Modulation of cell adhesion systems by prenatal nicotine exposure in limbic brain regions of adolescent female rats

Junran Cao1, Jennifer B. Dwyer2, Jamie E. Mangold1, Ju Wang1, Jinxue Wei1, Frances M. Leslie2 and Ming D. Li1

1 Department of Psychiatry and Neurobehavioral Sciences, University of Virginia, Charlottesville, VA, USA
2 Department of Pharmacology, University of California, Irvine, CA, USA

Abstract
Maternal smoking during pregnancy (MS) has long-lasting neurobehavioural effects on the offspring. Many MS-associated psychiatric disorders begin or change symptomatology during adolescence, a period of continuous development of the central nervous system. However, the underlying molecular mechanisms are largely unknown. Given that cell adhesion molecules (CAMs) modulate various neurotransmitter systems and are associated with many psychiatric disorders, we hypothesize that CAMs are altered by prenatal treatment of nicotine, the major psychoactive component in tobacco, in adolescent brains. Pregnant Sprague–Dawley rats were treated with nicotine (3 mg/kg.d) or saline via osmotic mini-pumps from gestational days 4 to 18. Female offspring at postnatal day 35 were sacrificed, and several limbic brain regions (the caudate putamen, nucleus accumbens, prefrontal cortex, and amygdala) were dissected for evaluation of gene expression using microarray and quantitative RT–PCR techniques. Various CAMs including neurexin, immunoglobulin, cadherin, and adhesion-GPCR superfamilies, and their intracellular signalling pathways were modified by gestational nicotine treatment (GN). Among the CAM-related pathways, GN has stronger effects on cytoskeleton reorganization pathways than on gene transcription pathways. These effects were highly region dependent, with the caudate putamen showing the greatest vulnerability. Given the important roles of CAMs in neuronal development and synaptic plasticity, our findings suggest that alteration of CAMs contributes to the neurobehavioural deficits associated with MS. Further, our study underscores that low doses of nicotine produce substantial and long-lasting changes in the brain, implying that nicotine replacement therapy during pregnancy may carry many of the same risks to the offspring as MS.

Introduction
Maternal smoking during pregnancy (MS) produces adverse effects on offspring that persist or emerge after the initial tobacco exposure (Rogers, 2008; Shea & Steiner, 2008). In addition to the MS-linked deficits during the prenatal and neonatal stages (Fantuzzi et al. 2007), MS is significantly associated with neuropsychiatric disorders that emerge or change symptomatology during adolescence, a period of continuous development of the central nervous system (CNS) (Spear, 2000). Children whose mothers smoke during pregnancy are more likely to develop attention deficit hyperactivity disorder (ADHD), conduct disorder, depression, and autism (Indredavik et al. 2007; Weissman et al. 1999). Those exposed to prenatal tobacco also are more vulnerable to various drug addictions (Fergusson et al. 1998; Weissman et al. 1999). Moreover, the intensity of MS is inversely related to offspring intelligence (IQ) and cognitive ability (Batty et al. 2006; Olds et al. 1994). Many of these disorders are thought to be mediated by dysfunction of the...
limbic system (Drevets et al. 2008; Feltenstein & See, 2008), a collection of brain nuclei that mature during adolescence (Spear, 2000). The delayed onset of MS-related neurobehavioural disorders suggests that alterations during prenatal development manifest only as the limbic circuitry matures.

Animal studies have evaluated the neurochemical mechanisms underlying the effects of prenatal exposure to nicotine, the major psychoactive component of tobacco. Gestational nicotine exposure (GN) modulates cholinergic receptor expression, which remains altered into adolescence (Chen et al. 2005; Tizabi & Perry, 2000). Deficits in monoamine transmission including those of dopamine, norepinephrine, and serotonin also are observed in GN-treated adolescent animals (Kane et al. 2004; Seidler et al. 1992; Xu et al. 2001). Additionally, GN has lasting effects on the glutamatergic system, producing alterations in AMPA receptor function (Vaglenova et al. 2008). Many of the neurotransmitter systems impacted by GN are regulated at the structural and functional levels by cell adhesion systems (Craig & Kang, 2007; Hulley et al. 1998; Yamagata et al. 2003).

Cell adhesion molecules (CAMs) have broad functions, modulating cell–cell, cell–matrix interactions, and intracellular signal transduction (Juliano, 2002). In the CNS, CAMs such as the cadherins, neurexins, integrin, and immunoglobulin superfamilies have been identified at synapses (Yamagata et al. 2003). Cell adhesion systems play important roles in the development, maturation, and plasticity of the CNS by regulating neuronal migration, neurite outgrowth, axon fasciculations, axon guidance, synaptogenesis in the developing brain, and synaptic formation and function in the mature brain (Sudhof, 2008). Abnormal expression of CAM genes is associated with psychiatric and cognitive disorders such as autism, schizophrenia, bipolar disorder, and Alzheimer’s disease (Liu et al. 2006; Rujescu et al. 2009; Sudhof, 2008). Recent Genome-Wide Association Studies (GWAS) also suggest that CAM genes are related to drug abuse (Li & Burmeister, 2009; Liu et al. 2006). As the prevalence of many of these disorders is increased by MS, it is possible that changes in CAM function underlie the alterations in neurotransmission and the behavioural phenotypes in GN animal models.

