Early life exposure to low levels of AHR agonist PCB126 (3,3',4,4',5-pentachlorobiphenyl) reprograms gene expression in adult brain

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Abstract

Early life exposure to environmental chemicals can have long-term consequences that are not always apparent until later in life. We recently demonstrated that developmental exposure of zebrafish to low, non-embryotoxic levels of 3,3',4,4',5-pentachlorobiphenyl (PCB126) did not affect larval behavior, but caused changes in adult behavior. The objective of this study was to investigate the underlying molecular basis for adult behavioral phenotypes resulting from early life exposure to PCB126. We exposed zebrafish embryos to PCB126 during early development and measured transcriptional profiles in whole embryos, larvae and adult male brains using RNA-sequencing. Early life exposure to 0.3 nM PCB126 induced *cyp1a* transcript levels in 2-dpf embryos, but not in 5-dpf larvae, suggesting transient activation of aryl hydrocarbon receptor with this treatment. No significant induction of *cyp1a* was observed in the brains of adults exposed as embryos to PCB126. However, a total of 2209 and 1628 genes were differentially expressed in 0.3 nM and 1.2 nM PCB126-exposed groups, respectively. KEGG pathway analyses of upregulated genes in the brain suggest enrichment of calcium signaling, MAPK and notch signaling, and lysine degradation pathways. Calcium is an important signaling molecule in the brain and altered calcium homeostasis could affect neurobehavior. The downregulated genes in the brain were enriched with oxidative phosphorylation and various metabolic pathways, suggesting that the metabolic capacity of the brain is impaired. Overall, our results suggest that PCB exposure during sensitive periods of early development alters normal development of the brain by reprogramming gene expression patterns, which may result in alterations in adult behavior.

Key words: zebrafish, DOHaD, RNAseq, latent effects, brain, males

Introduction

Epidemiological and experimental studies have clearly established that exposure to stressors during preconception and perinatal periods of development can have long-term implications that are seen well after the exposure has occurred (Gluckman *et al.*, 2016). This is a growing field of research investigating the latent effects of early life exposure to stressors, known as the developmental origins of adult health and disease (DOHaD). A number of early life events such as exposure to nutritional, psychological and chemical stressors have been shown to have later life consequences (Gluckman, *et al.*, 2016). Such early life exposures have been linked to disease outcomes such as cardiovascular and metabolic disorders (Gilbert, 2016; Heindel *et al.*, 2017), hypertension (Gilbert and Nijland, 2008), cognitive disabilities (Lester *et al.*, 2012), respiratory disorders (Turner, 2016) and various types of cancers (Ho *et al.*, 2016; Walker and Ho, 2012). DOHaD research in the past decade has focused on characterizing the molecular basis of the relationship between developmental exposure and later life diseases.

The list of environmental chemicals investigated for latent effects of early life exposure is increasing rapidly and includes persistent organic pollutants such as polychlorinated biphenyls (PCBs). Even though PCBs have been banned for many decades, they are ubiquitously distributed in the environment and are present in detectable levels (0.4-1.9 parts per billion) in human blood samples (Xue *et al.*, 2014). The effects of prenatal exposure to PCBs on the offspring are well documented. For example, there is a strong association between prenatal exposure to PCBs and lower intelligence in children (Stewart *et al.*, 2008). Similar associations have been observed between PCB levels in school buildings and behavioral changes such as learning and memory deficits in children (Schantz *et al.*, 2003) and adolescents (Newman *et al.*, 2009). It is also increasingly being recognized that exposure to low levels of PCBs that do not cause overt acute toxicity can have long-term consequences on behavior, growth and metabolism (Jensen *et al.*, 2014; Patandin *et al.*, 1998; Vreugdenhil *et al.*, 2002; Winneke *et al.*, 2014); however, the underlying molecular basis is not well understood.

A wide range of species have been utilized as models in DOHaD research including sheep, rats, mice, guinea pigs (Dickinson *et al.*, 2016), and more recently zebrafish (Bailey *et al.*, 2016; Bailey *et al.*, 2015; Baker *et al.*, 2014a; Knecht *et al.*, 2017; Wirbisky *et al.*, 2016a; Wirbisky *et al.*, 2015). Zebrafish are ideal for DOHaD studies because of short generation time (~3-4 months to reach adulthood), relatively large clutch sizes and external fertilization. This allows the exposure of embryos to toxicants very early during embryogenesis, have a large number of biological replicates and conduct multigenerational studies in a relatively short period of time. In addition, the availability of genomic and bioinformatic resources enables investigating mechanisms of action. Several studies have recently demonstrated that exposure to environmental chemicals during early zebrafish development can have latent effects. For instance, exposure of zebrafish embryos to TCDD for 1h at two critical developmental time points impaired reproductive performance in adults (Baker *et al.*, 2014b; King Heiden *et al.*, 2009). Some of these effects were even shown to be observed in subsequent generations. Similarly, benzo[a]pyrene (BaP) and atrazine exposure during zebrafish development was shown to cause reproductive defects in adults and morphological defects in subsequent generations (Corrales *et al.*, 2014; Wirbisky *et al.*, 2016b).

We recently demonstrated that exposure of early zebrafish embryos to low levels of a dioxin-like PCB (PCB126) had no overt toxicity during early development, but as adults, PCB126-exposed fish showed impaired habituation to a novel environment (Glazer *et al.*, 2016). All of these studies clearly demonstrate latent phenotypes associated with early life exposures to a variety of environmental chemicals. However, very few studies have investigated the transcriptional changes in the adults following developmental exposure to toxicants (Baker *et al.*, 2016; Wirbisky*, et al.*, 2016a; Wirbisky*, et al.*, 2015). These studies provided important information about the latent effects of early life exposure to toxicants. Because gene expression changes are dynamic, it is important to determine the effects of exposure at multiple time points. Hence, in this study we investigated the transcriptional responses associated with exposure to two different doses (0.3 and 1.2nM) of PCB126 using RNA-sequencing at three different time points (embryo, larvae and adult brain). We used

PCB126 as a model toxicant because its mode of action and developmental toxicity are well understood. PCB126 acts through the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor that mediates the toxic effects of chlorinated dioxins, planar PCBs, and other dioxin-like compounds. We hypothesized that the latent behavioral effects observed in adult fish that were developmentally exposed to low levels of PCB126 are due to altered programming of gene expression patterns in the brain.

Methods

Animals and experimental design

Tupfel-longfin (TL) strain of zebrafish were used in this study. All experiments conducted were approved by the Woods Hole Oceanographic Institution's animal care and use committee. The experimental design and sampling regime followed are described in (Glazer*, et al.*, 2016). Briefly, zebrafish embryos were exposed to either solvent control (DMSO) or different concentrations of PCB126 (0.3, 0.6 and 1.2 nM) starting from 4 hours post-fertilization (hpf) to 24 hpf. These PCB126 concentrations were chosen because they do not cause overt morphological phenotypes such as pericardial and yolk sac edema, heart deformities and lower jaw malformation typically observed with concentrations above 3nM. Due to lipophilic nature and bioaccumulative properties of PCBs, short term exposure of zebrafish embryos is sufficient for its uptake and persistence throughout embryonic development. At the end of the exposure period, embryos were thoroughly rinsed and raised in clean water until 6 months of age. Embryos and larvae were sampled at 2 and 5 days post-fertilization (dpf) respectively, for measuring acute gene expression changes. Whole brain samples were collected from adult male zebrafish to determine latent effects of developmental exposure to low levels of PCB126. The adult males were the same fish used in the behavioral assays described in (Glazer*, et al.*, 2016); the brains were sampled after completion of the behavioral testing. We only did transcriptional profiling on 0.3 nM and 1.2 nM PCB126 exposed groups.

