Hypothalamic transcriptomic alterations in male and female California mice (Peromyscus californicus) developmentally exposed to bisphenol A or ethinyl estradiol

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Abstract
Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) prevalent in many household items. Rodent models and human epidemiological studies have linked this chemical to neurobehavior impairments. In California mice, developmental exposure to BPA results in sociosexual disorders at adulthood, including communication and biparental care deficits, behaviors that are primarily regulated by the hypothalamus. Thus, we sought to examine the transcriptomic profile in this brain region of juvenile male and female California mice offspring exposed from periconception through lactation to BPA or ethinyl estradiol (EE, estrogen present in birth control pills and considered a positive estrogen control for BPA studies). Two weeks prior to breeding, P₀ females were fed a control diet, or this diet supplemented with 50 mg BPA/kg feed weight or 0.1 ppb EE, and continued on the diets through lactation. At weaning, brains from male and female offspring were collected, hypothalamic RNA isolated, and RNA-seq analysis performed. Results indicate that BPA and EE groups clustered separately from controls with BPA and EE exposure leading to unique set of signature gene profiles. Kcnd3 was downregulated in the hypothalamus of BPA- and EE-exposed females, whereas Tbl2, Topors, Kif3a, and Phactr2 were upregulated in these groups. Comparison of transcripts differentially expressed in BPA and EE groups revealed significant enrichment of gene ontology terms associated with microtubule-based processes. Current results show that perinatal exposure to BPA or EE can result in several transcriptomic alterations, including those associated with microtubule functions, in the hypothalamus of California mice. It remains to be determined whether these genes mediate BPA-induced behavioral disruptions.

Introduction
Exposure to endocrine-disrupting chemicals (EDCs), including bisphenol A (BPA), may increase the risk of neurobehavioral disorders typified by social impairments, such as autism spectrum disorders (Grandjean and Landrigan 2006; Landrigan 2010; Dietert et al. 2011; Miodovnik et al. 2011; Braun 2012; de Cock et al. 2012; Braun et al. . . . )
2014; Kalkbrenner et al. 2014; Kaur et al. 2014; Stein et al. 2015). Most EDCs are manufactured chemicals (Diamanti-Kandarakis et al. 2009) with BPA being one of the most ubiquitous (He et al. 2009; Biedermann et al. 2010; Galloway et al. 2010). In 2013, production of this chemical was estimated to be approximately 15 billion pounds (GrandViewResearch, 2014). Its stability and pervasiveness (Environment Canada, 2008) has ensured continual exposure (Vandenberg et al. 2009). This chemical is detectable in the urine of 93% of the U.S. population (Calafat et al. 2008), as well as in fetal plasma, placenta (vom Saal et al. 2007), and breast milk (Vandenberg et al. 2007). In 2012, the FDA banned the production of baby bottles and sippy cups containing BPA https://www.federalregister.gov/documents/2012/07/17/2012-17366/indirect-food-additives-polymers. (accessed 23 January 2017). However, this restriction fails to address transfer of BPA across the placenta and through the milk (Ikezuki et al. 2002; Kawamoto et al. 2007; Balakrishnan et al. 2010; Nishikawa et al. 2010; Vandenberg et al. 2010). Moreover, fetuses and neonates lack many enzymes needed to metabolize BPA and may experience greater levels of active BPA than the mother (Ikezuki et al. 2002; Kawamoto et al. 2007; Nishikawa et al. 2010).

The fetus is especially vulnerable to endocrine disruption. Sex steroid hormones guide brain sexual differentiation throughout gestation and into the neonatal period (Arnold and Breedlove 1985; Adkins-Regan 2005; McCarthy 2008). Environmental chemicals that mimic or interfere with sex hormone action may disturb this development (Palanza et al. 1999; Jasarevic et al. 2012; Kundakovic et al., 2013b). Broad ranges of behaviors are influenced by BPA exposure. Affected behaviors include reproductive, emotional, cognitive, and social behaviors and spatial reasoning (Berenbaum and Hines 1992; Mueller et al. 2008; Puts et al. 2008; Geary 2010). Many of the sociosexual deficits associated with BPA exposure in children and animals models are reviewed in Rosenfeld (2015). In our own studies, we tested two *Peromyscus* species, one that is polygynous and female uniparental (deer mice, *P. maniculatus bairdii*) and the other that is monogamous and biparental (California mice, *Peromyscus californicus*). In a mate choice experiment, females selectively reject deer mice males developmentally exposed to BPA (Jasarevic et al. 2011). Adult male California mice perinatally exposed to BPA show reduced territorial marking, a form of communication needed to protect the home range and mate from intruders, whereas exposed females demonstrate decreased exploratory and voluntary physical activity behaviors (Williams et al. 2013; Johnson et al. 2015c). Both male and female California mice developmentally exposed to BPA or ethinyl estradiol (EE, estrogen present in birth control pills) exhibit compromised parental care (Johnson et al. 2015b). These adult behavioral disruptions likely trace their origins to disturbances in normal brain programming during the perinatal period.

