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Environmental Toxicant Effects on Neuroendocrine Function

Andrea C. Gore

Kastor Neurobiology of Aging Laboratories, Fishberg Research Center for Neurobiology, and Schwartz Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, New York, NY

Exposure to environmental toxicants can have profound effects on normal growth and development. However, the mechanisms by which these toxicants exert these effects are not well understood. Many environmental toxicants alter reproductive function and have effects on the central nervous system and behavior, yet the link between these reproductive and neurologic phenomena has not been systematically investigated. The neuroendocrine (hypothalamic-pituitary-gonadal) axis, which integrates inputs to and outputs from the nervous and reproductive systems, is functionally and anatomically situated to mediate effects of environmental toxicants, particularly those that are endocrine-disrupting chemicals (EDCs), on developmental processes. This article reviews the current literature on EDC effects on the neuroendocrine system, particularly at the level of hypothalamic gonadotropin-releasing hormone (GnRH) neurons, the key cells involved in the regulation of reproductive function. The focus of this article is on two polychlorinated biphenyl mixtures (Aroclor 1221, Aroclor 1254) and two organochlorine pesticides (methoxychlor and chlorpyrifos). Some experimental data are presented for each of the four urban environmental toxicants on GnRH cells in vitro and in vivo. The results of in vitro experiments indicate that all four of the toxicants profoundly affect hypothalamic GnRH gene expression, cell survival, and neurite outgrowth, demonstrating direct effects of EDCs on a GnRH cell line. In in vivo experiments, three of the toxicants (Aroclor 1221, methoxychlor, and chlorpyrifos) caused significant alterations in GnRH mRNA levels in female rats. Both the in vitro and in vivo findings support the novel concept of chlorpyrifos as an EDC. The results, taken together with the literature, support the hypothesis that the neuroendocrine axis, and specifically GnRH neurons, are sensitive to urban environmental toxicants, and that reproductive and neurologic effects of EDCs may be mediated at this level of the hypothalamic-pituitary-gonadal axis.

Key Words: Gonadotropin-releasing hormone; puberty; reproduction; chlorpyrifos; methoxychlor; PCB.

Effects of Environmental Toxicants on Reproductive and Neuronal Development

Reproductive System Effects of Environmental Toxicants: Polychlorinated Biphenyls

Numerous toxic substances that are found in the urban environment cause abnormalities in normal growth and development. Important examples of such toxicants are polychlorinated biphenyls (PCBs), which were used as industrial sealants, electrical insulators, and dielectrics and in carbonless paper (Fig. 1). PCBs have been a continuous problem in urban environments because they persist for many years and can bioaccumulate *(1,2)*. These substances are endocrine-disrupting chemicals (EDCs) in that they can bind to hormone (usually estrogen or androgen) receptors to either activate or inactivate them. The ability of PCBs to affect the estrogen receptor (ER) is generally related to their degree of chlorination, with more lightly chlorinated PCBs acting in an estrogenic manner, and those PCBs with higher (>48%) chlorination acting as weak estrogens or as ER antagonists *(3,4)*.

In the reproductive system, PCBs can increase or decrease uterine weight, depending on the PCB (Fig. 2), and affect basal and gonadotropin-releasing hormone (GnRH)-induced gonadotropin release from the anterior pituitary gland *(4,5)*. In female rats, perinatal exposure to PCBs alters sexual behavior *(6)*. Neonatal PCBs can affect aromatase levels in the brain, the enzyme that is responsible for the conversion of testosterone to estradiol and plays a critical role in the development of gender-appropriate sexual behavior *(7)*. Male rats exposed to PCBs have altered testis and other reproductive tissue weights, and decreased serum testosterone levels (*7,8)* (Fig. 3). Exposure of male rats to Aroclor 1254 neonatally causes reduced mating and reproductive success in adulthood *(8)*. Recently, PCBs have been reported to inhibit estrogen sulfotransferase, the

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Author to whom all correspondence and reprint requests should be addressed: Andrea C. Gore, Ph.D., Neurobiology of Aging Laboratories, Box 1639, Mount Sinai School of Medicine, New York, NY 10029. E-mail: andrea.gore@mssm.edu

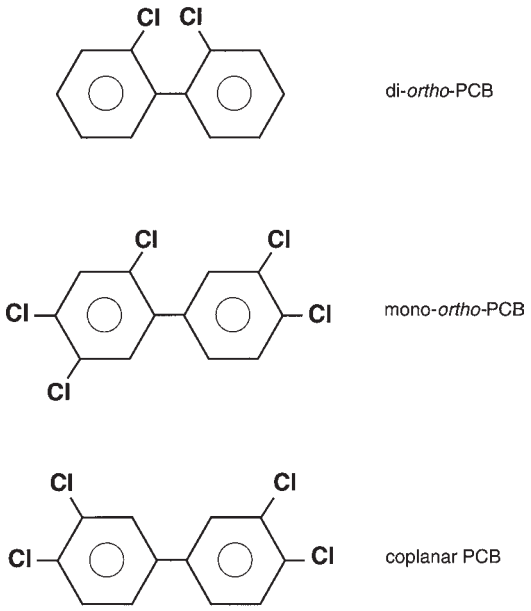

Fig. 1. Chemical structures of some representative PCBs. (Modified from ref. *13*.)

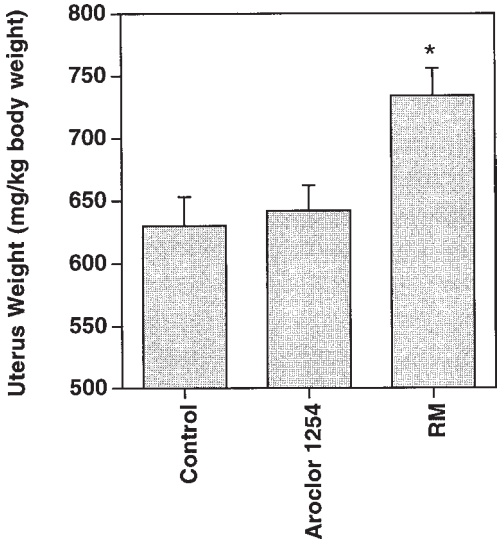

Fig. 2. Effects of control (oil), Aroclor 1254, or a reconstituted mixture of PCBs similar to that in breast milk (RM) on uterine weight in female offspring of dams treated during pregnancy and lactation. Experiments were performed on female offspring at postnatal d 21. *$p < 0.05$ vs control (oil). (Modified from ref. *7*.)

