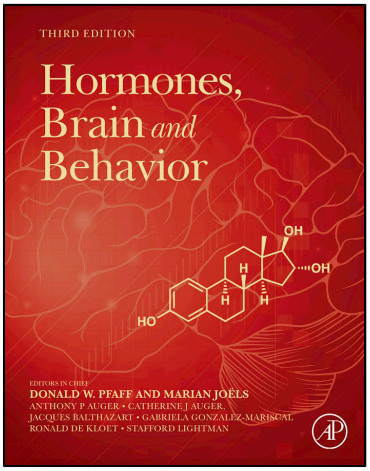

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5.04 Environmental Endocrine Disruption of Brain and Behavior

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Glossary

Endocrine disruption The process by which exogenous or endogenous chemicals cause perturbations in endocrine and/or reproductive systems.

Epigenetics The interactions between the environment and the genome by mechanisms, independent of genetic mutation, resulting in modifications of gene expression.

Fetal basis of adult disease The scientific discipline studying the interactions of an individual with its fetal environment (e.g., the uterus or the egg), its external environment (e.g., exposures to environmental endocrine-disrupting chemicals), and its genome and epigenome, resulting in an individual predisposition (or lack thereof) for the development of a disease later in life. An important component of this hypothesis is that early developmental organisms are particularly vulnerable to external perturbations.

Gonadotropin-releasing hormone (GnRH) The hypothalamic 10-amino-acid peptide that controls reproductive function in vertebrates.

Hypothalamic–pituitary–gonadal axis The reproductive system of vertebrates, comprising the hypothalamus at the base of the brain, the anterior pituitary gland, and the gonads (ovary or testis).

No observed adverse effect level (NOAEL) It is a commonly used, but flawed, approach in risk assessment.

Sexual differentiation of the brain The process by which hormones, chromosomes, and the environment interact in

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the developing nervous system to permanently affect sexual behavior and physiology in adulthood.

Transgenerational effects Effects that are observed not only in an exposed organism but also in that organism's offspring and future generations.

Xenobiotic Literally, a foreign biological agent; an exogenous substance that exerts biological effects.

5.04.1 Introduction to Endocrine Disruption

Endocrine-disrupting chemicals (EDCs) comprise a wide variety of natural and synthetic compounds that exert actions upon hormonally sensitive pathways, resulting in endocrine and/or reproductive dysfunctions (Diamanti-Kandarakis et al., 2009; Gore et al., 2015). There are numerous industrial chemicals, pesticides, fungicides, plasticizers, pharmaceuticals, phytoestrogens, and other compounds that are endocrine disrupting (**Table 1**). Although their mechanisms of action vary and are in some cases not fully understood, they may include any or all of the following: nuclear hormone receptors, membrane hormone receptors, enzymes involved in the biosynthesis/metabolism/degradation of hormones, coregulatory factors, and neurotransmitter systems in the brain that control neuroendocrine functions, among others (reviewed in Gore et al., 2006; Gore, 2007; Patisaul and Adewale, 2009; Frye et al., 2012). It is not atypical for EDCs to simultaneously act via multiple mechanisms. Our bodies and environment continuously contain a complex, dynamic mixture of EDCs arising from a myriad of sources. EDCs enter our bodies and the environment via varied routes including hormonally active foods (phytoestrogens are a prime example of naturally occurring EDCs), use of personal care products, leaching from food contact materials, discharges from municipal wastewater treatment plants, within effluents from industrial processes, runoff from agricultural fields, atmospheric deposition, and landfill leachate (Frye et al., 2012; Orlando and Ellestad, 2014; Annamalai and Namasivayam, 2015). EDCs and other chemicals in the barrage of products we use every day are excreted or washed away quite literally down the drain and into municipal wastewater treatment plants where they are not efficiently removed before the disinfected water is discharged or reclaimed (Ramos et al., 2008; Sun et al., 2013). Some, including polychlorinated biphenyls (PCBs), perfluorinated compounds (e.g., PFOA), polybrominated diphenyl ether (PBDE) flame retardants, and other persistent, lipophilic compounds, have the capacity to bioaccumulate and biomagnify in higher trophic levels. Others, such as dichlorodiphenyltrichloroethane (DDT), can biotransform into other, even more bioactive metabolites (dichlorodiphenyldichloroethylene (DDE)). Still others, such as bisphenol A (BPA), are rapidly metabolized and can be virtually eliminated from the body if exposure ceases.

PCBs are a well-characterized prototypical EDC example. PCBs are a family of highly stable organochlorides. Initially synthesized in 1881, they entered commercial production in 1927 and were sold as mixtures for a wide range of applications including the use as coolant fluids in electric motors, transformers, and capacitors and as plasticizers and stabilizers in paints and cement, fire retardants, hydraulic fluid, adhesives, pesticide mixtures, and sealants. PCBs were banned from use in the United States in 1977 for their toxicity and ability to biomagnify up the food chain. As a class, the PCBs are one of the most persistent and ubiquitous environmental

Table 1 Common sources of endocrine-disrupting chemicals (EDCs)

Source	*EDCs*
Children's products	Cadmium, phthalates, lead, fire retardants, formaldehyde
Cookware	Perflurochemicals (PFCs) including perfluorooctanoic acid (PFOA or C8 which is used to make Teflon)
Electronics, building materials, and furniture	Bisphenol A (BPA), D4, fire retardants, glycol ethers, polychlorinated biphenyls (PCBs), polyvinyl chloride (PVC)
Food contact materials	BPA, perfluorinated chemicals (PFCs), phthalates, ultraviolet (UV) filters
Food contaminants[a]	Arsenic, dichlorodiphenyltrichloroethane (DDT), dioxins (TCDD), fire retardants, PCBs, BPA, pesticides, herbicides, fungicides, and other persistent organic pollutants
Hormonally active foods	Soy and other legumes (genistein, daidzein), lignins, red wine (resveratrol), clover, and alfalfa (coumestrol)
Lawn and garden chemicals	Pesticides, herbicides, and fungicides including atrazine, malathion, synthetic pyrethroids, vinclozolin, organophosphates, and organochlorines
Personal care products and sunscreens	Parabens, phthalates, polycyclic musks, triclosan, UV filters
Pharmaceuticals	Ethinyl estradiol (EE), diethylstilbestrol (DES), and other synthetic hormones; tamoxifen, finasteride, letrozole, phthalates in the coating of capsules and pills
Textiles, clothing, camping gear	PFCs including perfluorooctanesulfonic acid (PFOS), which is the key ingredient of Scotchgard, and related compounds including C6 and C8 PFCs

[a]The US FDA is tracking 500+ chemicals in food at trace levels.

pollutants ever developed and are present in every ecosystem on Earth and in the bodies of virtually all living organisms, including humans, making them one of the most devastating environmental pollutants of the twentieth century (Rosner and Markowitz, 2013). At high doses (levels typical of occupational exposure or an industrial accident) PCBs are toxic, are carcinogenic, and can cause distinctive skin lesions called chloracne, but also liver disease, lethargy, suppressed appetite, and depressed libido. At lower doses (more typical of environmental exposure), PCBs are endocrine disrupting. For example, PCB congeners can interact with the aryl hydrocarbon receptor, nuclear hormone receptors (estrogen, androgen, thyroid), and neurotransmitter receptors (acetylcholine, dopamine, GABA, serotonin) (e.g., Kuiper et al., 1998; Safe, 1984; Dickerson and Gore, 2007; Winneke, 2011). PCBs also exert actions on other steroid-regulatory or steroid-sensitive pathways including inhibition of estrogen sulfotransferase (Connor et al., 1997; Kester et al., 2000; Ohtake et al., 2003). With specific regard to neuroendocrine systems, PCBs can disrupt aspects of reproductive physiological and behavioral functions controlled by the preoptic area (POA) of the hypothalamus (Chung and Clemens, 1999; Chung et al., 2001; Steinberg et al., 2007). Some forms have also been shown to interfere with intracellular calcium homeostasis by enhancing the activity of ryanodine receptors, thereby interrupting signal transduction (Fischer et al., 1998), and activate the nuclear receptors PXR and CAR (Al-Salman and Plant, 2012). Along with other persistent organic pollutants, PCBs have now also been linked to cognitive impairments, behavioral problems in children, low birthweight, diabetes, obesity, dyslipidemia, and insulin resistance (Lee et al., 2011; Winneke, 2011; Paule et al., 2012). The PCBs thus serve as a well-characterized example of how widely and via a diversity of mechanisms EDCs can impact reproductive and endocrine functions.

This chapter summarizes the evidence of EDC effects on vertebrate neuroendocrine systems, focusing on the consequences for the development of sexually dimorphic neural pathways, behaviors, and physiological functions. Although a formal definition for what constitutes 'neuroendocrine disruption' has not yet been established, we and others have used the term to broadly describe chemical impacts on endocrine-related brain development and function (Gore and Patisaul, 2010; Waye and Trudeau, 2011). Importantly, neuroendocrine disruption is distinct from, and should not be considered synonymous with, neurotoxicity, which focuses primarily on neuronal cell death and related downstream consequences (e.g., dopaminergic cell death and Parkinson's like symptoms) and effects resulting in peripheral neuropathies. EDCs are not necessarily neurotoxic.

The ability of organisms to exhibit sex-appropriate reproductive physiological and behavioral functions in adulthood is, in most species, contingent upon highly coordinated exposures (or the lack thereof) to specific endogenous hormones during critical developmental windows (reviewed in Gore, 2008; McCarthy et al., 2009; and see Chapter 2.10, Hormones, Brain, and Behavior in Reptiles; Chapter 2.11, Neuroendocrine Regulation of Reproductive Behavior in Birds; Chapter 5.01, Sexual Differentiation of the Brain: A Fresh Look at Mode, Mechanisms, and Meaning; Chapter 5.09, Sexual Differentiation of Brain and Behavior in Birds; and Chapter 5.10, Sexual Differentiation of Behavior in Nonhuman Primates). This concept of timing is particularly relevant to the field of neuroendocrine disruption, as exposure to exogenous substances during these critical periods, or even to endogenous hormones (e.g., from the maternal environment), may have transient or permanent effects, including multi- or even transgenerational effects. This chapter will highlight the most vulnerable neuroendocrine systems and critical windows of exposure. We acknowledge that the different vertebrate classes are differentially affected by EDCs and have thus taken a comparative approach to the field of neuroendocrine disruption to some degree, but, because the EDC literature is so large and human-related effects are of particular interest, this chapter focuses most heavily on mammals. Importantly, research across the evolutionary spectrum has been particularly enlightening about the mechanisms by which EDCs exert their actions, and for more information on that aspect of EDC work and sex differences in EDC actions in invertebrates, the reader is referred to McClellan-Green et al. (2007) and Waye and Trudeau (2011).

5.04.1.1 Critical Issues about Endocrine Disruption

Several key topics have emerged in an effort to define endocrine-disrupting activity/mechanisms of action and distinguish them from other classes of toxicants. While fundamental to endocrine disruption they are now also keystone principles of the 'developmental origins of adult disease' (DoHAD) concept (Schug et al., 2011; Haugen et al., 2014).

5.04.1.1.1 Life Stage and Timing

Life stage is a key consideration in interpretation of EDC data. There are critical windows of developmental sensitivity to natural or exogenous hormones or hormonelike compounds. In mammals, the late embryonic/early postnatal period is considered a critical period for brain sexual differentiation, during which exposure to a hormone, or the lack thereof, causes permanent molecular changes in the brain (Gore, 2008; Phoenix et al., 1959; Wright et al., 2010). The peripubertal period is a less explored, but also potentially vulnerable time for organizational EDC-related impacts (Sisk and Zehr, 2005; Ahmed et al., 2008; Sisk, 2016). Later in life, in response to increased gonadal steroid hormones during puberty, these organizational effects of hormones on the brain are manifested as appropriate masculine or feminine reproductive physiology and behaviors (Dohler, 1991; Rhees et al., 1990). Endocrine disruption, during the critical periods of brain sexual differentiation, is therefore particularly detrimental. For example, the risk of reproductive maladies common to children born to mothers who took the diethylstilbestrol (DES) during pregnancy varies substantially depending on the timing of the mother's first exposure, total dose, and length of exposure (Robboy et al., 1981; Robboy et al., 1984; Faber et al., 1990).

