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Significant modulation of mitochondrial electron transport system by nicotine in various rat brain regions

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Abstract

The mitochondrion is the organelle responsible for generation of most usable energy in a cell. It also plays an important role in a series of physiological processes such as apoptosis and proliferation. Although previous studies have demonstrated that nicotine modulates the morphology and function of mitochondria, the mechanism(s) underlying these effects is largely unknown. In this study, using a microarray consisting of 4,793 clones derived from a mouse dopamine cDNA library, we profiled the gene expression patterns for six brain regions (amygdala, hippocampus, nucleus accumbens, prefrontal cortex, striatum, and ventral tegmental area) of female Sprague-Dawley rats subjected to nicotine treatment for 7 days through osmotic minipump infusion. We identified a number of genes and pathways, including components of the electron transport system of mitochondria, such as cytochrome c oxidase subunit I (*Mt-co1*), *Mt-co2*, *Mt-co3*, cytochrome b (*Mt-cyb*), mitochondrial NADH dehydrogenase 4 (*Mt-nd4*), and *Mt-nd6*, that were significantly modulated by nicotine in multiple brain regions. Bioinformatics analysis provided evidence that Gene Ontology categories related to the electron transport system were overrepresented in each brain region. Finally, the results from the microarray analysis were verified by quantitative RT-PCR for four representative genes. Together, our findings imply that mitochondria are involved in neuronal adaptation to chronic nicotine exposure.

Keywords

nicotine; microarray; gene expression; brain; mitochondria

INTRODUCTION

Nicotine is the primary addictive component of tobacco smoke (Dani and Harris 2005; Wonnacott et al. 2005). Through interactions with nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS), nicotine modulates various genes and cellular pathways

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in target neurons. For example, nicotine upregulates the expression of immediate early genes such as *c-Fos*, *c-Jun*, *Nurr77*, and *Egr1* (Ichino et al. 1999; Ichino et al. 2002; Shim et al. 2001). Also, mitogen-activated protein kinase (MAPK) signaling, phosphatidylinositol phosphatase signaling, growth factor signaling, and ubiquitin-proteasome pathways are modulated by chronic nicotine treatment (Konu et al. 2001; Li et al. 2004; Tang et al. 1998). Further, animal studies indicate that nicotine, like other drugs of abuse, stimulates the mesocorticolimbic dopamine system in the outer shell of the nucleus accumbens (NA) (Pontieri et al. 1996; Robbins and Everitt 1999) and modulates the release of other neurotransmitters such as norepinephrine, serotonin, acetylcholine, glutamate, and GABA (Barik and Wonnacott 2006; MacDermott et al. 1999; Schilström et al. 2000; Vizi and Lendvai 1999; Wonnacott 1997). Through its interaction directly or indirectly with these genes, pathways, and neurotransmitter systems, nicotine is involved in the regulation of various physiological processes (Dajas-Bailador and Wonnacott 2004; Dasgupta and Chellappan 2006; Dasgupta et al. 2006; Harkness and Millar 2002; Hwang and Li 2006; Kane et al. 2004; Robinson and Kolb 2004), as well as the morphology and function of different organelles (Cucina et al. 2000; Landais et al. 2005; Roy and Sabherwal 1998; Sallette et al. 2005).

Among the organelles affected by nicotine treatment, the mitochondrion is of particular interest because it is responsible for the generation of most usable energy and is the principal source of reactive oxygen species (ROS) in a cell. In addition, mitochondria play an important role in many physiological processes such as apoptosis and proliferation. However, the effect of nicotine on mitochondria appears to be complicated. For example, rats receiving nicotine showed alterations in mitochondrial ultrastructure such as irregular cristae and an electron-dense matrix in testis (Aydos et al. 2001), and dilated mitochondria were observed in nicotine-treated rat gingival fibroblasts (Lahmouzi et al. 2000). Fetal and neonatal exposure to nicotine also leads to detrimental changes in the mitochondria of postnatal pancreatic tissues, such as volume swelling, reduced respiratory chain activity, and increased oxidative stress (Bruin et al. 2008a; Bruin et al. 2008b; Bruin et al. 2008c). When colon adenocarcinoma-derived HCT-116 cells were treated with nicotine at a concentration similar to that in a smoker, an alteration in mitochondrial membrane potential and an increase in oxidative stress and apoptosis were noted (Crowley-Weber et al. 2003). In another study, nicotine proved to be antiapoptotic, modulating mitochondrial signaling pathways and inhibiting chemotherapy-induced apoptosis in lung cancer (Zhang et al. 2008). Nicotine treatment also can regulate the activity of mitochondria in the brain. Both acute and chronic nicotine treatment lead to increases in the expression of malate dehydrogenase and succinate dehydrogenase of the mitochondrial Krebs cycle in the rat frontoparietal cortex, but not in other regions of the brain (Turegano et al. 2001). There also is evidence that chronic nicotine treatment induces oxidative stress greatly and increases the amount of mitochondrial glutathione S-transferase, the major cellular antioxidant component, in rat brain (Bhagwat et al. 1998).

