weight and serum leptin concentrations (568), and intermediate doses of phthalates decrease serum cholesterol levels (569). Thus, although understanding the mechanisms operating at the cellular level of organization has not yet led to definitive knowledge of the mechanisms producing NMDRCs in human populations, there appear to be strong similarities in cells, animals, and humans that support a call for continued work focusing on metabolic disease endpoints at each level of biological organization.

D. NMDRC summary

We have demonstrated that nonmonotonicity is a common occurrence after exposures to hormones and EDCs in cell culture and animals and across human populations. Because of the abundance of examples of NMDRCs, we expect that if adequate dose ranges are included in animal and cell culture studies, including the use of negative and well-chosen positive controls, NMDRCs may be observed more often than not. Here, we have focused mainly on studies that examined a wide range of doses, including many that examined the effects of doses that span the low-dose and toxicological ranges. We also discussed several mechanisms that produce NMDRCs. Each of these mechanisms can and does operate at the same time in a biological system, and this cooperative action is ultimately responsible for NMDRCs.

Understanding nonmonotonicity has both theoretical and practical relevance. When a chemical produces monotonic responses, all doses are expected to produce similar effects whose magnitude varies with the dose, but when a chemical produces a NMDRC, dissimilar or even opposite effects will be observed at different doses. Thus, monotonic responses can be modeled using the assumption that each step in a linear pathway behaves according to the law of mass action (43, 570); high doses are always expected to produce higher responses. In contrast, NMDRCs are not easy to model (although they are quite easy to test for), requiring detailed knowledge of the specific mechanisms operating in several biological components. From a regulatory standpoint, information from high doses cannot always be used to assess whether low doses will produce a biological effect (38).

IV. Implications of Low-Dose Effects and Nonmonotonicity

Both low-dose effects and NMDRCs have been observed for a wide variety of EDCs as well as natural hormones. Importantly, these phenomena encompass every level of biological organization, from gene expression, hormone production, and cell number to changes in tissue architecture to behavior and population-based disease risks. One conclusion from this review is that low-dose effects and NMDRCs are often observed after administration of environmentally relevant doses of EDCs. For both hormones and EDCs, NMDRCs should be the default assumption absent sufficient data to indicate otherwise. Furthermore, there are well-understood mechanisms to explain how low-dose effects and NMDRCs manifest *in vitro* and *in vivo*. Accepting these phenomena, therefore, should lead to paradigm shifts in toxicological studies and will likely also have lasting effects on regulatory science. Some of these aspects are discussed below. Additionally, we have briefly explored how this knowledge should influence future approaches in human and environmental health.

At a very practical level, we recommend that researchers publishing data with low-dose and nonmonotonic effects include key words in the abstract/article that identify them as such specifically. This review was unquestionably impeded because this has not been standard practice. We also strongly recommend that data showing nonmonotonic and binary response patterns not be rejected or criticized because there is no dose response.

A. Experimental design

1. Dose ranges must be chosen carefully

To detect low-dose effects or NMDRCs, the doses included for testing are of utmost importance. Most of the studies we examined here for nonmonotonicity tested

doses over severalfold concentrations. Unfortunately, regulatory guidelines only require that three doses be tested. Both low-dose effects and NMDRCs can be observed when examining only a few doses, but some studies may detect significant results purely by luck, because a small shift in dose can have a large impact on the ability to observe differences relative to untreated controls.

In the multitude of chemicals that have never been tested at low doses, or in the development of new chemicals, to determine whether a chemical has low-dose effects in laboratory animals, we suggest setting the NOAEL or LOAEL from traditional toxicological studies as the highest dose in experiments specifically designed to test endocrine-sensitive endpoints. We suggest setting the lowest dose in the experiment below the range of human exposures, if such a dose is known. Several intermediate doses overlapping the range of typical human exposures should be included also, bringing the total number in the range of five to eight total doses tested. Importantly, although the levels of many environmental chemicals in human blood and/or urine have been reported by the CDC and other groups responsible for population-scale biomonitoring, it is often not known what administered doses are needed to achieve these internal exposure levels in animals (4, 253); thus, toxicokinetic studies are often needed before the onset of low-dose testing. This is important because the critical issue is to determine what effects are observed in animals when circulating levels of an EDC match what is measured in the typical human. Due to differences in metabolism, route of exposure, and other factors, a relatively high dose may need to be administered to a rodent to produce blood concentrations in the range of human levels; however, this should not be considered a high-dose study.

