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Endocrine-disrupting chemicals alter the neuromolecular phenotype in F2 generation adult male rats

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ABSTRACT

Endocrine-disrupting chemical (EDC) exposures to the fetus have long-lasting effects on health and disease in adulthood. Such EDC exposure to the F1 fetuses also reaches the germ cells that become the F2 generation. Previously, we demonstrated that adult social and communicative behaviors such as ultrasonic vocalizations and mating behaviors were altered by EDCs in F2 rats, especially males. In the current study, we used the brains of these F2 males to ascertain the underlying molecular changes in the hypothalamus related to these behavioral outcomes. Their progenitors were Sprague-Dawley rat dams, treated on pregnancy days 8 to 18 with one of three treatments: a polychlorinated biphenyl (PCB) mixture, Aroclor 1221, selected because it is weakly estrogenic; the anti-androgenic fungicide vinclozolin (VIN); or the vehicle, 6% dimethylsulfoxide in sesame oil (VEH). In adulthood, F1 male and female offspring were bred with untreated partners to generate paternal or maternal lineages of the F2 offspring, the subjects of molecular work. Quantitative real-time PCR was conducted in the medial preoptic area (POA) and the ventromedial nucleus (VMN) of the hypothalamus, selected for their roles in social and sexual behaviors. Of the genes assessed, steroid hormone receptors (estrogen receptor α, androgen receptor, progesterone receptor) but not dopamine receptors 1 and 2 or DNA methyltransferase 3a expression were altered, particularly in the VIN males. Several significant correlations between behavior and gene expression were also detected. These results suggest that preconceptional exposure of male rats to EDCs at the germ cell stage alters the neuromolecular phenotype in adulthood in a lineage-dependent manner.

1. Introduction

The health of humans and wildlife has been permanently altered by environmental chemicals from industry, agriculture, manufacturing, and many other sources. Some of these chemicals are categorized as endocrine-disrupting chemicals (EDCs) because they interfere with hormone action [1]. Polychlorinated biphenyls (PCBs) are a class of legacy EDCs, no longer manufactured but with persistent effects from environmental contamination and subsequent bioaccumulation and biomagnification up the food chain. Although banned since the 1970s in the U.S., recent epidemiological data shows that PCBs are still detectable in human tissue [2] and are associated with impaired reproductive and neurological health in humans [3–6]. Contemporary chemicals such as the common-use fungicide vinclozolin (VIN) also cause impairments in human fertility [7–9] as well as physiology and behavior of various species [10–13].

Several brain regions are sexually dimorphic and organized by endogenous steroid hormones during sensitive developmental phases of early postnatal life. These neural circuits are subsequently activated by hormones of puberty and adulthood that lead to the manifestation of sex-appropriate behaviors and physiology [14,15]. The exquisite sensitivity of the developing brain to hormones means that exogenous EDC exposures may perturb these processes, and increase the predisposition for disease and dysfunction later in life [16], including sexually dimorphic behaviors: juvenile play, adult learning, anxiety, and social and sexual behavior [17–20]. Other studies investigating the molecular and neurobiological substrates underlying these behavioral changes caused by EDCs have reported alterations in metabolic activity, steroid hormone receptor expression, transcriptional activity, and epigenetic marks in the brain and other tissues [12,21–23].

Prenatal EDCs given to the F1 fetus also exposes the germ cells that become the F2 generation. This means that any observed effects of

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EDCs on F2 descendants are presumably due to programming effects during the preconceptional period. In a companion study [24], we reported that in F2 rats, EDCs altered adult physiology, sexual behavior, and ultrasonic vocalizations, the latter important for communicating affective state and social/sexual interest [25–27]. Although the effects varied based on sex and lineage (paternal or maternal descent), male rodents descended from the paternal lineage were particularly vulnerable to EDC disruption.

Here, we examined outcomes of preconceptional exposure to two classes of EDCs: Aroclor 1221, a weakly estrogenic PCB mixture, and VIN, an anti-androgenic fungicide, on the F2 generation. These particular EDCs have been the focus of study in our laboratory and were selected because they act via different hormonal pathways (estrogenic vs. anti-androgenic) and represent different classes of EDCs to which humans and wildlife are exposed today (legacy vs. modern). Our choice of the ventromedial nucleus (VMN) and preoptic area (POA) was based on their roles in the control of sociosexual behaviors [28–31], with genes implicated in these functions.

2. Methods

2.1. EDCs

As described [24], Aroclor 1221 (PCB mixture; AccuStandard, New Haven, CT, C-221N-50MG, 083-166) and Vinclozolin (VIN; Chem Service Inc., West Chester, PA N-13745-250MG, 4054200), each at 1 mg/kg, were dissolved in a vehicle (VEH) of 6% dimethylsulfoxide (Sigma number D4540; Sigma, St Louis, Missouri) in sesame oil. The rationale for dosages was detailed in our companion study [24]; in brief, they were selected to model circulating concentrations of these chemicals in humans, and were used at or below the acceptable daily intake level.

2.2. Animal husbandry and EDC treatments

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin's Institutional Animal Care and Use Committee (IACUC). Sprague-Dawley rats were purchased from Harlan Laboratories (Houston, Texas & Indianapolis, Indiana) and housed in humidity-controlled rooms on a 10:14 partially reversed light cycle (lights off at 11:30 a.m.) and maintained at 21–23 °C. Two to three animals were housed together in polycarbonate cages (43 × 21 × 25 cm) with aspen bedding (PJ Murphy Forest Products, Sani-Chip), with a PVC tube for enrichment. Cages were changed weekly, and rats were fed a low phytoestrogen diet (Harlan-Teklad, Indianapolis, Indiana) ad libitum. These rats were handled once a week for 5 min to acclimate them to the experimenters. Mating began two weeks after their arrival.