Animals may retain responsiveness to EDCs throughout life (Brezner et al., 1984), although, in general, effects in the adult are less likely to be permanent than those which occur in a developing organism. For example, excessive phytoestrogen intake has repeatedly been shown to suppress

ovulation and fertility in a diverse range of vertebrates (Patisaul and Jefferson, 2010), including women, but is reversible if consumption decreases (Chandrareddy et al., 2008).

In vertebrates, developmental exposure is ubiquitous and multifaceted. Placental and lactational transfer of hundreds of EDCs is well documented in a wide range of species, including humans (for example, see Jacobson et al., 1984; Doerge et al., 2001, 2011). Since 2001, the Centers for Disease Control (CDC) has been annually publishing a list of the chemicals it could detect in human fluids, including cord blood, a list which has 300+ chemicals and is growing. Thus humans and other vertebrates are born 'prepolluted' with a cocktail of EDCs and other chemicals with suspected neurodevelopmental activity (for a relevant review see Grandjean and Landrigan, 2014). In nonmammalian species, the embryo may be exposed to EDCs through the yolk. Bern (1990) first noted the significance of maternal steroid accumulation in yolking follicles and that these hormones can influence offspring phenotype in a number of species, a finding now supported in studies with fish, amphibians, reptiles, and birds (Bowden et al., 2000; Cariello et al., 2006; Eising et al., 2001; Elf, 2004; Elf et al., 2002; Gil et al., 1999; Groothuis and Schwabl, 2008; Lipar and Ketterson, 2000; Martin and Schwabl, 2007; Ottinger et al., 2005a; Pilz et al., 2005; Radder et al., 2007; Rhen et al., 2006; Schwabl, 1993, 1996; Sockman et al., 2006; Verboven et al., 2003). Numerous studies have established that, both for aquatic and groundwater environments, contamination penetrates into the egg resulting in EDC profiles in the yolk that match those of the surrounding environment (Bishop et al., 1998; Gale et al., 2002; Halldin, 2005; Heinz et al., 1991; Peterman et al., 1996; Podreka et al., 1998).

5.04.1.1.2 Differential Sensitivity to EDCs

In addition to age, vulnerability to EDCs varies across species and strain (Crews et al., 2000; Spearow et al., 1999; Thigpen et al., 2007). This should not be surprising considering the substantial differences among species in the nature and amount of circulating concentrations of steroid hormones, with differences of similar magnitude existing between inbred strains (Shire, 1976). In some species, testosterone may be the predominant steroid in males, but in other species it may be dihydrotestosterone or 11-ketotestosterone. In females, estrone (E1), 17β-estradiol (E2), and estriol (E3) are the three primary forms of steroidal estrogen with E2 considered the most potent. While this may be true for humans, it does not follow for all other mammals and other vertebrate classes. For example, in the red-eared slider turtle sex-determination assay, E3 is far more potent than E2 in overcoming the effects of a male-producing incubation temperature (Bergeron et al., 1999). Similarly, *o,p′*-DDE and its metabolites have been implicated in a variety of reproductive anomalies, but not in all species. In the red-eared slider turtle, DDE causes female development when applied in concentrations found in contaminated environments (Willingham and Crews, 1999), but it has no detectable effect in the green sea turtle, *Chelonia mydas* (Podreka et al., 1998), and is antiestrogenic in the tiger salamander, *Ambystoma tigrinum* (Clark et al., 1998). In the rat, methoxychlor causes delayed anovulatory syndrome (Gray et al., 1989), but in hamsters it is without apparent effect on estrous cycling (Gray et al., 1985). On the other hand, the pesticide Kepone (chlordecone) masculinizes the behavior of female hamsters (Gray, 1982) but, in rats, seems to inhibit female sexual behavior when administered at proestrus (Brown et al., 1991), fails to induce lordosis in ovariectomized females (Uphouse et al., 1986), and accelerates puberty (Gellert, 1978). Disruption of testicular development by phthalates is also notoriously species specific. In the rat, prenatal phthalate exposure causes male reproductive tract malformations, primarily via inhibition of fetal Leydig cell androgen production. This effect is not observed in the mouse, despite mouse phthalate pharmacokinetics being similar to the rat (Johnson et al., 2012). Species-specific differences have made assessing risk to humans difficult for phthalates and other EDCs.

Species and individual differences in behavioral sensitivity to a steroid hormone are also due to the abundance of the associated receptor in the brain, which is inversely proportional to the typical levels in the circulation (Young and Crews, 1995). This has implications for EDCs in that species with relatively low levels of endogenous estrogen(s) in their circulation will be more sensitive to chemicals that mimic the action of estrogen (Crews et al., 2000). Thus, rather than concentrating on a few species to serve as standards for defining a chemical as an EDC, a more instructive approach is to document the effects and then understand why different species vary in their sensitivity to specific compounds. In toxicology, multistrain approaches are being developed to identify fundamental mechanisms of acute toxicity and biomarkers of susceptibility. For example, a panel of 36 inbred mouse strains was used to reveal that acetaminophen toxicity-induced liver injury was associated with polymorphisms in four genes, but susceptibility to hepatotoxicity was associated with a different subset (Harrill et al., 2009). Similar types of approaches are being used to understand individual differences in susceptibility to DES, PCBs, and other EDCs. Published examples of these kinds of toxicogenomic approaches for EDC effects remain rare but potentially powerful for understanding the basic biology of interindividual differences in disease and chemical sensitivity.

Clearly, genetics also plays a role in resistance to EDC-related effects. For example, Ozburn and Morrison (1962) produced *o,p′*-DDT-tolerant mice after eight generations of selection, and Poonacha et al. (1973) fed selected lines of quail a diet containing 200 ppm of DDT during the quails' first 30 days of life. The development of resistance was evident after the third generation of selection, as indicated by lower mortality among the selected lines when compared to a control line that was fed DDT. Interestingly, there was a sex difference in resistance, with females being less resistant than males. Numerous examples of EDC-driven evolution have also been observed in fish living in water heavily contaminated by PDBs and dioxins and other persistent organic pollutants. In some instances this adaptation comes with reduced tolerance to other stressors including temperature fluctuations and hypoxia. Tolerance can allow for high body burdens which, consequently, lead to greater exposure for predators. Whether or not evolution of EDC-resistance comes with unanticipated costs to fitness in other species is unclear and poorly explored but not out of the realm of possibility.

5.04.1.1.3 Sex Differences in Sensitivity

It is remarkable that although the issue of sex differences has been a dominant topic in behavioral neuroendocrinology over 50 years, and within the larger biomedical community since 1993 (Food and Drug Administration, 1993), it is only relatively recently that sex differences are coming to the fore as an important factor in EDC research, or in neuroscience research more generally. Because the EDC field essentially emerged from a community focused on reproductive endpoints, sex-specific effects have always been a cornerstone of EDC research, but impacts on sexually dimorphic neuroendocrine pathways, behaviors, and functions outside the reproductive system remain poorly explored (Rebuli and Patisaul, 2015). Epidemiological data in children have repeatedly associated exposures to EDCs such as PBDEs and PCBs with a higher risk of sex-biased psychosocial disorders including autistic spectrum disorder, attention deficit hyperactivity disorder, and anxiety disorders (Eubig et al., 2010; Sagiv et al., 2010; Braun et al., 2011; Cheslack-Postava et al., 2013), but the mechanisms by which EDCs may heighten risk are unclear, and supporting evidence in appropriate animal models remains minimally available. Understanding the sex-specific neural outcomes of developmental endocrine disruption is fundamental for identifying the degree to which EDCs may be contributing to mental health and other neuroendocrine disorders, and a knowledge gap that remains a priority for future study. Sex matters, and readers are recommended to peruse the following reviews for more in-depth information (Burger et al., 2007; Cummings et al., 2007; Dickerson and Gore, 2007; Gochfeld, 2007; Orlando and Guillette, 2007; Walker and Gore, 2007; Patisaul and Adewale, 2009; Frye et al., 2012; Patisaul, 2013; Rebuli and Patisaul, 2015). This list, while not exhaustive, also provides examples of all the key concepts highlighted in this subsection including the need to consider sex, mixtures, timing of exposure, dose, 'nonclassical' mechanisms of action, and the potential for multigenerational effects when considering the health impacts of EDCs.

5.04.1.1.4 Latency of Effects

The DoHAD hypothesis postulates that early exposures to EDCs may not have any immediate apparent effects, but can predispose an organism to the latent development of a disease or disorder (Barker, 2003; Gore et al., 2006; Haugen et al., 2014). A compelling example comes from humans, as millions of women who took the estrogenic pharmaceutical DES under physicians' misguided advice to avert miscarriage inadvertently exposed their fetuses to a potent estrogen. At birth, the infant girls appeared externally normal, but later in life they were found to have a disproportionately high level of reproductive-tract abnormality and increased incidence of rare vaginocervical cancers (Newbold, 2004). Laboratory rodent models of DES are quite consistent with the human data, as fetal DES is associated with the latent development of uterine cancer (Newbold et al., 2006). Animal models consistently demonstrate that fetal exposure to EDCs often has no discernible effects at birth, but result in infertility, abnormalities, and cancers much later (years to decades) in life (Welshons et al., 2006). This DoHAD concept also applies to the context of hormones and reproductive behaviors (Gore, 2008; Gore et al., 2006; Steinberg et al., 2007, 2008).

5.04.1.1.5 Consequential Effects versus Adverse Outcomes

Historically, developmental toxicology has centered on the detection of 'adverse outcomes' which are primarily gross, morphological malformations to specific structures or systems. Examples related to EDC exposure include the development of supernumerary, split, and duplicated limbs in frogs; stunted growth resulting in smaller individuals; and malformed external genitalia (for a classic example see Guillette et al., 1995). Such individuals are more likely to die early in life than those not affected. A second category includes effects on those animals that appear otherwise normal externally, but are grossly abnormal internally, either structurally or in physiology. Examples include hermaphroditic fish with ovotestes and/or abnormal accessory sex structure development, delayed or absent puberty, and high vitellogenin levels (examples include Guillette et al., 1994; Hayes et al., 2002). Such individuals usually are sterile or severely reproductively compromised. Most EDC-related effects are not this overt but rather more subtle and include behavioral impairments and other aspects of neuroendocrine function including timing of puberty, age at reproductive senescence, and regularity of the ovulatory cycle (Crain et al., 2008). For example, female fertility is disrupted in a myriad of species following developmental exposure to the phytoestrogen genistein, but this may not be initially apparent as severity changes with age. Female mice treated with low doses of genistein (0.5 and 5 mg kg^{-1}) showed no difference in the numbers of mice delivering live pups compared to controls at 2 and 4 months of age. By 6 months of age, however, the percentage of mice delivering live pups in the genistein-exposed groups dropped, as did litter size (Jefferson et al., 2005). Subsequent studies conducted to characterize the source of infertility found that the oviductal and uterine environments were not suitable to maintain pregnancy. There was a 50% loss of embryos during transit through the oviduct of genistein-treated mice, and embryo transfer experiments showed that the uterus of genistein-treated mice is not capable of sustaining pregnancy even if the blastocysts are from control mice (Jefferson et al., 2009). These data suggest that developmental genistein exposure caused a permanent change in the function of the female reproductive tract ultimately manifesting as complete infertility with advancing age (Jefferson et al., 2012). This infertility appeared earlier than in unexposed, typically aging mice.

