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A Comparison of Molecular Alterations in Environmental and Genetic Rat Models of ADHD: a pilot study

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Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is the most common neurobehavioral disorder in school-aged children. In addition to genetic factors, environmental influences or gene × environmental interactions also play an important role in ADHD. One example of a well studied environmental risk factor for ADHD is exposure to polychlorinated biphenyls (PCBs). In this study, we investigated whether the well-established genetic model of ADHD based on the Spontaneously Hypertensive Rat (SHR) and a well established PCB-based model of ADHD *exhibited* similar molecular changes in brain circuits involved in ADHD. The brains from 28 male rats (8 SHR, 8 Sprague-Dawley (SD) controls, 8 Wistar-Kyoto (WKY) controls, and 4 PCB-exposed SD rats) were harvested at postnatal day 55-65 and RNA was isolated from six brain regions of interest. The RNA was analyzed for differences in expression of a set of 308 probe sets interrogating 218 unique genes considered highly relevant to ADHD or epigenetic gene regulation using the Rat RAE 230 2.0 GeneChip (Affymetrix). Selected observations were confirmed by real time quantitative RT-PCR. The results show that the expression levels of genes Gnal, COMT, Adrbk1, Ntrk2, Hk1, Syt11 and Csnk1a1 were altered in both the SHR rats and the PCB-exposed SD rats. Arrb2, Stx12, Aqp6, Syt1, Ddc and Pgk1 expression levels were changed only in the PCB-exposed SD rats. Genes with altered expression only in the SHRs included Oprm1, Calcyon, Calmodulin, Lhx1 and Hes6. The epigenetic genes Crebbp, Mecp2 and Hdac5 are significantly altered in both models. The data provide strong evidence that genes and environment can affect different set of genes in two different models of ADHD and yet result in the similar disease-like symptoms.

Keywords

microarray; real-time PCR; SHR; PCB; epigenetics

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Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most common cognitive, behavioral and emotional disorder diagnosed among school age children (American Academy of Pediatrics, 2000). The symptoms of ADHD include deficits in attention and problem-solving, hyperactivity and difficulty inhibiting incorrect responses. Overall, ADHD affects an estimated 8-12% of children (Faraone and others 2003). Moreover, ADHD can persist into adulthood (Faraone and others 2006), where it affects an estimated 4% of the population (Faraone and Biederman 2005; Kessler and others 2005).

Reduced dopamine (DA) transmission is believed to be a causal mechanism for ADHD (Johansen and others 2005). DA plays a critical role in strengthening synaptic connections following successful behavioral outcomes that lead to primary reinforcement and attenuating such connections following unrewarded outcomes (Johansen and others 2005; Schultz 2002). In ADHD, significant associations have been reported with genes involved in catecholamine signaling and metabolism (Faraone and Khan 2006). If such changes lead to functional alterations, they could impair a critical mechanism underlying behavioral reinforcement (Sagvolden and others 2005).

A 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 (Faraone and others 2005). However, the less then complete heritability of ADHD indicates that the prevalence of ADHD cannot be explained by genetics alone. Indeed, several environmental factors have been identified as potential risk factors for ADHD, including prenatal exposure to alcohol, cigarette smoke and environmental pollutants including lead and polychlorinated biphenyls (PCBs) (Banerjee and others 2007; Sagvolden and Sergeant 1998).

There are numerous cognitive and behavioral abnormalities reported in children exposed to PCBs (Rice 1997; Rice 1998; Rice 1999). PCBs are a family of 209 manufactured compounds (congeners) that were once produced on a large scale (from 1929-1977) in the United States for use as electrical insulators, brake liners, paints, sealing compounds, etc. (Ross 2004). In 1977, manufacturing of PCBs was banned in the US because of evidence that they posed persistent and significant health risks. It is now well-established that PCBs are highly-stable, bioaccumulate and, because of their stability, have entered the food chain (even in Arctic areas) (Berger and others 2001; Fonnum and others 2006). Thus, PCBs have the potential to affect reported rates of ADHD-like behaviors throughout much of the world.

Several animal models, based on genetic and environmental theories, have been developed to study the pathophysiology of ADHD (Johansen and others 2005). One of the most well-characterized genetic models of ADHD *is* the Spontaneously Hypertensive Rat (SHR). SHRs display all behaviors characteristics of ADHD: impaired sustained attention, motor impulsiveness, and hyperactivity that is not present in novel situations but develops over time when reinforcers are infrequent (Johansen and others 2005) (Sagvolden 2000) and also imitate behavioral symptoms of children with ADHD like increased behavioral variability, deficient response reengagement, and make significantly more errors than controls (Sagvolden 2000) (Wiersema and others 2005). The PCB-exposed rat is also a behaviorally well-characterized environmental model of ADHD, which reproduces the hyperactivity and impulsivity seen in children with ADHD but no impaired sustained attention (Berger and others 2001; Holene and others 1998).

Despite their widespread use, little is known about the mechanisms through which these rat models mimic ADHD. To begin to address this issue, we conducted a pilot study to examine the gene expression profiles of the SHR rat and PCB-exposed SD rat in brain regions

relevant to ADHD. We hypothesized that this comparison might shed light on possible mechanisms underlying ADHD, and help determine if genetic and environmental risk factors for ADHD converge on common molecular pathways. The differential gene expression results of PCB-exposed SD rats and SHR rats point to different pathways involved in the genetic and environmental models of ADHD and reinforce the notion that neuropsychiatric disorders are multifactorial in nature.