The breeding strategy, numbers of litters, and numbers of F2 experimental subjects, is shown in Fig. 1a. Female virgin rats (3–4 months old) were bred with sexually experienced male rats (~6 months old). The day of mating was designated as embryonic day 0 (E0). Following confirmation of the presence of sperm in the vagina, dams were single-housed for the duration of their pregnancy. From embryonic day (E) 8 to E18, dams were weighed daily and injected with ~0.1 ml (based on body weight) of VEH, PCB, or VIN (i.p.). This timeframe encompasses not only the beginning of brain sexual differentiation [32] but also primordial germ cell migration and reprogramming [33].

On the day after birth, the F1 litters were culled to 10 pups of equal sex ratio by euthanizing those with extreme anogenital index measures (AGI (anogenital distance)^3√ body weight) [34]. After weaning on P21, individuals were housed two to three per cage with same-sex littermates.

In adulthood (~P80), two F1 females and two F1 males per litter were bred with untreated stimulus animals (purchased from Harlan) to create the F2 generation. The F2 generation male individuals from both the maternal and paternal lineage were the focus of this experimental design based on our observed alterations in behavior and developmental milestones in this sex. These behavioral data have been published [24], and animals' brains stored as described below. Animals derived from 5 to 8 litters per group, with 1–2 males per litter used for behavior and brain work (Fig. 1a).

2.3. Tissue collection

Approximately two weeks after behavioral characterization was completed (between postnatal days (P) 90–120), experimental males were weighed and euthanized by rapid decapitation. Brains were immediately removed and flash-frozen in isopentane, and stored at −80 °C. Coronal sections were obtained by slicing the brain on a cryostat at 450 μm. Slices were mounted on slides, placed on a freezing stage and allowed to equilibrate to −16 °C. The medial preoptic area (POA) and ventromedial nucleus (VMN) of the hypothalamus were taken bilaterally using a 1 mm punch (Stoelting; Fig. 1b). Tissue punches were placed in 1 ml cold Eppendorf tubes and stored at −80 °C until RNA isolation.

2.4. RNA extraction

Frozen tissue punches were lysed and homogenized using 22 gauge needles and syringes. RNA was extracted using the Denville Scientific Inc. SpinSmart Total RNA Mini Purification kit (CM-610-50, CM-610-250) according to manufacturer instructions. RNA was eluted with 50 μl of nuclease-free water (Applied Biosystems Cat No AM9937). Samples were stored at −20 °C in 66% ethanol and 0.5 M NaCl for 5–7 days before being concentrated. To pellet the RNA, samples were placed in −80 °C for 10 min, then centrifuged at 14000 × *g* for 20 min at 4 °C. The pellet was washed with 70% ethanol and centrifuged again for 10 min, after which the supernatant was discarded. The samples were then dried first by inversion at room temperature for 10 min, then in a speedvac at 43 °C for 5 min. The dried pellets were then resuspended in 12 μl nuclease-free water. RNA quantity was determined by the Nanodrop 2000 Spectrophotometer according to manufacturer instructions. 130–1200 ng of RNA was isolated, and the quality of our samples was assessed by randomly selecting ~10% of our samples to run on the Bioanalyzer 2100 (Agilent RNA 6000 Nano Kit, Cat 5067-1511, Agilent Technologies, Santa Clara, California); all tested samples had an RNA integrity number of 8.5 and above. The small sizes of the dissections limited us to 4 (VMN) or 5 (POA) genes per region for qPCR.

2.5. Gene expression quantification

Using a high-capacity cDNA reverse transcription kit with RNase inhibitor (Life Technologies, Cat. No. 4368814), 170 ng of RNA per sample were converted to cDNA in 20 μl reactions according to manufacturer instructions. Samples went through the following cycles on the Applied Biosystems 2720 Thermocycler: 25 °C for 10 min, then 37 °C for 120 min, and finally 85 °C for 5 min. cDNA was stored at −20 °C for 2–5 days before use. Gene expression primer and probe assays were purchased predesigned from LifeTech to identify genes of interest (FAM/MGB-NFQ, Cat No 4351372) and reference genes (VIC/MGB-NFQ, Cat No 4448489), as shown in Table 1. Assays were prevalidated on a test plate for duplexing to run both target and calibrating genes together. Each sample was run in triplicate, and Taqman Gene expression master mix (Cat No 4369016) was used in a 20 μl reaction with 10 ng of cDNA.

qPCR was conducted on the Applied Biosystems ViiA7 with the following parameters: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. Quantification threshold (Ct) was determined automatically by the ViiA7. *Gapdh* was chosen as a reference gene, based on pilot work showing that this gene is not significantly affected by EDC treatments. Relative expression of targets

