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DISRUPTED FEMALE REPRODUCTIVE PHYSIOLOGY FOLLOWING NEONATAL EXPOSURE TO PHYTOESTROGENS OR ESTROGEN SPECIFIC LIGANDS IS ASSOCIATED WITH DECREASED GNRH ACTIVATION AND KISSPEPTIN FIBER DENSITY IN THE HYPOTHALAMUS

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Abstract

It is well established that estrogen administration during neonatal development can advance pubertal onset and prevent the maintenance of regular estrous cycles in female rats. This treatment paradigm also eliminates the preovulatory rise of gonadotropin releasing hormone (GnRH). It remains unclear, however, through which of the two primary forms of the estrogen receptor (ER α or ER β) this effect is mediated. It is also unclear whether endocrine disrupting compounds (EDCs) can produce similar effects. Here we compared the effect of neonatal exposure to estradiol benzoate (EB), the ER α specific agonist 1,3,5-tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), the ER β specific agonist diarylpropionitrile (DPN) and the naturally occurring EDCs genistein (GEN) and equol (EQ) on pubertal onset, estrous cyclicity, GnRH activation, and kisspeptin content in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei. Vaginal opening was significantly advanced by EB and GEN. By ten weeks post-puberty, irregular estrous cycles were observed in all groups except the control group. GnRH activation, as measured by the percentage of immunopositive GnRH neurons that were also immunopositive for Fos, was significantly lower in all treatment groups except the DPN group compared to the control group. GnRH activation was absent in the PPT group. These data suggest that neonatal exposure to EDCs can suppress GnRH activity in adulthood, and that ER α plays a pivotal role in this process. Kisspeptins (KISS) have recently been characterized to be potent stimulators of GnRH secretion. Therefore we quantified the density of KISS immunolabeled fibers in the AVPV and ARC. In the AVPV, KISS fiber density was significantly lower in the EB and GEN groups compared to the control group but only in the EB and PPT groups in the ARC. The data suggest that decreased stimulation of GnRH neurons by KISS could be a mechanism by which EDCs can impair female reproductive function.

Keywords

genistein; equol; soy; isoflavones; gonadotropin; puberty; development; endocrine disruption; DPN; PPT

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1. Introduction

It has been hypothesized that developmental exposure to compounds with estrogen-like action may be a factor in the advancement of puberty and the decrease in human fecundity (Freni-Titulaer et al., 1986; Schoental, 1983). The mechanism by which this may occur remains unclear but both are regulated primarily by the hypothalamic-pituitary-gonadal (HPG) axis suggesting that disruption within this system may underlie both outcomes. Within the HPG axis, reproductive maturation and function is coordinated by the release of gonadotropin releasing hormone (GnRH) (Elkind-Hirsch et al., 1981; Gorski et al., 1975) from a neuronal population surrounding the vascular organ of the lamina terminalis (OVLTA) a region just rostral to the anterior ventral periventricular nucleus (AVPV). The neural components of the HPG axis that regulate GnRH secretion are sexually differentiated by endogenous gonadal hormones, primarily estradiol, through a series of pre- and perinatal critical periods (Cooke et al., 1998). Therefore it is possible that disruption of this sex-specific organization by exposure to endocrine disrupting compounds (EDCs) during one or more of these critical periods could affect both the timing of puberty and the maintenance of regular estrus. In rats, it is well established that the administration of steroid hormones during the neonatal critical period can advance puberty and permanently eliminate the ability to generate regular preovulatory GnRH surges (Arai and Gorski, 1968; Kauffman et al., 2007a; Simerly, 2002). Therefore, for the present experiments EDC exposure was confined to the neonatal period. The goals of the present study were to (1) determine if advanced puberty and impaired estrus function in female rats following neonatal exposure to EDCs is associated with impaired GnRH activation within the hypothalamus and (2) explore the estrogen receptor dependent mechanisms by which this may be occurring.

Although GnRH neurons express the beta form of the estrogen receptor (ER β) throughout development (Herbison and Pape, 2001; Hrabovszky et al., 2000; Hrabovszky et al., 2001) and thus may respond to estrogenic compounds directly, it is generally accepted that hormonal and other environmental signals are largely conveyed to GnRH neurons from other hormone-responsive neurons within the hypothalamus, particularly the AVPV. The AVPV appears to be the primary regulator of GnRH neuronal activity in females as lesions to this region abolish estrogen-induced LH surges and disrupt the estrous cycle (Terasawa et al., 1980; Wiegand et al., 1980). We have previously shown that neonatal exposure to the EDCs genistein (GEN) or Bisphenol-A (BPA) can affect sexually dimorphic gene expression in the AVPV (Patisaul et al., 2006) and AVPV volume (Patisaul et al., 2007) suggesting that this region may be particularly sensitive to disruption by EDCs. Estrogen receptor containing neurons that send axonal projections to GnRH neurons are abundant in the AVPV (Gu and Simerly, 1997; Polston et al., 2004; Polston and Simerly, 2006; Shughrue et al., 1997; Simerly et al., 1990). Of these, a subset also express the KiSS-1 gene, which has recently been found to play a key role in both the timing of puberty and the regulation of the estrous cycle (Kauffman et al., 2007a; Smith et al., 2006a; Smith et al., 2006b). The KiSS-1 gene codes for a family of proteins called kisspeptins (KISS), also known as metastatins (Navarro et al., 2004), which act as endogenous ligands for the G protein-coupled receptor GPR54 (Kotani et al., 2001; Lee et al., 1999; Muir et al., 2001). GnRH neurons constitutively express GPR54 (Irwig et al., 2004) and activation of this receptor appears to be vital for triggering the onset of puberty as humans lacking GPR54 are hypogonadal and fail to enter puberty (de Roux et al., 2003; Seminara et al., 2003). The number of KiSS-1 neurons in the AVPV is markedly sexually dimorphic with females having substantially more than males (Clarkson and Herbison, 2006). Neonatal androgen exposure has recently been shown to result in a male pattern of KiSS-1 gene expression in female rats (Kauffman et al., 2007b). However, this masculinizing effect was likely mediated by estrogens, aromatized in the brain from the administered androgens, because it is well established that estrogens are primarily responsible for masculinizing the hypothalamic nuclei of the rodent HPG axis (Gorski, 1985; Simerly, 2002). Therefore, we hypothesized that neonatal exposure

