Molecular and Cellular Endocrinology 479 (2019) 133–146

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Social and neuromolecular phenotypes are programmed by prenatal exposures to endocrine-disrupting chemicals

Viktoria Y. Topper[a,1], Michael P. Reilly[b,1], Lauren M. Wagner[b], Lindsay M. Thompson[b], Ross Gillette[a], David Crews[a,c], Andrea C. Gore[a,b,*]

[a] The Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA
[b] Division of Pharmacology and Toxicology, The University of Texas at Austin, Austin, TX 78712, USA
[c] Department of Integrative Biology, The University of Texas at Austin, Austin, TX 78712, USA

ARTICLE INFO

Keywords:
Endocrine-disrupting chemicals (EDCs)
Polychlorinated biphenyls (PCBs)
Aroclor 1221
Ultrasonic vocalization
Sociosexual behavior
Sex difference
Gene expression
Hypothalamus
VMN
MPN

ABSTRACT

Exposures to endocrine-disrupting chemicals (EDCs) affect the development of hormone-sensitive neural circuits, the proper organization of which are necessary for the manifestation of appropriate adult social and sexual behaviors. We examined whether prenatal exposure to polychlorinated biphenyls (PCBs), a family of ubiquitous industrial contaminants detectable in virtually all humans and wildlife, caused changes in sexually-dimorphic social interactions and communications, and profiled the underlying neuromolecular phenotype. Rats were treated with a PCB commercial mixture, Aroclor 1221 (A1221), estradiol benzoate (EB) as a positive control for estrogenic effects of A1221, or the vehicle (4% DMSO), on embryonic day (E) 16 and 18. In adult F1 offspring, we first conducted tests of ultrasonic vocalization (USV) calls in a sociosexual context as a measure of motivated communications. Numbers of certain USV call types were significantly increased by prenatal treatment with A1221 in males, and decreased by EB in females. In a test of sociosexual preference for a hormone-vs. a non-hormone-primed opposite sex conspecific, male (but not female) nose-touching with opposite-sex rats was significantly diminished by EDCs. Gene expression profiling was conducted in two brain regions that are part of the social decision-making network in the brain: the medial preoptic nucleus (MPN) and the ventromedial nucleus (VMN). In both regions, many more genes were affected by A1221 or EB in females than males. In female MPN, A1221 changed expression of steroid hormone receptor and neuropeptide genes (e.g., *Ar, Esr1, Esr2,* and *Kiss1*). In male MPN, only *Per2* was affected by A1221. The VMN had a number of genes affected by EB compared to vehicle (females: *Kiss1, Kiss1r, Pgr;* males: *Crh*) but not A1221. These differences between EB and A1221 indicate that the mechanism of action of A1221 goes beyond estrogenic pathways. These data show sex-specific effects of prenatal PCBs on adult behaviors and the neuromolecular phenotype.

1. Introduction

Polychlorinated biphenyls (PCBs) are a family of industrial chemicals that were banned 40 years ago in the U.S., yet are still highly relevant to humans and wildlife due to their environmental persistence. The Centers for Disease Control of the U.S. and the Canadian Health Measures Survey have detected PCB congeners in tissues of virtually all people (Haines and Murray, 2012; Xue et al., 2014; Zong et al., 2015). Similar results were made for wildlife, especially apex predators [bald eagles (Elliott et al., 2009), polar bears (Bytingsvik et al., 2012), and orcas and dolphins (Jepson et al., 2016)]. In Antarctic icefish, PCB concentrations are actually higher today than 20 years ago, presumably due to "increased volatilization and release of persistent organic pollutants (POPs) trapped in glaciers, sea- or pack-ice, thereby leading to an ongoing contamination of the Southern Ocean and its biota" (Strobel et al., 2016). This phenomenon is particularly alarming in light of climate change effects on ice depots and the potential for chemicals to be liberated and enter the food chain, resulting in biomagnification and bioaccumulation.

As endocrine-disrupting chemicals (EDCs), PCBs perturb or mimic hormone actions through their interactions with nuclear hormone receptors and enzymes involved in steroidogenesis [reviewed in (Dickerson and Gore, 2007; Gore et al., 2015)]. PCBs are also known neurotoxicants, acting upon several neural signaling systems

* Corresponding author. University of Texas at Austin, 107 W. Dean Keeton, C0875, Austin, TX, 78712. USA.
E-mail address: andrea.gore@austin.utexas.edu (A.C. Gore).
[1] Co-first authors.

https://doi.org/10.1016/j.mce.2018.09.010
Received 6 July 2018; Received in revised form 25 August 2018; Accepted 28 September 2018
Available online 01 October 2018
0303-7207/

(dopamine, glutamate, calcium channels, etc.) (Gore et al., 2015). The brain's neuroendocrine systems are therefore highly vulnerable targets of EDCs, as neurons of the hypothalamus expresses steroid hormone receptors and steroidogenic enzymes, and actions of both endogenous hormones and EDCs converge on steroid-sensitive hypothalamic neurotransmitter systems [reviewed in (Walker and Gore, 2017)]. These neurobiological effects of EDCs in laboratory studies are reflected by epidemiological data showing associations between PCB body burden, cognition, and neurobehavioral problems (Bouchard et al., 2014; Grandjean and Landrigan, 2006; Winneke et al., 2014).

While the body and brain are vulnerable to EDCs at any life stage, exposure during late gestation and early postnatal life is particularly problematic. This is a critical period of brain development and sexual differentiation of the hypothalamus, during which sex differences in endogenous ovarian or testicular hormones set the trajectory of the developing brain in a male- or female-typical direction. Exposures to PCBs during these life stages (Bell et al., 2016a, 2016b; Dickerson et al., 2011a, 2011b; Reilly et al., 2015), as well as to other EDCs such as bisphenol A (BPA) and vinclozolin (Colbert et al., 2005; Jasarevic et al., 2011; Ogi et al., 2013) cause organizational, structural, and molecular changes to the hypothalamus that lead to behavioral abnormalities later in life. Here, we conducted sexually-dimorphic behavioral tests of communication and social behavior in laboratory rats, together with neuromolecular profiling of hypothalamic genes, to determine the effects of ecologically-relevant exposures to an industrial PCB mixture, Aroclor 1221 (A1221). The medial preoptic nucleus (MPN) and ventromedial nucleus (VMN) of the hypothalamus were selected because of their roles as key nodes in the regulation of social and sexual behaviors (Gore et al., 2018; Hoshina et al., 1994; Malsbury et al., 1977; Mathews and Edwards, 1977; O'Connell and Hofmann, 2012).

2. Materials and methods

2.1. Experimental rats

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas at Austin. Adult (two-three months of age) virgin female (n = 40) and male (n = 15) Sprague-Dawley rats were purchased from Harlan Laboratories (Houston, TX). On arrival, animals were housed in same-sex groups, 2–3 animals per cage and provided low phytoestrogen Harlan-Teklad Extruded 2019 Global Rodent diet and water *ad libidum*. Animals were acclimated to laboratory housing conditions: temperature (21–22 °C) with a partially reversed 12:12 light cycle (lights on at 12:00 a.m.). After two weeks of vaginal smearing to confirm regular estrous cycles, the female rats were mated with the males from the same cohort. Their offspring (F1 generation) were the experimental animals, exposed prenatally to PCBs or vehicles.

2.2. EDC treatments

Breeding pairs were established, and the day after mating (confirmed by presence of sperm in the vagina) was designated as embryonic day 1 (E1). On E16 and 18, dams were weighed and randomly assigned to one of four treatment groups and injected intraperitoneally with either 0.1 ml of vehicle (4% dimethylsulfoxide (DMSO), catalog no. D4540; Sigma-Aldrich, St. Louis, MO; diluted in sesame oil, catalog no.156621, MP Biomedicals, Carlsbad, CA), 50 µg/kg estradiol benzoate (EB; catalog no. E8515; Sigma-Aldrich) as a positive estrogenic control, 0.5 mg/kg A1221 [henceforward A1221 (0.5)] or 1 mg/kg A1221 [henceforward A1221 (1.0); catalog no. C-221N, AccuStandard, New Haven, CT]. The dosages are estimated to be within the range of PCB body burdens found in humans (Fitzgerald et al., 2008). Previous work in the laboratory using these dosages showed they are nontoxic to dams and do not cause fetal loss (Dickerson et al., 2011b; Steinberg et al., 2007). On the day after birth [postnatal day (P) 1], litter composition was recorded and the litters culled to equal sex ratios of 4 males and 4 females (8 pups) per litter or as close as possible. Pups were weaned at P21 and rehoused 4 per cage in same-sex groups. At P49 they were split into two cages of same-sex dyads. Rats were monitored daily for signs of pubertal development: vaginal opening (VO) in females and preputial separation (PPS) in males (Steinberg et al., 2007; Walker et al., 2012). Following VO, daily vaginal smears were taken and cell cytology examined as a measure of estrous cyclicity in the females. There were 10 litters per treatment, with 1 male and 1 female per litter contributing to behavioral and molecular endpoints. Thus, the litter was the unit of analysis.

2.3. Stimulus rats

An additional set of adult rats was purchased and used as stimulus rats. For the ultrasonic vocalization experiment, female stimulus rats were ovariectomized (OVX) and implanted with an estradiol (E2) capsule during the OVX surgery, per standard lab protocols (Garcia et al., 2017a). On the day of testing they were injected with progesterone (0900) to induce sexual receptivity ~4–6 h later. Male stimulus rats were sexually experienced intact males. For the sociosexual preference experiment two novel gonadectomized opposite-sex animals were used. Experimental males were given a choice between two OVX females, one with an E2 capsule, the other without hormone. Experimental females were given a choice between castrated males, one implanted with a testosterone (T) capsule, the other without hormone, per lab protocols (Wu and Gore, 2010).