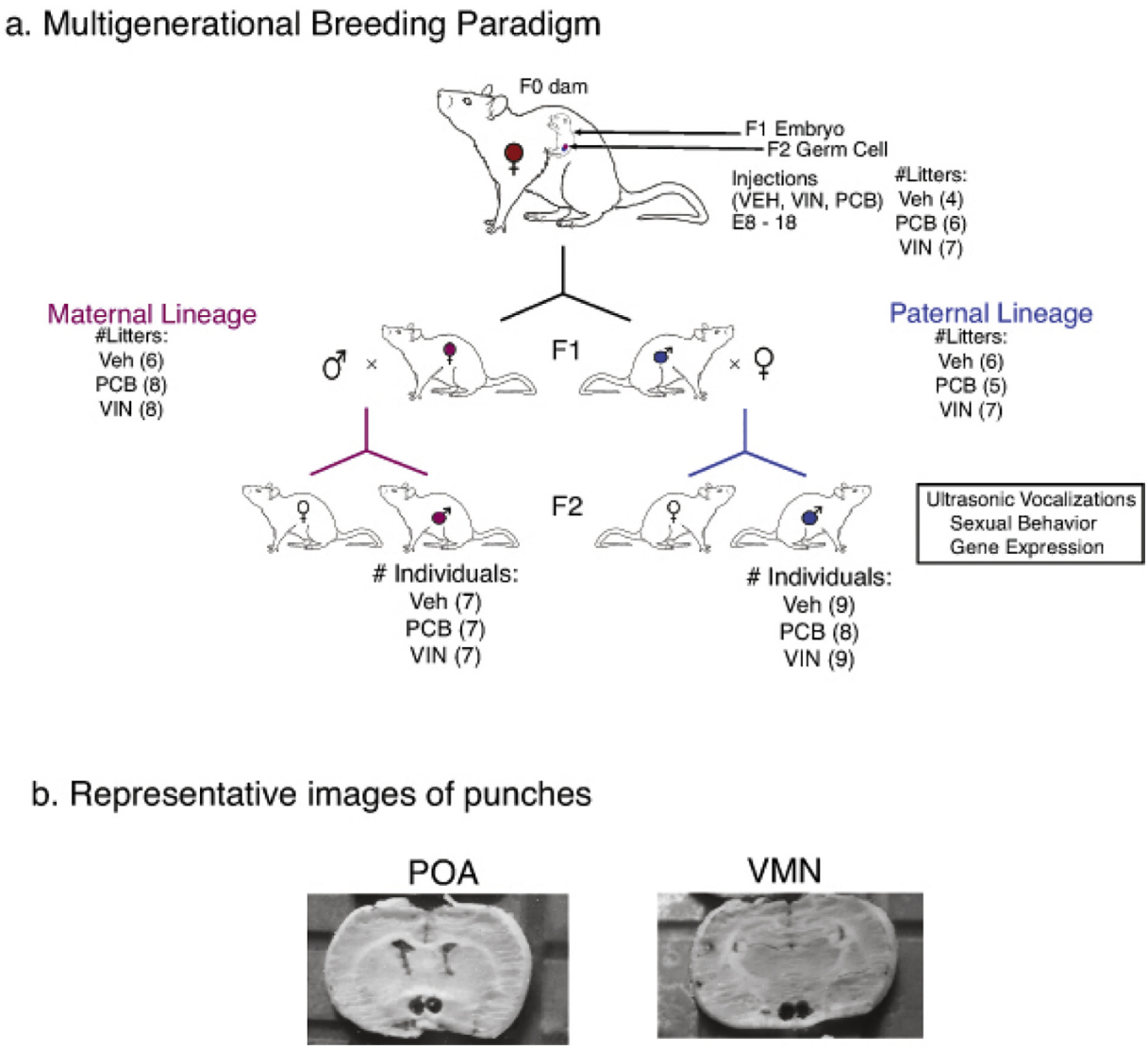

Fig. 1. A) The breeding paradigm and experimental design is shown, beginning with pregnant rats injected with either the vehicle (VEH), vinclozolin (VIN) or Aroclor 1221 (PCB) from E8-18. The F1 female offspring were bred with untreated males to generate a maternal F2 lineage. Similarly, F1 male offspring were bred with untreated females to generate a paternal F2 lineage. The F2 generation was behaviorally characterized, and their brains used in the current study. Numbers of litters are indicated in parentheses for F0 and F1 generations, and numbers of F2 male individuals are indicated in parentheses. B) Photographs of representative punches from the medial preoptic area (POA) and the ventromedial nucleus (VMN) of the hypothalamus are shown.

Table 1
PCR target and assay information.

Gene name (Abbreviation)	Brain region assayed	Life Technologies assay ID
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	POA, VMN	Rn01775763_g1
Androgen receptor (*Ar*)	POA, VMN	Rn00560747_m1
Estrogen receptor alpha (*Esr1*)	POA, VMN	Rn01640372_m1
Dopamine receptor D1 (*Drd1*)	POA	Rn03062203_s1
Dopamine receptor D2 (*Drd2*)	POA	Rn00561126_m1
DNA methyltransferase 3 alpha (*Dnmt3a*)	POA, VMN	Rn01027162_g1
Progesterone receptor (*Pgr*)	VMN	Rn01448227_m1

was calculated using the comparative Ct method: reference Cts were subtracted from target Cts to determine delta Ct within each sample well. Samples were run in triplicate, with triplicate delta Cts averaged together. To normalize the data for each target gene, the median delta Ct (ΔCt) of all VEH males (maternal and paternal lineages) was calculated and subtracted to determine ΔΔCt. From this, fold change in gene expression for each individual was calculated as $2^{-\Delta\Delta Ct}$.

2.6. Analysis and statistics

Litter was tested as a covariate, and since no effects were found, we used individual rats (n = 7–9 per group) as the unit of statistical analysis (Fig. 1a). The Grubb's test was used to determine significant outliers, and the number of outliers removed was limited to two per group. Gene expression measures were analyzed using a factorial analysis of variance (ANOVA) to determine main effects of lineage or treatment as well as interactions. Main effects and interactions were investigated using Tukey's HSD post-hoc tests, since they correct for multiple comparisons. The data were tested initially for normality and homoscedasticity using the Shapiro-Wilk and Bartlett's test, respectively. All data presented in this study were normally distributed and homoscedastic. Effect sizes for each factor of the ANOVAs were calculated as partial eta-squared (η_p^2), which represents the proportion of variance that is accounted for by the factor being tested. Effect sizes of 0.14 or greater are considered to be large, 0.06 to 0.13 medium, and below

0.06 small. Significance was determined at $p < .05$.

