Title: **Transcriptome-wide Gene Expression in a Rat Model of Attention Deficit Hyperactivity Disorder Symptoms: Rats Developmentally Exposed to Polychlorinated Biphenyls**

Abbreviated Title: Genomewide gene expression and PCBs

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ABSTRACT

Objective: PCB exposure in rodents provides a useful model for the symptoms of Attention deficit hyperactivity disorder (ADHD). The goal of this study is to identify genes whose expression levels are altered in response to PCB exposure.

Method: The brains from 48 rats separated into two age groups of 24 animals each (4 males and 4 females for each PCB exposure level (control, PCB utero and PCB lactational)) were harvested at postnatal day 23 and 35 respectively. The RNA was isolated from three brain regions of interest and was analyzed for differences in expression of a set of 27,342 transcripts.

Results: 279 transcripts showed significant differential expression due to PCB exposure mostly due to the difference between PCB lactational and control groups. The cluster analysis applied to these transcripts revealed that significant changes in gene expression levels in PFC area due to PCB lactational exposure. Our pathway analyses implicated 27 significant canonical pathways and 38 significant functional pathways.

Conclusions: Our transcriptome-wide analysis of the effects of PCB exposure shows that the expression of many genes is dysregulated by lactational PCB exposure, but not gestational exposure and has highlighted biological pathways that might mediate the effects of PCB exposure on ADHD-like behaviors seen in exposed animals. Our work should further motivate studies of fatty acids in ADHD, and further suggests that another potentially druggable pathway, oxidative stress, may play a role in PCB induced ADHD behaviors.

Attention deficit hyperactivity disorder (ADHD) is a common disruptive behavior disorder, which affects 8 to 12% of children and 4% of adults (Faraone and others 2003). Our review of 20 twin studies indicates that ADHD has an estimated heritability of 0.76, and meta-analyses of linkage and association studies have implicated several candidate genes: DRD4, SLC6A3, DRD5, HTR1B, SNAP25, DBH and SLC6A4 (Faraone and Mick 2010). Meta-analyses suggest that these genes have small but statistically significant effects on susceptibility to ADHD and all have been implicated by neurobiological studies implicating monoamine pathways in the etiology of the disorder. However, despite these encouraging findings, genomewide association studies of ADHD have not implicated any gene at a genome wide level of significance (Lasky-Su and others 2008; Neale and others 2008).

The less than complete heritability of ADHD, and the difficulty confirming susceptibility genes, indicates that the prevalence of ADHD cannot be explained by genes alone. Several environmental factors have been identified as potential risk factors for ADHD, including prenatal exposure to alcohol, cigarette smoke and environmental pollutants such as pesticides and polychlorinated biphenyls (PCBs) (Banerjee and others 2007; Sagvolden and Sergeant 1998).

PCBs are a family of 209 manufactured compounds once produced on a large scale from 1929 to 1977 in the USA for use as heat resistant electrical insulators, brake liners, paints, sealing compounds etc. (Safe 1994). In 1977, the manufacturing of PCBs was banned in the US because of evidence that they were persistent and posed health risks. Indeed, we now know that PCBs are highly stable, they bioaccumulate, and because of their stability have entered the food chain as far north as the Arctic Circle (Berger and others 2001). The potential exposure routes for PCBs to affect human development are numerous. Patandin et al. (1997) and Koopman-Esseboom et al. (1994a; Koopman-Esseboom and others 1994b) found a significant correlation between levels of PCBs in maternal plasma and umbilical cord plasma. Studies of breast milk of women throughout the world find a relatively consistent pattern. PCB congeners 138, 153 and 180 were found in the breast milk of women in New Zealand (Bates and others 1994). PCB congeners 138, 153, 180 and 170 were found in the breast milk of Inuit women, a group that depends heavily upon fish consumption for their existence (Dewailly and others 1994). High levels of PCB congeners 153, 138, 118 and 180 were also found in the breast milk of women from the Akwesasne reservation in northern New York State, which borders the St. Lawrence River. According to the EPA (2000), all of the Great Lakes have fish and wildlife advisories for PCBs. The number of advisories increased 120% from 1993 to 1998 (319 to 703). The number of other states issuing advisories rose to 38 in 1999. In that year alone, the US government issued 1285 no-consumption fish advisories for sensitive sub-populations (defined as pregnant women, nursing mothers and children). Clearly, PCB exposure is a rampant environmental health problem that poses serious health risks.

Many studies of humans show that PCBs produce the behavioral symptoms and neuropsychological impairments associated with ADHD. Rice showed that exposure to PCBs produces a neurotoxic effect in the developing human at environmentally relevant levels (Rice 1997a; Rice 1997b; Rice 1997c), and she articulated the similarities between children with ADHD and monkeys exposed to PCBs. Rice (2000) indicated that both have problems learning from the consequences of their behavior, and both show an inability to organize the temporal sequencing of behavior. In the two incidents of mass poisoning by PCB mixtures in Japan and Taiwan (Chang and others 1981; Rogan and others 1988) the most significant effects of PCB

exposure appeared in children born to exposed women. Since many of the children were born years after the exposure of the mother, the effects appear to be mediated via exposure of the fetus from the PCB stores in the mother's body fat. In addition to prenatal exposure, children may have been exposed to PCBs via breast feeding. The children showed abnormalities on behavioral assessment and increased activity level similar to those seen in ADHD youth (Chen and others 1994). They also showed significant delays in behavioral milestones, deficits in formal developmental testing and lower scores on several tests of cognitive function (Lai and others 1994; Rogan and others 1988). The Taiwanese children were more active and had more behavior problems than unexposed controls (Chen and others 1994). These exposed children (tested at ages between 7 and 12) also had significantly lower verbal and full-scale IQs, which are also reduced in ADHD (Antshel and others 2006; Bridgett and Walker 2006; Frazier and others 2004).

In addition to these studies on Asian populations, three groups have investigated the development of PCB-exposed US children. Jacobson and colleagues (Jacobson and Jacobson 1996; Jacobson and others 1990; Jacobson and others 1985) studied children of Great Lakes fish eaters, where PCBs are assumed to be the major contaminants. Higher cord serum PCB level was associated with poorer performance on the Fagan Test of Infant Intelligence. Cord serum PCB level was related to Fagan scores in a dose dependent fashion (Jacobson and others 1985). When tested at four years of age, higher cord PCB levels were associated with poorer performance on the Verbal and Memory Scales from the McCarthy Scales of Children's Abilities (Jacobson and others 1990). When tested at 11 years of age, the most highly exposed children were found to have a 6.2 point decrement of full scale and verbal IQ scores, and were also two years behind in reading comprehension. Boucher et al. (2009), reviewed nine prospective longitudinal birth cohort studies of the neuropsychological effects of PCBs in humans. The most consistent effects from these studies were findings of impaired executive functioning subsequent to prenatal PCB exposure. This is particularly relevant for ADHD studies because impaired executive functioning is common among ADHD patients (Biederman and others 2011; Biederman and others 2004; Biederman and others 2008a; Biederman and others 2008b; Biederman and others 2007; Doyle and others 2005; Faraone and others 2005; Makris and others 2007; Seidman and others 2005; Willcutt and others 2005; Fischer and others 2005).

Several studies show that PCB exposure in studies of rodents provides a useful model for some of the symptoms and neuropsychological impairments of ADHD. Daly et al. (1989) showed ADHD-like deficits in rats prenatally exposed to ADHD. Like ADHD children (Scheres and others 2007; Tripp and Alsop 1999; Tripp and Alsop 2001), these animals showed greater aversion toward delayed or reduced rewards. Berger et al. (2001) showed that adult rats exposed to either Aroclor 1248 or PCB-contaminated fish show hyperactivity and impulsiveness and increased frustration. Holene et al. in (1998) showed that rats when postnatally exposed to PCB congeners 153 and 126 showed higher frequency of lever presses which denoted hyperactivity. In two studies, Branchi et al. (2002; Branchi and others 2005) found that prenatal exposure to the Aroclor 1254 led to behavioral hyperactivity in mice but did not affect sustained attention. Although exposure to Aroclor congeners leads to hyperactivity and impulsiveness of ADHD, this exposure does not affect attentional tasks. This dissociation of inattention from hyperactivity and impulsivity is consistent with the clinical subtyping of human ADHD into inattentive, hyperactive-impulsive and combined subtypes (Faraone and others 2000; Todd and others 2008; Todd and Lobos 2002) and with human and animal studies showing these are

separate dimensions of ADHD that likely have separate etiologies (Blondeau and Dellu-Hagedorn 2006; Hudziak and others 1998; Hudziak and others 2000; Sagvolden and others 2008; Sagvolden and others 2009).