In the current study, we have undertaken a systematic evaluation of the relationship between CAM systems and GN in rats. First, we used quantitative real-time PCR to examine the expression pattern of the 29 CAM-related genes that are suggested to play a significant role in drug addiction based on human genetic studies (Li & Burmeister, 2009; Liu et al. 2006) in four limbic brain regions of adolescent female rats subjected to GN. Then, we investigated the regulation pattern of the biochemical pathways related to CAM systems in these brain regions based on microarray data by focusing on most of the genes involved in the system. To our knowledge, this represents the first report that CAMs and CAM-related intracellular signal transduction pathways are significantly modified by GN in limbic brain regions of adolescent female offspring.

Materials and methods

Animals and tissue collection

Sprague–Dawley rats were maintained in a temperature-(21 ºC) and humidity-(50%) controlled room on a 12-h light/dark cycle (lights on 07:00 hours) with unlimited access to food and water. Pregnant rats (Charles River, USA) were treated with nicotine or saline as previously described (Park et al. 2006). Each rat was given either nicotine at a concentration of 3 mg/kg.d or saline via an osmotic mini-pump from gestational days 4 to 18. After birth, litters were culled to ten and pups were cross-fostered to drug-naive mothers to minimize the effects of abnormal maternal rearing behaviours. Blood concentrations resulting from this dose of nicotine are equivalent to levels found in humans who smoke about 1.5 packs of cigarettes per day (Matta & Elberger, 2007), approximately (15–45 ng/ml; Benowitz & Jacob, 1984). As previously reported (Franke et al. 2007), GN treatment at this moderate dose did not influence dam weight gain, litter size, or pup weight gain during postnatal development. Pups were weaned at postnatal day 21 (PD 21) and sacrificed at PD 35 via rapid decapitation, and brains were immediately removed. Using a rat brain matrix, 2-mm slices were taken that contained the prefrontal cortex (PFC), caudate putamen (CPu), nucleus accumbens (NAc), and amygdala (Amy), which were identified with reference to a rat brain atlas (Paxinos & Watson, 1998). Using a 1-mm-diameter punch, tissue was collected bilaterally from each brain region from each pup and stored at -80 ºC until use. Tissue of ten female pups from different litters was used for microarray with five animals in gestational saline treatment (GS) and GN groups, respectively. To get sufficient mRNA for quantitative real-time polymerase chain reaction (qRT–PCR), total mRNA from each brain region of two animals per litter was combined to yield a total of five litters in each experimental group. All experiments were performed in accordance with the Institutional Animal Care and
Use Committee at the University of California, Irvine, and were consistent with Federal guidelines.

**Microarray production**

A pathway-focused oligoarray designed specifically for drug addiction and brain-related research was used. Briefly, 3565 genes including those implicated in the maintenance of neuronal homeostasis and associated with the neuronal responses to addictive substances were selected on the basis of an earlier version of a pathway-focused cDNA microarray (Konu et al. 2004) and an extensive literature survey. The oligonucleotide for each gene was designed using OligoWiz (http://www.cbs.dtu.dk/services/OligoWiz/) with a final length of 59.2 ± 3.8 (mean ± s.d.), guanine cytosine (GC) content of 0.53 ± 0.05, and Tm 76.4 ± 1.7 °C. Then, the designed oligonucleotides and 10 control clones were synthesized and spotted at a concentration of 40 µM in 3× SSC and 1.5 M Betaine buffer onto CMT-GAPS II slides (Corning, USA), using OmniGrid MicroArrayer OGR-03 (GeneMachines, USA).

**RNA isolation and amplification, cDNA probe synthesis and microarray hybridization**

RNA was isolated from each brain region using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions and amplified as described previously for adequate cDNA probe labelling (Gutala et al. 2004; Konu et al. 2004; Li et al. 2004). Briefly, 2 µg total RNA was reverse-transcribed into the first-strand cDNA with an introduction of a T7 promotor region. The RT product was then mixed with 5× second-strand buffer (30 µl), 10 mM dNTP (3 µl), DNA polymerase (4 µl), RNase H (0.5 µl), E. coli DNA ligase (1 µl), and H2O (92.5 µl) and incubated at 16 °C for 3 h to synthesize double-stranded cDNA, which was then amplified using AmpliScribe™ T7 Transcription kits (Epicentre, USA).

cDNA probes were synthesized and hybridized to microarray slides as described previously (Gutala et al. 2004; Li et al. 2004). Briefly, 4 µg of amplified RNA were reverse-transcribed. The product was dissolved in H2O (28 µl) and mixed with 10× buffer (4 µl), 10 mM dTTP-free dNTP (4 µl), 10 mM dTTP (1 µl), 1 mM cytidine 3-dUTP or cyanine 5-dUTP (2 µl, Enzo, USA), and Klenow fragment (1 µl, 50 units/µl). The mixture was then incubated at 37 °C for 3 h. After purification, cyanine 5-labelled sample cDNA probes were mixed with cyanine 3-labelled control probes and applied in a total of 50 µl volume containing 20× SSC (7.5 µl), Cot1 DNA (3 µg), polyA (3 µg), and 10% SDS (0.5 µl). The mixture was applied to the pathway-focused oligonucleotide microarray described above and hybridized overnight at 60 °C. Slides were washed in 1× SSC and 0.2% SDS at 60 °C for 5 min followed by washing in 0.1× SSC and 0.2% SDS and in 0.1× SSC at room temperature for 10 min. Hybridized slides were scanned using the ScanArray Gx microarray scanner, and the intensity of each probe was quantified with the ScanArray Express microarray analysis system (PerkinElmer, USA).