Materials and Methods

Animals

A total of 28 male rats were used in this study. These consisted of 12 Sprague-Dawley rats (NTac:SD), including 8 control SD rats and 4 PCB-exposed SD rats, 8 SHR rats (SHR/NCrl) from Charles River (Germany/US) and 8 Wistar/Kyoto (WKY/NHsd) from Harlan (UK). The 8 male SD controls were randomly selected offspring of dams not exposed to PCB. The 4 male PCB-exposed SD rats were randomly selected offspring of dams from the PCB-exposed group. The 8 male SHR rats and WKY rats were randomly selected offspring from their respective dams.

Dams were maintained on a 12-h light: 12-h dark cycle, at 20–22 °C, with a relative humidity of 60–63%. They were housed in pairs from the same group in 24×18×18 cm (height) cages. Animals had free access to food and water during gestation. The PCB exposure period was from gestational day (GD) 5 to 19. On each of those days, in addition to their LabDiet ProLab RMH 3000, the PCB-exposed dams received one-half of a Nilla Wafer cookie onto which had been added 0.1 ml of corn oil containing 4.0 μg/g (body weight) of a 1:1 mixture of Aroclor 1254/1260. The wafers given to the control, WKY and SHR dams contained 0.1 ml of corn oil alone. As a consequence of the dams' exposure during gestation (during which time the PCBs tend to bioaccumulate in fatty tissues), their offspring were exposed to PCBs in utero and also during nursing. The 28 male rats used in this study were weaned on postnatal day (PND) 22.

Tissue sampling

Brains from all 28 male rats were harvested at PND 65 following euthanasia with an overdose of isofluorane and submerged in RNAlater® (Ambion, Austin, TX). We then dissected six brain regions- the medial prefrontal cortex, ventral striatum, dorsal striatum, hippocampus, vermis, and ventral mesencephalon (including the substantia nigra and ventral tegmental area, SN/VTA). We chose brain regions that had been implicated in ADHD by a meta-analysis of structural imaging studies in humans (Valera and others 2007). For example, the prefrontal cortex (PFC) is particularly relevant to ADHD as imaging studies indicate smaller PFC volumes, especially on the right side for ADHD patients (Castellanos and others 1996). The PFC is also the center of executive functions such as attention span, organization, problem solving and self-monitoring and supervision, all of which are impaired in ADHD. Damage to the striatum is also thought to be associated with the etiology of ADHD (Lou 1996). This is because striatum is one of the richest sources of dopaminergic synapses and dopamine is important in the regulation of striatal functions, and also experimental striatal lesions in animals mimic symptoms of hyperactivity and poor performance on tasks of response inhibition (Alexander and others 1986). Imaging studies have shown significantly smaller caudate volumes, either on the left or right side (Castellanos and others 1994), (Mataro and others 1997). Researchers studying the cerebellum in ADHD children have observed structural abnormalities in specific regions of the vermis (posterior inferior lobules) in both boys and girls (Bussing and others 2002), (Hill and others 2003). A cross-sectional case-control study of the morphology of hippocampus in children with ADHD, reported the presence of enlarged hippocampus in children and

adolescents with ADHD (Plessen and others 2006), which, the authors reasoned, may represent a compensatory response to the presence of disturbances in the perception of time and stimulus seeking associated with ADHD.

RNA Purification

Total RNA was extracted from all of the brain samples using the RNeasy® Mini Kit and QiaShredder™ (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 for the microarray-based screen did not include a DNase treatment step. However, in our experience we obtain greater than 99% pure RNA from the RNeasy columns (Qiagen). Moreover, our cDNA synthesis and labeling protocol (Ovation, NuGen) utilizes an oligo-dT based first strand synthesis reaction which specifically allows the amplification of poly-adenylated RNA but not DNA (DNA sequences only contain the poly A signal (usually the sequence AATAAA) upstream of where the ~40 nucleotide poly A tail is added specifically to mRNA molecules when they are synthesized from the DNA. In the course of our real-time quantitative RT-PCR studies, however, where the oligo-dT selection was not exclusively used in the first strand synthesis, we did eliminate DNA contamination by using the Quantitect Reverse Transcription Kit from Qiagen, which includes a DNase treatment step.

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 (RAE230 2.0, Affymetrix). For these assays, we pooled equal quantities of RNA from each of the eight rats in each group for each brain area and ran a single GeneChip for that area and treatment group. In total, 24 GeneChip hybridizations were performed to compare the changes in expression across six brain areas in four groups of rats: SD, PCB-exposed SD, SHR and WKY. 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 GeneChip Operating System (GCOS) software to obtain performance metrics, and normalized using the RMA method. Individual transcript levels in each of the two sets of experimental groups and six brain regions were compared using a two-way analysis of variance comparing rat group × area (2×6 ANOVA). These analyses were performed only for comparisons involving SD vs PCB-exposed SD rats and for SHR vs WKY rats. In each case, the nominal significance obtained by the two-way ANOVA was adjusted for multiple testing using the Benjamini-Hochberg False Discovery Rate (BH-FDR) correction (with α=0.05). Because our primary aim was to determine if the genetic and environmental rat models showed similar or different changes in expression in systems relevant to ADHD, we focused our analysis on ADHD candidate genes identified as a part of the International Multi-center ADHD Gene project (IMAGE) project, and their biological neighbors (hereafter referred to as IMAGE genes). We defined the IMAGE gene biological neighbors as any gene which was part of the same gene or protein family as an IMAGE gene, or had a well-established direct relationship with an IMAGE gene. The entire list of IMAGE gene neighbors was curated by hand after systematic review of the array content by two of the study team members. These genes were selected by the senior investigator from the IMAGE project through knowledge of the pathophysiology of the disorder. A total of 274 probes were present on the RAE 230 2.0 GeneChip interrogating 186 unique IMAGE genes (see Table S1). We also examined genes involved in epigenetic mechanisms because it is plausible that PCB exposure could lead to

epigenetic changes that exert master regulatory influences on gene expression. A total of 32 probes and unique epigenetic regulatory genes were present on the array. Although other genes may be relevant to ADHD, by focusing on the IMAGE and epigenetic gene sets, we greatly reduced the number of transcripts studied, and increased our statistical power for the initial screen. The raw microarray data has been submitted at the Gene Expression Omnibus website of NCBI (Accession number: GSE12457).