In a review of monkey, rodent and human studies, Sable and Shantz (2008) summarized the evidence that prenatal PCB exposure led to impairments in a wide range of executive functions: 1) cognitive flexibility as measured by spatial reversal learning in monkeys and rats and the Wisconsin Card Sorting Test in humans; 2) working memory as measured by the delayed spatial alternation task in monkeys and rats and the McCarthy Memory Scale, the Fagan test, WISC-R digit span and the California Verbal Learning Test in humans; and 3) inhibitory control as measured by perseverative errors in rats, monkeys and humans, disinhibition on fixed interval operant trials in monkeys and rats and Continuous Performance Task errors of commission in humans.

In a pilot study, we examined the effects of in utero exposure to the PCB mixture of Aroclor 1254/1260 on expression of ADHD candidate genes in Sprague-Dawley (SD) rats when they reached adulthood. Our first report from this project focused on candidate genes of ADHD implicated in catecholaminergic and dopaminergic transmission along with genes involved in epigenetic dysregulation. We found evidence that PCBs dysregulated the expression levels of several of these genes GNAL, COMT, ADRBK1, NTRK2, HK1, SYT11, CSNK1A1, ARRB2, STX12, AQP6, SYT1, DDC and PGK1. In a second experiment, we reported that lactational exposure to A1254 had a greater effect on gene expression of ADHD candidate genes compared to gestational exposure DasBanerjee et al., in revision. The magnitude of difference between the two exposures, however, was not modulated by age, gender and brain area. In this report, we extend our prior work by reporting the results of genomewide comparisons rather than candidate genes.

Methods

Animals

Eighteen timed pregnant Sprague-Dawley (SD) rats were obtained from Charles River Laboratory (Kingston, NY) for arrival on gestational day 4 (G4) (day of insemination was G0), and housed in the American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility at SUNY Upstate Medical University's Department of Laboratory Animal Resources (DLAR). The animals were housed individually in standard (24 cm x 18 cm x 18 cm) plastic hanging cages with sterilized pine shavings as bedding. Food (Purina Laboratory Rodent Diet 5001 pellets) and water were provided ad libitum. Temperature was maintained at 21±2 °C and relative humidity at 50±10% with a 12 h light/dark cycle (6:00–18:00 h). All experiments were approved in advance by SUNY Upstate's Committee for Humane Use of Animals.

Animal dosing

On G5, all pregnant dams were weighed and divided into three weight-matched groups-group I (6 dams-control), group II (6 dams-control) and group III (6 dams-A1254-exposed) **(Fig.1)**. Group II control dams were to serve as surrogate mothers for the pups from the A1254-exposed dams after birth. During the exposure period from G5-G19, the group III dams were fed a diet of Purina Laboratory Rodent Diet 5001 pellets augmented with half a Nilla Wafer cookie onto which 0.1 ml of corn oil containing 4.0 µg/gram body weight of A1254 (Sigma) was

placed. The control dams in groups I and II were fed the same Purina pellets, but their diet was augmented with half a Nilla Wafer cookie with 0.1 ml of corn oil alone. The cookies were rapidly consumed by the dams. This procedure is a reliable and nonstressful means of exposing the dams to PCB and has been used by others (Seegal and others 1997). All dams delivered on G21 (P0), within a few hours apart and the litter size varied between nine and 15 pups. Litters were culled to four male and four female pups within the first eight hours of birth. Within the first 12 hours of birth, pups born to the A1254-exposed dams were cross-fostered to group II control dams. These pups were exposed to A1254 in utero and thus formed the gestation-only A1254 experimental group. Pups born to group II control dams were cross-fostered to the A1254-exposed dams. Since these pups did not receive any A1254 gestationally, but were exposed to A1254 during lactation through breast milk of A1254-exposed dams (as PCBs tend to bioaccumulate in fatty tissues), these pups formed the lactation-only A1254 exposure group. Since the cross fostering paradigm was implemented within the first 12 hours of birth, it is possible that some of the pups that were exposed to A1254 gestationally were also exposed to A1254 lactationally during this 12-hour time period. To control for any effects of fostering alone, the pups from the control dams in group I were fostered by exchanging litters within group I itself, and formed the control group of animals. All the offspring used in this study were weaned at P22.

Tissue sampling

We wanted to determine genome-wide alterations in gene expression due to A1254 exposure at P23 and P35, ages corresponding to juvenile and adolescent time points in the rat. At P23, eight males and eight females from the 6 control litters were randomly selected. Of these, four males and four females were deeply anaesthetized using isofluorane, and perfused transcardially with phosphate buffered saline followed by 4% buffered paraformaldehyde. The brains were then serially immersed in 10% sucrose and 30% sucrose for 24hours each, and finally frozen in Tissue-Tek Pre-Processing solution (Sakura Finetek, Europe) on dry ice and stored at -80°C for later histological staining. The remaining four males and four females were euthanized with an isofluorane overdose, decapitated, with brains removed immediately. Three regions were dissected from these brains—the medial prefrontal cortex (PFC), vermis, and ventral mesencephalon (including the substantia nigra and ventral tegmental area, SNVTA). The dissected tissues were stored in RNAlater solution for gene expression analyses. Brains were harvested in a similar manner from eight male and eight female rats of the gestation-only A1254 exposure group and eight male and eight females from the lactation-only group at P23. At P35 we followed the same protocol as P23 to obtain the required number of brains from each exposure group for further analyses.

RNA purification

Total RNA was extracted from all of the brain samples using the RNeasy Mini Kit and QiaShredder™ Kit (Qiagen®, Valencia, CA). RNA concentrations were determined by UV spectrophotometry and RNA quality assessed by 1% agarose gel electrophoresis. Only RNA samples with greater 28S than 18S intensity and no obvious degradation or contamination were used (all samples had to show 260:280 ratios exceeding 1.6). Our RNA isolation procedure did not include a DNase treatment step. However, we obtain greater than 99% pure RNA from the RNeasy columns (Qiagen®).

The input microarray data contained 27,342 transcripts (rows) and 72 samples (columns).

Samples were represented as 36 experimental conditions with 2 replicates each. Experimental conditions were combinations of 4 factors listed in Table 1 with their corresponding levels, which gave the following total of 36 combinations: (3 Treatment)x(2 Time)x(3 Brain)x(2 Gender).

Microarray screen of changes in gene expression across brain areas

Comparative gene expression analysis of each brain area in each group of animals was performed using oligonucleotide microarrays (Rat ST Gene Array, Affymetrix). For these assays, we pooled RNA from four male or four female rat brain samples in each treatment group into two pooled RNA samples (consisting of two individual RNA samples in each pooled sample). This 2:1 pooling was repeated for all samples collected at P23 and P35. We then ran a total of 4 ST Gene Arrays for each area and treatment group (2 per gender). In total, 36 array hybridizations were performed to compare the changes in expression across three brain areas in three groups of rats: SD, PCButero, and PCBlac. Amplification and labeling of the pooled RNA samples were performed using the WT-Ovation™ RNA Amplification System (NuGen), and processing of the GeneChips performed according to standard protocol (GeneChip Expression Analysis Technical Manual 701021 rev 5, Affymetrix). After scanning, the microarray images were analyzed using Gene Expression Console software (Affymetrix) to obtain performance metrics, quantile normalized using the RMA method, and reported in $\log_2$ scale.