to estrogenic EDCs could interfere with the female-typical organization of KISS neurons resulting in reduced numbers of KISS efferent fibers projecting rostrally from the AVPV towards GnRH neurons.

A second population of KISS neurons resides in the arcuate nucleus (ARC). In contrast to the AVPV, KiSS-1 expression in the ARC does not appear to be sexually dimorphic or affected by neonatal androgen treatment (Kauffman et al., 2007b). In adult rodents, gonadal steroids amplify the expression of KiSS-1 mRNA in the AVPV but inhibit its expression in the ARC (Irwig et al., 2004; Navarro et al., 2004; Smith et al., 2005; Smith et al., 2006b) suggesting that the two populations play distinct roles in the regulation of gonadotropin secretion. It has previously been hypothesized that the AVPV population is critical for steroid positive feedback while the ARC population regulates steroid negative feedback (Kauffman et al., 2007a; Kauffman et al., 2007b; Smith et al., 2006b). To determine if neonatal EDC exposure could affect either population, KISS fiber density was quantified in the ARC as well as the AVPV.

The EDCs selected to test this hypotheses were the isoflavone phytoestrogens equol (EQ) and genistein (GEN). Many EDCs bind to at least one form of the estrogen receptor and affect transcription but the phytoestrogens are particularly potent compared to most synthetic EDCs (Kuiper et al., 1997; Kuiper et al., 1998; Mueller et al., 2003). GEN is commonly present in soy based foods while EQ is a metabolite of the soy isoflavone daidzein (Setchell et al., 2003) and is generated entirely from intestinal microflora. Not all humans are capable of biotransforming daidzein to EQ (Setchell et al., 2002). For example, infants cannot generate EQ, but EQ produced by the mother, as well as GEN and other phytoestrogens, readily cross the placenta (Todaka et al., 2005). Phytoestrogens have previously been shown to disrupt reproductive physiology in both laboratory animals and humans. For example, neonatal exposure to phytoestrogens has been associated with advanced pubertal onset in Puerto Rican girls (Freni- Titulaer et al., 1986; Schoental, 1983) and a retrospective cohort study found that young women fed soy-based infant formula as infants reported longer menstrual bleeding and menstrual discomfort (Strom et al., 2001). Studies in mice have shown that neonatal exposure to GEN can advance puberty, lengthen the reproductive cycle, and alter ovarian morphology (Jefferson et al., 2006a; Jefferson et al., 2005). In contrast, relatively little is known about the consequences of neonatal exposure to EQ in either rodents or humans.

For the present experiments, the synthetic estrogen estradiol benzoate (EB) was used as a positive control because neonatal exposure to EB has previously been shown to advance puberty and suppress ovarian cyclicity (Aihara and Hayashi, 1989; Nagao et al., 1999). We also used the ER α specific agonist 1,3,5-tris(4-Hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) and the ER β specific agonist diarylpropionitrile (DPN) as additional controls to gain insight as to which form of the ER might mediate the effects of EDCs on HPG organization and function. It is widely hypothesized that ER α and ER β play distinct roles in estrogen-dependent brain organization. Use of synthetic agonists specific for each ER subtype (Harris et al., 2002) and knockout animals lacking ER α , ER β , or both (Couse et al., 1995a; Couse et al., 1995b; Rissman et al., 1999) have proven to be very useful for identifying and defining these roles. In terms of behavior in the adult animal, ER α has generally been shown to be essential for the regulation of sexual behavior and reproductive function (Hewitt and Korach, 2003; Ogawa et al., 1998; Rissman et al., 1999), while ER β plays a more significant role in the modulation of estrogen action on anxiety and stress (Lund et al., 2005) although by no means do they do so exclusively of the other (Choleris et al., 2006). Therefore we hypothesized that the ER α agonist PPT would have the most significant impact on female reproductive physiology, GnRH neuronal activation, and KISS fiber density.

