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Prenatal exposure to endocrine disrupting chemicals: effects on behavioral development

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Abstract

Numerous chemicals released into the environment by man are able to disrupt the functioning of the endocrine system by binding to hormonal receptors. Exposure to estrogenic endocrine disruptors during critical periods in fetal life can alter the development of reproductive organs, the neuroendocrine system and subsequent behavior. We present a series of studies on the effects of exposure during fetal life to low, environmentally relevant doses of two pesticides, *o,p'*DDT and methoxychlor, and of low doses of the synthetic estrogen, diethylstilbestrol on subsequent neuro-behavioral development in house mice. The main findings can be summarized as follows: (1) Mice prenatally exposed to methoxychlor showed changes in reflex development. Exposure to a very low dose of methoxychlor appeared to produce an increased reactivity during early postnatal life. (2) Methoxychlor exposed periadolescent mice showed a decreased reaction time exploring both a novel environment and a novel object. (3) The onset of male intrasex aggression appeared to be delayed in males prenatally exposed to low doses of methoxychlor, since exposed males showed low levels of aggressive interactions during early adolescence but not after they reached adulthood. (4) The rate of depositing urine marks in a novel environment was increased in males prenatally exposed to DES, and also to *o,p'*DDT and methoxychlor. (5) The proportion of both males and females attacking a same-sex conspecific was increased in mice prenatally exposed to low doses of DES and, marginally, to *o,p'*DDT. This effect appeared to be related to a decreased latency to attack. However, males prenatally exposed to *o,p'*DDT displayed a decreased intensity of aggression. The possible implications of perturbing the hormonal milieu during fetal development on the modulation of developmental turnpoints and future behavioral responses are discussed.

Keywords: Estrogens; Estrogenic pesticides; Development; Behavior; Aggression; Novelty; House mice

1. Introduction

A variety of man-made chemicals which animal and human populations encounter in the environment, such as DDT and its derivatives, are able to disrupt the functioning of the endocrine system in animals by binding to hormone receptors in responsive cells and acting as agonist or antagonist of endogenous hormones. At this time, the best characterized endocrine-disrupting chemical are those able to bind to estrogen receptors in cells, although there are endocrine disrupting chemicals that can interfere with androgen receptors [1], thyroid hormone function [2,3], and operate via other mechanisms [4]. While estrogen is a critical hormone with regard to functioning of the reproductive system in adult females, estrogen is now also recognized to play an important role in normal fetal development and the functioning of male reproductive organs [5–8]. So far scientific interest in the effects of exposure of man and animals to environmental estrogens has focused on the potential for effects on male fertility and gross reproductive disturbances, while the wider effects of exposure to these compounds has received less attention. During fetal life, sex steroids, such as estradiol, have marked effects on the development of the neuroendocrine system and subsequent behavior. The focus of the research we will describe is on behavioral effects of endocrine disrupting chemicals which mimic the action of estrogen, emphasizing consequences of exposure to low, environmentally relevant doses, within the range of exposure of humans and animals, during critical periods in brain and behavioral development.

1.1. The hormonal control of brain and behavioral development

Sex steroids exert potent influences on the nervous system during critical developmental periods and on into adulthood by organizing and reorganizing the neuronal circuitry involved in neuroendocrine and behavioral

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functions [9]. The developmental effects of steroids are typically irreversible and are referred to as "organizational", while effects in adults are typically reversible and are referred to as "activational" [10,11]. Specifically, estrogen or aromatizable androgens (that are converted to estrogen by the enzyme aromatase in cells) play a significant role in regulating neuronal development and neural circuit formation during the perinatal period, and these organizational actions of sex steroids can induce permanent sexual dimorphisms in certain brain regions, in synaptic formation, in dendritic length, in the distribution patterns of serotoninergic fibers, and in neuronal connectivity [9].

There are a wide array of behavioral differences, in addition to sexual behaviors, between males and females due to sex differences in sex hormones during fetal life. Specific behavioral differences between males and females include differences in infant play, aggression, learning, exploration, activity level, food intake and preference, and many more [12]. While some of these differences reflect activational effects of estradiol and testosterone in the blood of adult males and females, many also are due to differences in brain functions that were organized during early life by differential actions of sex steroids. In rodents, as in other mammals, most non-reproductive behaviors have been described to show sex differences in quantity of performance expressed rather than being present in one sex and absent in the other [13]. An example of a behavior which is organized by sex hormones during the period of brain development in rodents and primates is aggressiveness [10]. Similarly, maze learning and the acquisition of shuttle-box avoidance responses also are "organized" by sex hormones during development [14]. Naturally occurring variation in the levels of testosterone and estradiol in female mouse, rat and gerbil fetuses (due to being positioned in utero between male or female fetuses) leads to marked differences in a wide range of reproductive and behavioral traits: genital morphology, timing of puberty, length of estrus cycle, aggressive and territorial behavior, sexual behavior and sexual attractiveness [15]. Intrauterine position can affect steroid metabolism and steroid receptors of reproductive organs in male mice [16]. At this time, however, few studies have addressed the role of sex steroids exposure during perinatal life in rodents in influencing behaviors typically studied using the ethological approach, such as responses to changes in the socio-ecological environment and, more generally, behavioral strategies in coping.

Selective pressures appear to have operated during vertebrate evolution such that all vertebrates share a set of homologous neuroendocrine control mechanisms mediating socio-sexual behaviors and reproductive functions [17]. Of great importance with regard to the emerging field of ethotoxicology [18], the underlying mechanisms of the action of hormones such as estradiol are fundamentally identical across vertebrates [19–21]. The hormone estradiol is identical in all vertebrates; all steroid hormones are identified by their precise structure, unlike protein hormones, which can vary in amino acid sequences but have the same name in different species. In addition, the region of the classical estrogen receptor (ER alpha) that binds estradiol in fish is fundamentally the same as that in birds and in women [19,20]. Species and tissue distribution, and binding characteristics of the recently discovered ER beta are now being investigated, as are the possibility of unique mechanisms of response to estrogens and other steroids in different vertebrates [22,23]. However, the high degree of conservation of the alpha form of the estrogen receptor over hundreds of millions of years of vertebrate evolution has profound implications with regard to estrogenic endocrine disruptors [21]. If a chemical can bind to the estrogen receptor in one vertebrate, it should be expected to bind to estrogen receptors in any other vertebrate, including humans. This is not intended to suggest that the outcome of binding to the receptor will be the same in different species. In addition, this does not imply that in different tissues within a species, nor even within a tissue at different times in life, will the effects of binding of a chemical to estrogen receptors be the same.

The outcome of binding of any estrogenic chemical to the estrogen receptor depends on the conformational change induced in the receptor, the interaction of the receptor with tissue-specific proteins associated with the transcriptional apparatus, and the specific genes associated with estrogen response elements (EREs) to which the transcriptional regulating complex of ligand, receptor and associated proteins binds, thus regulating the process of transcription [24,25]. The issue is that some physiological consequence of the event of binding of a chemical to estrogen receptors, and some change in cell function, will occur. A change in what would have been the normal course of development can occur, and the specific nature of the change (disruption) will differ from species to species, from tissue to tissue, and as a function of the time in development that exposure occurs.

The timing of exposure to hormones, and thus endocrine disruptors, is critical. During the period when the central nervous system is undergoing rapid change and before homeostatic (protective) mechanisms have developed, endocrine disrupting chemicals, at environmentally relevant concentrations within the range of exposure of human and wildlife populations, can lead to irreversible alterations in brain development. This can occur during development at exposure levels that might produce little effect in an adult [26–28]. Exposure to estrogen, androgenic or thyroid hormone disrupting chemicals in the environment during critical developmental periods in fetal life has the potential to produce permanent changes in the structure and functioning of the brain, leading to changes in behavior [4].

1.2. Effects of endocrine disrupting chemicals in wildlife and humans

A substantial literature has reported that manmade endocrine disrupting chemicals may alter development, leading to altered behavior and reproductive capacity in wildlife.