There is strong conservation in brain development and function across taxa, including in rodents and humans (Rice and Barone 2000; Howdeshell 2002). The hypothalamus is one of the primary brain areas governing many of these sociosexual behaviors. Thus, BPA might induce global transcriptomic changes in this brain region that is essential for guiding sociosexual behaviors. By using in situ hybridization, cDNA expression array, Northern blot, and qPCR approaches, prior rodent and zebrafish (*Danio rerio*) animal model, and in vitro cell culture studies report that BPA can alter individual candidate genes in hypothalamic regions or isolated neurons (Funabashi et al. 2001; Ceccarelli et al. 2007; Fukushima et al. 2007; Monje et al. 2007; Cao et al. 2012, 2013, 2014; Kundakovic et al., 2013a; Warita et al. 2013, 2014; Chen et al. 2014; Cano-Nicolau et al. 2016). Collectively, these studies suggest BPA disrupts the hypothalamic expression of aromatase B (*Cyp19a1b*), nerve growth factor (*Ngf*), glucocorticoid receptor (*Gr*), estrogen receptor α (*Esr1* and transcript variants), estrogen receptor β (*Esr2*), DNA methyltransferases (*Dnmt1, 3a, 3b*), methyl-CpG-binding protein 2 (*Mecp2*), kisspeptin (*Kiss1*), transforming growth factor-β3 (*Tgfβ3*), and progesterone receptor (*Pr*). A recent study suggests that in utero exposure of rats to BPA can induce select gene expression differences on postnatal day (PND) 1 (Arambula et al. 2016). However, to our knowledge, no previous studies have examined the comprehensive transcriptomic profile changes in the hypothalamus of a rodent model exposed to BPA or EE throughout the pre- and postnatal period, with the latter period approximating the third trimester of neural development in the hypothalamus, hippocampus, amygdala, and other brain regions in humans (Rice and Barone 2000; Howdeshell 2002). Such transcriptomic alterations may provide useful biomarkers of early exposure to these EDC. With this notion in mind, we used RNA-seq to determine the global transcriptomic alterations in the hypothalamus of weanling (30 days of age) male and female California mice exposed to BPA throughout gestation and lactation. This time period was chosen as it represents the end of the exposure period to these chemicals and reflects the hypothalamic gene expression patterns prior to the observed adult-onset behavioral deficits. The hypotheses at the outset were that (1) developmental exposure through the maternal diet to BPA or EE would induce global gene expression changes in the hypothalamus of juvenile California mice and (2) BPA and EE transcriptomic alterations would be dependent on offspring sex.
Materials and Methods

Animals and treatments

Founder out-bred adult (60–90 days of age) California mice females and males were purchased from the Peromyscus Genetic Stock Center (PGSC) at the University of South Carolina (Columbia, SC), and placed in quarantine for a minimum of 8 weeks to ensure that they did not carry any common rodent pathogens. At the PGSC, *P. californicus* captive stocks have been bred to maintain their out-bred status. We currently have our own breeding colony at the University of Missouri. As needed, additional California mice are purchased to maintain their out-bred status. All experiments were approved by the University of Missouri Animal Care and Use Committee (Protocol #8693). Experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Two weeks prior to breeding, virgin females, 8–12 weeks of age were randomly assigned to receive one of three diets: (1) a low phytoestrogen AIN 93G diet supplemented with 7% by weight corn oil to minimize potential phytoestrogenic contamination that would otherwise be present with inclusion of soybean oil in the diet, (2) the same diet supplemented with 50 mg BPA/kg feed weight, which we have documented to lead to internal serum concentrations close to those measured in pregnant women unknowingly exposed to this chemical (Jasarevic et al. 2011; Sieli et al. 2011), or (3) AIN93G diet supplemented with 0.1 parts per billion of EE, as the U.S. Food and Drug Administration (FDA) required estrogen-positive control for BPA studies (vom Saal et al. 2005; Johnson et al. 2015a). The FDA has requested EE be included in BPA studies that may guide policy decisions based on the premise that BPA acts primarily as a weak estrogen (Vandenberg et al. 2009). The diets were started two weeks prior to breeding to span the periconceptional period. Females were maintained on these diets throughout gestation and lactation, as described previously (Jasarevic et al. 2011, 2013; Williams et al. 2013). To avoid any potential litter effects, only one male and one female offspring per litter were randomly chosen and examined in the current studies. At weaning, five replicates of each sex and group were euthanized, and brains were flash frozen on dry ice, and stored at −80°C until the hypothalamus was dissected, as described previously for mice (Kundakovc et al., 2013a). While no brain dissection guide for *Peromyscus* is available, the landmarks described in the Rat and Mouse Brain Dissection Guide Atlases (Paxinos and Franklin 2008; Paxinos 2013) can be used for California mice.

RNA isolation from hypothalamic samples

Hypothalamic RNA was isolated using Qiagen AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Valencia, CA). Isolated DNA and miRNA will be used in future studies. The quantity and quality of the RNA was determined using a Nanodrop ND1000 spectrophotometer (Nanodrop Products, Wilmington, DE). The results were further confirmed by analyzing the RNA on the Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA). Only RNA with a RQN score above 8.0 was used for RNA-seq analyses. Five different animals representing five different litters per each sex and each group were initially tested such that a total of 50 mice were initially screened. Similar numbers of replicates in other species have been successfully used to delineate transcriptomic changes following exposure to other environmental chemicals (Richter et al. 2014; Wood et al. 2014; Wirbisky et al. 2015; Arambula et al. 2016).

Illumina TruSeq RNA library preparation and sequencing

High-throughput sequencing was performed at the University of Missouri DNA Core Facility. Libraries were constructed following the manufacturer’s protocol with reagents supplied in Illumina’s TruSeq Stranded mRNA Library Preparation kit. Briefly, the poly-A containing miRNA is purified from total RNA (2 µg), RNA is fragmented, double-stranded cDNA is generated from fragmented RNA, and the index containing adapters are ligated to fragment ends. PCR amplification was performed as follows: 98°C[([0:30]+[98°C([0:10]+60°C([0:30]+72°C([0:30])]×15 cycles + 72°C(5:00)]. The amplified cDNA construct were purified with Ampure Mag PCR Clean-up beads. Purified libraries were evaluated using the Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA), quantified with the Qubit 2.0 Flurometer (Invitrogen, Carlsbad, CA) using the quant-iT HS dsDNA reagent kit, and diluted according to Illumina’s standard sequencing protocol for sequencing on the HiSeq 2500 with a single end, 50 base read length. To maximize the number of reads per sample, only three samples were included in each lane, and the groups of three samples were randomized across treatments to avoid any confounding effects due to sequencing lane.

