ETHOTOXICOLOGY: AN EVOLUTIONARY APPROACH TO THE STUDY OF ENVIRONMENTAL ENDOCRINE-DISRUPTING CHEMICALS

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The focus of most studies in behavioral toxicology has been on effects of chemicals on learning and memory, sensory function, activity, and neuromuscular function. Behavior has also been used as a biomarker of chemical exposure, often without concern for the context in which the behavior was being observed. We have applied the strategies employed in ethological analyses of behavior and developed an alternative, ethotoxicological, approach to the study of effects of endocrine disruptors on behavior. This involves examining whether a behavioral alteration may be pathological from the perspective of the adaptive significance of the behavior under investigation. Pathological changes in behavior lead to reduced social adaptation and impaired responsiveness to environmental demands, with consequences for social structure and population dynamics. It thus follows that the ecological context in which the behavior would normally occur and the function of the behavior are of paramount importance when studying the impact of endocrine disruptors.

INTRODUCTION

During fetal life, sex steroids, such as estradiol, have marked effects on the development of the neuroendocrine system and subsequent behavior (vom Saal et al., 1992). Exposure to estrogenic endocrine-disrupting chemicals in the environment during critical developmental periods in fetal life thus also has the potential to produce permanent changes in the structure and functioning of the brain, leading to changes in behavior (vom Saal, 1995). The timing of exposure is critical. During the period when the central nervous system is undergoing rapid change and before protective mechanisms have developed, estrogenic xenobiotic chemicals, at environmentally relevant concentrations, can lead to irreversible alterations in brain development at exposure levels that might produce little effect in an adult (Bern, 1992; vom Saal, 1995; vom Saal et al., 1995; Nagel et al., 1997; vom Saal et al., herein).

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2. Abbreviations: ADI, acceptable daily intake; DES, diethylstilbestrol; MRL, minimum risk level.

3. Key words: aggression, developmental toxicology, territorial behavior.

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Behavior is a very sensitive biomarker of exposure to endocrine-disrupting xenobiotics. Across a wide variety of vertebrate species, including humans, estrogen and other steroid hormones influence aggressive and parental behaviors, as well as other sociosexual behaviors, in both males and females. Importantly, the underlying mechanisms of action are similar across species (Nelson, 1995). Perturbation of hormonal systems during early development can alter brain neurochemistry, and consequently can lead to changes in social interactions and alter population dynamics (vom Saal, 1984). Our focus is on sociosexual behaviors that are critical with regard to reproductive success.

ETHOTOXICOLOGICAL STUDY METHODS

The House Mouse as an Experimental Animal

We have been investigating the effects of exposure to estrogenic chemicals during fetal life on the subsequent sociosexual behaviors of both male and female house mice (*Mus domesticus*). We use outbred stocks of mice (CD-1 and CF-1) in our studies (Parmigiani et al., 1989). The house mouse is widely distributed throughout the world and thus has been subjected to varied ecological pressures (Bronson, 1979). For this reason the house mouse is one of the most versatile mammalian species. House mice live in environments as diverse as fields, cold stores, warehouses, hayricks, Pacific atolls, and islands close to Antarctica. Although there are reports of feral populations of this species living totally apart from humans, house mice live most commonly as commensals of man, and mice are thus likely to be exposed to many of the same environmental pollutants as humans (Bronson, 1979; Berry, 1989). House mice serve as prey for both terrestrial and avian predators, and they may serve as vectors for pollutants encountered by mice in food, water, and air.

House mice exhibit different social organizations, based on prevailing local conditions of food distribution, available cover, and animal density. In particular, the social organization in different populations of house mice reflects variations in female and male social aggression. House mice thus show flexibility in social behavior and social structure, as well as rapid adaptation. This feature of house mice makes them an interesting model animal for studying the impact of environmental endocrine disruptors on behavior and social dynamics, since it may be possible to relate variation in the social structure of populations to specific pollutants in the environment.

RESULTS AND DISCUSSION

Behavioral Effects of Fetal Exposure to Diethylstilbestrol and Estrogenic Pesticides

One of the social behaviors that we have examined in male mice exposed during fetal life to estrogenic chemicals is territorial urine marking behavior. We fed pregnant CF-1 mice different doses of the drug diethylstilbestrol (DES), which served as a positive control for estrogen action, and two insecticides, o,p'-DDT (an estrogenic contaminant in commercial DDT) and methoxychlor. Fetal exposure to these chemicals influenced the rate at which males deposited urine marks in a novel environment in adulthood (vom Saal et al., 1995). In more detail, we dissolved each of these chemicals in corn oil and fed the chemical (using a pipette to deliver an

accurate volume) to pregnant female mice. Pregnant mice were allowed to drink the oil solution (which they readily consume) after placing the oil into their mouths, rather than being subjected to gavage. The objective was to minimize stress that would be associated with placing a tube down an animal's throat and into its stomach. We fed each pregnant mouse a chemical once a day from gestation day 11–17 (mating = day 0), during the time that the initial development of the brain and reproductive organs occurs in fetuses (vom Saal et al., 1992).