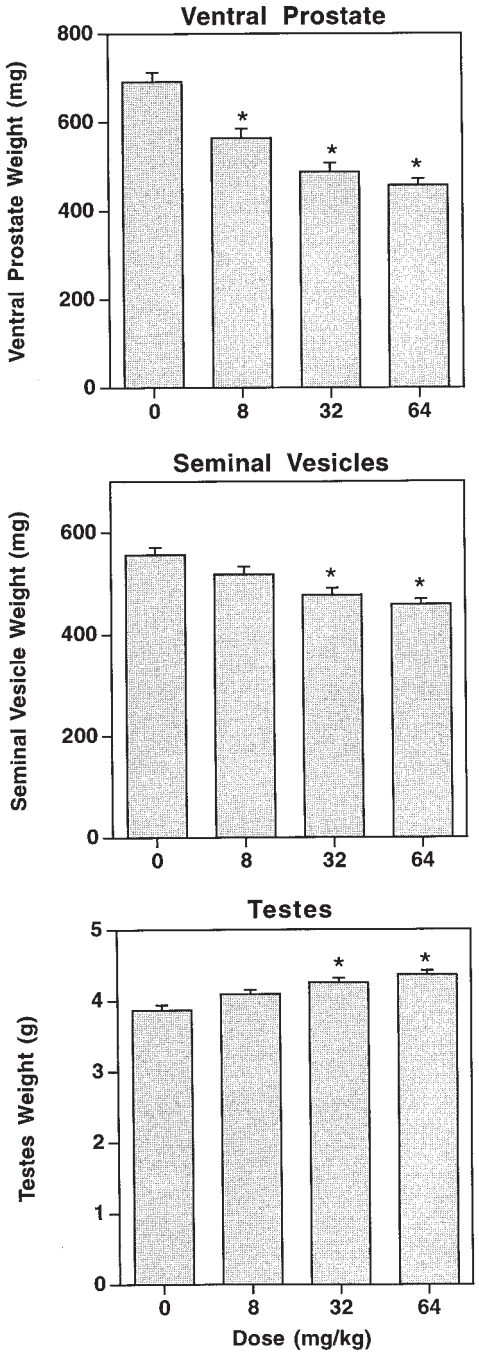

Fig. 3. Effects of treatment of pregnant rats with the PCB congener Aroclor 1254 on male offspring reproductive tissue weights. Dams had been treated on d 1, 2, 3, 5, 7, and 9 of lactation with Aroclor 1254 or vehicle, and their male offspring were used on postnatal d 165. The dose of Aroclor 1254 is shown on the *x*-axis. *$p < 0.05$ vs. control (oil). (Modified from ref. *8*.)

enzyme responsible for inhibition of estrogen metabolism *(3)*; this would have the net result of increasing estradiol levels. Since alterations in estradiol play a critical role in the dimorphism of sexual behavior, increases in neonatal estradiol exposure could have a potent effect on the masculinization of the brain (reviewed in ref. *9*).

Regarding the development of the reproductive system and the attainment of reproductive competency, PCBs can cause either precocious or delayed puberty, again depending on the nature of the PCB studied and the experimental model *(4,10)*. One laboratory reported that Aroclor 1254 advances the timing of vaginal opening in female rats, an indication that the first preovulatory hormone increase has occurred *(11)*, whereas other laboratories have found inhibitory effects of Aroclor 1254 on pubertal development *(12)*. Animals in the former experiment also had abnormal

Table 1
Summary of Effects of PCBs and Organochlorine Pesticides on Reproductive and Nervous System Functions

Reproductive system	Effects
PCB	
Aroclor 1254	Decreases GnRH-induced stimulation of LH release in Atlantic croaker *(5)*
	Disrupts estrous cycles, decreases size of uterus, diminishes fecundity in rats *(11)*
	Decreases reproductive success in male rats, decreases prostate weight *(8)*
	Increases duration of menses in rhesus monkeys *(91)*
	Decreases female sexual behavior in rats *(6)*
Aroclor 1221	Decreases female sexual receptivity in rats *(6)*
Aroclor 1242	Increases basal and GnRH-induced LH release, increases uterine weight *(4)*
PCB77	Antagonizes effects of estrogen on uterine weight *(4)*
Reconstituted mixture of PCBs	Increases uterine weight *(7)*
Hydroxylated PCB metabolites	Inhibit estrogen sulfotransferase *(3)*
Pesticide	
o,p'-DDT	Advances puberty, causes persistent estrus *(92)*
	Suppresses basal and GnRH-induced LH release in male rats *(69)*
	Increases uterine weight, stimulates uterine enzyme levels *(31,34)*
p,p'-DDE	Binds the androgen receptor *(36)*
Methoxychlor	Stimulates estrogen-responsive uterine genes *(35)*
	Increases uterine weight and development, increases DNA content in reproductive tissues *(30)*
	Increases GnRH content in mediobasal hypothalamus of male rats *(70)*
	Delays puberty, causes infertility in male rats *(37)*
	Stimulates ovarian and uterine growth *(38)*
	Accelerates vaginal opening in females, delays preputial separation in males *(93)*
HPTE (methoxychlor metabolite)	Decreases GnRH gene expression in GT1-7 cells *(85)*
Chlorpyrifos	Does not activate the estrogen receptor in MCF-7 cells *(56)*
	Does not affect aromatase activity in cell lines *(55)*
	Does not alter reproduction or fertility in rats *(54)*
Nervous system	**Effects**
PCB	
Aroclor 1254	Decreases serotonin and dopamine in hypothalamus of Atlantic croaker *(5)*
	Decreases serotonin and dopamine levels and metabolism in specific brain regions *(18,94)*
	Decreases norepinephrine levels in frontal cortex and hippocampus *(95)*
	Increases synaptic activity in hippocampus *(16)*
PCBs in humans	Decrease Bayley scores in young children *(25)*
Pesticide	
o,p'-DDT	Causes changes in urine marking, aggression *(39)*
Methoxychlor	Facilitates sexual behavior in female rodents *(38)*
	Causes effects on reflex development, reaction time, aggression, urine marking *(39)*
	Decreases male sexual arousal *(96)*
Chlorpyrifos	Blocks neurite outgrowth in PC-12 and C6 glioma cells *(53)*
	Inhibits acetylcholinesterase activity (*43,97,98*)
	Inhibits DNA synthesis and content in PC12 cells *(51)*
	Causes neuronal loss in brain stem and forebrain *(42)*
	Causes sensory neuropathy *(99)*
	Alters synaptic development and neurotransmitter turnover *(46)*

reproductive cycles and behavior in response to Aroclor 1254 *(11)*. Aroclor 1221, which is more lightly chlorinated than Aroclor 1254, causes the timing of vaginal opening to occur earlier than normal and increases uterine weight *(10)*. Although the exact results of these studies may be somewhat inconsistent, they are all in agreement that PCBs have endocrine-disrupting effects resulting in abnormal reproductive development, behavior, and physiology (Table 1).