These studies clearly show the importance of mitochondria in the biological response to nicotine. However, the molecular mechanism(s) underlying the involvement of nicotine or abused substances in mitochondrial activities remains largely unknown. On the basis of a cDNA library constructed from purified embryonic ventral mesencephalic dopaminergic neurons, Barrett et al. (2001) developed a microarray consisting of 960 clones that was used to study gene expression changes during methamphetamine neurotoxicity in mouse brain. Expression of genes encoded by the mitochondrial genome, especially that of the cytochrome c oxidase subunit I of complex IV (*Mt-coI*), proved to be regulated significantly by amphetamine.

In this study, we sequenced and annotated more than 12,000 clones from the same cDNA library and fabricated a microarray for the non-redundant clones. The primary purpose was to identify and characterize gene expression profiles in six brain regions, namely the amygdala,

hippocampus, NA, prefrontal cortex (PFC), striatum, and ventral tegmental area (VTA), of Sprague-Dawley rats in response to chronic nicotine treatment.

MATERIALS AND METHODS

Animals and nicotine administration

Twenty-two-month-old female Sprague-Dawley rats (250–300 g; Harlan Industries, Inc., Indianapolis, IN) were housed in wire-bottom cages on a 12-h light/dark cycle and were allowed food and water *ad libitum*. Rats received continuous subcutaneous infusions of either nicotine bitartrate (3.15 mg/kg/day; expressed as the base) in saline (treatment group) or an equal volume of saline alone (control group) via osmotic minipumps for seven days (Malin 2001). Ten animals were used for the microarray experiment and ten for real-time PCR (RT-PCR) with five animals per group (i.e., control and nicotine-treated). All experimental protocols were approved by the Institutional Animal Use Committee at the University of Virginia.

Brain punches and RNA isolation

At the end of nicotine treatment, animals were euthanized with an overdose of sodium pentobarbital, and the brains were dissected out immediately after decapitation. After brain slices were cut in a dish containing ice-cold saline using a Stoelting tissue slicer (Chicago, IL), bilateral punches were excised from the amygdala, anterior area of the hippocampus, NA, PFC, striatum, and VTA using a bilateral 2.0-mm-diameter brain punch tissue set (myNeuroLab.com, St. Louis, MO) according to the coordinates of Paxinos and Watson (1986). All tissues were stored at -80°C until RNA isolation.

Total RNA was isolated separately from each brain region using Trizol reagent (Invitrogen, Carlsbad, CA). Because the amount of RNA obtained from the punches of each brain region was insufficient for the cDNA probe labeling required for array hybridization, each RNA sample was amplified using the protocol we described previously (Gutala et al. 2004; Konu et al. 2004).

Sequencing analysis of cDNA clones and microarray production

All cDNA clones printed on an array were sequenced. Briefly, plasmid DNA was extracted from the clones using a TempliphilTM DNA Sequencing Template Amplification kit (Amersham Biosciences, NJ) according to the manufacturer's protocol. All clones were sequenced using standard dye primer chemistry by an ABI PRISM[®] 310 Genetic Analyzer (Applied Biosystems, CA). The resulting sequences from 12,000 cDNA clones (ca. 300–800 nucleotides in length) were searched against various public genomic databases to determine the function and category of each clone.