Another important category in regard to subtle but biologically meaningful adverse phenotypes includes effects in which exposed individuals might be capable of breeding but do not engage in normal courtship and copulatory behavior with opposite-sex individuals. Birds that form pair bonds, but with a member of the same sex, fall into this category. These individuals might live as long as normal, unexposed individuals, but they represent a form of behavioral infertility and, ultimately, evolutionary death in that they do not reproduce. For some species this may lead to extinction. A valuable example is the demonstration that low exposure to ethinyl estradiol (the synthetic estrogen in birth control pills) of a population of

fathead minnows (*Pimephales promelas*) in an experimental lake led to its near extinction (Kidd et al., 2007).

5.04.1.1.6 Dose–Response Curves and Thresholds (or the Lack Thereof)

EDC dose responses are not always linear, a phenomenon which has proved vexing for traditional risk assessment strategies to handle empirically and conceptually (Kendig et al., 2010; Vandenberg et al., 2012). Classically, toxicological studies have conceptually considered dose with the attitude that 'the dosage makes the poison' (modified from a statement made by the sixteenth-century German physician Paracelsus, considered the father of toxicology). Indeed, risk assessments for virtually all chemicals, except genotoxic chemicals, assume that, for any substance, there exists a threshold dose below which exposure is safe. This assumption underlies the concept of the no observed adverse effect level (NOAEL), an important benchmark for setting exposure levels considered 'acceptable' for humans. By definition, the NOAEL is the dose at which no 'adverse' effects are seen. As described above, these effects are typically gross malformations or death, not subtle outcomes more typical of EDC exposure such as obesity, early puberty, premature reproductive senescence, or loss of sex-typical behaviors. Extrapolating the NOAEL relies on the presumption that dose responses are linear. Once calculated, safety factors are applied (typically 10–1000) to obtain and set the reference dose or the dose considered 'safe' for human exposure (Bokkers and Slob, 2007). For example, the NOAEL for BPA is 50 mg kg^{-1} body weight (bw) per day (via oral exposure), but the reference dose in the United States (the dose considered safe) is 50 μg kg^{-1} bw per day. Importantly exposure levels considered safe are rarely tested empirically. This is primarily because doing so is too animal intensive. The number of doses needed to be tested as well as the number of individuals required to detect significant and valid deviations is prohibitively large. As a consequence, classical toxicological assessments use as few as two doses (typically high; close to lethal in most cases). Safety standards for most of the 85 000+ chemicals in commerce depend on the validity of the threshold assumption. EDC research has demonstrated that they are not universally applicable.

The threshold assumption was first challenged mathematically by Hoel (1980) who demonstrated that if an endogenous chemical normally produces a quantifiable response, then exogenous administration of that same chemical cannot produce a threshold because it automatically exceeds that which is present. For example, in any endocrine-mediated process, such as sexual development, the individual, during any stage of its normal development, is already producing the levels of the chemical(s) necessary for that process. In other words, the individual is already at its own threshold, and any additional added ligand from the internal or the external environment is necessarily above threshold and adverse. Subsequently, Daston (1993), Gaylord et al. (1988), Sheehan (2006), and Welshons et al. (2003) questioned the underlying assumptions of the NOAEL, specifically the threshold hypothesis, and suggested that it may not apply to chemicals that share a common mechanism with endogenous chemicals important to normal development. Specifically, they proposed that if the threshold for the exogenous chemical is already exceeded by the endogenous chemical, then administration of the same chemical should lead to a curve showing no threshold dose, no matter how low the background incidence caused by the endogenous chemical. The slider turtle enabled a strong test of this hypothesis because previous work demonstrated that estrogen is involved in normal ovarian sex determination and that exogenous estrogen can override incubation temperatures that, otherwise, would produce males. Using over 2400 individuals, Sheehan et al. (1999) established that even a dose of 40 ng kg^{-1} of E2 surpassed the endogenous threshold of 1.7 ng of E2 in an egg. This can be interpreted as indicating that, in the developing embryo, endogenous compounds such as E2, necessary for normal development, already exist at threshold levels and any additional ligand exceeds that threshold. Such biologically based dose–response models better describe the relationships between different components of the continuum between exposure to, and the adverse effects of, a chemical (Andersen and Dennison, 2001; Sheehan, 2006; Welshons et al., 2003) and have been extended to other EDC-associated phenomena (Sheehan, 2006; Welshons et al., 2006; Pottenger et al., 2011; Lagarde et al., 2015).

That the dose–response relationships for EDCs are not always linear is not atypical, as many substances, including hormones, vitamins, and other nutrients, are often characterized by nonmonotonic dose/response relationships (NMDRs). Examples of NMDRs include U-shaped curves, such as those for essential nutrients, where adverse effects can occur at high or low doses but differ at each end of the range. NMDRs can also resemble inverted U, sigmoidal, or even more complex curves (Kendig et al., 2010; Vandenberg et al., 2012). In many cases, it is unclear specifically how compounds can produce nonlinear dose effects, but they likely result from steroid feedback mechanisms or the integration of two or more different mechanisms of action, each of which optimally occurs at a different dose range. For example, EDCs such as the PCBs could potentially interfere with steroid hormone action at low doses but act as a potent neurotoxin at higher levels (Toppari et al., 1996). Similarly, receptor-selective pharmaceuticals can have analogous complex dose–response curves because of nonspecific lower-affinity effects on the same or other receptors or signaling systems. Unanticipated effects of even the most selective drugs are not unusual, sometimes resulting in their removal from the market. For EDCs, 'off target' effects are even more likely because unlike pharmaceuticals designed to be specific agonist or antagonist of a receptor, they were not specifically designed to target any particular receptor or pathway.

5.04.1.1.7 Low-Dose Effects

The term 'low dose' has been inconsistently applied across the EDC literature because there are different perspectives and disagreement regarding what is meant by 'low.' One approach is to consider all doses below the NOAEL to be low. This is only feasible, however, for the small fraction of chemicals that have undergone formal toxicity testing and thus for which a NOAEL is defined. Another perspective is to consider exposure at or below human exposure levels to be low dose. Again, this is only feasible for chemicals in which exposure levels are known. Operationally, 'low-dose effects' are considered to be biologic changes that occur in the range of human exposure or at doses

lower than those used in the standard testing paradigm of the US EPA for evaluating reproductive and developmental toxicity (Melnick et al., 2002). Numerous studies have shown that, because they can have NMDRs, EDCs can produce effects on neuroendocrine systems and behaviors in the low dose range (Diamanti-Kandarakis et al., 2009; Patisaul and Adewale, 2009; Kendig et al., 2010; Vandenberg et al., 2012), relevant to levels found in the bodies of humans and wildlife.

5.04.1.1.8 Transgenerational, Epigenetic Effects of EDCs

There is rapidly growing appreciation that EDCs may exert actions not only on the exposed individual but also on subsequent generations. The majority of emerging studies exploring the impact of EDCs continue to focus on the individual in its own lifetime. This is a valuable information, but says little about the impact of the chemical on the population through time (proximate or ultimate). A proof of principle example comes from experiments using high doses of the endocrine-disrupting fungicide, vinclozolin. Exposure of pregnant rats to vinclozolin resulted in latent development of reproductive dysfunctions, infertility, and cancers in their male F1 offspring (Anway et al., 2005; Anway and Skinner, 2006). Moreover, if the F1 males mated prior to the development of disease, their F2 male offspring developed a similar phenotype. To date, Skinner's lab has demonstrated that this effect carries at least as far as in the F5 offspring and that the mechanism involves (at least in part) an epigenetic modification caused by a change in methylation patterns to the male germ line (Anway et al., 2005). Male F3 descendants of vinclozolin-treated dams, compared to the F3 descendants of vehicle-treated dams, were significantly less attractive to a female rat in a mate preference test, even though the experimental F3 males did not yet express any obvious phenotypic dysfunction (Crews et al., 2007). This result suggests evolutionary consequences of EDCs on mate preference and the ability of an animal to successfully reproduce. Subsequent work by this group and others has evaluated the potential for vinclozolin, BPA, phthalates, methoxychlor, PCBs, and other EDCs to induce epigenetic changes at lower, environmentally relevant doses (Wolstenholme et al., 2011; LaRocca et al., 2014; Leon-Olea et al., 2014; Casati et al., 2015; Ziv-Gal et al., 2015). Further discussion of this subject is provided in Section 5.04.5.

5.04.1.1.9 Degradation and Metabolism, Mixtures, and Synergism

It is important to keep in mind that environmental and bodily contamination never consists of single chemicals but of chemical mixtures. Some chemicals are rapidly metabolized and removed but others persist. Over time, degradation and/or metabolism occurs such that the metabolized/degraded product may be even more biologically active than the parent chemical. For example, *o,p′*-DDT is biotransformed in the environment to form *o,p′*-DDE, a compound equally persistent and, in some instances, more biologically active than its parent. Similarly, the soy isoflavone daidzein can be biotransformed to the more bioactive metabolite equol in individuals containing the right mix of microflora to make the conversion (Pottenger et al., 2011).

The issue of ecologically relevant mixtures continues to be underappreciated. Many chemicals are used and sold as mixtures, as is the case of fire retardants (Stapleton et al., 2008; Stapleton et al., 2009) and PCBs that consist of different chlorinated biphenyls depending upon the industrial application, for example, industrial mixtures such as Aroclors. Relatively few studies have identified the active chemicals in such mixtures, nor have they demonstrated how they may combine or synergize to produce their effects in a complex biological system. When it is considered that, in nature, virtually all contamination is the form of mixtures, the importance of this aspect of endocrine disruption cannot be overestimated. While synergism is a commonly demonstrated phenomenon in endocrinology, work along these lines on EDCs has been limited (e.g., Christiansen et al., 2008; Sobolewski et al., 2014; Lichtensteiger et al., 2015). Classic examples come from work in slider turtles and alligators, species which show temperature-dependent sex but will develop as females if exposed to estrogen or estrogenic EDCs embryonically (see Crews, 1996 for review and also Willingham, 2004; Willingham and Crews, 1999). Knowledge about such impacts in mammalian species, including humans, remains sparse.

5.04.2 Neuroendocrine Effects of EDCs on the Hypothalamic–Pituitary Control of Reproduction

A substantial body of evidence strongly supports effects of EDCs on reproductive and endocrine function *in vivo*, and in *in vitro* models (reviewed in Dickerson and Gore, 2007; Walker and Gore, 2007; Patisaul and Adewale, 2009; Jefferson et al., 2012; Patisaul, 2013; Patel et al., 2015). Whereas much of the original endocrine disruption work showed effects on vitellogenesis (yolk formation in fish), reproductive tract, genitalia, serum hormone levels, and reproductive developmental landmarks (e.g., timing of puberty), little of this work in any species focused on the effects of EDCs on the neuroendocrine control of reproduction (representative examples include Walker et al., 2009, 2014; Losa et al., 2010; Patisaul et al., 2014). This is surprising for several reasons. First, neuroendocrine circuits in the brain, specifically the hypothalamus and POA, control much of reproductive function. The hypothalamic neurons that synthesize and secrete the neuropeptide, gonadotropin-releasing hormone (GnRH), provide the primary driving force of reproductive development and puberty and maintain reproductive function in adult males and females (Gore, 2002a; Yin and Gore, 2006; Semaan and Kauffman, 2010). Second, hypothalamic–preoptic brain regions are highly sensitive to steroid hormones throughout life and have robust expression of ERα and ERβ, androgen receptors (ARs), and progesterone receptors (PRs; Chakraborty et al., 2003a,b; McAbee and DonCarlos, 1999; Quadros et al., 2002), any of which are targeted by EDCs. Third, effects of hormones on the hypothalamic GnRH neural system are mostly mediated by afferent neural and glial inputs, rather than directly upon GnRH neurons, which do not express ERα, PR, or AR (Herbison and Theodosis, 1992; Huang and Harlan, 1993; Shivers et al., 1983; Skinner et al., 2001). Although GnRH cells coexpress ERβ, this is not sufficient to explain the effects of estrogen actions on GnRH function (Wintermantel et al., 2006). This point is important because other neurotransmitter factors that control GnRH release and synthesis, most notably kisspeptin neurons, do express steroid

hormone receptors, and therefore EDCs that act via nuclear hormone receptors may exert these actions through the neurotransmitter systems that regulate GnRH neuronal functions. Indeed, EDCs can act upon cells in the nervous system that synthesize/release kisspeptin, RFRP3, dopamine, serotonin, glutamate, norepinephrine, and others (e.g., Chakraborty et al., 2003b,c; Sar, 1984; Sar et al., 1990; Sar and Stumpf, 1981; Patisaul, 2013), and all of these systems are part of the central nervous circuitry controlling GnRH neuroendocrine functions. Thus, the neuroendocrine hypothalamus is a key node for the integration of the neurological and endocrine effects of EDCs.