Estimation of the power inherent in our microarray study was performed using The Power Atlas (http://www.poweratlas.org) which was developed specifically for microarray-based studies (Page and others 2006) power analysis and sample size estimation in the age of high dimensional biology. Overall, using our 2 way ANOVA design, with Benjamini-Hochberg false discovery rate correction; we estimate that we had an average of 65% power to detect true positive effects and 47% power to detect true negative effects at an alpha level of 0.05 across the two comparisons of interest. These values rose to 73% and 52% at an alpha level of 0.10.

Real-Time Quantitative RT-PCR

Selected changes in gene expression observed in the microarray screen were examined on individual RNA samples from each rat using the ABI7000 Real-Time Sequence Detection System with the Absolute™ QPCR SYBR® Green system (ABgene). Primers for specific genes of interest were custom-designed using Primer3 software to have a Tm of ~60°C and span an intron (see Table S2). The identity of each amplicon in a standard RT-PCR reaction was confirmed as a single band of the correct predicted size and end-point melt curve analysis of template on the ABI7000 was used to confirm single melt peaks. In the validation studies, we utilized 500 nanograms of RNA from each brain region of four different male rats of each strain as a template for reverse transcription (RT) reaction using the Quantitect Reverse Transcription Kit (Qiagen). RT-PCR reactions were performed on an equal number of samples from all the four different strains. Since we had only four PCB-exposed SD rats, we decided to choose four different animals from each strain for analyses. The above kit includes a DNase treatment step. After the RT, the reaction was diluted five-fold and 1 μl of the diluted reaction was used in triplicate or quadruplicate PCR reactions for each gene of interest for each rat and brain region on 96 well plates. Apart from 18S, we have also used TATA-binding protein (TBP) as another housekeeping control gene for our analyses. The results from either analysis were similar. The data for qRT-PCR using TBP as the reference gene has not been shown. These reactions were cycled according to standard conditions (95°C-15 min, then 40 cycles of 95°C-15s and 60°C-30s). Control reactions in the absence of template were included to quantify stray signal due to primer dimerization. Statistical analysis of the real-time data was performed using a repeated-measures ANOVA comparing the difference in the number of cycles to threshold (ΔCt) between the transcript of interest and the reference gene (18S rRNA). Group differences were calculated by determining the mean difference in the target gene ΔCt values for each strain and brain area (the ΔΔCt). For direct comparisons with the microarray data, all differences in the microarray and real-time experiments are shown in log2 scale.

Results

Gene expression analyses

The microarray screen across multiple brain areas indicated that only certain IMAGE genes showed significant changes in expression as a result of PCB exposure (Table I, Fig S1 **top**). Specifically, the transcripts for Gnal, Arrb2, Adrbk1, COMT, Stx12, Syt11 and Hk1 showed increased expression in PCB-exposed SD rats. The increased expression of Csnk1a1 was significant but not as robust as these other genes. In contrast to these IMAGE genes, the

transcripts for Syt1, Ntrk2, Ddc, Pgk1 and Aqp6 all showed significantly decreased expression in PCB-exposed SD rats.

The Spontaneously Hypertensive Rats (SHR) showed significant changes in a different set of IMAGE genes (Table II, Fig S1 **bottom**). Transcripts for Lhx, Vax2, Gnal, Aqp4, Csnk1a1, Camk1, Ntrk1, Per2, Syt3, Stxbp1, Grinl1a, Gria3 and Calm3 showed more than two-fold decreased expression in SHR rats compared to WKY rats. In contrast, the transcripts for Gria2, Hes6, Stx11, Strn, Oprm1, Chrna9, Stx17 and Aqp3 showed greater than two-fold increased (log 2 > 1.0) expression in the SHR rats compared to WKY rats.

By combining the results of Tables I and II we determined that the SHR rats and the PCB-exposed SD rats both showed significant alterations (decreases or increases) in the expression levels of Gnal, COMT, Adrbk1, Ntrk2, Hk1, Syt11 and Csnk1a1 compared to their appropriate controls.

We also observed significant changes in a small subset of genes involved in epigenetic regulation (Table III). Specifically, five of these genes showed changes across all brain areas in either PCB-exposed SD rats or the SHRs, including Crebbp, Hdac5, Mecp2 and Dnmt3a. Changes in Mecp2 expression in SHRs and Dnmt3a expression in PCB_SD, though greater than two-fold, were not significant.

Genes of interest examined by RT-PCR

From the genes showing significant changes in expression using pooled RNA in the microarray screen, we chose to confirm the expression changes (or lack thereof) of a total of 21 genes using real-time quantitative RT-PCR on individual RNA samples from the rats used to create the RNA pools. “Confirmation of microarray data by RT-PCR” implies that RT-PCR also shows a significant gene expression difference observed in the same direction as predicted by data from the microarray. Our PCR data (all reactions performed in triplicate or quadruplicate) confirmed (significant gene expression difference observed in the same direction predicted from microarray data) the microarray data for 4/7 genes in PCB-exposed SD rats and 6/14 genes in SHRs listed in Tables IV and V, respectively. The genes that showed a failure to confirm by RT-PCR did not involve any particular brain area.