Total RNA isolation and strand-specific RNA sequencing

Previously (Glazer*, et al.*, 2016), we reported that developmental exposure to PCB126 did not affect early development or behavior, but as adults the fish displayed behavioral changes. In order to understand the transcriptional basis for these responses, we measured gene expression patterns in the embryos (2 dpf), larvae (5 dpf) and in the adult brain (6 months). Each time point had three treatments (DMSO, 0.3 and 1.2nM PCB126) except at 2dpf, where only DMSO and 0.3nM PCB126 samples were sequenced. Each treatment had three biological replicates and each replicate is a pool of embryos (10 per pool) or larvae (15 per pool). For adult male brain samples, each replicate is from an individual fish. Total RNA was isolated from 2 dpf embryos and adult brain samples following a protocol for simultaneous isolation of DNA and RNA (Pena-Llopis and Brugarolas, 2013). Total RNA was isolated from 5 dpf larvae using the Aurum^{TM} Total RNA Mini Kit (Bio-Rad, Hercules, CA). Quality of the RNA was checked using Bioanalyzer (Agilent Technologies, Santa Clara, CA); the RNA integrity numbers of all samples used for RNAseq were above 9.2. Strand-specific RNAseq library preparation and sequencing were done at the Tufts University Core Facility. Library preparation was done using Illumina TruSeq with Ribozero total RNA library prep kit and 50 bp single-end, strand-specific sequencing was performed on the HiSeq2500 platform.

Data analysis

Raw data files were assessed for quality using FastQC (Andrews, 2010) prior to pre-processing. Pre-processing was done 1) by trimming the adaptor sequences using Trimmomatic and 2) removing any reads with low sequence quality (Phred score less than 20). Trimmed sequence reads were mapped to the zebrafish genome using the STAR aligner (Dobin and Gingeras, 2015). Mapping quality was checked using RSeQC pipeline (Wang *et al.*, 2012) and coordinate sorted BAM files were filtered using samtools (-F 256) to remove reads with poor mapping quality. The number of reads mapped to annotated regions of the genome were obtained using HTSeq-count (Anders *et al.*, 2015). We used Ensembl version 84 (GRCz10) of the zebrafish genome and annotations (gtf) in this

analysis (Yates *et al.*, 2016). Statistical analysis was conducted using edgeR, a Bioconductor package (Robinson *et al.*, 2010). We used the quasi-likelihood model in edgeR (glmQLFTest) to perform differential gene expression analysis. Only genes with false discovery rate (FDR) of less than 5% were considered to be differentially expressed. Raw data has been deposited in gene expression omnibus (Accession number GSE98741) and Dryad (Aluru *et al.*, 2017). We used BioMart (Smedley *et al.*, 2015) to obtain gene symbols and gene names. The complete list of differentially expressed genes in different treatment conditions is provided in supplemental information (**dge.xlsx**).

Gene Ontology (GO) classification and KEGG pathway analysis

Differentially expressed genes (DEGs) were functionally classified based on GO terms using the PANTHER (Protein Annotation THrough Evolutionary Relationship) classification system (pantherdb.org; (Mi *et al.*, 2013)) and gProfiler (Reimand *et al.*, 2016). PANTHER and gProfiler include comprehensive species-specific GO annotations directly imported from the GO database (Gene Ontology, 2015). We used zebrafish ensembl IDs as input and classified our DEGs using the GO molecular function complete database, which includes both manually curated and electronic annotations. Bonferroni correction for multiple testing was used while determining the fold enrichment of GO terms. Only GO terms with p-value of less than 0.05 were considered to be statistically significant and used in subsequent analyses. To understand the relationship between GO terms, Directed Acyclic Graphs (DAGs) of significantly enriched GO terms were drawn using GOView (webgestalt.org/GOView). We obtained similar results with both PANTHER and gProfiler software.

We did GO term and KEGG pathway analysis on three different groups of DEGs. First, we did the analysis on up and downregulated genes separately. A second analysis was done on the genes that were common to both exposure groups. The third analysis was done on all the DEGs combined (up and downregulated genes together). Results from the first two analyses are reported in the text; results from the third analysis are included in the supplemental information. To compare the results from the two PCB126 concentrations, gCocoa was used (Reimand*, et al.*, 2016). KEGG pathway

analysis of the differentially expressed genes (DEGs) was done using gProfiler and the pathways were visualized using the KEGG database (http://www.genome.jp/kegg/). We manually went through the list of genes represented under each enriched GO term and KEGG pathway and only the pathways with unique lists of genes are shown.

Results

Strand-specific RNA sequencing of embryos, larvae and adult male brain samples yielded an average of 31 million reads per sample, after pre-processing. Of these, 84% of the reads were uniquely mapped and this was consistent across all three developmental stages. The summary of mapping statistics is provided in supplemental Information **(summary_statistics.xlsx).**

Acute effects of exposure to low levels of PCB126

We exposed zebrafish embryos to 0.3nM and 1.2nM PCB126 from 4 to 24 hpf and collected samples at 2 and 5 dpf to determine the acute effects of exposure. In 0.3nM PCB126-exposed embryos, there was only one differentially expressed gene (DEG), cytochrome P4501A (*cyp1a*; FDR <5%) at 2 dpf (**Tables 1**). *Cyp1a* was induced 12.5-fold, evidence of modest AHR activation and consistent with our quantitative RT-PCR results reported earlier (Glazer et al. 2016). Changing the FDR cutoff to 10% showed two additional genes to be induced, the xenobiotic metabolism genes *cyp1c1* and *cyp1c2*, induced 2.5- and 4.5-fold respectively. At 5 dpf, 0.3 nM PCB126-exposed larvae had no DEGs, suggesting that AHR activation was transient at this concentration (**Table 1**). In contrast, in 1.2 nM PCB126-exposed larvae a total of 7 genes were differentially expressed (FDR <5%): *cyp1a, cyp1c1, cyp1c2, cyp1b1, ahrra, foxf2a* and one non-coding RNA (lincRNA) (**Table 1**). All these are AHR target genes and were upregulated. We observed a modest increase in AHR gene expression in both 0.3 nM (logFC of 1.2) and 1.2 nM (logFC of 1.43), which were not significant (FDR >0.05).