On the basis of these sequence analysis and annotation results, we developed a cDNA microarray consisting of 4,793 non-redundant clones. Prior to printing, we prepared a cDNA insert of each clone. Briefly, *E. coli* containing the clones was grown overnight in GS-96 medium (BIO 101 Systems, Carlsbad, CA) followed by incubation of 10 μl of cell culture plus 90 μl of water at 95°C for 10 min to release plasmid DNA. Ten microliters of supernatant liquid containing plasmid DNA was added to a PCR cocktail containing *Taq* DNA polymerase in a total volume of 100 μl . The mixtures were denatured at 95°C for 3 min and then subjected to 35 cycles of denaturation (95°C , 30s), annealing (55°C , 30s), and extension (72°C , 2 min). The products were ethanol-precipitated, washed, and reconstituted in 20 μl of TE buffer. Thirty microliters of 80% dimethyl sulfoxide was added, and the mixture was printed on CMT-GAPS-coated slides (Corning, NY) using an OmniGrid MicroArrayer OGR-03 (Genomic Solutions, San Carlos, CA). Each cDNA clone was printed in duplicate on each slide. After arraying,

slides were UV-linked, washed in 3× SSC twice and in H₂O once, then incubated with I-Block at 60°C for 30 min, followed by washing and storage in an airtight desiccator until use.

Microarray experiment design, probe labeling, hybridization

A balanced dye-swap design was adopted for our microarray experiment. For each brain region, every RNA sample from the control group was randomly paired with a sample from the treatment group. Then, each of the paired samples was split into two aliquots, which were labeled separately with either Cy3 or Cy5. Next, the Cy3-labeled control probes and Cy5-labeled treatment probes were combined and hybridized to the same slide; the Cy5-labeled control probes and Cy3-labeled treatment probes were combined and hybridized to another slide. The probe was labeled and hybridized with the cDNA microarray using a 3DNA Array 900™ Expression Array Detection kit (Genisphere, Hatfield, PA) according to the manufacturer's protocol. Hybridized slides were first washed in 1× SSC–0.1% SDS buffer at 60°C for 10 min and then in 2× SSC–0.2× SSC buffer for 10 min at room temperature prior to being spun down briefly and scanned on a GenePix 4000B scanner (Axon Instruments Inc., Union City, CA). A total of 60 arrays were processed for this study with 10 slides per brain region.

Data normalization and statistical analysis

By scanning the arrays, we obtained the raw hybridization intensities for each element and then used the background-subtracted median intensity of each spot for further statistical analysis. We initially analyzed two replicates of each clone within a chip separately and then discarded 5% of the weakest and saturated spots in each replicate. An intensity-dependent normalization method, namely, locally weighted linear regress (*Lowess*), was used to normalize the data for each replicate (Yang et al. 2002). Clones with ≤ 6 valid measurements were excluded from further statistical analysis, and two replicates per chip were averaged and used as the measurement of a clone for a given sample. Further, the normalized ratios of each dye-swap pair were averaged to get the final expression measurement of each gene. Significance Analysis of Microarray (SAM) (Tusher et al. 2001) was used to detect the significantly regulated genes, and a false discovery rate (FDR) value of 0.10 was used for all the brain regions.

A bioinformatics method, namely Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005), was used to detect overrepresented biochemical processes. The genes in our dataset were categorized by their Gene Ontology (GO) annotation. Briefly, the annotation file for mouse was downloaded from the GO website (www.geneontology.org) (Ashburner et al. 2000). All the sequence-verified genes included in the chip were searched against this annotation, and the biological process, molecular function, and cellular component categories of each gene were extracted. Each gene was assigned to one or more GO categories, and several highly related categories were combined because only a few genes were represented in each process. The constructed gene sets and the gene expression data were loaded into GSEA to detect enriched GO categories.

Validation of microarray results by quantitative real-time RT-PCR (qRT-PCR)

Real-time PCR was carried out for four representative genes, namely, ATP synthase (*ATP5j*; ATP synthase, H⁺ transporting, F0 complex, subunit F6), *Mt-co1*, mitochondrial NADH dehydrogenase 6 (*Mt-nd6*), and nuclear receptor subfamily 4, group A, member 1 (*Nurr1*) for all the six brain regions. The primers and *TaqMan* probes for the four genes were purchased from Applied Biosystems (Foster City, CA), and quantitative RT-PCR analysis was performed as described previously (Konu et al. 2004). The PCR was carried out in a volume of 20 µl using the *TaqMan* assay on an ABI 7000 Sequence Detection System. The 18S ribosomal RNA was used as an internal control to normalize the expression patterns of genes of interest. Data

analysis was performed using a comparative C_t method (Winer et al. 1999). For each verified gene, five independent replicates were analyzed for each experimental group.

