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PRENATAL EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE ALTERS GENE EXPRESSION IN THE DEVELOPING MURINE HIPPOCAMPUS

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Abstract

Background—Little is known about the effects of passive smoke exposures on the developing brain.

Objective—The purpose of the current study was to identify changes in gene expression in the murine hippocampus as a consequence of *in utero* exposure to sidestream cigarette smoke (an experimental equivalent of environmental tobacco smoke (ETS)) at exposure levels that do not result in fetal growth inhibition.

Methods—A whole body smoke inhalation exposure system was utilized to deliver ETS to pregnant C57BL/6J mice for six hours/day from gestational days 6–17 (gd 6–17) [for microarray] or gd 6–18.5 [for fetal phenotyping].

Results—There were no significant effects of ETS exposure on fetal phenotype. However, 61 “expressed” genes in the gd 18.5 fetal hippocampus were differentially regulated (up- or down-regulated by 1.5 fold or greater) by maternal exposure to ETS. Of these 61 genes, 25 genes were upregulated while 36 genes were downregulated. A systems biology approach, including computational methodologies, identified cellular response pathways, and biological themes, underlying altered fetal programming of the embryonic hippocampus by *in utero* cigarette smoke exposure.

Conclusions—Results from the present study suggest that even in the absence of effects on fetal growth, prenatal smoke exposure can alter gene expression during the “early” period of hippocampal growth and may result in abnormal hippocampal morphology, connectivity, and function.

Keywords

mouse; fetus; cigarette; sidestream smoke; hippocampus; microarray; environmental tobacco smoke; gene expression

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The authors declare that there are no conflicts of interest.

1. INTRODUCTION

Despite worldwide attention highlighting the deleterious consequences of cigarette smoking on overall health and reproduction, over 20% of adults in the United States smoke [1]. It has been estimated that one third to one half of all pregnant women are exposed to cigarette smoke via passive or involuntary means in their homes, in public, or in the workplace [2–4]. Moreover, 43% of children in the United States −2 months to 11 years of age – live in homes with at least one smoker, rendering cigarette smoke exposure a significant environmental hazard for this cohort [5,6].

'Passive' smoking refers to exposure to environmental tobacco smoke (ETS), also known as secondhand smoke. ETS is comprised of the smoke released from the end of the smoldering cigarette and the portion of the mainstream smoke that is exhaled by the smoker. It is a complex mixture of over 4,000 compounds, many of which, such as nicotine, arsenic, and lead are possible human teratogens [7–9]. While numerous components of cigarette smoke are present in lower concentrations in sidestream smoke than in mainstream smoke, incomplete combustion of tobacco products from the smoldering end of the cigarette results in the release of higher concentrations of toxic constituents such as nitrosamines and formaldehyde [10–12].

Adverse pregnancy outcomes such as fetal growth restriction, increased rates of spontaneous abortion, premature placental abruption, perinatal lethality, congenital malformations, and cognitive impairments have been linked to maternal smoking during pregnancy [13–18]. While the deleterious consequences of maternal smoking on infant development have been well documented, less is known about the effects of ETS on developmental outcomes. Nevertheless, increased risk for respiratory illnesses, sudden infant death syndrome, middle ear disease, low birth weight and long-term cognitive and behavioral deficiencies have been linked to ETS exposure [19,20].

An extensive body of literature supports the notion that the brain, particularly regions associated with learning and memory, is a developmental target for the constituents of cigarette smoke. For example, prenatal nicotine exposure of rodents resulted in decreases in cell size, cell layer thickness, and cell density as well as alterations in dendritic morphology in the hippocampus [21,22]. In addition, long-term impairments in attention, learning and memory follow developmental exposures to nicotine [23–28]. Moreover, prenatal carbon monoxide exposure in rodents has also been shown to disrupt long-term potentiation and alter hippocampal-dependent behaviors [29,30].

Preliminary microarray analysis of whole brain tissue collected from murine fetuses exposed to sidestream smoke during gestational days 6–18.5 (gd 6–18.5), revealed significant alterations in gene expression profiles when compared to controls[55]. Included among the categories of genes whose expression was differentially altered by smoke exposure were those known to be involved in neurodevelopmental, neuro-behavioral, and cognitive processes. These data support the notion that subtle smoke-induced changes in the developing fetal brain can result in long-term behavioral and cognitive deficits. The purpose of the current study was to identify changes in gene expression in the fetal mouse hippocampus following *in utero* exposure to sidestream cigarette smoke, a model of ETS exposure.

2. MATERIALS AND METHODS

2.1 Experimental Animals

C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME), were maintained in the American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility at the University of Louisville on a 12-hour light/dark cycle and *ad libitum* food and water. Timed pregnancies were obtained by overnight mating of a single mature male with two nulliparous females. The presence of a vaginal plug was considered to be evidence of mating and the time designated as gestational day 0 (gd 0). Pregnant mice were exposed to sidestream cigarette smoke (CSE) or ambient air (sham-exposed controls) for six hours per day on either gd 6–17 [for array] or gd 6–18.5 [fetal assessment]. Dams were weighed daily and monitored for overt signs of toxicity including weight loss, moribundity, mortality, ruffled fur, reluctance to ambulate, and chromodacryorrhea. Animals (dams and corresponding litters) were excluded from fetal outcome analysis if the dam was not pregnant (3 Sham and 1 CSE) or if the dam gave birth early (1 CSE).

On gd 18.5, dams were anesthetized with Avertin (500 mg/kg, i.p.; Aldrich; (2,2,2 – tribromoethylalcohol; St. Louis, MO) [31] and then euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Uteri were exteriorized and the number and location of implantations were recorded. Fetuses were then removed and the following outcomes were assessed: number of viable fetuses, fetal weight, number of abnormalities, and fetal crown-rump length. For microarray analysis, fetal brains were harvested**,** hippocampal tissue microdissected (Figure 1) pooled by litter, and processed as described below.

2.2 Whole Animal Inhalation System

A Teague TE-10C whole body smoke inhalation exposure system (Teague Enterprises; Davis, CA) was used to generate and deliver sidestream cigarette smoke. The Teague, TE-10C [32] is a microprocessor controlled instrument that produces sidestream smoke or a combination of mainstream and sidestream smoke. Dams were exposed to sidestream cigarette smoke generated from Philip Morris Marlboro Red cigarettes™ (Philip Morris; Richmond, VA; 15 mg of tar/cigarette; 1.1 mg nicotine/cigarette; additives). Philip Morris Marlboro Red cigarettes™ were selected because they represent the most popular brand of cigarettes consumed among 18–25 year olds, the age group containing the majority of maternal smokers [33]. The cigarettes were stored at 4°C until 48 hours prior to use when they were brought to a relative humidity of 60%. Cigarettes were smoked using the standard Federal Trade Commission method: a two second, 35 cm^3 puff, once a minute for a total of nine minutes [32]. For quality control purposes, paired exposure chambers (one receiving cigarette smoke and one receiving ambient air [sham]) were characterized twice during each daily exposure session for: total suspended particulates (TSP), temperature, carbon monoxide levels, and humidity. Tail blood was collected from each dam immediately following the six-hour exposure session on gestational day 6, 9, 12, and 15 for determination of plasma cotinine levels (described below).

2.3 Cotinine Assay

Cotinine, the principal metabolite of nicotine, is a well-documented marker of active tobacco smoking and passive/environmental tobacco smoke exposure [34]. Cotinine concentrations can be reliably measured in blood, urine, and hair [35–37]. At designated time points, tail blood was collected from each dam and plasma cotinine concentrations were determined utilizing a Cotinine One-Step ELISA Detection Kit (International Diagnostic Systems; St. Joseph, MI). In brief, 20 μl of plasma or cotinine standard was added to cotinine antibody-coated microtiter plates. Enzyme conjugate (100 μl) was added to each well and following a 30-minute incubation at room temperature the solution was removed and the plate washed. The substrate,

tetramethylbenzidine, (150 μl) was added to each well and the plate further incubated at room temperature until the wells developed a medium blue color. The reaction was terminated by adding 150 μl of stop solution (3 N sulfuric acid) to each well and the absorbance was read at 450 nm. Cotinine concentrations were quantified using a standard curve (0–50 ng/ml) and the data reported as mean ng/ml cotinine ± standard error of the mean.