Long-term effects of early life AHR activation on gene expression patterns in the male brain

In contrast to the acute effects, a large number of genes were differentially expressed in the whole brain samples of adult male fish exposed to PCB126 during early development. Volcano plots show the number of DEGs in the brain in comparison to 2 and 5 dpf animals **(Figure 1)**. A total of 2209 and 1628 DEGs were observed in 0.3 and 1.2 nM PCB126-exposed groups, respectively (FDR <5%). The number of up and downregulated genes at each concentration are shown in **Figure 2**. A total of 977 genes were common to both treatments. Among them, 415 genes were downregulated and 562 were upregulated (**Figure 2**). The list of 977 genes is provided in supplemental information (**977_commongenes.xlsx**).

Functional classification of DEGs using GO annotations

GO term enrichment analysis on up and downregulated genes revealed that the DEGs in 0.3 and 1.2 nM PCB126 groups are associated with the same GO terms. **Table 2** shows the statistically significant GO terms and the associated DEGs for up and downregulated genes. The list of genes represented in each GO term is provided in the supplemental information. Among the upregulated genes, two GO terms--cation channel activity and transcription factor activity--are enriched in both PCB126 groups (**Table 2**). Downregulated genes are enriched in GO terms such as structural constituent of ribosome, RNA binding, collagen binding, sulfur compound binding, phosphotidylinositol-4,5-biphosphate binding and potassium:chloride symporter activity. Of these, only structural constituent of ribosome is enriched in both treatment groups (**Table 2**). GO analysis on the 977 DEGs that are common to both treatment groups revealed enrichment of GO terms high voltage-gated calcium channel activity (GO:0008331) and structural constituent of ribosome (GO:0003735) (**Table 4**).

Enrichment of KEGG pathways

Functional annotation of DEGs revealed enrichment of important pathways in both up and downregulated gene sets. Among the upregulated genes, calcium signaling, MAPK signaling, lysine degradation, ErbB signaling and GnRH signaling pathways were significantly enriched in both treatment groups (**Table 3**). **Figure 3** shows the KEGG calcium signaling pathway with the genes that were upregulated in one or both treatment groups highlighted. The fold change values of these genes in 0.3nM and 1.2nM PCB126 groups are shown in **Table 5**.

The downregulated DEGs were enriched in pathways such as oxidative phosphorylation, ribosome, metabolic pathways, carbon metabolism, ECM-receptor interaction, glutathione metabolism, fatty acid metabolism and citrate cycle (**Table 3**). Of these, only oxidative phosphorylation and ribosome were enriched in both 0.3 and 1.2nM PCB126 groups. The remaining pathways were only enriched in the 0.3 nM PCB126 group. The key steps in the oxidative phosphorylation pathway and the genes that were downregulated in one or both treatment groups are shown in **Figure 4**. KEGG analysis on the 977 DEGs common to both exposure groups revealed enrichment of ribosome, oxidative phosphorylation, calcium signaling pathway and cardiac muscle contraction (**Table 4**). We have provided the list of genes represented under each GO and KEGG terms in the supplemental information (PathwayAnalysis_Up.xlsx and PathwayAnalysis_Down.xlsx).

Discussion

We recently demonstrated that low-dose PCB126 exposure during early embryonic development results in behavioral deficits in adults (Glazer*, et al.*, 2016). The current study was aimed at understanding the transcriptomic changes associated with these latent behavioral changes observed in the adults. Changes in gene expression and toxic effects associated with developmental PCB126 exposure in zebrafish are well documented (Grimes *et al.*, 2008; Jonsson *et al.*, 2007a; Jonsson *et al.*, 2012; Jonsson *et al.*, 2007b). In this study, exposure to 0.3 or 1.2 nM PCB126 did not cause any morphological changes typically seen with higher doses of dioxin-like PCBs (Jonsson*, et al.*, 2007a; Jonsson*, et al.*, 2012). We also did not observe any defects in behavior in the exposed

larvae at 7 and 14 dpf; however, as adults the fish exposed as embryos showed impaired habituation to a novel environment (Glazer*, et al.*, 2016). We hypothesized that these latent behavioral defects are due to AHR-induced altered programming of gene expression patterns in the brain during early development. Studies in mammals have demonstrated the importance of AHR in embryogenesis, particularly in the development of important tissues and organ systems (Fernandez-Salguero *et al.*, 1995; Lund *et al.*, 2006; McMillan and Bradfield, 2007; Schneider *et al.*, 2014; Singh *et al.*, 2009). Similar to our results, activation of AHR in pregnant dams by dioxin exposure was shown to cause behavioral abnormalities in the offspring as adults, suggesting that abnormal AHR signaling during development can alter the developmental trajectory leading to various behavioral phenotypes (Endo *et al.*, 2012; Kakeyama *et al.*, 2014; Markowski *et al.*, 2001; Schantz *et al.*, 1996; Thiel *et al.*, 1994). However, the underlying long-term transcriptional changes associated with developmental exposure to persistent organic pollutants such as PCBs have not been characterized.

Similar to mammals, induction of CYP1A is a classical response to PCB126 exposure in zebrafish. CYP1A induction was the only significant change in gene expression in 0.3nM PCB126-exposed embryos at 2 dpf, with no significant changes observed at 5 dpf, pointing to a transient AHR activation by this low concentration of the chemical. On the other hand, a higher concentration (1.2nM) of PCB126 caused induction of several AHR target genes at 5 dpf suggesting sustained AHR activation throughout early development. We also have previously reported induction of CYP1A in 1.2nM PCB126 group at 2 dpf (Glazer et al., 2016). In contrast to the results at 2 and 5 dpf when there were changes in expression of very few genes, there were large-scale transcriptional changes in the brain of adult fish that were developmentally exposed to PCB126.

Gene Ontology analysis of the genes upregulated in the brain shows enrichment of the GO term cation channel activity. The majority of genes included in this term are voltage-gated calcium channels (VGCCs) encoding alpha subunits that represent P/Q (*cacna1aa, cacna1ab*), N (*cacna1ba,*

cacna1bb), L (*cacna1c, cacna1da*) and T (*cacna1g, cacna1ha, cacna1i*) type calcium channels. All these genes were significantly upregulated in the brain (**Table 5**). VGCCs are important players in the transmission of electrical impulses, regulating many different physiological processes. Previous studies have shown that TCDD and non-dioxin-like PCBs, affect both the basal and stimulated (depolarization-evoked) increase in intracellular calcium levels (Kim and Yang, 2005; Langeveld *et al.*, 2012). Depolarization-evoked increases in intracellular calcium occur mainly via voltage–activated L-, N-, and P/Q-type and to a lesser extent by T-type channels. Acute exposure of rat neocortical cultures to Aroclor 1254, a commercial PCB mixture caused an increase in resting intracellular calcium levels which has been attributed to calcium ion influx (Inglefield and Shafer, 2000), suggesting altered calcium homeostasis. Indeed, KEGG pathway analysis of our data revealed that calcium signaling was one of the enriched pathways among the upregulated genes. In addition to VGCCs, we observed upregulation of genes encoding G-protein coupled receptors (glutamate (*grm1b, grm5a*), cholinergic (*chrm2a*), tachykinin (*tacr1a*), histamine (*hrh1*)) and important signal transduction molecules (adenylate cyclases *adcy1a, adcy2a*) in calcium signaling. The results thus suggest that dioxin-like PCBs affect calcium homeostasis in the brain *in vivo*.

