

Effects of Xenoestrogens on the Differentiation of Behaviorally Relevant Neural Circuits in Higher Vertebrates

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Several environmental chemicals have the capability of impacting endocrine function (endocrine disrupting chemicals [EDCs]), and therefore they may have long-term consequences, especially if exposure occurs during embryonic development. In this study we present data relative to two widely used animal models: the Japanese quail and the mouse. These two species have been used to understand neural, neuroendocrine, and behavioral components of reproduction and are optimal models to understand how these components are altered by precocious exposure to EDCs. In particular, we discuss the effects of embryonic exposure to diethylstilbestrol, genistein, or ethylene,1,1-dichloro-2,2-bis(p-chlorophenyl) on the sexually dimorphic parvocellular vasotocin system and male copulatory behavior in quail and the effects of bisphenol A on the nitrinergic and kisspeptin systems and their behavioral impact in the mouse. In both models the exposure to EDCs during the critical period (early embryonic period in birds, perinatal period in rodents) alters the differentiation of relevant sexually dimorphic pathways, often inducing the appearance of a sex-reversed neurochemical phenotype that is the most probable cause of the final alteration of sexually differentiated behaviors in the adult animal. In conclusion, the data presented here should stimulate a critical reanalysis of the way to determine the “safe” exposure levels to EDCs for wild species and humans, considering behavior and related neural circuits among the factors to be analyzed.

Key words: diethylstilbestrol; genistein; DDE; bisphenol A; vasotocin; nitric oxide synthase; kisspeptin

Introduction

The development of a living organism depends upon a large and intricate array of chemical signaling systems guiding cell proliferation and differentiation. Many of these signals are derived from the endocrine system. Among the hormonal signals with high impact on brain development, gonadal hormones, such as 17β -

estradiol (E_2) or androgens, play key roles in the development of primary and secondary sex characteristics in higher vertebrates, including several steroid-dependent behaviors and neural circuits.¹

In particular, estrogens may modulate neuronal differentiation, notably by influencing cell migration, survival and death, and synaptic plasticity of neurons both in hypothalamic and in extrahypothalamic circuits.² They are, therefore, essential for normal development and sexual differentiation of the central nervous system and reproductive behaviors.³ Several experimental studies demonstrated that disturbing

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this developmental milieu, via exogenous estrogen treatment or gonadectomy, during critical periods of the prenatal and/or postnatal development, may induce irreversible changes in the organization of the central nervous system as well as behavioral alterations in many species.⁴⁻⁶

In recent years several observations have shown that early exposure to industrial pollutants can induce adverse effects on endocrine structure development and therefore affect humans, farm animals, and wildlife.⁷ These environmental chemicals have been termed *endocrine disrupting chemicals* (EDCs) and have been defined as "Exogenous substances that change endocrine function and cause adverse effects at the level of the organism, its progeny, and of populations of organisms."⁸

Many of the EDCs are estrogens or estrogen-like molecules that have been classified as environmental estrogens or xenoestrogens.^{9,10} Because of their estrogenic effects, they could, even in very low concentrations, deeply influence the development and the function of estrogen-dependent neural circuits and behaviors. In fact, behavioral responses represent the culmination of several integrated systems. Therefore even small changes of neural or neuroendocrine components are likely to disrupt or modify behavior. Importantly, disturbances in normal behavior may influence the individual fitness and, therefore, acquire a real biological significance in both animal and human ecosystems.¹¹⁻¹³

Several studies investigated cellular effects of EDCs in *in vitro* systems or in lower vertebrates. In recent years, several laboratories, including ours, started to investigate the effects of precocious exposure to EDCs on both behavior and neural circuits in higher vertebrates. In particular we used two different experimental approaches for birds and mammals to try and mimic the real mode of exposure to EDCs that may happen during prenatal and postnatal development.

