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Developmental cigarette smoke exposure: Hippocampus proteome and metabolome profiles in low birth weight pups

Rachel E. Neal[a,d,*], Jing Chen[a], Rekha Jagadapillai[b], HyeJeong Jang[c], Bassam Abomoelak[a], Guy Brock[c,d], Robert M. Greene[b,d], M. Michele Pisano[b,d]

[a] Department of Environmental and Occupational Health Sciences, School of Public Health and Information Sciences, University of Louisville, Louisville, KY, USA
[b] Department of Molecular, Cellular, and Craniofacial Biology, ULSD, University of Louisville, Louisville, KY, USA
[c] Department of Biostatistics and Bioinformatics, School of Public Health and Information Sciences, University of Louisville, Louisville, KY, USA
[d] Birth Defects Center, University of Louisville, Louisville, KY, USA

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ABSTRACT

Exposure to cigarette smoke during development is linked to neurodevelopmental delays and cognitive impairment including impulsivity, attention deficit disorder, and lower IQ. However, brain region specific biomolecular alterations induced by developmental cigarette smoke exposure (CSE) remain largely unexplored. In the current molecular phenotyping study, a mouse model of 'active' developmental CSE (serum cotinine > 50 ng/mL) spanning pre-implantation through third trimester-equivalent brain development (gestational day (GD) 1 through postnatal day (PD) 21) was utilized. Hippocampus tissue collected at the time of cessation of exposure was processed for gel-based proteomic and non-targeted metabolomic profiling with Partial Least Squares-Discriminant Analysis (PLS-DA) for selection of features of interest. Ingenuity Pathway Analysis was utilized to identify candidate molecular and metabolic pathways impacted within the hippocampus. CSE impacted glycolysis, oxidative phosphorylation, fatty acid metabolism, and neurodevelopment pathways within the developing hippocampus.

1. Introduction

Cigarette smoke contains approximately 8000 toxic chemicals including nicotine, carbon monoxide, heavy metals, hydrogen cyanide, and polycyclic aromatic hydrocarbons (Borgerding and Klus, 2005; Rodgman and Perfetti, 2013). In the US, approximately 44 million adults smoke cigarettes including one fifth of all pregnant women (2012). Maternal cigarette smoking during pregnancy is associated with a variety of adverse pregnancy outcomes, of which low birth weight (LBW) is the most well documented (Abel, 1980; Cooke, 1998; Mitchell et al., 2002). Prenatal growth restriction and resultant LBW have long-lasting effects on infant and childhood growth and cognitive development (Botero and Lifshitz, 1999; Das and Sysyn, 2004; Gluckman et al., 2005). Epidemiological and case control studies suggest that children exposed to cigarette smoke during development exhibit aberrant behavioral and cognitive development including hyperactivity and impulsivity (Fried and Makin, 1987; Roy et al., 1998; Slotkin et al., 2002), impaired learning and memory (Batstra et al., 2003; Sexton et al., 1990), perception deficits (Fried and Watkinson, 2000; McCartney et al., 1994), and lower IQ with impaired intellectual development (Butler and Goldstei, 1973; Butler and Goldstein, 1973; Olds et al., 1994a,b,c).

The developmental toxicity of cigarette smoke, or its principal addictive components such as nicotine, has been widely investigated in animal models, including effects on neuro/cognition and behavior. While studies investigating the effects of developmental nicotine exposure are numerous, those examining the effects of actual inhalation exposures of cigarette smoke in animal models – as in the present study – are generally lacking. The preponderance of neurocognitive/behavioral data in mammalian animal models indicates that, when administered during fetal ontogenesis, nicotine is a potent developmental neurotoxicant with a diversity of effects on the central nervous system, including alterations in neurotransmitter signaling and resultant perturbations in the neuroanatomical and cytoarchitectural development of the brain (Ernst et al., 2001; Levin and Slotkin, 1998; Levin et al., 1996; Slikker et al., 2005; Slotkin, 1992). Prenatal exposure to nicotine in varied rodent models is known to: perturb early brain morphogenesis through excessive neuroepithelial cell apoptosis (Roy et al., 1998; Zhao and Reece, 2005); elicit shortfalls in neuronal cell numbers through decreased cell proliferation or enhanced apoptosis

* Corresponding author at: Department of Environmental and Occupational Health Sciences, University of Louisville, 485 E. Gray Street, Louisville, KY 40202, USA. Tel.: +1 502 852 3179; fax: +1 502 852 3304.
E-mail address: rachel.neal@louisville.edu (R.E. Neal).