2.4 Isolation of RNA from Fetal Hippocampal Tissue

Total RNA was isolated from fetal hippocampal samples using the RNeasy Protect Mini Kit (Qiagen; Valencia, CA) following the manufacturer's recommendations. The quality and quantity of the extracted total RNA were assessed by UV Absorbance (NanoDrop® ND1000 Spectrophotometer v3.1.2, NanoDrop Technologies, Wilmington, DE) and the RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA). Hippocampal RNA was isolated from three sham-exposed and three sidestream cigarette smoke-exposed litters and each sample was applied to an Affymetrix high-density mouse genome 430 2.0 GeneChip® array.

2.5 cDNA Target Synthesis, Biotin-Labeling of cRNA, and GeneChip® Hybridization

Double stranded cDNA was prepared using the GeneChip® One cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA). In brief, 5 μg of total RNA was denatured and annealed to 2 μl (50 μM) T7- Oligo-dT primer for 10 minutes at 70°C. The reaction was cooled to 4 °C in a thermal cycler and the RNA reverse transcribed for 1 hour by adding 1 μl Superscript II at 42 °C in reaction buffer containing, 10 mM dithiothreitol, and 0.5 mM dNTP mix, in a total volume of 27 μl. Second-strand cDNA was synthesized by adding 4 μl DNA polymerase I, 1 μl *E. coli* DNA ligase, 1 μl RNase H, 30 μl 5X second-strand buffer, 3 μl 10 mM dNTP mix, and water to a total volume of 130 μl and incubating for 120 min at 16 °C. Subsequently, 2 μl of T4 DNA polymerase was added and the incubation continued at 16 °C for 5 additional minutes. Second-strand cDNA synthesis was stopped by the addition of 10 μl of 0.5 M EDTA. The resulting double stranded cDNA was purified using the cDNA clean-up module (Affymetrix).

Twelve microliters (12 μl) of double stranded cDNA was *in vitro* transcribed using a GeneChip® Expression 3′-Amplification Kit (Affymetrix) with biotinylated CTP and UTP according to the manufacturer's instructions. Following a 16-hour incubation at 37°C, the resultant biotin-labeled cRNA was purified with the cRNA clean-up module (Affymetrix) and eluted in 21 μl of RNase-free water. The concentration of biotin-labeled cRNA was assessed by UV Absorbance (NanoDrop® ND1000 Spectrophotometer v3.1.2, NanoDrop Technologies, Wilmington, DE) and the RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA). Twenty micrograms (20 μg) of labeled cRNA was then fragmented in 40 μl 1X fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM potassium-acetate, 30 mM magnesium-acetate) for 35 min at 94°C and the effectiveness of fragmentation assessed by agarose gel electrophoresis. Fragmented cRNA was brought to a total volume of 300 μl with 1X hybridization buffer (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01% Tween 20), 100 μg/ml herring sperm DNA, 500 μg/ml acetylated BSA, 50 pM biotinylated control oligonucleotide B2 and 1X eukaryotic hybridization controls. cRNAs derived from fetal hippocampal tissue exposed to either sham or sidestream smoke conditions were hybridized for 16 hours to an individual GeneChip® from an identical lot of Affymetrix Mouse Genome 430 2.0 GeneChip® arrays. These arrays contain 45,101 genes and EST probes sets. GeneChip arrays were then washed and stained using antibody-mediated signal amplification and the Affymetrix Fluidics Station's standard Eukaryotic GE Wash 5′ protocol.

2.6 Reduction, Statistical Analysis, and Biological Interpretation of Microarray Data

Individual GeneChip® arrays were scanned with the GeneChip® Scanner 3000 (Affymetrix) and images were processed using Affymetrix GCOS 1.2 software, according to Affymetrix protocols. The CEL files, containing individual raw chip data (probe intensities), were imported

into GeneSpring GX 7.3.1 (Silicon Genetics, Inc.; Redwood City, CA) and pre-processed using Robust Multi-chip Average, with GC-content background correction (GC-RMA). The GC-RMA processed data were further normalized by the 'per gene normalization' step in which all the samples were normalized against the median of the control samples (i.e. the expression value for a gene across the different conditions is centered on 1. This is done by dividing the expression value of a given gene, by the median expression values for that gene across the conditions). This ensured that genes whose expression did not change across treatment conditions received a normalized expression value of 1, allowing for easy visual detection of differentially expressed genes. To define a set of statistically significant, differentially expressed genes, a one-way ANOVA (parametric test, assuming equal variances) was applied using the "Benjamini and Hochberg false discovery rate" as the multiple testing correction ($p = 0.05$). This restriction tested each of the 45,101 genes and ESTs on the microarray and generated a list of 3,109 genes with statistically significant expression values. A fold change "filter" (probes with fold differences ≥1.5 were considered significant) was then applied to the list of 3,109 genes. The gene/EST list resulting from this analysis includes those whose expression is either ≥1.5-fold up- (25 genes and ESTs) or down-regulated (36 genes and ESTs) in a statistically significant manner ($p < 0.05$) as a function of the treatment condition (sham versus sidestream smoke-exposed). Hierarchical clustering analysis was then performed using the GeneSpring 7.2 software (Silicon Genetics, Inc., Redwood City, CA) to generate a "condition tree" representing the functional category of genes based on their expression profile. A heat map (Figure 2) was generated by dividing each measurement by the 50th percentile of all the measurements in that sample. The average value of expression for each gene across the samples was then set to 1.0 and the normalized signal value for each sample was plotted (values less than 0.01 were set to 0.01). In order to establish an overview of different biological pathways impacted by *in utero* sidestream cigarette smoke, computational gene interaction predictions were made using Ingenuity Systems Pathway Analysis (Ingenuity Systems, Mountain View, CA; http://www.ingenuity.com). Several downregulated and upregulated genes from the study were used to construct gene association maps for predicting the effects of sidestream smoke on various cellular and molecular processes in the fetal mouse hippocampus.

2.7 Quantitative Real-Time PCR

Quantitative Real-Time PCR analysis was performed using an ABI Prism 7000 Sequence Detector System (Applied Biosystems; Foster City, CA). Primers and their corresponding fluorescent probes for each of the genes that were examined were purchased from Applied Biosystems. In all cases, both forward and reverse primers were used at a concentration of 900 nM, while the concentration of the probe was 250 nM. For the PCR reaction, 1 pg of cRNA template was mixed with 0.2 mM each of dATP, dCTP, and dGTP, 0.4 mM dUTP, and 0.625 units of AmpliTaq Gold (Applied Biosystems) in a final volume of 25 μl. Cycling parameters were as follows: 50°C for 2 min for probe and primer activation, 95°C for 10 min of DNA strand denaturation, followed by 40 cycles of denaturation at 95°C for 15 s and primer extension at 60°C for 1 min. Raw data were acquired and processed with ABI Sequence Detector System software, v1.0 (Applied Biosystems; UK). Each cDNA sample was tested in triplicate and mean Ct values are reported. For each cDNA template reaction, a parallel reaction lacking template was performed as a negative control. Each determination of mRNA amount for the genes analyzed was normalized to GAPDH mRNA present in the sample by using TaqMan™ GAPDH PCR primers and probe.

2.8 Statistical Analysis

Outcome data were analyzed using a mixed-model ANOVA followed by t-tests with the Statistical Package for Social Sciences© version 14.0 (SPSS; Chicago, IL). Since the dam was

the treated entity in the conducted studies, the litter rather than individual fetuses was regarded as the experimental unit [38]. Statistical significance was assigned a $p < 0.05$.