Indeed, AHR agonists TCDD and benzo(*a*)pyrene (BaP) have been shown to cause a transient increase in intracellular calcium levels, possibly by an AHR-independent mechanism (Kobayashi *et al.*, 2009; Mayati *et al.*, 2012; Puga *et al.*, 1992). The signal transduction pathway for induction of intracellular calcium concentration by BaP involves activation of β2-adrenergic receptor and induction of adenylyl cyclase and inositol 1,4,5-trisphosphate signaling cascade (Mayati*, et al.*, 2012). The genes associated with this signaling cascade were upregulated in the adult brains in both of our PCB exposure groups. The disruption in calcium homeostasis could affect downstream signaling pathways potentially affecting important cognitive functions such as learning and memory.

One of the pathways directly affected by altered calcium signaling is the mitogen-activated protein kinase (MAPK) signaling pathway, also enriched in our dataset. MAPK signaling influences a variety of cellular functions, including cell proliferation, senescence and apoptosis. One of the widely studied MAPK pathways is the Ras/Raf/MEK/ERK cascade; we observed upregulation of genes associated with each step of this signaling cascade. For instance, in 0.3nM PCB126 group, there was upregulation of Ras (*rasgraf2b*), Raf(*braf*) and MEK/ERK (*mapk2k5, mapk3k13, mapk8b, mapk2k4a, mapk10, mapk4k3b*) genes. MAPK signaling has been implicated in brain development (Jeanneteau and Deinhardt, 2011; Thomas and Huganir, 2004). It has been shown to play an important role in synaptic plasticity, long-term memory and in anxiety and depression-like behaviors (Jeanneteau and Deinhardt, 2011; Thomas and Huganir, 2004; Wefers *et al.*, 2012). AHR agonists such as TCDD and PCB126 have been shown to induce MAPK signaling in neuronal cells (Li *et al.*, 2013; Puga*, et al.*, 1992; Song and Freedman, 2005) but It remains to be determined if the latent behavioral effects of developmental exposure to AHR agonists are mediated by MAPK signaling.

One of the widely investigated mechanisms behind latent effects of developmental exposure to stressors is the epigenetic regulation of gene expression, which includes DNA methylation, chromatin modifications, and altered noncoding RNAs. In this study, we observed enrichment of genes associated with methylation of lysine (K) residues in histone proteins (KEGG: lysine degradation pathway). Lysine methylation exists in mono, di, and tri-methyl states and these modifications can regulate gene expression by changing chromatin structure and DNA accessibility. The most well-characterized lysine methylation residues are K4, K9, K27, K36, and K79 of histone H3. Methylation of H3K4, H3K36, and H3K79 is associated with transcriptional activation, whereas H3K9 and H3K27 are correlated with transcriptional repression (Martin and Zhang, 2005). The primary regulators of H3K4 methylation are histone lysine methyltransferases (KMTs); we observed upregulation of 7 genes belonging to this class of proteins (*kmt2a, kmt2bb, kmt2ca, setd1a, setd1ba, ash1 and whsc1l1*). In addition, we observed a H3K79 methyltransferase (*dot1l*) to be upregulated in PCB-

exposed fish. Previous studies have shown that PCBs target histone modifications (Casati *et al.*, 2012; Ovesen *et al.*, 2011) but the effects of altered expression of KMTs on chromatin accessibility and gene expression are not known. One recent study has characterized the persistent effects of developmental exposure to toxicants that involve histone modifications. For example, developmental exposure to bisphenol A (BPA) increased the H3K4 trimethylation mark at genes associated with prostate cancer, and these marks persisted into adulthood (Wang *et al.*, 2016). Similar functional studies should be conducted in order to characterize the consequences of upregulation of KMT genes in PCB126-exposed fish.

Another significant finding of this study is the downregulation of a large number of genes associated with oxidative phosphorylation (OxPhos). Oxidative phosphorylation takes place inside mitochondria, generating ATP necessary for cellular functions. Downregulation of OxPhos genes suggests mitochondrial dysfunction and defects in ATP generation. Energy metabolism in the brain is also mainly dependent on OxPhos for ATP generation (Belanger *et al.*, 2011), and reduced ATP generation in the brain is a hallmark of neurodegenerative disorders (Koopman *et al.*, 2013). The genes downregulated in our study belong to all four complexes (I-IV) and the final ATP synthesis step of the electron transport chain. Surprisingly, 64 OxPhos genes were downregulated in the 0.3nM PCB126-exposed group compared to only 20 genes in the 1.2nM PCB126 group, suggesting that the mechanisms of action might be different at these two concentrations. One potential explanation for more genes differentially expressed with 0.3 nM PCB126 than with 1.2nM could be the non-monotonic dose response effects. The acute non-monotonic effects of toxicants have been widely demonstrated (Birnbaum, 2012), but similar studies in understanding the DOHaD effects are lacking. Our results stress the need for investigating the non-monotonic effects in the DOHaD context.

Effects of AHR agonists on mitochondrial function have been documented previously (Biswas *et al.*, 2008; Shertzer *et al.*, 2006). For instance, TCDD-exposed mice have approximately

60% reduction in hepatic ATP production in the mitochondria (Shertzer*, et al.*, 2006). In murine myoblast cells, TCDD disrupts mitochondrial transmembrane potential, transcription and translation (Biswas*, et al.*, 2008). These are acute effects of TCDD exposure observed within a few days to a week, whereas in the current study the effects observed are 6 months after the developmental exposure, suggesting that effects of dioxin-like PCBs might be similar irrespective of the exposure regime, but that the mechanisms of action might vary. In addition to the effects on OxPhos, we observed enrichment of KEGG terms such as metabolic pathways, fatty acid metabolism, ribosome and carbon metabolism. These results suggest an overall reduction in metabolism in the brain as shown by reduction in glucose and fatty acid metabolism genes and ribosomal genes, which are essential for protein synthesis. The brain is a very important metabolic organ and the energy required for the generation of action potentials, maintenance of ionic gradients and neurotransmission is dependent on ATP generation. In addition, the intermediates of metabolic pathways are the precursors for neurotransmitter biosynthesis. As neurotransmitters are important players in the cognitive and learning behaviors, any effects on brain metabolism can have far reaching negative consequences. The behavioral defects observed in our study (Glazer*, et al.*, 2016) may also be the consequence of altered metabolic capacity of the brain initiated by changes during development.

AHR has been shown to play an important role in neurodevelopment in both invertebrates and vertebrates. For instance, AHR has been shown to regulate neuronal growth in *C.elegans* (Qin and Powell-Coffman, 2004), dendrite morphogenesis in *D.melanogaster* (Crews and Brenman, 2006) and neuronal differentiation in rodents (Dever *et al.*, 2016; Latchney *et al.*, 2013), suggesting an evolutionarily conserved role for AHR in neurodevelopment. However, the effects of AHR activation during development on cellular differentiation in the developing nervous system are only beginning to be understood. Recently, it has been demonstrated that 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exposure to dams at gestational day 12.5 disrupted dendritic branch growth in the hippocampus and amygdala in 14 day old offspring and significantly reduced spine densities at 16 months, suggesting

that AHR activation during development causes persistent changes in tissue morphology (Kimura *et al.*, 2015). To our knowledge this is the first study investigating the persistent effects of AHR activation during development in zebrafish. Our results concur with previous observations that AHR agonists alter intracellular calcium signaling and energy metabolism during brain development. Although we cannot directly compare the results from our study to these previous findings, our results provide evidence that transient AHR activation during critical periods of development may cause tissue remodelling, which could have far-reaching consequences on brain function later in life. Further studies are needed to characterize the cellular phenotypes and the molecular mechanisms associated with long-term changes in gene expression.