Reproductive system abnormalities in wildlife have been related to endocrine disruptors in fish, alligators and turtles, birds and mammals [4,29]. For example, alterations of reproductive and socio-sexual behaviors were reported in Herring gulls nesting in the Great Lakes. Gulls in these colonies had high incidence of supernormal clutches and exhibited disinterest and delays in breeding and reproduction, abnormal incubation behavior [30], loss of expression of territorial defence, poor parenting, female–female pairing, and feminization and demasculinization [31,32], resulting in severely reduced reproductive success. These effects were associated with effects on the endocrine system by organochlorine contaminants, such as dichlodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs) and their in vivo metabolites [32,33]. Recent studies are now confirming that the same chemicals implicated in the adverse effects observed in wildlife are also related to detrimental effects in humans [2,3,34,35]. The Jacobsons' studies showed an association between neurobehavioral deficits in the infants and their mother's consumption of Lake Michigan PCB-contaminated fish, based on umbilical cord blood levels of PCBs. Children with highest PCBs levels processed information more slowly, had lower quantitative and visual discrimination memory, reduced auditory and verbal skills [36] and an average 6.2 IQ point deficit [34]. In a study on rats eating contaminated fish during pregnancy or lactation, Daly et al. [37] reported that they showed an increased depression effect following frustrative non-reward; these findings suggest that similar types of neurobehavioral deficits can occur in rodents and humans due to developmental exposure to some endocrine disruptors.

There are trends in genital abnormalities in men, such as a 50-year decline in semen quality [38,39], a 20-year steady increase in genital tract malformations, such as cryptorchidism (undescended testes) [40], hypospadias (malformed penis and urethra) [41], and testicular and prostate cancer [42,43]. As yet, studies have not been conducted to determine whether there is a relationship between any of these trends and endocrine disruptors. However, environmental factors are thought to contribute significantly to these trends in genital tract abnormalities, which may be observed at birth but are more commonly not observed until much later in life (they thus represent latent birth defects). Also, a decrease in testicular sperm production, and developmental changes and cancer in the epididymides and prostate, as well as abnormalities such as cryptorchidism, have been experimentally produced in laboratory animals with high levels of estrogenic chemicals [44–46], and, more recently genital abnormalities have been produced by prenatal exposure to doses of estrogenic endocrine disruptors within an environmentally relevant range for human exposure [47–49].

1.3. Behavior as a biomarker of EDCs exposure: ethotoxicology

So far scientific interest in the effects of exposure of man and animals to environmental endocrine disruptors has, to a large degree, focused on effects on male fertility and gross reproductive disturbances, while the wider effects of exposure to these compounds has been largely unexplored. Behavioral indices may be particularly sensitive to perturbation of hormonal systems because they represent the end-point of integrated systems, and even subtle alterations in any of the component systems are likely to be reflected in the disruption or modification of behavior. Importantly, disturbances in behavior (revealed by studies focusing on effects on individuals) may be of biological significance in both human and animal ecosystems, due to impaired responsiveness to environmental demands that could result in a reduced social adaptability.

The functional capacity of the central nervous system cannot be determined by histological or even physiological studies independent of behavioral analysis. A central nervous system deficit may become evident only upon a specific kind of behavioral challenge, and consequences of exposure to environmental chemicals can be subtle. This is a critical issue since toxicological testing for developmental effects has focused on teratology (gross visible damage) and not on outcomes that could not be detected on gross physical examination [28]. Examination of both learned and unlearned (reflex and phylogenetically specialized) behaviors may reveal subtle deficits in CNS function, which may or may not be accompanied by demonstrable tissue pathology.

Of particular importance to understand possible adverse effects of EDCs are those behaviors critical for survival and reproduction, such as territorial aggression, sexual and reproductive behavior, exploration, parental behavior. Both the developmental organization and adult expression of these behaviors is regulated by the neuroendocrine system. These neuroendocrine systems were shaped by evolutionary processes to maximize fitness.

In our present studies, we have applied strategies employed in ethological analysis to the study of effects of estrogenic endocrine disruptors on behavior. We have designed our experiments based on our interest in predicting whether a behavioral alteration might be adverse from the perspective of the adaptive significance of the behavior under investigation. This focus on adaptation is quite different from the traditional experimental approach of behavioral toxicology, in which animals are typically used as "tools" to detect alterations in neural or endocrine mechanisms. This focus on animals as "detectors" has not involved considering whether the social and environmental situations in which animals are tested are ethologically appropriate and thus relevant in terms of the adaptive function of the behavior being examined. We have proposed that the context and function of behavior are of paramount importance when studying the underlying substrates and refer to this approach as ethotoxicology [18].

2. Effects of prenatal exposure to DES, *o,p'*DDT and methoxychlor on neuro-behavioral development

An approach that we have used to investigate the developmental effects of estrogenic chemicals is the analysis of differential developmental effects depending jointly on developmental stage at the time of exposure, age of testing, and response endpoint. We report here a series of experiments, in part previously published and in part original data, on the effects on behavioral development of the synthetic estrogenic drug, diethylstilbestrol (DES) and of two pesticides, *o,p'*DDT and methoxychlor, that have previously been shown to have estrogenic activity both in vitro and in vivo [27,50,51].

DDT (dichlorodiphenyl trichloroethane) is one of the most commonly detected environmental pollutants in human tissues. It has been known for over 40 years that DDT accumulates in body fat, and reaches particularly high levels in breast milk. The *o,p'*-isomer of DDT is a contaminant (11–29%) found in DDT. *o,p'*DDT appears to be the primary estrogenic component of technical grade DDT. *p,p'*DDE is the highly persistent in vivo metabolite found in animal (including human) tissues [52] that acts as a androgen receptor antagonist [1], but only shows very weak partial estrogenic activity at a dose just below that which is acutely toxic [51]. *p,p'*DDE is not thought to contribute to the estrogenic activity that is related to exposure to *o,p'*DDT associated with the use of DDT as an insecticide.

Exposure of female rats to high doses of *o,p'*DDT during development results in precocious puberty as well as acceleration of the loss of fertility, referred to as the delayed anovulatory syndrome [53,54]. In male rats, exposure to a high dose of *o,p'*DDT during early life leads to marked impairment of fertility and reduced weight of prostate and seminal vesicles [55], as well as neurobehavioral effects, such as an increase in locomotor activity associated with a decrease in muscarinic cholinergic receptors density in the cerebral cortex [56].

Methoxychlor [bis-*p*-methoxyDDT;1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane], is an analog of DDT, but methoxychlor and its in vivo metabolites are far less persistent in animal tissues. Methoxychlor exposure is due to its use as an insecticide on pets, in home gardens, and on crops and livestock. Methoxychlor only has estrogenic effects in vivo after demethylation in the liver to mono-hydroxy-methoxychlor (30% of administered dose) or bis-hydroxy-methoxychlor (23% of administered dose) [57], with the most potent estrogenic metabolite being bis-hydroxy-methoxychlor [53,54,58]. Estrogenic effects that have been observed after neonatal administration of high doses of methoxychlor in female rats and mice are acceleration of first vaginal estrus (cornified squamous epithelium) indicative of the pubertal ovulation, and persistent vaginal cornification (acyclicity) at an young age, which is indicative of an acceleration of the loss of fertility [59]. Also observed were abnormal cell types in the uterus and oviducts [50,53,55,60].

Exposure during development to methoxychlor also leads to changes in the reproductive system in male rats and mice. Administration of 1 mg/kg/day methoxychlor to male mice during the first week after birth led to reduced testosterone levels, and a reduction in DNA content in prostate and seminal vesicles in adulthood [61]. Administration of 50 mg/kg/day methoxychlor to female rats throughout pregnancy and lactation resulted in smaller testes, epididymides and reduced sperm counts in male offspring [50,60].