3. RESULTS

3.1 Exposure Conditions and Cotinine Levels

Chamber conditions (total suspended particulates, carbon monoxide levels, temperature, and humidity) were measured daily for quality control purposes and are reported in Table 1. Under the experimental paradigm utilized, the mean total suspended particulates (TSP) in the sidestream smoke chamber was 5.9 ± 0.2 mg/m^3. Measured TSP resulted in exposure levels where dam plasma cotinine levels approximated those of pregnant women who are 'passively' exposed to cigarette smoke [34,39]. In order to monitor cigarette exposure levels, tail blood was collected for cotinine determination by ELISA following exposures on gd 6, 9, 12, and 15. Average maternal cotinine levels in dams exposed to sidestream smoke were 35.3 ± 3.5 ng/ml.

3.2 Fetal Outcomes

In an effort to avoid any confounding effects that exposure to cigarette smoke might have on murine embryo implantation, which is typically completed by gd 5 [40–42], dams were exposed beginning on gd 6 and continued throughout gestation. This exposure period represents post-implantation development and includes organogenesis, late term growth, and early hippocampal neurogenesis [43–45]. Notably, no dams died as a result of the exposure regimen, nor were there any observed signs of maternal toxicity.

Fetal outcomes are shown in Table 2. Exposure to sidestream smoke resulted in no observable effects on dam weight gain, fetal viability, or fetal morphometric measures. One fetus from a litter designated for array analysis, and exposed to sidestream cigarette smoke, exhibited micrognathia and cleft palate. This fetus was excluded from hippocampal tissue analysis.

3.3 "Transcriptional Profiling" of the Fetal Mouse Hippocampal tissue

Of the 45,101 gene/EST probes on the Affymetrix high-density GeneChip® array, 3,109 demonstrated a detectable level of expression. Results indicated that 61 genes were differentially expressed in the hippocampus following *in utero* sidestream smoke exposure with 25 genes/ESTs being upregulated at least 1.5 fold and 36 genes/ESTs being down-regulated 1.5 fold or greater (Table 3). As shown in the heat map in Figure 2, expression of these genes was reproducible in the three biological samples taken from each treatment condition (smoke- and sham-exposed). Extensive literature mining of the differentially expressed genes identified numerous functional categories including cell cycling, neuronal transmission, neurogenesis, synaptogenesis, cellular organization, ion transport, lipid metabolism, protein metabolism/modulation, signal transduction, transcription/translation, and platelet aggregation. Figures 3 and 4 depict the functional categorization of genes differentially expressed within the hippocampus following sidestream smoke exposure during gestation.

3.4 Identification of Relevant Biological Networks for Differentially Expressed Genes within the Hippocampus as a result of in utero ETS Exposure

To determine how the differentially expressed genes might interact in the ETS-exposed hippocampus, an Ingenuity Pathway Analysis (IPA; Ingenuity Systems) was performed using the Ingenuity Systems program at www.ingenuity.com. IPA employs a knowledge base program to generate relevant biological networks. Four major networks (containing many genes already known to be expressed in the hippocampus) were discovered in the differentially-

expressed gene list. All of these networks have significant p-values, indicating that it is highly implausible that these networks were detected by chance. Three of the four highest scoring networks (1st Network with p-value 1.0E-25; Fig. 5A; 2nd Network with p-value 1.0E-21; Fig. 5B; and 3rd Network with p-value 1.0E-20; Fig. 5C) are presented as examples in Figure 5. The pathway analysis data suggest that novel, differentially expressed genes (identified in the present gene expression profiling study) that cluster in these networks (containing several known hippocampus-expressed genes) may be essential for sustaining key hippocampal functions.

3.5 TaqMan ™ Quantitative Real Time PCR Verification of Microarray Results

Twelve genes were randomly selected from the list of upregulated and downregulated genes (four upregulated and eight downregulated) and their expression patterns determined by quantitative real-time PCR using diluted cRNA as template. The overall expression profiles of the twelve mRNAs tested were found to be in agreement with the GeneChip microarray results (Table 4).

4. DISCUSSION

The purpose of the current study was to identify changes in gene expression in the murine hippocampus as a consequence of *in utero* exposure to sidestream cigarette smoke (an experimental equivalent of environmental tobacco smoke (ETS)). Exposures to ETS, also called secondhand smoke, are estimated to affect 60% of non-smokers [46]. These involuntary exposures pose a significant threat to the health of children and the developing fetus [47–50]. Cigarette smoke exposure has been shown to adversely affect implantation rates, [40,51,52] and exposure to low levels of cigarette smoke, typically through passive or involuntary means, has been linked to a number of adverse developmental outcomes [13–16,18,48–50]. Deleterious effects associated with exposure to ETS during development include impaired cognitive development and reduced vocabulary abilities in children [18,53,54]. Constituents of smoke exposure have been shown to target the developing brain [21,22,28,30]. Recent work within our laboratory has demonstrated significant changes in gene expression in the murine fetal brain following exposure to sidestream cigarette smoke during prenatal development [55]. In an attempt to better understand how the exposure to ETS during development modifies gene expression within the hippocampus, a brain region known to play a key role in learning and memory, microarray analysis was employed on hippocampal tissue isolated from fetuses exposed to ETS *in utero*.

As a consequence of exposure to ETS, expression of a number of genes regulating key processes within the hippocampus such as synaptic function, axon guidance, neurogenesis, and cell survival were significantly altered (Table 3 & Fig 5). These findings support the notion that prenatal exposure to sidestream smoke may negatively influence postnatal learning and memory tasks.

Serotonin and serotonergic innervations play a key role in central nervous system (CNS) development. Regulation of serotonin levels is, driven, in part, by serotonin uptake transporters, known as SERTs [56]. Serotonin has also been found in non-neuronal cells including platelets and in the placenta [57,58]. Altered platelet serotonin uptake and metabolism have been associated with Down Syndrome (DS), and SERT expression has been shown to be significantly increased in samples taken from the cortex of DS patients [59]. Increased SERT expression would result in altered serotonin signaling in the cortex of DS patients leading to abnormal neurogenesis (particularly within the cortex) which might be one of the responsible factors for learning disabilities seen in these patients [60]. Our results revealed a significant upregulation of the gene (*slc6a4*) encoding SERT**,** suggesting alteration of the serotonergic system in the hippocampus of mouse embryos following ETS exposure (Table 3). Since

serotonin has been reported to reduce apoptosis during neurogenesis [61], ETS exposure may result in SERT-induced hippocampal apoptosis. Thus, altered serotonergic signaling may be hypothesized as contributing to ETS-associated impaired cognitive development.

The Klarsicht/ANC-1/Syne homologue (KASH)-domain-containing proteins execute key functions in nuclear positioning during various cellular and developmental processes [62]. Syne-1, Syne-2 and Nesprin-3 belong to the Nesprin family of proteins which mediate nuclear membrane localization, and binding to actin [63]. Syne-1 is expressed in multiple tissues, including the CNS [64]. Mutations in *SYNE1* gene have been associated with autosomal recessive cerebellar ataxia type 1 [65]. Interestingly, nicotine has been reported to induce ataxia [66]. In the current study, significant upregulation of the *Syne1* gene was observed in the hippocampus of mouse embryos following ETS exposure (Table 3). It can thus be postulated that *in utero* ETS exposure may render the embryos susceptible to ataxia and related neuropathies through enhanced hippocampal expression of *Syne1* in conjunction with the ataxia-inducing effect of nicotine.

Several low fidelity mammalian polymerases, including Pol z, Pol h, Pol i, Pol k, and Rev1, function to evade unrepaired DNA lesions that otherwise inhibit replication by normal polymerases [67]. Pol-k is one such polymerase that is highly inaccurate when replicating undamaged DNA [68]. Indeed, transient expression of *PolK* in cultured mouse cells significantly increased the incidence of specific mutations [69]. Because of the inaccuracy of PolK in replicating undamaged DNA, overexpression may contribute to defective cell cycle control leading to anomalous embryogenesis [70]. Markedly enhanced expression of *Polk* within the hippocampus of *in utero* ETS exposed embryos (Table 3) may therefore lead to abnormal hippocampal neurogenesis via aberrant cell cycle control or anomalies in genome stability pathways, eventually affecting long-term behavioral and cognitive function.

