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Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (*Peromyscus maniculatus bairdii*) offspring

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ABSTRACT

Bisphenol A (BPA) is a widely produced, endocrine disrupting compound that is pervasive in the environment. Data suggest that developmental exposure to BPA during sexual differentiation of the brain leads to later behavioral consequences in offspring. Outbred deer mice (*Peromyscus maniculatus bairdii*) are an excellent animal model for such studies as they exhibit well-defined sex- and steroid-dependent behaviors. Here, dams during gestation and lactation were fed with a phytoestrogen-free control diet, the same diet supplemented with either ethinyl estradiol (0.1 ppb), or one of the three doses of BPA (50 mg, 5 mg, 50 μg/kg feed weight). After weaning, the pups were maintained on control diet until they reached sexual maturity and then assessed for both spatial learning capabilities and anxiety-like and exploratory behaviors. Relative to controls, males exposed to the two upper but not the lowest dose of BPA demonstrated similar impairments in spatial learning, increased anxiety and reduced exploratory behaviors as ethinyl estradiol-exposed males, while females exposed to ethinyl estradiol, but not to BPA, consistently exhibited masculinized spatial abilities. We also determined whether dams maintained chronically on the upper dose of BPA contained environmentally relevant concentrations of BPA in their blood. While serum concentrations of unconjugated BPA in controls were below the minimum level of detection, those from dams on the BPA diet were comparable (5.48 ± 2.07 ng/ml) to concentrations that have been observed in humans. Together, these studies demonstrate that developmental exposure to environmentally relevant concentrations of BPA can disrupt adult behaviors in a dose- and sex-dependent manner.

Introduction

Endocrine disrupting compounds (EDCs) are a concern, given their potential to cause reproductive dysfunction and behavioral abnormalities (Vandenberg et al., 2009; Wolstenholme et al., 2011a). Of these chemicals, bisphenol A (BPA) is among the most pervasive (Biedermann et al., 2010; Galloway et al., 2010) and can act either as an estrogen receptor agonist or antagonist, but may also mediate effects through other steroid receptor pathways as well (Charles et al., 2007; Kuiper et al., 1998; Moriyama et al., 2002; Prasanth et al., 2010; Ryan et al., 2010; Stump et al., 2010). Given this broad range of potential molecular targets, developmental exposure to BPA might be expected to influence an assortment of phenotypic features in a sex- and dose-dependent way but not necessarily in a predictable manner.

BPA exposure has been inferred to disrupt reproductive processes, cognitive abilities, social and emotional behaviors, and the neural networks that support these phenotypic aspects (Frye et al., 2011; Rubin, 2011), although not all authorities agree with these conclusions (Ryan et al., 2010; Stump et al., 2010). Of these studies, only a few have reported whether or not prenatal and early developmental exposure to BPA influences sexually dimorphic adult behaviors (Cox et al., 2010; Jašarević et al., 2011; Xu et al., 2011). Such traits, if under

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sexual selection and controlled by sex hormones, are expected to be exaggerated (Andersson and Simmons, 2006), but highly sensitive to endocrine disruption (Jašarević et al., 2011).

Sex differences in spatial learning, generally favoring males, have been reported for a variety of species, including humans (Galea and Kimura, 1993; Gaulin, 1992; Williams et al., 1990). In the laboratory, spatial learning is assessed by measuring latency to reach the escape hole, path length, errors (i.e., entering a blind hole), and search strategy. The latter is classified into three categories: 1) random/mixed strategy characterized as unorganized hole searches and multiple crossings of the maze center before locating the escape hole; 2) serial/thigmotaxic strategy, where the animal moves around the periphery of the maze in a clockwise or counter-clockwise direction and enters at least two incorrect (blind) holes prior to locating the escape hole; 3) direct strategy, where the animal moves directly to the quadrant containing the escape hole and either immediately enters this hole or moves to an adjacent blind hole prior to entering the correct hole. Predictably, animals employing the direct strategy exhibit shorter latencies and path lengths and commit fewer errors than animals using the other two strategies (Harrison et al., 2006b; Jašarević et al., 2011; Mueller and Bale, 2007; Rodriguez et al., 2010).

For many of these laboratory tasks, males exhibit shorter latencies, commit fewer search errors, and adopt the direct search strategies more quickly than females (e.g., Galea et al. 1996; Galea et al. 1994; Jašarević et al., 2011; Jašarević et al., 2012; Rodriguez et al., 2010). However, individual differences in anxiety appear to modulate performance and sex differences on spatial learning tasks (Herrero et al., 2006), with the least anxious animals tending to perform the best (Carr et al., 2003; Cox et al., 2010; Eliam-Stock et al., 2012; Jašarević et al., 2011; Kim et al., 2011; Ryan and Vandenbergh, 2006; Xu et al., 2010; Xu et al., 2011). Although the length and route of exposure to an EDC such as BPA differed widely in these studies, the common outcome has been that exposed males exhibit longer latencies and path lengths, and higher search errors during spatial learning tasks, as well as higher anxiety-like behavior than controls (Carr et al., 2003; Cox et al., 2010; Eliam-Stock et al., 2012; Jašarević et al., 2011; Kim et al., 2011; Ryan and Vandenbergh, 2006; Xu et al., 2010; Xu et al., 2011). Given the interaction between anxiety and spatial learning, it is possible that BPA induces elevated anxiety levels in BPA-exposed animals, with compromised performance on a spatial learning tasks a secondary outcome.