Analysis of Variance

For each combination of the PCB exposure level, time point and gender there were four animals, each sampled at three different brain regions. The RNA samples from the same brain region of each two animals were pooled to create two replicates. Thus, the experiment fitted a split-plot ANOVA design with the following factors: the whole-unit treatment is given by the combination of 3 factors: Treatment, Time and Gender, resulting in 3x2x2 = 12 levels; the subunit treatment is the Brain (brain region) factor. For each replicate the model included all possible interactions and was as follows:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\beta\gamma)_{jk} + (\alpha\gamma)_{ik} + (\alpha\beta\gamma)_{ijk} + \delta_{ijk} + \\ + \theta_l + (\alpha\theta)_{il} + (\beta\theta)_{jl} + (\gamma\theta)_{kl} + (\alpha\beta\theta)_{ijl} + (\beta\gamma\theta)_{jkl} + (\alpha\gamma\theta)_{ikl} + (\alpha\beta\gamma\theta)_{ijkl} + \varepsilon_{ijkl}$$

μ is the general mean
α_i is the effect of the i^{th} level of factor Treatment
β_j is the effect of the j^{th} level of factor Time
γ_k is the effect of the k^{th} level of factor Gender
$\alpha\beta_{ij}$ is the interaction effect of the i^{th} level of factor Treatment and the j^{th} level of factor Time
$\beta\gamma_{jk}$ is the interaction effect of the j^{th} level of factor Time and the k^{th} level of factor Gender
$\alpha\gamma_{ik}$ is the interaction effect of the i^{th} level of factor Treatment and the k^{th} level of factor Gender
$\alpha\beta\gamma_{ijk}$ is the interaction effect of the i^{th} level of factor Treatment, the j^{th} level of factor Time and the k^{th} level of factor Gender
δ_{ijk} is the whole plot random error effect for the combination of the i^{th} level of factor Treatment, the j^{th} level of factor Time and the k^{th} level of factor Gender
θ_l is the effect of the l^{th} level of factor Brain
$\alpha\theta_{il}$ is the interaction effect of the i^{th} level of factor Treatment and the l^{th} level of factor Brain
$\beta\theta_{jl}$ is the interaction effect of the j^{th} level of factor Time and the l^{th} level of factor Brain
$\gamma\theta_{kl}$ is the interaction effect of the k^{th} level of factor Gender and the l^{th} level of factor Brain

$\alpha\beta\theta_{ijl}$ is the interaction effect of the i^{th} level of factor Treatment, the j^{th} level of factor Time and the l^{th} level of factor Brain
$\beta\gamma\theta_{jkl}$ is the interaction effect of the j^{th} level of factor Time, the k^{th} level of factor Gender and the l^{th} level of factor Brain
$\alpha\gamma\theta_{ikl}$ is the interaction effect of the i^{th} level of factor Treatment, the k^{th} level of factor Gender and the l^{th} level of factor Brain
$\alpha\beta\gamma\theta_{ijkl}$ is the interaction effect of the i^{th} level of factor Treatment, the j^{th} level of factor Time, the k^{th} level of factor Gender and the l^{th} level of factor Brain
ε_{ijkl} is the subplot random error effect associated with the y_{ijkl} subplot unit

The above design was applied to the full data (full ANOVA) and then to partial data which included only two treatments at a time (out of the three: ctrl, PCButero, PCBlac). These partial ANOVAs will be subsequently called two-way contrasts, and designated ctrl-PCBlac ANOVA, ctrl-PCButero ANOVA and PCButero-PCBlac ANOVA. We corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (BH-FDR) set at 5% (Thissen and others 2002).

Cluster Analyses

Two-way cluster analysis was performed on the data by separately applying the clustering algorithm to cluster genes and then to cluster experimental conditions. Hierarchical clustering was done using Ward's method, where the Pearson correlation coefficient was used to measure the distance between genes/treatments. We used the R package **oompa** (in particular, its library "ClassDiscovery") to perform the cluster analysis and present the results. The major drawback of the two-way cluster analysis approach is that clustering of genes does not distinguish between conditions and, likewise, clustering of conditions is performed across all genes at the same time. Another approach called biclustering is aimed at finding groups of genes that are co-regulated under some conditions (which also need to be determined). Such genes and conditions together comprise a cluster. We used a recently developed R package **biclust** (Kaiser and Leisch 2008) to apply it to our data. Package **biclust** offers a good selection of biclustering methods depending on the specific purposes of a study. We applied the Plaid model of Lazzeroni and Owen (2002) and Turner and others (2005) which offers no limit to the number of clusters and is based on fitting the linear model with gene and condition effects.

Pathway Analyses

We used Ingenuity Pathways Analysis (IPA) software to determine if the top genes associated with exposure effects were over-represented in known canonical biological networks or functional networks that had been manually curated by IPA scientists (Ingenuity Systems®, www.ingenuity.com). A dataset containing Affymetrix gene identifiers for all nominally significant transcripts ($p<.05$) was analyzed. We conducted three analyses based on the source of the p-values: 1) main effect of treatment; 2) treatment by time interaction and 3) treatment by time by brain region interaction. The top genes for each analysis, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity knowledge base.

For each curated pathway, a Fisher's Exact test was computed by classifying all genes in the genome into a two by two table with rows defined by the presence/absence of the gene in the focus list and columns by presence or absence of the gene's product in the canonical pathway. The p-value for each pathway is calculated by considering (1) the number of focus molecules that participate in that function, pathway, or list and (2) the total number of molecules that are

known to be associated with that pathway in Ingenuity's knowledge base. The more focus molecules involved in a given pathway, the more likely the association is not due to chance and the more significant the p-value. Similarly, the larger the total number of molecules known to be associated with the process, the greater the likelihood that an association is due to random chance, and the p-value accordingly becomes less significant. We used the Benjamini-Hochberg method of correcting for multiple comparisons to account for the fact that we tested multiple pathways.

Results

Effects of Exposure, Time and Brain Region on Gene Expression

After applying the BH-FDR correction, we identified 279 transcripts which showed a significant effect of treatment (PCB exposure) on their expression level. The 20 transcripts with the lowest p-values are listed in Table 2. The transcripts showing significance for each interaction effect are listed in Table 3. Among 279 transcripts differentially expressed for the treatment effect only one transcript also appeared among those differentially expressed for all interaction effects (as given in Table 3): this transcript with ID 10867026 corresponds to the Bhlhb3 gene. Gene annotations were downloaded from affymetrix.com. We also performed two-way contrast ANOVAs by considering the data for only two out of three levels of treatment effect (ctrl, PCBlac, PCButero) to obtain p-values for pairs of comparisons. Similar to the full ANOVA, selection of significantly expressed genes for each of the partial ANOVAs was done using the BH-FDR correction with FDR set at 5%. Fig. 1 shows three volcano plots for two-way comparisons of treatment effect levels. The horizontal axis in each plot shows the fold-change as the difference between the log-transformed mean expression levels of two groups. The vertical axis shows negative log-transformed p-values from the corresponding two-way contrast ANOVA. Points highlighted in green were significant in the full ANOVA. Points highlighted in red were significant in the two-way contrast ANOVA.

The distribution of colored genes in the volcano plots from Fig. 1 indicates that the major contribution to the differential expression between Treatment levels is due to differences between the control and PCBlac groups: the Ctrl-PCBlac plot shows almost all significant genes identified in the full ANOVA (green circles) located in the upper part of the plot whereas in the Ctrl-PCButero plot most of the green circles are, actually, located in the lower part and the magnitudes of the two-way contrast ANOVA p-values are too high to select any significantly differentially expressed genes; in the PCButero-PCBlac plot these genes are much less concentrated in the upper part with only one gene selected in the two-way contrast ANOVA as significantly differently expressed.

Table 4 lists the differentially expressed genes identified in the two-way contrast ANOVAs, stratified into two groups: under-expressed (negative fold change) and over-expressed (positive fold change) relative to control (for PCBlac and PCButero) or to PCButero (for PCBlac).

