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Regulation of Arcuate Genes by Developmental Exposures to Endocrine-Disrupting Compounds in Female Rats

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Abstract

Developmental exposure to endocrine-disrupting compounds (EDCs) alters reproduction and energy homeostasis, both of which are regulated by the arcuate nucleus (ARC). Little is known about the effects of EDC on ARC gene expression. In Experiment #1, pregnant dams were treated with either two doses of bisphenol A (BPA) or oil from embryonic day (E)18-21. Neonates were injected from postnatal day (PND)0-7. Vaginal opening, body weights, and ARC gene expression were measured. *Chrm3* (muscarinic receptor 3) and *Adipor1* (adiponectin receptor 1) were decreased by BPA. *Bdnf* (brain-derived neurotropic factor), *Igf1* (insulin-like growth factor 1), *Htr2c* (5-hydroxytryptamine receptor), and *Cck2r* (cholescystokinin 2 receptor) were impacted. In Experiment #2, females were exposed to BPA, diethylstilbestrol (DES), di(2-ethylhexyl)phthalate, or methoxychlor (MXC) during E11-PND7. MXC and DES advanced the age of vaginal opening and ARC gene expression was impacted. These data indicate that EDCs alter ARC genes involved in reproduction and energy homeostasis in females.

Keywords

arcuate nucleus; bisphenol A; diethylstilbestrol; phthalates; methoxychlor; energy homeostasis; puberty; endocrine-disrupting compounds

1. Introduction

Recent research has begun to elucidate the perturbations of central nervous system regulators of homeostatic functions including reproduction and energy homeostasis by endocrine-disrupting compounds (EDCs). These two functions are controlled, in part, through the hypothalamus, which is regarded as a key brain region integrating hormonal control of energy homeostasis and reproduction [1;2]. Many hypothalamic nuclei are involved in this integration include the arcuate nucleus (ARC), the paraventricular nucleus

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(PVH), the lateral hypothalamus (LH), and the ventromedial nucleus (VMH) [3]. Of these, the ARC is near an area of local permeability in the blood brain barrier and is readily exposed to circulating steroids, hormones, and nutrients including insulin, leptin, and glucose [4]. ARC neurons, in particular proopiomelanocortin (POMC), neuropeptide Y (NPY), and KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons, respond to both peripheral signals, reflecting the energy status of the body, and to inputs from the hindbrain to integrate sensory attributes, emotional states, motivated behaviors [4-6].

The effect on energy homeostasis from developmental exposures to common EDCs has potential relevance in the increase in childhood and adulthood obesity in the industrialized world. Evidence suggests that EDCs, especially bisphenol A (BPA), may affect normal energy homeostasis by altering homeostatic functions, glucose homeostasis, and peripheral gene expression [7-9]. Recent evidence also suggests that perinatal exposure to BPA alters hypothalamic melanocortin neurocircuitry and ARC neuropeptide gene expression in response to high fat diets [10]. The mechanisms behind these effects are unknown but potentially involve organizational and epigenetic effects in the hypothalamus during development, which potentially is mediated by the classical estrogen receptors (ER) α/β. Little is known about the effects of perinatal exposure to other estrogenic and non-estrogenic EDC on expression of ARC genes involved in energy homeostasis.

Developmental exposures to estrogenic EDCs alter many neuroendocrine reproductive functions [11;12]. BPA and other estrogenic EDCs (diethylstilbestrol (DES), methoxychlor (MXC)) are reported to alter the timing of puberty onset [11-15], however not consistently [16;17]. Phthalates are typically considered anti-androgenic and are known to alter male reproduction including puberty [18;19], but there is limited data with mixed results on phthalates (e.g., di(2-ethylhexyl)phthalate, dibutyl phthalate) in females. A few studies showed that the exposure advanced pubertal onset [20;21], and other studies suggested the exposure delayed puberty [22]. Differences in the timing of exposure may explain, in part, the contradictory results with phthalates. Puberty and the control of reproduction are initiated by interactions between peripheral energy signals (leptin, insulin, adiponectin, etc.) with hypothalamic neurons, in particular, NPY/AgRP [23] and KNDy [24]. Activity of neurotransmitters [25-28] and expression of growth factors (IGF-1) [29] in the hypothalamus are associated with the onset of puberty and the reproductive cycle in females. However, little is known about the effects of perinatal exposure to EDCs on expression of the genes associated with these signals in the hypothalamus and especially in the ARC.

There are numerous ARC genes that have been characterized for their role in controlling reproduction and energy homeostasis. These genes include receptors for hormones and neurotransmitters, signalling molecules, transcription factors, and neuropeptides (reviewed in [6;30;31]. Peripheral hormonal receptors, including members of G-protein coupled receptor (GPCR) and cytokine receptor families, are vital for the hypothalamic sensing of the body's energy status, which is then communicated to other hypothalamic neurons to control reproduction, especially during puberty. GPCR are common receptors for neurotransmitters involved in reproduction and energy homeostasis including serotonin receptors. ARC neuropeptides (e.g., POMC, NPY, AgRP, Kisspeptin) are integral to the hypothalamic control of feeding, energy expenditure, and puberty and are modulated by both

peripheral and central signals of energy homeostasis. Therefore, to address the question of whether these genes are altered in the ARC by developmental exposures to EDCs, we designed a Taqman Low-Density Array (TLDA) to analyze relative gene expression.

In order to determine the organizational effects of EDCs, dams and neonates were treated during fetal and neonatal periods when steroid receptor expression and the development of the melanocortin neurocircuitry in the rodent hypothalamus occur (E10-P14) [32-37]. In addition, EDCs alters hypothalamic gene expression [38], including steroid hormone receptor expression (ERα/β and AR) [7;39] during similar developmental windows. Furthermore, this exposure window encompasses critical gonadal differentiation events that start mid-gestation (i.e., E11) and subsequent early ovarian developmental events, including oocyte nest breakdown and follicular assembly in rats (i.e., E18-PD7), which can influence reproductive health later in life [40].