It has also been suggested that animal studies that are used to understand the potential effects of a chemical on humans should use a relevant route of administration to recapitulate human exposures (571, 572) because there may be differences in metabolism after oral and nonoral administration. Many chemicals that enter the body orally undergo first-pass metabolism and are then inactivated via liver enzymes, whereas other routes (*i.e.* sc) can bypass these mechanisms and lead to a higher concentration of the active compound in circulation (573). Studies indicate, however, that inactivation of chemicals via first-pass metabolism is not complete and also that deconjugation of metabolites can occur in some tissues allowing the re-release of the active form (574, 575). Additionally, for some chemicals, it is clear that route of administration has little or no impact on the availability of the active compound in the body (241, 384), and other studies show that route of administration has no impact on the biological effects of these chemicals; *i.e.* regardless of how it enters the body, dioxin has similar effects on exposed individuals (384), and comparable results have been observed for BPA (141). Although understanding the typical route of human exposure to each environmental chemical is an important task, it has been argued that any method that leads to blood concentrations of a test chemical in the range they are observed in humans is an acceptable exposure protocol, and this is especially true with gestational exposures, because fetuses are exposed to chemicals only via their mothers' blood (31, 576).

2. Timing of exposures is important

Rodent studies indicate that EDC exposures during development have organizational effects, with permanent effects that can manifest even in late adulthood, whereas exposures after puberty are for the most part activational, with effects that are abrogated when exposures cease. For example, the adult uterus requires relatively large doses of BPA (in the parts-per-million range) to induce changes associated with the uterotrophic assay (555, 577), whereas parts-per-trillion and ppb exposures during the fetal period permanently and effectively alter development of the uterus (279, 310, 578). Thus, the timing of exposures is profoundly important to detect low-dose effects of EDCs.

Human studies also support this conclusion. The 1976 explosion of a chemical plant in Seveso, Italy, which led to widespread human exposure to large amounts of TCDD, a particularly toxic form of dioxin, and the deposition of this chemical on the land surrounding the chemical plant, provided evidence in support of the organizational and activational effects of endocrine-active chemicals in humans (579). Serum TCDD concentrations showed correlations between exposure levels and several disease outcomes including breast cancer risk, abnormal menstrual cycles, and endometriosis (580–582), but individuals who were either infants or teenagers at the time of the explosion were found to be at greatest risk for developing adult diseases (583, 584). Importantly, many scientists have argued that organizational effects can occur during puberty, *i.e.* that the period where hormones have irreversible effects on organ development extends beyond the fetal and neonatal period (585), and for some endpoints this appears to be the case (586, 587).

It has also been proposed that the endocrine system maintains homeostasis in the face of environmental insults (210). The adult endocrine system does appear to provide some ability to maintain a type of homeostasis; when the pharmaceutical estrogen DES is administered to pregnant mice, the circulating estradiol concentrations in the dam respond by decreasing linearly (224). In contrast, fetal concentrations of estradiol respond nonmonotonically in

a way that is clearly not correlated with maternal levels. Similarly, there is evidence that BPA can induce aromatase and therefore increase estradiol levels *in situ* in the fetal urogenital sinus (588). This is an example of a feed-forward positive-feedback effect rather than a homeostatic response. The effects of EDCs on adult subjects, both animal and people, suggest that diseases often result from low-dose adult exposures (589–595); this argues against a view of the endocrine system as a means to maintain homeostatic control. Instead, individuals can be permanently changed, in an adverse way, after EDC exposures.