2.4. Behavior testing

Beginning at P60, experimental males and females were tested first in an ultrasonic vocalization (USV) test followed by sociosexual behavioral test. Testing was conducted under dim red light, 2–6 h after lights off, with experimental females tested on proestrus when confirmed sexually receptive. Due to the large number of animals necessary for the study, the animals were tested in eight cohorts of 10 animals each with treatments equally represented across each cohort. The entire study was conducted with the observer blind to experimental rats' treatments. Animals were coded, and the code broken only after all data analysis was completed.

Ultrasonic vocalizations (USVs): USVs were recorded over 3 consecutive days in a sound-attenuated Plexiglas apparatus, using a chamber (23l X 29w X 40 h cm) divided into two equal compartments by a wire mesh, following published protocols (Garcia et al., 2017a, 2017b). To habituate the rat, on the first two days the experimental rat was placed alone into the apparatus, and baseline USVs were recorded for 10 min using a CM16/CMPA microphone (Avisoft, Germany). Because of the low number of calls emitted by rats during the baseline, we did not include the baseline data in the analysis. On the third day, the rat was returned to the chamber and an unfamiliar opposite-sex stimulus animal was placed on the other side of the wire mesh. After 5 min, the stimulus animal was removed and USVs recorded from the experimental animal for 10 min. This protocol reliably elicits high levels of callings in the animal left in the chamber(McGinnis and Vakulenko, 2003), and thus the USVs on day 3 were the focus of analysis. USVs were recorded with UltraSoundGate hardware at a sampling rate of 96 kHz, and analyzed with Saslab Pro (all Avisoft). Recordings were Fourier-transformed using a window size of 512 samples and 75% overlap, then analyzed with Avisoft's “whistle-tracking element separation” algorithm, optimized to testing conditions, was used to automatically detect and characterize calls. The number of flat and frequency-modulated USVs in the 50 kHz range or higher were quantified each day. Frequency-modulated USVs were further characterized as rises, trills, and steps, based on the pattern of frequency modulation (Wright et al., 2010).

Sociosexual preference behavior: The sociosexual behavior test used a

Stoelting Any-Maze three-chamber Plexiglas apparatus (100L X 100 W × 34.5H cm total size), with restraint cages containing the stimulus animals (opposite sex gonadectomized rats, one given hormone treatment, the other untreated) placed in opposite corners of the side chambers (Crews et al., 2012; Moy et al., 2004). The test took place on day 3 of USV testing, immediately after the USV test was completed. The experimental animal was habituated in the center compartment for 5 min with the side chamber doors closed. After habituation, the doors were opened, and the experimental animal was allowed to explore the entire arena for 10 min. Stoelting's Any-Maze software quantified distance travelled, average speed, time standing still, and time spent near each stimulus animals. Other behaviors (time at Plexiglas, time spent investigating the stimulus animal's enclosure, rearing, grooming, and nose-to-nose touching) were manually scored from videos by a trained observer blind to treatment.

2.5. Tissue collection and storage

Testing was completed by P90 and animals were weighed and euthanized 1–3 h before lights out (females on proestrus). Immediately after decapitation, trunk blood samples were collected, allowed to clot, and centrifuged to obtain serum samples. Brains were removed quickly, chilled on ice, and sliced into 1 mm coronal sections using a chilled rat brain matrix. The 1 mm sections were transferred to microscope slides, frozen on top of dry ice, and stored at −80 C. After all tissues were collected, the slides containing sections with the MPN and VMN were placed on a freezing stage at −18 C, punched with a Palkovits punch, collected in chilled microfuge tubes, and stored at −80 C until extraction (Walker et al., 2013, 2014).

2.6. Serum hormone assays (corticosterone, testosterone)

Serum testosterone was quantified in 10 µl duplicate samples from male rats in a single assay. MP Biomedicals' MP Biomedicals' testosterone ^{125}I RIA #07189102 radioimmunoassay was used following manufacturer's protocols, and as published (Gillette et al., 2017; Mennigen et al., 2018). Assay sensitivity was 0.03 ng/ml, and intra-assay variability was 1.5%. Serum corticosterone was assayed in 10 µl duplicate samples from both sexes in a single assay. MP Biomedicals' corticosterone ^{125}I RIA #07120103) radioimmunoassay was used following manufacturer's protocols and as published (Reilly et al., 2015). Assay sensitivity was 7.7 ng/ml, and intra-assay variability was 2.9%.

2.7. RNA isolation

Total RNA was isolated from frozen MPN and VMN punches of behaviorally tested male and female rats using the mirVana microRNA isolation kit, following manufacturer's protocols for total RNA extraction. All RNA samples were analyzed for quantity by Nanodrop spectrophotometry and run on a Bioanalyzer 2100 nanodrop kit (catalog no. 5067–1511, Agilent Technologies, Santa Clara, CA) to assess RNA purity and integrity. Only RNA samples with the RIN of 8 or higher were used in subsequent experiments. Based on this criterion, 5 samples were excluded from the analysis in the MPN, and 8 were excluded from the analysis in the VMN, resulting in a sample size of 8–10 animals per group for molecular work.

2.8. cDNA synthesis and taqman microfluidic real-time qPCR cards

Total RNA (200 ng total) was used to generate cDNA using high-capacity cDNA reverse transcription kit with RNase inhibitor (catalog no. 4374966, all Life Technologies) according to the manufacturer's recommended protocols. For Taqman Low Density Array (TLDA) analysis, custom-designed microfluidic 48-gene qPCR cards (Life Technologies) were used to analyze selected mRNAs as published and validated by our laboratory (Walker et al., 2009, 2013, 2014). Genes on the card were steroid hormone receptors (*Ar, Esr1, Esr2, Gper, Pgr, Nr3c1*), transporters (*Slc6a3* (dopamine transporter)), neurotransmitters and receptors (*Drd1a, Drd2, Drd3, Grin1, Grin2a, Grin2b*), enzymes (*Th*), neuropeptides and receptors (*Crh, Kiss1, Kiss1r, Lepr, Oprd1, Oprl1, Oprk1, Oprm1, Oxt, Oxtr*), neurotrophic factors and receptors (*Bdnf, Igf1, Igf1r*), genes implicated in USVs (*Foxp1, Foxp2*), circadian genes (*Arntl, Clock, Per1, Per2*) and others (*Egr1, Fmr1, Nlgn3, Rapgef4, Shank1, Shank2, Shank3*), along with 3 normalizing genes (18s, *Gapdh* and *Rpl13a*).

Real-time PCR was carried out on an ABI ViiA7 using Taqman universal master mix (catalog no. 4324018, Life Technologies) and the following run parameters: 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. Relative expression was determined for each sample using the comparative cycle threshold (Ct) method (Pfaffl, 2001). The Cts were further calibrated to the mean Δ-Ct of the vehicle females to determine the relative Ct value for each mRNA. The selection of normalizing genes was based on inclusion of 18s, *Gapdh* and *Rpl13a* on the TLDA cards, and determination of any treatment effects. The MPN was normalized to the geometric mean of *Gapdh* and *Rpl13a*, and the VMN to the geometric mean of *Gapdh*, *Rpl13a*, and 18s.

2.9. Statistics and functional landscapes

All statistical analyses were conducted using R software (version 3.3.2) with the following packages: stats, outliers, ggplot2, stringr, lsr. Sex differences in vehicle control (VEH) animals were determined by *t*-test. Subsequently ANOVA or Kruskal-Wallis was used to identify any potential cohort, litter, and treatment effects, based on the normality and variance of each dataset. When appropriate, a repeated measures multiple comparisons MANOVA (which addresses the false discovery rate) was used. Grubb's test was used to exclude up to 2 outliers per group in the dataset. *Post hoc* analyses were conducted by Tukey HSD for treatment effects and interaction. All data are presented at mean ± SEM. Effect sizes were also calculated (Reilly et al., 2015, 2018), for ANOVAs as partial eta-squared (η_p^2), with η_p^2 of 0.09–0.24 medium, and 0.25 or greater large. A similar calculation was done for nonparametric Kruskal-Wallis tests, using the epsilon-squared (ε^2) calculation. For sex differences, effect sizes were determined by Cohen's d, with a medium effect from 0.5 to 0.7, and large at 0.8 and above.

Functional landscapes were also created: this is a topological visualization of how chosen components spanning different levels of biological organization can be combined to reveal relationships that cannot be represented by bar graphs or tabular data. The method in its final form accommodates different scales of measurement to illustrate how diverse factors such as gene expression, hormones, and behaviors are related and change in response to treatment (Crews et al., 2012; Scarpino et al., 2014). This method was used to generate a 3-dimensional representation of the composite phenotype for each group. The peak or valley of each node in the landscape is calculated as the percent of maximum from the highest group mean. The width or slope of each node is the result of the number of nodes in each landscape (in arbitrary units).

3. Results

Throughout the results, we present p-values for significant effects in the prose and figures, with full statistics for significant effects provided in Tables 1–4, including F-values, post-hoc results, and effect sizes.