It is becoming increasingly apparent that insulin and insulin-like growth factor-I (IGF-I) play previously unrecognized crucial roles in the developing and adult brain where they promote myelinization of neuronal axons, contribute to the formation, maintenance and repair of synaptic networks, and are involved in learning and memory, cell survival, neurogenesis and longevity [71,72]. Moreover, stimulation of neuronal nicotinic acetylcholine receptors (nAChRs) through exposure to nicotine resulted in time- and dose-dependent upregulation of insulin receptor substrate (IRS)-1 and IRS-2 mRNAs and proteins and enhanced insulin-induced activation of PI3K and ERK pathways [71]. This highlights the importance of the IRS family of proteins in regulating synaptic plasticity, learning, memory, and cell survival. Thus, significantly enhanced expression of the genes encoding Irs-4 and Insulin-like growth factor 2 mRNA binding protein 1 (Igf2bp1) in the hippocampus of mouse embryos following gestational exposure to ETS (Table 3), may result in functional compromise of hippocampal neuronal nicotinic acetylcholine receptors resulting in long-term cognitive impairments.

Apolipoprotein D (apoD), a member of the lipocalin family, is generally found in plasma associated with plasma high density lipoproteins (HDL) [73,74]. While tissue distribution of apoD mRNA is species-specific, all species investigated exhibit strong expression in the central and peripheral nervous systems [75]. It has been observed that apoD levels were significantly increased in the hippocampus and cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) [76]. Alterations in apoD expression within the brain have also been reported in a number of neurodegenerative diseases as well as patients with schizophrenic and bipolar disorders [77]. Exposure to cigarette smoke has also been reported to affect apoD expression [78]. Collectively, these findings suggest that altered apoD levels in neural tissues, including the hippocampus, could be considered as a marker of a range of neuropathologies associated with prenatal tobacco smoke exposure. Thus, significantly downregulated expression of *ApoD* in the embryonic hippocampal tissue observed in the present gene expression profiling

study (Table 3), could be a contributing factor to generation of neuropathologies associated with *in utero* ETS exposure.

Epigenetic modifications of DNA or associated proteins are essential in controlling key processes such as cellular differentiation, development and behavior – including learning and memory [79]. Dysregulation of the epigenome has been widely accepted as a contributing factor towards the emergence of a range of neurodevelopmental disorders [80]. Inhibitors of class 1 HDACs (HDACs 1, 2, 3 and 8) and class 2 HDACs (HDACs 4, 5, 6, 7, 9 and 10) have been considered as targets for therapeutic intervention of neurodegenerative diseases and cognitive deficits associated with scores of neurodevelopmental disorders [80–82]. HDAC2 is highly expressed in the hippocampus and is also, a developmental target for the constituents of cigarette smoke [21,22]. We demonstrate, in the present study, that *Hdac2* expression is significantly diminished in the hippocampus of murine embryos exposed *in utero* to ETS (Table 3). This observation is supported by the study of Yang et al. [83] which demonstrated reduced activity and levels of HDAC1, HDAC2, and HDAC3 in a human macrophage-like cell line exposed to cigarette smoke extract. These data implicate various histone deacetylases, including HDAC2, in the pathogenesis of a range of neurodegenerative and neurodevelopmental disorders, and emphasize that constituents present in cigarette smoke can modulate the expression and activity of HDAC2. Further, they highlight a possible linkage between prenatal exposure to cigarette smoke and postnatal development of certain neurodegenerative diseases with associated problems of depression, anxiety and cognitive deficits.

Semaphorins represent a large family of 20 different, secreted, GPI-linked, or transmembrane proteins characterized by the presence of a conserved 500-amino-acid- “Sema” domain at their amino-terminus. Semaphorins bind to two major families of receptors**,** neuropilins and plexins**,** to act as chemorepellents or inhibitors of growth cones [84,85] during regeneration of injured nerve fibers [86] and axon guidance [87]. These proteins are also thought to play a role in cell migration, morphogenesis and angiogenesis, during development [88], as well as contributing to distorted structural plasticity in neurodegenerative disorders such as Alzheimer’s disease [89]. Exposure to psychomotor-stimulant drugs induces changes in the expression of axon guidance molecules such as Sema5A [90] and this may contribute to cognitive deficits associated with drug abuse. Thus, the ETS-induced inhibition of Sema5A expression in the hippocampus (Table 3) could be a factor contributing to aberrant wiring of neural networks and axon guidance within the developing hippocampus leading to long-term postnatal deficiency in learning, memory and cognition.

Several neural-specific RNA-binding proteins (RBPs), such as Musashi1 (Msi1) and Musashi2 (Msi2), affect splicing, transport, translation and stability of target mRNAs [91]. Expression of Msi1 and Msi2 is developmentally regulated and coexpression of Msi1 and Msi2 has been detected in proliferating embryonic pluripotent neural precursors and in precursor cell populations of postnatal and adult CNS stem cells [92–94]. Indeed, it has been suggested that these two proteins contribute to the development and maintenance of CNS stem cells [95]. Inhibition of expression of *msi1* and *msi2* inhibited the ability of CNS stem cells to proliferate and form neurospheres [96]. Generation of specific neural populations takes place primarily in the subventricular zone (SVZ) of the lateral ventricles**,** and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus [97]. Based on enhanced expression of Musashi-1 and -2 in proliferating neural progenitor cells in the brain of patients with subarachnoid hemorrhage**,** Sgubin et al., [98] concluded that Msi-2 may function as an activator of neural progenitor cell proliferation. In the present study, we demonstrate that the gene encoding Msi-2 was significantly downregulated in the embryonic murine hippocampus subsequent to ETS exposure (Table 3). It can thus be hypothesized that diminished expression of the *Msi2* gene may result in altered hippocampal neurogenesis in fetuses exposed (*in utero*) to sidestream

smoke, rendering them susceptible towards various neurological disorders associated with prenatal ETS exposure.

The Ingenuity Pathway Analysis (IPA; Ingenuity Systems) program allows generation of gene networks based on the biological associations of specific genes and various functions controlled by those genes. Based on the differentially expressed genes in the fetal hippocampus subsequent to maternal ETS exposure, pathway analysis revealed three statistically significant networks. One network (Fig. 5A) is noteworthy for including signaling pathways such as axon guidance, ephrin receptor signaling and calcium signaling, all crucial for hippocampal function. Other pathways include those regulating cell cycle and DNA damage, apoptosis, TGFβ-, Wnt/β-catenin-, PI3K/AKT-, Aryl hydrocarbon receptor- and p53- signaling, fatty acid and xenobiotic metabolism, NRF2-mediated oxidative stress and hypoxia-inducible factor signaling (Fig. 5A). Intriguingly, two neurological disease process interactomes, Huntington's disease and Reelin signaling**,** are also present within this network (Fig. 5A). A second network (Fig. 5B) includes pathways crucial for neural cell formation, differentiation, viability, and transmembrane potential, as well as cell adhesion, cell migration, inflammatory response, nitric oxide production, calcium entry, G protein and osmosensory signaling. Similar to network 1, network 2 included the interactomes of two neurological disease process**,** bipolar affective disorder and Prader-Willi syndrome (Fig. 5B). A third network (Fig. 5C) includes pathways instrumental in regulating apoptosis and/or survival of various types of neurons and astrocytes, methylation of DNA, DNA repair and cellular aging. This network also links to a neurodevelopmental disease e.g. Costello Syndrome.