In the present study, animals were exposed to either vehicle, EB, GEN, EQ, DPN or PPT in the first 4 days of life, then monitored for changes in reproductive physiology, GnRH activation

and hypothalamic KISS fiber density. In mammals, puberty is manifested by changes in sex-specific physiology and behavior. The primary mechanism for this awakening is still unclear (Sisk and Foster, 2004; Terasawa and Fernandez, 2001) but in rodents vaginal opening is a hallmark of puberty and was therefore used as the physiological marker of pubertal onset. The ability to generate and maintain a regular estrous cycle was assessed using vaginal cytology. The animals were then ovariectomized, hormone replaced and sacrificed at a time point when GnRH activation is maximal. The brains were then collected, sectioned, and stained by immunofluorescent immunohistochemistry. GnRH activation was determined by quantifying the co-expression of GnRH and Fos in the OVLT. KISS content was determined by quantifying the relative density of KISS fibers in the AVPV and ARC using confocal microscopy.

2. Materials and Methods

2.1 Animals and neonatal treatment

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 10:00 to 22:00) at 23°C and 50% average relative humidity at the Biological Resource Facility at North Carolina State University (NCSSU). 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 for the duration of the experiment (AIN-93G, Test Diet, Richmond, IN). Eight of the 10 dams littered on the same evening, therefore, female pups from these 8 dams were cross fostered and treated within 4–6 hours of birth (6–10 females per dam). Within each litter, only two females that were biologically related to the mother were kept with that litter and the rest were obtained from other litters. All remaining females were culled. Although they were not used for this study, some males were kept with their mothers (a maximum of 5 per dam) to reduce maternal anxiety and the risk of cannibalism. In all cases, the cross fostered litter was smaller than the original biological litter and all of the pups within a cross fostered litter were given the same treatment to avoid cross contamination. There were two vehicle treated litters (6 treatment conditions; 8 total litters) but all litters were cross fostered.

Beginning on the day of birth, females were subcutaneously (sc) injected with sesame oil (0.05ml, Sigma, St. Louis), estradiol benzoate (EB, 50 μ g, Sigma), the ER α agonist propylpyrazole-triol (PPT; 1 mg/kg bw, Tocris Biosciences, Ellisville, MS), the ER β agonist diarylpropionitrile (DPN; 1 mg/kg bw, Tocris Biosciences), racemic equol (EQ; 10 mg/kg bw, generously supplied by Mike Adams of Wake Forest University) or genistein (GEN; 10 mg/kg bw, Indofine Chemical Company, Hillsborough, NJ). All compounds were dissolved in ethanol, and then sesame oil at a ratio of 10% EtOH and 90% oil as we have done previously (Patisaul et al., 2006). The vehicle was also prepared with this ratio. We have found this vehicle to cause less skin irritation than the alternative vehicle, DMSO. This dose of GEN is similar to the total amount of soy phytoestrogens consumed daily by children fed soy infant formula (Setchell et al., 1997) and the dose of EQ was chosen to match. The dose of DPN and PPT was approximately equivalent to what has been used in 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 ER β selective agonist with a 70-fold greater relative binding affinity and 170-fold greater relative potency in transcription assays for ER β than ER α (Meyers et al., 2001). Additionally, PPT, is selective for ER α , with a 400-fold preference for ER α and minimal binding to ER β (Stauffer et al., 2000; Sun et al., 2002). The animals ($n = 8$ –16 per group) received injections daily from the day of birth (PND 0) through PND 3 (4 injections total). Not all animals were used for the present study.

All pups were weaned into littermate pairs on PND 22, ear tagged, and maintained on a reverse light schedule (lights off from 10:00 to 22:00). Upon weaning, the animals were checked daily for vaginal opening. Monitoring of the estrous cycle by vaginal lavage commenced approximately two weeks after vaginal opening and all samples were collected in the morning, just after lights off. Because frequent lavage can induce pseudopregnancy, all animals observed to have stopped cycling were resampled 2–3 weeks later to confirm that the irregular cycle was not due to a pseudopregnancy. Sampling proceeded for 10 weeks with an additional 2–3 weeks for validation.

Animal care and maintenance were conducted in accordance with the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services “Guide for the Care and use of Laboratory Animals.” All experimental procedures involving animals were approved by the NCSU Institutional Animal Care and Use Committee.