Therefore, the current study sought to determine if developmental (gestational and neonatal) exposures to a selection of known EDCs will alter puberty (vaginal opening), body weight, and ARC gene expression in adult female rats. As an initial EDC screen, we exposed dams and neonate females to two doses of BPA, a known disruptor of reproduction and energy homeostasis [10;14] and analyzed for gene expression using the TLDA. In the subsequent experiment, dams and neonate females were exposed to either BPA, MXC, di(2-ethylhexyl)phthalate (DEHP), or DES. We hypothesize that important genes involved in reproduction and energy homeostasis would be differentially regulated in the ARC by developmental exposures to these compounds, which may contribute to the dysregulation of reproduction and energy homeostasis in juveniles and adults.

2. Materials and Methods

2.1. Animals

Eight- to twelve-week-old Fischer CDF female rats (Charles River, Wilmington, MA) were maintained on a 14-hour light/10-hour dark cycle and fed, *ad libitum*. A reduced isoflavone diet was provided in order to minimize possible effects of phytoestrogens (Purina, 5V01, Brentwood, MO). The estrous cycles of the rats were followed daily, and individual females were mated with untreated males starting on the day of proestrus. A sperm-positive vaginal smear was designated embryonic day 0 (E0). All animal care and treatment protocols were carried out in accordance with institutional guidelines and were approved by Rutgers University Institutional Animal Care and Use Committee.

2.2. Chemicals

All chemicals (BPA, MXC, DES, DEHP, dimethyl sulfoxide (DMSO), sesame oil, and corn oil) were purchased from Sigma-Aldrich.

2.3. Experimental Design and Treatments

In Experiment #1, the timed-pregnant females received one of two different treatment dosages of BPA: 50 μg/kg/day (Lo-BPA; n = 3 litters; 10 pups) and 50 mg/kg/day (Hi-BPA; n = 4 litters; 8 pups) in 1 ml vehicle/kg BW. Control animals (n = 5 litters; 12 pups) received

an emulsion of 10% absolute ethanol (EtOH) and 90% corn oil. Daily treatments were administered (i.p.) to the pregnant dams beginning on E18 and sub-cutaneously (in the folds of the neck) to the neonates within 8 hours of birth (considered PND 0) and continued until PND 7. Starting PND 28, females were followed for their vaginal opening as an indication of pubertal age. Vaginal opening was followed to determine if developmental exposures to EDCs alters the age of puberty [41-43]. After vaginal opening, the stages of estrous cycles of all females were followed by vaginal cytology, and 8 randomly selected females from each treatment were weighed and euthanized between PND 50 and 60 on proestrous day after their third estrous cycle for hypothalamic tissue isolation and gene expression analysis.

In Experiment #2, the timed-pregnant females were divided into groups with each group receiving one of 4 different EDCs or two control vehicles. The EDCs and doses were as follows: BPA (50 mg/kg/day; n = 8 litters, 26 pups); DES (0.1 μg/kg/day; n = 6 litters, 20 pups); and DEHP (500 mg/kg/day; n = 5 litters, 18 pups) in a vehicle of EtOH:oil (1:9) (1 ml vehicle/kg BW volume). Another group received MXC (75 mg/kg/day; n = 8 litters, 16 pups) in a vehicle of dimethylsulfoxide (DMSO): sesame oil (1:2). There were two sub-groups of control animals receiving either the EtOH:corn oil vehicle (n = 5 litters, 15 pups) or the DMSO:sesame oil vehicle (n = 5 litters, 13 pups). Daily treatments were administered (i.p.) to the pregnant dams beginning on E11 and sub-cutaneously (in the folds of the neck) to the neonates within 8 hours of birth (considered PND 0) and continued until PND 7. The vaginal opening of all female offspring and the stages of estrous cycle were followed, similar to Experiment #1. One to two females from each dam (*n* = litter) were randomly chosen, weighed, and euthanized on proestrous day between PND 70 and 90 for isolation of hypothalamic tissues and analysis of gene expression. The age of sacrifice was determined by estrous cyclicity and due to perturbations in estrous cycles by EDC treatments, the range in ages is ~20 days. See Table 1 for a list of the samples sizes (litters) and number of total pups for each experiment, treatment, and endpoint.

Doses of compounds were chosen from previous experimentation demonstrating reproductive effects or from the literature. BPA doses (high (50 mg/kg/day) and low (50 μg/kg/day)) represent the EPA LOAEL and reference doses, respectively [7;39]. DEHP was administered at 500 mg/kg/day, which is higher than the highest dose that human infants are exposed in neonatal intensive care units (~23 mg/kg/day) [44;45], but was shown to be effective at inducing transgenerational effects in male mice [46]. The environmental levels of MXC range from 160 mg/l in waters down-stream of MXC-sprayed areas to 0.1 ng/kg/day, the FDA's calculated average daily intake of MXC in general adult population. In addition, the 75 mg/kg/day is within the dose range that is used in many studies and has been previously shown to be effective in producing reproductive effects without inducing complete sterility in females [47]. DES (0.1 μg) is a lower dose than what is used in previous studies on pregnant women [48], but is effective in rodents as a model estrogenic EDC [49]. In addition, while oral exposure is considered more common for some EDCs (e.g., MXC or BPA), for certain cases parenteral exposure is more relevant (e.g., DES and exposure to DEHP via medical devices). Thus, to be consistent between EDCs, and to precisely control the doses of the chemicals that were administered, we choose to use a parenteral route. See Figure 1 for a timeline of Experiment #1 and #2.

2.4. Tissue Dissection and RNA Extraction

Vehicle- or EDC-treated adult females were decapitated quickly and the brain was extracted and transferred to ice-cold Sorensen's Buffer. Hypothalamic nuclei were microdissected for RNA extraction and quantitative real-time analysis. The basal hypothalamus was cut using a brain slicer (Ted Pella, Redding, CA) into 1-mm thick coronal rostral and caudal blocks. The slices corresponded to Nissl-stained figures in *Chemoarchitectonic Atlas of The Rat Brain* by Paxinos and coauthors [50] using pages 183–232. The tissue blocks were placed in RNAlater for 24 h (Life Technologies, Inc.), and the rostral and caudal parts of the ARC were dissected from the rostral and caudal blocks, respectively, using a dissecting microscope. Dissected tissue was stored at −80 ºC. From the combined nuclei (rostral and caudal ARC), total RNA was extracted and DNase I-treated using Ambion RNAqueous Micro Kits (Life Technologies, Inc.) according to the manufacturer's protocol. RNA samples were tested for quantity and purity using the NanoDrop ND-2000 spectrophotometer (ThermoFisher, Waltham, MA). RNA quality was assessed by applying 1 μl of each RNA sample to a channel of an Agilent Nanochip and analyzing on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Only samples with RIN > 6 were used in the experiments.