Gene expression analyses

Previously described methods (Givan et al. 2012) were modified to create a custom transcriptome and determine differential transcriptome expression. Specifically, 50-mer RNA-Seq reads from the Illumina HiSeq were first cleaned using scripts from the Fastx Toolkit (https://
The remaining distribution of values satisfied the Anderson-Darling test for normality, \( P = 0.06 \), as implemented in the nortest package in R (https://cran.r-project.org/web/packages/nortest/index.html). Subsequent, the distance values for EE.M1 and EE.M2 were determined to be outliers using the Grubbs test for two outliers, \( P = 2.2 \times 10^{-16} \), as implemented in the outliers package in R (https://cran.r-project.org/web/packages/outliers/index.html). Therefore, samples EE.M1 and EE.M2 were removed from all subsequent analyses discussed below. Subsequent to the removal of EE.M1 and EE.M2, no additional outliers were detected using the Grubbs test. Additionally, results from one of the EE female replicate samples were discarded due to 3X lower yield during the library generation. Lower yield may be due to a variety of reasons, but the potential for bias is high, and thus, this sample was not considered in subsequent analyses.

Differential expression of the transcripts in the remaining replicates was determined using RSEM version 1.2.15 (Li and Dewey 2011) and EdgeR version 3.8.2 (Robinson et al. 2010), as implemented in TrinityRNaseq. A transcript was considered differentially expressed between two conditions if the FDR value associated with the expression ratio was \(<0.05\). Visualization of the gene expression results via the heatmap was done using ggplot2 version 2.1.0 (Springer-Verlag, 2009) and rgl version 0.96.0 (Adler 2016). Visualization of the differential expression via the heatmap was done using the ComplexHeatmap (Gu et al. 2016) package. Functional assessments of sets of differentially expressed genes such as pathway analyses and enrichment analyses were done using the TargetMine website (Chen et al. 2016). Before doing functional analyses, de novo-assembled transcripts were mapped to human proteins based on extended amino acid similarity.

**Results**

**General characterizations**

The goal was to generate \( \sim 50 \) million reads per sample by loading three samples to each lane. The average number of raw reads was 59,965,113 and mapped reads was 43,462,565. Table 1 summarizes the alignment of RNA-seq reads for each individual sample to the \( P.\) *californicus* genome sequence. For all three groups (BPA, EE, and controls) and both sexes, similar results were obtained in all of the categories. This number of reads and gene annotations is considered more than sufficient for a eukaryotic genome (Givan et al. 2012).

**Gene expression, association, and pathway differences**

The PCA plot based on FPKM values shows a nonrandom distribution of points associated with samples (PC1
24.6%, PC2 10.2%, PC3 9.7%, PERMANOVA = $2e^{-04}$ from 10,000 permutations; Fig. 1A). Groupings of points are relatively obvious for BPA-treated males, BPA-treated females, EE-treated males, and control males, whereas control females and EE-treated females are intermingled. Even though the EE female samples cluster with the control female samples, it does not necessarily mean that the effects of EE on the female hypothalamus transcriptome are minor as further detailed below. A heatmap based on the hierarchical clustering of expression ratios for the differentially expressed genes revealed four main clusters containing primarily samples of BPA treatment, EE treatment, control females, and control males (Fig. 1B). There was no clear separation based on sex in either the BPA- or EE-treated samples.

Volcano plot comparisons revealed that our approach led to sufficient coverage to detect gene expression differences in BPA- or EE-exposed males and females relative to their respective control groups (Fig. 2). Venn diagram comparison for females revealed select genes that were either increased or decreased in both BPA- and EE-exposed individuals (Fig. 3A and B, Supporting Information File 1). However, each group possessed unique signature profile of genes that include transcripts that were increased and decreased in both groups. Likewise, BPA- and EE-exposed males had select genes that were up- and downregulated in both groups (Fig. 3C and D, Supporting Information File 1), but more genes were either increased or decreased in one of the two treatment groups.

As described earlier, EdgeR was used to identify the genes that differed in the following comparisons: control females versus control males, control females versus BPA females, control females versus EE females, control males versus BPA males, control males versus EE males, BPA females versus BPA males, and EE females versus EE males (Supporting Information File 2). Sex differences in hypothalamic gene expression between the three treatment groups are further addressed below. Tables 2 and 3 show the top 20 genes that are down- and upregulated, respectively, in BPA females versus control females. Tables 4 and 5 include the top 20 genes that are down- and upregulated, respectively, in EE females versus control females. Tables 6 and 7 show the top 20 genes that are down- and upregulated, respectively, in BPA males versus control males. Tables 8 and 9 include the top 20 genes that are down- and upregulated, respectively, in EE males versus control males. These collective tables indicate that each