2.6.1. Gene-behavior-hormone correlations

Prior to tissue collection, F2 males had been assessed for ultrasonic vocalizations, mating behavior, and serum hormone levels, reported in [24]. A principal components analysis to determine the underlying factors contributing to the variance within each behavior showed that EDC exposure affected the acoustic properties and number of vocalizations, latency to and frequency of mating behaviors, and concentrations of serum estradiol and testosterone in these males. That study showed that PCB males from the paternal lineage in particular were most affected. These data were used here in Pearson correlations to determine relationships between gene expression with serum estradiol and testosterone, and the principal components of each behavioral outcome. Because a large number of behaviors were measured, a behavior score transformed by the eigenvectors of the principal components was calculated and used for correlations with serum hormones (estradiol, testosterone) and with gene expression. All endpoints were selected for analysis according to a priori hypotheses, and accordingly, significance levels were not adjusted.

3. Results

3.1. POA gene expression

Significant EDC treatment effects were found for the two steroid hormone receptors (Fig. 2). For *Ar,* a main effect of treatment was found in F2 males ($F_{2,\ 43} = 4.30$, $p = .02$, $\eta_p^2 = 0.17$; Fig. 2a), with PCB animals significantly lower than VIN animals ($p = .005$). A treatment × lineage interaction was also found for *Ar* ($F_{2,\ 43} = 6.44$, $p = .004$, $\eta_p^2 = 0.23$). VEH males from the paternal lineage had higher *Ar* expression than the maternal lineage ($p = .05$). By contrast, maternal VIN animals had higher *Ar* expression than paternal lineage VIN males ($p = .02$). Within the maternal lineage, *Ar* was higher in VIN than VEH ($p = .02$), and lower in PCB than VIN ($p < .001$) rats.

Esr1 expression had a significant treatment × lineage interaction ($F_{2,\ 36} = 6.25$, $p = .005$, $\eta_p^2 = 0.26$; Fig. 2b). *Post-hoc* analysis showed that maternal PCB males had significantly lower *Esr1* expression than paternal PCB males ($p = .01$). An opposite pattern (MAT > PAT) was found in the VIN males ($p = .05$). Within the maternal lineage, *Esr1* was significantly higher in VIN than PCB males ($p = .04$).

The dopamine receptors 1 and 2 (*Drd1, Drd2*), and DNA methyltransferase 3a (*Dnmt3a*) genes, were unaffected by treatment or lineage in the POA (Fig. 2c, d, e).

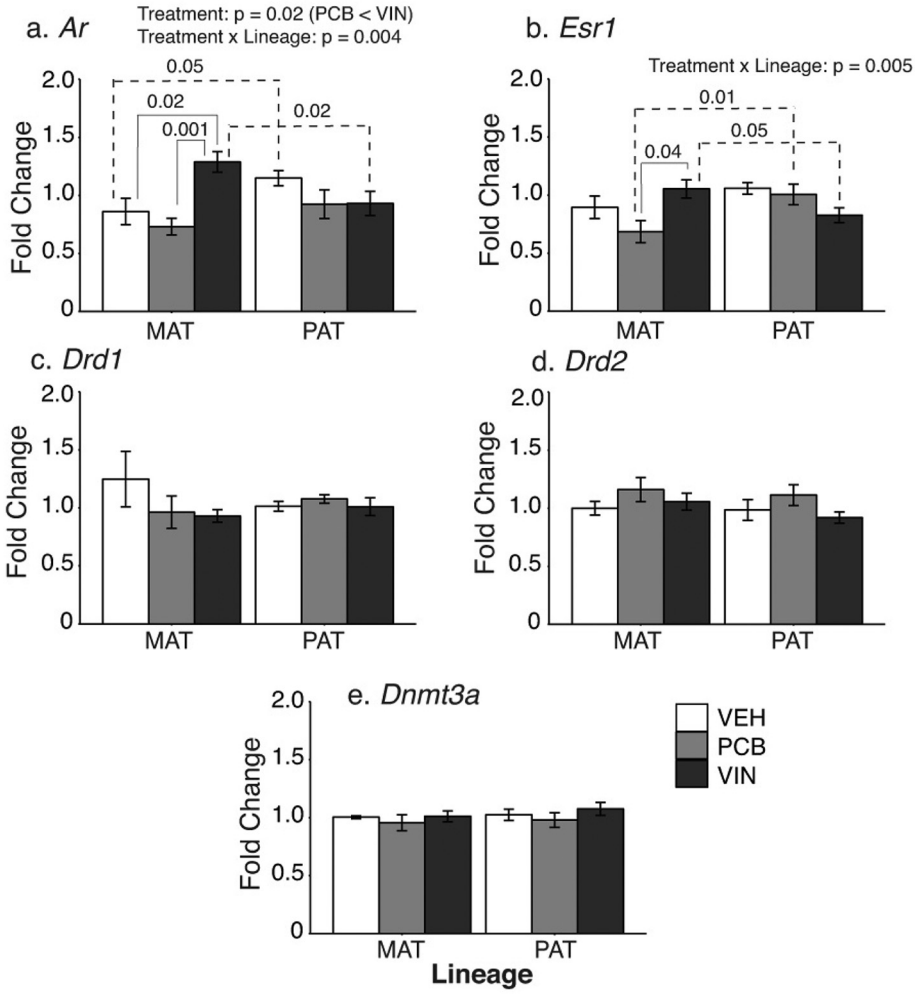

Fig. 2. POA gene expression results are shown for a) *Ar*, b) *Esr1*, c) *Drd1*, d) *Drd2* and e) *Dnmt3a.* Of these, *Ar* and *Esr1* expression were significantly changed, with main effects and interactions indicated. Significant treatment differences within a lineage are indicated with solid brackets; significant differences between lineages are indicated with dashed brackets. Data are graphed as mean ± SEM.