To further visualize the differential effects of PCB exposure, we applied cluster analysis to the 279 transcripts implicated by the significant Treatment effects (PCB exposure). The resulting data matrix of 279 rows was reduced to contain 18 experimental conditions: (3 Treatment)x(2 Time)x(3 Brain). Reduction of the data was performed by averaging out gene expression levels corresponding to the same experimental condition. Thus, the data matrix for clustering contained 279 rows (transcripts) and 18 columns (experimental conditions).

Figure 2 shows the two-way clustering results as a color-coded data matrix with rows (transcripts) and columns (treatments) reordered according to the sequence of clusters in the dendrogram. The top of Figure 2 shows the clustering of experimental conditions. The clustering by experimental conditions shows a clear partition into 3 clusters according to brain area: PFC, Vermis and SNVTA. This is expected as it is well known that genes are differentially expressed in different brain regions. On the lower level of the cluster hierarchy for the columns there are sub-clusters that emphasize major effects: "PCBlac PFC P23" and "PCBlac PFC P35" are grouped into a distinct cluster indicating significant change in overall gene expression levels in PFC area between PCBlac group and the other two PCB exposure groups (PCButero and control); the time factor is emphasized in a sub-cluster of Vermis region containing "ctrl Vermis P35", "PCBlac Vermis P35" and "PCButero Vermis P35".

The right side of Figure 2 shows the clustering of genes, which only makes sense in connection to specific conditions. As shown in Fig. 2 it is fairly easy to visually identify genes representing the brain region clusters; at the same time finding genes co-regulated within the sub-clusters of the brain region clusters is quite difficult. Thus, to better understand the co-regulation of genes, we applied bicluster analysis.

Overall Co-regulation of PCB Exposure Effects: Bicluster Analysis

The purpose of bicluster analysis is to find groups of genes associated with groups of conditions (together called a cluster) so that each such group of genes is co-regulated with the group of conditions from the same cluster. The bicluster analysis algorithm found 10 clusters: most of the clusters emphasize genes co-regulated in specific brain areas. The only cluster related to PCB exposure was cluster number 6 shown in Fig. 3, where the data set is represented as 279x18 green matrix with red cell indicating cluster components. The parallel coordinates plots in Fig. 4 show expression levels of conditions across the genes (Fig.4(a)), and expression levels of genes across conditions from the same cluster (Fig.4(b)). This cluster included condition labeled as "PCBlac PCF P23", "PCBlac PCF P35", "PCBlac Vermis P23" and 18 genes are given in the Fig. 4 plots.

In Fig. 4(a) experimental conditions (combinations of treatment group, brain region and time, i.e., the 12 columns of Fig. 3) are represented by lines: the black lines are the three conditions from cluster 6, gray lines are the other 12 conditions. Thus, for each condition its line shows gene expression values across the 18 genes from cluster 6. In Fig. 4(b), the lines represent genes: black lines indicate the 18 genes from cluster 6, gray lines are the rest of the genes. Each line shows gene expression levels for a given gene across the three experimental conditions from cluster 6. Visual inspection of parallel coordinates plots in Fig. 4(a) shows that genes involved in cluster 6 are over-expressed in the three conditions highlighted in the column label of Fig. 3, when compared to the expression levels for the rest of the conditions. In addition, among the three conditions two PCBlac conditions from the PFC brain region seem to be closest together in terms of the expressions levels of included genes. This simply reflects the brain region effect (see Fig. 4(b)). In fact, overall, among the 279 genes with a significant effect of treatment, the vast majority of variance (>74%) was still attributable to brain region (see Supplemental Fig. 1), with age accounting for the second largest source of variance (13.3%). In contrast, the effect of treatment alone only accounted for less than 1.4% of the overall variance, although there were also numerous contributions from the effects of treatment interactions. (Suppl. Fig. 1).

Functional Analysis of PCB Exposure Effects: Pathway Analysis

In our pathway analyses, no significant pathways were found for the main effect of treatment or the treatment by time by brain region interaction. In contrast, for the treatment by time interaction results, even after correcting for multiple comparisons, we found 27 significant canonical pathways and 38 significant functional pathways. The top 10 canonical pathways are given in Table 8 and the top 10 functional pathways are given in Table 9.

Discussion

Our transcriptome-wide analysis of the effects of developmental PCB exposure allows for three broad conclusions. First, the expression of many genes is dysregulated by lactational PCB exposure, but not gestational exposure. Second, the bi-cluster analyses show that although the similarity of gene expression across observations is mostly influenced by brain regions, the effects of PCB exposure and time of observation are also evident within the brain region clusters. Finally, our pathway analyses show that the effects of PCBs are evident in many biological pathways, some of which are clearly relevant to the effects of PCBs in causing ADHD like symptoms and neuropsychological impairment.

Our finding that the transcriptome-wide effects were much stronger for lactational compared with gestational exposure is consistent with our prior studies of catecholaminergic genes, which had been selected as risk factors for ADHD based on prior human genetic and pharmacologic studies (DasBanerjee, et al., in revision). These results highlight the importance of considering the effects of developmental, toxic exposures that occur following gestation. Notably, the ADHD candidate genes we studied in our prior work are not implicated by the most significant findings from our transcriptome-wide analyses. This highlights the importance of taking a transcriptome-wide view for implicating new biological pathways, in addition to focusing on candidate gene-based approaches.

The cluster analyses show that mechanisms specific to each brain region account for most of the variance in the co-regulation of gene expression. In contrast, there was no large effect of PCB exposure on co-regulation that would indicate many genes responding in a coordinated fashion to PCB exposure. Instead, we found only one small cluster of 18 genes systematically co-regulated by PCB exposure across all brain regions. Of interest to the effects of PCBs on ADHD-like symptoms, many of these genes regulate synaptic plasticity and cytoskeletal architecture (NLGN2,SHANK1, WIPF2, ABBA-1, CTNND2), and others regulate transcription (GSK3, EP300, NCOR2,). Thus, PCB exposure may activate a transcriptional cascade, leading to changes in synaptic plasticity. Disruption of synaptic plasticity might account for the neuropsychological deficits observed in animals after developmental exposure to PCBs.

The pathway analyses indicate which groups of genes in biologically meaningful pathways show changes subsequent to PCB exposure. This indicates whether co-regulation is occurring in biologically meaningful pathways. We found significant enrichment of gene expression dysregulation only for the treatment by time interaction effect. This suggests that the time course of PCB effects is regulated by biologically meaningful pathways. The canonical and functional pathway analyses provide two perspectives on the gene expression changes in or data. The canonical pathways define well characterized cell signaling and metabolic pathways. The functional pathways define gene sets involved in the same biological and disease processes.

The functional pathways provide a broad overview of the predicted effects of

developmental PCB exposure. It is notable that some of the most significant functional pathways are clearly relevant to the types of neuropsychological phenomena seen in PCB exposed animals. Among the top 10 pathways were Neurological Disorder, Psychological Disorder and Neurodegenerative Disorder. This indicates that functional pathways involved in known central nervous system disorders are also dysregulated by developmental PCB exposure. Also, consistent with the co-regulation analyses, the functional pathway analyses implicate genes regulating transcription.

The canonical pathway analyses provide several intriguing results giving more detail about the biological processes that may underlie the effects seen in the functional analyses. Although, as Table 8 shows, PCB exposure has a wide range of effects, many of the processes implicated by the top 10 pathways are relevant to the effects of PCBs on ADHD-like behaviors in exposed animals. Consistent with the bi-cluster findings (which implicated synaptic plasticity), one of the most significant pathways was Long Term Depression (LTD). The molecular interactions in this pathway lead to LTD and synaptic plasticity in the cerebellum (Hemart and others 1995; Kashiwabuchi and others 1995), which has been implicated in ADHD in humans by neuroimaging studies (Monuteaux and others 2008; Seidman and others 1999; Zang and others 2005). Given the role of LTD in learning and memory, this pathway could be one mechanism underlying PCB associated neuropsychological impairments.