3.1. Ultrasonic vocalizations (USV)

Examples spectrograms for the four call types are shown in Fig. 1A–D, and quantification of call numbers on day 3, immediately after removal of an opposite-sex rat, is shown for females (Fig. 1E) and males (Fig. 1F). Female and male rats in all treatment groups emitted flat and frequency-modulated USVS, with flat and rise calls most

Table 1
USV statistics for significant effects of (A) treatment within each sex, or (B) sex differences in the vehicle group.

A. Treatment Effects

Measure	Treatment	Mean ± SEM	Statistical Test	P	F (df)	Effect size η_p^2	Post-Hoc (Tukey HSD)
Females							
# USV Step Calls on Day 3	VEH	21.1 ± 4.9	Sqrt; ANOVA	0.0003	F(3,33) = 5.72	0.34	EB < VEH, p = 0.03; EB < A (0.5), p = 0.02; EB < A (1.0), p = 0.01
	EB	6.9 ± 2.2					
	A1221 (0.5)	28.0 ± 6.8					
	A1221 (1.0)	22.7 ± 4.3					
Males							
# USV Rise Calls on Day 3	VEH	19.8 ± 7.1	Sqrt; ANOVA	0.0008	F(3,36) = 7.03	0.37	VEH < A (0.5), p = 0.02; VEH < A (1.0), p = 0.005; EB < A (1.0), p = 0.02
	EB	20.6 ± 5.1					
	A1221 (0.5)	53.6 ± 10.9					
	A1221 (1.0)	60.7 ± 8.9					
# USV Step Calls on Day 3	VEH	10.9 ± 4.4	Sqrt; ANOVA	0.0013	F(3,36) = 6.46	0.86	VEH < A (0.5), p = 0.02; VEH < A (1.0), p = 0.05; EB < A (0.5), p = 0.02; EB < A (1.0), p = 0.05
	EB	9.1 ± 3.4					
	A1221 (0.5)	29.7 ± 5.7					
	A1221 (1.0)	26.2 ± 4.8					

B. Sex Differences in the vehicle group

Measure	Sex	Mean	Statistical Test	N	P	Effect Size (Cohen's d)	Sex Differerence
# USV Flat Calls on Day 3	Females	46.5 ± 9.0	Student's t-test	21	0.05	0.92	F > M
	Males	25.8 ± 4.3					

Statistical analysis was done on transformed (Square root (SQRT)) data by ANOVA. F (ANOVA) values with degrees of freedom (df) are shown. Effect sizes by partial eta squared are SMALL 0.01, MEDIUM 0.09, and LARGE 0.25. Effect sizes by Cohen's d are SMALL 0.2, MEDIUM 0.5, and LARGE 0.8.

Table 2
Sex differences in gene expression in the MPN (A) and VMN (B) of vehicle rats.

Gene	Sex	Mean ± SEM	N	P	Effect Size (Cohen's d)	Direction of sex difference
A. MPN sex differences						
Ar	Females	0.99 ± 0.09	19	0.046	−0.98	M > F
	Males	1.38 ± 0.16				
Drd3	Females	1.09 ± 0.14	18	0.005	−1.51	M > F
	Males	1.84 ± 0.18				
Egr1	Females	1.05 ± 0.12	20	0.012	1.28	F > M
	Males	0.66 ± 0.07				
Esr2	Females	1.00 ± 0.08	20	0.044	−0.82	M > F
	Males	1.31 ± 0.14				
Oprd1	Females	0.87 ± 0.07	18	0.022	−1.18	M > F
	Males	1.18 ± 0.10				
Per1	Females	1.02 ± 0.06	20	0.029	1.26	F > M
	Males	0.85 ± 0.04				
Per2	Females	1.03 ± 0.08	20	0.015	1.27	F > M
	Males	0.77 ± 0.04				
Slc6a3	Females	0.88 ± 0.15	17	0.007	−1.47	M > F
	Males	2.13 ± 0.36				
B. VMN sex differences						
Crh	Females	1.15 ± 0.30	16	0.027	1.25	F > M
	Males	0.33 ± 0.07				
Drd3	Females	1.08 ± 0.13	18	0.027	1.28	F > M
	Males	0.73 ± 0.04				
Igf1	Females	1.01 ± 0.05	18	0.020	1.20	F > M
	Males	0.84 ± 0.04				
Oprk1	Females	1.01 ± 0.06	19	0.005	1.47	F > M
	Males	0.77 ± 0.05				
Oprm1	Females	1.01 ± 0.04	19	0.014	1.26	F > M
	Males	0.85 ± 0.04				
Per2	Females	1.01 ± 0.04	19	0.006	1.44	F > M
	Males	0.82 ± 0.04				
Shank3	Females	1.00 ± 0.09	19	0.007	−1.39	M > F
	Males	1.12 ± 0.02				

All statistical comparisons were done by Student's t-test. Effect sizes by Cohen's d are SMALL 0.2, MEDIUM 0.5, and LARGE 0.8.

abundant, followed by step and trill USVs. Comparisons of calls between the sexes was done for the VEH groups, with flat calls being significantly greater in number in females than males ($p < 0.05$; Table 1). Frequency-modulated USVs (rise, step, trill) were not sexually dimorphic in number.

Effects of treatment were then determined for each call type. For females, only the number of step USV calls was affected (Fig. 1E; $p = 0.0003$), with the EB group calling significantly less than other groups (Table 1). For males, flat and trill USVs were not affected by treatment. Rise USVs were significantly affected (Fig. 1E; $p = 0.0008$), with the A1221 (0.5) group calling significantly more than VEH, and A1221 (1.0) calling more than both VEH and EB (Table 1). Step calls were similarly affected in males (Fig. 1F; $p = 0.0013$), with both A1221 groups calling more than VEH and EB (Table 1).

3.2. Sociosexual preference behavior

The full dataset and statistical analysis of the sociosexual behavior test is shown in Supplemental Table 1. Female experimental rats were given a choice between two stimulus rats: a castrated male without any hormone replacement, and a castrated male treated with a testosterone capsule. Females of all treatment groups showed the expected preference for the testosterone-treated over the no hormone castrated males, with significantly more time spent near the former than the latter. There were no significant differences between treatment groups in females (Fig. 2A; Supplemental Table 1). Male experimental rats were given a choice between two OVX females, one with estradiol plus progesterone (EP), the other with no hormone. Similar to females, males spent significantly more time near the EP-treated over the no-hormone females. However, males had a significant treatment effect for time spent engaged in nose-to-nose touching. This was found for total time spent nose touching with the two stimulus females combined (Fig. 2B; Supplemental Table 1; $p < 0.002$, $\varepsilon^2 = 0.26$), with post-hoc analysis showing that the vehicle males did more nose touching than all other treatment groups (VEH vs. EB: $p < 0.05$; VEH vs. A1221 (0.5): $p < 0.01$; VEH vs. A1221 (1.0): $p < 0.05$). When nose touching time was analyzed separately toward each stimulus rat, an effect of treatment on nose touching was found for A1221 (0.5) males spending less time nose-touching with the EP female ($p = 0.03$, $\varepsilon^2 = 0.15$). A trend with the same directionality was also seen for time spent with the no-hormone treatment (lower in A1221 (0.5) than VEH) but this did not

Table 3
MPN genes significantly affected by treatment in females (A) or males (B).