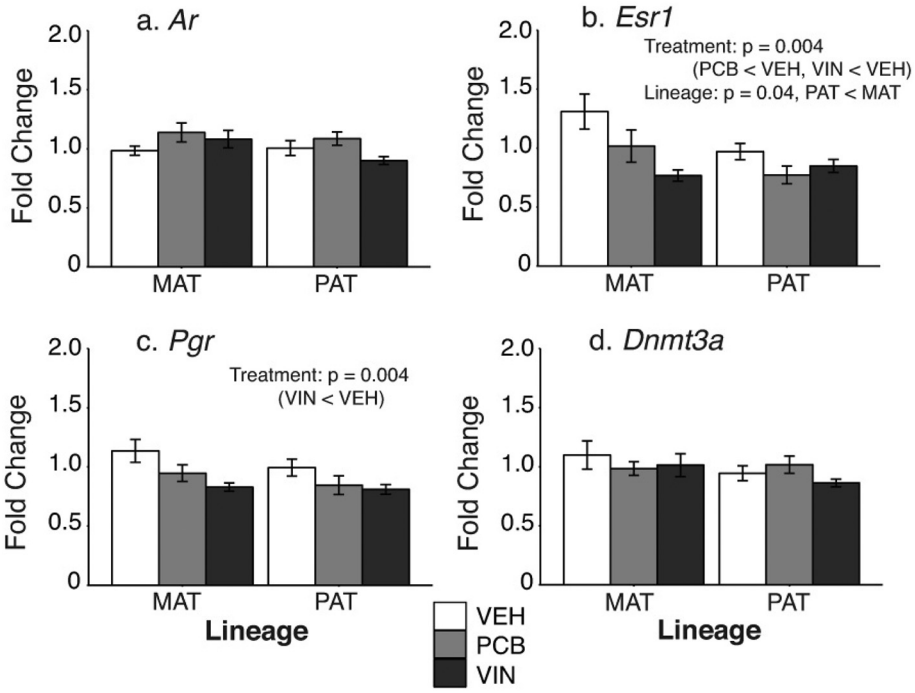

Fig. 3. VMN gene expression results are shown for a) *Ar,* b) *Esr1*, c) *Pgr,* and d) *Dnmt3a*. *Esr1* had significant main effects of both treatment and lineage, and *Pgr* had a significant treatment effect. *Ar* and *Dnmt3a* were unaffected. Significant treatment differences within a lineage are indicated with solid brackets. Data are graphed as mean ± SEM.

3.2. VMN gene expression

In the VMN, *Ar* and *Dnmt3a* were unaffected by treatment or lineage (Fig. 3a, d). *Esr1* was significantly affected by treatment ($F_{2,\ 42} = 6.49$, $p = .004$, $\eta_p^2 = 0.24$; Fig. 3b). *Post-hoc* tests showed that both PCB ($p = .01$) and VIN ($p = .001$) males had significantly lower *Esr1* expression than VEH males. There was also a main effect of lineage ($F_{2,\ 42} = 4.71$, $p = .04$, $\eta_p^2 = 0.10$), with males from the paternal lineage having lower *Esr1* than those in the maternal lineage ($p = .04$).

Pgr expression in the VMN was significantly affected by treatment ($F_{2,\ 41} = 6.43$, $p = .004$, $\eta_p^2 = 0.23$; Fig. 3c). Post hoc tests revealed that VIN males had significantly lower *Pgr* than VEH males ($p = .02$).

3.3. Correlations

In [24], we examined the behavioral and physiological phenotype of these same F2 male rats, investigating serum hormone concentrations (estradiol, testosterone) and communicative and reproductive behaviors. A principal components analysis determined the primary underlying factors in each behavior, and we subsequently analyzed the data transformed by the eigenvectors of the principal components (PC) for treatment differences. The following measures were significantly affected in adult males [24] and included in the Pearson's correlation analyses: 1) ultrasonic vocalization (USV) acoustic properties, 2) total USV call counts, 3) the latency to engage in sex behavior, 4) female-elicited sex behavior, and 5) circulating serum estradiol and 6) testosterone concentrations.

To determine whether the observed molecular changes in the POA and VMN could be responsible for EDC-induced behavioral and physiological changes in males, we conducted pair-wise Pearson's correlations between expression of significantly affected genes (POA: *Ar, Esr1*; VMN: *Esr1, Pgr*) with serum hormones (estradiol, testosterone) and the eigenvector-transformed score of the principal components of the behaviors (Table 2).

3.3.1. Serum hormones-gene expression correlations

Circulating estradiol levels showed a few significant correlations in EDC groups relative to the VEH group (Table 2). In the POA, circulating estradiol concentrations were negatively correlated with *Ar* in maternal VIN males ($r = -0.89$, $p = .008$), with no relationship in the VEH group. In the VMN, *Pgr* and estradiol were negatively correlated in both maternal VIN males ($r = -0.82$, $p = .03$) and in paternal VEH males ($r = -0.74$, $p = .04$). For testosterone, only one correlation was found. In the maternal PCB lineage males, testosterone was negatively correlated with *Esr1* in the POA ($r = -0.78$, $p = .04$), with no such relationship observed in the VEH group (Table 2).

3.3.2. Sex behavior-gene expression correlations

Few significant correlations were found between gene expression and male sexual behavior (Table 2). In paternal VIN males, there was a significant positive correlation between POA *Ar* and female-elicited sex behavior ($r = 0.74$, $p = .04$; Fig. 4a). Similarly, maternal VIN males showed a positive correlation in the POA ($r = 0.77$, $p = .04$, Fig. 4a). By contrast, neither paternal nor maternal VEH or PCB males had significant correlations between *Ar* and these behaviors.