Conclusions

In conclusion, we observed significant changes in gene expression in the brains of adult male zebrafish that were developmentally exposed to low doses of PCB126 and in which we previously had observed behavioral deficits. These results provide a basis for DOHaD effects caused by persistent organic pollutants. The low doses of PCB126 used in this study only induced AHR signaling transiently during development providing an opportunity to investigate the latent effects of AHR activation during development. Genes upregulated by PCB126 are enriched in calcium signaling, MAPK signaling and lysine degradation pathways. Both calcium and MAPK signaling play an important role in neurodevelopment and cognitive functions such as learning and memory, and enrichment of lysine methyltransferase (KMT) genes implicates histone modifications. Among the downregulated genes, there is an overrepresentation of genes involved in oxidative phosphorylation suggesting that developmental exposure to PCB126 altered energy homeostasis in the brain. Futher studies are necessary to characterize the functional significance of these changes.

Supplementary Data Description

1. dge.xlsx contains the complete list of differentially expressed genes in different treatment conditions.
2. summary_statistics.xlsx provides summary of mapping statistics.
3. 977_commongenes.xlsx contains the list of 977 genes common to both treatment conditions.
4. PathwayAnalysis_Up.xlsx and PathwayAnalysis_Down.xlsx contains GO and KEGG pathway analysis results.

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Figure legends

Figure 1. Transcriptional changes associated with developmental exposure to PCB126. Volcano plots showing gene expression changes in zebrafish exposed to 0.3 nM (top panel) and 1.2 nM PCB126 (bottom panel). A. 2 dpf embryos; B and D. 5 dpf larvae and C and E. Adult brains (6 months). Each spot in the graphs represents one gene. Red spots represent significant differentially expressed genes (False discovery rate less than or equal to 5%). The few significant DEGs are highlighted in (A) and (D).

Figure 2. Number of differentially expressed genes in adult zebrafish brain. Venn diagram shows the number of unique and common DEGs in the adults that were developmentally exposed to 0.3 or 1.2 nM PCB126. Arrows represent Up- and down-regulated genes.

Figure 3. Effect of PCB126 exposure on the calcium signaling pathway. There was upregulation of several genes associated with calcium signaling in the brain of adult fish that were developmentally exposed to PCB126. These genes include voltage dependent calcium channels, glutamate and cholinergic receptors and members of downstream signaling. Genes that are differentially expressed are highlighted in green (0.3nM PCB126), red (1.2nM PCB126) and blue (both concentrations).

Figure 4. Effect of PCB126 exposure on oxidative phosphorylation. There was downregulation of a number of genes associated with oxidative phosphorylation in the brain of adult fish that were developmentally exposed to PCB126. These genes are associated with electron transport chain and ATP synthase. The figure shows all components of the oxidative phosphorylation pathway and the DEGs corresponding to each component are listed below. Genes that are differentially expressed are highlighted in green (0.3nM PCB126), red (1.2nM PCB126) and blue (both concentrations).

List of tables

Table 1. List of differentially expressed genes and their expression levels (fold change) in the embryos and larvae exposed to 0.3 nM and 1.2 nM PCB126, respectively. The entire list of genes is provided in supplemental information (dge.xlsx).

PCB	dpf	Gene	Fold change	FDR
0.3 nM	2	cytochrome P4501a *(cyp1a)*	12.49	0.00455
0.3 nM	5	(none)		
1.2 nM	5	cytochrome P4501a *(cyp1a)*	23.98	0.00025
		cytochrome P4501c2 *(cyp1c2)*	3.08	0.00516
		cytochrome P4501b1 *(cyp1b1)*	3.62	0.00699
		cytochrome P4501c1 *(cyp1c1)*	5.59	0.00747
		forkhead box F2a *(foxf2a)*	1.83	0.0237
		si:ch1073-384e4.1 (lincRNA)	4.3	0.0331
		aryl hydrocarbon receptor repressor a *(ahrra)*	10.08	0.0379

Table 2. Gene Ontology (GO) term enrichment analysis of differentially expressed genes in the brain. Only significantly enriched GO child terms are shown. The number of up and downregulated genes represented under each GO term are listed for both PCB126 concentrations. The list of gene names associated with each GO term are provided in the supplemental information (PathwayAnalysis_up.xlsx; PathwayAnalysis_down.xlsx). A dash (-) indicates that the indicated GO term was not significantly enriched in that exposure group.

GO Term	0.3nM PCB126	1.2nM PCB126	Adjusted p.value
Upregulated genes			
GO:0005261 Cation channel activity	27	25	4.52E-06
GO:0000982 Transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	10	9	1.62E-03
GO:0043565 Sequence specific DNA binding	-	18	2.20E-02
GO:0019905 Syntaxin binding	9	-	4.81E-02
GO:0005096 GTPase activator activity	13	-	5.00E-02
Downregulated genes			
GO:0003735 Structural constituent of ribosome	23	51	1.85E-43
GO:0003723 RNA binding	-	35	6.04E-09
GO:0005518 collagen binding	6	-	2.34E-04
GO:1901681 sulfur compound binding	10	-	2.03E-02
GO:0005546 phosphotidylinositol-4,5-bisphosphate binding	3	-	2.75E-02
GO:0015379 potassium:chloride symporter activity	-	2	3.19E-02

Table 3. KEGG pathway analysis of DEGs in the brain. KEGG pathway analysis of DEGs from 0.3nM and 1.2nM PCB groups was conducted using gProfiler and the enriched KEGG terms were compared using gCocoa. KEGG pathways and the number of up and downregulated DEGs associated with each KEGG term from both treatments are listed below. The list of gene names associated with each pathway are provided in supplemental information (PathwayAnalysis_up.xlsx; PathwayAnalysis_down.xlsx). A dash (-) indicates that the KEGG term was not significantly enriched in that exposure group.