In birds we used the Japanese quail as a model in which neural circuits and hormones

controlling reproductive behavior have been identified during numerous experimental studies (for reviews see Refs. 14, 15). In particular, embryonic exposure to E₂, prior to day 12 of incubation, irreversibly demasculinizes both adult male copulatory behavior¹⁶ and the parvocellular vasotocin (VT) system of the bed nucleus of the stria terminalis, pars medialis (BSTm),¹⁷ a peptidergic system that is involved in the control of male copulatory behavior.¹⁸ Typically, exogenous EDCs are eaten by the mother and accumulated within eggs,¹⁹ therefore we injected different xenoestrogens within the eggs at day 3 of incubation and tested their estrogenic effects on both male sexual behavior and VT immunoreactivity.

In rodents, perinatal exposure to gonadal hormones irreversibly alters both sexual behavior and some sexually dimorphic circuits as the sexually dimorphic nucleus of the pre-optic area.²⁰ Therefore we applied a widely used procedure that allowed oral administration of a controlled amount of the EDCs to the pregnant/lactating female mouse from gestation day 11 to postpartum day 8 (perinatal exposure). Their offspring were examined for exploratory and sexual behaviors before and after puberty and for alterations of some behaviorally relevant neural circuits in adulthood.

The Effects of Estrogenic EDCs in Japanese Quail

Female quail given E₂ implants are known to transfer E₂ to offspring via the yolk.²¹ Similarly, soy phytoestrogens and especially genistein readily transfer to the yolk when ingested by the hen.¹⁹ Therefore the accumulation of steroids and steroid-like compounds in the yolk in birds is providing a primary route of exposure to EDCs during embryonic development (for a review see Ref. 22).

Earlier studies showed that some pesticides interfere with the avian hypothalamic-pituitary-gonadal axis (Ref. 23). More recent studies demonstrated that insecticides, such as *o,p'*-dichlorodiphenyltrichloroethane

(DDT),²⁴ dichlorodiphenyldichloroethylene (DDE),²⁵ or methoxychlor (MXC),^{26,27} as well as other compounds, such as ethinylestradiol (EE₂),²⁸ diethylstilbestrol (DES),²⁹ and genistein,³⁰ significantly decrease male sexual performance when administered during embryonic development. Besides the effects on behavioral differentiation, these chemicals may affect differentiation of gonads, accessory sexual organs, and brain circuits.

Brain changes were specifically analyzed in our studies. In particular we observed a significant demasculinizing effect of DES, genistein, and DDE on the sexually dimorphic parvocellular VT system of BSTm and on its projections to medial preoptic nucleus and lateral septum.^{25,29,30} For other EDCs the effects on this neural system were not significant or absent. For example, EE₂ and MXC both affect male sexual behavior²⁶ but did not induce alterations of the VT system. Recent experiments with the estrogen receptor (ER) α -selective agonist, propyl pyrazole triol, suggested that estrogen-induced effects on reproductive organ differentiation are mediated by ER α , whereas demasculinization of male copulatory behavior and of the VT-immunoreactive system appears not to be induced by activation of ER α alone,³¹ that ER β may have a primary role for the development of these circuits. This hypothesis is also supported by the observation that, during development of hypothalamic and limbic regions of Japanese quail, ER α appears later than ER β .³²

Several studies also investigated the effects of *in ovo* administration of bisphenol A (BPA), a xenoestrogen used to manufacture polycarbonate and numerous plastic articles that is widely diffused in the environment and in animal and human food.³³ Administration of BPA to chicken embryos induced Mullerian duct (embryonic oviduct) malformation in female embryos and feminization of the left testis (ovotestis) in male embryos³⁴ by the age of 15 or 19 embryonic days. In two different experiments we administered 50, 100, or 200 μ g of BPA per egg. The result of these embryonic treatments was a dra-

matic decrease in the number of hatched animals: the percentage of living chicks was in fact ranging from 8–11% of injected eggs and the BPA-injected young quail did not survive after 1 week of life. The dissection of nonhatched embryos revealed that the large majority of the embryos died immediately after the BPA administration (from 36–63%), and in embryos that died later, we observed a high incidence of malformations of the gut, abdominal wall, and legs. With these experiments we were, obviously, unable to study any alteration in the brain.³⁵ Therefore we may conclude that, contrary to what happens in mammals (see below), BPA, even at low doses, has robust adverse effects in birds, inducing several malformations also involving the reproductive tract that ultimately result in a strong reduction in survival in exposed subjects.