RESULTS

Identification of genes significantly modulated by nicotine

The number of differentially expressed genes differed greatly among the six brain regions. At 10% FDR, we found that 16, 28, 12, 15, 8, and 27 genes were significantly regulated in the amygdala, hippocampus, NA, PFC, striatum, and VTA, respectively (Table 1). In the amygdala, several genes involved in mitochondrial function, specifically electron transport, were upregulated, including *Atp5j*, *Mt-co1*, *Mt-cyb*, and *Mt-nd6*. Multiple genes involved in transcription also were modulated, such as chromodomain helicase DNA-binding protein 6 (*Chd6*), zinc finger protein CCHC domain containing 12 (*Zcchc12*), and zinc finger protein interacting with K protein 1 (*Zik1*). We also observed regulation of protein modification- and degradation-related genes, which included chaperonin subunit 5 (*Cct5*) and proteasome (prosome, macropain) activator subunit 4 (*Psmc4*). In the hippocampus, mitochondrial electron transport genes were upregulated; e.g., *Atp5j*, *Mt-co1*, *Mt-nd2*, and *Mt-nd4*, as were several genes involved in protein translation, such as acidic ribosomal phosphoprotein P0 (*Arbp*), eukaryotic translation initiation factor 3, subunit 8 (*Eif3s8*), and tumor protein, translationally controlled 1 (*Tpt1*). We also observed alterations in the expression of genes related to signal transduction, such as CD24a (*Cd24a*), neurotrophic tyrosine kinase receptor type 2 (*Ntrk2*), and polo-like kinase 2 (*Plk2*).

In contrast to the amygdala and hippocampus, in the NA, mitochondrial electron transport genes were downregulated by nicotine, as evidenced by the suppression of *Mt-co1*, *Mt-cyb*, and *Mt-nd6*. The expression of genes related to other functions also was reduced. In the PFC, we detected the induction of genes involved in electron transport (e.g., *Atp5j*, *Mt-co1*, *Mt-cyb*, and *Mt-nd6*), protein modification or transport (*Canx*, *Nedd4*, and *Stx12*), and signal transduction (*Ywhae*). In the striatum, some genes were suppressed by nicotine, such as *Mt-nd6*, secretogranin II (*Scg2*), and voltage-gated sodium channel type III beta (*Scn3b*), whereas others were induced, such as insulin-like growth factor binding protein 5 (*Igfbp5*), nuclear receptor subfamily 4 group A member 1 (*Nurr1*), and Stathmin 1 (*Stmn1*). Among the genes detected in the VTA, those related to electron transport (e.g., *Atp5j*, *Mt-co1*, *Mt-cyb*, and *Mt-nd6*), as well as mRNA processing (e.g., heterogeneous nuclear ribonucleoprotein H1 [*Hnrph1*], PRP18 pre-mRNA processing factor 18 homolog [*Prpf18*], and RNA-binding motif protein 16 [*Rbm16*]) were upregulated. Whereas a number of transcription regulators such as forkhead box A1 (*Foxa1*), mortality factor 4 like 1 (*Morf4l1*), and melanocyte-specific gene-related gene 1 (*Mrg1*) were upregulated, melanoma antigen family D member 1 (*Maged1*) was suppressed by nicotine. Despite the relative diversity of genes differentially regulated by nicotine in each brain region, it is clear that one or more genes related to the mitochondrial electron transport system were significantly regulated in all these regions.