| Table 1. Summary of California mice hypothalamic RNA-seq data generated and aligned to the reference genome sequence. |
| --- |
| Sample type | Sample | Raw reads | QC | Mapped reads | Mapping efficiency |
| --- | --- | --- | --- | --- | --- |
| Control ♀ | 1 | 69,130,197 | 55,971,358 | 51,865,339 | 92.7 |
| 2 | 59,953,540 | 46,892,283 | 43,166,939 | 92.1 |
| 3 | 63,537,487 | 49,756,404 | 46,292,764 | 93 |
| ... | ... | ... | ... | ... | ... |
| BPA ♀ | 1 | 45,592,976 | 36,777,665 | 33,894,109 | 92.2 |
| 2 | 62,081,241 | 48,332,836 | 44,626,692 | 92.3 |
| 3 | 48,640,791 | 37,908,444 | 34,912,852 | 92.1 |
| ... | ... | ... | ... | ... | ... |
| EE ♀ | 1 | 55,370,002 | 43,910,820 | 40,531,812 | 92.3 |
| 2 | 65,056,368 | 42,583,645 | 39,390,432 | 92.5 |
| 3 | 56,097,553 | 42,583,645 | 39,390,432 | 92.5 |
| ... | ... | ... | ... | ... | ... |
| Control ♂ | 1 | 46,376,849 | 34,835,622 | 33,238,813 | 95.4 |
| 2 | 65,673,113 | 51,651,522 | 47,823,627 | 92.6 |
| 3 | 52,258,679 | 42,073,456 | 38,938,021 | 92.6 |
| ... | ... | ... | ... | ... | ... |
| BPA ♂ | 1 | 66,308,474 | 49,724,724 | 45,792,562 | 92.1 |
| 2 | 56,296,984 | 44,408,114 | 40,927,183 | 92.2 |
| 3 | 62,267,149 | 49,653,807 | 45,769,399 | 92.2 |
| ... | ... | ... | ... | ... | ... |
| EE ♂ | 1 | 60,772,693 | 48,331,755 | 44,729,572 | 92.6 |
| 2 | 51,359,489 | 40,459,368 | 37,291,216 | 92.2 |
| 3 | 65,978,847 | 52,139,890 | 48,467,572 | 93 |

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Treatment × Sex combination led to a unique signature set of genes. In other words, different genes were differentially regulated by BPA and EE and these depend on offspring sex. The exception is Kcnd3, which was downregulated in the hypothalamus of BPA- and EE-exposed females relative to control females (bold in Tables 2 and 4). Tbl2, Topors, Kif13a, and Phactr2 were in the top 20 genes upregulated in BPA- and EE-exposed females relative to control females (bold in Tables 3 and 5). No genes were shared in the top 20 genes differentially regulated in both BPA- and EE-exposed males versus control males. Supporting Information File 3 provides gene differences between the three comparisons (BPA vs. control, EE vs. control, and BPA vs. EE) that were in common for both sexes.

Analysis of the gene expression differences to examine for gene ontology (GO) and pathways that were significantly different only revealed one term GO: 0007017: microtubule-based processes, where there were 42 genes and FDR < 0.05. Genes involved in this process were significantly enriched in the EE males compared to control males, and BPA males compared to control males. No other GO terms or pathways were significantly enriched in any of the other comparisons.

**Sex differences in hypothalamic gene expression**

Tables S1 and S2 list the top 20 genes that are down- and upregulated, respectively, in control males versus control females. Tables S3 and S4 include the top 20 genes that are down- and upregulated, respectively, in BPA males versus BPA females. Tables S5 and S6 include the top 20 genes that are down- and upregulated, respectively, in EE males versus EE females. Similar to above, sex differences in down- and upregulated genes varied based on the treatment. In the case of the top 20 genes that were downregulated in males, the only ones that overlapped include Celf4 in control and BPA groups (shaded in Tables S1 and S3), and Kcnip4 in control and EE groups (bold in Tables S1 and S5). However, comparison of the top 20 genes that are downregulated in males compared to females in BPA and EE revealed no overlapping transcripts (Tables S3 and S5). For the top 20 genes that are upregulated in males versus females, Tbkbp1 was included in the control and BPA groups (shaded in Tables S2 and S4). No genes were shared among the top 20 genes that are upregulated in males versus females for control and EE and BPA and EE groups (Tables S2, S4, and S6).
Discussion

The goals of the current study were to determine if developmental exposure through the maternal diet to BPA or EE induces global gene expression changes in the hypothalamus of juvenile California mice. Second, we sought to determine whether the BPA and EE transcriptomic alterations were dependent on offspring sex. Since BPA is considered a weak estrogen (Vandenberg et al. 2009), the notion at the outset was that within sex similar gene expression pattern changes would be seen in BPA- and EE-exposed individuals. The final aim was to examine for sexually dimorphic gene expression patterns within the control, BPA, and EE groups to determine if early exposure to one of the EDCs altered naturally occurring sex differences in hypothalamic gene expression in juvenile animals.

In relation to the first goal, RNA-seq analysis identified several genes that were differentially expressed in the hypothalamus of BPA- and EE-exposed females and males relative to their respective controls. Predictably, the specific gene expression patterns induced by each treatment varied based on offspring sex. A recent study examined gene expression patterns in the hippocampus and hypothalamus of PND1 rats exposed in utero to varying doses of BPA and EE, and reported only minimal gene expression changes (Arambula et al. 2016). As detailed above, this study design, however, failed to consider the postnatal exposure, which may be even more critical in rodent hypothalamic and other brain region development. It is this time period that approximates the third trimester in humans (Rice and Barone 2000; Howdeshell 2002). Additionally, in the previous study, the average number of reads per sample, presumably raw (although not explicitly specified) was only 29.2 million for the hypothalamus, which is about half of the total number of reads that were generated in the current studies. This difference is likely because the earlier study included more samples per lane, although no indication was provided on how many samples per lane were sequenced. In the current study, only three samples were sequenced per lane to ensure sufficient coverage per sample. Of the genes listed in the article, there does not appear to be any overlap with the current studies.