2.5. Reverse Transcription and Quantitative Real-Time PCR

Complementary DNA (cDNA) was synthesized from 200 ng total RNA using Superscript III (Life Technologies, Inc.), 4 μl 5x buffer, 25 mM $MgCl_2$, 10 mM dNTP, 100 ng random hexamer primers (Promega), 40 U/μl Rnasin (Promega), and 100 mM dithiothreitol (DTT) in diethylpyrocarbonate (DEPC)-water (GeneMate) in total volume of 20 μl. RT was conducted using the following protocol: 5 min at 25 °C, 60 min at 50 °C, 15 min at 70 °C. The cDNA was diluted to 1:20 with Nuclease-free water (GeneMate) for a final cDNA concentration of 0.5 ng/μl and stored at −20 °C. Basal hypothalamic (BH) test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

For qPCR, 5 μl cDNA template (an equivalent of 2.5 ng total RNA) was amplified using Taqman® Gene Expression Master Mix (Life Technologies) on a StepOnePlus™ Real-Time PCR system. For Experiment #1, samples were analyzed using a Taqman® Custom Low-Density Gene Array (TLDA) of 40 genes selected for their roles in energy homeostasis, reproduction, and hypothalamic neuronal activity. Genes and assays identification numbers are presented in Table 2. The gene assays were arrayed in duplicate on each plate along with endogenous controls (*Actb*, 3 wells; 18s, 1 well). One of the *Actb* wells was used for a sample of pooled controls to determine inter-plate variation. One experimental sample was run per plate including an untreated male sample used as calibrator for determining relative gene expression. The amplification protocol for the plates was as follows: 95 °C for 10 min (initial denaturing) followed by 40 cycles of amplification at 94 °C for 15 sec (denaturing), 60 °C for 60 sec (annealing). The relative mRNA expression data were analyzed using the $\Delta\Delta C_T$ method, where C_T is cycle threshold [51].

For Experiment #2, samples were analyzed for relative gene expression only in those genes that had a statistically significant alteration in gene expression from the TLDA. Individual

assays (FAM dye) for each gene (identical to those used in the array) were used to analyze the samples (see Table 2). Each gene assay was tested using BH cDNA while being multiplexed with a primer-limited endogenous control (*Actb*-VIC). Each gene was analyzed in one plate by multiplexing the gene assay with the endogenous control. The relative mRNA expression data were analyzed using the $\Delta\Delta C_T$ method, where C_T is cycle threshold [51]. The amplification protocol for all the genes was as follows: 95 °C for 10 min (initial denaturing) followed by 40 cycles of amplification at 94 °C for 15 sec (denaturing), 60 °C for 30 sec (annealing). Positive and negative BH cDNA controls were added to each amplification run including a water blank.

2.6. Data analysis

All data are expressed as mean ± SEM. We analyzed expression data from Experiment #1 using Data Assist® software (Life Technologies) to determine significant differences between EtOH:oil- and BPA-treated females. The false discovery rate was calculated (Benjamini-Hochberg method, $P < 0.05$) for Experiment #1. Secondary one-way ANOVA analysis was conducted on all significant data from the Data Assist analysis. All the data from the body weight measurements, pubertal age, and quantitative real-time PCR from Experiment #2 were analyzed using a one-way ANOVA followed by a *post-hoc* Bonferroni multiple comparison tests for each gene assay. All data were analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) or Statistica (Dell Software, Aliso Viejo, CA, USA). Because multiple genes were analyzed using the TLDA, we adjusted the alpha for Experiment #1 to $p \leq 0.01$ for the ANOVA. For Experiment #2, the alpha was set at $p \leq 0.05$ within each gene. A Grubb's test was used to detect outliers in all experiments.

3. Results

3.1. Vaginal opening and body weight

In Experiment #1, neither low dose (37.1 ± 0.6 d; n = 3) nor high dose (36.8 ± 0.8 d; n = 4) BPA altered the day of the vaginal opening as compared to control (36.4 ± 0.6 d; n = 5) (Figure 2A; $F(2, 9) = 0.2853$; not significant (ns)). In Experiment #2, there was an effect of EDC treatment on pubertal age (Figure 2B; $F(5, 31) = 9.064$; $p < 0.0001$). Pubertal age was advanced by both MXC (33.5 ± 0.5 d; n = 8; $p < 0.01$) and DES (35.0 ± 0.4 d; n = 6; $p < 0.05$) compared to their respective controls (DMSO:oil – 36.6 ± 0.4 d; n = 5, and EtOH:oil – 36.8 ± 0.4 d; n = 5). In contrast, DEHP delayed pubertal age (40.2 ± 2.0 d; n = 5; $p < 0.05$) vs. EtOH:oil control. As in Experiment, #1, BPA did not alter the pubertal age (37.9 ± 0.5; n = 8).

In Experiment #1, female rats were weighed at the age of 50-60 days. There was no effect of treatment on age-matched body weights (Figure 2C; $F(2, 9) = 0.6023$; ns). Vehicle–treated females weighed an average of 135.5 ± 4.3 g (n = 5). Females exposed to the 50 μg/kg/day BPA dose weighed 135.7 ± 6.6 g (n = 3). Females exposed to 50 mg/kg/day BPA dose weighed 127.8 ± 7.0 g (n = 4). In Experiment #2, there was an effect of EDC treatment on body weights (Figure 2D; $F(5, 22) = 4.624$; $p < 0.01$). Control females were divided into those treated with EtOH:oil (1:9) (n = 3) and those treated with DMSO:oil (1:2) (n = 4). The average age-matched body weight for the two control groups did not differ (EtOH:oil –

165.6 ± 4.3 g; DMSO:oil - 162.9 ± 1.8 g). BPA (164.4 ± 1.5 g; n = 6), DES (181.8 ± 6.8 g; n = 6) and DEHP-treated (152.3 ± 3.6; n = 4) females were compared to EtOH:oil control and MXC-treated (185.8 ± 9.6 g; n = 5) was compared to DMSO:oil control. However, no individual EDC treatment altered body weights compared to controls.