Gene Ontology categories enriched in each brain region

Although identification of significantly modulated genes provides clues about the expression pattern of each brain region's response to nicotine, we were more interested in identifying the biological pathways potentially regulated by nicotine in these regions. By searching the annotated database with the GSEA algorithm for all genes included in our chip on the basis of GO information, we identified the overrepresented categories (Table 2). For the amygdala, nine enriched GO categories were identified, among which seven (i.e., electron transport, microtubule-based movement, mitochondrial function, protein folding, protein polymerization, DNA-dependent regulation of transcription, and zinc ion binding) were positively associated with nicotine exposure. Conversely, the GO categories related to cell

proliferation and protein biosynthesis were negatively related to the treatment. For the hippocampus, eight GO categories were overrepresented, including three related to mitochondria (i.e., mitochondrial respiratory chain complex I, mitochondrion, and electron transport) and two others (namely, regulation of transcription from RNA polymerase II promoter and signal transduction), all of which were positively related to nicotine treatment. On the other hand, the GO categories related to protein biosynthesis and modification and nervous system development were negatively related to nicotine exposure. For the NA, eight processes were overrepresented; two of them (i.e., proteolysis and protein biosynthesis) were upregulated, and six (i.e., calcium ion binding, electron transport, mitochondrion, mRNA processing, organ morphogenesis, and RNA splicing) were downregulated. For the PFC, eight GO categories (i.e., ATP synthesis-coupled proton transport, electron transport, mitochondrion, mitochondrial respiratory chain complex I, ion transport, neuron migration, protein folding, and response to stress) were positively associated with nicotine treatment, whereas only protein biosynthesis was negatively regulated by nicotine. For the striatum, five GO categories (i.e., axon guidance, cell proliferation, mitochondrial respiratory chain complex I, mitochondrion, and protein biosynthesis) were suppressed, and three (i.e., microtubule-based process, protein polymerization, and protein amino acid phosphorylation) were induced. For the VTA, eight GO categories were upregulated, including those related to mitochondrial function (i.e., electron transport, mitochondrial respiratory chain complex I, mitochondrion, and proton-transporting two-sector ATPase complex), ion transport, small GTPase-mediated signal transduction, and two others (axon and cell adhesion). We also identified a few downregulated GO categories, including protein biosynthesis, protein complex, ribonucleoprotein complex, ribosome biogenesis and assembly, RNA binding, RNA splicing, and structural constituent of ribosome.

Similar to the expression patterns of genes modulated by nicotine, the overrepresented GO categories were region specific. This indicates the differential responses of these regions to nicotine treatment. Despite these differences, the GO categories related to the mitochondrion were enriched in all six brain regions. In the amygdala, hippocampus, PFC, and VTA, the GSEA scores (Table 2) for mitochondria-related GO categories all were positive, indicating that nicotine exposure preferentially upregulated the expression of a subset of genes, the leading-edge subset (Subramanian et al. 2005), whereas in the NA and striatum, the GSEA scores for mitochondria-related GO categories were all negative, indicating the genes of leading-edge subset were suppressed by nicotine. Specifically, in each brain region, the mitochondrial electron transport system or part of it (such as complex I or ATP synthase) were among the enriched GO categories detected by GSEA.

Regulation of the electron transfer system by chronic nicotine treatment

As shown above, both SAM and GSEA analyses indicated the mitochondrial electron transport system is regulated by chronic nicotine exposure in different brain regions. Figure 1 provides a summary of the expression changes of ten genes in these six regions. In the amygdala, two subunits of NADH dehydrogenase (complex I), *Mt-nd4* and *Mt-nd6*, were significantly regulated, whereas two other subunits (*Mt-nd1* and *Mt-nd2*) were slightly upregulated. Similarly, the expression of these genes was elevated in other brain regions, namely the hippocampus, PFC, and VTA. However, in the NA and striatum, their expression was decreased by nicotine. *Mt-cyb* of complex III was significantly induced by nicotine in the amygdala, PFC, and VTA, whereas it was suppressed in the NA. We also observed a slight elevation and inhibition of its expression in the hippocampus and striatum, respectively. The expression of three mitochondrial-DNA encoded subunits of complex IV (i.e., *Mt-co1*, *Mt-co2*, and *Mt-co3*) was differentially elevated in the amygdala, hippocampus, PFC, and VTA, whereas their expression was somewhat suppressed in the NA and striatum. We also identified two upregulated subunits of ATP synthase, *Atp5b* and *Atp5j*, in the amygdala, hippocampus,

PFC, and VTA. *Atp5j* was downregulated or slightly suppressed in the NA or striatum. Consistent with the pattern of individual genes, the electron transport pathway was overrepresented in the amygdala, hippocampus, PFC, and VTA and was underrepresented in the NA and striatum.