Nervous System Effects of Environmental Toxicants: Polychlorinated Biphenyls

Along with their effects on the reproductive system, PCBs have neurologic and other actions (Table 1). Epidemiologic studies on humans have demonstrated the effects of PCBs on the thyroid hormone system, body weight, psychomotor development, and neurobehavioral and cognitive function (reviewed in refs. *13* and *14*). Animal studies

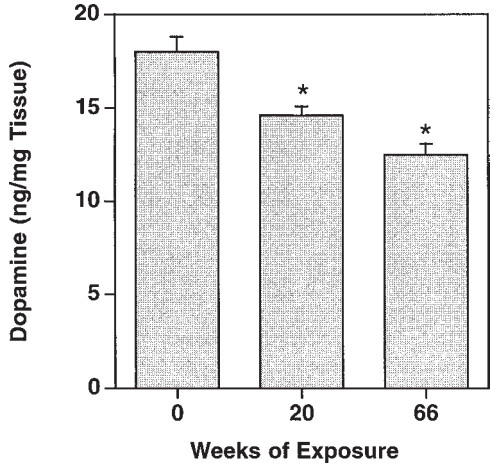

Fig. 4. Effects of PCB exposure on dopamine levels in the caudate nucleus of adult male nonhuman primates. Animals were treated with 3.2 mg/(kg·d) of Aroclor 1260 for 20 or 66 wk. *$p < 0.05$ vs control. (Modified from ref. *18*.)

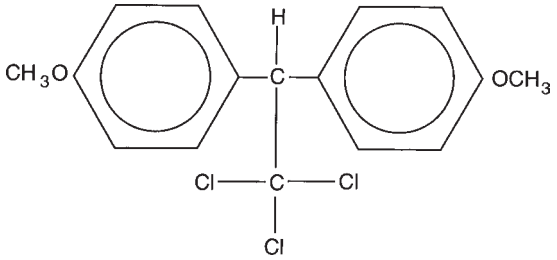

Fig. 5. Chemical structure of methoxychlor. (Modified from ref. *33*.)

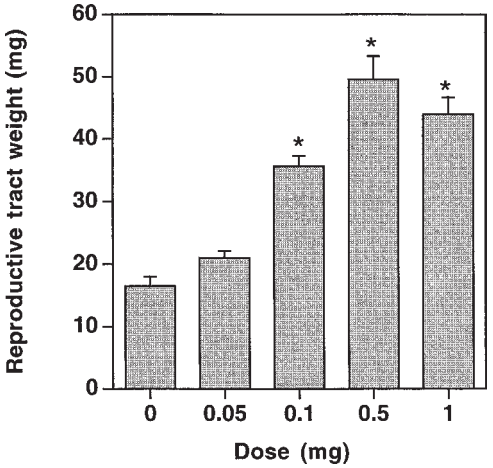

Fig. 6. Reproductive tract weights in 10-d-old female mice treated daily with methoxychlor or vehicle (sesame oil) beginning on the day of birth. The dose of methoxychlor is indicated on the *x*-axis. *$p < 0.05$ vs vehicle. (Modified from ref. *30*.)

show that PCBs alter spatial learning and other cognitive and behavioral measures (reviewed in ref. *13*). These effects are similar to those of estrogenic substances on cognitive function, learning, and memory (reviewed in ref. *15*) and may be mediated by the ER in the brain. Consistent with this finging, Aroclor 1254 was reported to alter synaptic transmission and plasticity in the hippocampus, the site of learning and memory, of rats *(16)*, and estrogen has similar actions *(15,17)*. Other nonsteroid hormone receptor–mediated effects of PCBs on the central nervous system (CNS) have been studied, and it was reported that PCBs change levels of the neurotransmitter dopamine and its metabolites (*18)* (Fig. 4). Alterations in the dopaminergic system have broad-ranging implications, because this neurotransmitter system plays crucial roles in numerous systems, including affective behavior, motor function, addictive behaviors, and neurodegenerative diseases *(19–21)*. Dopamine is also implicated in the control of reproduction *(22)*, and this could be another mechanism by which PCBs alter neuroendocrine function.

The effects of PCBs on the nervous system can be particularly potent in the developing organism, which is quite susceptible to the neurotoxic effects of environmental toxicants (*18,23,24*). Thus, prenatal or childhood exposure can have profound long-term consequences. This has been observed in humans, in which early transplacental exposure of the fetus to PCBs causes a small delay in maturation of motor function that was not seen in infants exposed after birth by lactation *(25,26)*.

Reproductive System Effects of Environmental Toxicants: Pesticides

Chlorinated pesiticides, including dichlorodiphenyltrichloroethane (DDT) and its analogs (e.g., methoxychlor) (Fig. 5), can cause abnormal stimulation of reproductive tract development *(27,28)*. Exposure to methoxychlor causes hypertrophy of the uterus and alterations in uterine and vaginal cytology and stimulates reproductive tract development (*29,30)* (Fig. 6). Gray et al. *(28)* found that methoxychlor accelerates the timing of vaginal opening but decreases fertility overall in female rats. In male rats, unlike females, methoxychlor decreases reproductive tissue weights, delays puberty, and inhibits testosterone production *(28)*. The reproductive system effects of methoxychlor are summarized in Table 1.

Methoxychlor and other members of the DDT family appear to act through an estrogenic mechanism in their uterotrophic and other effects *(31–34)*. Thus, these pesticides appear to fit the description of EDCs. However, methoxychlor's cellular site of action may differ from that of estradiol in that its effects are not blocked by the estrogen antagonist, ICI 182,780 *(33,35)*. Methoxychlor also does not appear to act through the classic ERα because its effects persist in the ERα-knockout mouse *(35)*, and, moreover, these effects may also be independent of the ERβ *(35)*. Another DDT metabolite, p,p'-DDE, is an androgen receptor antagonist *(36)*; this is a potential mechanism by which chlorinated pesticides act as EDCs.