While progress has been made on investigating the effects of BPA exposure on behavior, the dose-dependent effects of developmental exposure to BPA through the maternal diet on sex differences in spatial learning and anxiety-like behaviors remain unclear. Deer mouse males exposed to 50 mg/kg feed weight (fw) BPA exhibited increased anxiety-like behavior and deficits in spatial learning compared with unexposed males, with no effect observed in exposed females (Jašarević et al., 2011). However, the possibility remains that BPA exposure exerts sex-dependent effects on anxiety and cognition at lower exposures. In most studies where the effects of developmental BPA exposure have been assessed in rodents after consumption of food by the mother, an upper dose of 50 mg BPA/kg fw has been considered to be environmentally relevant (Anderson et al., 2012; Cox et al., 2010; Dolinoy et al., 2007; Wolstenholme et al., 2011b) and provides serum concentrations of BPA in laboratory mice that are close to those measured in humans unknowingly exposed to the chemical (Sieli et al., 2011). However, serum concentrations have not been measured in deer mice, which are the focus of our experiments.

Outbred deer mice were chosen as the animal for the present studies, because deer mice have maintained naturally occurring sex differences, despite being housed in standardized laboratory environments (Layne, 1968). Inbreeding may obscure naturally occurring sex differences (Gray, 1971; Harker and Whishaw, 2002; O'Leary et al., 2011; Thompson, 1953), and, as a result, use of inbred strains may underestimate the effect of BPA exposure on sex-dependent behaviors. The present study sought to address these gaps by measuring levels of circulating BPA in the blood of adult breeder females chronically exposed to the 50 mg BPA/kg fw dose and also by determining whether the effects of development BPA exposure on sex differences in spatial learning, exploration, and anxiety-like behaviors are dose-dependent. Two behavioral mazes, Barnes maze and elevated plus maze (EPM), were used to assess the adult behavioral responses. The Barnes maze measures spatial learning and memory, while the EPM analyzes exploratory and anxiety-like behaviors that are generally correlated with spatial cognition, as an animal has to demonstrate motivation and confidence to explore and learn about the surrounding environment.

Material and methods

Animal husbandry

Outbred adult male and female deer mice (50 of each), free of common rodent pathogens, were purchased from the *Peromyscus* Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC), and placed in quarantine for a minimum of 8 weeks to ensure that they did not carry any transmittable and zoonotic diseases. From the time the animals had been captured in 1948 from the wild, *Peromyscus maniculatus bairdii*, captive stocks have been carefully bred by the PGSC to maintain their outbred status. All experiments were approved by University of Missouri Animal Care and Use Committee and performed in accordance with National Institutes of Health Animal Care and Use Guidelines. Sentinel testing for common rodent pathogens was performed on a quarterly basis and no rodent pathogens have been detected in the colony.

Virgin females, 8 to 12 weeks of age, were randomly assigned to receive one of five diets: (i) a low phytoestrogen AIN 93G diet with 7% corn oil (CTL; Control); (ii) AIN93G supplemented with 50 mg of BPA/kg fw (upper dose; U); (iii) AIN93G supplemented with 5 mg of BPA/kg fw (middle dose; M); (iv) AIN93G supplemented with 50 μg of BPA/kg fw (low dose; L); or (v) AIN93G diet supplemented with 0.1 ppb of ethinyl estradiol (EE), as the FDA required positive control for BPA studies (vom Saal et al., 2005). Diets were administered 2 weeks prior to mating, and dams remained on the diet throughout pregnancy and lactation, as sexual differentiation of the brain extends into the early postnatal period (McCarthy, 2008). Litters with singleton births were excluded from these studies because social isolation during development and adolescence may confound cognitive and social behavior in a sex-dependent manner in rodents (Einon, 1980). To obtain sufficient numbers of offspring, some dams were bred more than once. The sample sizes of adult offspring in relation to their sex and diet exposure, plus the number of different litters sampled and the male and female offspring within each litter are summarized in Supplemental Table 1.

After weaning, all offspring were placed on the CTL diet and housed with no more than 3 same-sex siblings per cage until sexual maturity (age ≈ 60 d). In contrast to common laboratory rodent species, *P. maniculatus bairdii* does not respond well to handling (Martin et al., 2007), and thus animals were only handled during weekly cage changes and behavioral testing. To minimize background exposure to BPA beyond treatment regimen, deer mice were housed in white polypropylene cages (32 cm × 18 cm × 24 cm) under standard conditions (25 ± 2 °C and 50% ± 10% humidity), with ad libitum access to BPA-free water provided in glass bottles and diet specific to each treatment group. All animals were maintained on a long day light cycle (16 h light:8 h dark) to induce sexual maturity in males and females (Layne, 1968). To reduce any potential social housing and accompanying dominance/subordinate effects, adult deer mice were moved into single-housing conditions one week prior to behavioral testing.