5.04.2.1 Endocrine Disruption of GnRH Neurons

5.04.2.1.1 Mammals

That EDCs can disrupt the organization and/or function of the HPG axis is clear, and while early studies could not easily differentiate whether the target of action was hypothalamic, pituitary, ovarian, or some combination of the three, recent technological advances have allowed for the specific interrogation of impacts on GnRH neurons.

Early evidence of disruption at the level of the GnRH neuron was indirect. A strong example of hypothalamic disruption of the HPG axis is provided by a study on two phytoestrogens: coumestrol (a coumestan) and genistein (a soy isoflavone). In this report by McGarvey et al. (2001), adult female rats were treated intravenously with coumestrol, genistein, E2, or a vehicle for an 8-h period, during which blood samples were collected frequently for subsequent radioimmunoassay of LH (as a proxy of GnRH activity). Coumestrol and E2, but not genistein or the vehicle, caused significant suppressions of parameters of pulsatile LH release (McGarvey et al., 2001). That this effect is at least, in part, due to a hypothalamic action was elegantly proven through multiunit activity recording in the hypothalamus during the experiment. Multiunit activity volley frequency was significantly suppressed by coumestrol in concert with the suppression of LH pulses. Another part of the effect of coumestrol is also integrated at the pituitary gland because the pituitary response to GnRH challenge was attenuated in these animals (McGarvey et al., 2001). Thus, a specific class of phytoestrogen has suppressive actions on both the hypothalamus and pituitary in the suppression of HPG-axis function. Other phytoestrogens also suppress gonadotropin release and GnRH activity including soy isoflavones (Patisaul and Jefferson, 2010). A 2009 meta-analysis concluded that, in women, isoflavone intake increases cycle length and suppresses LH and FSH levels (Hooper et al., 2009). Phytoestrogen-rich foods confer many benefits but it is important to be mindful of their endocrine-disrupting properties.

Other *in vivo* studies have reported effects of EDCs on serum LH concentrations. Lyche et al. (2004) treated goat kids with PCBs during gestation, and exposure presumably continued postnatally through lactational transfer. Blood samples were collected regularly through the pubertal period for an assay of serum LH. Results showed that PCB153 exposure was associated with significantly lower prepubertal LH concentrations, along with a delay in the timing of puberty. Again, these findings are consistent with effects of EDCs on HPG-axis function, and this latter study is particularly interesting since exposure occurred early in life during a critical developmental window, but effects were not manifested until much later in postnatal life.

More direct evidence that early-life EDC exposure could impact GnRH function has since emerged. For example, female rats treated with PCB congeners on embryonic day 16 had upregulated GnRH mRNA levels in the POA when assayed at P40, and similar exposure to organochlorine pesticides was also associated with elevated GnRH mRNA levels at P50 (Gore, 2001). Similarly, exposure to BPA over just the first 4 days of life was shown to alter the density of RFRP3 (RFamide-related peptide 3; the mammalian ortholog of GnIH) synapses on GnRH neurons at peripuberty in female rats (Losa-Ward et al., 2012) and hypothalamic expression of kisspeptin RNA (Navarro et al., 2009). Aspects of the medial preoptic and arcuate kisspeptin signaling pathways have now been shown to be vulnerable to BPA, PCBs, and the phytoestrogen genistein (Patisaul, 2013). Using a microdialysis method, BPA has been shown to suppress GnRH and kisspeptin release from the female (late pubertal) rhesus macaque hypothalamus providing some of the best evidence to date that BPA and, by extension, estrogenic EDCs can directly impact GnRH output (Kurian et al., 2015). An elegant series of experiments have shown that DDT can amplify GnRH pulse frequency via a mechanism involving crosstalk with ERα, aryl hydrocarbon receptors, and AMPA receptors (Bourguignon et al., 2013). Collectively these data show that EDCs can have acute and/or long-lasting consequences on the sexual differentiation and function of GnRH signaling pathways.

5.04.2.1.2 Nonmammalian Species

As reviewed in this book series previously by Ottinger and Vom Saal (2002), the GnRH neurosecretory system is highly conserved across the vertebrate classes. While direct evidence of EDC-related effects on GnRH secretion is scarce, that EDCs affect neurotransmitter systems known to affect GnRH cells has been shown for birds (see Ottinger et al., 2005b for a review) and fish (Khan and Thomas, 1997; Khan and Thomas, 2001). For example, male Atlantic croaker (*Micropogonias undulatus*) exposed for 30 days with the PCB mixtures Aroclor 1254 displayed diminished pituitary gonadotropin responsiveness to treatment with a GnRH analog (LHRHa) *in vitro* and reduced serum concentrations of the major fish androgens, 11-ketotestosterone and testosterone (Khan and Thomas, 1997). Further impacts of EDC exposure on GnRH and related neuroendocrine systems in teleost fish have recently been reviewed (Le Page et al., 2011). Evidence of GnRH effects in amphibians and reptiles remains extremely limited (Sower et al., 2000).

5.04.2.1.3 In Vitro Studies

In vitro studies using the hypothalamic GnRH GT1–7 cell lines (Mellon et al., 1990) have also supported direct effects of EDCs on GnRH neurons. Low doses of PCBs significantly increased GnRH mRNA levels through a posttranscriptional mechanism that was partially, but not entirely, mediated by nuclear estrogen receptors (ERs) (Gore et al., 2002). GnRH-peptide release from GT1–7 cells was also stimulated by Aroclor 1221, a PCB cocktail (Gore et al., 2002). In a similar study

on organochlorine pesticides, treatment with methoxychlor or chlorpyrifos affected GnRH mRNA levels with an inverted U-shaped dose–response curve: low doses stimulated GnRH mRNA while higher doses suppressed this endpoint (Gore, 2002b). The methoxychlor metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) suppressed both GnRH transcription and mRNA levels (Roy et al., 1999). Finally, a study of the phytoestrogen, coumestrol, showed a suppression of GnRH mRNA levels, an effect that was prevented in the presence of a selective ERβ-antagonist (Bowe et al., 2003). These *in vitro* studies on GT1–7 GnRH cells are consistent with these neurons being direct targets of EDCs through mechanisms that are at least partially mediated through nuclear ERs. By contrast, a study by Petroff et al. (2003) on effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; the most potent dioxin congener) on HPG-axis function found *in vivo* effects (described above) but no effect on GT1–7 cell numbers, GnRH peptide in the medium, or GnRH promoter activity in a promoter–reporter assay. Thus, effects of EDCs may be limited to certain categories of compounds. Consistent with this, Petroff et al. (2003) could not detect the aryl hydrocarbon receptor mRNA in the GT1–7 cells, explaining the lack of effect of TCDD in this model.

Direct effects of BPA on GnRH neuronal activity were recently shown using an elegant explant model in which large numbers of primary GnRH neurons were maintained under conditions which preserved expression of many of the receptors found *in vivo* (Klenke et al., 2016). Assessment of oscillations in intracellular calcium via calcium imaging revealed that BPA suppressed electrical activity in GnRH neurons. That the effect was not blocked by GABA- or glutamatergic input supports the conclusion that GnRH neuronal activity is directly influenced by BPA. Inhibition of GnRH neuronal activity occurred independent of ERs, GPER, or estrogen-related receptor-γ, suggesting that activity was via a noncanonical pathway. These results provide robust evidence of a direct effect of BPA on GnRH neurons and the potential for GnRH neurons to be the target of other EDCs.

Together, *in vivo* and *in vitro* studies strongly support hypothalamic GnRH neurons as both a direct and indirect target of endocrine disruption. These hypothalamic cells provide key links between central nervous actions of EDCs, including on steroid hormone receptors and neurotransmitter receptors and synthetic/degradatory enzymes in the brain, and endocrine outputs to the anterior pituitary gland. An understanding of EDC effects on neuroendocrine systems is, therefore, critical to discerning the mechanisms by which EDCs alter sexual differentiation, puberty, fertility, and adult reproductive functions.

5.04.3 Endocrine Disruption of the Sexual Differentiation of the Brain

As reviewed in numerous chapters, including elsewhere in this work (see Chapter 2.10, Hormones, Brain, and Behavior in Reptiles), the brain undergoes sexually dimorphic developmental processes due to influences of hormones, the environment, and chromosomes. These processes are highly subject to perturbation during critical developmental windows through exposures to EDCs (Dickerson and Gore, 2007; Gore, 2008; Patisaul and Adewale, 2009; Rebuli and Patisaul, 2015; Panzica and Melcangi, 2016). There is an extensive literature showing specific effects of EDCs on the development of sexually dimorphic limbic brain regions and on the expression of molecules in the brain that are involved in the control of sexually dimorphic sexual and nonsexual behaviors. Although it is impossible to include all of the examples from the literature, in the following section we provide some representative examples of these effects of EDCs, focusing on hypothalamic–preoptic brain regions and the expression of hormone receptors in these areas.

5.04.3.1 Mammals

Numerous studies in experimental laboratory animals, principally rats, show that low levels of EDC exposure during gestation and early postnatal life, the critical period for brain sexual differentiation, cause permanent morphological changes in sexually dimorphic, steroid-sensitive hypothalamic–limbic brain regions. As reviewed in Dickerson and Gore (2007) and Patisaul and Adewale (2009), much research has focused on developmental effects of EDCs on subregions of the hypothalamus–preoptic systems that control reproductive physiology and behavior in adulthood. To follow is a brief presentation of evidence for endocrine disruption of hypothalamic morphology and cellular phenotype.

5.04.3.1.1 Anteroventral Periventricular Nucleus

The anteroventral periventricular nucleus (AVPV) is a preoptic region that is important for the neuroendocrine control of ovulation in female rodents (Simerly, 2002; Wiegand et al., 1978), and it is sexually dimorphic in size, being larger in female than male rats. Research on EDCs shows consistent demasculinizing and/or feminizing effects of EDCs in AVPVs of neonatally exposed males and defeminizing and/or masculinizing effects in their female counterparts. For example, one of us (Andrea C. Gore; Salama et al., 2003) showed that fetal exposures of rats to low levels of PCBs in early life (late embryonic through early postnatal) resulted in a significant (50%) suppression, in adulthood, of numbers of cells expressing ERβ-immunoreactivity in the AVPV. Patisaul et al. (2006) reported that early postnatal treatment of rat pups with BPA or genistein demasculinized the sexually dimorphic expression of tyrosine hydroxylase in the AVPV of male rats. Such treatment also defeminized the coexpression of ERα and tyrosine hydroxylase in the AVPV of females. Similarly, a diminution of the normal sex difference in both AVPV size, and in tyrosine hydroxylase-positive cell numbers in AVPV, was reported by Rubin et al. (2006). In this same brain region, Petersen's group showed a sex difference in the expression of mRNA for glutamic acid decarboxylase 67, the enzyme that synthesizes the neurotransmitter GABA and the expression of which indicates that a cell is GABAergic. They further reported that this sex difference was abolished by the dioxin TCDD given during prenatal life (Hays et al., 2002), presumably through actions on the aryl hydrocarbon receptor that is coexpressed in GABAergic cells. Together, these studies show that both the morphology of the AVPV and the phenotypes of its specific cells are significantly perturbed by early-life exposures to EDCs.