Maternal VEH males had a significant positive correlation between *Esr1* in the VMN, and latency to sex behavior ($r = 0.79$; $p = .03$; Table 2), while the EDC males had no significant relationship.

3.3.3. Ultrasonic vocalizations-gene expression correlations

Several correlations were found between USVs and gene expression (Table 2). *Esr1* in the POA of paternal lineage VEH and PCB males was significantly positively correlated with total USV call counts (VEH: $r = 0.75$, $p = .03$; PCB: $r = 0.83$, $p = .02$; Fig. 4b), while VIN males had no significant relationship. In the VMN, paternal PCB males showed a significant positive correlation between *Esr1* and total USV counts ($r = 0.83$, $p = .02$; Fig. 4c), an effect not observed in VEH males.

4. Discussion

The results of this study provide novel information about how preconceptional EDCs reprogram the molecular phenotype of the POA and VMN of F2 male rats, and relationships to hormones and behaviors that had previously been characterized in these animals. The basis of this present work was that numbers and characteristics of USVs, as well as dyadic sexual interactions between the males with untreated female rats, differed substantially between EDC and vehicle F2 males, especially of the paternal lineage [24]. This led us to choose the POA and

Table 2
F2 male gene-behavior-hormone correlation.

Group		Behavior				Serum hormones	
Maternal VEH		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	0.00	0.47	−0.27	0.45	0.45	−0.50
	Esr1	−0.25	0.11	0.59	0.08	0.11	0.25
VMN	Esr1	0.01	−0.74	0.79*	0.04	−0.38	0.21
	Pgr	0.13	−0.19	0.26	0.75	0.20	−0.38
Maternal PCB		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	−0.42	−0.36	0.08	−0.26	0.28	−0.25
	Esr1	−0.24	−0.20	−0.12	0.47	−0.31	−0.78*
VMN	Esr1	−0.27	−0.08	0.24	0.36	0.18	−0.61
	Pgr	−0.15	0.03	0.42	0.31	0.35	−0.16
Maternal VIN		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	−0.33	0.14	−0.63	0.77*	−0.89**	0.31
	Esr1	0.21	0.22	−0.54	0.36	−0.62	0.35
VMN	Esr1	0.32	0.26	−0.42	−0.04	0.06	0.10
	Pgr	0.03	0.44	−0.70	0.75	−0.82*	0.18
Paternal VEH		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	0.23	0.51	−0.10	−0.37	−0.49	−0.10
	Esr1	0.02	0.75*	0.21	−0.01	−0.68	−0.13
VMN	Esr1	−0.33	0.19	0.68	0.19	−0.21	0.47
	Pgr	0.39	0.65	−0.23	−0.02	−0.74*	−0.56
Paternal PCB		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	0.60	0.07	0.52	−0.40	0.01	−0.48
	Esr1	0.62	0.83*	0.05	−0.48	0.34	−0.23
VMN	Esr1	0.30	0.82*	−0.55	−0.48	0.42	0.28
	Pgr	0.48	0.33	0.34	−0.36	0.10	−0.74
Paternal VIN		USV acoustic properties	Total USV call counts	Latency to sex behavior	Female-elicited sex behavior	Estradiol	Testosterone
POA	Ar	−0.43	−0.34	−0.70	0.74*	−0.19	−0.29
	Esr1	−0.48	−0.25	−0.44	0.48	−0.09	−0.16
VMN	Esr1	−0.24	−0.12	0.16	−0.07	0.26	0.24
	Pgr	0.13	−0.51	0.20	−0.03	−0.10	−0.09

Correlations were conducted for each treatment and lineage between behaviors (left) and hormones (right) and gene expression in the F2 male rats. The correlation coefficient is shown. Significant correlations are indicated with asterisks as $_{*}p < .05$, $_{**}p < .01$.

VMN as regions of interest based on their roles in sociosexual behaviors and as part of the USV neural pathway [35–37]. Here, to determine the underlying molecular phenotype, we selected as our gene targets steroid hormone receptors (*Ar*, *Esr1, Pgr*), dopamine receptors 1 and 2, and the epigenetic modifier DNA methylation transferase 3a.

4.1. Lineage is important to intergenerational effects of EDCs

The lineage of the F2 males – maternal or paternal – was revealed as a key factor in determining behavioral and molecular phenotypes in the current and companion study [24]. Lineage is rarely considered as a variable in most other EDC studies due to methodological approaches such as sibling or same-treatment breeding (i.e., EDC animals bred with similarly treated EDC animals), or focus on a single lineage (e.g. paternal [21] or maternal [38–40], but not both).

In our work, we specifically isolated maternal and paternal lineages by breeding our experimental progenitors with untreated partners. Our results showed that expression of *Esr1* (POA, VMN) and *Ar* (POA) differed by lineage, as did certain behaviors in these rats [24]; these data will be discussed in more detail below. Notably, our lab has published evidence for lineage differences in both EDC- and vehicle-treated animals [24,41,42]. These lineage differences are biologically plausible and may be attributable to at least 6 possible factors, none mutually exclusive. First, maternal and paternal F2 lineages differ due to the genotype (XX vs. XY) of the F1 parents. Second, maternal stress may occur in the F0 dams due to the EDC or vehicle injections; this may differentially influence outcomes in F1 male and female offspring. Third, the epigenetic state of the F2 generation germ cells within the F1 embryos differs in the timing of demethylation and remethylation during the exposure period (E8 to 18), with these processes completed in males by birth, but incomplete until puberty in females [43]. Fourth, the gonadal steroid hormone milieu of the F1 progenitors in utero differed quite profoundly with sex (male rat gonads have more active steroidogenesis than those of females), and this influences exposure of the F2 germ cells to differential hormones [32]. Fifth, behaviors of the F1 mothers to their offspring may differ. The F1 maternal lineage dams received exposure to EDCs or vehicle, whereas the F1 paternal lineage dams were untreated, and bred with F1 EDC males. Sixth, information may be imparted by sperm via microRNAs, exosomes, epigenetic factors, and others, that differ between F1 EDC males of the paternal lineage (exposed) vs. F1 male partners (unexposed) to the F1 EDC females [44–46]. With respect to the F2 offspring, these differences in genetics, epigenetic programming, behavior, hormones, and other factors would play out as lineage differences.