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(Jang et al., 2002; Navarro et al., 1989; Slotkin, 1999; Slotkin et al., 1986, 1987, 1997); alter cell size, packing density or cortical thickness (Gospe et al., 1996; Roy et al., 2002); promote abnormal gliosis at the expense of neurogenesis (Roy et al., 2002); and interfere with the development of neural circuitry (Levin and Slotkin, 1998; Levin et al., 1996; Slawecki and Ehlers, 2002; Slawecki et al., 2000) (Slotkin, 1992, 1999) – all in the *developing* brain.

The nicotine-induced "structural" alterations in the brain, noted above, correlate with neurobehavioral/cognitive "functional" deficits in exposed animals which mimic deficits seen in children whose mothers smoked during pregnancy (Cornelius and Day, 2000; DiFranza et al., 2004; Ernst et al., 2001) including: increased locomotor activity (Fung, 1988; Koehl et al., 2000; Nagahara and Handa, 1999; Shacka et al., 1997), hyperactivity (Ajarem and Ahmad, 1998; Newman et al., 1999; Sobrian et al., 2003; Vaglenova et al., 2004), impulsivity (Sobrian et al., 2003), and anxiety (Vaglenova et al., 2004). In addition, long-term impairments in attention, learning, and memory in smoke exposed animals have been observed through the aid of various paradigms such as, avoidance acquisition (Genedani et al., 1983; Peters and Ngan, 1982; Vaglenova et al., 2004), radial arm maze tasks (Levin et al., 1993; Sorenson et al., 1991), and operant learning behaviors (Martin and Becker, 1971). In the animal model employed in the current study, developmental CSE altered offspring neurobehavioral maturation and outcomes (Amos-Kroohs et al., 2013). Specifically, prenatal and early postnatal inhalation exposure of mice to cigarette smoke induced subnormal anxiety in various novel environments, impaired spatial learning and reference memory while sparing other behaviors (i.e., route-based learning, fear conditioning, and forced swim immobility) – supporting mounting evidence that developmental cigarette smoke exposure has long-term adverse effects on the brain, including hippocampus-mediated memory function (Amos-Kroohs et al., 2013).

Across species, the hippocampus plays an important role in orchestrating learning and memory processing (Nishitani, 2003; Nishitani et al., 1998; Olton and Feustle, 1981; Olton and Papas, 1979; Romijn et al., 1991), including declarative memory, spatial cognition, memory consolidation, multimodal sensory integration, habituation and novelty detection, as well as temporal information processing and sequencing (d'Hellencourt and Harry, 2005; Eichenbaum, 1999,2004a,b; Fortin et al., 2004; Hammond et al., 2004; Levenson and Sweatt, 2005; Shapiro and Eichenbaum, 1999; Sweatt, 2004; Whishaw and Jarrard, 1996; Yamaguchi et al., 2004). In view of the association between pre- and peri-natal exposure to cigarette smoke/nicotine (in humans and rodents) and deficits in attention, perception, learning and memory, studies detailed in the present report investigate the effects of murine inhalation exposure to cigarette smoke during development on the molecular ontogenesis of the hippocampus. The main objective of the current study was to determine the impact of developmental CSE on the hippocampus biomolecular phenotype *at the time of cessation of exposure*. Utilizing a mouse model simulating "active" maternal cigarette smoking (Esposito et al., 2008) which is characterized by attendant low birth weight in offspring and altered neurobehavioral phenotypes at maturity (Amos-Kroohs et al., 2013), hippocampus proteome and metabolome profiles were examined. The offspring were exposed to CSE (6 h/day, 7 days/week) from gestational day (GD) 1 through postnatal day (PD) 21 using commercial Marlboro Red cigarettes, the most common brand of cigarettes smoked by young women. In parallel studies from identical offspring, developmental cigarette smoke exposure altered liver and kidney proteome profiles of the low birth weight offspring (Canales et al., 2012; Jagadapillai et al., 2012). The current study forms the basis of future studies on the persistence of these alterations past the cessation of exposure at the time of development of aberrant neurobehavioral phenotypes.