Effects of BPA Administration in Rodents

BPA is known to have a weak estrogenic action because of its low affinity for the ER α .³⁶ In spite of this weak binding activity, very low doses of BPA administered during the perinatal period have functional consequences in mice; in male mice it specifically increases prostate weight and decreases sperm production,^{37,38} whereas in female mice it significantly increases the body weight, accelerates puberty onset,³⁹ and alters maternal behavior of adults.⁴⁰

In the last few years several studies investigated the effects of early administration of BPA on specific neural pathways in the rodent brain. In particular they demonstrated that the monoaminergic system is an important target for BPA action. The functional impact of dopamine (DA) system alterations has been demonstrated by investigating potential changes in the reinforcing effects of amphetamine (AMPH)⁴¹ and morphine.⁴² These effects appear to be sexually differentiated. When compared to unexposed female mice, BPA-exposed female mice failed to show AMPH-induced conditioning whereas male mice showed no changes from the same

prenatal treatment. Thus, prenatal exposure to BPA was apparently responsible in female mice of the impairment of brain reward pathways targeted by the drug. These functional alterations have been confirmed by other studies demonstrating that mice or rats perinatally exposed to low doses of BPA show alterations in sexually dimorphic populations of tyrosine hydroxylase (TH) neurons in the anteroventral periventricular preoptic area (Refs. 43, 44) and in the locus ceruleus,⁴⁵ two brain regions characterized by a larger volume and larger number of TH neurons in female rats than in male rats.

It is recognized that DA regulates male sexual behavior in rodents through its cooperation with the nitric oxide (NO)-producing system.^{46,47} This last system may also be influenced by perinatal exposure to BPA in a very specific way. The NO-producing system (identified through the immunohistochemical detection of the NO synthesizing enzyme) is widely present within the rodent brain,⁴⁸ but BPA selectively alters this system at the level of the medial preoptic nucleus and of the bed nucleus of the stria terminalis,⁴⁹ two key nuclei for the control of reproductive behaviors. These data indicate that the NO system is another potential and important target for the action of EDCs in mammals and may explain data showing that male rats perinatally exposed to BPA display less efficient sexual behaviors than controls.^{50,51}

Recently we investigated the effects of perinatal administration of BPA on another key peptidergic system for the control of mammalian reproduction, the kisspeptin producing system. Kisspeptin (and related peptides identified in several vertebrates⁵²) is universally recognized as an essential activator of the gonadotropic axis, with key roles in puberty onset and in the control of gonadotropin secretion. While these fundamental functions are now well established, novel aspects have emerged, including the involvement of kisspeptin in the neuroendocrine control of ovulation and the metabolic gating of reproductive function.⁵³ Our data demonstrate that early ex-

posure to BPA induces the disappearance of parts of the sexually dimorphic distribution of the kisspeptin system. In fact, in control mice kisspeptin immunoreactivity is higher in females than in males, and BPA exposure increases kisspeptin expression in male arcuate, periventricular, and anteroventral periventricular nuclei.⁵⁴ All together these results suggest that alterations of sexually dimorphic behaviors and of reproduction from early exposure to BPA may be linked to modifications of DA, NO, and kisspeptin–gonadotropin-releasing hormone circuits.

Conclusions

Data reported in this brief review clearly indicate that EDCs acting at low concentrations can exert subtle effects by interfering with gene expression and other cellular activities that can cause transient activational responses or permanent impairment.^{55–57} Thus, the impact of EDCs will vary depending upon a variety of factors, including the doses that subjects are exposed to, when in the life cycle of an organism exposure occurs, as well as the duration of the exposure. In particular, developmental stages are typically far more vulnerable to signal disruption than adult stages and the consequences of fetal exposure may be drastically different from those of adult exposure. Therefore the search for the maximal acceptable doses should differentiate exposure levels during development, prepuberal period, and adulthood. One clear implication of this focus on low-level exposure during fetal and neonatal development is that levels of exposure that have been considered as “background,” and thus “safe,” can have deleterious effects early in life. Many laboratory studies on animal models now support the conclusion of a higher sensitivity of the embryo and neonate,^{58–60} and this notion is also consistent with some epidemiological data from human studies.⁶¹