Gene	Treatment	Mean ± SEM	Statistical Test	p-value	F (df) for ANOVA; ChiSq (df) for KW	η_p^2 for ANOVA; ε^2 for KW	Post-Hoc (Tukey HSD for ANOVA, Steel-Dwass for KW)
A. Females							
Ar	VEH	0.99 ± 0.09	ANOVA	0.004	F(3, 33) = 5.44	0.33	VEH < A (1.0) p = 0.01; EB < A (1.0) p = 0.02
	EB	0.96 ± 0.10					
	A1221 (0.5)	1.29 ± 0.09					
	A1221 (1.0)	1.38 ± 0.09					
Avp	VEH	0.96 ± 0.23	Kruskal-Wallis	0.024	C^2(3,35) = 9.43	0.28	None identified
	EB	1.71 ± 0.46					
	A1221 (0.5)	0.16 ± 0.05					
	A1221 (1.0)	0.51 ± 0.18					
Crh	VEH	1.06 ± 0.08	Kruskal-Wallis	0.006	C^2(3,36) = 12.50	0.36	EB < A (0.5) p = 0.02; EB < A (1.0) p = 0.05
	EB	0.67 ± 0.13					
	A1221 (0.5)	1.29 ± 0.09					
	A1221 (1.0)	1.67 ± 0.30					
Drd3	VEH	1.09 ± 0.14	Kruskal-Wallis	0.012	C^2(3,34) = 10.93	0.31	VEH < A (0.5) p = 0.05
	EB	1.24 ± 0.18					
	A1221 (0.5)	1.89 ± 0.17					
	A1221 (1.0)	1.74 ± 0.20					
Esr1	VEH	1.06 ± 0.14	ANOVA	0.001	F(3,32) = 6.94	0.39	VEH < A (1.0) p = 0.02; VEH < A (0.5) p = 0.03; EB < A (0.5) p = 0.01; EB < A (1.0) p = 0.01
	EB	0.96 ± 0.13					
	A1221 (0.5)	1.86 ± 0.13					
	A1221 (1.0)	1.82 ± 0.28					
Esr2	VEH	1.00 ± 0.08	ANOVA	0.001	F(3,32) = 6.73	0.39	VEH < A (0.5) p = 0.003; EB < A (0.5) p = 0.003
	EB	1.00 ± 0.08					
	A1221 (0.5)	1.50 ± 0.10					
	A1221 (1.0)	1.29 ± 0.14					
Foxp1	VEH	0.99 ± 0.06	ANOVA	0.017	F(3,33) = 3.89	0.26	A (0.5) < EB p = 0.02
	EB	1.12 ± 0.07					
	A1221 (0.5)	0.86 ± 0.01					
	A1221 (1.0)	0.91 ± 0.06					
Igf1	VEH	1.03 ± 0.09	LOG; ANOVA	0.049	F(3,33) = 2.91	0.21	None identified
	EB	0.81 ± 0.08					
	A1221 (0.5)	1.16 ± 0.14					
	A1221 (1.0)	1.08 ± 0.06					
Igf1r	VEH	1.00 ± 0.03	ANOVA	0.025	F(3,30) = 3.60	0.26	A (0.5) < EB p = 0.02
	EB	1.21 ± 0.07					
	A1221 (0.5)	0.95 ± 0.06					
	A1221 (1.0)	1.02 ± 0.06					
Kiss1	VEH	1.19 ± 0.35	LOG; ANOVA	0.000	F(3,29) = 9.47	0.49	VEH, EB, A (1.0) < A (0.5) p = 0.01
	EB	0.54 ± 0.12					
	A1221 (0.5)	8.20 ± 2.87					
	A1221 (1.0)	0.77 ± 0.21					
Oprk1	VEH	1.09 ± 0.05	ANOVA	0.006	F(3,32) = 4.94	0.32	EB < A (0.5) p = 0.006
	EB	0.97 ± 0.08					
	A1221 (0.5)	1.36 ± 0.10					
	A1221 (1.0)	1.24 ± 0.07					
Oprl1	VEH	1.07 ± 0.04	Kruskal-Wallis	4.30E-03	C^2(3,35) = 13.15	0.39	VEH < A (0.5) p = 0.04; EB < A (1.0) p = 0.03
	EB	1.06 ± 0.05					
	A1221 (0.5)	1.10 ± 0.05					
	A1221 (1.0)	1.26 ± 0.02					
Oprm1	VEH	1.02 ± 0.06	ANOVA	0.002	F(3,33) = 6.41	0.37	VEH < A (1.0) p = 0.05; EB < A (0.5) p = 0.01; EB < A (1.0) p = 0.009
	EB	0.94 ± 0.08					
	A1221 (0.5)	1.32 ± 0.06					
	A1221 (1.0)	1.31 ± 0.10					
Pgr	VEH	1.01 ± 0.05	Kruskal-Wallis	0.003	C^2(3,36) = 13.15	0.40	EB < A (0.5) p = 0.03; EB < A (1.0) p = 0.02
	EB	0.80 ± 0.06					
	A1221 (0.5)	1.25 ± 0.10					
	A1221 (1.0)	1.22 ± 0.12					
Shank2	VEH	1.00 ± 0.03	ANOVA	0.011	F(3,31) = 4.35	0.30	EB < A (1.0) p = 0.007
	EB	0.91 ± 0.05					
	A1221 (0.5)	1.04 ± 0.05					
	A1221 (1.0)	1.13 ± 0.02					
B. Males							
Drd1a	VEH	0.89 ± 0.05	Kruskal-Wallis	0.030	C^2(3,34) = 8.99	0.27	None identified
	EB	0.97 ± 0.07					
	A1221 (0.5)	1.10 ± 0.16					
	A1221 (1.0)	0.70 ± 0.03					
Per2	VEH	0.77 ± 0.04	LOG-ANOVA	0.003	F(3,29) = 5.81	0.38	VEH < A (0.5) p = 0.01; A (1.0) < A (0.5) p = 0.008
	EB	0.90 ± 0.05					
	A1221 (0.5)	1.01 ± 0.07					
	A1221 (1.0)	0.71 ± 0.02					

Statistical analysis was done on raw or transformed (Log or Square root (SQRT)) by ANOVA, or by non-parametric (Kruskal-Wallis (KW). F (ANOVA) or Chi-square (ChiSq; Kruskal-Wallis)) values with degrees of freedom (df) are shown. Effect sizes by partial eta squared for ANOVA or episolon-squared for Kruskal Wallis are SMALL 0.01, MEDIUM 0.09, and LARGE 0.25.

Table 4
VMN genes significantly affected by treatment in females (A) or males (B).

Gene	Treatment	Mean ± SEM	Statistical Test	P	F (df) for ANOVA; ChiSq (df) for KW	η_p^2 for ANOVA; ε^2 for KW	Post-Hoc (Tukey HSD for ANOVA, Steel-Dwass for KW)
A. Females							
Avpr1a	VEH	1.02 ± 0.06	ANOVA	0.032	F(3,33) = 3.3128	0.24	VEH < A (1.0) p = 0.04
	EB	1.02 ± 0.04					
	A1221 (0.5)	1.09 ± 0.03					
	A1221 (1.0)	1.20 ± 0.04					
Clock	VEH	1.00 ± 0.02	ANOVA	0.012	F(3,30) = 4.3541	0.31	EB < A (0.5) p = 0.05; EB < A (1.0) p = 0.03
	EB	0.97 ± 0.02					
	A1221 (0.5)	1.08 ± 0.02					
	A1221 (1.0)	1.09 ± 0.05					
Egr1	VEH	1.01 ± 0.05	ANOVA	0.011	F(3,31(= 4.3748))	0.30	EB < A (0.5) p = 0.01
	EB	0.85 ± 0.06					
	A1221 (0.5)	1.13 ± 0.04					
	A1221 (1.0)	0.92 ± 0.08					
Foxp1	VEH	0.97 ± 0.03	ANOVA	0.010	F(3, 32) = 4.48	0.30	EB < A (0.5) p = 0.008
	EB	0.88 ± 0.04					
	A1221 (0.5)	1.10 ± 0.05					
	A1221 (1.0)	1.04 ± 0.06					
Igf1r	VEH	1.01 ± 0.04	LOG, ANOVA	0.048	F(3,34) = 2.97	0.23	EB < A (1.0) p = 0.04
	EB	0.93 ± 0.04					
	A1221 (0.5)	1.08 ± 0.07					
	A1221 (1.0)	1.14 ± 0.06					
Kiss1	VEH	1.14 ± 0.17	LOG, ANOVA	0.025	F(3,31) = 3.60	0.26	VEH < EB p = 0.02
	EB	2.09 ± 0.29					
	A1221 (0.5)	1.26 ± 0.08					
	A1221 (1.0)	1.78 ± 0.35					
Kiss1r	VEH	1.02 ± 0.06	LOG, ANOVA	0.001	F(3,31) = 7.25	0.42	EB < VEH p = 0.003; EB < A (0.5) p = 0.004;
	EB	0.74 ± 0.04					EB < A (1.0) p = 0.004
	A1221 (0.5)	1.02 ± 0.03					
	A1221 (1.0)	1.03 ± 0.08					
Nlgn3	VEH	0.98 ± 0.02	Kruskal-Wallis	0.030	C^2(3,33) = 8.95	0.28	VEH < A (0.5) p = 0.01
	EB	0.94 ± 0.05					
	A1221 (0.5)	1.08 ± 0.01					
	A1221 (1.0)	1.00 ± 0.03					
Oprk1	VEH	1.01 ± 0.06	Kruskal-Wallis	0.038	C^2(3,32) = 8.42	0.27	None identified
	EB	0.90 ± 0.03					
	A1221 (0.5)	1.09 ± 0.06					
	A1221 (1.0)	0.91 ± 0.03					
Oprm1	VEH	1.01 ± 0.04	ANOVA	0.011	F(3, 31) = 4.38	0.30	EB < VEH p = 0.007
	EB	0.81 ± 0.03					
	A1221 (0.5)	0.94 ± 0.02					
	A1221 (1.0)	0.96 ± 0.06					
Per1	VEH	1.01 ± 0.04	Kruskal-Wallis	0.040	C^2(3,34) = 8.34	0.25	A (1.0) < A (0.5) p = 0.02
	EB	1.03 ± 0.03					
	A1221 (0.5)	1.13 ± 0.01					
	A1221 (1.0)	1.01 ± 0.03					
Pgr	VEH	1.03 ± 0.08	ANOVA	0.043	F(3,31) = 3.08	0.24	VEH < EB p = 0.03
	EB	1.38 ± 0.11					
	A1221 (0.5)	1.29 ± 0.07					
	A1221 (1.0)	1.23 ± 0.09					
Rapgef4	VEH	1.01 ± 0.04	ANOVA	0.012	F(3,33) = 4.26	0.29	EB < A (0.5) p = 0.01
	EB	0.87 ± 0.06					
	A1221 (0.5)	1.11 ± 0.05					
	A1221 (1.0)	1.06 ± 0.05					
Shank1	VEH	1.01 ± 0.04	ANOVA	0.016	F(3,32) = 3.99	0.28	EB < A (1.0) p = 0.01
	EB	0.90 ± 0.03					
	A1221 (0.5)	1.04 ± 0.05					
	A1221 (1.0)	1.09 ± 0.02					
B. Males							
Crh	VEH	0.33 ± 0.07	ANOVA	0.024	F(3,29) = 3.67	0.28	VEH < EB p = 0.02
	EB	0.81 ± 0.12					
	A1221 (0.5)	0.77 ± 0.19					
	A1221 (1.0)	0.66 ± 0.12					
Drd3	VEH	0.73 ± 0.04	ANOVA	0.048	F(3,32) = 2.95	0.22	None identified
	EB	0.90 ± 0.09					
	A1221 (0.5)	0.66 ± 0.06					
	A1221 (1.0)	0.91 ± 0.07					
Egr1	VEH	1.04 ± 0.09	ANOVA	0.012	F(3,32) = 4.30	0.29	A (1.0) < EB p = 0.02
	EB	1.11 ± 0.07					
	A1221 (0.5)	0.84 ± 0.07					
	A1221 (1.0)	0.79 ± 0.06					