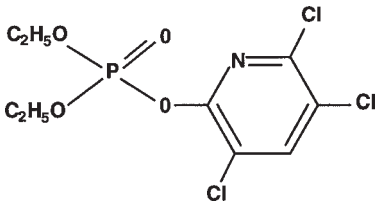

Fig. 7. Chemical structure of the bioactivated form of chlorpyrifos, the chlorpyrifos oxon (*O*,*O*-diethyl *O*-[3, 5, 6-trichloro-2-pyridyl] phosphate). (Modified from ref. *53*.)

Nervous System Effects of Environmental Toxicants: Pesticides

Although methoxychlor has primarily been studied as an EDC, it also has neurotoxic and other neurologic effects (Table 1). Mating behavior and success is decreased in male rats exposed to methoxychlor, and this toxicant appears to exert some of these effects through direct actions on the CNS *(37)*. Methoxychlor is able to induce mating behavior in ovariectomized female rats and hamsters, again indicating a nervous system effect *(38)*. Other behaviors, including development of reflexes, exploration of a novel environment or object, aggression, and urine marking, are altered in male and female mice exposed to methoxychlor prenatally *(39)*.

Other organophosphate pesticides such as chlorpyrifos (Dursban) (Fig. 7) have been reported to cause severe neurologic defects (Table 1) *(40–42)*. Until it was banned recently (summer 2000), chlorpyrifos was the most commonly used pesticide in urban apartments as well as in rural farms. Nevertheless, the neurotoxic effects of chlorpyrifos have been documented for many years. In 1980, it was reported that chlorpyrifos can cause toxicity in the embryos of pregnant mice fed chlorpyrifos and alters enzyme levels involved in neurotransmitter (acetylcholine) biosynthesis, as measured by cholinesterase levels *(43)*. Another laboratory found embryotoxicity and neurotoxicity of chlorpyrifos exposure in rats, the latter assessed by behavioral tests (motor behavior and performance; *[44]*). The mechanism by which chlorpyrifos alters behavior may be mediated, at least in part, by the neurotransmitter acetylcholine, because chlorpyrifos can alter synthesis and degradation of acetylcholine, and this in turn can modify neuronal development *(45)*. Numerous studies indicate that chlorpyrifos exerts many of its neuronal actions through the acetylcholine system (*46–48)* (Fig. 8). Although the effects of acetylcholine on neuroendocrine function have not been extensively studied, there are several reports indicating that acetylcholine can stimulate neuroendocrine cells, possibly through the muscarinic receptor *(49,50)*. Other effects of chlorpyrifos include an inhibition of DNA synthesis *(51)*, transcription factor activity *(52)*, protein synthesis *(41)*, and neurite outgrowth *(53)* in cell lines.

As is the case for PCBs, the effects of organochlorine pesticides such as chlorpyrifos are more potently exerted when exposure occurs early in life. Early exposure to chlorpyrifos (immediately after birth) has a much greater neurotoxic effect (cell loss, lack of neuronal growth) than later exposure (2 wk or more after birth) in rats *(42)*. Another study comparing juvenile and adult rats found much greater sensitivity of the juveniles than the adults to chlorpyrifos, as measured by behavioral changes and cholinesterase activity *(48)*.

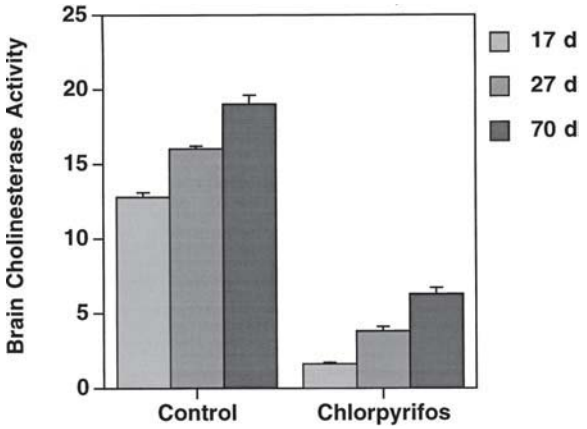

Fig. 8. Effects of a single dose of chlorpyrifos (20 mg/kg) in female rats age 17, 27, and 70 d on cholinesterase activity. Animals were killed 3.5 h after dosing. Cholinesterase activity is presented as micromoles of substrate hydrolyzed/(minute·g of tissue). (Modified from ref. *48*.)

Although numerous studies indicate neurotoxic effects of chlorpyrifos, particularly through the cholinergic system, few have evaluated reproductive effects of this pesticide. One laboratory reported that chlorpyrifos administered to a pregnant rat had no effect on the reproductive tract of the offspring *(54)*. In vitro assays did not detect an effect of chlorpyrifos on aromatase activity, suggesting no effect on the conversion of testosterone to estradiol *(55)*, and no effect on estrogen receptor activation *(56)*. Nevertheless, these studies are quite limited in the end points studied, and more in vivo evidence is necessary to determine whether chlorpyrifos is indeed an EDC as well as an environmental toxicant.

Neuroendocrine System Integrates Reproductive and Neuronal Function

The effects of environmental toxicants on the development of the nervous and reproductive systems are well documented. However, many of the mechanisms by which these substances exert their effects, particularly during development, are still unknown. The reproductive effects of toxicants are likely to occur at one or more of the three levels of this axis: the hypothalamus; the anterior pituitary gland, and the gonad (ovary or testis). Although the hypothalamic-pituitary-gonadal axis is a feedback loop, the gonadotropin-releasing hormone (GnRH) neurons are the principal regulators of this axis. These cells integrate information about the external environment and internal home-

ostatic milieu, as well as feedback from other reproductive levels (particularly from estrogen and testosterone from the ovary and testis, respectively) to control reproduction. Therefore, it is critical to determine the effects of environmental toxicants on GnRH neurons if we are to treat exposed children in an optimal manner.

The GnRH (also called luteinizing hormone [LH]-releasing hormone) neurosecretory system is the primary regulator of reproductive development and function. GnRH neurons are located in the preoptic area–anterior hypothalamus (POA-AH) of rodents, and the basal hypothalamus of primates *(57,58)*. They project to the portal capillary system, where the decapeptide is released to travel to the anterior pituitary gland. There, GnRH stimulates the gonadotropes to synthesize and secrete the gonadotropins, LH, and follicle-stimulating hormone, which are carried through the general circulation to effect development of the gonads and synthesis and release of sex steroid hormones, estrogen and progesterone in females, and testosterone in males.