The two animal models that we have outlined here (the VT parvocellular system and

the male copulatory behavior of the Japanese quail and the circuits controlling sexual behavior in rodents) seem to be particularly useful for understanding the mechanism through which EDCs may act during the prenatal or postnatal critical periods. In both models exposure to EDCs during the critical period (early embryonic period in birds, perinatal period in rodents) alter the differentiation of relevant sexually dimorphic pathways, inducing the appearance of a sex-reversed neurochemical phenotype that is the most probable cause of the final alteration of sexually differentiated behaviors in the adult animal.

The neuroendocrine determinants of the development of sex differences in brain circuits and behavior in Japanese quail and galliforms are well known. The strong sex dimorphism of some brain circuits (e.g., the VT parvocellular system¹⁴) and of male copulatory behavior (testosterone-dependent behavior present only in male Japanese quails and not in females⁶²) are powerful tools for analyzing effects of EDCs on the nervous system. In particular as demonstrated in several experiments, exposure to low levels of estrogens during early phases of male embryonic development induces a total loss of male copulatory behavior¹⁶ and a female-like pattern of expression of the VT parvocellular system.¹⁷ Therefore EDCs characterized by a xenoestrogenic activity (such as DES, DDE, genistein) may act during early phases of embryonic development by mimicking E2 effects and inducing a partial or total demasculinization of both male copulatory behavior and the VT parvocellular system.^{25,29,30} As suggested by studies with the ER α agonist propylpyrazole-triol,³¹ this action is probably mediated through the binding of ER β , which is expressed more precociously than ER α in the galliform embryos.³²

The situation is more complex in mammals. According to the classic “hormonal” theory, during the critical period of rodent brain development (from late pregnancy until the first 1–2 weeks after birth), E₂, actively synthesized in the brain from testosterone by neurons

expressing the enzyme aromatase,⁶³ permanently and irreversibly organizes male-typical specific circuits.⁶⁴ The brain of the female fetus is protected from circulating estrogens by a steroid-binding protein called alpha fetoprotein (AFP; abundant during fetal and early neonatal life⁶⁵). AFP also protects the male fetus brain from an excess of maternal estrogens. This hypothesis has been recently confirmed by elegant experiments in AFP-knockout mice.⁶⁶

This protection mechanism may be bypassed by EDCs, such as BPA, that generally exhibit a lower affinity for plasma estrogen-binding proteins, including AFP.⁶⁷ EDCs can thus interfere with the male- and female-typical development of brain areas (e.g., dopaminergic, NO-producing, and kisspeptin-producing systems) that control the occurrence of a wide range of behaviors required for reproduction, such as sexual, social, and nonsocial behaviors in adult life.

Recent evidence has pointed out that sex differences in neural structures not directly related to reproduction (i.e., the hippocampus) may also be the results of nongenomic mechanisms.² EDCs may also partly interfere with these mechanisms, in fact some effects of BPA seem to be partly related to cell signaling systems involving serial activation of kinases via ligand binding to cell membrane estrogen receptors at very low concentrations.⁵⁵ In addition, a number of studies have suggested that some of the neurobehavioral effects of xenoestrogens, such as BPA, cannot be simply explained by an estrogenic action of this compound.^{50,68,69} MacLusky and co-workers have recently reported antiestrogenic effects of BPA on hippocampal synaptogenesis of mice brain,⁷⁰ whereas other studies have demonstrated that BPA may exert estrogenic or antiestrogenic effects in the rat cerebellum, according to its concentration.⁷¹

In conclusion, the data collected in our and other laboratories should stimulate a critical re-analysis of the way to determine the “safe” exposure levels to EDCs for wild species as well as

humans, considering behavior and related neural circuits among the factors to be analyzed. On the other hand an increased knowledge of mechanisms involved in the sexual differentiation of brain and behavior is necessary in order to understand genetic and molecular mechanisms that are at the basis of the adverse effects of EDCs.

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Conflicts of Interest

The authors declare no conflicts of interest.

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