(*continued on next page*)

Table 4 (*continued*)

Gene	Treatment	Mean ± SEM	Statistical Test	P	F (df) for ANOVA; ChiSq (df) for KW	η_p^2 for ANOVA; ε^2 for KW	Post-Hoc (Tukey HSD for ANOVA, Steel-Dwass for KW)
Shank1	VEH	1.01 ± 0.02	ANOVA	0.005	F(3,30) = 5.20	0.35	VEH < EB p = 0.007
	EB	1.11 ± 0.02					
	A1221 (0.5)	1.03 ± 0.02					
	A1221 (1.0)	1.02 ± 0.02					

Statistical analysis was done on raw or transformed (Log or Square root (SQRT)) by ANOVA, or by non-parametric (Kruskal-Wallis (KW). F (ANOVA) or Chi-square (ChiSq; Kruskal-Wallis)) values with degrees of freedom (df) are shown. Effect sizes by partial eta squared for ANOVA or episolon-squared for Kruskal Wallis are SMALL 0.01, MEDIUM 0.09, and LARGE 0.25.

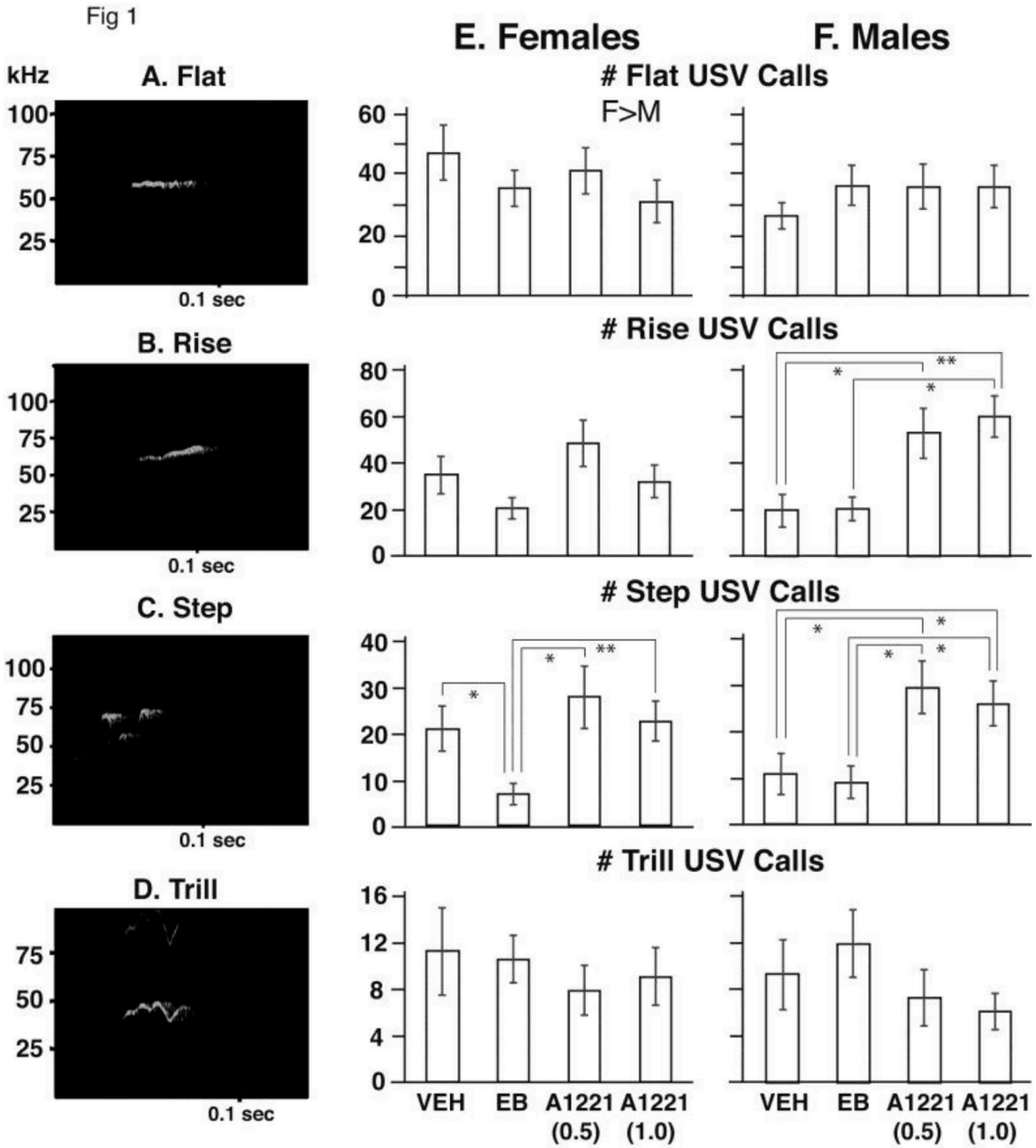

Fig. 1. Spectrograms for the four call types are shown: A) Flat; B) Rise; C) Step; and D) Trill. Numbers of each type of USV are shown for female (E) and male (F) rats on Day 3, recorded in a sociosexual context. The sexual dimorphism in flat calls is indicated as F > M. Effects of treatment within each sex are shown as: *, $p < 0.05$, **, $p < 0.01$. Data are mean ± SEM.

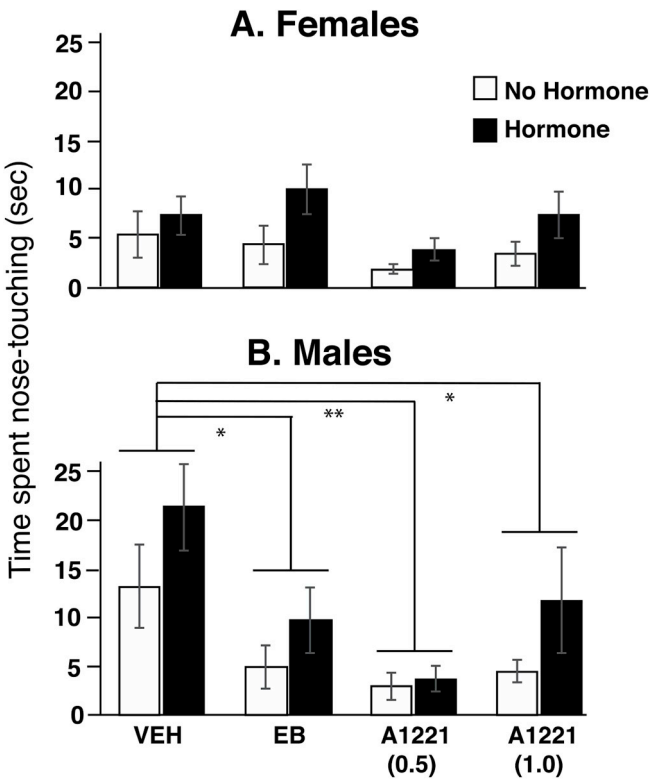

Fig. 2. Time spent nose-touching in the sociosexual preference test is shown for females (A) and males (B) towards the opposite-sex rat. The stimulus rats were gonadectomized (no hormone; white bar) or gonadectomized plus hormone replacement (black bar; females given estradiol + progesterone; males given testosterone). Nose-touching time was significantly decreased by EDCs in males but not females. Significant differences are shown as: *, $p < 0.05$, **, $p < 0.01$. Data are mean ± SEM.

reach significance ($p < 0.08$, $\varepsilon^2 = 0.11$). No other behaviors were significantly affected in males (Supplemental Table 1).

3.3. Gene expression in the MPN

Sex differences in gene expression in the MPN were determined in the control (VEH) rats, with a total of 8 genes identified as significantly sexually dimorphic (Table 2). Gene expression of *Ar, Drd3, Esr2, Oprd1,* and *Slc6a3* was higher in males compared to females. *Egr1, Per1*, and *Per2* were higher in females than males.

In females, a total of 15 genes were significantly affected by treatment (Table 3). Eleven of these genes were significantly affected by treatment, had a 20% or greater change in expression, and an identified significant post-hoc effect (Fig. 3A). Of this latter group, A1221 at one or both dosages significantly up-regulated *Ar, Esr1, Esr2, Drd3, Kiss1,* and *Oprm1*. In several cases, EB and at least one A1221 dosage group differed (Higher in A1221: *Ar, Crh, Esr1, Esr2, Kiss1, Oprk1, Oprm1, Pgr;* or higher in EB: *Foxp1, Igf1r*). In males, of the two genes affected by treatment (*Drd1a, Per2*; Table 3) only one (*Per2*) met criteria of having a significant effect by post-hoc analysis, and > 20% expression change. *Per2* was significantly affected by treatment in the MPN, with expression highest in A1221 (0.5) (Fig. 3B).

3.4. Gene expression in the VMN

Seven genes were sexually dimorphic in the VMN of VEH rats, of which 6 (*Crh, Drd3, Igf1, Oprk1, Oprm1,* and *Per2*) were higher in females, and one (*Shank3*) higher in males.