Pathway analysis thus reveals that genes differentially expressed within the hippocampus following gestational ETS exposure, may be functionally associated with several neurodegenerative diseases. Notably, a common theme underlying the pathologies of each of the aforementioned disorders is that the affected individuals have cognitive impairments and hippocampal dysfunction. Types of cognitive deficits common to all these disorders are attention deficits, and loss of short-term memory and sequential information processing. Furthermore, the neuroanatomical abnormalities seen in all such disorders localize to the hippocampus and cerebellum [99]. Collectively, our data strongly support the notion that prenatal ETS-exposure can trigger detrimental changes in normal hippocampal function leading to postnatal behavioral anomalies and cognitive deficits.

In the current study, pathway analysis enabled identification of a range of biological processes that, while indispensable for normal hippocampal function**,** could result in neuropathies when aberrantly regulated. For example, we showed ETS-induced downregulation in the fetal hippocampus of a number of genes that play key roles in cell survival and/or apoptosis (*Tulp4*, *Pla2g6*) [100,101]. Moreover, several p53-, Hras-, β-catenin-, RhoA- and Hydrogen peroxide-mediated neuronal apoptosis and/or cell survival pathways were detected within the networks generated with genes differentially-expressed within the prenatal ETS-exposed hippocampus (Fig 5A, 5B, 5C). Accordingly, these findings point to potential activation of various apoptosis and/or cell survival pathways within the hippocampus as a result of *in utero* sidestream cigarette smoke-exposure.

Several of the genes whose expression was altered in the fetal hippocampus by exposure to cigarette smoke are known to influence proliferation and growth. For example, genes encoding Centrosomal protein 250 (Cep 250) and Centromere-associated protein E (Cenp-E) are known regulators of cellular proliferation, and are significantly downregulated in the prenatal ETS-exposed hippocampus (Table 3). Decreased hippocampal cell proliferation has been linked to neurological diseases such as Huntington's disease [102]. Thus, ETS-induced diminished expression of genes encoding the aforementioned proliferation markers could be associated with abnormal neurogenesis within the developing hippocampus.

The connection between cigarette smoke exposure and DNA damage has been established by a number of studies [103,104]. Our pathway analysis, wherein various p53- and hydrogen peroxide-mediated DNA repair pathways were detected (Fig 5A, 5B, 5C), provides further support for this connection.

Growth inhibition is the single most important factor affecting infant mortality in humans [105]. Maternal smoking decreases infant birth weight by ~150–200 grams and accounts for over 20% of the incidence of low birth weight [50,106,107]. Moreover, exposure to ETS is also associated with reduced birth weight in humans**,** albeit (30–100 g) less than that observed with 'active' maternal smoking [49,108]. We thus anticipated that our experimental paradigm of exposure to low levels of cigarette smoke during gestation would result in fetal growth restriction. However, *in utero* exposure to sidestream cigarette smoke during gd 6–18.5 had no significant effect on fetal weight or crown-rump length. Several studies are in agreement with our data, demonstrating that exposure to ETS during gestation failed to result in decreases in fetal weight [21,109–111]. In contrast, other studies demonstrate a link between exposure to sidestream smoke during gestation and reduced fetal weight [112–114]. These conflicting data may be in part due to differences in exposure parameters, including the period of gestational exposure. In the current study, exposures were begun on gestational day 6, and continued throughout gestation. Although this developmental period encompasses the period of organogenesis [115], it begins after implantation which is completed on gd 5 [40–42]. Recent studies in our laboratory have shown that exposure of murine dams to mainstream/sidestream tobacco smoke during the first five days of gestation (pre/peri-implantation period gd 1–5) resulted in decreases in fetal weight and crown-rump length, suggesting a temporal window of vulnerability for cigarette smoke-induced fetal growth restriction [112]. As women are typically exposed to environmental tobacco smoke both during and prior to pregnancy, the experimental paradigm used in this study may not adequately reveal true exposure risks.

However, it is important to note that even in the absence of observable effects on such phenotypic measures as term fetal-weight, -length and overall morphology, significant changes in the expression of genes associated with the development and function of the hippocampus were observed. These results are consistent with previous reports demonstrating alterations in brain development following prenatal exposure to nicotine, even in the absence of a low birth weight phenotype [22,116]. Collectively, data from the present study raise the possibility that subtle smoke-induced changes in the expression of genes within the developing hippocampus could affect long-term behavioral and cognitive function. Currently, studies are underway in our laboratory to determine whether exposure to low levels of cigarette smoke during gestation, in our animal model of *in utero* ETS exposure, induces permanent gene expression changes in the postnatal animal, and if such changes are accompanied by altered hippocampal-dependent behaviors.

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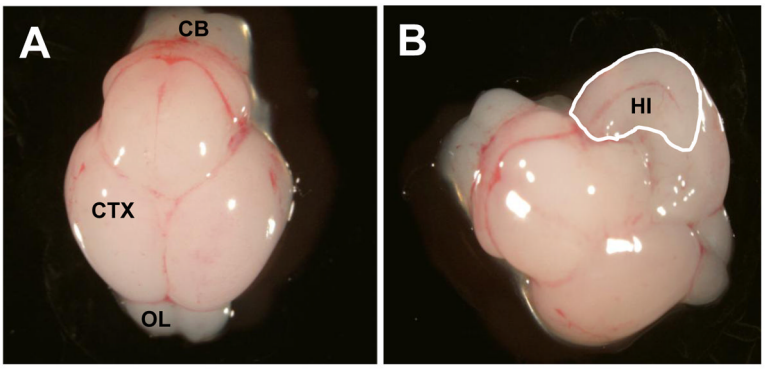


Figure 1.

Photographs of the dorsal view of the gestational day 17 murine brain. **(A)** The intact fetal brain on gestational day 17. **(B)** A unilateral view of the interior portion of the fetal cerebral cortex on gestational day 17. The cerebral cortex was opened along the midline and the hippocampal region was identified, removed and frozen on dry ice. The region demarcated by the white line represents the hippocampal tissue that was bilaterally excised from the cerebral cortex of the gestational day 17 murine fetus for RNA isolation. (CB) Cerebellum; (CTX) Cerebral cortex/Cerebrum; (OL) Olfactory lobes; (HI) Hippocampus.

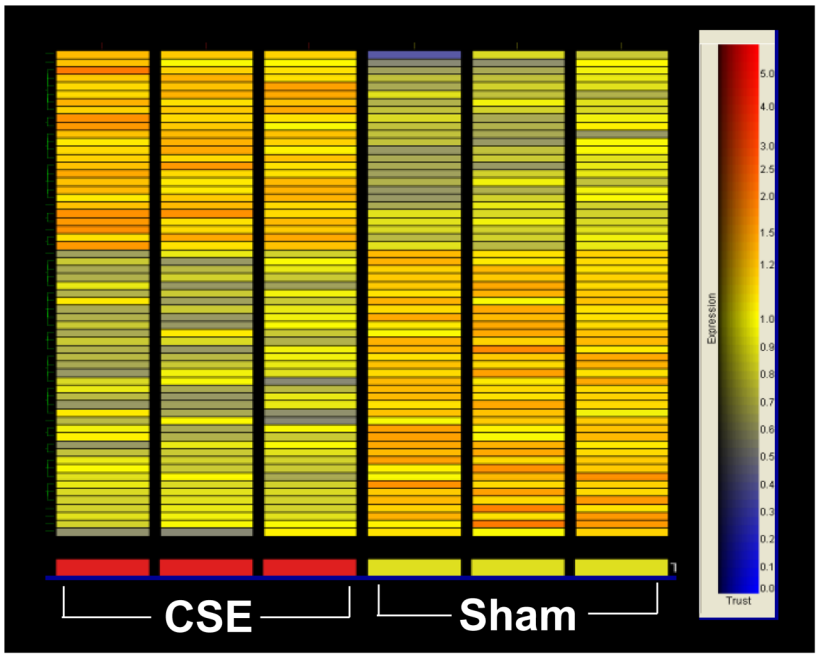


Figure 2.