**Microarray data analysis and Gene Set Enrichment Analysis**

After scanning each array, we obtained the raw hybridization intensity of each element and used the background-subtracted median intensity of each spot for further statistical analysis. Two replicates of each gene on a chip were analysed separately. To minimize spot variations and reduce experimental error, we discarded spots that were either over-saturated or poorly expressed (i.e. 5% of the weakest spots in each replicate of an array). We used an intensity-dependent normalization method (locally weighted linear regression; Lowess) to normalize the data for each replicate (Yang et al. 2002). After removing spots with fewer than six valid measurements per experimental group, we averaged two replicates per chip to be used as the measurement of the expression of a gene in a given sample.

Then, a bioinformatics tool, called Gene Set Enrichment Analysis (GSEA; Subramanian et al. 2005) was utilized to determine the pathways showing expression differences in each brain region. GSEA is a bioinformatics tool that computationally identifies whether an a priori-defined set of genes (pathways in our case) shows statistically significant and concordant differences between two biological states. For each predefined gene set (pathway in our case), a Normalized Enrichment Score (NES) is calculated by considering all the gene sets tested and a p value is assigned to determine whether this gene set is statistically enriched in the input genes when compared with random distribution. The pathways included in GSEA database were collected from multiple public domains (e.g. http://www.sigmaaldrich.com/; http://www.biocarta.com; http://www.genome.jp/kegg/). The software and the curated pathway database was downloaded and implemented locally in our laboratory.

For more details about the database, please refer to http://www.broadinstitute.org/gsea/.
Quantitative real-time PCR array

Representative CAMs and key genes in CAM-related intracellular signalling transduction pathways were examined with qRT–PCR using a different set of samples from those in microarray. Primers used in the qRT–PCR array were designed using Primer Express (v. 3.0) software. The sequences were subjected to a BLAST search to ensure specificity of the primers for the target gene and synthesized by Fisher Scientific (USA). All the primers were tested before addition to the qRT–PCR array. The primer sequences are listed in Supplementary Table S1 (available online).

qRT–PCR was conducted as described previously (Gutala et al. 2004; Li et al. 2004). Briefly, RT product was amplified in a volume of 10 μl containing 5 μl 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems, USA), and combined sense and antisense lysted using a comparative 3-phosphate dehydrogenase (GAPDH) and then ana-

Expressions of all genes were plate using the 7900HT Fast Real Time PCR system (Applied Biosystems). Expressions of all genes were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then analysed using a comparative Ct method (Winer et al. 1999). The relative gene expression was compared between GN and GS using the Student’s t test. Genes considered to be significant are those with a p value of <0.05 and fold change of >25%. Because all these genes are from the CAM system and highly functional, we chose not to perform correction for multiple testing in this report. Therefore, it is possible some of the genes identified as significantly expressed may be false positives. However, considering the genes evaluated in our work are so functionally related to each other in the CAM system and many of them showed consistent regulation by GN (see Results section for details), we believe that the conclusions drawn from our data are reasonable and reliable.

Results

Cell adhesion genes were modified by GN

Genes encoding CAMs and their intracellular anchor proteins were modified by GN at the mRNA level in four limbic brain regions of the female adolescent rats (Table 1). Using the qRT–PCR array, we examined 29 cell adhesion-related genes, which fell into six categories: neurexin, immunoglobulin, integrin, cadherin, and adhesion G protein-coupled-receptor (GPCR) superfamilies, with four genes that do not belong to any of the superfamilies grouped into the sixth category. We also included cadherin-associated proteins (catennins), vinculin (Vcl), actin (Actn1), Fyn proto-oncogene (Fyn), and zyxin (Zyx), which encode intracellular anchoring proteins that connect CAMs to the cytoskeleton. Among the 29 cell adhesion-related genes, we observed 17 significantly modified by GN in one of the four brain regions examined. Three genes, namely, contactin 4 (Cntn4), Down syndrome cell adhesion molecule (Dscam), and latrophilin 3 (Lphn3), were significantly changed in two of the four brain regions. Periostin (Postn), an extracellular CAM, was modified by GN in three brain regions. Most of the affected CAM-related genes were down-regulated, with only Postn exhibiting significant up-regulation in the NAc and PFC.

In the CPu, all 29 genes showed at least a trend for down-regulation by GN, and more genes were significantly changed in this region than in any other examined. Those showing significant down-regulation included Neurexin 1 (Nlgn1) [0.60 ± 0.16 (fold change ± S.D.); p = 0.027] in the neurexin superfamily; Ctnn4 [0.45 ± 0.01, p = 6.0 × 10⁻³], Ctnn5 [0.72 ± 0.07, p = 0.024], Ctnn6 [0.66 ± 0.15, p = 0.035], and Dscam [0.66 ± 0.17, p = 0.040] in the immunoglobulin superfamily; cadherin 13 (Cdh13) [0.51 ± 0.21, p = 0.013], catenin α1 (Ctnna1) [0.63 ± 0.07, p = 1.2 × 10⁻³], catenin α2 (Ctnna2) [0.67 ± 0.03, p = 1.7 × 10⁻³], catenin β1 (Ctnnb1) [0.71 ± 0.05, p = 6.5 × 10⁻³], catenin δ2 (Ctnnd2) [0.61 ± 0.09, p = 2.3 × 10⁻³] in the cadherin superfamily; and adhesion GPCRs such as brain-specific angiogenesis inhibitor 3 (Bai3) [0.59 ± 0.16, p = 0.037] and Lphn3 [0.66 ± 0.18, p = 0.047]. Postn [0.65 ± 0.03, p = 1.6 × 10⁻⁴] and genes encoding intracellular anchor proteins such as Actn1 [0.46 ± 0.13, p = 0.014] and Fyn [0.62 ± 0.10, p = 5.4 × 10⁻⁵] were significantly down-regulated. In addition, CUB and Sushi multiple domains 1 (Csmd1), a gene suggested to be involved in drug addiction (Liu et al. 2006) was significantly down-regulated (0.66 ± 0.04, p = 3.3 × 10⁻⁴) by GN in the CPu.