2.2 Ovariectomy, tissue collection, and immunohistochemistry

Animals were gonadectomized (GNX) under isoflurane anesthesia (completed over 10 days beginning on postnatal day 146) and allowed 6 weeks to recover to provide adequate time for endogenous hormone levels to diminish. The OVX females were then injected sc with 10 μ g EB dissolved in sesame oil at 0900 h, followed 48 hours later by a sc injection of 500 μ g of progesterone dissolved in the same vehicle. All animals were sacrificed by transcardial perfusion with 4% paraformaldehyde 6–8 hours after the progesterone injection. It has previously been established that this is the point at which GnRH activity following sequential steroid hormone administration is maximal (Finn et al., 1998; Wu et al., 1992). Brains were removed, post-fixed in 20% sucrose paraformaldehyde, cryoprotected overnight in potassium phosphate buffer containing 20% sucrose, rapidly frozen in powdered dry ice, and stored at -80°C . Brains were then sliced into 35 μ m coronal sections, and divided into two series of free-floating alternating sections. Tissue was stored in antifreeze solution (20% glycerol, 30% ethylene glycol in KPBS) at -20°C until immunohistochemical processing. One set of tissue was immunolabeled for GnRH and Fos while the other set was immunolabeled for KISS using immunofluorescent immunohistochemistry (IHC) methods described in detail elsewhere (Patisaul et al., 2008). Briefly, the first set of tissue was incubated in a cocktail of primary antibodies directed against GnRH (raised in rabbit, 1:3000, Chemicon LHRH, AB1567) and Fos (raised in goat, 1:250, Santa Cruz, SC-52-6) followed by the secondary antibodies Alexa-Fluor donkey anti-rabbit 568 and Alexa-Fluor donkey anti-goat 488 at 1:200. The second set of tissue was incubated in a primary antibody directed against kisspeptin-10, raised in rabbit and generously gifted by Alain Caraty of Institut National de la Recherche Agronomique/Centre National de la Recherche Scientifique, Université de Tours, at 1:6,000 and the Alexa-Fluor donkey anti-rabbit 568 secondary antibody. After secondary antibody incubation, sections were counterstained with Hoechst 33258 (Invitrogen), rinsed, mounted onto slides (Suprefrost Plus, Fisher, Pittsburgh, PA), and coverslipped using a standard glycerol mountant.

2.3 Quantification of GnRH and Fos immunoreactivity in the OVLT

GnRH/Fos double-immunofluorescent label was visualized in the organum vasculosum of the lamina terminalis (OVLT). Anatomical identification of each section was made by comparing the counterstained sections to a brain atlas (Paxinos and Watson, 1998; Paxinos and Watson, 2004). GnRH and Fos immunostaining was observed to be consistent and evenly distributed throughout the OVLT. Two midlevel sections per animal were selected and analyzed using a Leica 5000DM microscope fitted with 20X, and 40X objective lenses and filter cubes for Cy3 and FITC. To quantify double label, first GnRH immunostaining was observed with the FITC filter and photographed at 20X and 40X with a Retiga 1800 digital camera. Second, Fos immunostaining was observed in the same region with the Cy3 filter and photographed. The images were then merged using the MCID elite Image Analysis (Interfocus Imaging Ltd.,

Cambridge, England) software package. Cells immunostained for GnRH only, and cells immunolabeled for both GnRH and Fos were then hand counted by an individual blind to the treatment groups and verified by a second independent observer.

2.4 Quantification of KISS fiber density in the AVPV and ARC

KISS immunoreactivity (-ir) has previously been shown to be localized to extended lengths of fibers throughout the AVPV and within cell bodies located in the medial and caudal AVPV (Clarkson and Herbison, 2006). We aimed to quantify the density of AVPV KISS fibers projecting rostrally from the AVPV to the OVLT GnRH neurons, therefore one rostral AVPV section was selected for each animal using a brain atlas (Paxinos and Watson, 1998; Paxinos and Watson, 2004). In the ARC, KISS-ir has been shown to be localized to fibers and cell bodies throughout the ARC so one midlevel section of the ARC was selected for imaging and quantification. Thus, the selected regions are representative of the AVPV and ARC but do not include all KISS fibers present in either. KISS-ir was visualized on a Zeiss LSM 510 Meta confocal microscope fitted with a 63x oil corrective objective lens housed at the CHIT Centers for Health Research at the Hamner Institute (Research Triangle Park, NC). For each scan, a set of serial image planes (z-step distance = 1 μ m) was collected through the entire thickness of the section. Individual images were acquired sequentially for light emitted from each fluorophore and parsed into separate stacks of images for analysis using the Image J software package (National Institutes of Health (NIH), Bethesda, MD) as previously described (Patisaul et al., 2008). To control for variations in tissue thickness that would result in unequal numbers of image planes, substacks of consecutive image planes that excluded the rostral and caudal edges of the tissue sections were created for each set of scans. Substacks consisted of 16 sequential image planes for the AVPV and 22 sequential images planes for the ARC. Only data from sections with consistent staining throughout the entire thickness were included in the analysis. Individual images contained within each substack were first binarized and depixelated to minimize the inclusion of background fluorescence. Fibers were then skeletonized to a thickness of one pixel to compensate for differences in individual fiber thickness and brightness. The number of resulting bright pixels in each image plane was then quantified using the Image J Voxel Counter plug-in (NIH). The voxel counts were averaged within the substack to obtain a single measure for each section that was then used as a quantitative representation of average KISS fiber density within the volume sampled (Patisaul et al., 2008).

2.5 Statistical Analysis

Day of vaginal opening was compared across treatment groups by one-way analysis of variance (ANOVA) with treatment as a factor and followed up with Fisher's Least Significant Difference (LSD) post-hoc test for individual comparisons (SYSTAT) with the significance level set at $p < 0.05$. The percentage of immunopositive GnRH cells co-labeled with Fos within each group was also compared using a one-way ANOVA followed by LSD post hoc tests. As anticipated (Wray and Gainer, 1987; Wray and Hoffman, 1986) the number of GnRH neurons counted did not significantly differ between groups. Sample sizes for the KISS analysis in the AVPV were small, particularly in the DPN and PPT groups, due to the strict selection criteria used to ensure that each analyzed section was anatomically matched. Therefore, the data was divided into two groups and analyzed separately to address two specific hypotheses: (1) does neonatal exposure to phytoestrogens affect the density of AVPV KISS fibers and (2) through which ER subtype is this effect likely mediated. To address the first hypothesis, the density of voxels containing KISS fibers for the OIL, EB, EQ and GEN groups were compared by one-way ANOVA followed by LSD post hoc tests. To address the second, the OIL, EB, DPN and PPT groups were compared using the same methods. Samples sizes for the KISS analysis in the ARC were sufficient, therefore all groups were compared by one-way ANOVA followed by LSD post hoc tests. In all cases, the significance level was set at $p < 0.05$.