We found that a low dose of DES (0.02 ng/g maternal body weight; 20 ppt) significantly increased urine marking behavior relative to control males, and as the dose increased to 2 ng/g (2 ppb), rates of urine marking increased (Figure 1). However, males whose mothers were fed a 200 ng/g dose of DES showed significantly lower rates of urine marking than did males produced by mothers fed 2 ng/g (vom Saal et al., 1995). This type of inverted-U dose-response function has also been observed with other endpoints, such as adult prostate weight, in other studies of the effects of fetal exposure to estrogenic chemicals (vom Saal et al., 1997).

Based on an *in vitro* study with MCF-7 cells (vom Saal et al., 1995; Nagel et al., 1997), we predicted similar effects on urine marking behavior in response to maternal ingestion of o,p'-DDT at a dose between 1000–10 000 times higher relative to DES. Methoxychlor was expected to exert an estrogenic effect at a dose between 10 000–100 000 times higher than DES, which is exactly what we found (Figure 1). The doses of these chemicals that resulted in changes in behavior are within the range of human exposure and are thus environmentally relevant (ATSDR, 1993, 1994).

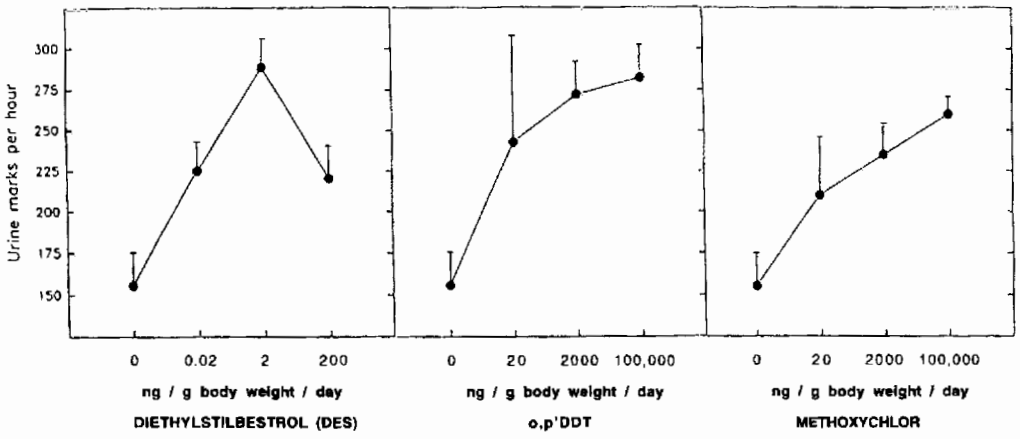

FIGURE 1. Mean (± SEM) number of urine marks deposited on filter paper during a 1 hour test in a novel cage by adult male offspring of pregnant mice fed DES, o,p'-DDT, or methoxychlor. The doses indicated are per gram body weight of the pregnant mice fed a chemical from gestation day 11–17. Urine of mice contains chemicals that are fluorescent under ultraviolet light, which allows individual urine marks to be counted. From vom Saal et al., 1995.

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 urine marking behavior. This suggested that an increase in intermale aggression might also be observed in male mice exposed prenatally to estrogenic chemicals. This prediction was recently confirmed in a study in which 2 and 200 ppt doses of DES and 20 and 200 ppb doses of o,p'-DDT were administered using another stock of outbred mice. Pregnant CD-1 female mice were fed a chemical as described above, and the male offspring in each group were examined in adulthood for aggressive behavior (biting and chasing the opponent) during a 10 minute test where an intruder was placed into the home cage of the experimental animal. Relative to the proportion of controls that attacked the intruder (14/26; 54%), the proportion of males exhibiting aggression was significantly increased by exposure to the 20 ppt DES dose (12/14; 86% attacked) as well as the 200 ppt DES dose (13/13; 100% attacked). Exposure to the 20 ng/g dose of DDT resulted in 10/12 (83%) of males attacking the intruder ($p = 0.08$ relative to controls), while exposure to 200 ng/g DDT resulted in 9/14 (64%) of the males attacking the intruder ($p > 0.1$) (manuscript in preparation).

Urine Marking and Aggressive Behavior

Urine marking and intermale aggression are behaviors that influence competition for mates and thus reproductive success in male mice. Males mark their territories to advertise their dominance status, based on pheromonal cues contained in the urine. The rate of urine marking is also a function of dominance status. Intermale aggression in this species is a primary determinant of reproductive success, with virtually all young within a territory being produced by the one dominant male (Bronson, 1979; vom Saal, 1984).