We use as a model animal for these studies an outbred stock of Swiss house mice (*Mus domesticus*). Previous research has shown that, relative to many other laboratory stocks of mice, CD-1 Swiss mice are very similar to wild mice in their behavior and social organization in seminatural environments [62]; we have also used another stock of Swiss mice (CF-1) in some of our studies. The house mouse is widely distributed throughout the world and thus has been subjected to varied ecological pressures. Although there are reports of feral populations of this species living totally apart from humans, most commonly house mice live associated with human communities as commensals of man, thus potentially being exposed to some of the same environmental factors, including pollutants, as humans [63,64].

In the studies described here, we examined the effects of exposure during fetal life to a wide range of doses of three estrogenic chemicals (DES, *o,p'*DDT, and methoxychlor) on behavioral development of mice challenged with several behavioral tests at different developmental stages: after birth, at weaning, at puberty, and as adults. It is critical to note here that in assessing the safety of these chemicals, toxicologists have only examined very high doses and used models to predict "safe" exposure levels that, to our knowledge, have never before been directly tested for developmental effects. We report here on effects of *o,p'*DDT and methoxychlor below the doses currently predicted to be without risk of adverse effects for each of these chemicals [65,66].

3. Part 1. Effects on early neurobehavioral development

The mouse is an altricial species, that is, the pups are born in a highly immature condition after a short pregnancy (19–20 days). Several reflexes and responses appear at successive postnatal stages in parallel with somatic changes, progressively increasing the pup's sensory and motor capabilities. The time of occurrence of specified somatic changes and the time of first appearance and subsequent complete maturation of various reflexes and responses show considerable regularity, thus providing an effective tool to assess whether somatic and neurobehavioral development are modified by prenatal exposure to hormone-mimicking drugs or chemicals [67]. We administered *o,p'*DDT, methoxychlor and DES, which served as a positive control for estrogen action, to pregnant CF-1 mice. Pregnant females were fed different doses of these chemicals (in tocopherol-stripped corn oil) for 7-days (gestation

day11–17; time-mating = day 0), during the time that the fetal reproductive system and brain begin differentiating [68], 1992). A 5-log range of doses for each chemical was administered to pregnant females (N = 6–10/group): 0.02, 0.2, 0.2, 20, 200 μg/kg body weight/day of DES; 20, 200, 2000, 20,000, 100,000 μg/kg/day o,p'DDT or methoxychlor. The doses of DES, o,p'DDT and methoxychlor administered to pregnant mice in this study were based on predictions of estrogenic potency of these chemicals using methods described in detail in Nagel et al. [47]. Females fed oil alone and unhandled females (i.e. left undisturbed) served as controls (N = 6–10/group).

The offspring were examined for changes in morphological and neurobehavioral development [69], and in adulthood for urine marking behavior (number of urine marks deposited) when placed into a novel environment [27]. Specifically, within 12 h of delivery, the number of pups per litter, number of male and female pups (sex ratio), weight and anogenital distance (i.e. the distance between the anus and the genital papilla) of each pup was determined. Litters were culled to 8 pups (3–5 males and 3–5 females) and returned to their mothers. The combined weight of all 8 pups within each litter (litter weight) was recorded daily from day 1 to day 12. On postnatal day 2, 5 and 12 all of the pups in each litter were individually weighed and tested for reflexes.

Fetal exposure to estrogenic chemicals altered anogenital distance at birth, which correlates with prenatal exposure to testosterone and is interfered with by estrogen, possibly due to inhibition of 5alfa-reductase [7]. A very complex relationship between prenatal dose of estrogenic chemicals and AGD was found, revealing a non-monotonic dose–response function in males, which was less apparent in female offspring. For males, relative to controls, as dose increased we first recorded an increase in AGD (at DES 0.02, 0.2 and 2 μg/kg/day; at DDT 200 μg/kg/day), but with a further increase in dose, AGD significantly decreased (at DES 200 μg/kg body weight/day and DDT 20 000 μg/kg/day). Finally, AGD showed again an increase at the highest doses of DDT and methoxychlor (100 000 μg/kg/day). A similar trend was found in female offspring, where exposure to higher doses of the two pesticides (but not to DES) increased AGD, while low-intermediate doses decreased AGD.

While methoxychlor exposure did not influence body weight of newborns, complex dose–response functions were observed for DES and o,p'DDT, with body weight showing an increase or a decrease at different doses. Exposure to low and high doses of DES decreased body weights, while intermediate dose (DES 2 μg/kg/day) increased body weight. DDT exposure decreased body weights at intermediate doses (DDT 200 and 2000 μg/kg/day) and tended to increase body weight at the highest dose (100,000 μg/kg/day). In general there were similarities in terms of differences in body weight between DES and DDT exposed animals, but generalizations concerning these findings are not possible. The mixed effects on body weight for the three componds may be due to the fact that DES is very potent and across the dose range we were using would likely interact with other steroid receptors leading to multiple responses. This could be true also for the high o,p'DDT dose. Methoxychlor is now known to be an antiandrogen as well as an estrogen, so its effects are more complicated than is true for DES and o,p'DDT, which are just estrogens [70].

We then analyzed the effects of exposure to these chemicals on two reflexive responses, righting reflex (i.e. the time taken for a pup placed on its back to turn over) and cliff drop avoidance (i.e. the time taken for a pup placed on the edge of a table to turn away). We found that only methoxychlor-exposed animals showed changes in reflex development. Exposure to a very low dose of methoxychlor (20 μg/kg/day) appeared to produce an increase in reactivity during early postnatal life, in that the exposed pups performed both righting and cliff avoidance reflexes more quickly than control pups. These tests can provide information concerning physical and motor development as well as sensory function and/or processing [71]. Since this effect disappeared in the following days, with increasing age, this finding possibly reflects an effect on reactivity to environmental stimuli rather than to acceleration of physical (and hence motor) development. In contrast, cliff avoidance was retarded on day 2 by exposure to a high methoxychlor dose (20,000 μg/kg/day), and on day 5 by exposure to the lowest (20 μg/kg/day) and highest (100,000 μg/kg/day) doses. Since righting reflex, that is also indicative of physical and motor development, was not affected by these doses of methoxychlor on these days, it is possible that the observed delay in response might result from reduced reaction time rather than a delay in maturation. The complexity of the dose–response relationship makes interpreting these findings very difficult. However, it is possible that unique effects of EDCs can occur at one dose that are not observed at other doses [8]. Further research will be required to determine the reliability and significance of these complex findings.

After weaning, the prenatally treated offspring were housed in unisexual sibling groups. Male offspring were then examined as adults (3 months old) for their rate of urine marking in a novel territory. Marking the environment with urine is a common behavior in the house mouse and serves different social functions, such as to advertise the dominant territorial status of a male to other male competitors and to potential mates. Urine marking thus serves to regulate social interactions between conspecifics [64]. We found that a low dose of DES (0.02 μg/kg/day) significantly increased urine-marking behavior relative to control males, and as the dose increased to 2 μg/kg/day, rates of urine marking increased. Interestingly, the dose–response curve formed an inverted-U in that males whose mothers were fed the 200 μg/kg/day dose of DES showed significantly lower rates of urine marking than did males produced by mothers fed the 2 μg/kg/day dose. This type of inverted-U

dose–response function has also been observe in other studies examining the effects of fetal exposure to estrogens on development of the reproductive organs [8]. For both *o,p'*DDT and methoxychlor, the rate of urine marking in males increased as a function of dose [27].

In summary, using much lower (ppt to ppb) doses of estrogenic chemicals than have previously been examined, prenatal exposure to DES, *o,p'*DDT, and methoxychlor produced alterations in body weight at birth, anogenital distance, reflex development, and urine marking behavior, although in many cases different effects were seen for the different chemicals, and markedly different effects occurred at different doses. The conclusion from these studies, as well as from other findings, is that responses to endocrine disrupting chemicals cannot be assumed to be monotonic across a wide dose range. Our findings suggest that unique outcomes may occur in response to low, environmentally relevant doses of endocrine disruptors that will not be observed at higher doses. Since it has recently been reported that the in vivo metabolite of methoxychlor (bis-hydroxy-methoxychlor) binds with equal affinity to the androgen receptor (and acts as an androgen antagonist) and estrogen receptor (and acts as an estrogen agonist) in rats [70], it is now not unexpected that *o,p'*DDT and methoxychlor would not produce identical effects on development.