4.2. Preconceptional effects of EDCs on gene expression in the POA

In the POA, preconceptional VIN significantly affected expression of *Ar,* with VIN males of the maternal lineage having higher expression than both VEH and PCB males. These differences were not found in the paternal lineage, illustrating the point raised above that lineage is a key factor in determining outcomes. Furthermore, specific lineage differences in *Ar* were observed in the VEH and VIN groups, albeit in opposite directions. Other studies on EDCs have demonstrated that the *Ar* is affected by prenatal EDCs [47], but to our knowledge this has not been studied in the F2 generation. The other steroid hormone receptor measured in the POA, *Esr1,* was significantly affected by the interaction of treatment and lineage in the POA of F2 males, due to differences

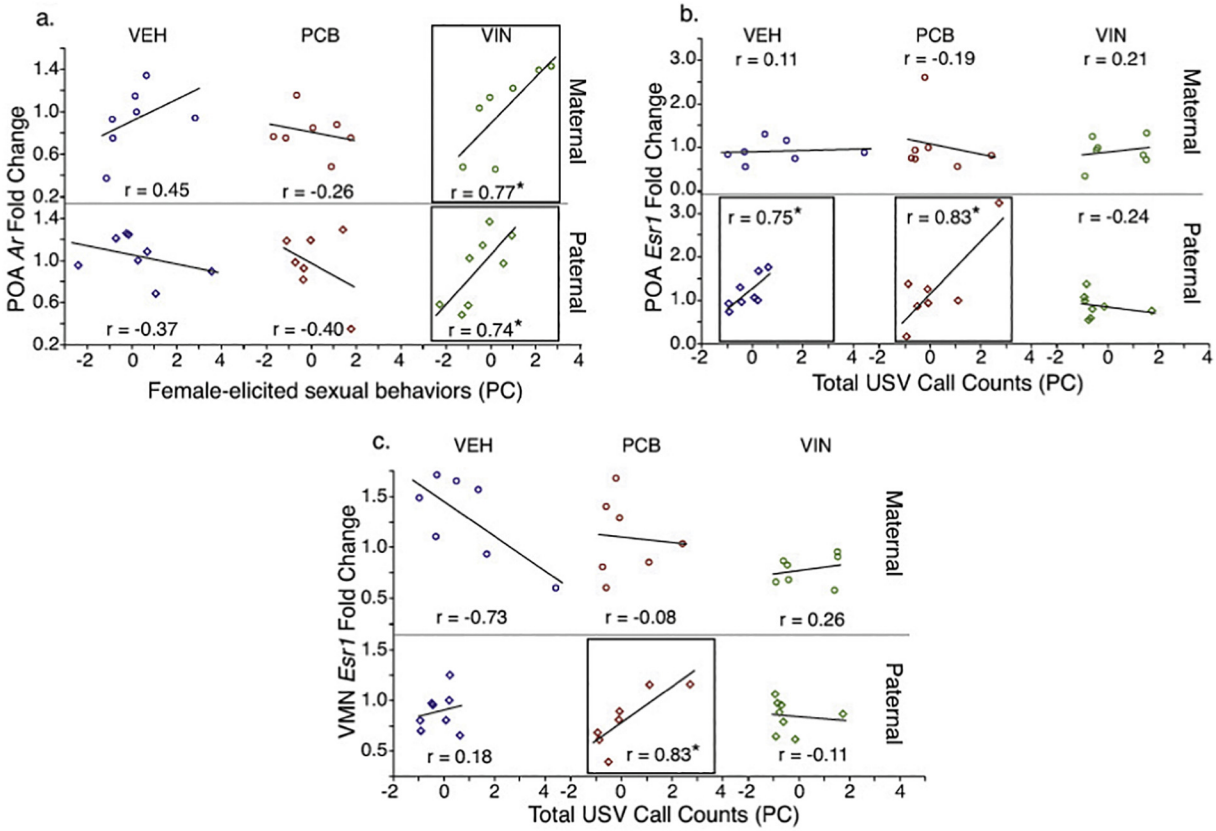

Fig. 4. Within-animal Pearson r correlations between F2 male behaviors and gene expression for select measures are shown separately for each EDC. Eigenvalues for behaviors that were altered by EDC exposure in F2 males were correlated with POA and VMN gene expression levels. Shown are: (a) POA *Ar* expression and female-elicited sex behaviors, (b) POA *Esr1* expression and total USV call counts, and (c) VMN *Esr1* expression and total USV call counts. Note the different y-axis scales between graphs. Those correlations that are significant at $p < .05$ are boxed and indicated by *. Pearson r correlation coefficients are provided for all graphs.

between PCB and VIN groups in the maternal lineage, and lineage effects in the PCB and VIN groups. The lack of treatment effect of PCBs on hypothalamic *Ar* and *Esr1* differs from studies on F1 rats showing effects of PCBs [19,47,48]. It is unsurprising that F1 and F2 generations would differ based on their very different life stages during exposure. In addition, the PCB mixture we selected (Aroclor 1221) is mainly weakly estrogenic, but it also has anti-estrogenic and other properties that could affect gene expression outcomes [49].