An increase in GnRH release and gene expression is the critical event resulting in the onset of puberty *(59–61)*. However, while GnRH neurons themselves are morphologically and functionally mature at birth with respect to cell number and localization *(62,63)*, the endogenous release of GnRH is low until the pubertal process begins. Nevertheless, GnRH cells of immature animals are capable of responding to external stimuli by increasing GnRH release (*61,64–67*). This is a likely mechanism by which EDCs can alter the reproductive axis.

Effects of Environmental Toxicants on GnRH Gene Expression In Vivo

Reports on the effects of environmental toxicants on GnRH neurons are extremely limited. This is particularly surprising because many of the developmental abnormalities in reproduction observed in humans and animals are secondary to a failure of the GnRH neurosecretory system to develop normally *(68)*. To our knowledge, no published studies have investigated the direct effects of PCBs on GnRH neuronal function, although there are a few reports on the effects of PCBs, as well as pesticides such as DDT, on GnRH-stimulated LH release, indicating a pituitary effect of these EDCs *(4,69)*. There is also little information on the effects of pesticides on GnRH cells. Goldman et al. *(70)* found that treatment of juvenile male rats with methoxychlor increased GnRH levels in the mediobasal hypothalamus, the site of GnRH neuroterminals (Fig. 9). They also reported an increase in KCl-stimulated GnRH release in methoxychlor-treated animals *(70)*. This is the extent of the literature on the effects of EDCs on GnRH cells in vivo.

Materials and Methods

My laboratory has begun to evaluate the effects of environmental toxicants directly on GnRH neurons. In initial studies, we have administered these toxicants either prenatally to pregnant rats or postnatally to developing pups, and the progression of puberty and GnRH gene expression were evaluated in the developing animals.

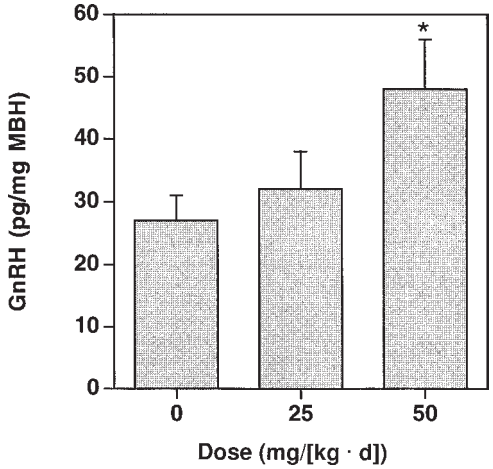

Fig. 9. Mean GnRH levels in the medial basal hypothalamus from rats treated daily with vehicle (corn oil) or methoxychlor (25 or 50 mg/[kg·d]) from d 21 through 77 d of age. $*p < 0.05$ vs control. (Modified from ref. *70*.) MBH, mediobasal hypothalamus.

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine.

Postnatal Administration of Endocrine Disruptors on GnRH mRNA Levels in Female Rats

It is well established that an increase in GnRH release and gene expression are important events for the normal progression of puberty *(59,60)*. Two PCB mixtures, Aroclor 1221 (1 or 10 mg/kg) and Aroclor 1254 (1 and 10 mg/kg) were tested for their effects on GnRH gene expression in developing female rats. Animals were injected daily with one dose of a toxicant or vehicle (dimethyl sulfoxide [DMSO]) beginning on postnatal day 2 and continuing through d 14, and they were killed at postnatal d 40 (postpuberty). GnRH mRNA levels were measured in the POA-AH, the site of GnRH perikarya in rats, by RNase protection assay *(71,72)*.

As shown in Table 2, GnRH mRNA levels were highest in female rats treated with the low dose of Aroclor 1221 (1 mg/kg). Statistical analysis (analysis of variance) demonstrated that levels of GnRH mRNA were significantly higher in the Aroclor 1221 (1 mg/kg) group than in control-treated animals ($p < 0.05$). No other treatments, including the higher dose of Aroclor 1221, affected GnRH mRNA levels. These doses were not high enough to influence the reproductive tract or the timing of puberty, both of which were normal. This stimulatory effect of a low dose of Aroclor 1221 on GnRH gene expression is consistent with its effects on reproductive development reported by other laboratories *(4,10)*. Why the higher dose of Aroclor 1221 did not have a similar effect to the lower dose is unknown, but it is possible that higher doses of EDCs may have dif-

Table 2
Effects of Postnatal Treatment with PCBs on GnRH Gene Expression in Female Rats[a]

Treatment	GnRH mRNA (fg GnRH/pg cyclophilin mRNA)
Vehicle (DMSO)	2.2 ± 0.1
Aroclor 1221 (1 mg/kg)	2.8 ± 0.3[b]
Aroclor 1221 (10 mg/kg)	1.8 ± 0.2
Aroclor 1254 (1 mg/kg)	1.8 ± 0.3
Aroclor 1254 (10 mg/kg)	2.2 ± 0.2

[a]Female rat pups were treated with Aroclor 1221, Aroclor 1254, or vehicle daily from postnatal d 2–14 and killed on postnatal d 40. GnRH mRNA levels were measured in the POA-AH by RNase protection assay. n = 4–6 animals per group.
[b]$p < 0.05$ vs vehicle (DMSO).

Table 3
Effects of Prenatal Treatment with PCBs on Puberty in Female Rats[a]

Treatment	Day of vaginal opening	Day of first diestrus
Vehicle (DMSO)	32 ± 0.6 (n = 9)	33 ± 0.7 (n = 8)
Aroclor 1221	32 ± 1.4 (n = 9)	33 ± 1.6 (n = 9)
Aroclor 1254	30 ± 1.3 (n = 10)	34 ± 0.8 (n = 11)
Methoxychlor	35 ± 0.8[b] (n = 11)	36 ± 0.8[b] (n = 7)
Chlorpyrifos	27 ± 0.9[b] (n = 16)	28 ± 1.1[b] (n = 8)

[a]Pregnant rat dams were treated with toxicant or vehicle on gestational d 16. Female offspring were monitored daily for the timing of vaginal opening or first diestrus. Data are expressed as mean ± SEM of age in postnatal days. The number of animals per group is in parentheses.
[b]$p < 0.05$ vs control.

ferential estrogenic effects from lower doses. Indeed, the effects of PCBs and other EDCs often occur in a limited range of doses, with an inverted U-shaped dose-response curve (*4,6,73–75*).