3.2. Developmental exposures to BPA alters ARC gene expression

Because the TLDA was used initially as a screen for potential gene candidates and to reduce false discovery rate, we set the ANOVA at $p \leq 0.01$ as the cutoff. Of the 40 genes examined, BPA treatment altered the gene expression in two of these genes in a dose-dependent manner (ANOVA: $p \leq 0.01$) (See Table 3). These two genes were *Chrm3* and *Adipor1*. *Chrm3*, the muscarinic acetylcholine receptor 3 gene, was suppressed by the low ($p < 0.05$) and high ($p < 0.01$) doses of BPA ($F(2, 8) = 15.07$; $p < 0.01$). *Chrm3* was also lower in the Hi-BPA than the Lo-BPA ($p < 0.05$). The adiponectin receptor 1 gene, *Adipor1*, was also suppressed by the low ($p < 0.05$) and high ($p < 0.01$) doses of BPA in the ARC ($F(2, 8) = 9.868$; $p < 0.01$).

There were 4 other genes in the TLDA that were significantly altered by BPA treatment with ANOVA p values between 0.05 and 0.01. These genes included *Bdnf*, *Cck2r*, *Htr2c*, and *Igf1* (See Table 2). *Bdnf* (brain-derived neurotrophic factor; $F(2, 8) = 7.279$; $p < 0.05$) was significantly suppressed by the high dose of BPA ($p < 0.05$). The cholecystokinin receptor 2 (*Cck2r*; ($F(2, 8) = 6.770$; $p < 0.05$) was significantly suppressed by the high BPA dose ($p < 0.05$). The serotonin receptor 5HT2c receptor (*Htr2c*; ($F(2, 8) = 7.329$; $p < 0.05$), was suppressed by the high dose of BPA ($p < 0.05$). *Igf1* (insulin growth factor 1; $F(2, 8) = 4.547$; $p < 0.05$) was suppressed only by the low dose of BPA ($p < 0.05$). These six genes were examined again in the ARC samples generated from Experiment #2.

3.4. Endocrine disrupting compounds alter ARC gene expression

In Experiment #2, females were exposed during development to 4 EDCs - BPA, DES, DEHP, or MXC - and sacrificed at 70-90 days in age. Of the six genes, five were affected by EDC treatment (ANOVA: $p \leq 0.01$) (See Figure 3). As in Experiment #1, BPA ($p < 0.05$) suppressed the expression of *Htr2c* in the ARC ($F(5, 25) = 4.524$; $p < 0.01$). MXC treatment also suppressed *Htr2c* expression ($p < 0.05$) (Figure 3A). Another gene that was suppressed by EDC treatment was *Adipor1* (Figure 3B; $F(5, 25) = 4.697$; $p < 0.01$). This gene was suppressed by DEHP ($p < 0.05$) and MXC ($p < 0.01$) treatment in the ARC but not by BPA as it was in Experiment #1. Unlike in Experiment #1, there was not an effect of EDC treatment on *Cck2r* gene expression (Figure 3C; $F(5, 25) = 2.067$; $p = 0.1036$).

Only DES significantly affected *Bdnf* gene expression in the ARC (Figure 3D; $F(5, 25) = 4.041$; $p < 0.01$). EDC treatment also augmented *Igf1* expression in the ARC (Figure 3E; $F(5, 25) = 7.095$; $p < 0.001$). Both BPA ($p < 0.05$) and DES ($p < 0.05$) significantly increased the expression of *Igf1* by more than twofold and MXC treatment increased the expression of *Igf1* compared to DMSO controls ($p < 0.01$). MXC also increased *Chrm3* expression (Figure 3F; $F(5, 25) = 2.868$; $p < 0.05$) by about 50% in the ARC with no other compounds having a significant effect. Because of their involvement in reproduction (puberty) and energy homeostasis, we also examine gene expression of *Kiss1*, *Igf-1r*,

Gabbr1, *Esr1*, and *Esr2* in these samples. No effect of EDC treatment was found for any of these genes (data not shown).

4. Discussion

The goal of this study was to characterize the effects of developmental exposures to EDC (BPA, DES, DEHP, and MXC) on adult female ARC gene expression involved in reproduction, energy homeostasis, and hypothalamic neuronal functions. We selected a range of ARC genes including many GPCR, neuropeptides, signaling molecules, and transcription factors (reviewed in [6;30;31] and employed TLDA technology to screen for potential candidate genes using two doses of BPA. Of the 40 genes selected, six genes were affected by developmental BPA exposures. Four of these genes were receptors for neurotransmitters (serotonin, acetylcholine) and peripheral hormones (adiponectin) or brain-gut peptides (cholecystokinin). The remaining two genes were the neurotrophin BDNF and the paracrine hormone IGF-1. Five of these genes (*Ht2cr*, *Adipor1*, *Bdnf*, *Igf1*, and *Chrm3*) were significantly regulated in the second experiment, which examined the effects of 4 EDCs on ARC gene expression in adult female offspring. The inconsistency in *Cck2r* gene regulation between Experiment #1 and #2 by BPA may be due to age-dependent processes (> PD60) or may be ameliorated by adult influences on developmental programming.

Recent evidence has indicated that developmental exposures to EDC can affect hypothalamic functions, especially reproduction [38;39;52-54]. Many of these effects include changes in gene expression of relevant neuropeptides and hormone receptors. For example, developmental PCB exposures in rats suppressed POA expression of IGF-1, androgen receptor, and NDMA glutamate receptor subunit NR2b in females [54]. Developmental MXC exposures altered expression of ERα and other genes in the POA of female rats [38]. Neonatal and prepubertal exposures to dibutyl phthalates (DBP) increased ARC kisspeptin expression while suppressing GPR54/Kiss1r and ERα gene expression in female rats [21]. Finally, gestational exposures to BPA and 17α-ethinylestradiol increased neonatal expression of ERα in the ARC of male and female pups [39;52].