Heat map illustrating the differentially regulated genes in the murine hippocampus following exposure to sham and sidestream smoke exposure. Each row of the heat map represents a gene while each column represents the experimental treatment (labeled at the bottom). The color saturation scale, shown to the right, represents the level of gene expression, with red indicating an increase in gene expression and blue indicating a decrease in gene expression. Only genes whose expression demonstrated a 1.5 fold or greater increase or decrease are depicted. The list of genes comprising the heat map is found in Table 3.

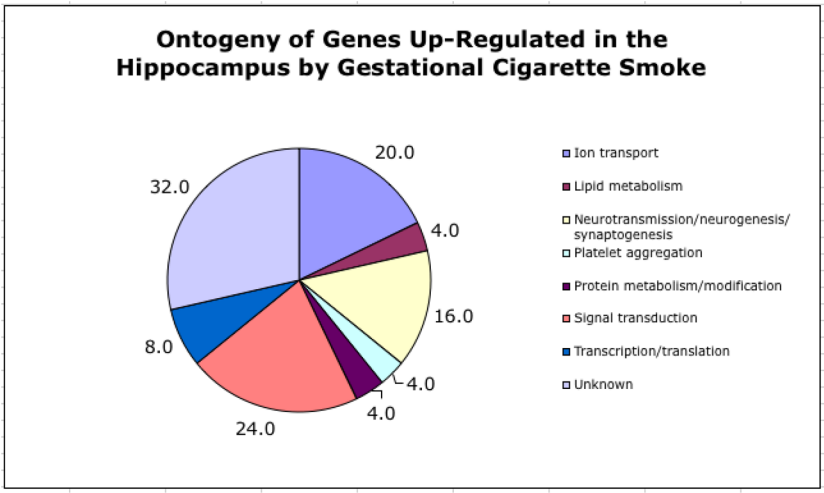


Figure 3.

Ontogeny of up-regulated genes in the hippocampus following exposure to ETS. The pie chart depicts the predicted function of genes that demonstrated an increase in expression in the hippocampus following ETS exposure. Numbers beside each pie segment indicate the percentage of the total number of upregulated genes assigned to a given functional category. Functional gene category assignments were determined through an extensive literature search in PubMed. Note that individual genes may be present in more than one category.

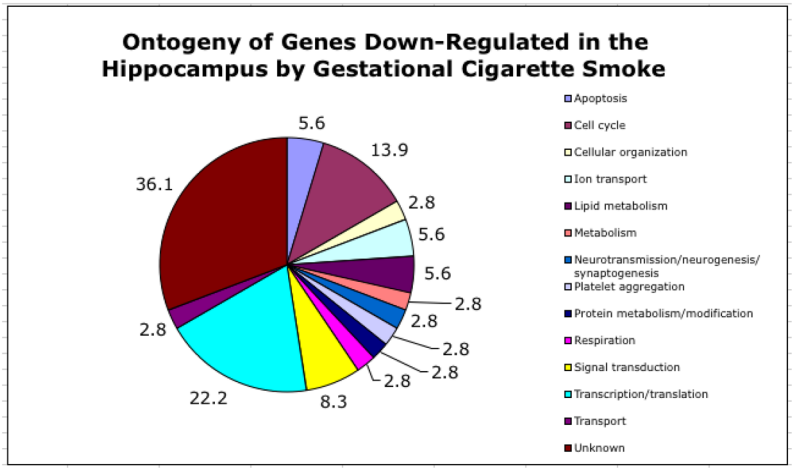
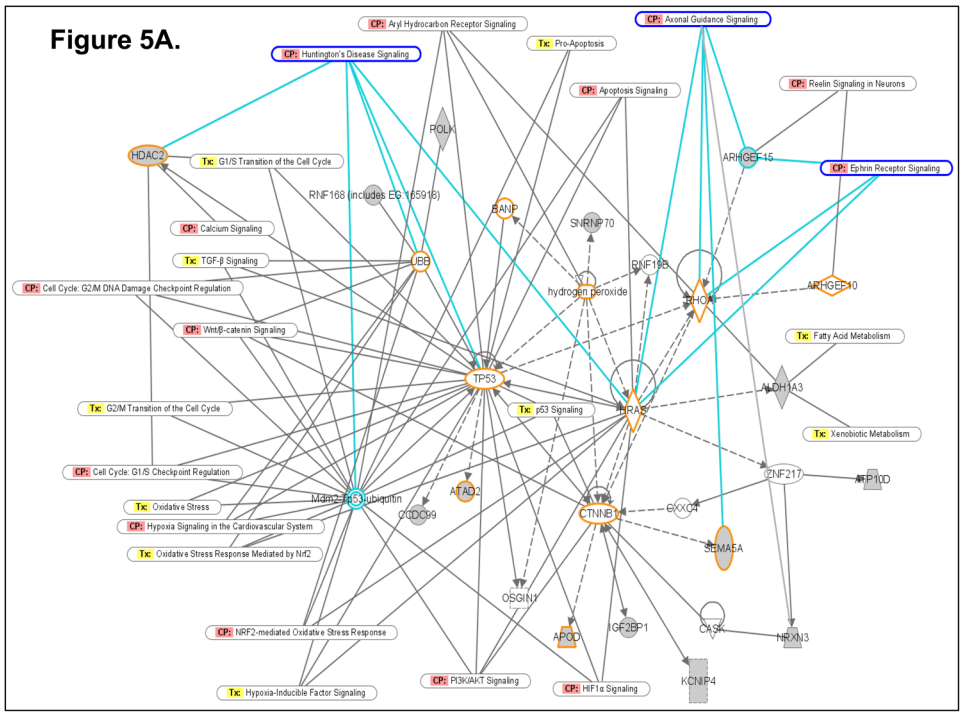
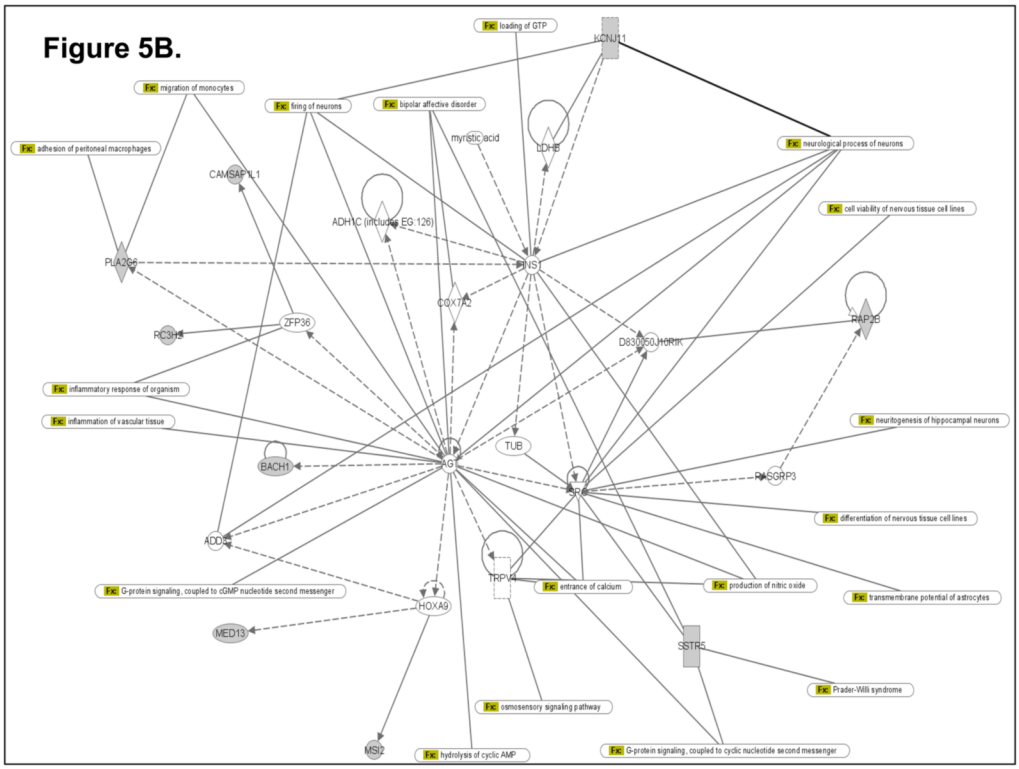


Figure 4.