Urine marking and intermale aggression are mediated by similar neuroendocrine and genetic mechanisms (Parmigiani et al., 1989; Parmigiani and Palanza, 1991; Ferrai et al., 1996). Urine marking is influenced by a male's social status and testosterone levels, both in terms of qualitative differences (urine composition) and quantitative differences (rate of urine deposition). Male mouse urine contains olfactory cues (pheromones) that affect both the behavior and the physiology of other mice. For example, male urine contains signaling pheromones that elicit intermale aggressive behavior, are attractive to females, and also stimulate interfemale aggression. In addition, male urine contains primer pheromones that accelerate puberty, induce estrus, and block pregnancy in unfamiliar females (Palanza et al., 1994). Males urine mark to advertise their dominance status, and it is likely that this plays a role in territorial defense against potential intruders. The rate of urine marking in response to social stimuli can thus be a useful indicator of a male's social rank and territorial defense potential, which provides one explanation for the females' use of pheromonal cues in male urine to assess the reproductive fitness of males (Bronson, 1979).

Further Evidence for Non-Monotonic Dose-Response Functions

We recently completed another study in which CF-1 mouse pups exposed prenatally to the same doses of DES, o,p'-DDT, and methoxychlor described above in the urine marking study underwent a series of tests to examine postnatal physical and behavioral development. On postnatal day 2, 5, and 10, all pups in each litter were weighed and tested for reflexes (righting and cliff drop aversion reflex) to assess development of the neuromuscular system and sensory reactivity. We

found complex dose-response functions for DES, o,p'-DDT, and methoxychlor on all measures examined. At some doses a behavior was increased while at other doses the same behavior was decreased; in all cases the dose-response functions were thus non-monotonic. In other words, a response might first increase, then decrease and then increase across dose (Palanza et al., submitted). These results provide further support for the finding by vom Saal et al. (1997) that exposure to high doses of an endocrine-disrupting chemical cannot be assumed to be predictive of effects at lower doses.

Implications for Non-Monotonic Dose-Response Functions for Risk Assessment

Taken together, our findings show that the actual impact of the release of man-made hormonally-active substances into the environment is not accurately predicted by current testing strategies in which only a few high doses of chemicals are tested (Nagel et al., 1997; vom Saal et al., herein). For example, the results of prior high dose studies with methoxychlor, coupled with extrapolation procedures [the application of uncertainty factors (Beck et al., 1994) to estimate risk of exposure to low doses], led to the calculation that 25 ng/g is safe for humans to consume on a daily basis, referred to as either the acceptable daily intake (ADI) or minimum risk level (MRL) (ATSDR, 1994). Specifically, the MRL for methoxychlor was based on results from a study in which the lowest dose of methoxychlor administered was 25 μg/g body weight even though this dose altered the timing of puberty and hormone levels in female rats. This dose was divided by an uncertainty factor of 1000, based on the procedure of multiplying an uncertainty factor of 10 for extrapolation from animals to humans, times another uncertainty factor of 10 to account for variability in response among humans, times a third uncertainty factor of 10 because this lowest dose tested actually led to an adverse outcome rather than no effect.

In contrast to the prediction that 25 ppb of methoxychlor would be safe for daily human consumption (no adverse health effects are expected to occur), we found that feeding pregnant mice a 20 ppb dose of methoxychlor once a day for seven days significantly increased urine marking behavior in male offspring (vom Saal et al., 1995). We consider a significant change in territorial behavior due to fetal exposure to an environmental chemical to represent an unacceptable, and clearly adverse, outcome.

The Ethotoxicological Approach to the Study of Endocrine Disruptors

In our studies of endocrine disruptors, we have combined the evolutionary approach to the study of behavior, which is the "core" of ethology, with developmental toxicology. This new experimental approach to behavioral toxicology is referred to as ethotoxicology. The loss of potential (that is, not developing to one's fullest potential) in humans and wildlife due to fetal exposure to endocrine disruptors can be expressed as reduced social adaptation and impaired responsiveness to environmental demands.

The traditional approach to the study of behavior in toxicology has involved focusing on behavioral test batteries designed to investigate effects of chemicals on learning and memory, sensory function, activity, and neuromuscular function. This experimental approach uses animals as tools to detect alterations in neural mechanisms. To date, the issue of whether the social and environmental situations in which animals are tested are appropriate for the animal and thus ecologically relevant

has not been considered. In contrast, ecological relevance is a central aspect of the ethological approach to the study of behavior. An attempt to apply more relevant ethological testing procedures to toxicological analyses has also been proposed by others (Alleva et al., herein).

In the ethotoxicological approach to the study of endocrine disruptors, we examine the proximal causes of behaviors (such as genetic, physiological, environmental, and experiential factors) in order to understand if a behavioral alteration may be pathological. The study of the proximate causes of behavior always takes into account the functions (the adaptive significance) of the behavior under investigation. In fact, the ultimate causation (the adaptive significance) of any phenotype, including behavior, is the result of selection pressures that have acted upon proximal factors, such as neural and endocrine substrates. It thus follows that context and function of behavior are of paramount importance when studying the impact of endocrine disruptors upon such underlying substrates.

Sociosexual behaviors of particular species are adapted to specific environmental (ecological) conditions (Krebs and Davies, 1981). However, there are many factors that give rise to individual differences in social behaviors, such as aggressiveness. 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, can influence social structure and population dynamics (vom Saal, 1984; Brain and Parmigiani, 1990). Alteration in social behaviors that can lead to changes in social structure in affected populations represents an unacceptable outcome of exposure to man-made chemicals.

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