4. Part 2. Effects of prenatal exposure to DES and *o,p'*,DDT on aggression in male and female mice

We have approached the issue of the effects of environmental chemicals and drugs on reproductive function and behavior from an evolutionary perspective. Our specific research strategy has involved designing experiments to determine the degree to which endogenous hormones, drugs, and environmental chemicals that act as endocrine disruptors can perturb development, thus impacting reproduction and social behaviors. A primary concern is with the long-term effects of developmental exposure to endocrine disruptors on the behavioral interactions within the species and with their environment (referred to as ethotoxicology). In social species, such as house mice, intrasex aggression plays a crucial role in determining the reproductive success of both males and females, as reproduction is generally restricted to dominant animals as population density and competition for resources increases [63,64]. Intrasex aggression also serves to regulate the density of animals, leading to appropriate spacing. Since sex steroids play a critical role in regulating the development of the neural areas mediating aggression, as well as the expression of aggression in adulthood (in species that have the genetic predisposition for aggressiveness), environmental chemicals that interfere with the normal actions of sex steroids have the potential to alter levels of aggressiveness in exposed animals. We have reported that prenatal exposure to a low dose of *o,p'*DDT increased the rate of territorial urine marking in male mice; a 1000-times lower dose of DES produced the same effect [27]. Since urine marking is correlated with dominance status, we subsequently examined the hypothesis that prenatal exposure to doses of either *o,p'*DDT or DES that resulted in equivalent estrogenic activity would alter intermale aggressive behavior in males and possibly also interfemale aggression in female mice. We also examined the behavior of male mice toward unrelated mouse pups [72].

From gestation day 11–17 pregnant female CD-1 mice were fed an approximate average DES concentration (dissolved in oil) of either 0.02 μg/kg/day or 0.2 μg/kg/day. The doses of *o,p'*DDT were approximately 20 and 200 μg/kg/day, based on the prediction from our prior experiment that the in vivo potency of *o,p'*-DDT would be approximately 1000-times lower than DES [27]. Two levels of control were used: a group of pregnant females remained undisturbed (unhandled controls), and a group of pregnant females were administered oil alone (vehicle controls). There were 12–14 pregnant females in each treatment group. Within 12 h after delivery litters were culled to 8 pups, 3–5 males and 3–5 females. Nursing females and their litters were left undisturbed until weaning, when the offspring were housed in same-sex sibling groups. As adults (60–90 days of age), males and females underwent behavioral tests.

In the first experiment we examined the behavior of adult male mice from each treatment group toward an unrelated newborn pup. Specifically, at 3 months of age two males were randomly selected from each litter and housed individually, and 24 h later the males were tested for their behavior toward a single 2-day-old pup ($n = 24$–28/group). Prenatal treatment did not influence a male's response to a pup. In any group, 50 to 60% of males exhibited infanticide, while 5–6% behaved parentally towards the pup.

In the second experiment, one male from each litter (not used in the infanticide study) was selected to be tested for aggression ($n = 12$–14/group). These males were individually housed for 7 days in large cages in order to have this become the established home territory of the resident experimental male. An untreated sexually naive male, matched for age and weight with the resident test animal, was introduced into the cage for 10 min. The two groups of control males (produced by oil exposed and unhandled mothers) did not differ, and 14/26 (53%) of the control males attacked the intruder. In contrast, for the 0.02 and 0.2 μg/kg/day DES doses, 12/14 (86%) and 13/13 (100%) of the males attacked the intruder, respectively ($p < 0.05$ relative to controls for both comparisons). For males exposed to the 20 μg/kg/day dose of *o,p'*DDT, 10/12 (83%) of males attacked the intruder ($p = 0.08$) relative to controls, while exposure to 200 μg/kg/day *o,p'*DDT resulted in 9/14 (64%) of the males attacking the intruder ($p > 0.1$). Males exposed to the 0.02 μg/kg/day dose of DES and the 0.2 μg/kg/day dose of DES showed a significantly shorter latency to attack the intruder than control

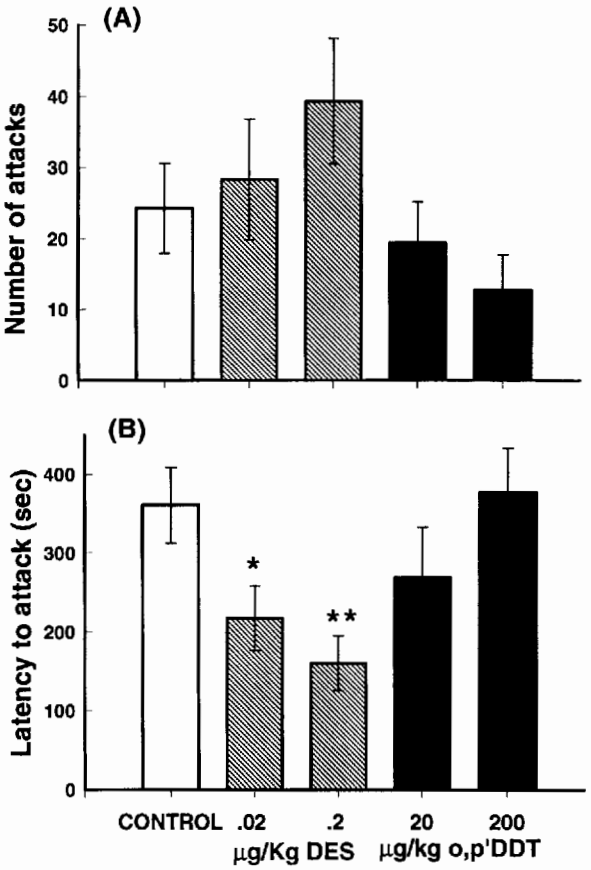

Fig. 1. Mean (±SEM) number of attacks (Panel (a)—$F(4) = 1.7$, $P < 0.15$) and mean (±SEM) latency to attack (Panel (b)—$F(4) = 3.15$, $P < 0.05$) same-sex intruders by adult males prenatally exposed to diethystilbestrol (DES), o,p'DDT or no chemical (control). $^{*} = P < 0.05$, $^{**} = P < 0.01$ vs. control.

males (Fig. 1(b)). Neither doses of o,p'DDT affected the latency to attack. There were no significant differences between control and prenatally treated males on two of the intensity of aggression measures: total attack time and number of attacks (Fig. 1(a)), although for DES, as dose increased, intensity of aggression appeared to increase. In contrast, for o,p'DDT, intensity of attack appeared to decrease as dose increased. However, a separate analysis (1-factor ANOVA) on data for intensity of aggression measures (bite frequency, total number of attacks and tail rattling) that was based only on the data from the males that attacked the intruder during the 10-min test (14/26 controls; 12/14 DES 0.02 μg/kg/day; 13/13 DES 0.2 μg/kg/day; 10/12 DDT 20 μg/kg/day; 9/14 DDT 200 μg/kg/day) showed a significant decrease ($p < 0.05$) in the intensity of aggression in males exposed prenatally to both doses of o,p'DDT relative to controls.

Males were also examined for the effects of prenatal exposure to estrogenic chemicals on the subsequent weight of testes and preputial gland in adulthood. These organs were examined because they both play a role in the regulation of sociosexual behaviors in mice. Testosterone is secreted by the testes and influences male aggressive and sexual behaviors; they, of course, also are the site of production of spermatozoa [68]. Preputial glands produce pheromones involved in social communication and preputial gland secretions influence aggressiveness between males [73,74]. At 6 months of age 12 control males and 8 males (not used in behavioral tests) from each estrogenic chemical dose group were individually housed for a week and then killed by CO2 asphyxiation. Males exposed to the 0.02 μg/kg/day dose of DES tended to have larger preputial glands relative to control males ($p < 0.06$). Neither dose of DES significantly affected testes weight. In contrast, testes weight was influenced by prenatal exposition to DDT: males exposed to a 20 μg/kg/day, but not 200 μg/kg/day; males exposed to the 20 μg/kg/day dose of o,p'DDT had smaller testes than controls ($P < 0.05$).