Discussion

In this preliminary study we compared the expression differences of a set of genes involved in epigenetic regulation and a set of genes thought to be highly-relevant to the pathophysiology of ADHD in an environmental model and a genetic model of ADHD. Overall, we note that the two models show some similarities in gene expression alterations, but many differences. In the space that follows, we briefly highlight some of the more intriguing observations supported by both the microarray and PCR data and their biological significance.

Genes with Expression Changes in Both Models

The genes whose expression levels were altered in both the PCB-exposed rats and SHR rats included Gnal, COMT, Adrbk1, Ntrk2, Hk1, Syt11 and Csnk1a1. Gnal (or G_{olf}) is known to be coupled to the DA receptor, DRD1, and plays a major role in excitatory DA transmission in the striatum. Herve and colleagues reported that disappearance of striatal DA increased G_{olf} levels by 40-50% (Herve and others 1993). Since it is known that exposure to PCBs reduces DA levels (Bemis and Seegal 1999) by inhibiting its uptake into synaptic vesicles (Mariussen and others 1999), it is reasonable to infer that a similar reduction in DA in our PCB-exposed SD rats (which we did not measure) could lead to increased G_{olf} expression. Although G_{olf} has not been previously studied in the SHR model, significant relationships

have been observed between certain SNPs in G_{olf} and symptoms of inattention and hyperactivity/impulsivity in ADHD children (P=0.003 and P=0.008) (Laurin and others 2008). Thus, future work could investigate if G_{olf} variants are present in the SHR rat.

The COMT gene encodes a well-studied enzyme that inactivates catecholamine neurotransmitters (including DA, epinephrine, and norepinephrine) by methylation. Since COMT is involved in DA breakdown, higher levels of COMT would indicate less DA in the brain, which is a characteristic feature of ADHD as well as the PCB and SHR rat models of ADHD. A polymorphism in human COMT (Val158Met) is known to result in reduced COMT activity, higher brain DA levels, and better performance on tests of executive function (Malhotra and others 2002). However, in children with ADHD, the strength of this association is weaker (Mills and others 2004; Taerk and others 2004).

In our lab we have not observed any sequence differences in the COMT gene in our rat groups through comprehensive re-sequencing of the gene (data not shown). Thus, we speculate that the link between PCBs and increased COMT expression is indirect, and may involve activation of the aryl hydrocarbon receptor (AhR) (Kodavanti and Tilson 1997; Tilson and Kodavanti 1997). AhR activation leads to induction of the cytochrome P450 enzymes CYP1A1, CYP1A2 and CYP1B1. In addition to their activity on PCBs, these enzymes also hydroxylate estrogen into highly reactive and genotoxic catechol estrogens (CEs). CEs, when oxidized to quinines, can either interact with DNA to produce mutations, or lead to increased production of reactive oxygen species (ROS) and cell death (Hayes and others 1996; Yager 2000). COMT plays a crucial role in the detoxification of CEs via methylation (Mannisto and Kaakkola 1999). Thus, in a scenario where increased activation of AhR due to PCBs leads to higher amounts of CEs, the system may compensate by increasing COMT expression to reduce cytotoxicity. Since the brain expresses moderately high levels of estrogen and estrogen receptors, this mechanism may explain the increased COMT expression across all brain areas in PCB-exposed SD rats. However, as already discussed, a byproduct of increased COMT expression is not only elimination of CEs but also higher rates of DA catabolism. Thus, COMT may prove to be a crucial gene linking PCB exposure with the reduced DA found in ADHD or the PCB model of ADHD.

Neurotrophic factors (NTFs) play well-established roles in the neurodevelopmental and synaptic changes that take place in the CNS during childhood, adolescence, and early adulthood and thus make attractive candidates for the pathogenesis of ADHD. A recent population-based association study in 546 ADHD patients (216 adults and 330 children) and 546 gender-matched unrelated control subjects suggested a childhood-specific contribution of the BDNF receptor Ntrk2 ($p = .0084$, OR = 1.52) to ADHD (Ribases and others 2008). Moreover, mice with forebrain-specific knockout of Ntrk2 showed stereotypic hyperlocomotion with reduced explorative activity and impulsive reactions to novel stimuli, behaviors consistent with the ADHD phenotype (Zorner and others 2003). The mechanism through which PCB exposures might causes a decrease in Ntrk2 expression is, however, unknown.

PCB-exposed SD rats and SHRs also showed significant changes in the expression of Hexokinase1 (Hk1) and Casein kinase 1, alpha 1 (Csnk1a1). Hk1 catalyzes the first step in glucose metabolism, which is phosphorylation of glucose to glucose-6-phosphate. The 5′ UTR of Hk1 has several binding sites for SP1. Since PCBs activate aryl hydrocarbon receptors (AhRs), and AhRs in turn could interact with an imperfect xenobiotic response element (XRE) present in the 5′-upstream region of SP1 (Krishnan and others 1995), it is possible that the increased expression of Hk1 is a fairly direct effect of PCB exposure. Expression levels of Hk1 in SHR animals may be a reflection of the basal physiological glucose metabolism, and thus basal neuronal activity. The neuronal energetics aspect of our

findings is supported by a recent article which discusses in detail a common observable feature of ADHD- marked moment-to-moment fluctuation in task performance- and proposes that this fluctuation arises from deficient lactate production and supply by astrocytes to rapidly firing neurons (Russell and others 2006). The link between increased glucose metabolism and ADHD needs to be further explored.