In the NAc, there were only two genes significantly regulated by GN. Postn mRNA was 40% up-regulated by GN [1.40 ± 0.11, p = 0.033], whereas receptor-type protein tyrosine phosphatase D (Ptprd), a gene suggested to be involved in drug addiction (Liu et al. 2006), was significantly down-regulated (0.57 ± 0.11, p = 0.021).

In the PFC, neurexin 3 (Nrxn3) [0.75 ± 0.02, p = 0.046], Ctnn4 [0.66 ± 0.04, p = 3.6 × 10⁻³], and Dscam [0.66 ± 0.12, p = 0.044] were significantly down-regulated by GN. In contrast, Postn (1.58 ± 0.42, p = 0.029) was significantly up-regulated.

In the Amy, Lphn3 [0.62 ± 0.02, p = 0.015], Sarcoglycan zeta (Sgcz) [0.65 ± 0.05, p = 7.6 × 10⁻⁵], and two genes in the immunoglobulin superfamily,
Table 1. Gestational nicotine exposure modified mRNA expression of cell adhesion molecules and intracellular anchoring proteins

<table>
<thead>
<tr>
<th>Cell adhesion family</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Fold change (± S.D.)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPu</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PFC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Amy</td>
</tr>
<tr>
<td>Neurexin</td>
<td>Nrnx1a</td>
<td>Neurexin1</td>
<td>0.79 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>Nrnx2</td>
<td>Neurexin2</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Nrnx3b</td>
<td>Neurexin3</td>
<td>0.62 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Ngln1</td>
<td>Neurogliin1</td>
<td>0.60 ± 0.16*</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>Ncam1</td>
<td>Neural cell adhesion molecule 1</td>
<td>0.84 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>Cntn4a</td>
<td>Contactin 4</td>
<td>0.45 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>Cntn5a</td>
<td>Contactin 5</td>
<td>0.72 ± 0.07*</td>
</tr>
<tr>
<td></td>
<td>Cntn6a</td>
<td>Contactin 6</td>
<td>0.66 ± 0.15*</td>
</tr>
<tr>
<td></td>
<td>Dscam1</td>
<td>Down syndrome cell adhesion molecule</td>
<td>0.66 ± 0.17*</td>
</tr>
<tr>
<td></td>
<td>Pecam1</td>
<td>Platelet/endothelial cell adhesion molecule 1</td>
<td>0.97 ± 0.45</td>
</tr>
<tr>
<td>Integrin</td>
<td>Itgb1</td>
<td>Integrin beta 1</td>
<td>0.97 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>Postn</td>
<td>Periostin, osteoblast specific factor</td>
<td>0.65 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>Vclb</td>
<td>Vinculin</td>
<td>0.74 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Actnb</td>
<td>Actinin, alpha 1</td>
<td>0.46 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>Zyxb</td>
<td>Zyxin</td>
<td>0.54 ± 0.62</td>
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<tr>
<td>Cadherin</td>
<td>Cdhl3a</td>
<td>Cadherin 13</td>
<td>0.51 ± 0.21*</td>
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<tr>
<td></td>
<td>Pcdh9a</td>
<td>Protocadherin 9</td>
<td>0.89 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>Cttnalb</td>
<td>Catenin (cadherin associated protein), alpha 1</td>
<td>0.63 ± 0.07**</td>
</tr>
<tr>
<td></td>
<td>Cttnalb</td>
<td>Catenin (cadherin associated protein), alpha 2</td>
<td>0.67 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>Cttnalb</td>
<td>Catenin (cadherin associated protein), alpha 3</td>
<td>0.67 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Cttnblb</td>
<td>Catenin (cadherin associated protein), beta 1</td>
<td>0.71 ± 0.05**</td>
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<tr>
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<td>Catenin (cadherin associated protein), delta 2</td>
<td>0.61 ± 0.09**</td>
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<td></td>
<td>Vclb</td>
<td>Vinculin</td>
<td>0.74 ± 0.28</td>
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<tr>
<td></td>
<td>Actnb</td>
<td>Actinin, alpha 1</td>
<td>0.46 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>Fynb</td>
<td>Fyn proto-oncogene</td>
<td>0.62 ± 0.10**</td>
</tr>
<tr>
<td>Adhesion GPCR</td>
<td>Bat3a</td>
<td>Brain-specific angiogenesis inhibitor 3</td>
<td>0.59 ± 0.16*</td>
</tr>
<tr>
<td></td>
<td>Lphnsa</td>
<td>Latrophilin 3</td>
<td>0.66 ± 0.18*</td>
</tr>
<tr>
<td>Others</td>
<td>Lrrnalb</td>
<td>Leucine rich repeat neuronal 1</td>
<td>0.75 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Ppyrlb</td>
<td>Receptor-type protein tyrosine phosphatase D</td>
<td>0.30 ± 0.37</td>
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<tr>
<td></td>
<td>Csmdl1a</td>
<td>CUB and Sushi multiple domains 1</td>
<td>0.66 ± 0.04***</td>
</tr>
<tr>
<td></td>
<td>Sgcza</td>
<td>Sarcoglycan zeta</td>
<td>0.69 ± 0.03</td>
</tr>
</tbody>
</table>

CPu, Caudate putamen; NAc, nucleus accumbens; PFC, prefrontal cortex; Amy, amygdala. GPCR, G protein coupled receptor. Cell adhesion molecules and intracellular anchor proteins were modified by gestational nicotine exposure (GN). Fold change > 1 indicates up-regulation of gene expression, whereas fold change < 1 indicates down-regulation by GN. Data are from qRT–PCR array.