3. Results

3.1 Age at Vaginal Opening

There was a significant effect of treatment on age at vaginal opening ($F(5,53) = 29.401, p \leq 0.0001$). Compared to the controls ($n = 12$), vaginal opening was significantly advanced by EB ($n = 15, p < 0.001$) and GEN ($n = 8, p < 0.03$) but not EQ ($n = 10$, Fig. 1.A.). Neither the ER α agonist PPT nor the ER β agonist DPN had a significant effect on the day of vaginal opening. The data suggest that EDC exposure during the neonatal critical period in which the HPG axis is undergoing steroid directed organization advance pubertal onset in females but the relative roles each ER subtype may play in the mediation of this effect remains unclear.

3.2 Estrous Cycle Data

Beginning approximately two weeks after vaginal opening, regularity of the estrous cycle was assessed weekly in a cohort of animals by vaginal lavage. Regular 4 day estrous cycles commenced in all treatment groups. As expected (Aihara and Hayashi, 1989), all of the EB females ($n = 10$) stopped cycling within three weeks after testing began and entered persistent estrus. By 10 weeks, less than 30% of the EQ ($n = 8$) and GEN ($n = 7$) treated females displayed regular estrous cycles (Fig. 1.B.). The remaining 70% were in either persistent estrus or diestrus. Smears from these animals contained a mixture of cornified cells, nucleated epithelial cells and leukocytes. The rate at which the EQ and GEN treated animals became acyclic was also very similar. All of the PPT treated animals were in persistent estrus by 8 weeks of test onset. In contrast, 57% of the DPN treated animals were still displaying regular estrous cycles 10 weeks after monitoring began. Smears from DPN animals that had stopped cycling primarily contained cornified cells but a number of nucleated cells were also present in most cases. The data suggest that neonatal EDC exposure can result in impaired ovarian cyclicity and that both ER subtypes may play a mechanistic role in mediating this effect.

3.3 GnRH Activation

The animals were then hormonally replaced by the sequential administration of EB and progesterone and sacrificed 6 hours after the progesterone injection. This is the point at which Fos expression in GnRH neurons is greatest (Finn et al., 1998; Wu et al., 1992). GnRH activity was determined by quantifying the percentage of OVLT GnRH neurons that co-expressed Fos. There was a significant effect of treatment on the percentage of GnRH neurons immunoreactive for Fos ($F(5,25) = 8.458, p \leq 0.0001$). Hormone administration successfully induced Fos expression in the GnRH neurons of the control females ($n = 7$) (Fig. 2). In contrast, less than 2% of GnRH neurons were colabeled with Fos in the EB females ($n = 8, p < 0.001$). GnRH activation was 50% lower in the EQ animals ($n = 4, p < 0.022$) and 70% lower in the GEN ($n = 4, p \leq 0.002$) animals compared to the controls. None of the GnRH neurons in the PPT animals contained Fos ($p \leq 0.001$) suggesting that agonism of ER α during the neonatal period may be a critical mechanism by which neonatal estrogen or EDC treatment suppresses GnRH activation in response to female gonadal hormones. GnRH activation was also reduced in the DPN group compared to the control group (although not significantly, $p \leq 0.057$) suggesting that ER β may also play a mechanistic role in this process.

3.4 AVPV KISS Fiber Density

The observed depletion of GnRH activation may not necessarily result from disruption at the level of the GnRH neuron itself, but rather from changes to the hormone sensitive neuronal inputs on GnRH neurons including KISS-producing neurons in the AVPV. KISS-ir was localized to extended lengths of fibers throughout the AVPV (Fig. 3A and B) as has been observed previously (Clarkson and Herbison, 2006). Only sections from animals which had consistent staining throughout the entire thickness of the AVPV were selected for the KISS-ir

analysis, a constraint that resulted in relatively small sample sizes in some groups. Therefore the data were divided into two sets for analysis. The first contained the OIL, EB, EQ and GEN groups (Fig. 3C) and tested the hypothesis that neonatal phytoestrogen treatment could alter KISS fiber density in the AVPV. There was a significant effect of treatment in this set ($F(3,21) = 3.502, p \leq 0.033$). Compared to the control females ($n = 8$), neonatal treatment with EB ($n = 4$) decreased average KISS fiber density 64%. ($p < 0.006$) and GEN reduced KISS content by 60% ($n = 5, p < 0.05$). Neonatal treatment with EQ reduced KISS fiber density by 25% compared to the controls, an effect that was not statistically significant ($n = 8, p < 0.17$). The second analysis was conducted to gain insight as to which ER subtype might be mediating the effect of EB and GEN on KISS-ir and contained the OIL, EB, DPN and PPT groups (Fig. 3D). Sample sizes in the DPN and PPT groups were very small and thus diminished the statistical power of this analysis. As a result, there was no significant main effect of treatment on average KISS fiber density in the AVPV within this set ($F(3,15) = 2.435, p = 0.105$). It has previously been shown that neonatal administration of steroid hormones can reduce the kisspeptin content of the anterior hypothalamus and suppress the GnRH surge associated with ovulation in rodents (Kauffman et al., 2007a). Therefore, our data suggest that decreased KISS expression may be a mechanism by which GnRH activation is impaired in animals neonatally treated with phytoestrogens but do not yield insight as to which ER subtype might mediate this effect.