Ontogeny of down-regulated genes in the hippocampus following exposure to ETS. The pie chart depicts the predicted function of genes that demonstrated a decrease in expression in the hippocampus following ETS exposure. Numbers on each pie segment indicate the percentage of the total number of down-regulated genes assigned to a given functional category. Functional gene category assignments were determined through an extensive literature search in PubMed. Note that individual genes may be present in more than one category.





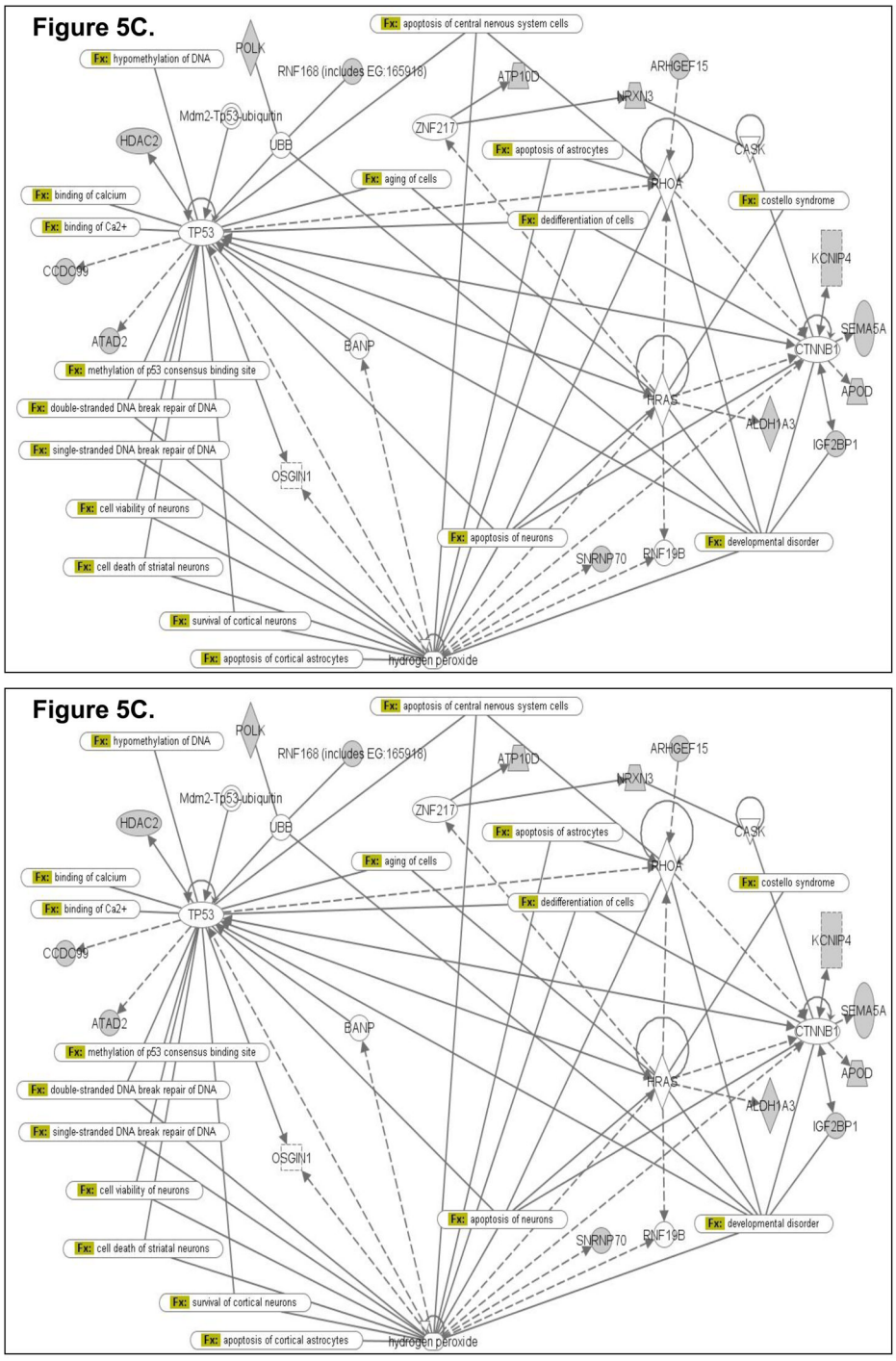


Figure 5C.
Fx: apoptosis of central nervous system cells
Fx: hypomethylation of DNA
POLK
RNF168 (includes EG:165918)
ATP10D
ARHGEF15
NRXN3
Mdm2-Tp53-ubiquitin
ZNF217
HDAC2
UBB
Fx: apoptosis of astrocytes
CASK
Fx: aging of cells
RHOA
Fx: binding of calcium
Fx: costello syndrome
Fx: binding of Ca2+
TP53
Fx: dedifferentiation of cells
KCNIP4
CCDC99
ATAD2
BANP
SEMA5A
CTNNB1
Fx: methylation of p53 consensus binding site
HRAS
APOD
Fx: double-stranded DNA break repair of DNA
ALDH1A3
IGF2BP1
Fx: single-stranded DNA break repair of DNA
OSGIN1
Fx: cell viability of neurons
Fx: apoptosis of neurons
SNRNP70
RNF19B
Fx: developmental disorder
Fx: cell death of striatal neurons
Fx: survival of cortical neurons
Fx: apoptosis of cortical astrocytes
hydrogen peroxide

Figure 5.

Computational gene interaction predictions: selected gene networks (A, B, C) in the fetal hippocampus following ETS exposure. Gene networks were constructed with Ingenuity Systems Pathway Analysis (IPA) software. Several differentially regulated genes from the study were used to construct gene association maps for predicting effects of prenatal exposure to ETS on various cellular and molecular events in the developing mouse hippocampus. The first statistically significant network (Fig. 5A) that was generated, includes p53 tumor

suppressor (TP53), Hras oncogene, β-catenin (CTNNB1) and Ubiquitin-B (UBB), and also consists of several genes such as those encoding HDAC2, Semaphorin-5A, Aldehyde dehydrogenase 1A3 (ALDH1A3), Rho guanine nucleotide exchange factor 15 (ARHGEF15), Apolipoprotein D, Polymerase kappa, and Neurexin-3 (NRXN3) among others, which demonstrated significant differential expression within the hippocampus as a consequence of prenatal ETS exposure (Table 3). The second statistically significant network (Fig. 5B) consists of – Rous sarcoma oncogene (SRC), Angiotensinogen (AGT), Zinc finger protein 36 (ZFP36) and Hoxa9, in addition to a number of genes encoding proteins such as Somatostatin receptor 5 (SSTR5), Potassium inwardly rectifying channel, subfamily J, member 11 (KCNJ11), Musashi-2 (MSI2), and Phospholipase A2, group VI (PLA2G6) among others, which are either up- or down-regulated in the developing hippocampus exposed to *in utero* sidestream tobacco smoke (Table 3). The third statistically significant network (Fig. 5C) is composed of – p53, RhoA, β-catenin, Hras, as well as several differentially expressed genes – detected in the present study – such as those encoding – Insulin-like growth factor 2 mRNA binding protein 1 (Igf2bp1), HDAC2, Apolipoprotein D (APOD), ATPase Class V, type 10D (ATP10D), Ring finger protein (RNF168), ATPase family, AAA domain containing 2 (ATAD2), etc. (Table 3). Solid lines specify direct relationships whereas dotted lines indicate indirect interactions.