In one example, pregnant mice were exposed to low concentrations of BPA, and their male offspring had altered pancreatic function at 6 months of age (158). Surprisingly, however, the mothers (exposed only during pregnancy) were also affected, with altered metabolic machinery and body weight at 4 months postpartum, long after exposures had ended. The increased incidence of breast cancer in women that took DES during pregnancy also illustrates this point (596, 597). These studies suggest that even the adult endocrine system is not invariably capable of maintaining a so-called homeostatic state when exogenous chemicals affecting the endocrine system are present. Thus, although adult exposures to EDCs have been given some attention by bench scientists (29), more work of this kind is needed to better understand whether and how EDCs can have permanent organizational effects on adult animals.

At the beginning of this review, we justified the need to critically examine the low-dose literature because of recent epidemiological findings linking EDC exposures and diseases. Yet there is inherent difficulty in examining neonatal exposures to EDCs and their connection to diseases due to the length of time needed for these studies; thus, many studies of this type have examined high doses of pharmaceuticals (*i.e.* DES) or accidental exposures to industrial chemicals (*i.e.* dioxin) (66, 398, 399, 581, 597–601).

Only recently, with the availability of biomonitoring samples from large reference populations, have lower doses begun to receive widespread attention from epidemiologists. Many recent studies have examined adult exposures to EDCs and correlated exposures with disease statuses (see for example Refs. 15, 16, and 602–604). Human studies examining fetal/neonatal exposures to low-dose EDCs and early life effects have also begun to be studied (6, 333, 605–607), although studies linking these early life exposures to adult diseases are likely to be decades away. More than anything, these studies support our view that the effects of low-dose exposures should be considered when determining chemical safety.

3. Importance of endpoints being examined

Traditional toxicology testing, and in particular those studies performed for the purposes of risk assessment, typically adhere to guideline studies that have been approved by international committees of experts (608). The endpoints assessed in these guideline-compliant studies are centered around higher-order levels, including death, weight loss, mortality, and changes in organ weight, and a limited number of histopathological analyses (609, 610). When pregnant animals are included in toxicological assessments, the endpoints measured typically include the ability to maintain pregnancies, the number of offspring delivered, sex ratios of surviving pups, and measures regarding maternal weight gain and food/water intake (610).

Yet low-dose EDCs are rarely toxic to the point of killing adult animals or causing spontaneous abortions, and traditional tests such as the uterotrophic assay have been shown to be relatively insensitive (72, 577). It has been argued that this type of testing is insufficient for understanding the effects of EDCs (31, 70, 495, 611). Many EDC studies have instead focused on examining newly developed, highly sensitive endpoints that span multiple levels of biological organization, from gene expression to tissue organization to organ systems to the whole animal (612), which may not be rapidly lethal but which nonetheless have enormous importance for health, including mortality. Thus, for example, studies designed to examine the effects of chemicals on obesity no longer focus on body weight alone but also analyze gene expression; fat content in adipose cells and the process of adipogenesis; inflammation, innervation, and vascularization parameters in specific fat pads; conversion rates of white and brown adipose tissues; systemic hormone levels and response to glucose and insulin challenges; and food intake and energy expenditures, among others (314, 613–615). As our knowledge of EDCs and the endocrine system continue to grow, the most sensitive endpoints should be used to determine whether a chemical is disrupting the development of organisms (70).

In moving beyond traditional, well-characterized health-related endpoints like mortality and weight loss, an important question has been raised: how do we define endpoints as adverse? This is an important point, because it has been suggested that the creative endpoints examined in independent EDC studies are not validated and may not represent adverse effects (609). There is also debate over whether the mechanism (or mode) of action must be explained for each effect to determine whether a relevant pathway is present in humans (616, 617). Yet, when originally assessing the low-dose literature, the NTP expert panel chose to examine all effects of EDC exposure, re-

gardless of whether the endpoint could be deemed adverse (2). From the perspective of developmental biology, any change in development should be seen as adverse, even if the change itself is not associated with a disease or dysfunction. Some of these developmental changes, in fact, may increase sensitivity or susceptibility to disease later on in life but will otherwise appear normal. Furthermore, studies of heavy metals have shown that small shifts in parameters like IQ may not have drastic effects on individuals but can have serious repercussions on the population level (618), and therefore changes in the variance/observable range of a phenotype should also be considered adverse (52).