3.5 ARC KISS Fiber Density

KISS-ir was localized to extended lengths of fibers and a small number of cell bodies throughout the ARC which is consistent with what has been observed previously (Clarkson and Herbison, 2006). One midlevel section per animal from animals which had consistent staining throughout the entire ARC was selected for analysis. There was a significant main effect of treatment on KISS fiber density in the ARC ($F(5, 37) = 11.644, p \leq 0.001$, Fig. 4). Compared to the control females ($n = 10$), KISS fiber density was lowest in the EB treated group ($n = 8$, 72% lower than the controls, $p \leq 0.001$) and also significantly lower in the PPT group ($n = 6, p \leq 0.001$). Neither EQ ($n = 7$) nor GEN ($n = 5$) treatment had a significant effect on KISS fiber density in the ARC nor did DPN ($n = 7$) suggesting that this measure is primarily sensitive to ER α agonism.

4. Discussion

EB and GEN were the only two compounds that significantly affected both the timing of vaginal opening and the ability to maintain a regular estrous cycle. GnRH activation was strongly impaired in these animals, as well as the PPT animals, suggesting that the mechanism of disruption is ER α dependent. Because GnRH neurons do not express ER α , this observation supports the hypothesis that endocrine disruption occurs within ER α expressing neurons that send efferent projections to GnRH neurons, such as KISS neurons. KISS fiber density was significantly lower in both the AVPV and ARC of the EB treated animals but only in the AVPV of the GEN treated animals. The AVPV population of KISS neurons is thought to regulate steroid positive feedback in mammals, including humans (Navarro et al., 2004; Pompolo et al., 2006; Roa et al., 2006; Shibata et al., 2007; Smith et al., 2006b; Tena-Sempere, 2006). Therefore, collectively the observations in the present study suggest that impaired organization of the steroid positive feedback circuitry on GnRH neurons might be the primary mechanism underlying the observed changes in reproductive physiology following neonatal exposure to EDCs.

Prior studies have repeatedly demonstrated that developmental exposure to estrogenic compounds, including phytoestrogens and other EDCs, can alter the timing of puberty, disrupt ovarian cycle function, and masculinize the female hypothalamus (Colborn et al., 1993; Jefferson et al., 2006b; Lephart et al., 2005; McLachlan et al., 2006; Patisaul, 2005; Patisaul

and Polston, 2007). To our knowledge, this is the first time that similar effects for the isoflavone metabolite EQ have been reported. Advanced puberty following exposure to GEN, or soy protein containing GEN and other isoflavones, has been observed previously (Badger et al., 2001). For example, Leaf et al, found that exposure to four days of 10 mg/kg bw per day of GEN, beginning on gestational day 15, (via administration by sc injection to the pregnant dam) also resulted in accelerated vaginal opening and altered ovarian cycle frequency (Levy et al., 1995). A similar outcome was reported for rats exposed to 40 mg/kg bw GEN per day from birth to weaning (Lewis et al., 2003) but not 4 mg/kg bw GEN per day. This may indicate that the dose of 10 mg/kg bw used in the present study is approaching the minimum level for which an effect of GEN on these endpoints is observed. In the present study, we found no significant effect of EQ on the timing of vaginal opening, but it remains to be seen whether or not there is a dose effect of EQ. One study failed to find advanced pubertal onset in the rat following oral administration of GEN across the first five days of life in doses ranging from 12.5 mg/kg bw to 100 mg/kg bw (Nagao et al., 2001), which may indicate that the route of exposure may also affect outcome. In the present study we opted to administer our compounds by sc injection because a thorough literature search indicated that this was the exposure route most frequently used in other studies using PPT and DPN. Therefore we administered all of the test compounds by sc injection to be consistent and ensure effective delivery of PPT and DPN. A newly published study has also now demonstrated that subcutaneous injection of GEN is a suitable model for oral exposure indicating that route of exposure may not be a serious concern (Jefferson et al., 2007).