Verification of the genes by quantitative real-time PCR

Quantitative RT-PCR was performed on four representative genes in different brain tissues from an independent animal experiment involving the same treatments as the corresponding groups in the microarray experiment. Three of these genes (*Atp5j*, *Mt-co1*, and *Mt-nd6*) are related to the electron transport system and were modified in multiple brain regions. Another gene, *Nurr1*, which is essential for differentiation of dopaminergic neurons, was significantly regulated by nicotine in the striatum and VTA. Figure 2 presents the fold change along with the standard deviation of the four genes in response to nicotine treatment relative to the control samples, as detected by microarray and quantitative RT-PCR. This analysis revealed significant regulation of *Atp5j* in all the brain regions except the striatum, which is consistent with the result from the microarray analysis. Furthermore, comparison of the fold changes of each gene detected by the two molecular techniques across the six brain regions revealed a correlation coefficient of 0.85 ($p = 0.032$), 0.92 ($p = 9.7 \times 10^{-3}$), 0.90 ($p = 0.014$), and 0.82 ($p = 0.044$) for *Atp5j*, *Mt-co1*, *Mt-nd6*, and *Nurr1*, respectively. Although only four representative genes were selected for verification by RT-PCR, a comparison of the two methods demonstrates that the results from our microarray analyses were reproducible and reliable.

DISCUSSION

Previous studies have shown that nicotine treatment not only causes swelling and structural remodeling of mitochondria in cells of various tissues, including the brain (Jin and Roomans 1997; Maritz and Thomas 1994; Onal et al. 2004; Zimmerman and McGeachie 1987), but also regulates processes such as protein turnover (Katyare and Shallom 1988), enzyme activity (Barbieri et al. 1989; Galvin et al. 1988), and generation of ROS (Bhagwat et al. 1998; Soto-Otero et al. 2002). Here, we further demonstrate that the genes of each complex in the electron transfer system are modulated by chronic nicotine treatment. The consistent changes in the genes included in complexes I, III, IV, and V of the electron transport system in multiple brain regions indicate the importance of this system in the interaction of the CNS with nicotine.

The differential expression of the electron transport system in the six brain regions is consistent with the concept that these regions have different cellular and physiological functions. Because these regions are divergent in their cellular compositions and behaviors (e.g., neurotransmitter release, receptor expression, intracellular signaling cascades) in order to manage specific physiological activities, and because they have different energy consumption and reaction mechanisms in response to external disturbances, a region-specific transcriptional response of the electron transport system to chronic nicotine treatment would be expected. The different responses of the electron transport system in these regions to nicotine may be associated with the specific role of each region in the development of nicotine dependence. For example, the upregulation of the expression of this system in the amygdala, hippocampus, PFC, and VTA may represent the neuron's effort to increase its energy-generating capacity or an acceleration of protein turnover. On the other hand, the suppression of the electron transport system in the NA and striatum may reflect a reduction in energy requirements in these regions or other slowdown in metabolism.

Defects of any gene in the electron transport system can disturb this system and interrupt the activity of mitochondria. For example, mutations of *Mt-cyb* have been associated with deficiency in complex III function and impaired mitochondrial activity (Dumoulin et al. 1996; Legros et al. 2001). Moreover, dysfunction of the mitochondrial electron transport

system is implicated in several pathogenic conditions, including neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Lin and Beal 2006). A significant and selective reduction of complex I activity was found in the substantia nigra of patients with PD. It has been reported also that a null mutation of *Mt-cyb* results in deficiency of complex III and an increase in free radical production in brain tissues of patients with PD (Rana et al. 2000). A deficiency of cytochrome c oxidase (complex IV) also can cause defects in energy metabolism in the brain and peripheral tissue, which may contribute to the neurodegenerative condition in AD patients (Grazina et al. 2006; Qiu et al. 2001). In these diseases, the damage to mitochondria or their components may increase production of ROS, which leads to the death of the neuron.