Casein kinase 1 (CK1) represents a subset of serine/threonine specific protein kinases. CKI is known to modulate Wnt signaling which plays a crucial role in development. The exact relationship between CKI and ADHD and how PCBs cause an increase in CKI expression is not understood. CK1 might be part of a novel pathway connecting PCBs to ADHD, but further studies are required to establish a connection.

Genes with Distinct Expression Patterns in the Two Models

The PCB-exposed SD rats demonstrated altered expression of beta-arrestin2 (Arrb2), syntaxin-12 (Stx12), aquaporin-6 (Aqp6), synaptotagmin-1 (Syt1), dopamine decarboxylase (Ddc) and phosphoglycerate kinase-1 (Pgk1). Ddc catalyzes the decarboxylation of L-DOPA to DA. PCB-exposed rats showed decreased expression of Ddc. Thus, less Ddc might indicate less initial production of DA. A previous in vitro study supports our finding (Angus and others 1997). The manner in which PCB exposure might inhibit Ddc is unclear. Reduced DA production in PCB-exposed rats, due to inhibition of Ddc by PCBs, could down-regulate pre-synaptic vesicular transport and thus down-regulate Syt1. An increase in Stx12 could indicate higher rates of endocytosis of surface DA receptors due to increased beta-arrestin and Grk2. There are no reports in the literature about a direct link of ADHD or PCB exposure to Aqp6 and Pgk1.

The SHR rats indicated robust alterations in a different set of genes. Mu opioid receptor 1 (Oprm1) is located on the plasma membrane of neurons. Experiments in knockout mice and humans indicate that Oprm1 is associated with substance abuse disorders (Berrendero and others 2002; Zhang and others 2006). Individuals with ADHD, depending on the subtype, also show strong substance dependence (Faraone and others 2007; Rodriguez and others 2007). Thus, it is possible that substance dependence in ADHD may be modulated by Oprm1.

The Calcyon gene encodes a brain-specific DRD1-interacting protein involved in DRD1/DRD5 receptor-mediated calcium signaling. In our data, the SHRs had two-fold increased expression of calcyon compared to WKY rats, which is in agreement with a recent study which examined calcyon mRNA expression in the frontal-striatal circuitry of three-, five-, and ten-week-old SHR and WKY rats (Heijtz and others 2007). In another study it was reported that mice overexpressing calcyon displayed a range of abnormal behaviors including hyperactivity, reduced anxiety, and/or impaired restraint (harm avoidance) that would indicate that calcyon up-regulation leads to deficits in control over behavioral output (Trantham-Davidson and others 2008).

Also related to calcium signaling, we observed that the calmodulin transcript was robustly downregulated in SHRs. These data support those of a previous study reporting lower calmodulin levels in the medial neostriatum of SHR rats compared to WKY (Akiyama and others 1992). Calmodulin activates CaMKII and is involved in LTP (Malenka and Nicoll 1999). A downregulation of calmodulin in the SHR supports the results showing that LTP is reduced in the SHR (Tang and others 2008).

The transcripts Lhx1 and Hes6 show evidence of significant modulation in SHRs. Hes6 belongs to the basic helix-loop-helix family (bHLH) of transcription factors and is known to be involved in cortical neurogenesis. Lhx1 plays an important role in Purkinje cell

generation and differentiation (Zhao and others 2007). Thus, changes in the expression of these genes could affect forebrain and hindbrain circuitry in the SHR model. It is known that the frontal cortex of SHRs shows decreased neurons and reduced laminar volume at four and six months of age (Mignini and others 2004). Although there is no known evidence connecting these genes directly to ADHD, it can be speculated that altered expression levels of these genes might affect cortical and cerebellar volumes in SHRs by influencing neurogenesis and differentiation. Such changes would be consistent with those reported in individuals with ADHD (Valera and others 2007).

Genes Involved in Epigenetic Mechanisms

We observed significant changes in expression of four epigenetic genes in either the PCB-exposed SD rats or the SHRs. Creb binding protein (Crebbp), histone deacetylase-5 (Hdac5), and methyl CpG binding protein-2 (MeCp2) showed changes in the PCB-exposed SD rats. An alternate transcript of MeCp2 and DNA methyltransferase 3a (Dnmt3a) showed changes in the SHR model. DNA methylation is not known to play a causal role in ADHD, but these observations suggest that some of the gene expression changes observed in SHRs or PCB-exposed SD rats may be modulated by the degree of promoter methylation. For example, hypomethylation of membrane-bound COMT is a major risk-factor for schizophrenia and bipolar disorder (Abdolmaleky and others 2006), and it is possible that the six-fold increase in COMT expression that we observed in PCB-exposed SD rats could have been produced by a toxin-induced hypomethylation of the COMT promoter. In contrast, the activation of epigenetic genes in the SHR was not expected. Instead, it is possible that such epigenetic transcript differences represent regulatory mechanisms induced by their ADHD-like behaviors.

The present study has certain limitations. First, the low number of animals used in this study, especially in the PCB-exposed SD group (n = 4), reduced our statistical power. Further more, the SHRs and the WKY rats used in this study were obtained from different suppliers in different countries which make any existing differences in housing conditions and the stress of transport to the laboratory as potential confounding factors. We have not taken the above differences into consideration in our analyses. The above aspects coupled with the differences in sample preparations (pooled vs individual RNAs) for the microarray and real time PCR experiments might have contributed to the observed discrepancies in the two sets of data. Another explanation for the observed discrepancies could be that for most of these genes there exist at least two known splice variants in rats. Subsequent inspection of the target sequences used in the RT-PCR reactions and the target sequences interrogated by the microarray probes for each gene indicates that different regions of several transcripts were being assayed in the two techniques. Thus, it is possible that splice variant effects could greatly influence the validation data. A second major limitation of the present study is that we examined gene expression differences in the two models of ADHD at a single time point, postnatal days 55-65, which makes it difficult to speculate if the gene expression changes observed are short-term, long-term or merely compensatory. Future studies are required to address this issue and to determine the degree to which these gene expression changes serve as a cumulative factor in causing ADHD. For this pilot study, we have not measured PCB levels in the brain or any other tissues in the offspring and hence cannot draw conclusions about any correlation between degree of change in gene expression and accumulation of PCB congeners in the different tissues. However, another study involving gestational exposure of rats to Aroclor 1254 have shown that at similar doses PCB congeners were detected above background levels in brains of adult females and males (Dziennis and others 2008).