2. Materials and methods

2.1. Animals

Adult C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). Animals were housed in ventilated racks and maintained in a controlled temperature/humidity environment with a 12 h light/dark cycle and free access to Purina Lab Diet 5015 and water in the University of Louisville Research Resources Center, an AAALAC accredited vivarium. All exposure procedures were approved by the Institutional Animal Use and Care Committees of the University of Louisville and conformed to the NIH *Guide for the Care and Use of Laboratory Animals*. Timed pregnancies were established by overnight mating of a single mature male with two nulliparous females. The presence of a vaginal plug on the following morning was considered evidence of mating and the time considered gestational day (GD) 0.

2.2. Murine cigarette smoke exposure model

Dams were randomly assigned to either the SHAM or cigarette smoke exposure (CSE) groups. Exposure to cigarette smoke was performed using the Teague TE-10C cigarette smoke inhalation exposure system (Teague Enterprises, Davis, CA). Animals were exposed to cigarette smoke (CSE; commercial Marlboro Red cigarettes) or filtered ambient air (SHAM) for 6 h/day throughout the entirety of gestation, and following parturition were exposed with their offspring until PD 21, as described in detail (Amos-Kroohs et al., 2013). Aged, diluted side- and mainstream smoke was delivered to the CSE chamber under conditions providing total suspended particulates (TSPs) in the range of 20–30 mg/m^3 – an exposure that elevates dam/pup plasma cotinine levels (Koren et al., 1992, 1998) to those resembling levels in pregnant women who are 'active' smokers (i.e., plasma cotinine greater than 12 ng/ml, Jarvis et al., 2008). TSP levels represent the "dose" of cigarette smoke to which the animal is exposed, while plasma cotinine (primary metabolite of nicotine) serves as a biomarker of the internal dose. Control animals were sham exposed to ambient air in whole body inhalation chambers under identical conditions (temperature, humidity, flow rate) to CSE mice. Chamber conditions including total suspended particulates (TSPs), carbon monoxide levels, humidity, and temperature were measured twice daily during the GD 1 through PD21 exposure period. On PD21, pups were euthanized by asphyxiation with carbon dioxide followed by thoracotomy and cardiac puncture. Tissues were harvested and stored at −80 °C until analysis. The tissues utilized for the current study were from identical offspring as those utilized for our prior studies with a total of 9 offspring from individual litters representing each group (n = 9 for CSE and Sham groups) (Canales et al., 2012; Jagadapillai et al., 2012).

2.3. Proteome profiling

Individual offspring hippocampus tissue samples were homogenized in an ice cold methanol/chloroform matrix, washed with methanol/chloroform, and then pelleted by centrifugation. The dried pellets were homogenized in sample preparation buffer [7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT)] and stored at −80 °C for a short time until analysis. Protein concentration for each of the samples was determined using the Bradford Assay (Bradford, 1976). For each individual hippocampus tissue protein extract (n = 9 per group), four hundred micrograms of protein in rehydration buffer (8 M urea, 2% CHAPS, 2 μl IPG buffer pH 3–10, 2.5 mg/ml DTT, 0.002% bromophenol blue) was applied to IPGphor Drystrips (Nonlinear, 3–11, 180 mm × 3 mm × 0.5 mm, GE Healthcare, Piscataway, NJ). First dimension separation by isoelectric focusing at 22,000 Volt hours (Vh) was performed with a hold at 100 V until further processing. The IEF strips were stored at −80 °C for 1 h followed by: (1) equilibration for 60 min in reducing buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue with 3.5 mg/ml DTT) and (2) equilibration in alkylating buffer (same buffer with 45 mg/ml iodoacetamide instead of DTT) for an additional 60 min. Second dimension SDS-PAGE separation (25 cm × 20.5 cm, 15% polyacrylamide gels) was performed overnight (18 h; 100 V). Protein spots were visualized by Colloidal Coomassie Blue G-250 (Candiano et al., 2004).

2.3.1. Image analysis

Gels were scanned using an Epson Expression 10,000 XL scanner with transparency attachment. Densitometric analysis of gel images was performed with Progenesis SameSpots software (Nonlinear Dynamics; New Castle-on-Tyne, UK). Protein spots were detected automatically and manually adjusted (if required) for accuracy. For each protein spot, the intensity was measured, background was subtracted, and individual spot density was normalized by total pixel density of each gel. Spots with average normalized pixel depth ≤1000 relative abundance units and non-normalized areas with pixel depth below 100 were removed as noise. The averaged normalized spot abundance was compared between groups to determine fold differences in abundance. Two gel images were removed from analysis due to high non-specific background resulting in a total of 7 CSE and 9 Sham 2D gels utilized for image analysis.