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Neonatal exposure to endocrine active compounds or an ERβ agonist increases adult anxiety and aggression in gonadally intact male rats

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Abstract

Endocrine active compounds (EACs) have been shown to influence a number of reproductive endpoints but less is known about how they might affect other hormone dependent behaviors including anxiety and aggression. Recent evidence suggests that these effects may be mediated through the beta form of the estrogen receptor (ERβ). Using male Long Evans rats, we sought to determine how neonatal exposure to EACs affects anxiety and aggression in adulthood. Anxiety was assessed using the elevated plus maze and aggression was assessed 8weeks later using the resident intruder test. To gain insight into which ER subtype (ERα vs ERβ) might be mediating these effects we used agonists specific for ERα (1,3,5-*tris*(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT)) or ERβ (Diarylpropionitrile (DPN)) as additional treatment groups. For these experiments the synthetic EAC bisphenol-A (BPA) and the phytoestrogen metabolite equol (EQ) were used. Male neonates were injected with either 0.05 ml sesame oil (control), 50 μg estradiol benzoate (EB), 1 mg/kg DPN, 1 mg/kg PPT, 50 μg/kg BPA, or 10 mg/kg EQ daily for 4 days beginning on the day of birth (PND 0). Compared to the oil treated controls, significantly fewer open arm entries were made by the males neonatally treated with DPN, EQ, or BPA. The DPN and EQ treated males were also more aggressive compared to the controls. These findings suggest that neonatal exposure to EACs with agonistic activity on ERβ may influence affective behavior in adulthood, including anxiety and aggression.

Keywords: DPN; PPT; Bisphenol; Equol; Estrogen; Phytoestrogens; Soy isoflavones; EAC; Endocrine disruptors

Because they bind to steroid receptors, endocrine active compounds (EACs) have the potential to affect physiology and behaviors that are sensitive to the effects of sex steroid hormones, including anxiety and aggression. To date, the vast majority of studies examining the potential impact of these compounds have focused largely on reproductive physiology and behavior (Buck Louis et al., 2006; Fisher, 2004; Guillette and Gunderson, 2001; Hess-Wilson and Knudsen, 2006; McLachlan et al., 2006; Safe, 2000). In contrast, far less is known about how these compounds might affect other hormone dependent behaviors, particularly in males. The present study examined anxiety-related and aggressive behaviors in adult, gonadally intact male rats following neonatal exposure to the synthetic EAC Bisphenol-A (BPA), the natural EAC equol (EQ), the estrogen receptor alpha (ERα) specific agonist 1,3,5-*tris*(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) or the estrogen receptor beta (ERβ) specific agonist diarylpropionitrile (DPN).

The mechanisms by which neonatal exposure to steroid hormones or EACs may influence adult affective behavior, including anxiety and aggression, are largely unknown but likely involve estrogen receptors. In rodents it is well established that manipulation of androgens during development, either by castration or direct administration, significantly affects aggression in males and females (Compaan et al., 1992; Gandelman, 1980; Giammanco and La Guardia, 1979a,b; Klein and Simon, 1991; Motelica-Heino et al., 1993; Peters et al., 1972; Ryan and Vandenbergh, 2002). Therefore, the postnatal period is a critical window for the modulation of adult behaviors by steroids and, potentially, EACs. The conversion of testicular androgens to estrogens by the aromatase enzyme is hypothesized to be the primary mechanism by which androgens influence the etiology of sexually dimorphic behaviors, including aggression and anxiety (Compaan et al., 1994a,b; Naftolin et al., 1972; Trainor et al., 2006). Estrogens can have either anxiolytic or anxiogenic

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effects depending on a number of physiological and environmental factors including age, stage of the reproductive cycle, and sex (Diaz-Veliz et al., 1997; Imhof et al., 1993; Johnston and File, 1991; Morgan and Pfaff, 2001). This dualistic and dynamic influence of estrogen could result, at least in part, from the respective functional roles of ERα and ERβ.

Agonism of ERβ has been shown in a pair of studies to reduce anxiety in adult gonadectomized male and female rats (Lund et al., 2005; Walf et al., 2004), while mice lacking ERβ (ERβKO mice) have been observed to be more aggressive towards conspecifics (Nomura et al., 2002; Ogawa et al., 1999). A previous study has also shown that gonadectomized, estrogen replaced female ERβKO mice spend less time on the open arms of the elevated plus maze as their conspecific WT counterparts (Imwalle et al., 2005). Collectively these studies suggest that ERβ may have a mechanistic role in the ontogeny of anxiety and aggression. We therefore hypothesized that manipulation of ERβ activity during the neonatal period could affect anxiety and aggression in adulthood.

To test this hypothesis we used three compounds that have previously been shown to bind and activated ERβ: BPA, EQ and DPN. BPA is a chemical component of polycarbonate plastic and epoxy resins. The list of products containing BPA includes the lining of food and beverage cans, syringes, plastic containers, and dental products. The U.S. Centers for Disease Control recently estimated that 95% of Americans have detectable levels of BPA in their bodies (Calafat et al., 2005). EQ is a metabolite of the isoflavone phytoestrogen daidzein (Setchell et al., 2003) and is generated from intestinal microflora. It appears that not all humans are capable of biotransforming soy isoflavones to EQ (Setchell et al., 2002), an observation that may have clinical relevance as soy consumption has been associated with improved bone, cardiovascular and menopausal health. There is growing speculation that the therapeutic benefit of soy consumption may greatly depend on the ability to produce EQ (Adlercreutz and Mazur, 1997; Rowland et al., 2000; Setchell et al., 2002). Other species, including rodents, non-human primates, and domestic farm animals, readily generate EQ. Therefore EQ can also be directly consumed in milk and possibly other dairy products (Antignac et al., 2003; King et al., 1998).