Neonatal administration of GEN or EQ also significantly abrogated GnRH neuronal activation following hormone priming in adulthood. This observation is consistent with a prior study which showed that GEN has a defeminizing effect on GnRH secretion (Faber and Hughes, 1993). The specific mechanism by which this occurs is unclear although activity on one or both ER subtype has long been hypothesized to be crucial. ER α and ER β are differentially distributed throughout the hypothalamus (Li et al., 1997; Shughrue et al., 1996; Shughrue et al., 1997) suggesting that they play distinct roles in GnRH activation and signaling. Therefore, selective disruption through one ER subtype may produce disparate outcomes on GnRH function. Phytoestrogens generally have a higher relative binding affinity for ER β than ER α *in vitro* (Kuiper et al., 1998; Mueller et al., 2004). However, *in vivo* they have been shown to alter both ER α and ER β dependent gene expression in the brain (Jacob et al., 2001; Patisaul et al., 2002; Patisaul et al., 1999) indicating that they at least have the potential to act through either subtype. The degree to which GnRH activation was suppressed by the phytoestrogens was more similar to the results seen with DPN than with PPT suggesting that the phytoestrogens may be operating through an ER β mediated mechanism. Alternatively, this observation may also indicate that the phytoestrogens are activating as weak agonists on ER α . This distinction is significant because GnRH neurons express ER β but not ER α throughout development (Herbison and Pape, 2001; Hrabovszky et al., 2000; Hrabovszky et al., 2001). Therefore, activity through ER β would suggest that phytoestrogens can act on GnRH neurons directly. Further work is necessary to resolve this critical issue.

In the present study, the ER β agonist DPN produced a less severe outcome than PPT on the ability to maintain a regular estrous cycle and the capacity to activate GnRH neurons in response to hormone priming. The observation that both PPT and DPN suppressed ovarian cycle function is consistent with effects previously reported following post-natal exposure to two other ER specific agonists (Patchev et al., 2004). In this prior study, agonism of ER β was found to be more potent on estrous cyclicity, an effect opposite to what was observed here. In the present study, although impairment of GnRH activation by ER β agonism did not reach statistical significance, it is possible that, within the DPN treated group, GnRH activation was lowest in the individual animals that entered persistent diestrus and higher in the animals that did not. This would then support the hypothesis that EDCs can directly interact with GnRH

through ER β . Unfortunately the sample sizes in the present study were too small to investigate this possibility but future studies will address this issue. The present data suggest that ER α plays a more significant mechanistic role because while the DPN effect did not quite reach statistical significance, ovarian cyclicity and GnRH activation were completely absent in the PPT group. Further studies using a range of DPN and PPT doses are needed to determine if this simply reflects a dose effect and that higher doses of DPN could also eliminate ovarian cyclicity and GnRH activation.

Since GnRH neurons do not express ER α it has been hypothesized that estrogen signaling is conveyed to GnRH neurons by ER α -expressing neurons elsewhere in the hypothalamus, particularly the AVPV (Simerly, 1998; Simerly, 2002). In the female AVPV, estrogen administration markedly increases KiSS-1 mRNA expression and the number of KiSS-ir cell bodies (Irwig et al., 2004; Navarro et al., 2004; Smith et al., 2005) but this effect can be eliminated by neonatal androgen treatment (Kauffman et al., 2007b). Here we have shown that neonatal exposure to EB or GEN had a similar effect on AVPV KiSS fiber density. AVPV KiSS neurons express ER α (Smith et al., 2005; Smith et al., 2006b) so presumably ER α plays a mechanistic role in this effect but unfortunately sample sizes were not sufficient to ascertain if DPN or PPT had an effect on KiSS fiber density in the AVPV. In the adult female, it has recently been demonstrated that neuronal ER α is required for the generation of the LH surge (Roa et al., 2008; Wintermantel et al., 2006) but the relative roles ER α and ER β play in the organization of the neural circuitry required to generate a gonadotropin surge in response to hormone priming are less well understood. The differential distribution of ER α and ER β in the neonatal rat AVPV have not yet been definitively described making it difficult to conclude that ER α is playing the most substantial role in mediating the organizing effects of estrogens. For example, a pair of studies conducted by the same research group (Orikasa et al., 2002; Orikasa and Sakuma, 2003) have clearly identified both ER β -ir and ER β mRNA in the AVPV of male and female rats on PND 7 and beyond (day of birth defined as PND 1 in these studies) but did not look earlier. In contrast a study conducted by a different research group observed no ER β -ir within the AVPV in the first 2 weeks of life (Perez et al., 2003). Therefore the neonatal distribution of ER α and ER β in the rat AVPV remains undefined.

We also found that neonatal exposure to EB reduced KiSS fiber density in the ARC following hormone priming in adulthood. ER α appears to play a mechanistic role in this process as KiSS fiber density was also reduced in the ARC of the PPT but not the DPN treated animals, an effect which is not unexpected given that ER α is abundant in the ARC while ER β is absent (Shughrue and Merchenthaler, 2001; Simerly et al., 1990). The significant effect of EB was somewhat surprising given that the number of KiSS-1 neurons nor the quantity of KiSS-1 expression in the ARC has not been found to be sexually dimorphic in rats (Kauffman et al., 2007b). Prior studies have shown that KiSS-1 expression and the number of cell bodies immunoreactive for kisspeptin in the ARC are significantly decreased by steroid hormone administration in adults (Kauffman et al., 2007b). Very few KiSS-ir cell bodies were present in any of our ARC sections, regardless of treatment, an observation that is consistent with these prior studies. Without an untreated control group, it is difficult to ascertain whether the absolute number of KiSS neurons in the ARC, or the AVPV, is reduced by EDC treatment or if their response to hormone priming in adulthood is modified. Experiments designed to distinguish between these two alternative hypotheses are underway. It will also be critical to determine if the KiSS fibers quantified in the present study make direct contact with OVLT GnRH neurons.