Few studies have examined the effects of EDC exposure on the expression of ARC genes involved in energy homeostasis and neuronal activity primarily focusing on the expression of the POMC and NPY neuropeptides. POMC neurons express the endogenous agonist for MC4 receptors, α-MSH, while NPY neurons express the endogenous MC4 antagonist, AgRP [55]. Recently, Mackay *et al.* (2013) reported that BPA and DES developmental exposures in CD-1 mice altered ARC expression of ERα in female offspring fed a control diet and augmented expression of NPY and AgRP in male offspring fed a high fat diet while suppressing the expression of POMC in females fed a high fat diet [10]. Additionally, the Mackay *et al.* (2013) study found alterations to hypothalamic melanocortin circuitry with a reduced POMC innervation of melanocortin receptor 4 (MC4) receptor-expressing PVH neurons.

Two of the genes regulated by BPA and EDCs in both experiments were receptors for neurotransmitters involved in reproduction and energy homeostasis. Serotoninergic neurons, originating from the dorsa raphe, are a major anorectic signal in the brain innervating both

ARC POMC and NPY neurons [56]. The serotonin 5HT2c receptor is the primary receptor involved in serotonin's anorectic actions in the ARC along with are the 5HT2a and 5HT1b receptors [57-59]. Agonists of the 5HT2c receptor reduce food intake and improve glucose homeostasis [60]. While the role of 5HT2c receptor in puberty is unknown, serotonin is involved in the initiation of puberty in rodents delaying puberty when injected systemically [26;61]. However, lesions of the dorsal raphe (containing serotonergic neurons) at PND 21 delayed vaginal opening and first vaginal estrus (ovulation) in rats while lesions after weaning (PND 24/27) did not [62], indicating that serotonin controls puberty onset. In our study, two compounds advanced puberty, DES and MXC, the latter also suppressed 5HT2c receptor expression. Conversely, in DEHP-treated females, there was a trend to elevated *Htr2c* expression ($p = 0.154$) and a delay in vaginal opening ($p < 0.05$).

There is no evidence that developmental or adult exposures to EDCs affect the expression of serotonin receptors and specifically the 5HT2c receptor in the hypothalamus. However, developmental exposures to estrogenic EDCs have been shown to alter serotoninergic innervation, release, and reuptake. Prenatal and lactational exposures to BPA increased the concentrations of serotonin and its metabolites in the hippocampus, thalamus, dorsal raphe, and substantia nigra [63;64]. MXC treatment in adulthood increased serotonin concentrations in the anterior hypothalamus while decreasing the concentrations in the posterior hypothalamus and the turnover in the anterior hypothalamus [65]. In our study, the reduction in 5HT2c receptor expression specifically in the ARC in BPA- and MXC-treated females could produce a decreased response to the anorectic serotoninergic signals originating from the dorsal raphe leading to hyperphagia and weight gain. Future studies should examine the expression and activity of 5HT2c receptors in ARC POMC neurons after developmental EDC exposure.

There are little data on the effects of EDC exposure on the activity and expression of *Chrm3*, an acetylcholine receptor, with this study being the first one to characterize a potential effect of developmental exposures on its expression in the hypothalamus. This receptor is thought to be involved in energy homeostasis due to genetic modification (*Chrm3* knockout] in mice. The subsequent alterations in energy homeostasis suggests that *Chrm3* has a role in glucose homeostasis, insulin sensitivity, food intake, and energy expenditure, which may be due to an increase in central sympathetic activity [66;67]. Although the role of M3 muscarinic receptor in ARC functions is unknown at this time, blocking muscarinic receptors with trihexyphenidyl or propantheline delays vaginal opening and first estrus in rats indicating cholinergic neurons are involved in the control of puberty [68]. The increase in *Chrm3* by MXC in the ARC in Experiment #2 correlates with an advance in vaginal opening (puberty), suggesting that changes in muscarinic receptors in the hypothalamus due to EDC exposure may contribute to the dysregulation of puberty.

Receptors for two peptide hormones, adiponectin receptor 1 (*Adipor1*) and cholecystokinin receptor 2 (*Cck2r*), were significantly regulated in Experiment #1. However, in Experiment #2, there was no effect on *Cck2r* expression in the ARC, although there was a trend for elevated *Cck2r* expression ($p = 0.07$) in DEHP-exposed females. Conversely, the adiponectin receptor was consistently regulated in the ARC by EDC exposure. Adiponectin is an adipocyte peptide hormone and is highly expressed in lean animals to control insulin

sensitivity and glucose homeostasis [69]. While controversial, adiponectin acts through its receptor in the hypothalamus to increase food intake and suppress energy expenditure by an AMPK-mediated pathway [70;71]. Adiponectin receptor 1 is expressed both in POMC and NPY neurons and the expression of this receptor is stimulated by fasting states [69]. While there are no data on the effects of EDC exposure on adiponectin receptor expression, both BPA and DEHP have been shown to suppress adiponectin expression in adipocytes in adult mice [72;73].

In our study, the reduction in *Adipor1* expression specifically in the ARC in EDC-treated females may contribute to advance the onset of puberty found in the MXC-treated females. Adiponectin, in part, via actions through its receptor, inhibits the HPG axis and GnRH secretion, and controls puberty [74;75]. In GT1-7 cells, adiponectin inhibits production and secretion of GnRH [74]. Adiponectin also inhibits hypothalamic *Kiss1* gene expression through AMPK-SP1 [76] and inhibits LH secretion from pituitary explants from rats by decreasing GnRH receptor expression in the pituitary [77]. In our study, MXC reduced *Adipor1* expression in the ARC, thus potentially reducing the inhibition of the HPG axis and *Kiss1* by adiponectin from peripheral adipose tissue to advance puberty and alter the estrous cycle.

Expression of two paracrine/autocrine neuropeptides was also differentially altered in our two experiments. Insulin-like Growth Factor 1 (*Igf1*) was suppressed by the low BPA dose in Experiment #1 while significantly increased by DES, MXC, and the high dose of BPA in Experiment #2. There are little to no data demonstrating developmental effects of EDC exposure on IGF-1 expression or activity in the brain. Recently, Huang *et al.* (2014) suggested that BPA exposure to human embryonic stem cells downregulated the expression of IGF-1 as well as other genes involved in neural development [78]. In the periphery, BPA exposure increased IGF-1 peptide expression in the liver of female rats [79] and MXC exposure altered proteins (e.g., *Igf1r* and *Igfbp5*) of the IGF-1 signaling in the ovary of rats [80].