In a third experiment, we examined females exposed to the 0.20 μg/kg/day body weight/day dose of DES ($n = 13$), to the 20 μg/kg/day of o,p'DDT and control females ($n = 12$) selected equally from both oil-treated and untreated litters. Only one female within a litter was randomly selected and individually housed for 24 h in a cage previously inhabited by a control male for 48 h ($N = 10$–13/group); the soiled bedding from the male remained in the cage. This procedure is known to stimulate female intrasex aggression [75]. An untreated virgin female matched for weight was introduced into this cage for 20 min. A significantly higher proportion of females exposed in utero to the 0.20 μg/kg/day dose of DES exhibited biting attacks toward same-sex intruders relative to control females (8/13 DES vs 2/10 controls, $P < 0.05$; o,p'DDT: 3/12, $P > 0.1$). Since so few control females (2/10) exhibited attacks, all of the measures of intensity of aggression by DES treated females differed significantly ($P < 0.01$) when the data for all females tested were analyzed (including zeros for the non-attacking females).

Altogether, the results of this study show that exposure during fetal life to low doses of the estrogenic chemicals DES, and more partially to o,p'-DDT, influence adult social behaviors in male and female mice [72]. Specifically, prenatal exposure to low, 20 or 200 part per trillion (ppt), doses of DES increased the proportion of both males and females responding aggressively to a same-sex intruder into an animal's home territory. The increase in the proportion of DES-exposed animals that responded aggressively to a same-sex intruder was not related to an increase in intensity of attack (total number or duration of attacks), but rather to a reduced time interval from first contact with the conspecific intruder to the onset of attack (latency to attack). This suggests that animals exposed in utero to this estrogenic compound may differ in their reactivity to aggression-inducing stimuli. Males exposed to the 0.02 μg/kg/day dose of DES also had larger preputial glands than did control males.

A slightly different set of effects on behavioral and reproductive organ development resulted from exposure to o,p'-DDT than occurred with DES. Although the lowest

*o,p'*DDT dose examined (20 μg/kg/day) tended to increase the proportion of males that attacked a male intruder, analysis of the behavior displayed by aggressive males (utilizing data only from those males that attacked the intruder) revealed that *o,p'*DDT-exposed males showed a lower intensity of attack than controls. Prenatal exposure to this low dose of *o,p'*-DDT thus appeared to result in a quantitative change in the aggressive behavior of males by reducing the intensity of attack (i.e. number of attacks and time spent in agonistic behaviors). The finding that males exposed to the lower *o,p'*-DDT dose had smaller testes suggests the possibility that exposure to *o,p'*-DDT during fetal life may have impaired normal testicular function, resulting in lower levels of circulating testosterone. This in turn could have also affected the intensity of attack. Since there is a correlation between intensity of aggression and social status [76,77], *o,p'*DDT-exposed animals may be less effective at achieving and/or maintaining dominance. However, the basis for and complete characterization of differences between control and *o,p'*DDT-exposed animals will require further investigation.

5. Part 3. Effects on periadolescent and adult behavior following prenatal exposure to methoxychlor

Early ontogeny is considered a markedly plastic and crucial stage in the organization and regulation of future behavioral responses. In addition to the well-characterized prenatal and neonatal critical periods during which the brain is "organized" by sex steroids, weaning and the onset of puberty also represents an important developmental period. It is at this point in life that mice begin to explore the surrounding environment by themselves, and to aggressively or sexually interact with conspecifics. Studies on altricial rodents indicate that the early social and non-social environment is of crucial importance to the organization of adult individual differences in behavior [78,79]. Thus, the hormonally mediated differentiation of brain structures per se is not the only factor involved in the sexual differentiation of adult behavior, and a number of mammalian species have been found to reach adult reproductive competence only following an adequate experience of sociosexual interactions. While this is widely known for a number of primate species, this also holds for rodents and several other mammalian species that have been examined [80]. All of these developmental processes can be viewed as part of an integrated system. Perturbations of the hormonal milieu during fetal/neonatal development may have a major role in modulating developmental trajectories that, in turn, will influence subsequent behavioral responses. The amount and characteristics of the behavior patterns actually expressed early in life—mostly depending upon social and non-social environmental conditions—can exert important neural, endocrine, physiological and psychological effects that can operate as a feedback on the still plastic CNS system, thus influencing neurobehavioral development [10,81].

In the following series of experiments described for the first time here, we examined changes in behavioral development of mice prenatally exposed to the estrogenic pesticide methoxychlor. Mice were tested during at least two critical phases in development: at weaning/pre-puberty and, only the male offspring, at puberty/post-puberty. Males were then tested as adults for territorial aggression.

6. General methods

6.1. Animals and husbandry

CD-1 mice (*Mus domesticus*) used in this experiment were born and reared in laboratories at the University of Parma. A breeding stock of males and females was originally purchased from Charles River Laboratories (Curno, Italy). Animals were housed in standard polypropylene mouse cages on sawdust bedding with food (MIL) and water available *ad libitum*. The light:dark cycle was 12 h light and 12 h dark, with lights on at 10:00 hrs. Room temperature was 23 ± 2°C. One-hundred and twenty adult females (3–4 month-old) were time-mated by being placed into the cage of a stud male for 4 h beginning at 08:00 hrs. When a vaginal plug was found (day 0 of pregnancy), females were housed three per cage ($40 \times 25 \times 15$ cm^3). Females were weighted at gestation days 0, 8, 11, 13, 15, 16 and 17. On gestation day 8 they were individually housed.

6.2. Maternal treatment

The estrogenic pesticide, methoxychlor, was dissolved in tocopherol-stripped corn oil (Cat# 901415, ICN, Aurora, OH). After being time mated, on day 1 of pregnancy, females were trained to spontaneously drink a small volume (0.01 ml) of corn oil from a modified syringe (without the needle and with a larger hole) introduced through the cage top every second day 2 h after light onset. When individually housed on day 8 of pregnancy, each female received a daily administration of corn oil alone at 12:00 hrs; the amount consumed was verified to ensure administration of the desired dose. All females easily learned to drink the oil as soon as the syringe was introduced, and this procedure allows accurate administration of chemicals or drugs without the stress associated with gavage or injection.

On gestation day 11 each female was randomly assigned to one of the following treatment groups ($N = 16 - 18$ females/group): vehicle controls received corn oil alone (we did not test unhandled controls as they did not differ from vehicle controls in prior experiments), and methoxychlor at 20, 200 and 2000 μg/kg/day body weight/day. From gestation day 11 to 17, 2 h after light onset (10:00 hrs), each female was allowed to drink 0.01 ml/50 g-body weight of oil with or without methoxychlor.

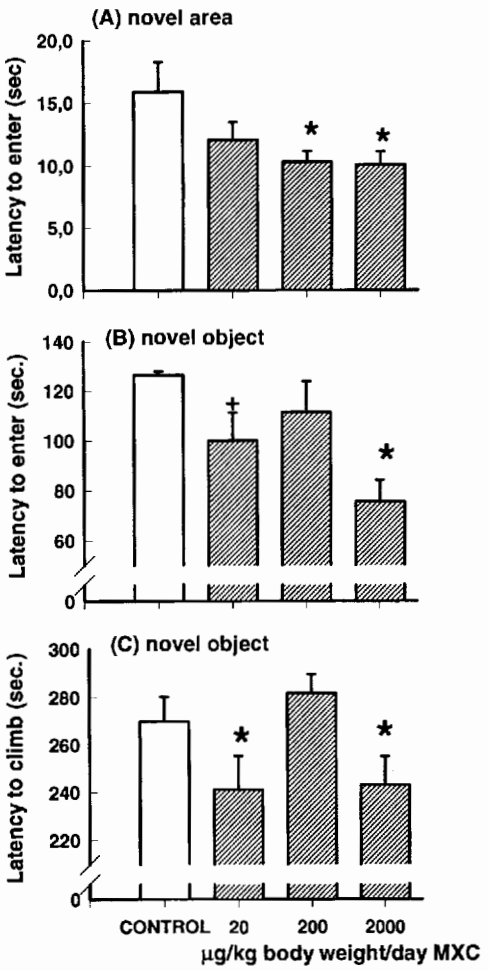

Fig. 2. Mean (±SEM) latency to enter a novel area (Panel (a)), latency to enter a novel object (Panel (b)) and, latency to climb on a novel object (Panel (c)) in both male and female mice prenatally exposed to different doses of methoxychlor. * = $P < 0.05$, + = $P < 0.08$ vs. controls.