Figure 2. Volcano plots. (A) Control females versus BPA-exposed females. (B) Control females versus EE-exposed females. (C) Control males versus BPA-exposed males. (D) Control males versus EE-exposed males. Significantly downregulated genes in the treatment group versus controls are depicted in blue, black genes indicate that they are not significantly different, and genes delineated in red are increased in expression relative to controls.
Although the authors indicate a full set of differentially expressed hypothalamic genes included in Table S2a and S2b, these could not be accessed from the journal website nor could any of the other supplemental material with the message on this site reading: "We are sorry, this page is not available." Thus, no additional comparisons to this earlier study can be made until the files become publicly available.
### Table 3. Top 20 annotated genes upregulated in BPA-exposed females compared to control females.

| Entrez ID | Gene symbol | Gene name | FDR               | Log2 fold change |
|-----------|-------------|-----------|-------------------|------------------|
| 26608     | TBL2        | Microtus ochrogaster guanine nucleotide binding protein (G protein), gamma 2 (Gng2), mRNA | 5.84E-05         | 14.060           |
| 23008     | KLHC10      | Kelch domain-containing protein 10 | 0.0473           | 13.042           |
| 170506    | DHX36       | ATP-dependent RNA helicase DHX36 isoform X2 | 0.0067           | 12.949           |
| 157378    | TMEM65      | Transmembrane protein 65 (Microtus ochrogaster) | 0.0071           | 12.571           |
| 10938     | EHD1        | EH-domain containing 1, partial | 0.0089           | 12.542           |
| 3920      | LAMP2       | Lysosome-associated membrane glycoprotein 2 isoform 2 precursor | 0.0092           | 12.409           |
| 4301      | MLT4        | Forkhead box protein K1 | 0.0102           | 12.165           |
| 9026      | HIP1R       | Huntingtin-interacting protein 1-related protein isoform X1 (Mesocricetus auratus) | 0.0086           | 12.098           |
| 221937    | FOXX1       | Forkhead box protein K1 | 5.23E-08         | 12.075           |
| 10210     | TOPORS      | Mustela putorius furo UDP-Gal:betaGalNAc beta 1,4- galactosyltransferase, polypeptide 5 (B4GALT5), partial mRNA | 0.0148           | 11.784           |
| 63971     | KIF13A      | Kinesin-like protein KIF13A isoform X4 (Peromyscus maniculatus bairdi) | 0.0004           | 11.690           |
| 23274     | CLEC16A     | Protein CLEC16A isoform X4 | 0.0015           | 11.641           |
| 120909    | SSGM1       | Small G protein signaling modulator 1 isoform X1 (Mesocricetus auratus) | 0.0133           | 11.569           |
| 9749      | PHACTR2     | Phosphatase and actin regulator 2 isoform X1 | 0.0160           | 11.394           |
| 55731     | FAM222B     | Protein FAM222B isoform X1 (Peromyscus maniculatus bairdi) | 0.0161           | 11.172           |
| 253959    | RALGAPA1    | Rat GTPase-activating protein subunit alpha-1 isoform X4 | 0.018            | 10.986           |
| 8314      | BAP1        | Ubiquitin carboxyl-terminal hydrolase BAP1 | 1.29E-05         | 10.90            |
| 375       | ARF1        | Peromyscus maniculatus bairdi PHD finger protein 20-like 1 (Phf20l1), transcript variant X3, mRNA | 0.0009           | 10.741           |
| 10401     | PIAS3       | E3 SUMO-protein ligase PIAS3 (Microtus ochrogaster) | 0.001            | 10.780           |
| 26035     | GLCE        | D-glucuronol C5-epimerase (Peromyscus maniculatus bairdi) | 0.0009           | 10.557           |
| 113402    | SFT2D1      | Peromyscus maniculatus bairdi SFT2 domain containing 1 (Sft2d1), mRNA | 0.0051           | 10.592           |

### Table 4. Top 20 annotated genes downregulated in EE-exposed females compared to control females.

| Entrez ID | Gene symbol | Gene name | FDR               | Log2 fold change |
|-----------|-------------|-----------|-------------------|------------------|
| 22864     | R3HDM2      | R3H domain-containing protein 2 isoform X9 | 1.33E-06         | −12.787          |
| 23017     | FAIM2       | Protein lifeeguard 2 isoform 1 | 7.67E-11         | −12.591          |
| 9671      | WSCD2       | W5C domain-containing protein 2 | 0.0055           | −11.717          |
| 3149      | HMGB3       | High-mobility group protein B3 isoform X2 | 6.54E-14         | −11.604          |
| 5905      | RANGAP1     | Ran GTPase-activating protein 1 isoform X3 | 0.0067           | −11.488          |
| 54982     | CLN6        | Peromyscus maniculatus bairdi CDP-diacylglycerol-inositol 3-phosphatidylinositol transferase (Cdip), transcript variant X2, mRNA | 0.0075           | −11.451          |
| 10390     | CEP1        | Choline/ethanolaminephosphotransferase 1 isoform 1 | 0.0225           | −11.439          |
| 59338     | PLEKHA1     | Pleckstrin homology domain-containing family A member 1 isoform X9 | 0.0109           | −11.437          |
| 9915      | ARNT2       | Aryl hydrocarbon receptor nuclear translocator 2 isoform X1 | 2.57E-14         | −11.130          |
| 3752      | KCND3       | Potassium voltage-gated channel subfamily D member 3 isoform 1 precursor | 0.0109           | −11.056          |
| 23378     | RRFP        | Ribosomal RNA-processing protein 8 isoform 2 | 2.05E-11         | −10.974          |
| 7690      | ZF131       | Zinc finger protein 131, isoform CRA_a | 5.85E-05         | −10.866          |
| 113829    | SLC35A4     | UDP-sugar transporter protein SLC35A4 | 0.0412           | −10.20           |
| 140609    | NEK7        | Microtus ochrogaster NIMA-related kinase 7 (Nek7), mRNA | 0.0116           | −10.181          |
| 10206     | TRIM13      | E3 ubiquitin-protein ligase TRIM13 | 0.0126           | −10.684          |
| 745       | MYRF        | Myelin regulatory factor isoform X6 | 0.0158           | −10.410          |
| 84915     | FAM222A     | Protein FAM222A | 0.0154           | −10.371          |
| 4728      | NDUFS8      | NADH dehydrogenase (ubiquinone) iron-sulfur protein 8, mitochondrial | 0.0198           | −10.334          |
| 2186      | BPTF        | Nucleosome-remodeling factor subunit BPTF isoform X6 | 0.0348           | −10.330          |
| 1973      | EFA4A1      | Eukaryotic initiation factor 4A-1 (Mesocricetus auratus) | 0.0348           | −10.289          |
### Table 5. Top 20 annotated genes upregulated in EE-exposed females compared to control females.