IGF-1 signaling in the brain is an important regulator of neuroendocrine control of reproduction and energy balance [81;82]. While the direct effect of increase in *Igf1* gene expression in the ARC on energy homeostasis is unknown, IGF-1 is a growth factor and an important hormone in the somatotropic axis [83]. Additionally, IGF-1 expression and activity is involved in the control of the HPG axis and the onset of puberty [81;82]. An increase in IGF-1 gene expression in the mediobasal hypothalamus is found in the neonate and in the adult (PND 60) [84]. Furthermore, peripheral administration of IGF-1 initiates the onset of puberty in prepubertal rats [85]. IGF-1 levels in ARC tanycytes is sexually dimorphic, is increased during the initiation of puberty, and is regulated by sex steroids including estradiol [86]. Previous studies have demonstrated that BPA and DES exposure advances puberty in female rodents [7;13;87]. However, in our study BPA did not advance vaginal opening while DES and MXC did. In the DES- and MXC-treated females, an increase in ARC IGF-1 expression may play a role in the effects on puberty and/or the estrous cycle.

The second paracrine/autocrine neuropeptide affected by EDC treatment was BDNF. In Experiments #1, *Bdnf* was suppressed by the high dose of BPA but not in Experiment #2 while being significantly increased by DES treatment in Experiment #2. In the hippocampus, developmental or neonatal exposures to DES increased *Bdnf* gene expression in male rats [66;88]. However, DES and BPA treatment suppressed BDNF protein expression in primary cultures of mouse cerebellar granule cells [89]. This suggests that EDC control BDNF expression through different mechanisms depending upon the species, the timing of exposure, and stage of development. During development, BDNF regulates neurogenesis and synaptic plasticity while during adulthood BDNF expression is associated with the control of food intake, body weight, and activity-dependent energy expenditure [53;90;91]. Deletion of BDNF in the VMH and DMH of adult mice increased food intake without affecting energy expenditure or locomotive behavior [92] although the involvement of ARC BDNF in energy homeostasis remains to be determined. As in the case of *Igf1*, the increase in *Bdnf* in the ARC with DES treatment is not associated with a higher weight gain in these females and therefore may be associated with the disruption of the HPG axis [93] or perhaps puberty, although the role of BDNF in puberty onset is unclear.

5. Conclusions

Our study indicates that solely examining the expression of anorectic and orexigenic neuropeptides or estrogen receptors and KNDy genes in the ARC is not sufficient to understanding the effects of developmental EDC exposures on homeostatic and reproductive functions. Many of the genes affected modulate energy homeostasis towards either a positive or negative balance (increase or decease food intake and/or energy expenditure) or are involved in the transmission of signals to initiate the onset of puberty. However, the effect of these changes in gene expression may offset each other and not disrupt reproduction or energy homeostasis, unless in an obesogenic environment [10]. The present study provides potential gene target for the developmental effects of EDCs on sexual maturity, estrous cycles, the HPG axis, and energy homeostasis that have been reported. Understanding these mechanisms of EDC toxicity is necessary in determining their respective effects on human and wild life health. For example, the consistent effect of BPA exposure on 5HT2c receptor has wide ranging potential to alter normal homeostatic functions increasing the propensity to obesity and Type II diabetes. Future experiments should examine these EDC effects in animal models that can target these receptors and neuropeptides at the level of specific neuronal cell types (POMC, NPY, KNDy, etc.). Due to small ARC tissue size, we did not measure protein levels, which are more functionally relevant. Because mRNA and protein levels may not correlate when comparing gene and protein expression [94], future studies will examine the expression and activity of the hormone and neurotransmitter receptors using electrophysiology and single cell gene expression assays in ARC neurons. Experiments such as those would determine if these effects on gene expression translate into neurological outcomes resulting in the dysregulation of reproduction and energy homeostasis.

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Abbreviations

Adipor1	adiponectin receptor 1
ARC	arcuate nucleus of the hypothalamus
Bdnf	brain-derived neurotropic factor
BPA	bisphenol A
Cck2r	cholescystokinin 2 receptor
Chrm3	muscarinic receptor 3
DEHP	di(2-ethylhexyl)phthalate
DES	diethylstilbestrol
Htr2c	5-hydroxytryptamine receptor
Igf1	insulin-like growth factor 1
MXC	methoxychlor
NPY	neuropeptide Y
POMC	proopiomelanocortin
TLDA	Taqman® Low-Density Array

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Highlights

- Developmental exposure to BPA did not affect body weights or puberty.
- Developmental exposure to DES & MXC advanced puberty and to DEHP delayed puberty.
- Developmental exposure to EDC altered arcuate gene expression in early adulthood

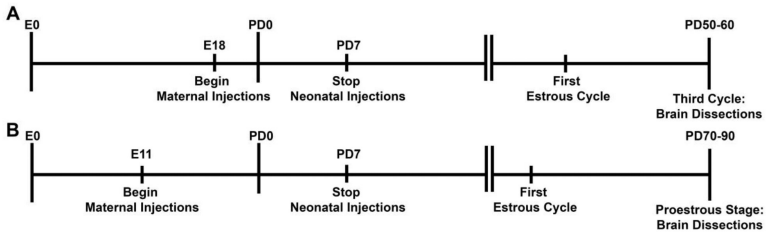

Figure 1.

A timeline illustrating the experimental protocol for Experiment #1 and #2. Pregnant dams were injected with the EDC or vehicle from embryonic day (E) 18 (Exp. #1, **A**) or E11 (Exp. #2, **B**) and the neonates were injected until postnatal day (PND) 7. Estrous cycles were monitored daily after puberty. Females were sacrificed in proestrus after the third estrous cycle in Experiment #1 or in proestrus in Experiment #2.