4. Importance of study size

National Institutes of Health guidelines require that the number of vertebrate animals used in experiments be as small as possible to show statistically significant effects based on power analysis. Yet many traditional toxicology studies have used large numbers of animals to draw conclusions about chemical safety. When the endpoints being assessed have binary outcomes (*i.e.* animal has a tumor *vs.* animal does not have a tumor) and the incidence of the phenotype is not high, a large number of animals is required to reveal statistically significant effects. In contrast, many of the endpoints examined in the field of endocrine disruption are more complex and are not binary; thus, power analysis allows researchers to determine how many animals are needed to observe statistically significant (and biologically relevant) differences between control and exposed populations. For this reason, arbitrary numbers set as cutoffs for determining whether a study is acceptable or unacceptable for risk assessments are not appropriate. Instead, the number of animals required for a study to be complete is dependent on the effect size, precision/variance, minimal meaningful difference to be considered between populations, and the α-value set in statistical tests.

B. Regulatory science

For decades, regulatory agencies have tested, or approved testing, of chemicals by examining high doses and then extrapolating down from the NOAEL, NOEL, and LOAEL to determine safe levels for humans and/or wildlife. As discussed earlier, these extrapolations use safety factors that acknowledge differences between humans and animals, exposures of vulnerable populations, interspecies variability, and other uncertainty factors. These safety factors are informed guesses, not quantitatively based calculations. Using this traditional way of setting safe doses, the levels declared safe are never in fact tested. Doses in the range of human exposures are therefore also unlikely to be tested. This has generated the current state of science, where many chemicals of concern have never been examined at environmentally relevant low doses (see Table 4 for a small number of examples).

Assumptions used in chemical risk assessments to estimate a threshold dose below which daily exposure to a chemical is estimated to be safe are false for EDCs. First, experimental data provide evidence for the lack of a threshold for EDCs (619). More broadly, the data in this review demonstrate that the central assumption underlying the use of high doses to predict low-dose effects will lead to false estimates of safety. The use of only a few high doses is based on the assumption that all dose-response relationships are monotonic and therefore that it is appropriate to apply a log-linear extrapolation from high-dose testing to estimate a safe reference dose (Fig. 4). The Endocrine Society issued a position statement on EDCs (620) and urged the risk assessment community to use the expertise of their members to develop new approaches to chemical risk assessments for EDCs based on principles of endocrinology. Undertaking this mission will represent a true paradigm shift in regulatory toxicology (79). The Endocrine Society statement was then supported in March 2011 by a letter to *Science* from eight societies with relevant expertise representing over 40,000 scientists and medical professionals (621).

Studies conducted for the purposes of risk assessment are expected to include three doses: a dose that has no effects on traditional toxicological endpoints (the NOAEL), a higher dose with effects on traditional endpoints (the LOAEL), and an even higher dose that shows toxicity. Although reducing the number of animals used for these types of studies is an important goal, more than three doses are often needed for a true picture of a chemical's toxicity. The examination of a larger number of doses would allow for 1) the study of chemicals at the reference dose, *i.e.* the dose that is calculated to be safe; 2) examination of doses in the range of actual human exposures, which is likely to be below the reference dose; and 3) the ability to detect NMDRCs, particularly in the low-dose range. The impact of testing more doses on the numbers of animals required can be mitigated by use of power analysis, as suggested above. Because no amount of research will ever match the diversity and reality of actual human experience, there should be ongoing epidemiological study of potential adverse effects of EDCs even after safe levels are published, with periodic reevaluation of those safe levels.

