

Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice

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Abstract

Humans and wildlife are exposed to numerous anthropogenic drugs and pollutants. Many of these compounds are hormonally active, and recent evidence suggests that the presence of these endocrine disruptors permanently alters normal development and physiology in a variety of vertebrate species. Here, we report on the effects of developmental exposure to two common estrogenic pollutants, bisphenol A and ethinyl estradiol on sexually dimorphic, non-reproductive behavior. Mice (*Mus musculus domesticus*) were exposed to environmentally relevant levels of these chemicals (2 and 200 $\mu\text{g}/\text{kg}/\text{day}$ for bisphenol A and 5 $\mu\text{g}/\text{kg}/\text{day}$ for ethinyl estradiol) throughout prenatal and early postnatal development. As adults, the animals were observed in a variety of tests measuring sexually dimorphic behaviors including short-term spatial memory (in a radial-arm maze and a Barnes maze) and anxiety (in an elevated-plus maze and a light/dark preference chamber). Developmental exposure to ethinyl estradiol was found to masculinize behavior in all of the assays used. Bisphenol A increased anxious behavior in a dose-dependent fashion but had no effect on spatial memory. These results indicate that non-reproductive, sexually dimorphic behavior is sensitive to endocrine disruption. In addition, these experiments suggest that both humans and wildlife are being exposed to levels of these endocrine disrupting compounds that are sufficient to disrupt the development of the nervous system and that may have permanent consequences on sexually dimorphic behaviors.

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Introduction

Humans and wildlife are exposed to numerous anthropogenic drugs and pollutants. Many of these pollutants are hormonally active and commonly referred to as “endocrine disruptors.” This study tested the effects of two estrogenic endocrine disruptors, bisphenol A (a component of plastic) and ethinyl estradiol (a pharmaceutical), on adult behavior in mice after developmental exposure.

Bisphenol A (BPA) is a synthetic compound which polymerizes to form polycarbonate plastic and epoxy resins. Over time, or with simple wear and use, these ester bonds degrade, freeing monomers of BPA (Howdeshell et al., 2003; Sajiki and Yonekubo, 2004). As a result, significant levels of BPA exist in surface and

drinking water (Furhacker et al., 2000; Kuch and Ballschmiter, 2001; Rippen, 1999). The prevalence of BPA in developed countries results in its presence in urine samples (Calafat et al., 2005; Matsumoto et al., 2003; Ouchi and Watanabe, 2002), placental tissue, amniotic fluid as well as maternal and fetal plasma (Ikezuki et al., 2002; Schonfelder et al., 2002).

In rodents, BPA passes quickly from mother to fetus (Shin et al., 2002) and can activate fetal estrogen receptors (Lemmen et al., 2004). Rodents developmentally exposed to BPA show earlier onset of puberty (Howdeshell et al., 1999) and altered meiosis (Hunt et al., 2003), prostate morphology (Timms et al., 2005; Welshons et al., 1999) and genitalia (Markey et al., 2005). BPA also disrupts behaviors such as exploration and pair-bond formation (Farabollini et al., 1999; Razzoli et al., 2005).

Ethinyl estradiol (EE) is an estrogenic drug synthesized to be identical to beta estradiol with the addition of an ethinyl group. This compound is most commonly used as the active estrogenic component of the contraceptive pill but is also prescribed to alleviate some effects of menopause. Widespread use has

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resulted in EE appearing in sewage effluents and surface water (Desbrow et al. 1998; Larsson et al., 1999; Routledge et al., 1998; Ternes et al., 1999).

EE is a strong estrogen, comparable in strength to 17 β -estradiol (Folmar et al., 2002). Rodents developmentally exposed to EE show alterations in the testis (Fisher et al., 1999), deficits in spermatogenesis (Atanassova et al., 1999), and altered prostate growth (Thayer et al., 2001) in males, altered morphology (Sawaki et al., 2003) and gene expression (Naciff et al., 2002) in the female reproductive system and increased activity and anxiety-like behavior (Arabo et al., 2005; Dugard et al., 2001).

We investigated two doses of BPA, 2 and 200 μ g/kg/day (BPA 2 and BPA 200) These doses were based on past research (vom Saal et al., 1998), and the lower dose of BPA is similar to human exposure levels (Nagel et al., 1997). One dose of EE was investigated, 5 μ g/kg/day (EE 5). A similar dose has been shown to be active in mice (Thayer et al., 2001) and was therefore an appropriate positive control.

Past research on estrogenic endocrine disruptors has focused primarily on anatomical, physiological and pathological reproductive endpoints. However, estrogens will masculinize the development and functioning of the nervous system in rodents. Behavior, being the end result of a variety of stimuli and body systems, may be uniquely sensitive to endocrine disruption. We chose to study two sexually dimorphic, non-reproductive behaviors: anxiety and short-term spatial memory.