The binding affinity of BPA for both ERα and ERβ in cell culture assays is about 10,000-fold lower than 17β-estradiol (Blair et al., 2000; Kuiper et al., 1998; Waller et al., 1996), but a number studies have found that exposure to BPA at levels far below the current No Observed Adverse Effects Limit (NOAEL) as defined by the US Environmental Protection Agency (EPA) may adversely impact reproductive health (Goodman et al., 2006; Kabuto et al., 2004; Kubo et al., 2003; Rubin et al., 2006, 2001; vom Saal, 2006; vom Saal and Hughes, 2005). Compared to most other EACs, including BPA, EQ has a higher relative binding affinity for ERα and ERβ and is more effective at activating transcription through the estrogen response element (Morito et al., 2001) which may make it more likely than many synthetic EACs to disrupt estrogen dependent physiology and behavior.

We have previously observed that consumption of soy supplements increased anxiety in gonadally intact adult male rats (Patisaul et al., 2005). These dietary preparations contained a mixture of phytoestrogens making it difficult to predict whether the observed effect resulted from exposure to a single compound within the supplement or the biogeneration of equol. Although not all humans are capable of generating equol, rats have been labeled "equol-producing machines" (Axelson and Setchell, 1981; Setchell et al., 2002) and it is therefore possible that the previously observed anxiogenic effect of the isoflavone rich diet resulted from EQ action on ERs.

The recent development of agonists specific for each of the estrogen receptor subtypes provides a new methodology for delineating the relative roles of ERα and ERβ within estrogen-sensitive systems. Therefore, in the present experiments an agonist specific for ERα (PPT) and an agonist specific for ERβ (DPN) were used as positive controls along with estradiol benzoate (EB). Neonatal male rats were exposed over four days, beginning on the day of birth, a period when endogenous androgen levels are high and sex specific brain organization is occurring (Baum et al., 1988; Motelica-Heino et al., 1988). The animals were then tested as adults for anxiety and aggression. For these experiments, the animals were left gonadally intact, a condition which allows the hypothalamic–pituitary–gonadal axis capable of responding to the exposure and mimics a naturalistic EAC exposure.

We hypothesized that neonatal exposure to either BPA, or EQ would increase anxiety and aggression in adulthood. In comparing the effects of these compounds to agonists specific for ERα and ERβ we sought to gain insight as to which nuclear ER these behavioral effects might be driven, and thus begin to parse out the mechanisms by which EACs affect hormone-dependent behaviors.

Methods

Animal care and maintenance

Timed pregnant Long Evans rats ($n=10$; Charles River, NC) were individually housed in a humidity and temperature controlled room with a 12-h light, 12-h dark cycle (lights on from 8:00 to 20:00) at 23 °C and 50% average relative humidity at the Biological Resource Facility at North Carolina State University (NCSU). Because standard lab chows are soy-based and thus contain significant amounts of phytoestrogens (Boettger-Tong et al., 1998; Brown and Setchell, 2001; Thigpen et al., 1999), all of the animals were fed a semi-purified, phytoestrogen-free diet ad libitum (AIN-93G, Test Diet, Richmond, IN). All dams except two littered on the same evening and littering took place within 4h. Male pups from these 8 dams were cross-fostered at birth (6–8 males per dam). Within each litter, only two males that were biologically related to the mother were kept in that litter and the rest were obtained from other litters. All remaining males were culled. Although they were not used for this study, the females were kept with their mothers (a maximum of 5 per dam) to reduce maternal anxiety and the risk of cannibalism. Maximum litter size was 12 pups. In all cases, the cross fostered litter was smaller than the biological litter. All of the pups within a cross fostered litter were given the same treatment to avoid cross contamination.

Beginning on the day of birth, the males were subcutaneously injected with either sesame oil (0.05 ml), estradiol benzoate (EB, 50 μg), the estrogen receptor α agonist propyl-pyrazole-triol (PPT; 1 mg/kg), the estrogen receptor β agonist diarylpropionitrile (DPN; 1 mg/kg), equol (EQ; 10 mg/kg bw) or Bisphenol-A (BPA; 50 μg/kg bw). All compounds were dissolved in ethanol, and then sesame oil at a ratio of 10% EtOH and 90% oil. The vehicle was also prepared with this ratio. The dose of BPA is equivalent to the Lowest Observed Adverse Effects Level (LOAEL) established by the Food and Drug Administration. The dose of EQ is similar to the total amount of soy phytoestrogens consumed daily by children fed soy infant formula (Setchell et al., 1997). The dose of DPN and PPT was based on previously published studies examining the effects of these compounds on estrogen mediated behavior and uterine weight in adults (Frasor et al., 2003; Harris et al., 2002; Lund et al., 2005; Rhodes and Frye, 2006; Walf et al., 2004). DPN is an ERβ