| Entrez ID | Gene symbol | Gene name | FDR     | Log2 fold change |
|-----------|-------------|-----------|---------|------------------|
| 26608     | TBL2        | Microtus ochrogaster guanine nucleotide binding protein (G protein), gamma 2 (Gng2), mRNA | 0.0022 | 12.258 |
| 78986     | DUSP26      | Dual specificity protein phosphatase 26 | 0.0303 | 11.953 |
| 64400     | AKTIP       | AKT-interacting protein isoform 1 | 0.0271 | 11.904 |
| 5909      | RAP1GAP     | Rap1 GTPase-activating protein 1 isoform 2 | 0.0272 | 11.887 |
| 54467     | ANKIB1      | Peromyscus maniculatus bairdii RAD23 homolog B (Saccharomyces cerevisiae) (Rad23b), mRNA | 0.0290 | 11.738 |
| 63971     | KIF13A      | Kinesin family member 13A isoform X4 | 0.0015 | 11.621 |
| 8266      | UBL4A       | Peromyscus maniculatus bairdii ubiquitin-like 4A (Ubl4a), mRNA | 0.0067 | 11.259 |
| 113178    | SCAMP4      | Secretory carrier membrane protein 4, isoform CRA_c | 0.0468 | 10.990 |
| 10210     | TOPORS      | Mustela putorius furo UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 (B4GALT5), partial mRNA | 0.0472 | 10.973 |
| 10150     | MBNL2       | Peromyscus maniculatus bairdii muscleblind-like splicing regulator 2 (Mbnl2), transcript variant X2, mRNA | 0.0044 | 10.917 |