In summary, this is the first broad gene expression analysis comparing a genetic model of ADHD (SHR) with an environmental model of the disorder (PCB-exposed SD rats).

Examination of the lists of ADHD candidate genes with significant expression changes in both models suggests a few common mechanisms that lead to the expression of ADHD-like symptoms in these animals. For example, both models exhibit a change in the levels of COMT, Gnal/Golf, Ntrk2, Hk1 and Csnk1a1 mRNA. Increased COMT expression would result in decreased dopamine levels in the brain which is a characteristic pathophysiology of ADHD (Oades 2002). Likewise, mice with forebrain-specific knockout of Ntrk2 show stereotypic hyperlocomotion with reduced explorative activity and impulsive reactions to novel stimuli (Zorner and others 2003), behaviors consistent with the ADHD phenotype. Finally, certain SNPs in Gnal/Golf have been observed to have a significant relationship with symptoms of inattention and hyperactivity/impulsivity in ADHD children (Laurin and others 2008). Thus, our observations of altered expression could be directly related to the production of ADHD-like behavior in several different ways. We also found that the expression of many genes differed between the PCB-exposed SD and SHR rats. It is quite possible that not all of these differences reflect ADHD-related behavior in the two models. A good example is the Aquaporin (Aqp) family of genes. The aquaporins are a family of water-selective membrane channels found in animals, plants, and microorganisms. Aqp4 is the predominant water channel in the brain and has an important role in brain water homeostasis. Aqp4 is significantly down regulated in the SHRs but not regulated in response to PCBs. While there are no known links between aquaporins and ADHD, Ishida et. al demonstrated that Aqp4 mRNA expression was lower in male stroke-prone spontaneously hypertensive rats (SHRSP) than in WKY rats at 20 weeks of age (Ishida and others 2006) and that is plays an important role in water distribution in the cerebral cortex of SHRSP with severe hypertension. Thus, the lower expression levels of Aqp4 in the SHR rats in our study could be an impression of the physiological homeostasis of the SHRs rather than an indication of ADHD-related behavior. These issues need to be explored in future investigations.

These findings suggest that there are some pathogenic mechanisms not shared by the two models. The different pathways involved in the genetic and environmental models of ADHD reinforce the notion that neuropsychiatric disorders are multifactorial and multidimensional in nature. Future studies comparing the two models are needed to elucidate these pathways, clarify the developmental timecourse of the underlying mechanisms and to propose new therapeutic measures for ADHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Genes with significant expression changes in microarray screen of PCB-exposed vs unexposed SD rat brain regions ranked in ascending order according to P value

qPCR	Gene Title	Symbol	Mean L2R	P value
»	Guanine nucleotide binding protein, alpha stimulating, olfactory type	Gnal	0.908	0.0034
»	Catechol-O-methyltransferase	Comt	3.7	0.0036
	Arrestin, beta 2	Arrb2	0.91	0.006
»	Synaptotagmin XI	Syt11	1.467	0.0162
	Syntaxin 12	Stx12	0.61	0.0181
»	Adrenergic receptor kinase, beta 1	Adrbk1	0.778	0.0189
	Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	-0.645	0.024
»	Hexokinase 1	Hk1	1.41	0.0284
»	Aquaporin 6	Aqp6	-1.478	0.0299
	Phosphoglycerate kinase 1	Pgk1	-0.523	0.0356
»	Dopa decarboxylase	Ddc	-1.023	0.0362
	Adrenergic receptor kinase, beta 1	Adrbk1	0.785	0.0365
»	Casein kinase 1, alpha 1	Csnk1a1	0.177	0.0367
	Guanine nucleotide binding protein, alpha stimulating, olfactory type	Gnal	0.738	0.0387
	Synaptotagmin I	Syt1	-1.235	0.0397

Mean L2R is mean log2 ratio of gene expression in PCB-exposed SD rats to SD rats across 6 brain areas.

P values are based on a pairwise T test and are corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate.

Genes appearing in multiple rows were interrogated by more than 1 probeset on the RAE230 GeneChip.

qPCR arrowheads indicate genes used in real-time RT PCR validation.