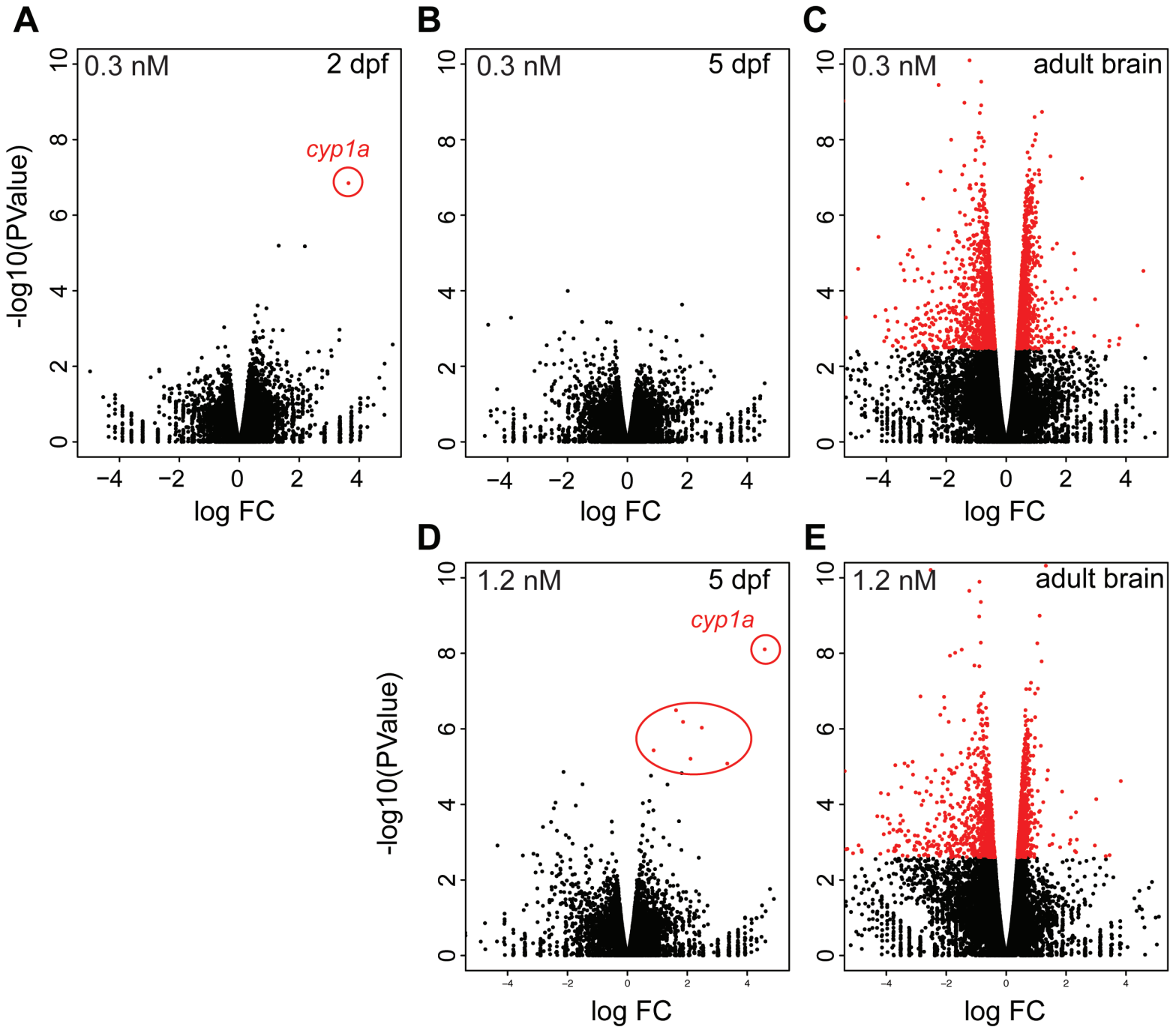
KEGG ID	KEGG pathway	0.3 nM PCB126	1.2 nM PCB126	Adjusted p.value
	Upregulated genes			
04020	Calcium signaling pathway	20	21	1.25E-06
04010	MAPK signaling pathway	23	23	4.44E-06
04330	Notch signaling pathway	12	-	5.61E-06
00310	Lysine degradation	8	8	2.36E-04
04012	ErbB signaling pathway	6	8	6.43E-03
04912	GnRH signaling pathway	12	6	7.04E-03
04320	Dorso-ventral axis formation	6	5	7.80E-03
04914	Progesterone-mediated oocyte maturation	8	6	9.52E-03
04068	FoxO signaling pathway	15	-	1.13E-02
	Downregulated genes			
00190	Oxidative phosphorylation	64	20	7.90E-61
03010	Ribosome	31	71	2.01E-46
01100	Metabolic pathways	124	-	4.93E-13
01200	Carbon metabolism	23	-	7.18E-04
04512	ECM-receptor interaction	15	-	3.04E-03
00480	Glutathione metabolism	8	-	3.67E-03
01212	Fatty acid metabolism	11	-	1.08E-02
00020	Citrate cycle (TCA cycle)	5	-	1.27E-02

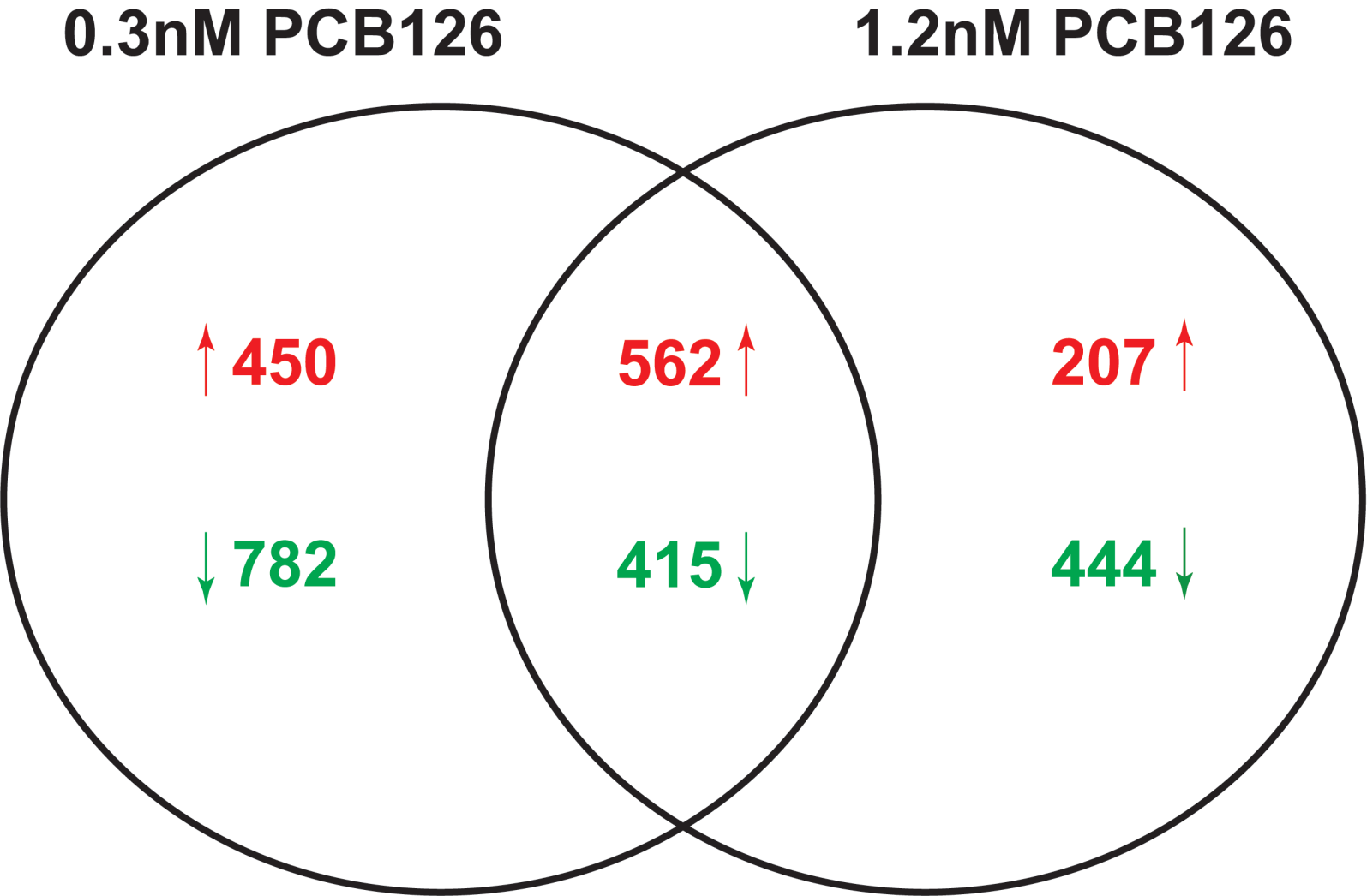
Table 4. Gene Ontology (GO) and KEGG pathway analysis of differentially expressed genes that are common to both treatment groups. Only significantly enriched GO child terms are shown. The number of genes represented under each GO term are listed and the list of gene names is provided in the supplemental information (977_commongenes.xlsx).