Within 12 h from delivery, the total number and sex of pups per litter, as well as weight of each pup was determined. Litters were culled to 8 pups (3–5 males and 3–5 females) to reduce litter-size induced variability in the growth and development of pups during the postnatal period. The pups were then returned to their mothers. Nursing females and their litters were left undisturbed until weaning, when the offspring were housed in same-sex sibling groups and underwent behavioral tests. For both sexes, the first test was conducted at weaning (26–27 days old). Male offspring were then tested for the onset of aggression at 39 and 54 days old and then at about 3 months of age for intermale territorial aggression.

7. Behavioral observations after weaning: response to novel stimuli

Animals pay more attention to novel information than to a familiar cues, and they actually seem to be both attracted and activated by novel stimuli as well as by variations in the set or the intensity of familiar ones [82–84]. Such enhanced response to environmental changes has adaptive value in that novel stimuli may pose a potential threat or, alternatively, become a possible resource. Individual differences in the response to novel stimuli, as with any other trait, may be caused by genetic and/or environmental factors, or, quite typically, result from their interaction. When compared to adult subjects, elevated levels of novelty seeking are expressed by mice during periadolescence [85], which has been defined as the ontogenetic period that encompasses the 7–10 days preceding the completion of the pubertal period (at about 40 days of age in rats and mice) and the first days thereafter [83].

7.1. Methods

As experimental apparatus transparent Plexiglas cage ($40 \times 25 \times 15$ cm^3) was divided in two compartments (A and B—20×25 cm^2 each) by a partition of white opaque polypropylene with a small opening in the middle (diameter 3 cm). On the side of the partition facing compartment B, a door could be opened and closed by the experimenter from outside of the cage and without moving the cage top. As a novel object, a 6-faced parallelepiped of $10 \times 13 \times 13$ cm^3 made by blue polypropylene was used. The object had a small opening on one side that allowed the animals to enter it, and a diagonal platform on the opposite side that allowed the animals to climb on it.

The animals were weaned when 26–27 days old. Same sex siblings (N = 14–16 sibling groups/prenatal treatment for each sex) were caged together in compartment A of the experimental apparatus with the door closed. Within each group of 3–4 same-sex siblings, each animal was individually marked on the tail with a non-toxic die. Each sibling group was housed in the experimental apparatus on test-day 0, and tested for the 2 following, consecutive days. After 24 h, on test-day 1, the door dividing the two compartments was opened thus allowing animals to enter compartment B, and the animals were observed for 5 min. The following variables were recorded for each sibling: latency to enter the novel compartment with all 4 paws, total time spent exploring the novel area, number of transitions between the two compartments. At the end of the 5-min period the door was then left open.

On the next day (test-day 2) animals were exposed to a novel object. The door was first closed keeping the animals in compartment A, and a novel object was placed into the center of compartment B. After 20 min the door was opened, and the experiment lasted 5 min. The following variables were recorded for each animal: latency to enter compartment B with all 4 paws, latency to enter the object with all 4 paws, latency to climb the object, number of transitions between compartments A and B, number of transitions inside–outside the novel object.

Observations on both days of testing were conducted

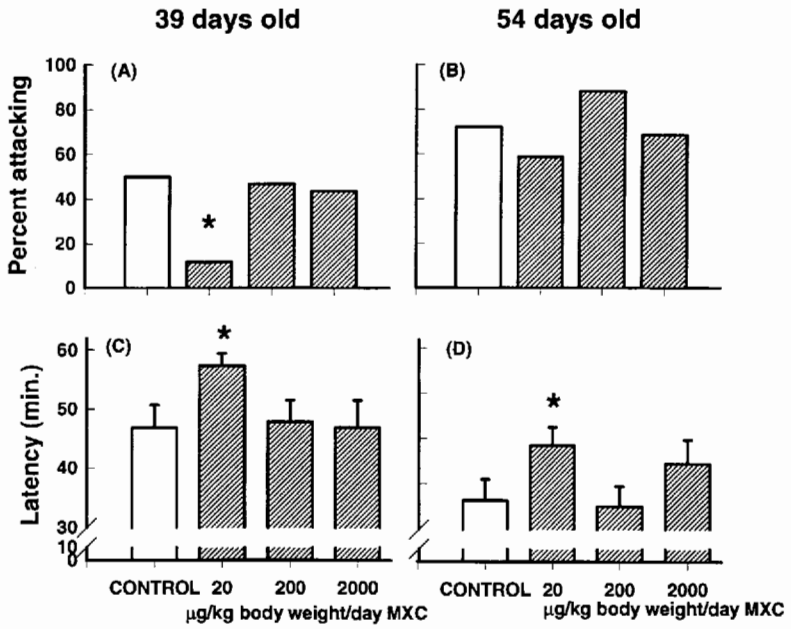

Fig. 3. Percent of animals showing attacks (Panels (a) and (b)) and mean (±SEM) latency to the first attack (Panels (c) and (d)) in male sibling groups placed into a novel environment at 39 and 54 days of age. * = $P < 0.05$ vs. controls.

during the 3 h following light onset. Male and female siblings from the same litter were observed simultaneously and all tests were videotaped. The novel objects were cleaned with alcohol and water prior being introduced into the cage. The data from each sibling group were analyzed by a two factor ANOVA (prenatal treatment and sex).

7.2. Results

There were no sex differences for any behavioral measure for mice in any treatment group, and data for male and female siblings are thus not presented separately.

Fig. 2(a) shows that prenatal treatment affected the mean latency for siblings to enter the novel area ($F(3) = 2.73, P < 0.05$). Periadolescent mice prenatally exposed to the 200 and 2000 µg/kg/day doses of methoxychlor entered the novel area earlier than control mice ($P < 0.05$). No difference was observed in the total time spent exploring the novel compartment or in the number of transitions between the two compartments (data not shown). Prenatal treatment influenced the readiness to enter ($F(3) = 2.78$, $P < 0.05$) and to climb on ($F(3) = 2.80$, $P < 0.05$) the novel object. When exposed to the novel object, mice prenatally exposed to the lower (20 µg/kg/day) and higher (2000 µg/kg/day) methoxychlor doses showed lower latencies to enter and to climb on the object (Fig. 2(b) and (c)). Total time spent exploring the object and number of transitions between the two compartments and inside outside the object did not differ in relation to the prenatal treatment (data not shown).

8. Behavioral observations during adolescence: onset of intermale aggression

The transition between the end of puberty and onset of adolescence, marked by the onset of testicular function, occurs in mice at about 40 days of age. Typically, male littermates at this age begin to show aggressive interactions that later on, lead to the establishment of a hierarchical order, with a dominant male, subdominant and/or submissive males [86,87]. It is known that in male mice, prior social experiences can profoundly affect the subsequent responses towards conspecifics [76]. The very first experience of fighting for a male may have long-lasting consequences and significantly alter future behavior (personal observations). The possible effects of prenatal exposure to methoxychlor on the development of aggressive interactions among male littermates during the onset of adolescence, were examined.

8.1. Methods

Male siblings from the same litter were grouped together (groups of 3–4) in Plexiglas cages ($40 \times 25 \times 15$ cm^3) with water and food ad libitum. For each experimental group, 16–18 male sibling groups were observed.