Table II

Genes with significant expression changes in microarray screen of SHR vs WKY rat brain regions ranked in ascending order according to P value

qPCR	Gene Title	Symbol	Mean L2R	P value
»	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	0.672	0.0006
	Glutamate receptor, ionotropic, NMDA2C	Grin2c	0.593	0.001
	Glucose phosphate isomerase	Gpi	0.838	0.0011
»	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	0.505	0.0011
	Lactate dehydrogenase B	Ldhb	0.492	0.0011
»	Monoamine oxidase A	Maoa	-0.65	0.0016
»	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	0.837	0.0023
»	Aldolase C	Aldoc	0.498	0.0027
	LIM homeobox protein 1	Lhx1	-1.197	0.0027
	Calmodulin 3	Calm3	0.755	0.0035
	Glutamate receptor, ionotropic, AMPA2	Gria2	1.823	0.0045
»	Dopamine receptor D1 interacting protein	Drd1ip	0.95	0.0047
»	Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	0.847	0.0051
	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	Camkk1	0.61	0.0052
	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	Grin1	0.472	0.0052
»	Guanine nucleotide binding protein, alpha stimulating, olfactory type	Gnal	-0.64	0.0053
»	Guanine nucleotide binding protein, alpha stimulating, olfactory type	Gnal	-1.033	0.0054
»	Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	Grinl1a	-0.527	0.0055
	Hairy and enhancer of split 6 (Drosophila)	Hes6	1.115	0.0058
	Cholinergic receptor, nicotinic, alpha polypeptide 6	Chrna6	0.588	0.0059
	Syntaxin 1B2	Stx1b2	0.785	0.0059
	Ventral anterior homeobox 2	Vax2	-1.523	0.0059
»	Synaptophysin	Syp	0.365	0.0062
	Triosephosphate isomerase 1	Tpi1	0.292	0.0063
	Enolase 1, alpha	Eno1	0.592	0.0074
	Casein kinase 1, alpha 1	Csnk1a1	-0.258	0.0082
	Syntaxin 11	Stx11	1.382	0.0094
»	Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	0.798	0.0096
	Aquaporin 4	Aqp4	-1.128	0.0097
	Aldolase A	Aldoa	0.567	0.0098
»	Protein kinase C, zeta	Prkcz	0.672	0.0099
	Fatty acid desaturase 1	Fads1	0.588	0.0126
	Transforming growth factor, beta 2	Tgfb2	0.593	0.013
	Aquaporin 3	Aqp3	1.305	0.0156
	Solute carrier family 2, member 5	Slc2a5	0.967	0.0156
	Syntaxin 17	Stx17	1.148	0.0156
	Calcium/calmodulin-dependent protein kinase I	Camk1	-1.548	0.0172
	Casein kinase 1, alpha 1	Csnk1a1	-1.032	0.0173
	Cholinergic receptor, nicotinic, alpha polypeptide 9	Chrna9	1.115	0.0177

qPCR	Gene Title	Symbol	Mean L2R	P value
	Neurotrophic tyrosine kinase, receptor, type 1	Ntrk1	-1.227	0.0209
	Ventral anterior homeobox 2	Vax2	-0.572	0.0211
	Opioid receptor, mu 1	Oprm1	0.99	0.0213
	Adrenergic receptor, beta 2	Adrb2	0.76	0.0215
	Glutamate oxaloacetate transaminase 2, mitochondrial	Got2	0.4	0.0218
	Transforming growth factor, beta 2	Tgfb2	0.278	0.0231
	Glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1 (glutamate binding)	Grina	0.223	0.0254
»	Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	-0.487	0.0257
»	Period 2 homolog	Per2	-1.033	0.0258
	Synaptotagmin XI	Syt11	-0.623	0.0261
	Synaptotagmin III	Syt3	-1.233	0.0264
	Calcium/calmodulin-dependent protein kinase II gamma	Camk2g	-0.688	0.0265
»	Catechol-O-methyltransferase	Comt	0.437	0.0265
	Syntaxin binding protein 1	Stxbp1	-1.538	0.0308
	Solute carrier family 6 (neurotransmitter transporter, dopamine) member 3	Slc6a3	0.65	0.0323
»	Adrenergic receptor kinase, beta 1	Adrbk1	-0.273	0.0326
	Syntaxin 6	Stx6	-0.495	0.0326
	Glutamate receptor, ionotropic, N-methyl D-aspartate 2D	Grin2d	-0.667	0.0331
	Malate dehydrogenase, mitochondrial	Mor1	0.57	0.0333
	Solute carrier family 6 (neurotransmitter transporter, noradrenalin) member 2	Slc6a2	-0.772	0.0336
	Adrenergic receptor, beta 1	Adrb1	-0.628	0.0357
»	Glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	Grinl1a	-1.577	0.0357
	Lactate dehydrogenase A	Ldha	0.387	0.0359
	Syntaxin binding protein 3	Stxbp3	-0.38	0.036
	Striatin, calmodulin binding protein 4	Strn4	0.67	0.0361
	Actin, beta	Actb	-1.307	0.0363
	Arrestin, beta 1	Arrb1	-0.697	0.0373
	Pyruvate dehydrogenase E1 alpha 1	Pdha1	-0.745	0.0375
	Fatty acid desaturase 1	Fads1	-0.817	0.0385
	5-hydroxytryptamine (serotonin) receptor 3b	Htr3b	0.96	0.0387
	Calcium/calmodulin-dependent protein kinase II gamma	Camk2g	-0.392	0.0387
	5-hydroxytryptamine (serotonin) receptor 2A	Htr2a	0.84	0.039
	Nuclear factor, interleukin 3 regulated	Nfil3	0.485	0.039
	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	Gria3	-1.132	0.0422
	Calcium/calmodulin-dependent protein kinase II, alpha	Camk2a	-0.848	0.0423
	Synaptotagmin binding, cytoplasmic RNA interacting protein	Syncrip	0.863	0.0424
	Hexokinase 1	Hk1	0.353	0.0425
	Phosphofructokinase, platelet	Pfkp	0.227	0.0426
	Synaptosomal-associated protein, 91kDa homolog (mouse)	Snap91	-0.375	0.0426
	Glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1 (glutamate binding)	Grina	-0.68	0.043
	Adrenergic receptor, alpha 1a	Adra1a	0.392	0.0477
	Brain derived neurotrophic factor	Bdnf	-0.557	0.048

qPCR	Gene Title	Symbol	Mean L2R	P value
	Striatin, calmodulin binding protein	Strn	1.027	0.0484
	Calmodulin 3	Calm3	-1.163	0.0496
	Pyruvate dehydrogenase E1 alpha 2	Pdha2	0.345	0.0496
»	Vesicle-associated membrane protein 1	Vamp1	0.958	0.0497

Conventions same as Table 1.