* Genes suggested as being associated with drug addiction in human studies.

b Genes encode intracellular proteins anchoring cell adhesion molecules.

* p < 0.05, ** p < 0.01, *** p < 0.001 significant difference between GN and control animals.

namely, neural cell adhesion molecule 1 (Ncam1) (0.47 ± 0.11, p = 9.5 × 10⁻³) and platelet/endothelial cell adhesion molecule 1 (Pecam1) (0.59 ± 0.03, p = 0.031), were significantly down-regulated by GN. In contrast, no genes were significantly up-regulated.

Intracellular signalling pathways related to CAMs were changed by gestational nicotine treatment

CAMs not only have adhesive functions that modulate cell–cell and cell–matrix interactions but also transmit signals to the cell interior (Juliano, 2002). CAMs can...
directly activate MAP kinase cascades and Rho small GTPases and are involved in Wnt/Frizzled pathways (Komiya & Habas, 2008) and signal through G protein-mediated pathways (Bjarnadottir et al., 2007). CAMs can also modulate signal transduction initiated by other receptor types, including GPCRs, growth factor receptors, and Notch receptors (Hu et al., 2006; Juliano, 2002; Maness & Schachner, 2007). To further examine whether GN modified CAM-related intracellular signalling pathways, we searched the annotated database with the GSEA algorithm on the basis of gene ontology (GO) information for all genes included on our

**Fig. 1.** Biological pathways significantly modified by gestational nicotine treatment in the adolescent brain regions analysed by GSEA (nominal p < 0.05). For each brain region, the pathways were plotted in descending order of the negative logarithm of their p values at base 10. The biological pathways related to cell adhesion molecules are shown in black columns, whereas others are shown in light grey. For each pathway, a short format of its name in the GSEA database is shown in the figure: for amygdala, Gamma_hexachlorocyclohexane and Mitochondrial_fatty_acid are short formats of Gamma_hexachlorocyclohexane_degradation and Mitochondrial_fatty_acid_betaoxidation, respectively; for NAc, Sig_pip3_signalling corresponds to Sig_pip3_signalling_in_cardiac_myoctes; for PFC, St_T_cell_signal and St_B_cell_antigen are short formats of St_T_cell_signal_transduction and St_B_cell_antigen_receptor, respectively; for PVN, Oxidative_phosphorylation, Glycerolipid_metabolism and Sa_B_cell_receptor_complexes are short formats of Oxidative_phosphorylation, Glycerolipid_metabolism and Sa_B_cell_receptor_complexes, respectively; for the other pathways, the word pathway has been omitted from their names.
microarray chip. Among the overrepresented categories that were significantly associated with GN, we found 2, 9, 7, and 11 major pathways were related to CAMs in the CPu, NAc, PFC, and Amy, respectively (Fig. 1). The CAM-related pathways generally belonged to five groups (Rho small GTPase-related, MAPK-related, GPCR-related signalling, Notch and Wnt/Frizzled, and growth factor-signalling) (Table 2). Rho small GTPase-related pathways (i.e. Rac1, Rho, Cdc42Rac, Eph4, integrin, Akap13) were modified by GN in all four brain regions. In contrast, MAPK-related pathways (i.e. MAPK, P38 MAPK, Cdk5, Pyk2) were modulated by GN in the PFC and Amy, but not in the CPu or NAc. GPCR-related signalling pathways (i.e. Gs, St_G_alpha_i, PLC, and Agpcr), Notch and Wnt/Frizzled pathways, and growth factor-signalling pathways (i.e. Pdgf, Edg1, EGF, Insulin, Met, Igf1, Erbb4) were associated with the treatment in the NAc, PFC, and Amy.

To further evaluate CAM-related signalling pathways, representative genes from the microarray with the addition of critical genes were examined by qRT–PCR in all four brain areas. Our results confirmed that genes in the CAM-related pathways were changed by GN in a brain region-dependent manner. Further bioinformatics analyses indicated that these genes generally play important roles in cytoskeleton reorganization (Fig. 2), gene transcription (Fig. 3), or both.