### Table 6. Top 20 annotated genes downregulated in BPA-exposed males compared to control males.

| Entrez ID | Gene symbol | Gene name | FDR     | Log2 fold change |
|-----------|-------------|-----------|---------|------------------|
| 125950    | RAVER1      | Peromyscus maniculatus bairdii ribonucleoprotein, PTB-binding 1 (Raver1), transcript variant X3, mRNA | 0.0453 | −11.309 |
| 58508     | KMT2C       | Histone-lysine N-methyltransferase 2C isoform X2 (Mus musculus) | 0.0358 | −11.129 |
| 1149      | CIDEA       | Cell death activator CID-E (Peromyscus maniculatus bairdii) | 0.0343 | −10.959 |
| 146547    | PRSS36      | Peromyscus maniculatus bairdii LysoM, putative peptidoglycan-binding, domain containing 4 (Lysmd4), mRNA | 0.0269 | −9.978 |
| 11346     | SYNP0       | Synaptotagin isoform X3 (Peromyscus maniculatus bairdii) | 0.0271 | −9.624 |
| 1307      | COL16A1     | Collagen α-(I/XVI) chain isoform X4 (Peromyscus maniculatus bairdii) | 7.14E-7 | −9.597 |
| 84893     | FBXO18      | F-box DNA helicase 1 isoform X1 (Peromyscus maniculatus bairdii) | 0.0270 | −9.524 |
| 1663      | DDX11       | Probable ATP-dependent RNA helicase DDX11 (Mesocricetus auratus) | 0.0079 | −9.499 |
| 6445      | SGC5        | Gamma-sarcoglycan (Peromyscus maniculatus bairdii) | 0.0096 | −9.498 |
| 7384      | UQCRC1      | Cytochrome b-c1 complex subunit 1, mitochondrial (Peromyscus maniculatus bairdii) | 0.0352 | −9.451 |
| 284098    | PIGW        | Phosphodiesterase-glycan biosynthesis class W protein (Peromyscus maniculatus bairdii) | 0.0163 | −9.404 |
| 131873    | COL6A6      | Collagen α-(IV) chain-like (Peromyscus maniculatus bairdii) | 0.0407 | −9.318 |
| 57698     | SHN1        | Shootin-1 isoform X1 (Peromyscus maniculatus bairdii) | 0.0005 | −9.285 |
| 8732      | RGNNT       | mRNA-capping enzyme isoform X4 (Peromyscus maniculatus bairdii) | 0.0149 | −9.261 |
| 2043      | EPHA4       | Low-quality protein: ephrin type-A receptor 10 (Lipotes vexillifer) | 0.0458 | −9.250 |
| 284403    | WDR62       | WD repeat-containing protein 62 isoform X4 (Peromyscus maniculatus) | 0.0250 | −9.239 |
| 284111    | SLCE13A5    | Solute carrier family 13 member 5 isoform X1 (Peromyscus maniculatus) | 0.0252 | −9.220 |
| 138881    | OR1L8       | Mesocricetus auratus weightless homolog (Drosophila) (Wls), transcript variant X1, mRNA | 0.0085 | −9.161 |
| 10114     | HIPK3       | Homeodomain-interacting protein kinase 3 isoform X4 (Peromyscus maniculatus bairdii) | 0.0043 | −9.083 |
| 11201     | POLI        | DNA polymerase iota isoform X3 (Peromyscus maniculatus bairdii) | 0.0035 | −9.023 |
Although select genes were up- and downregulated in both BPA and EE females and males, in general, for both sexes unique genes were altered in BPA and EE groups. The findings thus provide further evidence that BPA can induce effects by binding to other steroidogenic and nonsteroidogenic receptors besides ESRs. Another interpretation of
the H2AX protein and exerts an important role in females. During oxidative stress, TOPRS dissociates from greatest expression increase in BPA- and EE-exposed protein (Tsukumo et al. 2015). Topoisomerase I-binding unit with the net effect being alterations of specific protein factor 4 (ATF4), and associates with 60S ribosomal subunit with the net effect being alterations of specific proteins (Tsukumo et al. 2015). Topoisomerase I-binding protein (Topors) is also one of the genes that showed the greatest expression increase in BPA- and EE-exposed females. During oxidative stress, TOPRS dissociates from the H2AX protein and exerts an important role in chromatin reorganization during DNA repair and apoptosis (Seong et al. 2012). Little is known about the role of neural Kif13a, which is also upregulated in these two treatment groups. Deletion of this kinesin family motor protein in mice results in anxiety-like behaviors, likely due to reduced neuronal transport of 5HT(1A) receptor (Zhou et al. 2013). Phactr2 is also upregulated in these two groups. Phosphatase and actin regulators (PHACTRs) are abundant in the brain where they are reported to mediate activity of protein phosphatase 1 and actin-binding protein. Additionally, Phactr2 expression is elevated after traumatic brain injury (Kim et al. 2012). Taken together, the genes that are abundant in BPA- and EE-exposed females appear to be ones that in many cases are upregulated under varying stressful conditions and may help to partially alleviate further cellular damage and ensuing behavioral disturbances. In males, no genes were common among the 20 most highly differentiated genes expressed in both BPA- and EE-exposed females. However, looking outside of these 20 genes, 9 and 72 transcripts were down- and upregulated, respectively, in both of these groups (Fig. 2). These shared transcripts are involved in a variety of functions, as shown in Supporting Information File 2. In males, no genes were common among the 20 most highly differentiated genes expressed in both BPA- and EE-exposed females. However, looking outside of these 20 genes, 9 and 72 transcripts were down- and upregulated, respectively, in both of these groups (Fig. 2). These shared transcripts are involved in a variety of functions, as shown in Supporting Information File 2.

**Table 9. Top 20 annotated genes upregulated in EE-exposed males compared to control males.**

| Entrez ID | Gene symbol | Gene name | FDR | Log2 fold change |
|-----------|-------------|-----------|-----|-----------------|
| 30000 | TNP2 | Transportin-2 (Mesocricetus auratus) | 0.0016 | 12.5441 |
| 6196 | RPS6KA21 | Ribosomal protein S6 kinase a-2 isofrom X2 (Peromyscus maniculatus bairdii) | 2.59E-10 | 12.4266 |
| 2036 | EPB41L1 | Band 4.1-like protein 1 isofrom X12 (Peromyscus maniculatus bairdii) | 0.0022 | 12.2511 |
| 84918 | LRP11 | Low-density lipoprotein receptor-related protein 11 (Peromyscus maniculatus bairdii) | 0.0024 | 12.1854 |
| 386675 | KRTAP10-7 | Cricetus griseus protein kinase C, a (Prkca), transcript variant X1, mRNA | 0.0032 | 11.8746 |
| 84893 | FBXO18 | F-box DNA helicase 1 isofrom X1 (Peromyscus maniculatus bairdii) | 1.08E-05 | 11.7283 |
| 9026 | HIP1R | Huntingtin-interacting protein 1-related protein isofrom X1 (Mesocricetus auratus) | 0.0041 | 11.5967 |
| 79411 | GLB1L | ji-Galactosidase-1-like protein isofrom X1 (Peromyscus maniculatus bairdii) | 2.05E-5 | 11.3220 |
| 4043 | LRPAp1 | a-2-Macroglobulin receptor-associated protein (Peromyscus maniculatus bairdii) | 2.42E-5 | 11.2051 |
| 56927 | GPR10B | Protein GPR10B (Peromyscus maniculatus bairdii) | 0.0061 | 11.1658 |
| 22800 | RRAS2 | Ras-related protein R-Ras2 (Elephantulus edwardii) | 0.0001 | 11.1491 |
| 113178 | SCAMP4 | Secretory carrier membrane protein 4, isofrom CRA_c | 0.0068 | 11.0335 |
| 11346 | SYNPO | Synaptopodin isofrom X3 (Peromyscus maniculatus bairdii) | 4.62E-5 | 10.9740 |
| 51290 | ERGIC2 | Endoplasmic reticulum-Golgi intermediate compartment protein 2 isofrom X3 (Microtus ochrogaster) | 0.0004 | 10.9722 |
| 22826 | DNAJC8 | DnaJ homolog subfamily C member 8 isofrom X2 (Peromyscus maniculatus bairdii) | 0.0002 | 10.9581 |
| 396 | ARHGDA | Rho GDP-dissociation inhibitor 1 isofrom X2 (Cricetus griseus) | 0.0090 | 10.7457 |
| 8848 | TSC22D1 | Peromyscus maniculatus bairdii TSC22 domain family, member 1 (Tsc22d1), transcript variant X2, mRN | 0.0001 | 10.7014 |
| 11011 | Tlk2 | Serine/threonine-protein kinase tousled-like 2 isofrom X1 (Peromyscus maniculatus bairdii) | 0.0094 | 10.6909 |
| 2887 | GRB10 | growth factor receptor-bound protein 10 isofrom X2 (Peromyscus maniculatus bairdii) | 1.25E-5 | 10.6643 |
| N/A | N/A | Peromyscus maniculatus bairdii ArfGAP with SH3 domain, ankyrin repeat and PH | 0.0099 | 10.6337 |