TABLE 1

Chamber Conditions for Cigarette Smoke- and Sham-Exposed Dams[1]

Sidestream Smoke Exposure		
Condition	**Sham Chamber[2]**	**CSE[3] Chamber[2]**
Carbon Monoxide (ppm)	ND[4]	32.9 ± 1.0
Humidity (% RH)	40.0 ± 1.3	46.9 ± 0.1
Temperature (°C)	22.5 ± 0.1	21.9± 0.1
Total Suspended Particulates (mg/m^3)	0.2 ± 0.1	5.9 ± 0.2

[1] Chamber measurements were monitored prior to daily animal exposures and then twice during the daily six-hour exposure period.

[2] Data are reported as mean ± SEM.

[3] CSE = Cigarette Smoke Exposure

[4] ND = Not Detected

TABLE 2

Fetal Outcomes on Gestational Day 18.5[1]

Sidestream Smoke Exposure		
Outcome	**Sham[2]**	**CSE[2,3]**
Number of Implantations	8.2 ± 0.4	8.3 ± 0.5
Number of Resorptions	0.2 ± 0.1	0.5 ± 0.2
Number of Viable Fetuses	8.0 ± 0.4	7.7 ± 0.5
Number of Abnormalities	0.5 ± 0.3	0.6 ± 0.2
Fetal Weight (g)	1.15 ± 0.02	1.12 ± 0.03
Crown-Rump Length (mm)	27.5 ± 0.5	26.2 ± 0.4

[1] Fetal outcomes were assessed on gestational day 18.5.

[2] Data are reported as mean ± SEM for each measure (n = 10 – 11 litters).

[3] CSE = Cigarette Smoke Exposure

There were no significant effects of gestational sidestream smoke exposure on any of the fetal outcomes measured.

TABLE 3

Genes/ESTs that are Differentially Regulated in the Fetal Hippocampus Following Sidestream Cigarette Smoke Exposure on Gestational Days 1–17

Gene Probe ID	Gene Descriptor	Fold Change[1]
1436544_at	ATPase, Class V, type 10D	2.00
1438193_at	Neurexin III	1.99
1424995_at	Riken cDNA 6230410P16 gene	1.93
1417150_at	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 (Slc6a4; SERT)	1.85
1441429_at	Insulin receptor substrate 4	1.85
1430379_at	RIKEN DNA 5830411K21 gene	1.79
1450344_a_at	Prostaglandin E receptor 3	1.72
1444195_at	Required for meiotic nuclear division 5 homolog A (S. cerevisiae)	1.68
1440358_at	Rho guanine nucleotide exchange factor (GEF) 15	1.62
1429808_at	Hypothetical protein LOC68625	1.61
1429180_at	Guanosine monophosphate reductase 2	1.61
1455373_at	Transcribed locus	1.60
1455493_at	Synaptic nuclear envelope 1	1.60
1436593_at	RIKEN cDNA 1700016K19 gene = hypothetical protein LOC 74230	1.59
1459385_at	Calmodulin regulated spectrin-associated protein 1-like 1	1.56
1419501_at	Polymerase (DNA directed), Kappa	1.56
1427558_s_at	Asparagine-linked glycosylation 12 homolog	1.56
1445459_at	Somatostatin receptor 5	1.54
1450370_a_at	Kv channel interacting protein 4	1.54
1455223_at	Insulin-like growth factor 2 mRNA binding protein 1	1.53
1416275_at	Solute carrier family 26, member 6	1.53
1459143_at	Coiled-coil-helix-coiled-coil-helix domain containing 3	1.53
1449269_at	Coagulation factor V	1.52
1424989_at	Transmembrane protein 142A/ORAI calcium release-activated calcium modulator	1.51
1428948_at	RIKEN cDNA 5730414M22 gene	1.51
1423084_at	UDP-Gal:betaGlcNAcbeta 1,3- galactosyltransferase, polypeptide 2	−1.51
1450515_at	Potassium inwardly rectifying channel, subfamily J, member 11	−1.51
1438268_at	Ring finger and CCCH-type zinc finger domains 2	−1.51
1455586_at	Ring finger protein 168	−1.51
1458465_at	PAN3polyA specific ribonuclease subunit homolog (yeast)	−1.52
1444917_at	H3098E04-3 NIA Mouse 15K cDNA clone set Mus Musculus DNA clone H3098E04 3′, mRNA sequence	−1.52
1437372_at	Cleavage and polyadenylation specific factor 6	−1.52
1424971_at	Coiled-coil domain containing 99	−1.52
1443229_at	ATPase family, AAA domain containing 2	−1.53

Gene Probe ID	Gene Descriptor	Fold Change[1]
1440241_at	Melastatin 1/transient receptor potential cation channel, subfamily M, member 1	−1.53
1445484_at	Cytochrome c, somatic	−1.54
1429470_at	EST: similar to Speedy homolog A	−1.54
1456492_at	RIKEN cDNA 9130404D08 gene	−1.55
1450459_at	Signal peptide peptidase-like 2A/intramembrane protease3	−1.56
1429010_at	U1 small nuclear ribonucleoprotien polypeptide A	−1.56
1450188_s_at	Lipase, endothelial	−1.56
1421512_at	Centrosomal protein 250	−1.56
1429880_at	RIKEN cDNA 4921531C22 gene	−1.57
1439327_at	Collagen and calcium binding EGF domains 1	−1.58
1448885_at	RAP2B, (RAS oncogene family member)	−1.58
1449311_at	Basic Leucine zipper transcription factor 1	−1.59
1417642_at	Aldehyde dehydrogenase family 1, A3	−1.59
1453573_at	Histone cluster 1, H3d	−1.59
1458050_at	Transcribed Sequences	−1.60
1415927_at	Actin, alpha, cardiac	−1.60
1436904_at	Thyroid hormone receptor associated protein 1/mediator complex subunit 3	−1.61
14557787_at	Transcribed sequences	−1.61
1440282_at	Tubby-like protein 4	−1.64
1422167_at	Sema domain (semaphorin 5A)	−1.64
1439664_at	WD repeat domain 68	−1.69
1454354_at	RIKEN cDNA 8030476L19 gene	−1.70
1431278_s_at	Phospholipase A2, group VI	−1.73
1439704_at	Histone deacetylase 2	−1.74
1421230_a_at	Musashi Homolog 2 (Drosophila)	−1.74
1444564_at	Apolipoprotein D	−1.83
1439040_at	Centromere protein E	−1.90

[1]Only genes whose expression increased or decreased at least 1.5 fold (smoke exposed versus sham exposed) in the hippocampus are shown in this table. Positive numbers indicate an increase in expression, whereas negative numbers indicate a decrease in expression.

TABLE 4

GeneChip® Microarray Verification by TaqMan™ Quantitative Real-Time PCR[1]

Gene[2]	Entrez Gene	Affymetrix Probe ID	Concordance[3]
Downregulated Genes			
Apolipoprotein D	11815	1444564_at	+/+
HDAC-2	15182	1439704_at	+/+
Sema domain 5A (Semaphorin 5A)	20356	1422167_at	+/+
Musashi homolog 2	76626	1421230_a_at	+/+
Centromere protein E	229841	1439040_at	+/+
UDP-Gal:betaGlcNAcbeta 1,3-galactosyltransferase, polypeptide 2	26878	1423084_at	+/+
Aldehyde dehydrogenase family 1, A3	56847	1417642_at	+/+
Phospholipase A2, group VI	53357	1431278_s_at	+/+
Upregulated Genes			
Insulin Receptor Substrate 4 (IRS-4)	16370	1441429_at	+/+
Synaptic nuclear envelope 1	64009	1455493_at	+/+
Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	15567	1417150_at	+/+
Polymerase (DNA directed) Kappa	27015	1419501_at	+/+

[1]The differential expression of twelve genes in the murine hippocampus following exposure to sidestream cigarette smoke on gestational days 1–17, was compared using Affymetrix GeneChip® arrays and TaqMan™ quantitative real-time PCR.

[2]Target genes were selected randomly.

[3]Full concordance in the pattern or level of gene expression obtained using the Affymetrix GeneChip® arrays and the TaqMan™ quantitative real-time PCR is represented as '+/+'.