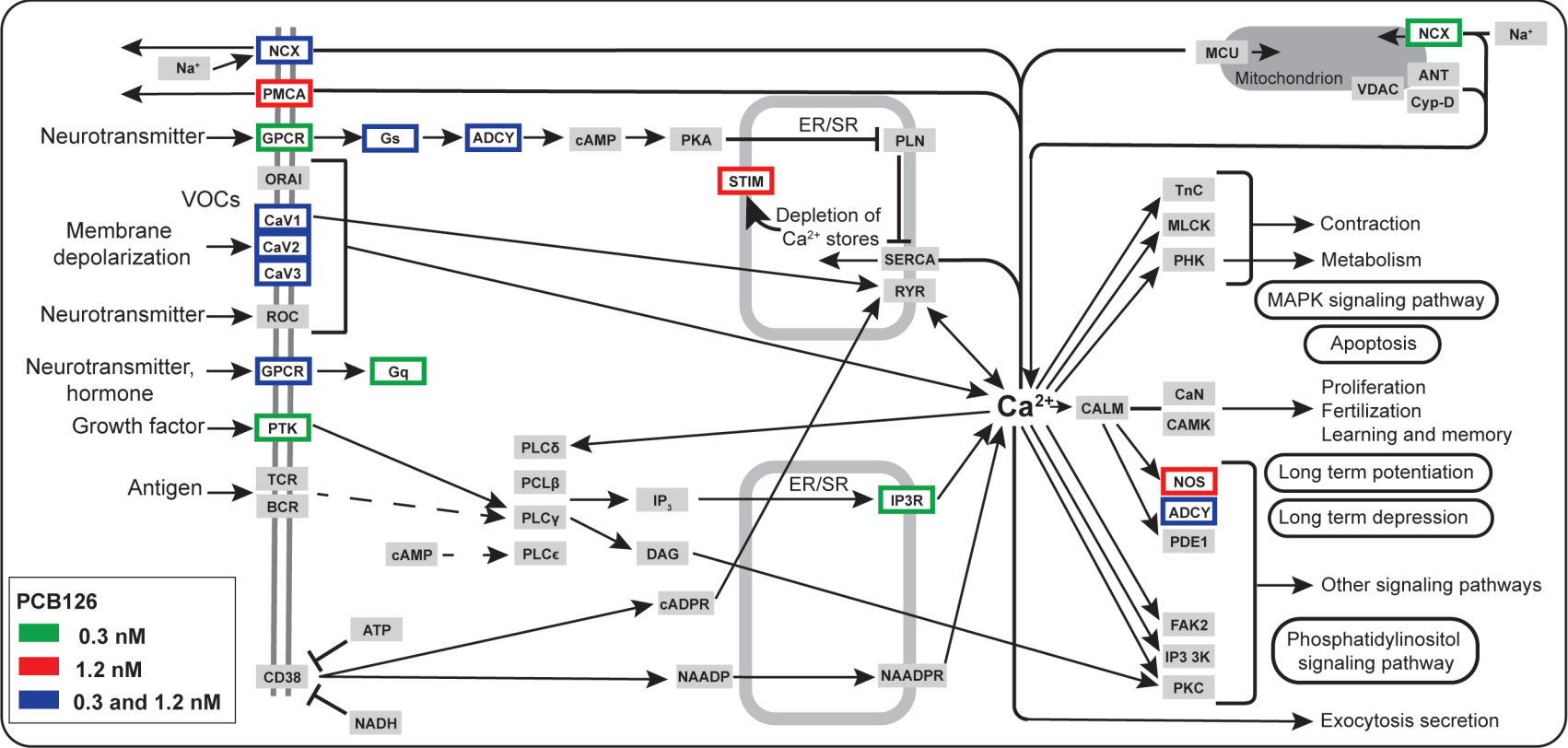
GO and KEGG terms	Number of genes	Adjusted p.value
GO:0008331 High voltage-gated calcium channel activity	6	0.01
GO:0003735 Structural constituent of ribosome	14	0.04
KEGG:03010 Ribosome	22	9.37E-07
KEGG:00190 Oxidative Phosphorylation	18	7.41E-04
KEGG:04020 Calcium signaling pathway	22	1.86E-02
KEGG:04260 Cardiac muscle contraction	12	2.03E-02

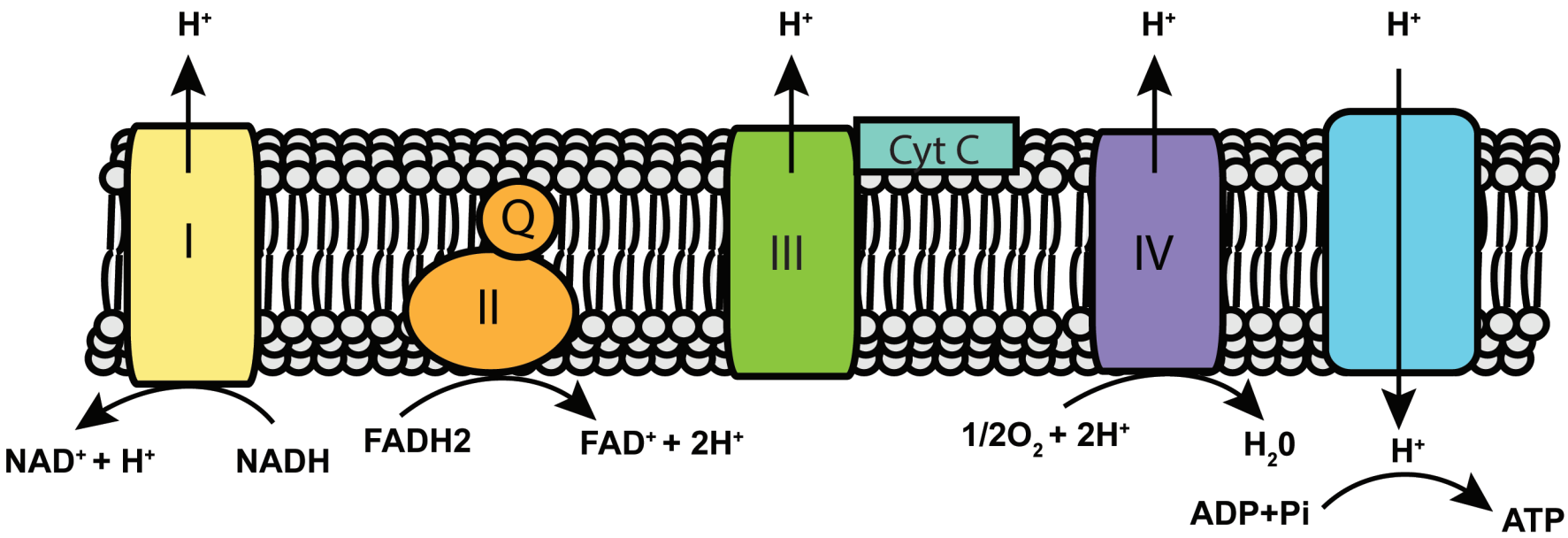
Table 5. List of differentially expressed genes associated with KEGG term calicum signaling. A dash (-) indicates that the gene was not differentially expressed in that exposure group.