5.04.3.1.2 Sexually Dimorphic Nucleus of the Preoptic Area

The SDN-POA of male rats is two to four times larger than that of females, and early developmental manipulations of sex steroid hormones diminish this sex difference (Gore, 2008; Gorski, 2002). Thus, it is often used as a biomarker of endocrine disruption in the developing brain due to its exquisite sensitivity to estrogen. It is important to mention that not all studies have reported effects of fetal exposure to EDCs on SDN-POA volume (Masutomi et al., 2003; Takagi et al., 2004), even for the same chemical, and that in many cases effects are sex specific or only observed at very high doses (Patisaul and Adewale, 2009; Patisaul and Jefferson, 2010). Volumetric and other impacts, however, have been shown for DES, BPA, genistein, and zearalenone (the latter is an estrogenic fungal metabolite). For example, one study found that injection of 250 μg BPA or genistein at 12-h intervals across the first 2 days of life did not alter SDN volume in males but enhanced the number of neurons immunopositive for calbindin (Patisaul et al., 2006). Although both masculinizing effects of EDCs in females and their demasculinizing effects in males have been reported, differences between these reports, as well as the lack of effects in others, are likely attributable to differences in dose, the timing of exposure, strain differences in sensitivity, and the nature of the EDC.

5.04.3.1.3 Ventromedial Nucleus of the Hypothalamus

The ventromedial nucleus of the hypothalamus (VMN) comprises diverse cell groups that are implicated in multiple behavioral and neuroendocrine systems. In rodents, the ventrolateral division of the VMN (VMNvl) is critically involved in the regulation of sexual behavior in both sexes including the lordosis display, a reflexive behavior indicative of female sexual receptivity. Synaptic organization in the VMN, particularly the VMNvl is sexually dimorphic and modified by steroid hormones in the early neonatal period (Matsumoto and Arai, 1983, 1986; Cao et al., 2012). Both the size of the VMN and its cellular phenotypes were disrupted through fetal and early postnatal exposures to EDCs, as well as exposures during puberty (Ceccarelli et al., 2007) and in adulthood (Funabashi et al., 2005). A good example in laboratory rats is provided by 4-methylbenzylidene camphor (4-MBC), an ultraviolet filter with actions both on estrogen and thyroid receptors (also known as Parsol 5000 or Eusolex 6300; not approved for use in sunscreen in the USA but listed as an 'inactive' ingredient in some personal care products; banned from EU products in 2015). Prenatal exposure to 4-MBC affected a suite of genes in the VMN at adulthood. Specifically, it decreased ERα mRNA in both males and females, decreased PR mRNA only in females (to male levels), and increased both insulin-like growth factor I (IGF-1) and steroid receptor coactivator-1 (SRC-1) in females only (Maerkel et al., 2007). VMN ER (both isoforms) levels were also disrupted by prenatal, but not neonatal, BPA exposure (Cao et al., 2012, 2013).

5.04.3.1.4 Medial Preoptic Area

The medial preoptic area (mPOA) is sexually dimorphic in many regards and plays key roles in controlling sexual behavior in adult females. Exposures to EDCs at various life stages, including perinatal and adult (Funabashi et al., 2001), affect expression of genes and proteins in this region. For example, prenatal BPA had latent effects on ERβ, but not on ERα, mRNA levels in the POA at adulthood (Ramos et al., 2003). As described above for the VMN, exposures to 4-MBC altered expression of several genes in the mPOA, including ERα (decreased in both sexes), PR (increased in males), and IGF-1 and SRC-1 (increased in females; Maerkel et al., 2007). BPA exposure has consistently and repeatedly been found to alter rodent mPOA levels of ERα and ERβ to varying degrees depending on sex and timing of exposure even at doses well below either the NOAEL or the reference dose (Ceccarelli et al., 2007; Monje et al., 2007; Monje et al., 2010; Adewale et al., 2011; Cao et al., 2012, 2013).

Intriguingly, emerging work in the POA is showing that the developing brain can be particularly vulnerable to multiple 'hits.' Exposure to PCBs prenatally and then again during juvenile development produces a different molecular and behavioral phenotypes than if exposure occurs during one of those periods. Males appear to be particularly vulnerable (Bell et al., 2016a,b). Two hit models using combinations of prenatal immune challenge and neonatal or juvenile stress have been long used to model schizophrenia and other psychosocial disorders (Bilbo, 2013; Deslauriers et al., 2016). It is only recently, however, that the EDC research community has leveraged this approach. Importantly, this more closely models 'real-world' exposures and situations, as humans are continuously exposed to a variety of EDCs and other chemicals and face stress and other challenges throughout life. Multihit models of EDC exposure using combinations including diet, stress, and other lifestyle factors are revealing profoundly different effects on the developing neuroendocrine system, including the POA, than single chemical exposures (Patisaul et al., 2012, 2014; Strakovsky et al., 2015).

5.04.3.2 Birds, Reptiles, and Amphibians

The considerable information on how EDC exposures modify gonadal differentiation and, in turn, influence adult brain–behavior relationships in various mammalian model systems, and the more limited but growing literature on birds, is absent in reptiles and amphibians. There is a literature on the sexual differentiation of the brain in reptiles, and the reader is referred to Chapter 2.10, Hormones, Brain, and Behavior in Reptiles. However, beyond the basic hormone-replacement studies per se, there is at present no information on the effects of EDCs on sexual differentiation of the reptilian brain. In amphibians, research continues to focus on the effects of EDCs on the development or activation of the reproductive system (intersex gonads, accessory sex structures, induction of vitellogenin production in males, etc.); a literature that has been reviewed by others (see recent such reviews by Crews et al., 2003; Hayes et al., 2006a; Kloas and Lutz, 2006; McLachlan, 2001; Milnes et al., 2006; Mosconi et al., 2002; Reeder et al., 2004; Vos et al., 2000). To date, there is no information on the effects of EDCs on sexual differentiation of the brain in these species.

5.04.3.3 Fish

Reports abound for effects of EDCs on reproduction in fish, with virtually all of the studies focusing on the gonad,

vitellogenesis in males, the manifestation of intersex gonads, alterations in secondary sexual characteristics, and/or reduced fertility (Bayley et al., 2003; Jobling et al., 2002; Koger et al., 2000; Toft et al., 2003; reviewed in Arukwe, 2001). Environmental EDCs are highly likely to exert effects on the fish brain, similar to those described above for mammals, but very few studies have probed for effects. Reports of EDC effects on gene expression in the fish nervous system date back at least a decade (e.g., Greytak and Callard, 2007; Lyssimachou et al., 2006; Gentilcore et al., 2013) with more recent studies showing effects on the pace of neurodevelopment and neural organization (Kinch et al., 2015). EDCs are also well known to alter aromatase expression in the zebrafish brain (Kishida et al., 2001; Le Page et al., 2011).

Although the above sections highlight the need for further research in nonmammalian systems, they provide strong evidence for neural substrates that underlie effects of EDCs on reproductive systems. The following section will take these observations to the next level, focusing on the implications of developmental endocrine disruption on the manifestation of sexual behaviors in adulthood.

5.04.4 Endocrine Disruption of the Sexual Differentiation of Behavior

Extensive and compelling evidence shows that exposure of vertebrates to EDCs during the critical period of brain sexual differentiation has permanent effects on the manifestation of these behaviors in adulthood. Historically, the literature has primarily focused on endocrine disruption of sexual behaviors, but over the past decade there has been a greater exploration of nonreproductive behaviors including anxiety, social recognition, and exploratory behaviors (Patisaul and Adewale, 2009; Wolstenholme et al., 2011). In response to increasing recognition that the developing brain may be one of the most sensitive EDC targets, high-throughput screening assays using zebrafish embryos are in development to better and more rapidly screen for an increasingly wide range of behavioral effects (Truong et al., 2014; Reif et al., 2015). Importantly, even small outcomes of EDC exposures on sexually dimorphic behaviors can be biologically relevant on both an individual and population scale because perturbation of behavior may alter social interactions, quality of maternal care, or even eliminate an animal from the mating pool if it is not selected by a conspecific mate. This may not be easily assessable or relevant in the laboratory setting, but is highly relevant in a natural setting. Use of 'nontraditional' animal models including *Microtus* may be useful in that regard (Engell et al., 2006; Sullivan et al., 2014). Readers are also referred to an excellent review by Zala and Penn (2004) for additional details, references, and information.

5.04.4.1 Mammals

Laboratory rodents have been used extensively in tests of behavioral effects, with most studies focusing on exposure to EDCs during the organizational period, followed by behavioral testing in adulthood. A subset of published research has utilized the model of exposure and testing in adulthood, and a handful have employed adolescent exposure. Available data provide overwhelming support for small but significant effects of EDCs on hormone-sensitive behaviors, particularly reproductive behaviors. These studies were designed and undertaken, in large part, to understand and inform the potential for human risk.

The PCBs are a good example of a chemical for which evidence of endocrine disruption of reproductive behaviors in mammals is particularly robust and multifaceted. Early-life exposures to PCBs alter paced mating behaviors in adult female rats, with effects varying slightly depending upon the age of exposure. The paced mating model enables females to pace the timing of mating and enhances reproductive success (Coopersmith and Erskine, 1994), so it better approximates a naturalistic mating experience. One experiment involved exposure of rats on embryonic day (E) 14, the day of parturition (postnatal day (P) 0), and P10 to either Aroclor 1221 or Aroclor 1254 (Chung and Clemens, 1999). In adulthood, rats were ovariectomized (OVX) and treated sequentially with EB plus progesterone (P_4) to induce receptivity. Aroclor 1221 decreased lordosis quotient and other aspects of paced mating, whereas Aroclor 1254 had fewer effects. A second report from this group tested effects of longer-term postnatal treatment with Aroclor 1221 or 1254 in a similar mating paradigm (Chung et al., 2001). In that case, rats were administered the toxicants daily from P0 to P6. In adulthood, significant effects of A1254, but not A1221, were found on mating behaviors in adult OVX + EB + P_4 rats, a result that suggests that A1254 is more effective when given postnatally (Chung et al., 2001), compared to A1221, effects of which are exerted prenatally (Chung and Clemens, 1999). In addition, neither A1221 nor A1254 given to adult OVX + EB + P_4 females had any effect on mating behavior (Chung et al., 2001), indicating that effects are limited to exposure earlier in life.

One of our laboratories (Andrea C. Gore) assessed paced mating behavior in the adult female offspring of pregnant rat dams dosed with Aroclor 1221 at days 16 and 18 of pregnancy (Steinberg et al., 2007). Unlike the studies from Clemens' group described above, mating tests were performed on ovary-intact adult females, used on the evening of proestrus, when females were anticipated to be receptive. The paced mating tests demonstrated several significant differences in mating behavior in the F1 adult females that had been prenatally treated with PCBs. They spent significantly more time away from the male, and they also took significantly more trials to successfully mate (Steinberg et al., 2007). Thus, this study showed significant effects of fetal PCBs on adult paced mating behavior in F1 female rats, and we interpret these data to mean that reproductive success is diminished by low-level fetal PCB exposure.

Phytoestrogens have also been the subject of considerable interest for their effects on sexual behaviors. Again, most studies involved perinatal treatments, with behavioral tests done in adulthood in OVX + E2 + progesterone-treated females. Neonatal exposure to prenatal resveratrol, a phytoestrogen found in grapes, decreased lordosis quotient and increased rejections, consistent with diminutions in sexual receptivity (Kubo et al., 2003). Another study by Henry and Witt (2006) showed that when resveratrol exposure occurred via lactation, the only effect on sexual behavior was on the latency of males to mount the females, which was shorter in

the resveratrol group. This surprising result suggests a potentially increased attractiveness of the resveratrol females, although the mechanism remains unknown. Genistein and daidzein, two soy isoflavones, were similarly tested for effects of early postnatal treatment at P0–P4 (Kouki et al., 2003). As adults, genistein-exposed rats had lower lordosis quotients on the third test compared to control rats. Daidzein had no influence on these behaviors. This result is interesting because it shows an interaction of sexual experience with the developmental treatment. This same lab performed a separate study for another class of phytoestrogen, coumestrol, a member of the coumestan family that is produced from clover and alfalfa sprouts (Kouki et al., 2005). When pups were exposed to a single injection of coumestrol at either a low (1 mg) or high (3 mg) dose on P4, in adulthood, the low dose of coumestrol resulted in higher lordosis quotient compared to control rats. By contrast, the higher dose of coumestrol (3 mg) almost completely suppressed the lordosis quotient to a similar extent as neonatal E2 (Kouki et al., 2005). These latter results emphasize the importance of dose in endocrine-disrupting effects of phytoestrogens.