Prenatal Administration of Endocrine Disruptors on GnRH mRNA Levels in Female Rats

The effects of prenatal (*in utero*) administration of environmental toxicants were also tested. This has particular relevance to humans, in which exposure to these substances often occurs during pregnancy. Pregnant rats received a single injection on gestational d 16 (parturition in our rat colony occurs on d 22 of pregnancy) with 1 mg/kg of Aroclor 1221, Aroclor 1254, methoxychlor, chlorpyrifos, or vehicle (DMSO). Animals were allowed to deliver litters, and pups were monitored for pubertal development and growth. They were killed at various stages of development, and GnRH gene expression was measured in hypothalamic dissections.

To monitor pubertal development in female rats, rats were checked daily for the timing of the day of vaginal opening (indicating that a preovulatory hormonal surge has occurred) and first diestrus (indicating that animals have begun reproductive cycles). In control rats, these phenomena occurred at an average of postnatal days 32 and 33, respectively (Table 3). This is somewhat earlier than normally occurs in the Mount Sinai rat colony (vaginal opening normally occurs between postnatal d 35 and 39; *[60,76]*) and thus it appears that the vehicle, DMSO, may have small effects on puberty in female rats. When using these low doses of toxicants, the timing of vaginal opening and first diestrus were not altered by Aroclor 1221 or Aroclor 1254 compared to DMSO vehicle. However, methoxychlor caused a slight delay of these events. While this result is not consistent with the effects of methoxychlor on pubertal development in females (*27,28,30*), an inhibitory effect of methoxychlor on reproductive maturation in male rats has been observed *(28)*. The slight inhibition of the progression of puberty in the present study may be owing to the dose or paradigm (e.g., age of exposure to methoxychlor). For the other pesticide studied, chlorpyrifos, a significant alteration in the timing of vaginal opening and first diestrus was seen: both occurred substantially earlier than in control rats. These ages of vaginal opening and first diestrus in female rats treated with chlorpyrifos are considerably earlier than those ever observed in my laboratory. This result indicates a potent effect of this pesticide on reproductive maturation.

GnRH mRNA levels were measured in the hypothalamus of these developing rats at postnatal d 20, 30, 40, and 50, as well as on the days of vaginal opening and first diestrus. My laboratory has previously reported that GnRH mRNA levels increase during postnatal development *(60,76)*, and that this is accelerated in rats in which precocious puberty has been induced by neurochemical stimulation *(60)*. In the present study, several of the endocrine disruptors caused increases in GnRH mRNA levels compared to control rats undergoing normal puberty (Fig. 10). Interestingly, two substances implicated in causing accelerated puberty, Aroclor 1221 and methoxychlor, although not accelerating vaginal opening and first diestrus in the present study (in fact, methoxychlor delayed these events), caused increased GnRH mRNA levels in POA-AH compared to control rats, particularly in the older (postnatal d 40 and 50) groups (Fig. 10). Additionally, chlorpyrifos caused significant increases in GnRH mRNA levels at postnatal d 50 compared to controls. These findings indicate that EDCs, as well as chlorpyrifos, which also appears to have endocrine-disrupting activities, affect the reproductive axis at the level of the GnRH neuron. Moreover, low-level exposure to endocrine disruptors appears to have implications for later adult reproductive cycles and subsequent fertility, because the GnRH system does not appear to be affected until after adult reproductive function has been attained (d 40–50).

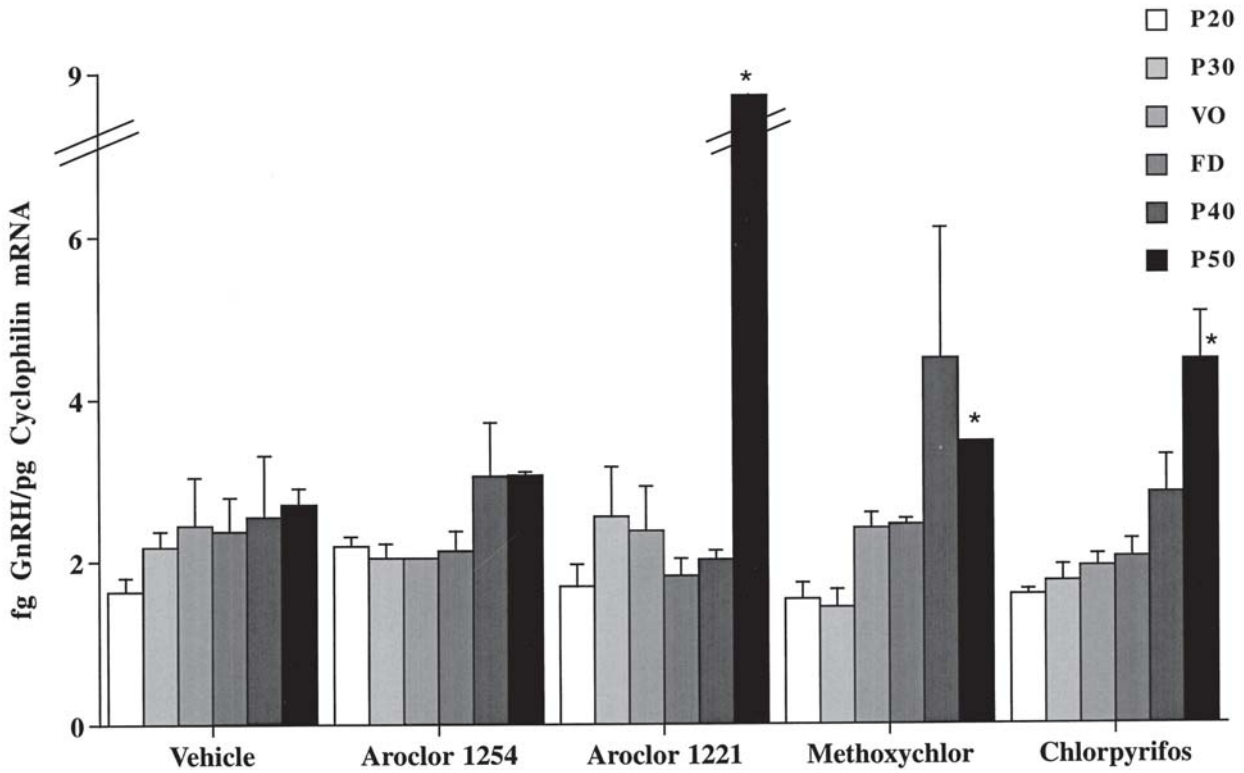

Fig. 10. Effects of vehicle (DMSO), Aroclor 1254, Aroclor 1221, methoxychlor, and chlorpyrifos injected into pregnant rats on embryonic d 16 on GnRH gene expression in the POA-AH of female offspring. The animals were killed on postnatal (P) d 20, 30, 40, 50, the day of vaginal opening (VO), or the day of first diestrus (FD). GnRH mRNA levels were significantly increased in rats treated with Aroclor 1221, methoxychlor, and chlorpyrifos compared with vehicle-treated controls. *$p < 0.05$ vs age-matched control.