Table III

Significantly changed epigenetic genes in PCB-exposed SD rats and SHR rats.

Gene Title	Symbol	PCB-SD Mean L2R	PCB-SD P value	SHR Mean L2R	SHR P value
CREB binding protein	Crebbp	-0.83	0.0024	-1.46	0.0012
DNA methyltransferase 3A	Dnmt3a	-1.39	0.0025	-0.42	0.2155
Histone deacetylase 5	Hdac5	0.96	0.0035	-1.09	0.0229
Methyl CpG binding protein 2	MeCp2	-0.48	0.1916	-1.86	0.0227

Conventions same as Table 1.

Table IV

Genes examined by qRT-PCR in the SD_PCB vs SD rat brain regions.

	Microarray Data						Real-time PCR Data*					
Symbol	**DS**	**Hipp**	**PFC**	**SN-VTA**	**VS**	**Vermis**	**DS**	**Hipp**	**PFC**	**SN-VTA**	**VS**	**Vermis**
Adrbk1	0.74	1.12	0.65	0.92	0.48	0.76	-0.24	-0.67	-1.39	-0.33	-0.19	-1.35
Adrbk1	0.55	1.15	0.31	0.86	0.86	0.98						
Arrb2	0.87	0.93	1.17	0.74	0.82	0.93	1.34	1.64	1.41	1.29	1.91	-0.74
Comt	4.04	3.86	4.01	4.08	3.86	2.35	2.06	1.95	1.52	2.65	2.17	1.59
Gnal	1.01	0.85	0.94	0.64	0.95	1.06	1.59	1.40	0.98	1.46	2.11	1.06
Gnal	1.06	0.93	0.42	0.33	0.9	0.79						
Ntrk2	-0.36	-0.46	-0.89	-0.66	-0.84	-0.66	1.73	2.35	1.50	2.00	2.12	1.11
Syt1	-1.93	-1.33	-1.62	-0.87	-0.97	-0.69	0.91	1.20	0.65	0.47	1.13	0.81

All differences are reported in log2 scale and reflect the change in the PCB-exposed SD rats compared to SD rats.

qPCR values are based on the relative differences in the cycle to threshold for the target gene and 18S RNA.

Genes appearing in multiple rows were interrogated by more than 1 probeset on the RAE230 GeneChip.

* $p<0.05$ for one-tail T-test.

DS, dorsal striatum; Hipp, hippocampus, PFC, prefrontal cortex; SN-VTA, substantia nigra-ventral tegmental area; VS, ventral striatum

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Table V

Genes examined by qRT-PCR in the SHR vs WKY rat brain regions.

	Microarray Data						Real-time PCR Data					
Symbol	**DS**	**Hipp**	**PFC**	**SN-VTA**	**VS**	**Vermis**	**DS**	**Hipp**	**PFC**	**SN-VTA**	**VS**	**Vermis**
Adrbk1	-0.32	-0.29	-0.17	-0.5	-0.05	-0.31	0.54	0.46	0.70	0.12	0.66	0.05
Aldoc	0.45	0.46	0.53	0.36	0.52	0.67	0.21	0.01	-0.15	-0.34	0.14	0.45
Comt	0.52	0.17	0.63	0.24	0.73	0.33	-0.06	0.26	-0.27	0.09	0.03	0.16
Drd1ip	0.95	0.99	0.8	1.24	1.14	0.58	0.33	0.22	0.20	0.73	0.72	0.42
Gapdh	0.7	0.63	0.7	0.69	0.77	0.54	0.40	0.34	0.43	0.48	0.53	0.28
Gapdh	0.57	0.41	0.53	0.42	0.53	0.57						
Gapdh	0.91	0.8	0.91	0.67	1.08	0.65						
Gapdh	0.47	0.71	0.98	0.85	1.03	1.04						
Gnal	-0.73	-0.61	-0.75	-0.29	-0.8	-0.66	-0.15	-0.28	0.22	-0.13	0.20	0.12
Gnal	-0.94	-1.09	-0.86	-0.77	-0.93	-1.61						
Grinl1a	-0.6	-0.53	-0.57	-0.7	-0.51	-0.25	0.84	0.75	0.54	0.49	0.84	0.01
Grinl1a	-1.8	-2.27	-0.52	-2.03	-0.35	-2.49						
Maoa	-0.76	-0.62	-0.76	-0.72	-0.49	-0.55	0.05	-0.13	-0.52	-0.06	0.02	-0.02
Ntrk2	1.1	0.94	0.31	1.03	0.72	0.69	0.49	0.58	0.95	0.67	0.80	0.25
Ntrk2	-0.32	-0.39	-0.81	-0.19	-0.47	-0.74						
Per2	-0.39	-1.32	-0.36	-1.25	-1.52	-1.36	0.02	-0.17	-0.04	0.36	-0.04	0.34
Prkcz	0.87	0.74	0.74	0.66	0.82	0.2	0.42	0.38	0.20	-0.39	0.44	0.06
Syp	0.52	0.24	0.26	0.4	0.46	0.31	-0.25	0.68	1.26	-0.43	0.80	1.18
Vamp1	1.31	1.76	1.27	0.53	-0.07	0.95	-0.24	-0.01	1.19	-0.10	0.49	0.02

Conventions same as Table 4.