Some experiments on sexual behavior have involved adult exposure. For example, one of our laboratories (Heather B. Patisaul) found that consumption of a commercially prepared isoflavone supplement by adult female rats, at a dose that results in serum levels between those seen in Western and Asian (human) adults, attenuated lordosis in OVX, hormone-primed females to the same degree as tamoxifen (Patisaul et al., 2001, 2004). The supplement also suppressed proceptive behaviors even more profoundly than tamoxifen suggesting that soy isoflavones can suppress female sexual motivation and solicitation. Administration of genistein alone did not recapitulate these effects (Patisaul et al., 2002). A similar experiment with resveratrol found no effects on lordosis behavior, but exposed rats showed a delay in the exhibition of rejection behaviors, possibly due to a decrease in interactions with the males (Henry and Witt, 2002). Similar studies have been done using zearalenone, a dietary estrogen produced by the *Fusarium* mold on cereal and grains, which acts upon both ERα and ERβ (Kuiper et al., 1998). While not technically a phytoestrogen, it is consumed in the diet. Adult female rats were OVX for 1 week and treated with zearalenone for three consecutive days, with P_4 administered on the fourth day (Turcotte et al., 2005). While low-dose zearalenone had no effect, higher-dose zearalenone treatment enabled P_4-facilitated sexual behavior to occur. This result suggests that zearalenone has enough estrogenic activity to substitute for endogenous E2 in the adult OVX rat.

Impacts on sexual behavior in males have also been explored for PCBs, phytoestrogens, and other EDCs. Overall, males may not be as sensitive as females to some of these compounds. For example, Wang et al. (2002) reported no effect of either PCB77 or PCB47 in males, despite this same group's findings of significant effects in females (Chung and Clemens, 1999; Chung et al., 2001). By contrast, Sager (1983) reported that "males exposed to PCBs showed a reluctance to mate when compared to control animals," and the latter group also showed an effect of sexual experience. In a series of four mating trials, 20 out of 32 males that had been exposed via lactation to Aroclor 1254 mated on their first trial (compared to 100% of control males), another seven on a subsequent trial, and five did not mate at all across the four trials (Sager, 1983). Thus, sexual experience may interact with prenatal exposure to determine a behavioral outcome. Another study evaluating effects of a single fetal exposure (on gestational day 15) showed that PCB 126 caused an increase in numbers of intromissions compared to the vehicle group in sexual-behavior tests of the males in adulthood, but no effects on other measured parameters (Faqi et al., 1998). These disparate results on PCBs indicate that the timing of exposure, the type of PCB, and the sensitivity of the behavioral assay have an effect on the result. Importantly, many of these studies did not use very sensitive outcomes, and much more careful and naturalistic behavioral tests are necessary to provide stronger conclusions.

Phytoestrogens have been extensively evaluated for decades for their effects on male sexual behaviors. A pioneering report by Whitten et al. (1995) determined effects of lactational coumestrol, given either during the first 10 days of life, on sexual behavior in the male rats in adulthood. Early postnatal-coumestrol-exposed male rats showed significantly fewer mounts and ejaculations, and the latency to the first mount and first ejaculation was significantly longer, compared to control males. Resveratrol treatment throughout gestation suppressed intromission rate without affecting other masculine behaviors in rats (Kubo et al., 2003). A more recent study by Henry and Witt (2006) evaluated lactational exposure to resveratrol in male rats and reported decreased mount frequency without affecting other behaviors. These reports suggest specific and modest effects of resveratrol that may differ in nature depending upon the timing of exposure (prenatal vs postnatal). Adult exposure to genistein, daidzein, and extracts from the legumes Mesquite (*Prosopis* sp.) and *Leucaena leucocephalam* have been shown to subtly impair aspects of male sexual behavior and result in increased testicular germ cell apoptosis, decreased sperm quality, decreased testicular weight, and lower circulating testosterone levels (Retana-Marquez et al., 2016). Neonatal exposure to genistein or the ERβ-selective agonist DPN has also been shown to have subtly emasculating effects on male sexual behaviors (Sullivan et al., 2011).

Other EDC classes given in gestational and early postnatal periods can affect sexual behaviors in adult male rats. Low-dose BPA treatment given during gestation caused a significant decrease in the intromission rate in male rats, although it did not alter other sexual behaviors, including numbers of mounts and timing of mating (Kubo et al., 2003). At least one other study has also reported sexual deficits in male rats perinatally exposed to BPA (Jones et al., 2011).

Fenvalerate, the pyrethroid insecticide, was administered to rat dams late in gestation and through postnatal life (Moniz et al., 1999). When male offspring matured, effects on a range of sexual behaviors were quantified. Of these, the only significant effects of fenvalerate were on the number of mounts prior to first ejaculation, which increased, and the number of ejaculations, which decreased (Moniz et al., 1999). These data suggest detrimental effects of perinatal fenvalerate on masculine mating behaviors in rats.

EDC effects on nonreproductive behaviors continue to be a growing area of focus. BPA, in particular, has been extensively tested in a variety of rodent species and consistently shown to

heighten behaviors associated with anxiety and, to a lesser degree, alter aspects of exploratory behavior (Wolstenholme et al., 2011). Importantly, there is evidence that external factors such as maternal care (Kundakovic et al., 2013), cross-fostering (Cox et al., 2010), and diet (Patisaul et al., 2012) can exacerbate or mitigate anxiogenic effects resulting from BPA exposure. For example, a study conducted by one of us (Heather B. Patisaul) found evidence of elevated anxiety in juveniles and diminished sex differences following perinatal (GD 6-PND 40) exposure to BPA via drinking water, but only in animals raised on a soy-free diet (Patisaul et al., 2012). The mechanism by which this occurs is unclear. Importantly, free serum BPA levels were in a human-relevant range ($\leq$2 ng ml^{-1} at all time points examined). Impacts on social behavior appear to be negligible, but at least one study using the prairie vole (*Microtus ochrogaster*), which is more prosocial than rats or mice, found evidence that postnatal BPA exposure, even at low doses, can sex reverse aspects of social interaction (Sullivan et al., 2014). No impacts on partner preference, however, were found. While a handful of rodent studies indicate that perinatal BPA exposures may compromise cognitive abilities, the results are not consistent. Data from cognitive tasks associated with novelty seeking are more concordant, with impairments reported in rats and *Peromyscus* following gestation and lactation oral exposures to the dam as low as 40 μg kg^{-1} bw per day of BPA. Notably, impaired cognition/spatial navigation was observed in polygamous deer mice, but not their monogamous California mice cousins, who do not rely on this behavior to locate prospective mates in the wild (Jasarevic et al., 2011, 2013; Williams et al., 2013). A recent study on prenatal PCB effects on social behavior in rats reported small but significant changes in a test of social novelty (Reilly et al., 2015). PCBs, fire retardants, and other persistent organic pollutants are associated with cognitive deficits in human populations, an effect which has been recapitulated in laboratory rodent models (Gore, 2008).

Clearly, there are effects of EDC exposures on brain and behavior. What remains incomplete is understanding of causal links and the underlying mechanisms. While disruption of sex steroid hormone-mediated pathways and systems is likely primary, emerging evidence suggests that alteration of neuropeptide systems (including oxytocin and/or vasopressin), synaptic spine density, and neurotransmitter signaling are also likely contributory (Patisaul et al., 2003; Leranth et al., 2008; Wolstenholme et al., 2011, 2012; Sullivan et al., 2014; Rebuli and Patisaul, 2015). For example, cognitive deficits and anxiety following BPA exposure have been linked to a sex-dependent reduction in neurogenesis and dendritic morphology in the prefrontal cortex and hippocampus (Weinstock, 2011). In male mice perinatally exposed to 250 ng kg^{-1} bw of BPA through subcutaneous injection, elevated anxiety was accompanied by increased dopamine levels and a decreased DOPAC/DA ratio in the limbic system and medulla oblongata (Matsuda et al., 2012), suggesting disruption of mesolimbic dopamine pathways (which are well known to influence mood, anxiety, and reward-based behaviors (Chen et al., 2004; Pandaranandaka et al., 2006)). There is also emerging evidence for region- and sex-specific epigenetic changes (Yeo et al., 2013) including altered hypothalamic DNMT expression (Wolstenholme et al., 2011; Kundakovic et al., 2013) and methylation of the ERα promoter (Kundakovic et al., 2013) in mice. Understanding epigenetic changes is a rapidly growing area of EDC research.

5.04.4.2 Birds, Reptiles, and Amphibians

Some of the first demonstrations of endocrine disruption in wildlife were on effects of pesticides (DDT and DDE) on reproductive measures in birds (Crews et al., 2003; McLachlan, 2001; Zala and Penn, 2004). Subsequent work documented EDC-induced alterations in nesting and/or courtship behavior in ring doves (*Streptopelia risoria*; Haegele and Hudson, 1977); Bengalese fishes (*Lonchura striata*; Jefferies, 1967); Japanese quail (*Coturnix c. japonica*; Adkins-Regan and Garcia, 1986; Bryan et al., 1989; Halldin et al., 1999); Western, California, and herring gulls (*Larus occidentalis*, *Larus californicus*, and *Larus argentatus*; Fry and Toone, 1981; Fry et al., 1987; Hunt, 1977); and tree swallows (*Tachycineta bicolor*; McCarty and Secord, 1999a,b).

The reports of the effects of EDCs on sexual differentiation on gross morphology in birds are extensive, and there is a limited but increasing literature on how exposure might influence behavior of the adult (see recent reviews of Halldin, 2005; Halldin et al., 1999; Ottinger et al., 2001, 2005a; Panzica et al., 2005a,b, 2007). In male quail, administration *in ovo* with estrogenic EDCs, such as BPA, DES, DDT, ethinyl estradiol, genistein, methoxychlor, and PCBs, reduced sexual behavior in adulthood (Bryan et al., 1989; Halldin, 2005; Halldin et al., 1999, 2001, 2003; Hoogesteijn et al., 2005; Ottinger et al., 2005b; Panzica et al., 2005a). Similar results were obtained following administration of antiestrogens and aromatase inhibitors (Adkins, 1976; Adkins and Nock, 1976; Balthazart et al., 1992). In field studies, correlations between contaminant burdens in food with reduced or abnormal reproductive behaviors have been documented in both fish-eating birds and seed-eating birds (Bosveld and van den Berg, 2002; Fox et al., 1978; Fry et al., 1987; Haegele and Hudson, 1977; McArthur et al., 1983).

Birds are particularly interesting in terms of EDC effects on brain and behavior because of the extensive work on the avian song system, an interconnected series of brain nuclei involved in the sensory integration and motor output of species-typical vocalizations (see Chapter 2.12, Neural and Hormonal Control of Birdsong). Although the role of steroid hormones in masculinizing sexual behavior in birds began in the mid-1970s (Adkins, 1976), it was the demonstration of Gurney and Konishi (1980) that early estrogen can alter the sexually dimorphic morphology of the song control system that provided the first direct link between early sex hormone exposure, brain development, and adult sexual behavior. The then-surprising result that administration of E2 to chicks would masculinize the brain and behavior of female offspring has since been documented by a number of laboratories (cf Noorden et al., 1987). Work by Adkins-Regan and colleagues has demonstrated that early estrogen treatment also altered the sexual-partner preference of adult female zebra finches, but only if they were raised in all-female colonies (Adkins-Regan, 1988, 1999; Adkins-Regan and Ascenzi, 1987; Mansukhani et al., 1996). Early estrogen treatment also led to a female-biased secondary sex ratio in this species (Von Englehardt et al., 2004;

Williams, 1999). Taken together, these studies show a strong parallel between this observation and that of Fry et al. (1987) and Hunt (1977) on female–female pairing in areas where DDT had skewed the sex ratio of adult gulls.