		0.3 nM PCB126		1.2 nM PCB126	
Gene symbol	**Gene description**	**logFC**	**FDR**	**logFC**	**FDR**
cacna1c	calcium channel, voltage-dependent, L type, alpha 1C subunit	0.71	0.00012	0.56	0.0025
cacna1g	calcium channel, voltage-dependent, T type, alpha 1G subunit	0.73	0.00013	0.54	0.0045
tacr1a	tachykinin receptor 1a	0.89	0.00036	0.67	0.0082
cacna1ba	calcium channel, voltage-dependent, N type, alpha 1B subunit, a	0.53	0.00172	0.40	0.0365
adcy1a	adenylate cyclase 1a	0.64	0.00197	0.59	0.0076
slc8a4a	solute carrier family 8 (sodium/calcium exchanger), member 4a	0.55	0.00202	0.53	0.0055
cacna1ab	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit, b	0.83	0.00394	0.63	0.0326
cacna1da	calcium channel, voltage-dependent, L type, alpha 1D subunit, a	0.58	0.00457	0.56	0.0093
erbb4a	erb-b2 receptor, tyrosine kinase 4a	0.64	0.00479	-	-
chrm2a	cholinergic receptor, muscarinic 2a	0.56	0.00691	0.71	0.0018
grm5a	glutamate receptor, metabotropic 5a	0.49	0.00753	0.83	0.0001
cacna1ha	calcium channel, voltage-dependent, T type, alpha 1H subunit, a	0.45	0.01142	0.43	0.0243
grm1b	glutamate receptor, metabotropic 1b	0.76	0.01179	-	-
adcy2a	adenylate cyclase 2a	0.47	0.01439	0.40	0.0486
cacna1bb	calcium channel, voltage-dependent, N type, alpha 1B subunit, b	0.43	0.01621	0.43	0.0215
cacna1i	calcium channel, voltage-dependent, T type, alpha 1I subunit	0.50	0.02267	0.55	0.0172
gna11b	guanine nucleotide binding protein (G protein), alpha 11b (Gq class)	0.41	0.02381	0.40	0.0394
hrh1	histamine receptor H1	1.14	0.02435	-	-
gnas	GNAS complex locus	0.40	0.02603	-	-
itpr1b	inositol 1,4,5-triphosphate receptor, type 1b	0.42	0.02829	-	-
atp2b3b	ATPase, Calcium transporting, plasma membrane 3b	-	-	0.55	0.0224
nos1	nitric oxide synthase 1 (neuronal)	-	-	0.69	0.0027
slc8a2b	solute carrier family 8 (sodium/calcium exchanger), member 2b	-	-	0.54	0.0389
trhrb	thyrotropin-releasing hormone receptor b	-	-	0.85	0.0360
trhra	thyrotropin-releasing hormone receptor a	-	-	0.82	0.0149
stim2b	stromal interaction molecule 2b	-	-	0.52	0.0473


A
0.3 nM
2 dpf
cyp1a
B
0.3 nM
5 dpf
C
0.3 nM
adult brain
D
1.2 nM
5 dpf
cyp1a
E
1.2 nM
adult brain
-log10(PValue)
log FC
0
2
4
6
8
10
−4
−2
0
2
4


0.3nM PCB126
1.2nM PCB126
450
782
562
415
207
444


Na+
NCX
PMCA
Neurotransmitter
GPCR
Gs
ADCY
cAMP
PKA
ER/SR
PLN
STIM
Depletion of Ca2+ stores
SERCA
RYR
ORAI
VOCs
CaV1
CaV2
CaV3
Membrane depolarization
Neurotransmitter
ROC
Neurotransmitter, hormone
GPCR
Gq
Growth factor
PTK
Antigen
TCR
BCR
PLCδ
PCLβ
PLCγ
PLCε
cAMP
IP3
DAG
ER/SR
IP3R
cADPR
ATP
CD38
NADH
NAADP
NAADPR
Ca2+
CALM
MCU
Mitochondrion
VDAC
ANT
Cyp-D
NCX
Na+
TnC
MLCK
PHK
Contraction
Metabolism
MAPK signaling pathway
Apoptosis
CaN
CAMK
Proliferation
Fertilization
Learning and memory
NOS
ADCY
PDE1
Long term potentiation
Long term depression
Other signaling pathways
FAK2
IP3 3K
PKC
Phosphatidylinositol signaling pathway
Exocytosis secretion
PCB126
0.3 nM
1.2 nM
0.3 and 1.2 nM



Complex I NADH dehydrogenase

ndufa1	ndufa2	ndufa4l
ndufa5	ndufa6	ndufa7
ndufa9a	ndufa11	ndufb2
ndufb3	ndufb4	ndufb5
ndufb6	ndufb7	ndufb8
ndufb10	ndufb11	ndufab1a
ndufab1b	ndufc1	ndufc2
ndufs3	ndufs4	ndufs5
ndufs6	ndufs7	ndufv2

Complex II Succinate dehydrogenase

sdhb
sdhc

Complex III

uqcr10
uqcrh
uqcrfs1
uqcrc2b
uqcrb
uqcrq

Complex IV Cytochrome C oxidase

cox4i1	cox5aa
cox5ab	cox5b
cox5b2	cox6a1
cox6b1	cox6b2
cox6c	cox7a2a
cox7a3	cox7b
cox7c	cox8a
cox8b	cox15
cox17	

ATP synthase

atp5c1	atp5d
atp5e	atp5f1
atp5g1	atp5g3b
atp5h	atp5ib
atp5j	atp5j2
atp5l	atp5o
atp6v0e1	atp6v0e2
atp6v1f	

PCB126

- 0.3 nM
- 1.2 nM
- 0.3 and 1.2 nM