**Actin cytoskeleton modified by Rho small GTPases**

Rho small GTPase-related pathways were modified by GN in all brain regions examined, and one of the principal functions of Rho small GTPases is to

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**Table 2. Gestational nicotine exposure modified intracellular signalling pathways related to cell adhesion molecules**

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Groups of pathways</th>
<th>Gene ontology categories identified by GSEA</th>
<th>No. of genes</th>
<th>NES</th>
<th>Permutated p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPu</td>
<td>Rho small GTPase-related</td>
<td>Eph4 pathway</td>
<td>10</td>
<td>−1.71</td>
<td>0.0118</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cdc42Rac pathway</td>
<td>14</td>
<td>−1.57</td>
<td>0.0352</td>
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<tr>
<td>NAc</td>
<td>Rho small GTPase-related</td>
<td>Rac1 pathway</td>
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<td>−1.86</td>
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<td>Cdc42Rac pathway</td>
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<td>−1.46</td>
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<td></td>
<td>Rho pathway</td>
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CPu, Caudate putamen; NAc, nucleus accumbens; PFC, prefrontal cortex; Amy, amygdala. NES, normalized enrichment score. The signalling pathways significantly modified by gestational nicotine exposure. Data are from microarray.
Fig. 2. Cell adhesion molecules modulate actin cytoskeleton via Rho small GTPases. Genes in red were up-regulated whereas those in green were down-regulated at the mRNA level by gestation nicotine exposure in (a) the caudate putamen, (b) nucleus accumbens, (c) prefrontal cortex and (d) amygdala. * Significantly modified compared with gestational saline treatment ($p < 0.05$ at least). ABI2, abl-interactor 2; ARP2/3, actin-related protein complex; Cdc42, cell division cycle 42; Cofilin, cofilin 1 (non-muscle); GDIA, Rho GDP dissociation inhibitor (GDI) alpha; Gelsolin, gelsolin (amyloidosis, Finnish type); LIMK, LIM domain kinase 1; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PAK, p21-activated protein kinase 1 (Pak1); PIP, 1-phosphatidyl-1D-myo-inositol 4-phosphate; PIP2, 1-phosphatidyl-1D-myo-inositol 4,5-bisphosphate; PIP5K, 1-phosphatidylinositol-4-phosphate 5-kinase; Rac, ras-related C3 botulinum toxin substrate 1 (Rac1); Rho, ras homolog gene family, member A (RhoA); ROCK, Rho-associated, coiled-coil containing protein kinase 1 (Rock1); WASP, Wiskott–Aldrich syndrome-like (N-WASP); WAVE, WAS protein family.
modulate the actin cytoskeleton. We therefore examined cytoskeleton reorganization using Rho small GTPase-related pathways as an example. Our data showed that GN modified key genes in these pathways, which implies a critical effect of GN on cytoskeleton reorganization (Fig. 2).
Fig. 3. Gene transcription pathways related to cell adhesion molecules. Genes in red were up-regulated whereas those in green were down-regulated at the mRNA level by gestation nicotine exposure in (a) the caudate putamen, (b) nucleus accumbens, (c) prefrontal cortex and (d) amygdala. * Significantly modified compared with gestational saline treatment ($p < 0.05$ at least). APC, adenomatous polyposis coli; Axin, axin 1; B-Raf, v-raf murine sarcoma viral oncogene homolog B1; c-Raf, v-raf-1 murine leukemia viral oncogene homolog 1; C3G, Rap guanine nucleotide exchange factor 1; Calpain, M calpain; CAS, breast cancer anti-estrogen resistance 1; CNTN1, contactin 1; CRKL, v-crk sarcoma virus CT10 oncogene homolog (avian)-like; Delta/Jagged, Notch ligand delta or jagged; Dsh, disheveled; FAK, PTK2 protein tyrosine kinase 2; Frizzled, frizzled homolog 1 (Drosophila); Fyn, FYN oncogene related to SRC, FGR, YES; GBP, frequently rearranged in advanced T-cell lymphomas; GRB2, growth factor receptor-bound protein 2; GSK3, glycogen synthase kinase 3; ILK, integrin-linked kinase; JNK1, mitogen-activated protein kinase 8; MKK4, mitogen-activated protein kinase kinase 4; MLCK, myosin light chain kinase; MLK3, mitogen-activated protein kinase kinase kinase 11; Notch, Notch gene homolog 1 (Notch 1); PAK, p21-activated protein kinase 1 (Pak1); PI3K,
In the CPu, all of the genes examined were down-regulated with significance for Cdc42 (0.61 ± 0.07, \( p = 2.5 \times 10^{-3} \)), Rho family member A (RhoA) (0.61 ± 0.06, \( p = 3.3 \times 10^{-3} \)), abl-interactor 2 (Abl2) (0.58 ± 0.16, \( p = 0.034 \)), and subunits of actin-related protein complex (Arp2/3). Although most genes also showed a trend for down-regulation in the NAc, Rac1, p21-activated protein kinase 1 (Pak1), and Rho-associated, coiled-coil-containing protein kinase 1 (Rock1) showed a trend for up-regulation with

phosphatidylinositol 3-kinase; PP2A, protein phosphatase type 2a; R-Ras, related RAS viral (r-ras) oncogene homolog; RAP, RAS-related protein 1a; Ras, Harvey rat sarcoma viral (v-Ha-ras) oncogene homolog; SHC, SHC (Src homology 2 domain containing) transforming protein 1; SOS, son of sevenless homolog; Src, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian); TACE, ADAM metallopeptidase domain 17; Wnt, wingless-type MMTV integration site family, member 1 (Wnt1); \( \beta \)-catenin, cadherin-associated protein, beta.
Our data also suggest that critical CAM down-regulation with significance for RAS-related protein 1a (Rap1a; 0.52 ± 0.18; p = 0.023), v-crk sarcoma virus CT10 oncogene homolog (avian)-like (CrkL; 0.67 ± 0.11; p = 9.0 × 10^{-4}), and adenomatous polyposis coli (Apc; 0.64 ± 0.07; p = 3.9 × 10^{-3}). In contrast, most genes in the NAc showed a trend for up-regulation, with significance for Rap guanine nucleotide exchange factor 1 (C3g; 2.31 ± 0.17; p = 0.014). Frizzled homolog 1 was significantly down-regulated in the PFC (Frizzled; 0.57 ± 0.18; p = 0.030), whereas wingless-type MMTV integration site family, member 1 (Wnt1; 0.70 ± 0.02, p = 0.011) and gene homolog 1 (Notch 1; 0.55 ± 0.04, p = 1.9 × 10^{-4}) were significantly down-regulated in the Amy.