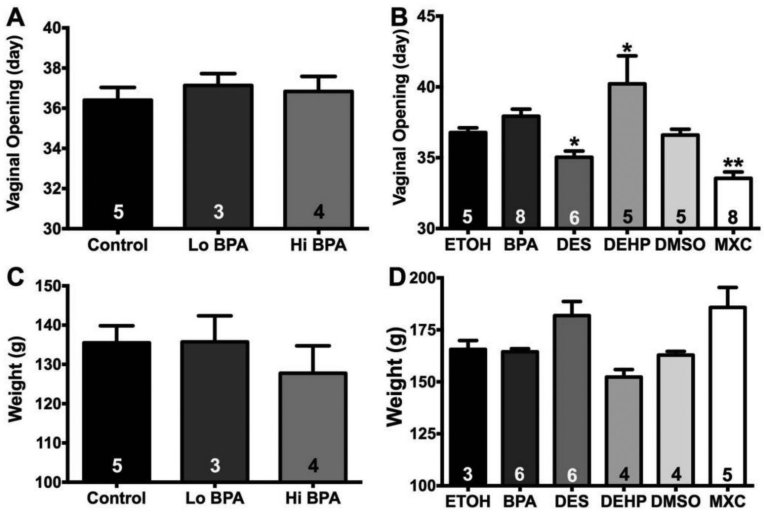

Figure 2.

Pubertal age (day of vaginal opening) and body weights (g) for female rats exposed to EDCs during gestation and neonatal development. A: Average day of vaginal opening in females from Experiment 1 ($F(2, 9) = 0.2853$, ns). B: Average day of vaginal opening in females from Experiment #2 ($F(5, 31) = 9.064$, $p < 0.0001$). C: Body weights from Experiment #1 ($F(2, 9) = 0.6023$, ns). D: Body weights from Experiment #2 ($F(5, 22) = 4.624$; $p < 0.01$). Data were analyzed by a one-way ANOVA with Bonferroni multiple comparison tests (* $p < 0.05$; ** $p < 0.01$). Numbers in column represent the sample size (n=litters) for each treatment.

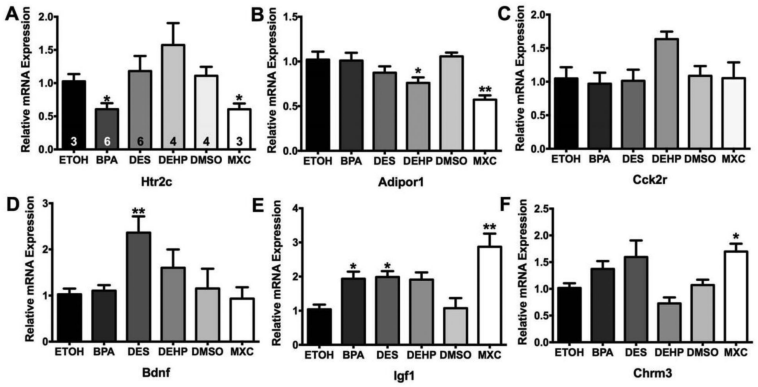

Figure 3.

Relative gene expression in ARC from young adult female rats exposed to EDC. A. *Ht2cr*, B. *Adipor1*, C. *Cck2r*, D. *Bdnf*, E. *Igf1*, and F. *Chrm3*. Data were analyzed using a one-way ANOVA followed by Bonferroni multiple comparison tests within each gene compared to the respective control samples (* $p < 0.05$; ** $p < 0.01$). Numbers in the columns of A are the sample sizes for each treatment (n = litters).

Table 1

Samples sizes (litters) and number of pups for each experiment, treatment, and endpoint.

	Vaginal Opening		Body Weight		QPCR	
	# of Litter	# of Pups	# of Litter	# of Pups	# of Litter	# of Pups
Experiment #1						
Control	5	12	5	12	3	8
50 μg BPA	3	10	3	10	3	8
50 mg BPA	4	8	4	8	4	8
Experiment #2						
ETOH	5	15	3	5	3	5
DES	6	20	6	10	6	10
DEHP	5	18	4	7	4	7
DMSO	5	13	4	4	4	4
MXC	8	16	5	8	3	5

Table 2

Taqman® Low Density Array Genes.

Gene Name	Gene	Assay ID
5HT2c Receptor	*Htr2c*	Rn00562748_m1
Adiponectin receptor 1	*Adipor1*	Rn01483784_m1
Agouti-regulated Protein	*Agrp*	Rn01431703_g1
Brain Derived Neurotrophic Factor	*Bdnf*	Rn02531967_s1
Cannabinoid Receptor 1	*Cnr1*	Rn02758689_s1
CART prepropeptide	*Cartpt*	Rn01645174_m1
Cholecystokinin Receptor 2	*Cck2r*	Rn00565867_m1
Estrogen Receptor α	*Esr1*	Rn01640372_m1
Estrogen Receptor β	*Esr2*	Rn00562610_m1
Forkhead box O1	*FoxO1*	Rn01494868_m1
GABA-B Receptor 1	*Gabbr1*	Rn00578911_m1
GABA-B Receptor 2	*Gabbr2*	Rn00582550_m1
Galanin Receptor 3	*Galr3*	Rn02132531_s1
Galanin-like Peptide	*Galp*	Rn00575275_m1
Gastrin-releasing peptide receptor	*Grpr*	Rn01420745_m1
Glucagon-like peptide 1 receptor	*Glp1r*	Rn00562406_m1
Glucokinase	*Gck*	Rn00561265_m1
Glutamate decarboxylase 1	*Gad1*	Rn00690300_m1
Glutamate decarboxylase 2	*Gad2*	Rn00561244_m1
Growth Hormone Secratogue Receptor	*Ghsr*	Rn00821417_m1
Insulin Receptor Substrate 1	*Irs1*	Rn02132493_s1
Insulin Receptor Substrate 2	*Irs2*	Rn01482270_s1
Insulin-like Growth Factor 1	*Igf1*	Rn00710306_m1
Insulin-like Growth Factor 1 Receptor	*Igf1r*	Rn00583837_m1
Kisspeptin 1	*Kiss1*	Rn00710914_m1
Leptin Receptor	*Lepr*	Rn01433205_m1
Melanocortin 3 Receptor	*Mc3r*	Rn02585847_s1
Metabotropic Glutamate Receptor 5	*Grm5*	Rn00566628_m1
Muscarinc Receptor 3	*Chrm3*	Rn00560986_s1
Neuropeptide Y	*Npy*	Rn01410145_m1
NPY Y2 Receptor	*Npy2r*	Rn04219516_g1
Opioid Receptor (μ)	*Oprm1*	Rn01430371_m1
Phosphatase and tensin homolog	*Pten*	Rn00477208_m1
Prodynorphin	*Pdyn*	Rn00571351_m1
Proopiomelanocortin	*Pomc*	Rn00595020_m1
Serine/threonine-protein kinases (Akt3)	*Akt3*	Rn00442194_m1
Signal transducer and activator of transcription 3	*Stat3*	Rn00562562_m1