As in the MPN, more significantly affected genes were found in females (14) than males (4; Table 4). Using the same criteria as in the MPN (post-hoc effect detected; > 20% change), 5 genes in females were identified, all due to differences with the EB group (Fig. 4A). *Kiss1* and *Pgr* expression were higher in EB than VEH rats; *Egr1* and *Rapgef4* were lower in EB than A1221 (0.5); and for *Kiss1r*, EB was significantly lower than the other 3 groups. For males, of 4 identified as significantly affected, 2 met criteria. Again, differences were always with the EB group: for *Crh,* EB increased expression compared to VEH; and for *Egr1*, expression in A1221 (1.0) was significantly lower than EB male rats.

3.5. Hormones and physiology

Serum corticosterone in both sexes, and testosterone in males, were assayed in terminal blood samples. No effect of treatment was found for any of these hormones (Fig. 5A–C). Age at vaginal opening (females) and preputial separation (males) were unaffected by treatment (Fig. 5D and E).

4. Discussion

As a whole, this study provides new data into effects of prenatal PCBs (A1221) on social, communicative, and neuromolecular properties in rats. A1221 is lightly chlorinated and has a short half-life in the body relative to other PCBs (Sundström et al., 1976); thus, exposure at E16 and E18 is enough to change the developing brain despite the chemical's transient presence in the body. It is notable that effects of A1221 were sex-specific, with the majority of gene expression changes observed in females, but more behavioral changes in males. In addition, while A1221 and EB often had similar actions, presumably mediated through estrogen receptors (Korach et al., 1988; Layton et al., 2002), differences between A1221 and EB also underscore non-estrogenic properties of A1221. We will discuss these findings and their implications in more detail below.

4.1. USVs in rats are sensitive to prenatal EDCs

Ultrasonic vocalizations are a primary mode of communication in rats and other rodents, conveying information about aversive and hedonic stimuli and enabling animals to make choices related to mates, food, and predators (Burgdorf et al., 2008; Fernández-Vargas and Johnston, 2015; Harding and McGinnis, 2003). In adults, USVs are often tested in a sociosexual context, in which an opposite-sex rat is first introduced and subsequently removed, a stimulus that reliably elicits USVs in the experimental subject (Ma et al., 2014; McGinnis and Vakulenko, 2003; Yang et al., 2013). USV calls are subdivided into two general classes: low-frequency (~20-kHz range, "non-hedonic") and high-frequency (~50 kHz range, "hedonic") calls (Maier et al., 2011; Takahashi et al., 2010; Wright et al., 2010). In recent work, calls were further subdivided into syllables, although the social salience is not known (Arriaga et al., 2012; Capela et al., 2018; Dombret et al., 2017; Ma et al., 2014; Takahashi et al., 2010; Wright et al., 2010).

In the current study, we detected 50 kHz but not 20 kHz USVs in our animals, consistent with other work in our lab using the same Sprague-Dawley rat colony (Bell et al., 2016b; Garcia et al., 2017a, 2017b). Flat calls were sexually dimorphic (females > males), but numbers of rise, step and trill calls were equivalent between the sexes. Effects of A1221 were limited to males, with numbers of rise and step, but not trill, calls increased in both A1221 dosage groups. The only treatment effect in females was that of EB, which diminished step calls compared to vehicle and A1221 groups.

Other work has shown sex differences in USV calls in response to rewarding stimuli. Wright et al. reported in rats that trill calls in particular were elevated by amphetamine compared to saline (Wright et al., 2010), and that pair-housed rats called about twice as much per animal as they did when on their own, emphasizing the importance of social context. In prairie voles, rewarding stimuli (amphetamine, exposure to an opposite-sex stimulus vole) increased call numbers in a

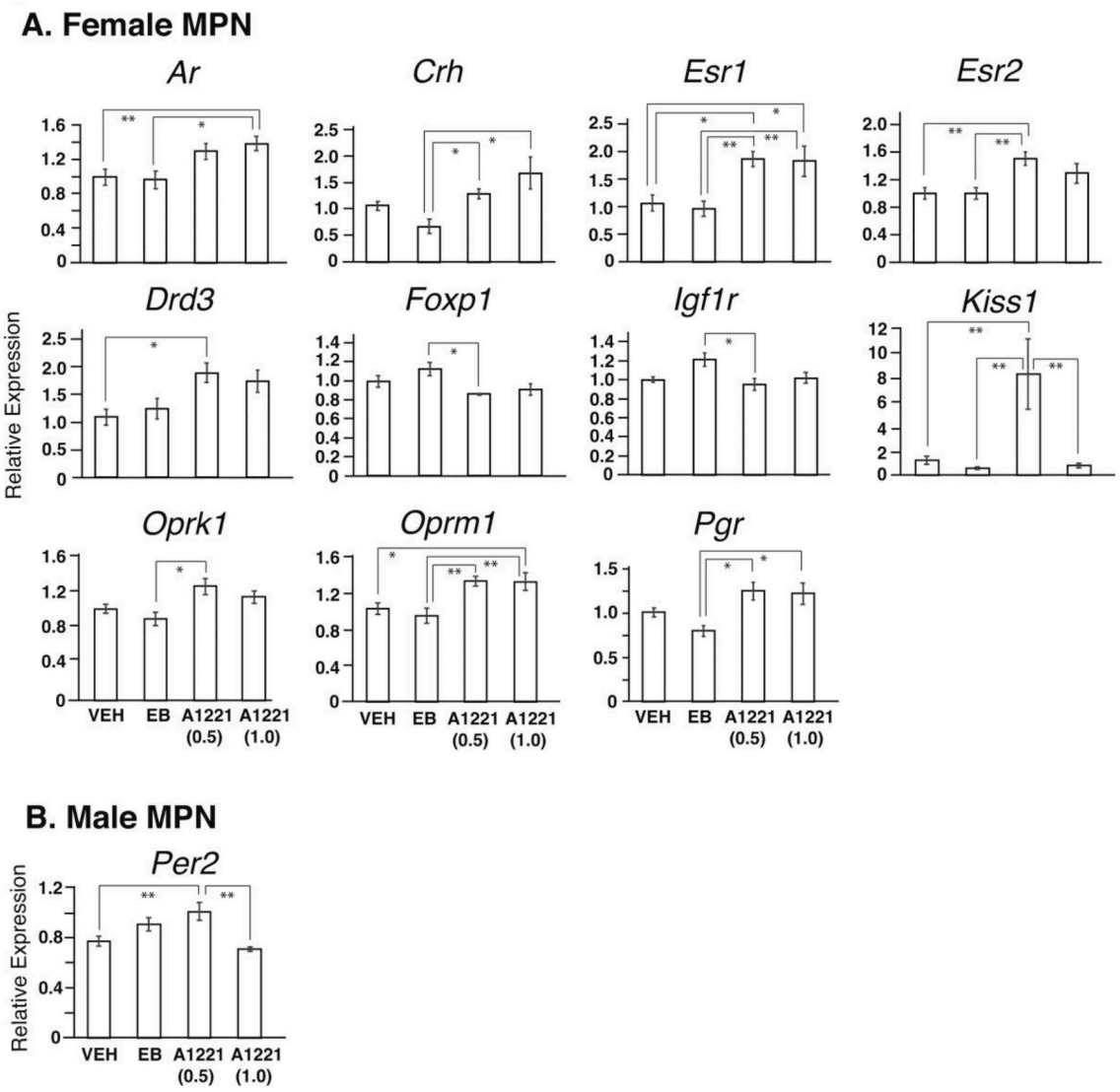

Fig. 3. Genes in the MPN meeting criteria of a main significant effect of EDCs, with > 20% change and a significant *post-hoc* effect, are shown. In females, 11 genes met criteria, and in males 1 gene met criteria. Significant differences are indicated as *, $p < 0.05$; **, $p < 0.01$. Data are mean ± SEM. The full dataset and statistics are in Table 3.

sex-dependent manner, with males increasing USVs more than females (Ma et al., 2014). This sex difference was also seen in our study: male rats exposed prenatally to A1221 increased both rise and step calls in response to removal of an opposite-sex stimulus rat, much more so than female A1221 rats.

Several recent studies have investigated effects of EDCs on adult USV calls. In our lab (Bell et al., 2016b) we showed effects of prenatal exposure, juvenile exposure, or both (2 hits), to A1221 (1 mg/kg) or vehicle. The group of Mhaouty-Kodja et al. has published two interesting papers on effects of adult exposure of male mice on USVs, one to nonylphenol and the other to di(2-ethylhexyl) phthalate (DEHP) (Dombret et al., 2017). In the nonylphenol study, exposure of adult males for 4 weeks at the middle dosage (0.5 µg/kg/day) increased numbers and duration of USVs (Capela et al., 2018). For DEHP, the highest dosage (50 µg/kg/day) decreased USV numbers (Dombret et al., 2017). These latter studies were conducted in an adult exposure model, but underscore the point that EDC exposures have effects on USVs, that are dependent upon the EDC, the exposure period, and the type of USV measured.