Because glutamatergic synapses are involved in LTD, it is not surprising that Glutamate Metabolism was one of the most significant canonical pathways. Also consistent with the idea that PCBs impact synaptic plasticity is the fact that Ephrin Receptor Signaling was one of the highly significant canonical pathways. This signaling network regulates cell behaviors involved in synaptic plasticity (e.g., cell migration, attraction and repulsion of neurons, adhesion and de-adhesion of neurons) (Ethell and others 2001). Ephrin Receptor Signaling also affects Axonal Guidance Signaling, another one of our significant canonical pathways. Because Axonal Guidance Signaling is clearly involved early in neurodevelopment, this suggests that PCB exposure affects both the initial development of the brain and also synaptic plasticity at the ages analyzed.

The NRF2-mediated Oxidative Stress Response pathway was also highly significant. Because oxidative stress can trigger apoptosis and necrosis, it has been implicated in many diseases such as Parkinson's disease and Alzheimer's disease. This finding is consistent with experimental evidence showing that Aroclor 1254 leads to oxidative stress and brain damage in animal models (Sridevi and others 2007; Venkataraman and others 2008). This may have implications for preventive interventions given that antioxidant treatment has been shown to protect against the neurotoxicity of Aroclor 1254 (Krishnamoorthy and others 2007; Muthuvel and others 2006; Zhang 2005).

The Peroxisome proliferator activated receptor α (PPARα)/Retinoid X receptor, alpha (RXRα) Activation pathway was also highly significant. This pathway is potentially related to the ADHD-like behaviors and neuropsychological impairments of PCB exposed animals because PPARα is involved in fatty acid oxidation and uptake in tissues. Colquhoun and Bunday (1981) proposed that a deficiency of essential fatty acids caused ADHD. They based this hypothesis on several sources of evidence: 1) Boys are more likely than girls to have ADHD and males are known to have much higher fatty acid requirements than females; 2) many of the ADHD children they studied had abnormal thirst, a cardinal sign of EFA deficiency; 3) Many of the children with ADHD they studied showed eczema, allergies and asthma which had been associated with fatty acid deficiencies; and 4) zinc deficiency has been reported in ADHD

(Arnold and Disilvestro 2005) and zinc is required for fatty acid metabolism. In a review of eight studies (Raz and Gabis 2009), two found no differences between ADHD patients and controls but six found ADHD to be associated with low omega-3 fatty acid levels and/or higher omega-6 to omega-3 ratios. The negative studies were small, and one did not make a formal ADHD diagnosis but instead assessed "maladaptive hyperactive" children. The fatty acid differences between ADHD and control youth do not appear to be due to dietary differences (Raz and Gabis 2009) and may reflect genetic differences in fatty acid metabolism (Brookes and others 2006). Our work now suggests that PCB exposure might lead to a cascade of events that affects fatty acid metabolism.

Related to these findings, it is particularly interesting that a recent large-scale pathway-based meta-analysis of transcriptome-wide changes in gene expression in Parkinson's disease brains singled out a set of genes controlling cellular bioenergetics that are expressed in response to PPARγ coactivator-1α as specifically underexpressed in Parkinson's disease patients (Zheng and others 2010). Therapeutic activation of the pathway was shown to increase expression of nuclear-encoded subunits of the mitochondrial respiratory chain and block the dopaminergic neuron loss induced by mutant α-synuclein or the pesticide rotenone in cellular disease models. Given that low doses of mitochondrial toxins can produce ADHD-like behavior in non-human primates, whereas high doses lead to parkinsonism, the PPAR pathways may represent common druggable targets for both disorders that can ameliorate pathological bioenergetic deficiencies.

In summary, our transcriptome-wide analysis of the effects of PCB exposure has shown that the expression of many genes is dysregulated by lactational PCB exposure, but not gestational exposure and has highlighted biological pathways that might mediate the effects of PCB exposure on the ADHD-like behaviors seen in animals exposed to PCBs early in life. Like any animal model, the relevance of this work to humans remains to be determined and our hypotheses about biological pathways need to be tested in experimental paradigms. Nevertheless, we have provided strong leads for future research. In particular, our work should further motivate studies of fatty acids and PPAR pathways in ADHD, and suggests that another potentially druggable pathway, oxidative stress, may play a role in the disorder.

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Table 1. Definition of Experimental Factors

Factor	Factor description	Levels	Levels description
Treatment	Treatment, exposure to PCB	ctrl PCBlac PCButero	Not exposed Exposed to PCB during lactation Exposed to PCB in utero (mother)
Time	Age, days since birth	P23 P35	23 days postnatal 35 days postnatal
Brain	Brain region	SNVTA PFC Vermis	
Gender	Gender	female male	

Table 2. The top 20 transcripts most affected byPCB exposure

Gene symbol	Transcript ID	ANOVA p-value (original)	Benjamini-Hochberg critical value
Rnf44	10794146	$2.55 \cdot 10^{-4}$	$2.55 \cdot 10^{-4}$
Src	10841624	$2.53 \cdot 10^{-4}$	$2.54 \cdot 10^{-4}$
Foxk1	10760502	$2.52 \cdot 10^{-4}$	$2.53 \cdot 10^{-4}$
-	10719304	$2.51 \cdot 10^{-4}$	$2.52 \cdot 10^{-4}$
Prkcc	10718572	$2.50 \cdot 10^{-4}$	$2.51 \cdot 10^{-4}$
Eltd1	10819770	$2.47 \cdot 10^{-4}$	$2.51 \cdot 10^{-4}$
Cd14	10803991	$2.44 \cdot 10^{-4}$	$2.50 \cdot 10^{-4}$
-	10802619	$2.43 \cdot 10^{-4}$	$2.49 \cdot 10^{-4}$
Zfyve20_predicted	10864158	$2.38 \cdot 10^{-4}$	$2.48 \cdot 10^{-4}$
Jmjd4_predicted	10733972	$2.37 \cdot 10^{-4}$	$2.47 \cdot 10^{-4}$
Mpzl1	10769538	$2.35 \cdot 10^{-4}$	$2.46 \cdot 10^{-4}$
Prr12	10721616	$2.30 \cdot 10^{-4}$	$2.45 \cdot 10^{-4}$
-	10816024	$2.30 \cdot 10^{-4}$	$2.44 \cdot 10^{-4}$
-	10778991	$2.29 \cdot 10^{-4}$	$2.43 \cdot 10^{-4}$
-	10937570	$2.28 \cdot 10^{-4}$	$2.42 \cdot 10^{-4}$
Tsn	10767058	$2.27 \cdot 10^{-4}$	$2.41 \cdot 10^{-4}$
Sfrs3-ps1	10793456	$2.27 \cdot 10^{-4}$	$2.40 \cdot 10^{-4}$
Elovl7_predicted	10812922	$2.26 \cdot 10^{-4}$	$2.40 \cdot 10^{-4}$
Mfap3	10733726	$2.25 \cdot 10^{-4}$	$2.39 \cdot 10^{-4}$
Prkcsh_predicted	10908576	$2.25 \cdot 10^{-4}$	$2.38 \cdot 10^{-4}$