Gene Name	Gene	Assay ID
Sulfonylurea Receptor 1	*Abcc8*	Rn00564778_m1
Tyrosine Hydroxylase	*Th*	Rn00562500_m1
Uncoupling Protein 2	*Ucp2*	Rn01754856_m1

Table 3

Relative gene expression in Experiment #1.

Gene Name	Vehicle	Lo-BPA	Hi-BPA	ANOVA p
Abcc8	1.47 ± 0.34	1.16 ± 0.14	1.14 ± 0.09	0.4391
Adipor1	1.18 ± 0.06	0.95 ± 0.03 *(a)*	0.82 ± 0.07 *(a)*	0.0069 **
Agrp	0.93 ± 0.29	0.72 ± 0.06	1.19 ± 0.49	0.6985
Akt3	1.42 ± 0.19	1.31 ± 0.12	1.37 ± 0.15	0.8784
Bdnf	1.12 ± 0.08	1.06 ± 0.12	0.60 ± 0.05 *(a), (b)*	0.0158 *
Cnr1	0.95 ± 0.10	0.72 ± 0.10	0.55 ± 0.10 *(a)*	0.0737
Cartpt	3.05 ± 0.13	3.88 ± 0.44	4.31 ± 0.84	0.4080
Cck2r	1.39 ± 0.16	0.91 ± 0.09	0.67 ± 0.16 *(a)*	0.0190 *
Chrm3	0.92 ± 0.07	0.70 ± 0.08 *(a)*	0.41 ± 0.03 *(a), (b)*	0.0019 **
Esr1	2.42 ± 0.51	2.53 ± 0.11	2.51 ± 0.22	0.9593
Esr2	1.81 ± 0.10	1.81 ± 0.18	1.66 ± 0.13	0.7128
FoxO1	1.60 ± 0.23	1.33 ± 0.11	1.46 ± 0.16	0.5508
Gabbr1	1.19 ± 0.18	1.18 ± 0.05	0.93 ± 0.07	0.1482
Gabbr2	1.22 ± 0.24	1.20 ± 0.11	1.21 ± 0.16	0.9951
Galr3	0.45 ± 0.13	0.56 ± 0.11	0.33 ± 0.05	0.2596
Galp	6.05 ± 1.33	5.52 ± 0.43	7.08 ± 0.60	0.3627
Grpr	2.06 ± 0.16	1.65 ± 0.07	1.88 ± 0.16	0.1742
Glp1r	2.68 ± 0.28	2.36 ± 0.14	2.66 ± 0.39	0.7331
Gck	1.37 ± 0.20	1.16 ± 0.06	1.12 ± 0.10	0.3593
Gad1	1.03 ± 0.25	0.97 ± 0.08	0.99 ± 0.12	0.9599
Gad2	1.23 ± 0.14	0.99 ± 0.09	1.12 ± 0.12	0.3736
Ghsr	2.71 ± 0.25	2.32 ± 0.15	2.48 ± 0.15	0.3596
Grm5	1.46 ± 0.15	1.00 ± 0.18	0.86 ± 0.11 *(a)*	0.0641
Htr2c	0.75 ± 0.11	0.58 ± 0.04	0.40 ± 0.04 *(a)*	0.0155 *
Irs1	0.71 ± 0.06	0.63 ± 0.04	0.57 ± 0.07	0.2858
Irs2	1.62 ± 0.14	1.55 ± 0.23	1.49 ± 0.09	0.1504
Igf1	1.25 ± 0.11	0.53 ± 0.05 *(a)*	1.18 ± 0.28	0.0480 *
Igf1r	1.54 ± 0.17	1.26 ± 0.10	1.52 ± 0.16	0.3345
Kiss1	2.83 ± 0.58	4.93 ± 1.51	3.13 ± 0.76	0.3890
Lepr	1.42 ± 0.16	1.08 ± 0.03	1.12 ± 0.16	0.2242
Mc3r	1.34 ± 0.18	1.01 ± 0.03	1.19 ± 0.26	0.5012
Npy	0.76 ± 0.07	0.72 ± 0.10	0.89 ± 0.24	0.7240
Npy2r	1.50 ± 0.06	1.35 ± 0.07	1.44 ± 0.23	0.7979
Oprm1	1.16 ± 0.15	1.09 ± 0.13	0.93 ± 0.07	0.4183
Pten	1.54 ± 0.23	1.24 ± 0.10	1.49 ± 0.13	0.3583

Gene Name	Vehicle	Lo-BPA	Hi-BPA	ANOVA p
Pdyn	1.82 ± 0.11	1.49 ± 0.20	1.35 ± 0.14	0.2026
Pomc	5.32 ± 0.15	5.50 ± 1.55	4.38 ± 0.76	0.7398
Stat3	1.76 ± 0.14	1.63 ± 0.10	1.80 ± 0.17	0.6721
Th	3.02 ± 0.34	2.82 ± 0.13	3.29 ± 0.54	0.6864
Ucp2	1.78 ± 0.43	1.40 ± 0.14	1.56 ± 0.17	0.5638

Data were analyzed by a one-way ANOVA followed by Bonferroni multiple comparison test with litter as the unit. Samples size: vehicle = 3, Lo-BPA = 3, Hi-BPA = 4. ANOVA p values:

* $p < 0.05$;

** $p < 0.01$.

$^{(a)}$ = $p < 0.05$ compared to vehicle;

$^{(b)}$ = $p < 0.05$ compared to Lo-BPA.