These data is that as a weak estrogen, BPA may not fully recapitulate the same gene expression changes as EE.

However, select genes were shared among highly differentially expressed genes in BPA- and EE-exposed females and males. These transcripts will be further considered. Kcnd3 was among the most highly downregulated genes in the hypothalamus of both BPA- and EE-exposed females. This gene encodes for the voltage-gated potassium 4.3 (Kv4.3) channel. In humans, mutation of KCND3 is associated with cerebellar ataxia, intellectual disability, epilepsy, attention deficit disorders, and other clinical signs (Smets et al. 2015). Similar to the current findings, estrogen decreases transcriptional expression of Kv4.3 in the myometrium (Song et al. 2001).

Tranducin (beta)-like 2 (Tbl2) was among the most highly upregulated genes for BPA- and EE-exposed females. This gene encodes a protein that localizes to the endoplasmic reticulum (ER). Under ER stress conditions, it interacts with PKR-like ER-resident kinase (PERK), modulates protein expression of activating transcription factor 4 (ATF4), and associates with 60S ribosomal subunit with the net effect being alterations of specific proteins (Tsukumo et al. 2015). Topoisomerase I-binding protein (Topors) is also one of the genes that showed the greatest expression increase in BPA- and EE-exposed females. During oxidative stress, TOPRS dissociates from the H2AX protein and exerts an important role in
processes. With their pleiotrophic roles, microtubules are essential for proper neuronal function. Microtubules must respond quickly to environmental changes to provide structural support for the Golgi apparatus, axon guidance, dendrites, neurite outgrowth, interkinetic nuclear migration, and separation of chromatids during mitosis to list a few of the functions ascribed to these structures in neurons (Prokop 2013; Breuss and Keays 2014; Liu and Dwyer 2014; Sainath and Gallo 2015). The microtubule cytoskeleton works in conjunction with microfilaments (actin) and intermediate filaments to facilitate intracellular transport. Previous studies suggest that BPA exposure can disrupt microtubule-associated proteins in hypothalamic neurons (Yokosuka et al. 2008; Iwakura et al. 2010) and other cell types (Nakagomi et al. 2001; Lehmann and Metzler 2004; Can et al. 2005; George et al. 2008). Ethinyl estradiol can also disrupt microtubular function (Epe et al. 1989; Sato et al. 1992).

In relation to the final aim, several genes were identified in all groups to be expressed in a sexually dimorphic pattern. However, there was minimal overlap of genes between the three groups, indicating that each treatment leads to a unique signature pattern within each sex. Alternatively, early exposure to BPA and EE altered the normal pattern of sex-specific neurobehavioral programming. Other EDCs lead to sex-dependent changes in the hypothalamus of largemouth bass (Micropterus salmoides) (Martyniuk et al. 2010, 2013) and rats (Walker et al. 2014).

While it would have been optimal to confirm the gene expression differences with qPCR, all of the RNA from the micropunch samples was used for the RNA-seq analysis. Such studies will be performed in follow-up studies, along with examining for protein expression differences. We also wish to examine other brain regions and non-neural tissues to determine if there are common transcripts altered by developmental exposure to BPA and EE. Furthermore, it would be of interest to examine for phenotypic changes that are consistent with BPA-induced microtubule dysfunction.

In conclusion, the current results show that perinatal exposure to BPA or EE mediates sex-dependent changes in the hypothalamus of juvenile California mice. Furthermore, BPA and EE exposure results in unique signature of hypothalamic transcripts in males and females. The one common target of BPA and EE exposure may be microtubule-based processes, which could lead to a wide range of pathophysiological effects in neuronal cells. The studies also demonstrate that even in juvenile animals there are normal sexually dimorphic differences in gene expression in the hypothalamus, but these may be disrupted by early exposure to either EDC tested. It remains to be determined whether the gene expression changes, including microtubule-associated genes, are responsible for behavioral deficits observed in California mice and possibly by translation to humans.

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**Conflict of Interest**

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Top 20 annotated genes downregulated in control males compared to control females. Shaded row is included in the BPA group (Table S3), whereas bold row is included in the EE group (Table S5).

Table S2. Top 20 annotated genes upregulated in control males compared to control females. Shaded row is included in the BPA group (Table 4).

Table S3. Top 20 annotated genes downregulated in BPA males compared to BPA females. Shaded row is included in the Control group (Table S1).

Table S4. Top 20 annotated genes upregulated in BPA males compared to BPA females. Shaded row is listed in controls (Table S2).

Table S5. Top 20 annotated genes downregulated in EE males compared to EE females. Bold row is included in the control group (Table S1).

Table S6. Top 20 annotated genes upregulated in EE males compared to EE females.

Data S1: Genes Increased or Decreased in Both BPA- and EE-Exposed Groups.

Data S2: Differentially Expressed Genes Based on Treatment and Sex.

Data S3: Differentially Expressed Genes Based on Treatment.