Table 3. Transcripts with significant interaction effects

Interaction effect	Gene symbol	Transcript ID	ANOVA p-value (original)	Benjamini-Hochberg critical value
Treatment:Time	Bhlhb3	10867026	$2.85 \cdot 10^{-7}$	$9.14 \cdot 10^{-7}$
Treatment:Brain	Usp21	10769854	$8.77 \cdot 10^{-6}$	$1.01 \cdot 10^{-5}$
	Sept14	10761388	$8.77 \cdot 10^{-6}$	$9.14 \cdot 10^{-6}$
	Znf532_predicted	10802294	$5.98 \cdot 10^{-6}$	$7.31 \cdot 10^{-6}$
	Cnksr3	10717813	$5.87 \cdot 10^{-6}$	$6.40 \cdot 10^{-6}$
	Dnm2	10908427	$5.37 \cdot 10^{-6}$	$5.49 \cdot 10^{-6}$
	Ankhd1-Eif4ebp3	10801024	$4.08 \cdot 10^{-6}$	$4.57 \cdot 10^{-6}$
	Med12	10934317	$2.75 \cdot 10^{-6}$	$3.66 \cdot 10^{-6}$
	Frmd5	10849156	$1.32 \cdot 10^{-6}$	$2.74 \cdot 10^{-6}$
	RGD1560888	10756521	$1.01 \cdot 10^{-6}$	$1.83 \cdot 10^{-6}$
	RGD1564887	10730492	$3.50 \cdot 10^{-7}$	$9.14 \cdot 10^{-7}$
Treatment:Gender	-	-	-	-
Treatment:Time:Brain	Plcg1	10841850	$8.99 \cdot 10^{-6}$	$1.28 \cdot 10^{-5}$
	Rfxdc1_predicted	10830105	$8.16 \cdot 10^{-6}$	$1.19 \cdot 10^{-5}$
	Pgrmc1	10936341	$6.64 \cdot 10^{-6}$	$1.10 \cdot 10^{-5}$
	Olr448_predicted	10846930	$6.07 \cdot 10^{-6}$	$1.01 \cdot 10^{-5}$
	Wdr48_predicted	10914251	$5.93 \cdot 10^{-6}$	$9.14 \cdot 10^{-6}$
	Kcnq2	10852437	$3.59 \cdot 10^{-6}$	$8.23 \cdot 10^{-6}$
	Cdc42se2	10742674	$3.08 \cdot 10^{-6}$	$7.31 \cdot 10^{-6}$
	Hist2h2be	10817529	$2.64 \cdot 10^{-6}$	$6.40 \cdot 10^{-6}$
	Pan3	10760047	$1.74 \cdot 10^{-6}$	$5.49 \cdot 10^{-6}$
	-	10909347	$7.18 \cdot 10^{-7}$	$4.57 \cdot 10^{-6}$
	Bmp1	10785063	$2.81 \cdot 10^{-7}$	$3.66 \cdot 10^{-6}$
	Hspa8	10889219	$2.26 \cdot 10^{-7}$	$2.74 \cdot 10^{-6}$
	Hspa8	10881303	$1.78 \cdot 10^{-7}$	$1.83 \cdot 10^{-6}$
	Hspa8	10749854	$1.24 \cdot 10^{-7}$	$9.14 \cdot 10^{-7}$
Treatment:Time:Gender	-	-	-	-
Treatment:Brain:Gender	-	-	-	-
Treatment:Time:Brain:Gender	-	-	-	-

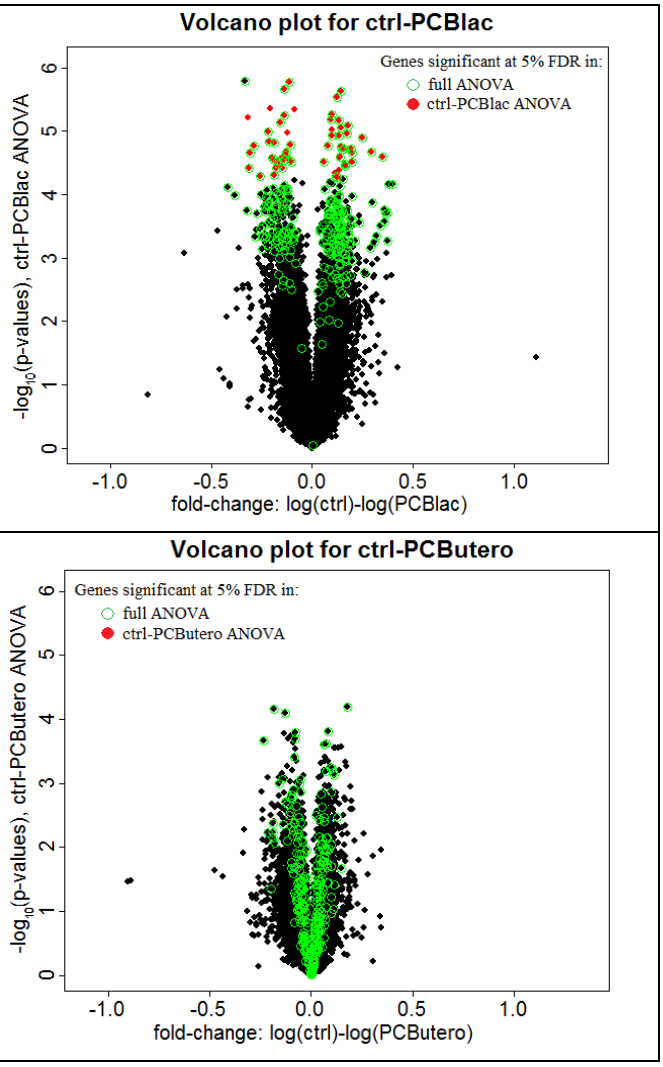
Table 4. Genes differentially Expressed in Pairwise Comparisons

ANOVA, Fold change	**Positive fold change (+)**		**Negative fold change (-)**	
	Gene	**Transcript ID**	**Gene**	**Transcript ID**
Ctrl-PCBlac ANOVA log(ctrl)-log(PCBlac)	Ctgf	10717233	LOC685941	10706836
	MGC93975	10721350	Brsk1	10718881
	Syt12	10727588	Gsk3a	10719798
	Cox11	10737438	-	10720506
	Lgals9	10745292	Ppp1r9b	10737587
	Fam134c	10747564	Cacng4	10748459
	Dgcr2	10752397	-	10761941
	Xab2	10756197	Nos1	10762455
	Slc19a2	10765248	R3hdm1	10763654
	Mat2a	10767001	Plekha6_predicted	10763976
	Gnb1	10788437	RGD1311575	10776361
	-	10791288	Zfp407	10805514
	Csnk1a1	10802114	Btbd14b	10806677
	LOC365643	10812468	Rps23	10812416
	Selt	10815552	Bcl9_predicted	10825228
	Bat5	10831208	Sobpl	10833823
	Gopc_predicted	10833337	Rnf208	10834157
	Tmem87b	10839696	RGD1310754	10847886
	Rab14	10844658	Ubn2	10854601
	Tmx2	10846821	-	10855946
	Pltp_predicted	10851670	Hipk2_predicted	10861997
	Gnai1	10853229	Atn1	10865487
	Zfp212	10855203	Ubap2_predicted	10876103
	Mat2a	10863221	Anks1b	10894812
	Slc31a1	10869310	Anks1b	10894814
	Kti12	10870845	Tcf20	10905765
	Slc2a1	10871521	Trak1	10914385
	Robo4	10909044	Ubqln2_predicted	10937464
	Dpy19l1_predicted	10915787	Arhgef9	10938467
	Arcn1	10916855		
	Rfk	10934662		
Ctrl-PCButero ANOVA log (ctrl)-log(PCButero)	-	-	-	-
PCButero-PCBlac ANOVA log(PCButero)-log(PCBlac)	Col4a1	10792955	-	-