In rodents, anxiety-like behaviors are sensitive to the developmental estrogen environment in general (Leret et al., 1994) and to BPA and EE specifically (Dugard et al., 2001; Farabolini et al., 1999). Two different anxiety assays were employed in this project, the elevated-plus maze and the light/dark preference chamber.

The elevated-plus maze has been used as a simple and reliable measure of general anxiety levels in rodents for 20 years (Pellow et al., 1985). The light/dark preference chamber is not as commonly used to measure anxiety as the elevated-plus maze but has been successfully used in mice (Crawley, 1999). Each assay provides both aversive and comfortable environments. Anxiety, therefore, can be quantified by measuring the amount of time an animal spends in each environment within the apparatus.

In addition to increasing general anxiety, estrogens can also masculinize short-term spatial memory (Luine et al., 1998; Wilson et al., 1999). To measure this, we used the radial-arm maze (Daniel et al., 1997, 1999; Fader et al., 1999) and the Barnes maze (Barnes et al., 1994; Inman-Wood et al., 2000). Both assays can be designed to specifically measure short-term spatial memory, a sexually dimorphic trait in rodents. Performance in these assays therefore may be sensitive to developmental estrogen exposure.

This study investigated the effects of developmental exposure to BPA and EE on sexually dimorphic, non-reproductive behaviors in adult mice. These endpoints are potentially sensitive to endocrine disruption but have not been extensively studied in these compounds. We hypothesize that both BPA and EE will masculinize female behavior in these assays and accelerate the onset of puberty.

Materials and methods

Animals

Nulliparous female C57/Bl-6 mice, obtained from Charles River Laboratories, were housed with a stud male and checked daily for vaginal plugs. When a plug was detected, the male was removed from the cage. The female was considered to be pregnant and on gestational day one (GD-1). On GD-3, each female was randomly assigned to a treatment group and the exposure period was initiated. Each pregnant female was orally gavaged once daily with the appropriate compound dissolved in tocopherol-stripped corn oil. Oral administration occurred from GD-3 through postnatal day 21 (PND-21). The compounds were dissolved in a low volume of oil (approximately 40 μ l per dose) and were introduced into the back of the mouth using a gavage needle. This method minimized spillage as the low volume was easily accommodated by the mouth and throat. This method also minimized stress as the gavage needle was never fully inserted down the throat, a procedure to which some mice do not acclimate. The doses investigated in this project were 2 and 200 μ g/kg/day BPA and 5 μ g/kg/day EE.

All mice were weaned on PND-21, and females were housed individually from this day forward. One week after weaning, the mice to be used in behavioral testing (one randomly selected mouse per litter) were surgically ovariectomized. After a 2-week recovery period, each mouse, now an adult, was tested in each behavioral assay.

Throughout the project, all mice were housed in standard polycarbonate cages on Beta-Chip bedding and were given Purina rodent chow 5001 and water ad-libitum. The cages and bottles, constructed of polycarbonate plastic, were monitored regularly to ensure that all materials were in good condition and showed no signs of degradation.

All aspects of this project complied with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were reviewed by the institutional animal care and use committee at North Carolina State University.

AGD, body weight and litter size

The litter size, anogenital distance (AGD) and the weight of each pup were recorded at weaning. The AGD was measured as described by Vandenbergh and Huggett (1995), and the measurement was taken with dial calipers accurate to 0.1 mm. An AGD index (AGDI) was then calculated by dividing AGD by body weight (Hotchkiss and Vandenbergh, 2005). The same experimenter, blind to the treatment groups, made these measurements in every mouse throughout the project.

Puberty

After the mice were weaned, 21 non-ovariectomized individuals were checked daily, and, once a vaginal opening was present, a vaginal smear was taken as described by Cooper et al. (1993). The first day of puberty was defined as the day on which cornified cells were first detected in the smear. Vaginal smears were taken for at least 2 days after the first cornification to verify that the animal was indeed cycling.

Anxiety-related behaviors

The elevated-plus maze was designed as described in Morgan and Pfaff (2001) with the runways constructed of clear Plexiglas. The closed arms were completely surrounded with walls, while the open arms were devoid of any ledges. All the animals were run with the maze in the same position in the testing room with the experimenter observing from outside the room. All mice were run in this apparatus once for 15 min during the beginning of the dark cycle, a period of high activity for mice.

At the start of the trial, the mouse was placed into the center of the maze under red-light illumination and allowed to freely explore the four arms. Mice were run in this apparatus once during the beginning of their dark cycle, and the amount of time spent in the enclosed arms, open arms and center area was recorded. In addition, unprotected head dips, defined as the head of the mouse

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