4.2. Sociosexual behaviors

When given a choice between two castrated opposite-sex rats, males and females of all treatment groups showed the expected preference for hormone-over a non-hormone-treated stimulus animal (Moy et al., 2004; Xiao et al., 2015). The most interesting data were for nose touching, which was sexually dimorphic, with males nose-touching significantly more than females. In addition, there were significant treatment effects: vehicle males spent more time nose-touching with stimulus animals, irrespective of hormone status, compared to both A1221 dosages or EB males. We found a similar effect in a previous study using the same prenatal EDCs but in animals given a choice between same-sex rather than opposite-sex conspecifics (Reilly et al., 2015). Specifically, males nose-touched more than females, and this was decreased by A1221 (0.5) (Reilly et al., 2015), which was also the treatment group most affected in the current sociosexual study. Nose-to-nose touching behavior, and chemoinvestigation more broadly, is important to rodents when discriminating properties of a conspecific (Brown et al., 1988; Crews et al., 2007; Pankevich et al., 2004). Several types of EDCs affect aspects of social behavior in a species-dependent manner (Kim et al., 2015; Rosenfeld, 2015; Rosenfeld and Trainor,

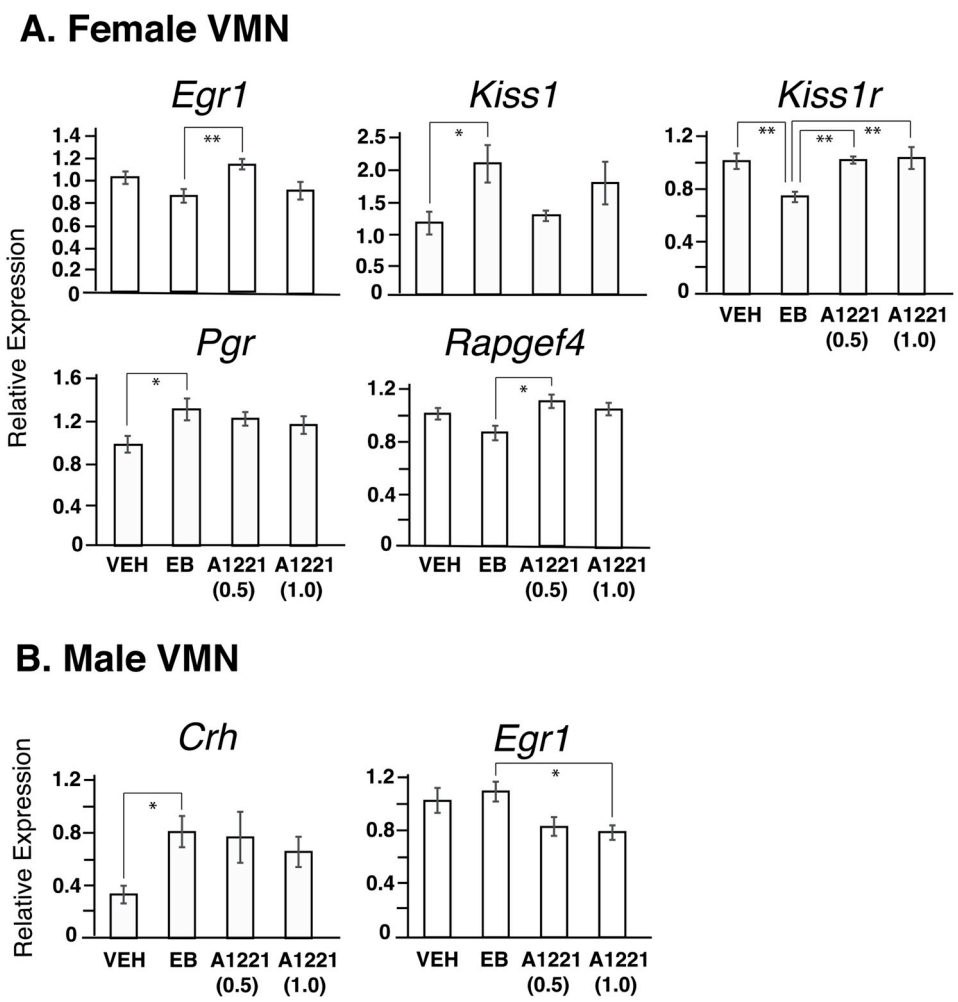

Fig. 4. Genes in the VMN meeting criteria of a main significant effect of EDCs, with > 20% change and a significant *post-hoc* effect, are shown. In females, 11 genes met criteria, and in males 1 gene met criteria. Significant differences are indicated as *, $p < 0.05$; **, $p < 0.01$. Data are mean ± SEM. The full dataset and statistics are in Table 4.

2014; Sullivan et al., 2014). However, the evidence for EDCs changing sensory processing of odorants, and/or olfactory preference is limited but results suggest that the ability to distinguish odorants is intact, at least based on adult exposure or transgenerational models (Capela et al., 2018; Crews et al., 2007; Dombret et al., 2017; Naule et al., 2014). Therefore, in our study it is likely to be a motivational or actual preference change, rather than perception of cues, that is affected by prenatal EDCs. We are currently conducting work to test whether this is the case.

It is informative to compare our nose-touching results to the USV results in the males: whereas nose-touching was decreased by prenatal EDCs, USV calls, especially rise and step calls, were increased. There are several possible explanations for this apparent discrepancy. First, it may not be a discrepancy at all. While studies of rewarding stimuli (sex, drugs) show increased 50 kHz USV calls (Ma et al., 2014; McGinnis and Vakulenko, 2003; Wright et al., 2010), and the interpretation is that these calls are hedonic, this may not always be the case. Although we do not know the neurobiological basis for increased calling in our model, when an opposite-sex stimulus is removed, the valence (hedonic vs. non-hedonic) is only inferred. In fact, when USV recordings were conducted in pairs of rats and related to behaviors (fighting, feeding, moving), Takahashi et al. (2010) found that calls in the 60 kHz range were most associated with moving, a behavior that may not have any particular hedonic value. Second, the decreased nose-touching in male rats may not indicate less social or sexual interest; in fact, it may represent improved social memory as indicated by a decreased need to interact with a stimulus animal that it already knows and remembers. Further work would be needed to distinguish these possibilities.

4.3. Neuromolecular profiling of hypothalamic genes

Neuroendocrine gene expression in the MPN and VMN, regions associated with social, sexual, and USV behaviors (Gore et al., 2018; Hoshina et al., 1994; Malsbury et al., 1977; Mathews and Edwards, 1977; McHenry et al., 2017), was quantified in our behaviorally-characterized rats. Here, we selected targets involved in steroid hormone signaling, neurotransmitters, neuropeptides, neurotrophic factors, and genes involved in social and sexual interactions. It was interesting that females were much more affected at the neuromolecular level than males, in contrast to the behaviors (males more affected than females). In the MPN, most effects were for females exposed to A1221. Six genes in the female MPN, *Ar, Drd3, Esr1, Esr2, Kiss1, and Oprm1*, were increased in one or both A1221 dosages. In males, only one gene was affected by A1221 (0.5) (*Per2*). Several points are notable. Three of the principal steroid hormone receptors in the hypothalamus, *Ar, Esr1,* and *Esr2* were targets of prenatal EDCs. These had increased expression in female A1221 rats, as did *Kiss1*. Prior work has implicated gonadal hormone receptors, and kisspeptin, as vulnerable to prenatal endocrine disruption (Bai et al., 2011; Bateman and Patisaul, 2008; Bellingham et al., 2009; Dickerson et al., 2011b; Tena-Sempere, 2010). Our current study supports those findings. The VMN showed a completely different result: EB, but not A1221, affected gene expression of *Kiss1* and *Pgr*

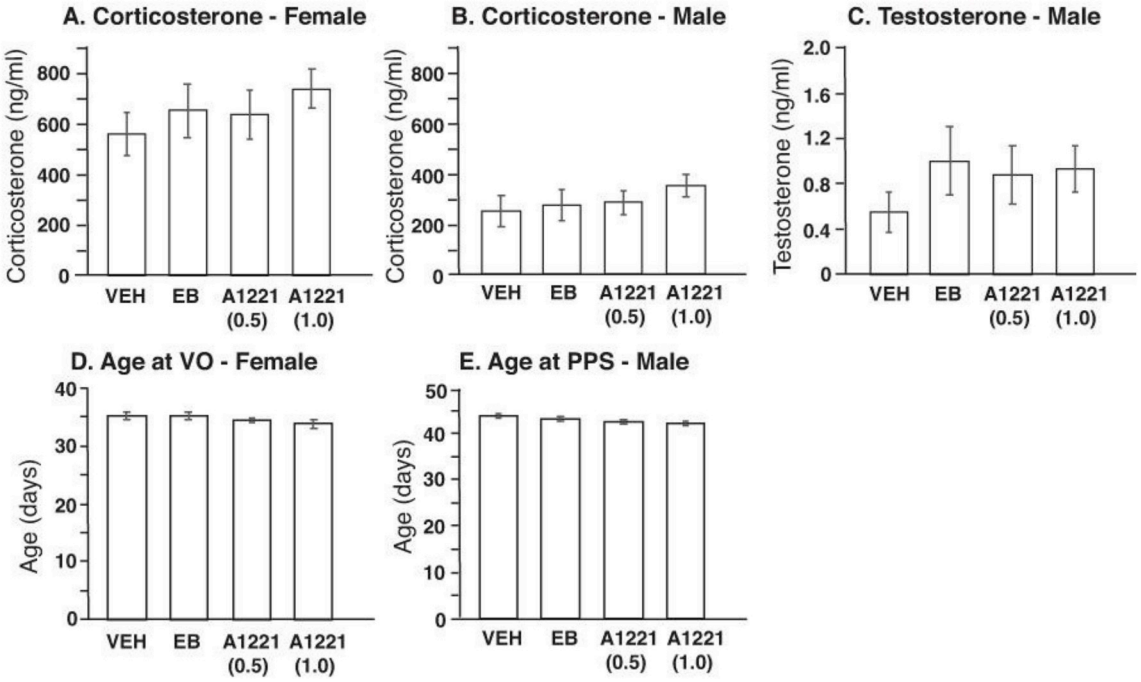

Fig. 5. Serum corticosterone in females (A) and males (B), and serum testosterone in males (C) are shown. There were no significant differences with treatment, although the expected sexual dimorphism in corticosterone (females > males) can be seen. Age at vaginal opening (VO) in females (D) and preputial separation (PPS) in males (E) were not affected by treatment. Data are mean ± SEM.