Table 8: Top 10 Significant Canonical Pathways from Treatment by Time Interaction Results		
Pathway	**p-value**	**Genes in the Pathway**
Huntington's Disease Signaling	.00006	GNAQ, GOSR1, MAPK3, PRKCH, PDPK1, SOS1, MTOR, EP300, HDAC10, ITPR1, PIK3C2B, GNG3, PRKCZ, PRKCA, HSPA8, MAPK9, CAPN2, HTT, DNM3, CAPNS1, HSPA5, ATP5B, CTSD, CREBBP, MAP2K4, ZDHHC17, ARFIP2, TCERG1, POLR2A, PLCB1, DLG4, PIK3CA, NCOR1, SOS2, RASA1, NCOR2, GRM5, HDAC3, NAPB, NAPG, CACNA1B, CAPN1, GNB1, AP2A2, CLTC, SGK1
Axonal Guidance Signaling	0.0001	PRKCH, SOS1, PIK3C2B, PRKAR1B, PRKCZ, PRKCA, UNC5D, CFL2, ROBO1, PFN2, SEMA3E, GNAL, GNAI1, SRGAP3, ITSN1, ADAM9, SEMA6D, ROCK2, PLXNB1, PLCB1, ADAM10, CXCL12, ITGA4, PIK3CA, DCC, RAP1B, RTN4R, PLXNA2, NTF3, EPHA4, SEMA4B, GNAQ, MAPK3, SEMA3D, SUFU, CRKL, GNAI2, WASL (includes EG:8976), L1CAM, ARPC4, EIF4E, GNG3, FZD6, EPHA5, WNT7B, VEGFB (includes EG:7423), ARPC2, EPHA6, PAK3, MRAS, PDGFB, BMP2, ARHGEF6, NRP1, PPP3CB, GLI3, SLIT2, DPYSL2, DOCK1, SOS2, ACTR3, RASA1, SEMA3C, NFAT5, SRGAP1, GNB1, NTRK2
Nuclear factor (erythroid-derived 2)-like 2 (NRF2)-mediated Oxidative Stress Response	0.0002	DNAJA3, ACTG1, MAPK3, PRKCH, BACH1, GCLC, DNAJC18, SQSTM1, HERPUD1, FKBP5, EP300, PIK3C2B, PRKCZ, TXNRD1, PRKCA, MAPK9, FOS, MRAS, DNAJC11, DNAJB11, MAP2K4, FOSL1, CREBBP, DNAJB12, MGST3, PIK3CA, DNAJC16, SOD1, MAP2K6, MGST2, CLPP, CDC34 (includes EG:997), DNAJC15, DNAJB9, GCLM, DNAJC6, DNAJC10
Endothelin-1 Signaling	0.0002	GNAQ, MAPK3, PRKCH, ECE1, PLA2G12A, MAPK6, SOS1, NOS1, ADCY5, GNAI2, ITPR1, PIK3C2B, PRKCZ, PRKCA, MAPK9, EDNRB, FOS, GUCY1A3, TMEM87B, MRAS, GNAL, PLD3, ECE2, EDN1, GNAI1, ITPR2, PTGS2, PLCB1, PIK3CA, PLCL1, PLA2G5, NOS3, PDIA3, YWHAZ, MAPK4, SPR
Glutamate Metabolism	0.0002	QARS, GCLC, CAD, GMPS, GFPT1, GFPT2, NADSYN1, GLUD1, GOT1, GCLM, GAD1, LGSN, GNPNAT1
Peroxisome proliferator activated receptor α (PPARα)/Retinoid X receptor, alpha (RXRα) Activation	0.0002	PPARA, GNAQ, MAPK3, GPD2, CAND1, SOS1, ADCY5, EP300, ADIPOR2, PRKAR1B, TGFB1, PRKCA, AIP, NFKBIA, CYP2C19, MED12, CLOCK, MRAS, TRAF6, CKAP5, INSR, MAP2K4, CREBBP, NR2C2, PLCB1, ABCA1, NCOR1, PLCL1, JAK2, GPD1, MAP2K6, SLC27A1, SOS2, NCOR2, PDIA3
Aldosterone Signaling in Epithelial Cells	0.0002	PIP4K2B, ITPR2, MAPK3, PRKCH, PLCB1, PDPK1, PLCL1, PIK3CA, SOS1, SOS2, SLC12A1, PIK3C2B, ITPR1, PRKCZ, PRKCA, HSPA8, DUSP1, PDIA3, PI4KA, HSPA5, SGK1
Ephrin Receptor Signaling	0.0003	GNAQ, MAPK3, CRKL, SOS1, GNAI2, WASL (includes EG:8976), STAT3, PTPN13, ARPC4, GNG3, EPHA5, CFL2, ARPC2, ABI1, VEGFB (includes EG:7423), EPHA6, PAK3, MRAS, GNAL, PDGFB, GNAI1, ITSN1, GRIN3A, SORBS1, ROCK2, GRINA, ADAM10, CXCL12, ITGA4, JAK2, ACTR3, SOS2, RASA1, RAP1B, EPHA4, GNB1
Epidermal growth factor Signaling	0.0004	MAP2K4, ITPR2, MAPK3, PIK3CA, SOS1, STAT3, SOS2, PIK3C2B, ITPR1, CSNK2A2, RASA1, PRKCA, JAK1, FOS
Synaptic Long Term Depression	0.0006	GNAQ, MAPK3, RYR2, PRKCH, PLA2G12A, CRHR1, NOS1, ADCY5, GNAI2, ITPR1, RYR1, PRKCZ, PRKCA, PPP2R3A, PPP2CA, GUCY1A3, TMEM87B, MRAS, GNAL, GNAI1, PRKG1, ITPR2, PLCB1, PLA2G5, NOS3, GRM5, YWHAZ, SPR, PPP2R1A, GRID2

Table 9: Top 10 Significant Functional Pathways from Treatment by Time Interaction Results		
Function	**p-value**	**Number of Molecules in Pathway**
Neurological Disorder	3.95e-19	697
Genetic Disorder	1.58e-18	1086
Huntington's Disease	1.34e-09	138
Dyskinesia	2.24e-09	140
Psychological Disorder	6.00e-09	290
Branching Of Neurites	4.51e-08	27
Metabolic Disorder	4.73e-08	457
Transcription Of Gene	1.54e-07	98
Development Of Plasma Membrane Projections	2.75e-07	58
Neurodegenerative Disorder	2.84e-07	177

Fig.1 Volcano plots for pairwise comparison of treatments: (a) control vs. PCBlac, (b) control vs. PCButero, (c) PCButero vs. PCBlac. Colored point are genes identified as significant in either the full ANOVA (green circles) or the corresponding pair-wise ANOVA (red).