One issue that has been raised by regulatory agencies is whether animal models are appropriate for understanding the effects of EDCs on humans. These arguments largely center around observed differences in hormone levels during different physiological periods in rodents and humans (57), and differences in the metabolic machinery and ex-

cretion of chemicals between species (622). To address the first issue, it should be noted that the FDA uses animals to test pharmaceuticals and other chemicals before any safety testing in humans because it is widely recognized that, although animals and humans do not have exactly the same physiologies, there is evolutionary conservation among vertebrates and specifically among mammals (62). Furthermore, animal studies proved to be highly predictive of the effects of DES on women, indicating that rodents are sufficiently similar to humans to reliably forecast affected endpoints in the endocrine system (64, 623). Thus, the default position must be that animal data are indicative of human effects until proven otherwise.

With regard to the second issue, BPA researchers in particular have examined species-specific differences in metabolism of this EDC. Interestingly, the pharmacokinetics of BPA in rodents, monkeys, and humans appear to be very similar (624), and regulatory agencies have subsequently concluded that rodents are appropriate models to assess the effects of this chemical (625, 626). Thus, researchers should select animal models that are sensitive to low doses of hormones and select appropriate species for the endpoints of interest. As the scope of our knowledge has broadened about how chemicals can alter the endocrine system, well beyond estrogens, androgens, and the thyroid, it is imperative that considerable thought be given to how to apply this for regulatory purposes.

C. Human health

As discussed several times throughout this review, there is now substantial evidence that low doses of EDCs have adverse effects on human health. Thus, although many epidemiological studies originally focused on occupationally exposed individuals and individuals affected by accidental exposures to high doses of environmental chemicals, these recent studies have suggested wide-ranging effects of EDCs on the general population.

Importantly, human exposures are examples of true mixtures; dozens if not hundreds of environmental chemicals are regularly detected in human tissues and fluids (91), yet very little is known about how these chemicals act in combination (627). Several studies indicate that EDCs can have additive or even synergistic effects (143, 323, 628–630), and thus these mixtures are likely to have unexpected and unpredictable effects on animals and humans. The study of mixtures is a growing and complex field that will require considerable attention in the years ahead as knowledge of EDCs in the laboratory setting are applied to human populations (631, 632).

How much will human health improve by testing chemicals at low, environmentally relevant doses and using the results to guide safety determinations? Current testing paradigms are missing important, sensitive endpoints; because they are often unable to detect NMDRCs, they cannot make appropriate predictions about what effects are occurring at low doses. At this time, it is not possible to quantify the total costs of low-dose exposures to EDCs. However, current epidemiology studies linking low-dose EDC exposures to a myriad of health problems, diseases, and disorders suggest that the costs of current low-dose exposures are likely to be substantial.

The weight of the available evidence suggests that EDCs affect a wide range of human health endpoints that manifest at different stages of life, from neonatal and infant periods to the aging adult. As the American population ages, healthcare costs continue to rise, and there are societal costs as well, with decreased quality of life concerns, decreases in work productivity due to illness or the need for workers to care for affected family members, and the psychological stresses of dealing with some outcomes like infertility. Thus, it is logical to conclude that low-dose testing, followed by regulatory action to minimize or eliminate human exposures to EDCs, could significantly benefit human health. This proposal effectively calls for greatly expanded research to give human communities feedback about themselves. It emanates from a view that human society benefits greatly from the many chemical compounds it uses but that extensive epidemiological surveillance and other focused research designs are needed to assure that the balance of risk/benefit from those chemicals is acceptable.

How much would human health benefit by a reduction in the use of EDCs? For some chemicals, minor changes in consumer habits or industrial practices can have drastic effects on exposures (633–636). Other chemicals like DDT that have been regulated in the United States for decades continue to be detected in human and environmental samples; the persistent nature of many of these agents suggests they may impact human health for decades to come. Even less-persistent chemicals like BPA are likely to remain in our environment long after a ban is enacted because of the large amounts of plastic waste leaching BPA (and other estrogenic compounds) from landfills into water sources (637) and its presence on thermal receipt paper and from there into recycled paper (638–640). Yet, despite these challenges, reducing human exposure to EDCs should be a priority, and one way to address that priority is to decrease the production and use of these chemicals. The Endocrine Society has called for such a reduction and the use of the precautionary principle, *i.e.* action in the presence of concerning information but in the absence of certainty to eliminate or cut the use of questionable chemicals even when cause-effect relationships are not yet established (620).