Finally, it is important to note that it is possible that GEN may produce its effects via mechanisms that are not dependent on ERs. GEN is a potent tyrosine kinase inhibitor so some of the observed effects may be attributable, at least in part, to this mechanism of action. GEN has also been found to alter aromatase production and steroid hormone secretion *in vivo* (Akingbemi et al., 2004; Brooks and Thompson, 2005; Kishida et al., 2001; Pelissero et al.,

1996; Whitehead et al., 2002). Although our findings suggest that ER α plays a mechanistic role, particularly in the suppression of the estrous cycle and GnRH activation, further biochemical and pharmacological experiments are required to conclusively determine the specific mechanisms by which GEN, EQ and other EDCs alter HPG organization. It is also essential to acknowledge that premature vaginal opening and estrous cycle loss could result from disruption anywhere within the HPG axis including the ovary. Disrupted ovarian morphology, including absence of corpora lutea, multi-oocyte follicles and inhibited oocyte nest breakdown (Jefferson et al., 2006a; Jefferson et al., 2002; Jefferson et al., 2006b; Kouki et al., 2003; Nagao et al., 2001) have been reported in both rats and mice following developmental exposure to GEN. By ovariectomizing and hormone replacing our animals prior to sacrifice we aimed to eliminate this potentially confounding effect of ovarian dysfunction.

Unlike rodents, the human hypothalamus is differentiated during gestation, therefore the critical period akin to the one explored in the present study occurs before birth in humans. The GnRH pulse generator is functional by the end of the first trimester (Grumbach, 2002), but it is currently unknown when or how KISS neurons differentiate across gestation and early development in humans. GEN, EQ and most other phytoestrogens readily cross the placenta (Todaka et al., 2005) as do many other EDCs suggesting that human hypothalamic development may be vulnerable to disruption by these compounds however human exposure levels may be too low to produce appreciable effects. Identifying the most sensitive and physiologically relevant critical periods as well as the most salient dose ranges of exposure is essential for environmental risk assessment. Our data support the hypothesis that the time during which the hypothalamus undergoes sexual differentiation is likely one of these critical periods. These findings emphasize the need for further studies aimed at uncovering the mechanisms by which exposure to EDCs, during pre- and postnatal development ultimately impact the health of exposed offspring.

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FIG. 1.

(A) Day of vaginal opening (indicative of pubertal onset) was significantly advanced in animals neonatally treated with EB ($n = 15$) or GEN ($n = 8$) but not EQ ($n = 10$) compared to the oil controls ($n = 12$). (B) Animals in all groups displayed regular estrous cycles in the first few weeks post-vaginal opening, however some individuals within each treatment groups except the control group (OIL, $n = 8$) eventually entered into a state of persistent estrus or diestrus. Females in the EB group ($n = 10$) entered persistent estrus earliest and all animals were in a state of persistent estrus by three weeks after the onset of testing. By ten weeks, 57% of the DPN animals ($n = 7$), 25% of the EQ animals ($n = 8$), 29% of the GEN ($n = 7$) animals and none of the PPT animals ($n = 7$) displayed a regular 4–5 day estrus cycle. The remainder had entered either a persistent estrus or diestrus. (Means $\pm$ s.e.m. * $P \leq 0.03$)

FIG 2.

(A–B) Immunolabeling of GnRH (red) and Fos (green) was readily observed. (A) A single labeled GnRH neuron (red) showing no co-expression of Fos and (B) a double labeled neuron immunopositive for GnRH (red) and Fos (green) are depicted here. (C) The percentage of GnRH neurons immunopositive for Fos was lowest in the EB treated animals (n=8). Neonatal EQ treatment (n=4) resulted in a 50% decrease in Fos colabeling and neonatal GEN treatment (n=4) resulted in a 70% decrease compared to controls (OIL). No colabeled cells were observed in any of the PPT (n=4) animals. GnRH activation was markedly lower in the DPN group but this effect did not quite reach statistical significance ($p = 0.057$). (Means $\pm$ s.e.m. * $P \leq 0.03$, ** $P \leq 0.002$, Scale bar = 20 μ m.)

FIG 3.

Representative confocal images (5 merged consecutive optical planes) depicting the extended lengths of immunolabeled KISS-ir fibers in the AVPV of an (A) Oil (B) EB (C) EQ and (D) GEN treated animal. (E) Compared to the OIL treated control animals KISS immunoreactivity was significantly reduced by neonatal administration of EB and GEN but not EQ. (F) Sample sizes in the DPN and PPT groups were too small to conduct a meaningful analysis but the quantification of KISS immunoreactivity is presented here (F) alongside the data from the OIL and EB groups for illustrative purposes. (Means $\pm$ s.e.m. * $p \leq 0.05$, ** $p \leq 0.006$, Scale bar = 20 μ m.)

FIG 4.

KISS-ir in the ARC was significantly lower in the animals neonatally treated with EB or the ER α agonist PPT compared to the oil treated controls (OIL). The phytoestrogens GEN and EQ did not significantly affect ARC KISS-ir nor did the ER β agonist DPN.