**Discussion**

These data suggest broad effects of GN on the cell adhesion system which modified genes in the neu-rexin, immunoglobulin, cadherin, and adhesion GPCR superfamilies in four limbic regions. In addition, GN indirectly regulates the integrin system by altering peristin, an extracellular integrin binding partner (Kudo et al. 2007), as well as the intracellular anchoring proteins actinin and vinculin.

Our data also suggest that critical CAM downstream pathways were significantly altered by GN.
Although these pathways are highly interconnected and have complicated intracellular functions, CAM signal transduction generally causes cytoskeleton reorganization and gene transcription. GN modified more genes in cytoskeleton reorganization-related pathways than in gene transcription-related pathways, suggesting enhanced interaction of GN with the CAM system in cytoskeleton reorganization. Since the CAM-related genes were evaluated only at the mRNA level, future studies will be needed to assess their regulation at the protein level.

The regional heterogeneity of GN-induced alterations in CAM gene expression and their related pathways within the limbic system is striking. Much is known regarding the roles of the CPu, NAc, PFC, and Amy in the neural circuitry implicated in neurobehavioral disorders. The present data provide compelling evidence for regionally selective vulnerability to GN in the adolescent limbic system, with important implications for the aetiology of MS-linked deficits. On the other hand, given that these brain regions closely interact with each other, alterations in one brain region may also indirectly change the functions of others, leading to abnormal functions of the whole limbic system.

CPu

GN modified more genes in the CPu than in any other region. Remarkably, all affected genes were downregulated, suggesting that GN negatively regulates CAMs in this region and that the CPu may be particularly vulnerable to the effects of GN.

The CPu regulates motor control, procedural learning, and memory (Herrero et al. 2002; Squire et al. 1993), and aberrant CPu processing has been linked to psychiatric disorders such as ADHD (Vaidya & Stollstorff, 2008), autism (Stanfield et al. 2008), and addiction (Hyman et al. 2006). Several CAMs downregulated by GN in this region have been implicated in these same disorders. For example, Cdh13 and Ctnna contain clusters of single nucleotide polymorphisms (SNPs) associated with ADHD (Lesch et al. 2008). Moreover, both Nlgn1 down-regulation and loss of function of Cntn4 have been linked to autism (Roohi et al. 2009; Ylisaukko-oja et al. 2005). GN effects on the CPu may relate to the link between MS and ADHD and autism (Hultman et al. 2002; Linnet et al. 2003).

Many of the altered CAMs in the CPu have been associated with addiction, including Bai3, Lphn3, and Csmd1, whose mechanisms are not known (Liu et al. 2006). Further inquiry into the molecular function of these genes in addiction-related regions is needed. In addition, catenin β-like 1 (CTNNBL1) is associated with obesity (Liu et al. 2006). Thus, altered CAM gene expression in the CPu may cause abnormal sensitivity to natural reward and vulnerability to addiction. Indeed, GN-treated adolescent rats exhibit abnormal responses to food and addictive drugs (Franke et al. 2007, 2008; Levin et al. 2006). Data from this model are consistent with clinical studies linking MS to obesity and addiction in the offspring (Kandel et al. 1994; Oken et al. 2008).

Animal studies of CAM function provide a more mechanistic framework for understanding how CAM alterations contribute to the behavioural and neurochemical phenotypes in the GN model. Many of the CAMs down-regulated by GN in the adolescent CPu are crucial for excitatory synaptic morphology and function, and their interaction with modulatory neurotransmitter systems. For example, catenins promote formation of dendritic spines and excitatory synapses (Arikkath, 2009). Neuroligin 1 (Nlgn1), located mainly at glutamatergic synapses, modulates synaptic assembly (Graf et al. 2004; Nam & Chen, 2005) and glutamate release (Futai et al. 2007). Both Dscam and actinin contribute to synaptic plasticity by recruitment and clustering of glutamate receptors (Cabello et al. 2007; Li et al. 2009; Schulz et al. 2004). Thus, reduction of these transcripts in the GN-treated CPu may alter dendritic spines and excitatory synapses. Fyn, a protein tyrosine kinase, is particularly important in glutamate-dopamine cross-talk, modulating redistribution of NMDA receptor in a D1 receptor-dependent way (Dunah et al. 2004). Behavioural testing of GN-treated adolescent animals suggests that glutamate–dopamine interactions are altered, as GN-treated, but not normal, adolescent animals exhibit behavioural sensitization to cocaine (Franke et al. 2007).

Pathway analysis also confirmed that GN modifies CAM-related pathways in the CPu. GN decreased Rho GTPase-related pathways and reduced expression of ARP2/3, a complex that regulates dendritic spine and excitatory synapse formation (Wegner et al. 2008). These data further suggest that GN alters excitatory synapse formation in the CPu.