GT1-7 Cell Line is a Model for Developing GnRH Neurons

GnRH neurons in the brain are few and are widely distributed, and, therefore, it is extremely difficult to study GnRH neurons using primary in vitro cultures of the POA-AH. However, it is advantageous to have an in vitro model for GnRH neurons to ascertain the cellular/molecular mechanisms by which neurotransmitters, growth factors, steroid hormones, or environmental factors affect this system. The immortalized hypothalamic GT1-7 cell lines that synthesize and secrete GnRH have contributed much to an understanding of GnRH neuronal function *(77)*. These cells have many similarities to immature GnRH neurons in vivo (*78–82*). Therefore, GT1-7 cells have great utility in understanding the GnRH system in a homogeneous cell line that is easily cultured and maintained, and that appears to respond to a similar complement of neurotransmitters that affect GnRH neurons in vivo. Furthermore, GT1-7 cells are one of a very few neuronal cell lines, and they have become an important model of an in vitro neuron in studies on neurotoxicity, cell survival, and proliferation. The observations that GT1-7 cells are responsive to stimulation by estrogen *(83)* and have ERs *(84,85)* make them an excellent model for studying the effects of endocrine disruptors on GnRH neurons.

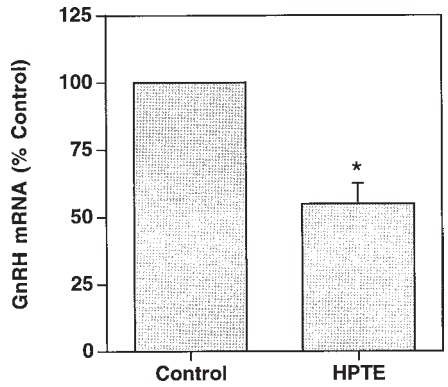

Fig. 11. Effects of HPTE, a methoxychlor metabolite, on relative GnRH mRNA levels in the GT1-7 cell line. Cells were harvested 24 h after application of HPTE and GnRH mRNA levels measured by Northern blot analysis. *$p < 0.05$ vs control. (Modified from ref. *85*.)

Experiments on Effects of Environmental Toxicants on GnRH Gene Expression in GT1-7 Cell Line

There are currently no published studies evaluating the effects of PCBs on GT1 cells. However, Roy et al. *(85)* recently tested the effects of the methoxychlor metabolite 2,2-bis-*p*-hydroxyphenyl-1,1,1-trichloroethane (HPTE) on GnRH gene expression in GT1-7 cells. They reported that HPTE at 100 n*M* for 24 or 36 h caused a decrease in GnRH mRNA levels in GT1-7 cells compared with vehicle-treated cells (Fig. 11). This indicates a direct sensitivity