Neither *Drd1* nor *Drd2* were altered by EDC exposure in the POA. These gene targets had been selected because D_1 and D_2 receptor activation in the POA have opposing actions on male sexual behavior ([50]; reviewed in [35]). Furthermore, the mesolimbic dopamine system is heavily implicated in the rewarding and appetitive aspects of 50 kHz USV calls [51], which were significantly lower in paternal lineage PCB males (as reported in our companion study [24]).

Early life perturbations can program the brain by modifying DNA methylation patterns [52–55]. PCB exposure in rats decreased global DNA methylation [56] and histone modification enzymes in the liver [57], but did not affect methylation of DNA repeats in the liver, spleen or thymus [58]. In our study, we measured mRNA levels of *Dnmt3a*, a DNA methyltransferase involved in de novo DNA methylation, and did not observe any effects of treatment or lineage. Other parts of the DNA methylation machinery, or additional epigenetic processes such as histone modifications to maintain methylation levels, or microRNAs to induce translational repression or degradation [59,60], should be investigated in future work. We reported that prenatal PCB exposure altered microRNA expression in the F1 adult male POA, and that these microRNAs target genes belonging to the nuclear hormone receptor family [47]. In fact, prenatal exposure of F1 male mice to a similar dose of VIN resulted in alterations in microRNA that are involved in F2 primordial germ cell differentiation [61], suggesting that these epigenetic factors could also play a role in the transmission of EDC effects across generations.

4.3. Preconceptional effects of EDCs on gene expression in the VMN

The VMN is a heterogeneous hypothalamic region abundant in steroid hormone receptors, and while best studied for its roles in sexual behavior in females, it also plays important roles in copulation and ultrasonic vocalizations in males [37,62–64]. In this region, there were significant effects of treatment on both *Esr1* and *Pgr*, which were lower in VIN than VEH F2 males. PCB males also had lower *Esr1* than VIN males. There was a main effect of lineage observed.

Esr1 in the VMN, especially the ventrolateral compartment, plays important roles in aggressive behaviors [65,66], as well mounting, sniffing, and close investigation behaviors in male mice [66]. Our previous study on F2 male rat behaviors demonstrated treatment- and lineage-specific effects on USV call numbers and frequency, intromission frequency, latency to first ejaculation, and behaviors received from the females [24], behaviors which may relate to the changes in *Esr1* in the VMN. Similarly, *Pgr* in the VMN, which was decreased by VIN, plays roles in masculine behaviors. Ablation of *Pgr*-positive cells in this region reduces mating and aggression of male mice, especially the consummatory aspects [65].

4.4. Relationships among hormones, behaviors and genes

Significant correlations with gene expression were found in a treatment- and lineage-specific manner, especially for USV call counts, female-elicited sex behavior, and serum estradiol concentrations. In the

maternal lineage, there were five significant correlations, three in the maternal VIN males (estradiol and *Ar* in the POA, estradiol and *Pgr* in the VMN, female-elicited behavior and *Ar* in the POA). In addition, *Esr1* was correlated with latency to sex behavior in the VMN of maternal VEH males, and testosterone was negatively correlated with *Esr1* in the POA of maternal PCB males. Two points are notable. First, correlations with genes were limited to *Esr1, Pgr,* and *Ar,* the three steroid hormone receptors. Second, most of the correlations were in EDC but not VEH males, and suggestive of the emergence of relationships in F2 descendants of EDC-exposed rats.

The paternal F2 males had five correlations, three of which were between *Esr1* and USV call counts. Although previous studies have found that VIN upregulates *Esr1* expression across multiple generations [21,67], our study did not observe the same pattern. Since *Esr1* expression is necessary for sociosexual behaviors such as ultrasonic vocalizations [68], and the upregulation of *Esr1* was not observed in our VIN animals, it would follow that there would be no relationship between the steroid hormone receptor and the USV behavior. However, we are careful not to over-interpret these relationships, as the VIN treatment was given to individuals two generations removed from the subjects of the gene expression analysis and the behavioral characterization.

One significant positive correlation, between *Ar* in the POA and female-elicited sex behaviors, was common to VIN males of both lineages. Based on our previous study [24], we speculate that this decrease could be related to the decreased number of lateral kicks received by the VIN male, which are often interpreted as rejection behaviors, and/or to the increased intromission frequency. Androgen receptor activation in the VMN and POA is necessary for copulatory behaviors [37,62], suggesting that the increased frequency of male sexual behavior (reported in our companion study) and subsequent female-elicited behavior could be driven by VIN mediated changes in *Ar* expression.

4.5. Conclusion

This study provides novel evidence that hypothalamic gene expression in F2 generation male rats was affected by preconceptional exposure to PCBs or VIN, in a lineage-dependent manner. We were limited to relatively few gene targets due to small sample size and limited RNA; future work should focus on other molecular targets that might underlie the observed changes in behavior. Furthermore, there are many brain regions beyond the POA and VMN that are part of the neural network that regulates sexual behavior, ultrasonic vocalizations, and the integration of rewarding inputs. Therefore, future work should include other brain regions of interest, and take a broader approach to gene expression (e.g. RNA sequencing) to explore the underlying neuromolecular targets of EDC disruption.

Our period of EDC administration was selected because it encompasses that of demethylation and remethylation of DNA in F2 male germ cells. Beyond DNA methylation, other epigenetic mechanisms such as histone modifications that affect chromatin state, and microRNA and other non-coding RNAs, might also be affected by EDCs during this sensitive developmental phase. Differences between maternal- vs. paternal-lineage males indicate that the sex of the F1 fetus, behavior of the F1 parent to the F2 offspring, and other mechanisms are important factors to be considered in future work. As a whole, these results demonstrate the need for toxicological testing to take epigenetic and non-epigenetic mechanisms of EDC transmission into account when considering the health of future generations of wildlife and humans.

Disclosure statement

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