NAc

The NAc, involved in motivational control and reward (Ikemoto, 2007), showed few GN-induced alterations in CAMs. Given that this region is regulated by inputs from both the PFC and the Amy (Berendse et al. 1992; Kelley et al. 1982), NAc may be indirectly influenced by GN-induced alterations in other limbic structures.
Pathway analysis showed that several pathways, including GPCR-related, growth factor signalling, and Notch and Wnt/Frizzled, were down-regulated by GN. These alterations suggest that CAM-initiated signal transduction is modified by GN in the NAc in spite of normal CAM transcript. As with the CPu, GN down-regulated Rho small GTPase-related pathways, specifically reducing expression of the Nwasp transcript, a brain-specific regulator of the ARP2/3 complex (Wegner et al. 2008). Loss of function of Nwasp significantly decreases dendritic spine density and the number of excitatory synapses (Wegner et al. 2008). Thus, GN may reduce excitatory synapses in the ventral striatum while compromising structural and functional aspects of glutamatergic signalling in the dorsal striatum.

**PFC**

The PFC serves an executive and decision-making role (Arnsten, 1997; Osada et al. 2008) and regulates limbic system activity via projections to the CPu, NAc, and Amy (Berendse et al. 1992). Some of the changes in CAMs in the PFC were similar to those of striatal regions, including down-regulation of Dscam and contactin 4. The contactin system not only regulates neuronal interactions but also contributes to axonal myelination (Boyle et al. 2001; Tait et al. 2000). Given that the adolescent PFC matures substantially with myelination-induced increases in white matter (Huttenlocher, 1979; Sowell et al. 2001), down-regulation of contactins by GN might disturb normal development. Neurexin 3 (Nrxn3), down-regulated only in the PFC, has been linked to alcohol, nicotine, and opiate addiction (Bierut et al. 2007; Hishimoto et al. 2007; Lachman et al. 2007; Li & Burmeister, 2009). Animal studies suggest that Nrxn3 plays a preferential role in GABAergic synapse formation and function (Craig & Kang, 2007). During adolescence, the function and regulation of GABAergic interneurons in the PFC continue to mature (Tseng & O'Donnell, 2007). Given that abnormal myelination and GABA signalling in the PFC is observed in various neuropsychiatric disorders (Feng, 2008; Lewis et al. 1999; Steketee, 2005), reduction of Nrxn3 and contactins may link the cognitive and neurobehavioural disorders (Fergusson et al. 1998; Weissman et al. 1999).

Pathway analysis further revealed GN-induced alterations in the PFC. GN down-regulated pathways related to GPCR, growth factor, MAPK, Notch, and Wnt/Frizzled signalling. During adolescence, the PFC undergoes extensive synaptic pruning, which refines the circuitry to produce adult-like executive function (Spear, 2000). In contrast to striatal regions, GN up-regulated genes in the WASP/ARP2/3 family, which may reflect resistance to excitatory pruning. This idea is supported by our observed finding of a decrease in cell death pathways in the PFC of GN-treated adolescents (data not shown).

**Amy**

The Amy is an important mediator of the stress response, fear and anxiety-like behaviour, and emotional learning (Herman et al. 1996; Koob, 1999), and provides input to the PFC and NAc (Cunningham et al. 2002; Kelley et al. 1982). GN altered a unique set of CAMs in the Amy. Ncam1, down-regulated nearly 50%, and plays a particularly important role in emotional behaviour, with its genetic deletion impairing Amy-dependent fear conditioning (Stork et al. 2000). GN reduction of Ncam1 suggests that emotional behaviours are altered in this model, which could provide a mechanism for the mood disorders linked to MS (Fergusson et al. 1998; Weissman et al. 1999). Recently, enhancement of fear conditioning has been reported in mice after maternal nicotine consumption in drinking water (Paz et al. 2007).

GN also caused alterations in CAM-related pathways in the Amy, with mixed effects on signal transduction pathways. Similar to the PFC, some members of the WASP/ARP2/3 family were up-regulated, suggesting positive regulation of spine formation. The Amy also undergoes significant synaptic pruning in adolescence (Zehr et al. 2006), which may be altered by GN treatment.

**Conclusions**

The present study has suggested that CAMs and their intracellular signal transduction pathways are modified in GN-treated adolescent female rats, although the genes were only examined at the mRNA level. Importantly, these changes are region-specific in the limbic system, which provide a novel framework for viewing GN-induced alterations at the neurochemical and behavioural levels (Fig. 4). Specifically, in striatal regions, CAMs related to glutamate synapse structure and function are down-regulated, with the CPu showing the greatest vulnerability. Conversely, in the PFC, CAMs related to GABAergic synapse formation appear to be compromised, while pruning of excitatory synapses are impaired. In both the PFC and the CPu, CAMs related to myelination are also down-regulated, suggesting a defect in glia–neuron interactions. In the Amy, CAMs related to emotional
learning and memory are altered, and synaptic pruning of excitatory synapses may also be modified.

This circuitry has been highly implicated in the MS-linked neurobehavioural disorders that are observed clinically in adolescents. The late onset of these deficits probably relates to the substantial maturation of the limbic system during adolescence. Alterations of CAMs at this critical age may disturb the development of the limbic system and therefore suggest a neuronal mechanism underlying MS-linked neurobehavioural deficits.

Note
Supplementary material accompanies this paper on the Journal’s website (http://journals.cambridge.org/pnp). (For an example of the melt curves for four representative genes relative to that for GAPDH, see Supplementary Figure S1, available online.)

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Statement of Interest

None.

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