(increased), and *Kiss1r* (decreased). In males, *Crh* was higher in EB than vehicle rats. That the kisspeptin system (both the neuropeptide and its receptor gene) were identified is consistent with the estrogen-responsiveness of kisspeptin neurons and their sexual dimorphism (Clarkson et al., 2009; Kauffman et al., 2007; Smith et al., 2005).

There are some limitations to the current study. Although we were able to profile 48 genes each in the MPN and VMN, we were unable to conduct follow-up protein work due to unavailability of such tissues. Another limitation is the biological relevance of gene (or protein) expression changes. Here, we focused on those genes that were not only affected by PCBs but were also changed by at least 20%, a relatively conservative cut-off. Of the genes identified in the current study, *Esr1, Esr2, Pgr, Ar* and *Kiss1* are of particular interest as they underwent substantial and consistent change; these same molecules have been reported to be affected by prenatal EDCs, including PCBs, in other studies (Dickerson et al., 2011b; Bateman and Patisaul, 2008; Bellingham et al., 2009).

5. Summary and conclusions

In considering the big picture of the hypothalamic gene expression results together with the behaviors, it is interesting that the developing brain is differentially sensitive to A1221 and EB exposures. Functional landscapes (Fig. 6) illustrate the different profiles for EB compared to A1221 in the female MPN. The steroid hormone receptor genes (*Esr1, Esr2, Ar, Pgr*) and *Kiss1* show robustly different effects of A1221 (increased expression) and EB (no effect) compared to vehicle. In the female VMN (Fig. 4), EB significantly decreased (*Kiss1r, Pgr*) or increased (*Kiss1*) gene expression, whereas A1221 did not differ from vehicle. Few genes were affected by either treatment in the male VMN and MPN. Rather, in males, differences between A1221 and EB are best illustrated by the behavioral results shown in the functional landscapes (Fig. 6). Whereas EB had little effect on USVs and nose touching compared to vehicle, A1221 changed these profiles considerably.

The explanation for differences between A1221 and EB may be the non-estrogenic mechanisms of the former. While lightly chlorinated PCBs found in the A1221 mixture are mainly known for their weakly estrogenic actions (Korach et al., 1988; Layton et al., 2002), A1221 has other modes of action including effects on androgen and thyroid hormone systems, and as an aromatase inhibitor (Woodhouse and Cooke, 2004; Kiliç et al., 2005). Not only do we see considerable differences between A1221 to EB here; our prior work has revealed both similarities and differences depending upon the sex, age of exposure, age at analysis, and endpoint (Dickerson et al., 2011a, 2011b; Walker et al., 2014). Previous data published from the same cohort of animals presented here also showed differential effects of A1221 and EB exposure on anxiety measures and body weight (Gillette et al., 2017). Taken together with the behavioral results, for which we found dissimilar (in fact sometimes opposite) effects of EB and A1221, we believe that the PCB mix is acting predominantly via non-estrogenic pathways.

Although many endpoints were affected similarly by the two A1221 dosages (0.5 and 1.0 mg/kg), this was not always the case. For example, *Kiss1* in the MPN, was increased almost 6-fold by A1221 (0.5), but it was unaffected by the 1.0 mg/kg A1221 dosage. Although this current study only utilized two doses and therefore did not include a dose-response curve, our finding is consistent with the non-monotonic, low-dose effects of EDCs, for which a lower dose can have a greater effect on a particular endpoint than a higher dose (Vandenberg et al., 2012).

As a whole, our study shows sex-specific effects of prenatal EDC treatment on the organization of neuromolecular networks in the hypothalamus, together with changes in social and communicative behaviors that are critical for forming affiliative relationships and for reproductive success. These results add to knowledge showing that prenatal EDCs reprogram hypothalamic development, lead to behavioral change, and impair the ability of an animal to behave in an appropriate social context.

Disclosure statement

Funding was provided by NIH grants from NIEHS (RO1 ES020662, R56 ES023254) to A.C.G. The authors have nothing to disclose.

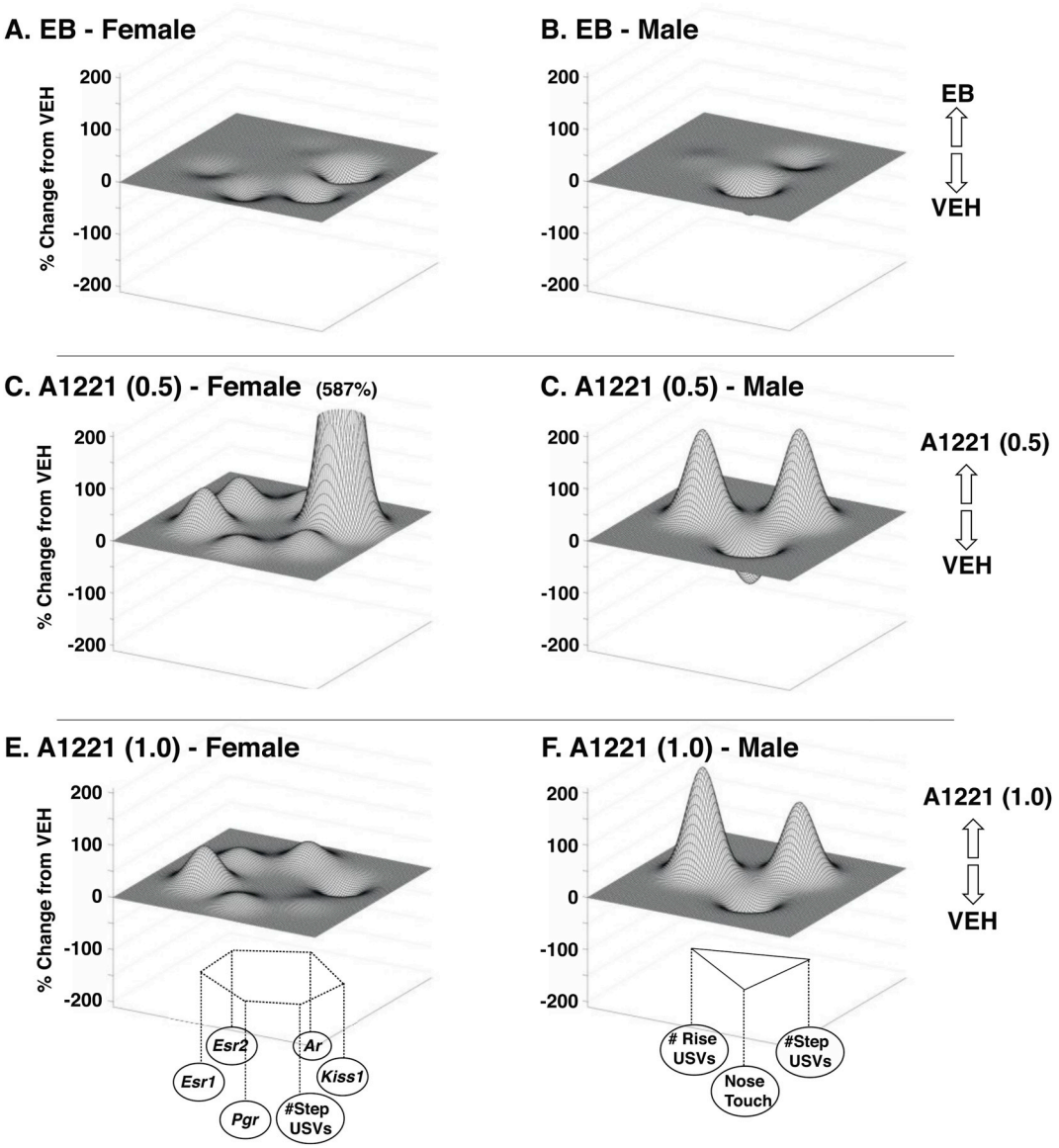

Fig. 6. Functional landscapes were generated for females (left) and males (right). The choice of nodes for the landscape, with the labels shown at the bottom, was based on gene and behavioral endpoints of interest, selected separately for each sex. Each landscape represents the difference between a treatment group and the vehicle, with data scaled to allow representation on the same y-axis. In the landscapes, upward deflections ("mountain") represent that endpoint being higher in the EDC group vs. vehicle, and downward deflections ("valley") indicate the vehicle group being higher than the EDC group for that sex. Separate landscapes are shown for each compound in relationship to its vehicle control: EB (A, B), A1221 (0.5) (C, D) and A1221 (1.0) (E, F). The node abbreviations in females are for 5 genes in the MPN (*Esr1, Esr2, Ar, Kiss1, Pgr*) and the number of step USVs. For males, numbers of rise USVs and step USVs, and time spent nose touching, are shown.

Acknowledgments

The authors thank Jayden Chen and Saazina Afsah for help with sociosexual behavior analysis, Andrea Chase for assisting with RNA extractions, and Dr. Krittika Krishnan with USV spectrograms.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2018.09.010.

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