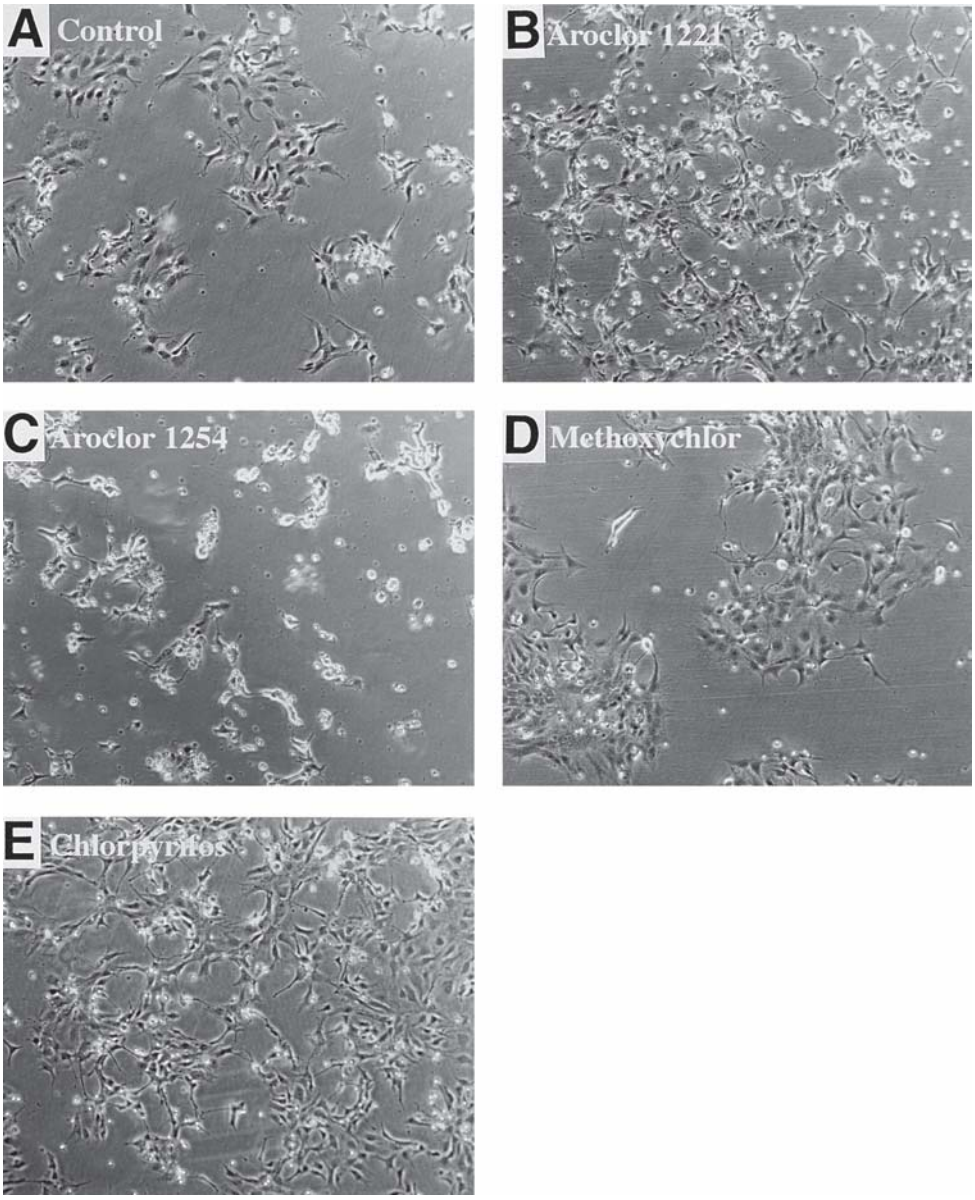

Fig. 12. Effects of environmental toxicants on GT1-7 cell morphology. Cells were treated for 24 hours with vehicle or toxicants (10 μ*M*). Photomicrographs were imaged with an SV Micro digital camera on a Nikon phase-contrast inverted microscope at a magnification of ×100. (**A**) control; (**B**) Aroclor 1221 caused an increase in cell extensions and connectivity compared with control; (**C**) Aroclor 1254 caused a retraction of processes and decreases in connectivity compared with control; (**D**) methoxychlor did not have a substantial effect on GT1-7 cell morphology, although it tended to be neurotoxic, especially at higher doses; (**E**) chlorpyrifos caused an increase in cell projections and connectivity compared with control. Effects of chlorpyrifos and Aroclor 1221 were similar to those of estradiol (data not shown).

of this GnRH neuronal cell line to EDCs and prompted us to test the effects of the EDCs under discussion using the GT1-7 cell line.

GT1-7 cells were grown as described previously (*80,86, 87*). Dose-response studies were performed in which each toxicant or vehicle was administered at 0, 0.01, 0.1, 1, 10, or 100 μ*M* (these doses were approximated for the Aroclor mixtures) for 24 h. GnRH mRNA levels were quantified by RNase protection assay (*72,86,88*).

Effects of Endocrine Disruptors on GT1-7 Cell Morphology

The four toxicants examined in the present study (Aroclor 1221, Aroclor 1254, chlorpyrifos, methoxychlor, all at 10 μ*M*) exerted effects on GT1-7 cell morphology. A photomicrograph of a representative control-treated GT1-7 cell culture is presented in Fig. 12A. Aroclor 1221 stimulated neurite outgrowth, with greater numbers of processes among GT1-7 cells (Fig. 12B). By contrast, Aroclor 1254 reduced the number of neuritic connections and decreased cell confluency (Fig. 12C); cells appeared to retract processes. Methoxychlor did not have much effect on GT1-7 cell morphology (Fig. 12D), although it tended to be neurotoxic. In some cultures, a large proportion of GT1-7 cells was killed by methoxychlor, although this varied between experiments. Chlorpyrifos appeared to cause an increase in

Table 4
Effects of Environmental Toxicants on GnRH mRNA Levels in GT1-7 Cells[a]

Treatment	Dose (μ*M*)	GnRH mRNA (fg/pg cyclophilin mRNA)
Control	0	170 ± 20
Aroclor 1221	0.01	205 ± 21
	0.1	315 ± 22[b]
	1	375 ± 10[b]
	10	160 ± 12
	100	165 ± 17
Aroclor 1254	0.01	475 ± 27[b]
	0.1	270 ± 32
	1	207 ± 84
	10	120 ± 17[b]
	100	82 ± 9[b]
Methoxychlor	0.01	448 ± 11[b]
	0.1	382 ± 28[b]
	1	89 ± 63
	10	104 ± 12[b]
	100	67 ± 6[b]
Chlorpyrifos	0.01	492 ± 102[b]
	0.1	702 ± 72[b]
	1	264 ± 52[b]
	10	139 ± 12
	100	74 ± 18[b]

[a]GT1-7 cells were treated with toxicant or vehicle at the indicated dose for 24 h. Cells were harvested and RNA extracted, and GnRH mRNA levels were measured by RNase protection assay. Data are expressed as mean ± SEM. n = 3–6 per group.
[b]$p < 0.05$ vs control.

cell density, with a greater number of projections and an increase in cell confluency (Fig. 12E).

Effects of Endocrine Disruptors on GnRH Gene Expression

All the toxicants studied had significant effects on GnRH mRNA levels in GT1-7 cells. In each case, the toxicant stimulated GnRH gene expression at a defined range of doses (Table 4). Some of the toxicants (methoxychlor, chlorpyrifos, Aroclor 1254) also caused a significant suppression of GnRH mRNA levels at the higher doses studied; however, this result may have been owing, at least in part, to neurotoxicity of these substances, which often occurred at the higher doses. It appears from the results that all four of the toxicants affected GnRH gene expression with an inverted U-shaped dose-response curve, similar to findings from other laboratories *(4,6,73)*.

These results, taken together with the results of Roy et al. *(85)*, indicate that the GT1-7 cells are highly sensitive to endocrine disruptors. Although the GT1-7 cells may have some differences from GnRH neurons in vivo *(*reviewed in refs. *89* and *90*), they appear to provide an excellent model for the evaluation of the direct effects of environmental toxicants on GnRH neurons in vitro. I would also like to emphasize the findings on the pesticide chlorpyrifos, which is potently stimulatory to GnRH mRNA levels and to neurite outgrowth of the GT1-7 cells, suggesting that it has endocrine-disrupting properties. Together with the animal studies discussed, these findings implicate chlorpyrifos as a regulator of reproductive neuroendocrine development and support its candidacy as another organochlorine pesticide EDC.

Conclusion

Environmental toxicants have been shown to disrupt neurologic and reproductive development. However, a common link between these phenomena has not been demonstrated. The neuroendocrine hypothalamus provides a likely integrative link between neuronal and reproductive effects of toxicants, since hypothalamic GnRH neurons integrate information from inputs arising from other CNS regions, as well as hormonal and environmental inputs. Animal and cell culture studies indicate that hypothalamic GnRH neurons are strongly affected by EDCs such as PCBs and pesticides. Moreover, in vivo results from a rat model indicate that these substances can affect GnRH gene expression, and that many of these effects are not manifested until adult reproductive function has been attained. Future studies will look toward further elucidating the mechanisms by which environmental toxicants alter these processes in the developing brain.

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