Several research groups have capitalized on the proven relationship between brain morphology and behavior in birds and are beginning to investigate how EDCs may act on specific brain regions to influence reproductive behaviors. Millam and colleagues focused most of their efforts on the zebra finch, demonstrating that oral administration (to mimic parental feeding) of exogenous estradiol benzoate administered shortly after hatching not only reduced fertility but also masculinized the song system in the female (but not in males; Millam et al., 2001; Quaglino et al., 2002). Interestingly, the administration of octylphenol, methoxychlor, and dicofol in the doses given had no effect on fertility. Male zebra finches, however, did show deficits in mating behavior if they received posthatch treatment with estrogen, as Adkins-Regan and colleagues have shown (Adkins-Regan and Ascenzi, 1987), a finding also supported by Millam et al. (2001). Administration of perchlorate by oral gavage affected various developmental behaviors, but did not alter song-control nuclei size (Rainwater et al., 2007), while methoxychlor diminished fertility and hatching success (Gee et al., 2004).

Iwaniuk et al. (2006) found that, in the American robin (*Turdus migratorius*) increasing levels of DDT and DDE (as determined by analysis of yolk content in eggs taken from the same nests shortly after laying) were correlated with a reduction in males in the size of two song nuclei, nucleus robustus arcopallialis (RA) and high vocal center (HVC), formerly known as the hyperstriatum ventrale, pars caudalis, and in both males and females with reduced neuronal size and overall volume of nucleus intercollicularis (ICo; see Reiner et al. (2004) for revised nomenclature of song-control nuclei); there is no change, however, in area X in either sex of the species. In the European starling (*Sturnus vulgaris*) a somewhat different picture emerges. Markman et al. (2008) reported that starlings foraging in the winter on the worms in sewage effluent filter beds received significantly higher amounts of synthetic and natural estrogens and other EDCs than those foraging on worms found in garden soil. The hypothesis that these contaminants might influence both the behavior and brain morphology was tested by feeding captive starlings mealworms containing 200 ng of E2 or a mixture of 200 ng of E2, 520 ng dioctylphthalate, 80 ng BPA, and 120 ng of dibutyl phthalate (EDCs that are also found in worms in contaminated sites). The following spring, both males and females were assessed for the amount and complexity of song by males and the size of song nuclei (HVC). Male song and HVC volume were increased in individuals receiving the mixture; males receiving E2 alone did not differ from control (peanut oil) in any of these trait measures (Markman et al., 2008). Treatment did not affect testosterone levels or body mass. In a separate experiment, female preference for male song was assessed by measuring the time females spent on the perch adjacent to song playback. Females preferred the more complex song of males that had received the EDC mixture. It should be noted that the females used in the preference tests were wild-caught and hence their ingestion of EDCs during the previous winter was not known. The authors also measured immunosuppression in the males using cell-mediated immune function (wing-web swelling after injection of phytohemagglutinin) and secondary humoral response (response to injection of sheep red blood cells), finding that both the E2-alone and mixture-group males showed significantly lower immune function. Thus, by selecting males with more complex song, the females were also selecting males who were immunocompromised.

The neurohypophysial hormone arginine vasopressin (arginine vasotocin (AVT) in nonmammalian vertebrates) is involved in a range of male-typical behaviors in vertebrates, including aggression and courtship, and its distribution in the brain is sexually dimorphic in all vertebrates studied to date (cf fish (Goodson and Bass, 2001); amphibians (Boyd, 1994; Boyd et al., 1992; Marler et al., 1999; Moore et al., 2000); reptiles (Hillsman et al., 2006; Propper et al., 1992; Smeets et al., 1990; Stoll and Voorn, 1985; Thepen et al., 1987); birds (Aste et al., 1998; Grossman et al., 2002; Jurkevich et al., 1997, 1999, 2001; Kimura et al., 1999; Panzica et al., 2001; Panzica and Viglietti-Panzica, 1999; Viglietti-Panzica et al., 1994; Voorhuis et al., 1988); and rodents (Aragona and Wang, 2004)). In the Japanese quail, the absence of, or reduced, sexual behavior of adult males exposed to EDCs *in ovo* is also reflected in disrupted AVT-ir in the bed nucleus of the stria terminalis, medial preoptic nucleus, and lateral septum (Panzica et al., 2005b, 2007).

Although amphibians are becoming symbolic of EDC effects in both field and laboratory studies, to the best of our knowledge there have been virtually no published studies at this time demonstrating that such compromised individuals show differences in behavior. However, the many demonstrations that EDCs alter gonadal differentiation and influence genes coding for steroidogenic factor-1 (SF-1) and other steroidogenic enzymes make this very likely. Strong evidence for such effects might be found in the *Xenopus*, in which both atrazine and PCB exposure inhibited laryngeal (both cartilage and muscle) development (Hayes et al., 2002; Qin et al., 2007), a sexually dimorphic structure important in the male calling behavior (Kelley and Brenowitz, 2002). Treating female red-spotted newts (*Notophthalmus viridescens*) with the insecticide endosulfan disrupted mate choice and lowered mating success (Park et al., 2001; Park and Propper, 2002). Helbing et al. (2007) demonstrated that perchlorate exposure to developing tadpoles modified gene expression profiles (using both cDNA array analysis and qPCR), with the greatest effect on the levels of mRNAs encoding proteins important in neural development and function.

5.04.4.3 Fish

Despite the considerable evidence for disruption and even complete failure of reproductive functions caused by EDCs (Arukwe, 2001; Bayley et al., 2003; Jobling et al., 2002; Kidd et al., 2007; Koger et al., 2000; Toft et al., 2003), the literature on effects of exposures on adult sexual behaviors is relatively sparse. There are many reports showing effects of exogenous hormones on sexual behaviors in male and female fish of many species (reviewed in Milnes et al., 2006), suggesting similar consequences of xenobiotics. One challenge in fish is studying behaviors of populations in the wild. The extent (dose, duration, nature, mixture, etc.) of contamination is

almost impossible to assess in these studies and results have sometimes been inconclusive. For example, courtship behavior in males was compared between mosquitofish in Lake Apopka, FL, which was polluted with DDT and metabolites and other EDCs such as dicofol, toxaphene, *trans*-nonachlor, dieldrin, and aldrin (Toft et al., 2003) and those in a reference lake, Orange Lake. Courtship behavior in the males was assayed by pairing with an uncontaminated adult female, and in particular, two behaviors, referred to as following behavior and close-following behavior in reference to the female's genital opening, were studied. Although certain sexual characteristics of the fish were depressed in the Lake Apopka fish, there were no statistical differences in following and close-following behaviors. A correlational study comparing sexual behaviors of mosquitofish (*Gambusia affinis holbrooki*) living upstream versus downstream to a paper mill showed aberrations in the behaviors of both the males and females (Howell et al., 1980). Other work on fish has assessed reproductive behaviors in a very generalized way, that is, whether animals mate or not, and the time taken by them to initiate mating. These reports show detrimental effects of EDC exposures on these behaviors (reviewed in Segner et al., 2003), such as in the Japanese medaka (*Oryzias latipes*) in which developmental exposure to ethinyl estradiol completely obliterated mating behaviors in adult females and virtually obliterated it in the males (Balch et al., 2004). A laboratory study was carried out in male guppies, testing effects of vinclozolin, a fungicide that acts at least in part as an antiandrogen, on secondary sex characteristics and on male courtship behavior (Bayley et al., 2003). Courtship was assayed through quantification of specific, stereotyped swimming patterns by the male toward the female. Vinclozolin-treated males had smaller first clutch sizes, reduced sperm count, and a significant decline in the number of sexual displays exhibited toward a nonreceptive adult female (Bayley et al., 2003). Interestingly, this effect was limited to the males, as exposure of the females to vinclozolin did not alter clutch size. Similarly, in male guppies, antiandrogenic pesticides decreased sigmoid displays toward females, with lower doses having more potent effects (Baatrup and Junge, 2001). In this same species, 4-*tert*-octylphenol, a xenoestrogen, decreased male sexual displays toward females (Bayley et al., 1999). Similar effects of octylphenol were found in Japanese medaka (Gray et al., 1999).

Over the past decade, interest in the value of fish as a model for neuroendocrine and behavioral aspects has exploded. Part of this heightened interest stems from growing unease surrounding the use of mammalian animal models for EDC testing because it is expensive, cumbersome, time-consuming, and at odds with a growing movement to reduce animal use in toxicological testing. Tanguay and colleagues have spearheaded large-scale efforts to construct high-throughput screening systems using embryonic zebrafish to characterize chemical-elicited behavioral responses at an early (24 h post-fertilization) stage that predict teratogenic consequences at a later developmental stage (Truong et al., 2014; Reif et al., 2015). His group has consistently found that, as an integrative measure of normal development, significant alterations in movement such as startle responses can identify neuroactive chemicals representing several modes of action including endocrine disruption (Saili et al., 2011; Garcia et al., 2016). In addition to this work, a recent important paper showed that treatment of zebrafish larvae with BPA, or the replacement chemical BPS, increased developmental neurogenesis in the hypothalamus, resulted in hyperactive behavior, via a mechanism involving androgen-receptor upregulation of the aromatase enzyme (Kinch et al., 2015).

5.04.5 Multi- and Transgenerational Effects of EDCs: A Role for Epigenetics

Evolution selects for outcomes, not mechanisms. The individual with its adapted morphological, physiological, and behavioral traits is both a result and a cause of evolutionary change. Only phenotypic change is subject to selection and novelty can arise via several processes. The ability of the genotype to produce different phenotypes in response to different environments has been termed plasticity. Epigenomic regulation of networks of genes and their products has emerged as a powerful mechanism by which this plasticity is conferred and, potentially, passed forward to future generations. In this sense it might be said that epigenomics is one way in which the 'genome learns from its experience' (Jaenisch and Bird, 2003). For the purposes of this chapter epigenetics is the study of cellular and physiological phenotypic trait variations that are caused by external or environmental factors that switch genes on and off and affect how cells read genes. Epigenetic effects are broadly defined as functionally relevant changes to the genome that do not involve a change in the nucleotide sequence (Jirtle and Skinner, 2007).

Rapidly emerging data reveal that EDCs can induce epigenomic changes in various tissues, including the brain (Yeo et al., 2013; Mileva et al., 2014; Rissman and Adli, 2014). Whether these molecular epigenetic effects and their transgenerational consequences are eventually incorporated in the genome so that selection might act is a question of great interest.

5.04.5.1 EDCs and Epigenetics

Changes in gene and/or protein expression in sexually dimorphic regions cannot necessarily explain all neuroendocrine and, especially, behavioral differences. Alternative mechanisms are beginning to be investigated including manipulation of DNA methylation. In one of the earliest studies along these lines Monje et al. (2007) showed that early postnatal (P1–P7) treatment of rat pups with BPA caused changes in the expression of the ERα gene in the POA, and immunohistochemical expression of the ERα protein in the AVPV, measured either at 8 or 21 days of age. Further, the methylation status of the ERα promoter was perturbed by BPA (Monje et al., 2007). This is a nice link between promoter utilization and gene and protein expression. Subsequent work identified BPA-related downregulation of ERα in the POA and VMN, regions critical for coordinating lordosis and other aspects of female reproductive behavior. Proceptive, but not receptive, behavior was found to be impaired (Monje et al., 2009).