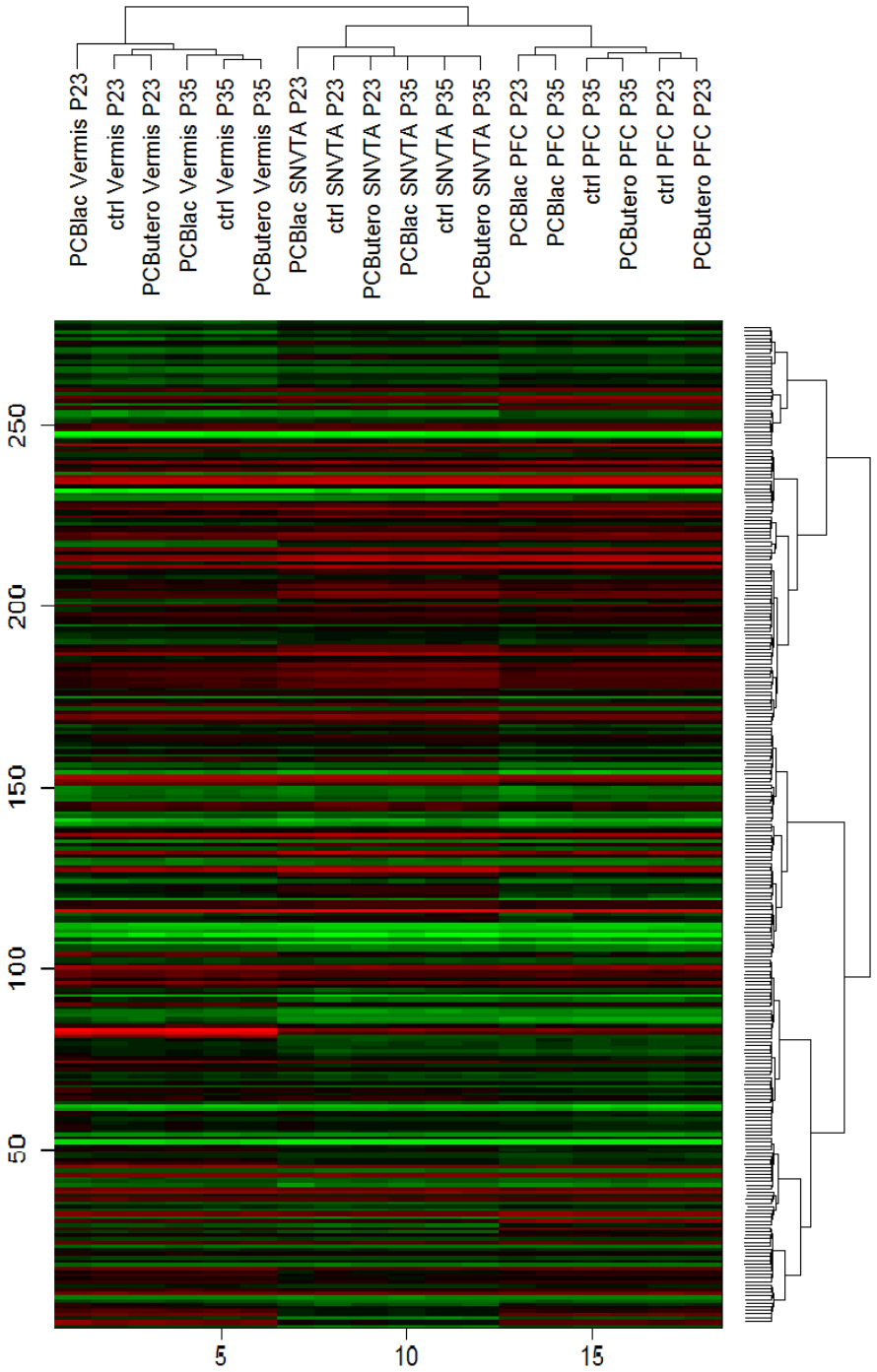


Fig. 2. Clustering of genes (rows) and experimental conditions (columns) of the PCB data set. Colors correspond to normalized mean expression levels (as an average across all replicates and Gender levels (males and females) within each combination of Treatm, Brain and Time factors) from low (green) to high (red).

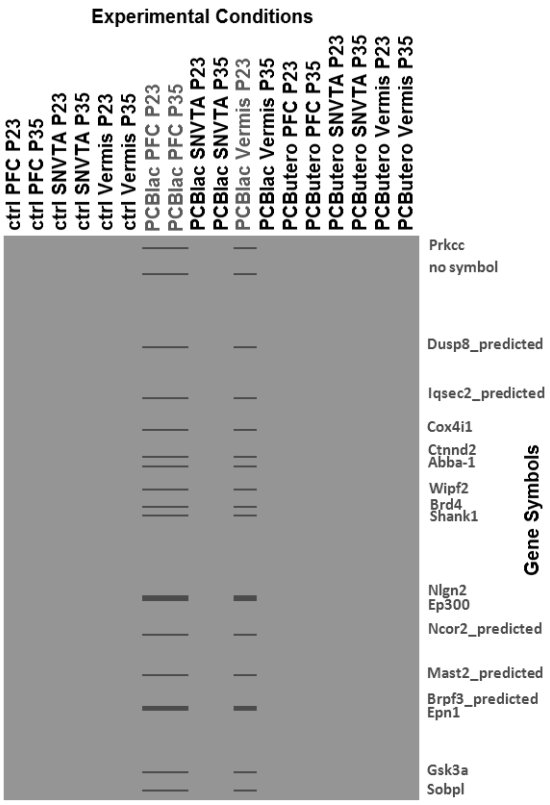


Fig. 3. Location of cluster 6 in the data matrix. Genes and conditions comprising cluster 6 are given in grey fonts.

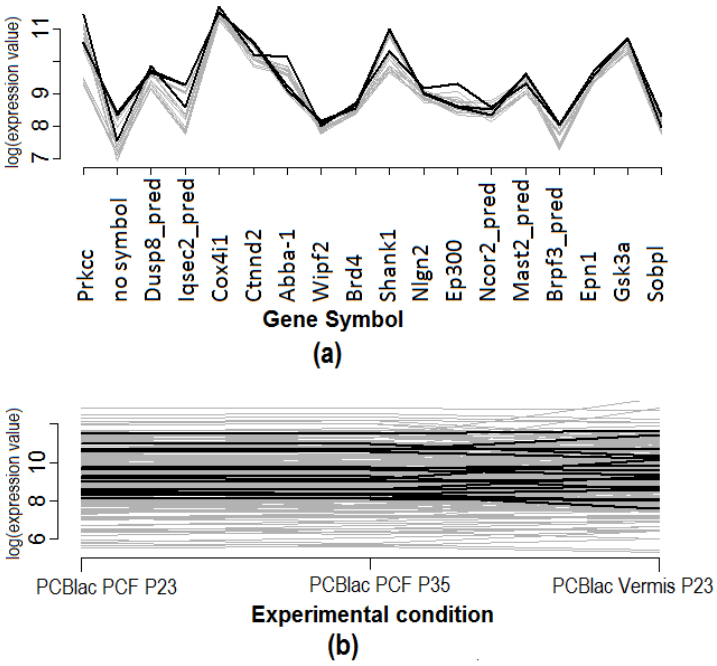
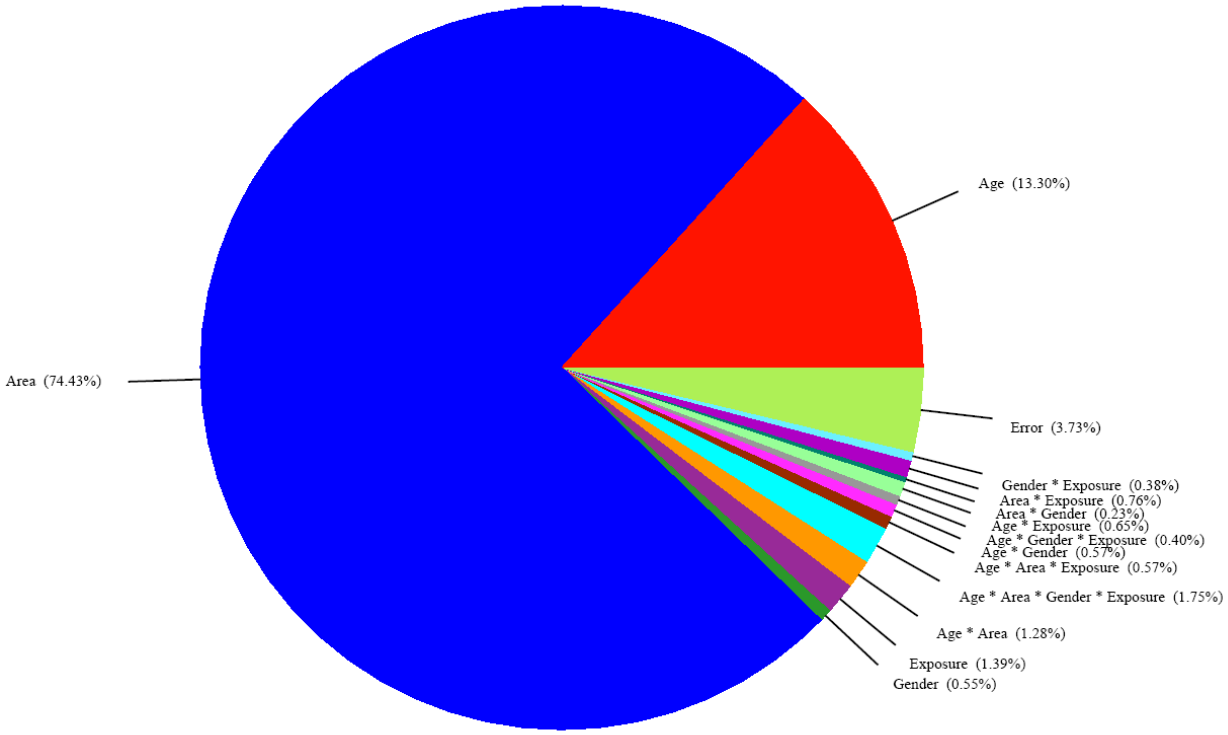


Fig. 4. Parallel coordinates plots for cluster 6: (a) expression levels of the three conditions across their genes, the rest of the conditions are represented by gray lines; (b) expression levels of the genes across the three conditions, the rest of the genes are represented by gray lines.



Supplemental Fig 1. Sources of variation (average sum of squares) for top 279 transcripts showing significant main effect of treatment after BH-FDR correction.