At 39 and 54 days of age, animals were moved into a novel cage with clean sawdust and were observed for the following 60 min to monitor the occurrence of aggressive interactions between siblings. Frequency of aggressive interactions and the latency to the first attack between siblings were recorded; a latency of 3600 s. was assigned

Table 1
Aggression by individually housed males prenatally exposed to different doses of Methoxichlor (MXC) or no chemical (control) confronting male intruders in a ten-minute test. Duration data are expressed as percent of time (Mean and SEM are given: $^{*}P < 0.05$ vs control)

Prenatal treatment	Proportion of attack (%)	Latency to attack (s)	Total attack time	Number of bites	Aggressive grooming	Social behavior	Defensive behavior
Control	21/36(58%)	289 ± 33	7.2 ± 1.3	16.8 ± 3.1	2.7 ± 0.7	33.3 ± 2.7	1.7 ± 1
MXC 20 ng/g	17/34(50%)	332 ± 38	6.4 ± 1.7	13.2 ± 3.9	1.2 ± 0.3*	30.6 ± 2.6	2 ± 1.3
MXC 200 ng/g	18/34(53%)	245 ± 31	7.1 ± 1.5	15 ± 3.3	3.6 ± 0.8	29.5 ± 2.4	0.3 ± 0.1
MXC 2000 ng/g	15/32(43%)	338 ± 36	4.6 ± 1.6	10 ± 3.2	1.4 ± 0.3	33.6 ± 2.8	0.5 ± 0.3

when no attack was observed within the observation time. Latency to attack data were analyzed to compare males from each prenatal treatment group using a repeated measures ANOVA. The proportion of animals per experimental group that attacked within the 60 min. observation were analyzed using Fisher's exact probability test.

8.2. Results

Fig. 3 shows the data for aggressive interactions and latency to the first attack in sibling groups during the 60 min following introduction of the animals into a new cage. In the first test, when males were 39 days old, a significantly lower proportion of aggressive interactions was recorded in male siblings prenatally exposed to the methoxychlor 20 μg/kg/day dose as compared to the control group ($P < 0.05$). However, this difference was not observed when animals were 54 days old. The analysis of variance for repeated measures showed a significant effect of age on the latency to attack ($F(2) = 71.78$, $P < 0.001$). When 54 days old, males started aggressive interactions more quickly as compared to when they were 39 days old. ANOVA showed no overall effect of prenatal treatment on latency to attack ($F(3) = 1.17$, $P = 0.17$). However, both when 39 and 54 days old, males prenatally exposed to the methoxychlor 20 μg/kg/day dose showed a significantly longer latency to display aggressive interactions relative to controls ($P < 0.05$).

9. Behavioral observations in adulthood: male territorial aggression

Male mice compete among themselves to establish and hold a territory and to achieve dominance. Since reproduction is largely confined to dominant, territorial males, a male's capacity to defeat male conspecifics intruding into its territory plays a crucial role in determining its reproductive fitness [64]. Male intrasex aggression is also thought to play an important role in spacing conspecifics, thus resulting in the regulation of the density of animals according to ecological conditions [76]. The behavior of rodents shown in a resident–intruder paradigm mimics territorial intermale aggression and appears to conform with what is believed to happen in wild populations of mice [76]. Therefore, in this experiment we examined whether fetal exposure to methoxychlor influenced intermale aggressive behavior measured during resident–intruder encounters as an indicator of territorial aggression.

9.1. Methods

The experiment animals were housed in groups of 3–4 siblings in 40 × 25 × 15 Plexiglas cages. When 80–85 days old, two males from each litter were randomly selected (n = 30–36/treatment group) to be tested for aggression. These males were individually housed for 3 days in Plexiglas

cages ($40 \times 20 \times 20$ cm^3) in order to have this become the established home territory of the resident experimental male. After 3 days of isolation, a resident–intruder test was conducted and was videotaped. An untreated sexually naive male, matched for age and weight with the resident test animal, was introduced into the cage for 10 min. The first attack was scored when the resident male attempted to bite the intruder. In only a few cases the resident did not attack first and, instead, was attacked by the intruder. Attacks also consisted of chasing and circling; in addition to biting, and the time (in s) spent exhibiting these behaviors was also included in the total attack time. The following variables were recorded: (1) number of males attacking an intruder (i.e. delivering at least one bite to the opponent); 92) latency to attack (i.e. time interval from the first contact to the first attack in s); (3) number of attacks; (4) total time spent attacking the intruder (in s); (5) social investigation (in s); (6) tail rattling (a behavior typically seen prior to an attack); (7) aggressive-grooming of the intruder (in s); and (8) defense (upright submissive posture, immobility or freezing behavior). Resident–intruder tests were carried out during the 3 h following light onset. Data were analyzed by ANOVA. Duration data were calculated as a proportion of the total session measures (i.e. percent of time measures). Percent time data were then arcsin transformed for statistical analysis.

9.2. Results

Table 1 shows the results for aggression and other variables recorded during the 10-min test. There were no significant differences between control and prenatally treated males on the proportion of attacking residents, intensity of aggression measures (total attack time, number of attacks, aggressive grooming), although methoxychlor 2000 μg/kg/day exposed males tended to show decreased intensity of attack. Methoxychlor exposure affected the amount of aggressive grooming shown by males ($F(3) = 3.31$, $P < 0.05$). Resident males exposed to the lower methoxychlor dose displayed lower levels of aggressive grooming as compared to controls. Prenatal treatment did not significantly affect any other behavioral measures recorded.

10. Discussion of periadolescent and adult behavioral observations of mice prenatally exposed to methoxychlor

Prenatal exposure to low doses of the estrogenic pesticide methoxychlor increased the reactivity of both male and female periadolescent mice to novelty. At weaning, methoxychlor exposed mice were more prompt in exploring both a novel area and a novel object; they appear to be less fearful and anxious than controls and/or more impulsive. A reduction in reaction time to novel stimuli could be a function of either direct or indirect effects of prenatal exposure to this chemical, such as a non-specific increase in the level of locomotor activity. The latter non-specific change could however be excluded since no significant changes in the number of transitions were observed. On the other hand, a more reactive profile to changes in the environment could be related to differences in emotionality or, alternatively, higher levels of novelty seeking behavior. Further studies are in progress to investigate these hypotheses.

Prenatal exposure to the lower methoxychlor dose (20 μg/kg/day) altered the onset of male aggressive behavior at the onset of adolescence. At 39 days of age, methoxychlor-exposed male mice showed a decreased frequency of aggressive interactions and a longer latency to attack. With increasing age, this effect of methoxychlor was still observed, although it was attenuated. It is possible that these effects are related either to delayed maturation or to decreased levels of social aggression. However, the last experiment showed that when tested as adults for territorial defence against a conspecific intruder, males prenatally exposed to methoxychlor did not differ from unexposed males. These findings suggest that a delay in maturation might be responsible for decreased aggressive behavior recorded in early adolescent males prenatally exposed to the lower methoxychlor dose. Further studies are required to test this hypothesis.

11. Conclusions and perspectives

The range of outcomes attributable to exposure to environmental relevant doses (within the range of exposure of wildlife and humans) of endocrine disrupting chemicals is not well known. In particular, functional changes, such as changes in behavioral responses or changes in organ function, as opposed to gross toxicity or teratology, have not typically been examined in toxicological studies. Disorders of neurobehavioral function can assume many different forms. In the present study we have reported a series of experiments on the effects of prenatal exposure to estrogenic endocrine disruptors on several behavioral responses in mice. The interplay among type and dose of the chemical, developmental stage at the time of exposure, age at testing, and response endpoint have been the subject of our investigations. We paid particular attention to the issue that is central to the emerging field of ethotoxicology, namely that social and environmental situations in which animals are tested should be ethologically appropriate and thus relevant in terms of adaptive function of the behavior being examined.