BPA has also been used to demonstrate not only hypomethylating effects of fetal exposure, but also the rescue of this effect by maternal nutrition. Dolinoy et al. (2007) used an

agouti mouse model (A^{vy} genotype), the gene of which is differentially methylated to result in a range of coat colors as an external phenotype, as well as differing in other phenotypic manifestations (reviewed in Jirtle and Skinner, 2007). BPA exposure of a pregnant mouse alters the expression of coat color concomitant with a shift in the A^{vy} site-specific methylation (BPA-caused hypomethylation), and further, this was compensated by supplementing the maternal diet with a methyl donor, such as genistein (Dolinoy et al., 2007). A follow-up study replicated this effect of BPA over multiple doses (Anderson et al., 2012). An additional study by this group associated high urinary BPA levels with less genomic methylation in preadolescent Egyptian girls demonstrating the capacity for EDCs to alter the human epigenome (Kim et al., 2013). Yet, another group reported epigenetic effects in the form of DNA methylation and altered gene regulation in the brain in response to very low-dose gestational BPA exposures, applying 2, 20, and 200 μg BPA/kg per day (Kundakovic et al., 2013). Genes of the epigenetic machinery were affected as well as sex-hormone nuclear receptors, some of them in a sex-specific manner. Offspring's behavior was significantly altered at all three doses. This study documents epigenetic effects on gene regulation in the brain by BPA at the low end of the dose and potential relationships to behavioral phenotype. Robust evidence of causal linkages between EDC-related epigenetic change and meaningfully altered physiological or behavioral phenotype remains rare in the available literature but a rapidly growing aspect of inquiry.

Via epigenetic reprogramming, BPA has also been shown to induce effects not considered 'typical' or 'classical' for endocrine disruption. *Kcc2/KCC2* is a critical neuronal-specific gene that codes for a chloride-extruding transporter molecule. Developmental upregulation of *Kcc2* expression is essential for proper migration of precursor neurons and synchronization of maturation at the cellular and neural network level. Deceleration/attenuation of the ontogenetic upregulation of *Kcc2* expression during the sensitive perinatal period can alter cortical organization. Liedtke and colleagues found that the histone-deacetylase inhibitor, trichostatin-A (TSA) can accelerate this process by upregulating *Kcc2* expression (Yeo et al., 2009) thereby demonstrating the importance of epigenetic regulation for *Kcc2* expression. Using cultured neurons, organotypic brain slice culture, and *in vivo* mouse models, this group then showed that BPA can enhance repression of *Kcc2* transcription, an effect which decelerated and delayed *Kcc2* transcription (Yeo et al., 2013). This effect is consistent with and a potentially explanatory mechanism for previously reported disruption of cortical layer architecture in brains of mouse pups exposed to BPA through gestational feeding to their dam (Nakamura et al., 2007; Komada et al., 2012). These neuroanatomical abnormalities also dovetail with the sequela of behavioral deficits previously reported in prenatally exposed animals (Wolstenholme et al., 2011).

5.04.5.2 Multigenerational Studies on Effects of EDCs

Most EDC-related work focuses on the affected individual, but environmental and bodily contamination by EDCs and other anthropogenic chemicals is ubiquitous, and thus species must adapt in response to this challenge. How likely is it that EDCs and other environmental signals could actually contribute to the process of evolution (Crews and Gore, 2011)? A striking but underappreciated aspect of environmental contamination is that some individuals are less affected than others (Orlando and Guillette, 2001), a common observation in centers of populations where there is greater variability. Even if fitness is compromised in all individuals, those that are less affected will have greater reproductive success than those who are rendered sterile or develop diseases. On the other hand, they may be epigenetically compromised such that their offspring are themselves affected. The 'thrifty phenotype' as originally proposed by Barker is a classic example and posits that via epigenetic or other mechanisms a person reared in a calorie-sparse environment will be developing a calorie-reserving phenotype and pass this capacity onto their offspring. This inherited adaptive response to starvation risk is actually deleterious in a calorie-rich world, such as the United States, because it heightens risk of obesity and related morbidities (Barker, 2007; Jones and Ozanne, 2009).

A plausible scenario for EDC influencing changes in methylation patterns leading to epigenetic inheritance has been proposed (Guerrero-Bosagna et al., 2005; Guerrero-Bosagna and Valladares, 2007). But evidence suggesting that EDCs might reprogram methylation patterns that are, in turn, incorporated into the germ line and hence transmitted to future generations is still sparse. The best evidence is probably provided in the work from Skinner's lab using the fungicide vinclozolin. This group showed that fetal exposure to high concentrations of vinclozolin caused latent reproductive and other dysfunctions later in life and, further, that these effects were transmitted via the paternal germ line for up to four generations (Anway et al., 2005, 2006; Anway and Skinner, 2006). Although there may be other mechanisms at work, at least one of these involved changes in DNA-methylation patterns in germ cells, such as within the lysophospholipase gene and the cytokine-inducible SH2 gene, both of which had altered methylation patterns across generations (Anway et al., 2005).

In order to apply the fetal basis of adult disease in a truly transgenerational manner, it is necessary to take experiments to the F3 generation. As previously reviewed (Skinner, 2008; Rissman and Adli, 2014), when the pregnant F0 dam is exposed to an EDC, her F1 pups are exposed directly *in utero*. At this developmental stage, the primordial germ cells of the fetuses (the future F2 generation) are developing and can have direct exposure to the EDC. Although they are unlikely to have an appreciable body burden as adults, nevertheless, the F2 generation may have been influenced by direct exposure to the EDC. Thus, only their F3 offspring are the first to be free of direct contact to the original insult.

This multigenerational approach has rarely been used in the laboratory setting, and although there are inevitable exposures of multiple generations in the wild, the lack of a controlled setting has made it virtually impossible to draw causal relationships. In the arena of reproductive neuroendocrinology, there is a wealth of evidence for effects of fetal and early postnatal exposures to both endogenous and exogenous hormones on the adult phenotype (reviewed in Gore, 2008). There is beginning to be interest in this field from the perspective of endocrine disruption, although most reports are limited to two-generational studies (up to the F2) and/or do not extend

to neuroendocrine or behavioral endpoints. We refer readers to Fernie et al. (2001), Keneko et al. (1974), and McCoy et al. (1999) and provide a brief discussion of a few for illustrative purposes. When Japanese and bobwhite female quail were exposed to dietary methoxychlor or vinclozolin, similar deficits were observed in the sexual behavior of the male offspring of the two generations when they reached adulthood (Ottinger et al., 2005b). In fish, lifelong exposures of zebrafish (*Danio rerio*) to ethinyl estradiol reduced reproductive success although affected males continued to try to mate (Nash et al., 2004). The latter result is important because it hearkens back to the concept of competition between affected and unaffected individuals and its detrimental effect on the population. DES has also been an informative model, as its transgenerational effects on the reproductive tract have been studied in models from mice to humans (Newbold, 2004; Newbold et al., 2006; Titus-Ernstoff et al., 2006; Walker and Haven, 1997). However, effects on the brain and behavior are still lacking in a transgenerational approach.

One of us (Andrea C. Gore) investigated effects of low-level fetal PCB (Aroclor 1221) exposure to F0 rat dams during the critical period of brain sexual differentiation (gestational days 16 and 18) on the reproductive development and behavior in the F1 generation of female offspring in adulthood (Steinberg et al., 2007, 2008). We also evaluated the female F2 generation of offspring in early adulthood for several reproductive physiological parameters. These F2 rats exhibited aberrant hormone profiles across the estrous cycle (Steinberg et al., 2008). In particular, LH and progesterone levels were significantly lower on proestrus in the F2-PCB females compared to the F2-vehicle group. These results show that exposure of the F2 rats' pregnant grandmother to PCBs had effects on the F2 descendants' reproductive physiology in adulthood (Steinberg et al., 2008). We are currently developing models to assess effects of F0 exposure to PCBs up through the F3 generation.

5.04.6 Summary and Recommendations for the Future

There is no question that certain chemicals disrupt endocrine systems essential to normal development. Although the field has matured in many aspects and discovered quite a bit about the what, why, where, and how neuroendocrine and behavioral anomalies result following EDC exposure, focus should not be on differences in susceptibility. Along those lines, greater understanding is needed for why some individuals that do not appear to be morphologically compromised do not breed. In these instances, we will be dealing with how such chemicals affect the brain mechanisms that process, produce, or integrate social signals necessary for successful reproduction that warrant research attention. For example, it is possible that such individuals are responsible for the widely reported precipitous declines in amphibian populations worldwide, and this may have similar effects in fish (Nash et al., 2004). The logic behind this revolutionary statement is quite simple. While it is true that disease caused by exogenous agents can eliminate the population within a single generation, this is not what happens typically in EDC-contaminated areas. Rather the incidence of disease and malformed amphibians increases and then stabilizes, yet the population is still in decline. The reason would appear obvious, namely that there are otherwise normal appearing individuals that simply are not breeding, leading to the precipitous declines observed. Understanding the mechanisms underpinning such phenomenon may ultimately be informative for understanding how EDCs and other chemicals might be contributing to rapidly rising rates of human psychosocial disorders. Such linkages need to be made with caution, however, given critical differences regarding the relative roles androgen and estrogen receptors play in the masculinization of the primate brain compared to rodents and other species. While reports in the popular press sometimes claim that prenatal exposure to chemicals such as BPA may increase the risk of autism (for example), such direct relationships cannot be drawn based on current knowledge. In fact, trying to link a single gene, a single chemical, or some other single 'hit' to a complex neurobehavioral or neurodevelopmental disorder is simply not plausible in humans.

Research into the effects of EDCs on wildlife and humans continues to grow and evolve. It might be useful to consider how this evolution reflects certain parallels in any newly emerging science, namely first the verification and validation of a phenomenon in a variety of organisms. This stage is fraught with controversy and denial from the both the parent field(s) and other interests as the inherent varieties of the phenomenon become evident (e.g., egg-shell thinning, gross morphological anomalies, hermaphroditism/intersex, and sex-atypical metabolism). Once deniability becomes specious, the guiding principles are developed, again with acrimony and contest (e.g., low dose, mixtures, synergy, and thresholds). There is the tendency, during this period, for research to become mainstream with most content to solidify major principles in its variety of forms, usually with one venue being the mechanistic, and ultimately the genetic, basis and the other capturing the complexity of the phenomenon. This arena might also be widened as proven techniques and test systems from other fields are implemented to deepen our understanding of the mechanisms that underlie the variety of EDC effects. It is curious that it always seems that behavioral modifications are the last to be discovered. Perhaps this is simply an ontogenetic process as disciplines mature, but for those of us who are principally interested in behavior, knowing as we do that behavior is at the leading edge of evolutionary change, it is perplexing that the ultimate consequences of global ecological change are not often studied from the behavioral standpoint.

Finally, it should be said that we will never know the true extent of the effects of EDCs simply by tallying grossly and obviously malformed individuals in a population any more than we will understand EDC effects on wildlife and human populations by detailing exactly the mechanism of their action. In the former instance, the poster-child approach, the emphasis is misplaced by focusing on such individuals, even if the broader interpretation is taken by noting that such individuals are a negative burden on the population because they consume what may be limited resources. Similarly, the focus on how a particular chemical reacts with chaperones to interfere with normal ligand-binding at the DNA will not lead to new therapeutic advances. Take, for example, atrazine, a widely used

herbicide. We now know that atrazine causes morphological abnormalities in amphibians in both nature and the laboratory and does so by affecting aromatase by acting on the SF-1 gene, thereby changing the relative production of androgen and estrogen during the time period when the reproductive system is forming (Fan et al., 2007a,b; Hayes et al., 2006a,b). The solution may seem quite simple. Stop making atrazine. It is important to acknowledge that the problem for which atrazine was developed is serious and should not be minimized, but new green chemistry might result in a chemical that equals, or betters, the performance of atrazine in the field without the endocrine-disrupting consequences (Thornton, 2000, 2007; Schug et al., 2013). Finally, the recently discovered phenomenon of transgenerational imprints by EDCs on DNA-regulatory mechanisms carried forward for four or perhaps more generations is a particularly sobering one. What this means is that simply cleaning up an environment and no longer using particular chemicals on a global level may have no ultimate effect. That is, the damage has already been done and the most we can do for remediation is to stop polluting, and thus maintain a status quo by employing green-manufacturing methods (Thornton, 2000, 2007).

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