D. Wildlife

Much of the recent focus on EDCs has been on the impact of these chemicals on human health. Yet the earliest studies of EDCs that focused on the impact of these chemicals on wildlife should not be forgotten. Rachel Carson's work on DDT and other pesticides provided some of the earliest warning signs that there were unintended consequences of chemical use. Carson's work was ahead of its time; she understood that exceedingly small doses of these chemicals produced adverse effects, that the timing of exposures was critical, and that chemical mixtures produced compounded effects (641). Now, decades after some of the most dangerous EDCs have been regulated, they continue to be measured in environmental samples as well as the bodies of wildlife animals.

Furthermore, it should be pointed out that humans, like wildlife, are not insulated from the environment, and effects in wildlife, including nonmammalian species, are indicative of and mirror effects in humans. For example, BPA has estrogen-like effects in fish (642–644), amphibians (645, 646), and reptiles (647, 648). A recent review showed that demasculinizing and feminizing effects of atrazine have been demonstrated in fish, amphibians, reptiles, birds, and mammals, *i.e.* every vertebrate class examined (326); and in fact, the first report to suggest that atrazine induced aromatase was conducted in reptiles (649). Similarly, perchlorate affects fish (650–653), amphibians (654–658), and birds (659–661) via mechanisms consistent with those described for humans, and some of the earliest reports on perchlorate's effects on thyroid function were conducted in amphibians (661, 662). Finally, ecological studies of dioxin and dioxin-like chemicals reveal effects on a range of exposed wildlife including birds (663, 664), fish (665, 666), and invertebrates (667). Although these studies have highlighted some of the species-specific effects of dioxin (389), and orders of magnitude differences in toxic equivalency factors between species (668), they also indicate the conservation of mechanisms for the effects of dioxin on a range of biological endpoints in wildlife, laboratory animals, and humans (384). In fact, in many cases, nonmammalian species are much more sensitive to EDC effects, and wildlife species serve as sentinels for environmental and public health (669–673). Thus, the effects of these chemicals on wildlife populations are likely to continue; for this reason, the low-dose effects of these chemicals are particularly worth understanding (674, 675).

V. Summary

In conclusion, we have provided hundreds of examples that clearly show that NMDRCs and low-dose effects are common in studies of hormones and EDCs. We have examined each of these issues separately and provided mechanistic explanations and examples of both. These topics are related, but they must be examined individually to be understood. The concept of nonmonotonicity is an essential one for the field of environmental health science because when NMDRCs occur, the effects of low doses cannot be predicted by the effects observed at high doses. In addition, the finding that chemicals have adverse effects on animals and humans in the range of environmental exposures clearly indicates that low doses cannot be ignored.

In closing, we encourage scientists and journal editors to publish data demonstrating NMDRCs and low-dose effects, even if the exact mechanism of action has not yet been elucidated. This is important because the study of EDC is a growing specialty that crosses many scientific fields, and scientists that work on or regulate EDCs should appreciate and acknowledge the existence of NMDRCs and low-dose effects and have access to this important information. We further recommend greatly expanded and generalized safety testing and surveillance to detect potential adverse effects of this broad class of chemicals. Before new chemicals are developed, a wider range of doses, extending into the low-dose range, should be fully tested. And finally, we envision that the concepts and empirical results we have presented in this paper will lead to many more collaborations among research scientists in academic and government laboratories across the globe, that more and more sophisticated study designs will emerge, that what we have produced herein will facilitate those making regulatory decisions, that actions taken in light of this information will begin to abate the use of EDCs, and ultimately that health impacts in people and in wildlife will be averted.

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Address requests for reprints to: Laura N. Vandenberg, Tufts University, Center for Regenerative and Developmental Biology, 200 Boston Avenue, Suite 4600, Medford, Massachusetts 02155. E-mail: laura.vandenberg@tufts.edu; or J. P. Myers, Environmental Health Sciences, 421 Park Street, Charlottesville, Virginia 22902. E-mail: jpmyers@ehsciences.org.

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