We focused here on our studies of the effects of low, environmentally relevant doses of two pesticides, *o,p'*DDT and methoxychlor, and of low doses of the synthetic estrogen, diethylstilbestrol. The main findings can be summarized as follows: (1) Animals prenatally exposed to methoxychlor showed changes in reflex development. Exposure to a very low dose of methoxychlor (below the current dose deemed safe to ingest per day

without risk; [66]) appeared to produce an increased reactivity during early postnatal life, in that the exposed pups performed both righting and cliff avoidance reflexes more quickly than control pups. (2) Methoxychlor exposed periadolescent mice showed a decreased reaction time exploring both a novel environment and a novel object. (3) The onset of male intrasex aggression appeared to be delayed in males prenatally exposed to low doses of methoxychlor, since exposed males showed low levels of aggressive interactions during early adolescence but not after they reached adulthood. (4) The rate of depositing urine marks in a novel environment was increased in males prenatally exposed to DES, and also to *o,p'*DDT and methoxychlor, suggesting an increase in territorial behavior. (5) The proportion of both males and females attacking a same-sex conspecific was increased in mice prenatally exposed to low doses of DES and, marginally, to *o,p'*DDT. This effect appeared to be related to a decreased latency to attack. This finding suggested that reactivity to a same-sex conspecific was influenced, but that aggressiveness, *per se*, may not have been directly affected. However, males prenatally exposed to *o,p'*DDT displayed a decreased intensity of aggression. Future research will be required to address this question.

Prenatal exposure to different doses of these chemicals can produce differential effects on a number of behavioral measures. However, a consistent finding is that prenatal exposure to low doses of different estrogenic chemicals, such as DES, *o,p'*DDT, methoxychlor, can produce increased reactivity at different developmental stages and in different experimental paradigms. In a variety of new situations, such as encountering a novel environment or a novel object (including an unfamiliar conspecific), the prenatally exposed animals were more reactive than controls. It is thus possible that the previously observed increase in urine marking behavior [27] may reflect an increased reactivity to novel environments, in addition to being an index of heightened territoriality.

Several hypotheses may be advanced to explain these findings. A possible explanation is that prenatal exposure to estrogenic chemical produces a non-specific increase in locomotor activity. It has been reported that pre- or perinatal exposure to PCBs, a mixture of chemicals that can result in the formation of in vivo metabolites with an affinity for estrogen receptors, results in behavioral hyperactivity in mice and rats [37,88,89]. Further experiments are in progress to examine the levels of general activity of prenatally exposed animals. On the other hand, a more reactive profile to changes in the environment could be related to differences in emotionality, since decreased reaction time responding to novel stimuli can be suggestive of a low anxiety profile [90,91]. It is well known that the behavior of animals exposed to novel situation results from a competition between an exploratory tendency (curiosity and novelty seeking) and a withdrawal tendency (Fear). Consequently, anxiety may be viewed not only as a factor that inhibits exploratory behavior, but also may stimulate, at moderate levels, the propensity to explore new environments. Steroid hormones are involved during fetal life in setting number and sensitivity of brain receptors for steroids and neurotransmitters [92,93]. Endocrine disruptors might thus interfere with the normal development of serotoninergic, dopaminergic and GABA-ergic receptor systems, and alter responses to novel or potentially stressful situations. Specific tests to measure anxiety responses (e.g. elevated plus maze, open field) and the use of specific drugs (i.e. ethopharmacological techniques) can help to identify the mechanisms of behaviors altered by environmental chemicals [94].

Explanations for the effects of prenatal exposure to estrogenic chemicals, such as DES, are complicated by the fact that hormones and hormone-mimicking chemicals do not show linear dose–response curves throughout a wide dose range. Instead, non-monotonic functions, such as U-shaped or inverted U-shaped curves are found [8]. This is a critical issue in toxicology, since only a few (typically 3) very high doses are examined in chronic toxicological studies, and the safety of low doses that are within the range of human exposure is not directly tested but, instead, is *estimated* based on models that assume a monotonic dose response curve. In studies in which we administered pregnant female mice a 5-log range of doses of DES, we obtained a non-monotonic, inverted-U dose–response function for anogenital distance and body weight at birth, reflex development [69], urine marking rates [27] and adult prostate size in male offspring [8]. While a non-monotonic, inverted-U dose–response curve may not occur for all responses to endocrine disruptors, numerous examples of non-monotonic functions have been reported for responses mediated by receptors for hormones and other intercellular signaling molecules [8,27,95,96]. The conclusion from our studies, as well as these other findings, is that responses to endocrine disruptors cannot be assumed to be monotonic across a wide dose range. Our findings suggest that unique outcomes may occur in response to low, environmentally relevant doses of endocrine disruptors that will not be observed at higher doses.

In the present studies we examined the developmental effect of estrogenic chemicals without a separation of the direct effects of treatment on the pups from those that can act through maternal behavior as a result of maternal exposure to chemicals during the last part of pregnancy. It is well known that variations in maternal care can affect the development of individual differences in behavior [81,97,98] and neuroendocrine responses to stress [99]. Estradiol plays an important role in priming and activating maternal behavior [100]. We have recently examined whether exposure to methoxychlor during pregnancy would alter maternal behavior in lactating mice. Preliminary results have shown that dams administered the lowest dose of methoxychlor examined in the experiments described here (20 μg/kg/day) tend to show lower levels of nursing behavior as compared to controls [101]. A further step in our studies will be to clarify which part of the behavioral effects we have described here

can be attributed to direct action on the fetus as opposed to effects mediated by disruption of normal maternal behavior, which can be determined by a cross-fostering procedure.

It is generally assumed that natural selection operates to create a phenotype that is optimum for a particular environment. If exposure to endocrine disruptors changes that phenotype, leading to a less than optimum set of traits, such as an altered level of reactivity to stimuli or altered aggressiveness for that environment, a negative impact on those individuals in the population is likely to occur, and changes in population dynamics will likely follow. While sociosexual behaviors of a particular species are adapted to specific environmental conditions [102], there are many factors that give rise to individual differences in these behaviors [7]. Consequently, there is an evolved range of social behaviors that occurs among animals within any population due to variation in genotype, hormone levels, experience, etc. Shifts in the proportion of animals within a population that show specific traits, such as increased aggressiveness or exploration, can influence social structure and dispersion, thus affecting population dynamics [76,103,104]. For instance, increased frequencies of males and females responding aggressively to conspecifics can change the social structure of a mouse population, potentially contributing to a decline or even a crash in population size [103,104]. In social species such as the house mouse, as well as many primate species that have been studied [105], intrasex aggression determines individual reproductive success and regulates the density of animals, leading to an appropriate spacing in relation to socio-ecological conditions [64,76]. Thus, our finding that low doses of DES significantly increased the proportion of male and female mice that attacked a same-sex intruder suggests that exposure to estrogenic chemicals during fetal life could influence population dynamics by changing sociosexual behaviors of females as well as males.

Natural and sexual selection operate at the level of phenotype, not directly on genes. Thus, even though environmental factors may not alter genes via mutations, they do alter phenotype and thus the course of evolution [106]. t is well recognized in toxicology that not all individuals are equally sensitive to environmental chemicals, and there has long been a concern with sensitive sub-populations that show a greater response to chemical exposure than other members of a population. Thus, when exposed to endocrine disruptors during development, some individuals will be shifted in phenotype to a greater degree than will other individuals. One consequence could be a decrease in the range of phenotypes in the population, thus possibly decreasing population variance. Our hypothesis is that this will also lead to a "feed forward" process leading to a change in genotype within the population, that is, a change in the course of evolution (see also Parmigiani et al. in this volume) The genetically predisposed individuals that show the greatest change in phenotype in response to endocrine disruptors could have had, in an uncontaminated environment, the optimum phenotype for the environment. Subsequent to developmental exposure to endocrine disruptors, this phenotype could be changed, and lost. In fact, the phenotype resulting from chemical exposure of these sensitive individuals may now be the least optimum for the environment. By this mechanism, pollutants operating on phenotype may eventually lead a change in gene frequency in the population.

Our ethotoxicological approach reveals the importance of evolutionary processes for the understanding of the potential effects both on individuals and on populations due to exposure to endocrine disruptors. This requires multidisciplinary studies in which analysis of the effects of these chemicals is undertaken using molecular, cellular, physiological, behavioral, ethological and ecological methods in order to understand the full range of their effects.

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