On the other hand, epidemiological studies have revealed an inverse correlation between smoking and the incidences of PD and AD (Picciotto and Zoli 2008; Quik 2004). Nicotine is believed to be the chemical responsible for such beneficial effects because it can stimulate the dopaminergic system and be neuroprotective in such disorders. Several mechanisms have been suggested to explain the relationship, and the most common one is that nicotine acts as an antioxidant (Obata et al. 2002; Soto-Otero et al. 2002). Also, nicotine interacts directly with the electron transport system. For example, nicotine may compete with NADH and inhibit mitochondrial NADH-ubiquinone reductase activity (Cormier et al. 2001, 2003) or regulate the electron leak at the site of complex I (Xie et al. 2005), with a consequent reduction in the amounts of ROS.

The simultaneous regulation of multiple complex genes in the electron transport system in the six brain regions observed in the current work may indicate a beneficial effect of nicotine on the nervous system. However, the mechanisms underlying the modulation of nicotine-electron transport system interactions are unclear. One of the plausible possibilities is that nicotine, which can enter cells by permeating the membrane (Sallette et al. 2005), acts as an antioxidant (Obata et al. 2002; Soto-Otero et al. 2002) and eliminates the ROS generated by mitochondria. Such interaction may eventually lead to modulation of the activity of the electron transport system, thereby affecting ATP production (Halestrap 1987, 1989; Lim et al. 2002). It also is possible that nicotine interacts directly with one or more complexes of the electron transport system (Cormier et al. 2001, 2003; Xie et al. 2005). Because the electron transport complexes are physically interdependent, regulation of any of their genes may result in modulation of the expression of genes of other complexes. For example, mutation of complex I genes results not only in complex I deficiency, but also in reduction of complex III activity (Budde et al. 2000). Further, mutations of the complex III gene *Mt-cyb* cause combined instability and reduced activity of complexes I and complex III (Blakely et al. 2005). On the other hand, nicotine may regulate other biochemical pathways in mitochondria through intracellular signaling pathways. In the nervous system, nicotine exerts its effect mainly through its interaction with nAChRs. Activation of nAChRs causes membrane depolarization and directly and indirectly increases the intracellular calcium concentration (McKay et al. 2007). The variation of cytosolic calcium concentration may influence the calcium homeostasis of mitochondria (Rimessi et al. 2008). The Ca^{2+} homeostasis is fundamental to the function of mitochondria and related to the generation of ATP, ROS, and a series of signaling pathways critical to cell life (Rego and Oliveira 2003; Rimessi et al. 2008; Szabadkai and Duchen 2008). Recent work also has demonstrated that these pathways, including the protein kinase A (PKA) and C (PKC) signaling cascades, are important regulators of mitochondrial energy production. In particular, they are able to act on the electron transport system through tyrosine phosphorylation (Huttemann et al. 2007; Salvi et al. 2005). Directly or indirectly, nicotine can trigger various intracellular signaling pathways, including PKC and MAPK, in neurons of different brain regions (Konu et al. 2001; Li et al. 2004; Quik 2004). Thus, the regulation of the intracellular signaling pathways in response to nicotine may lead to regulation of the electron transport system and, eventually, modulation of mitochondrial function.

In summary, we have demonstrated regulation of electron transport gene expression in multiple rat brain regions in response to chronic nicotine treatment. This suggests that chronic nicotine exposure modulates mitochondrial function, and likely disturbs cellular energy metabolism and the intracellular concentration of oxidative free radicals. The detection of this effect should help us to understand the molecular mechanisms underlying nicotine addiction, and may also suggest molecular targets to consider in human nicotine dependence mechanisms. Future challenges include defining the precise molecular mechanisms that underlie the response of the electron transport system to chronic nicotine treatment and to discover how these mechanisms interact with other cellular events, such as signal transduction, in addicted human smokers and nicotine-treated animals.

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Figure 1.

Expression of genes involved in electron transport system in six brain regions in response to chronic nicotine treatment. Ten genes are involved in four complexes; i.e., I, III, IV, and V (ATP synthase). The X axis shows gene(s) involved in each protein complex (given in four panels). For comparison, expressions of each gene in the six regions are grouped. The Y axis represents fold expression change relative to the control in six regions for genes in four complexes. (A) Complex I, four genes; i.e., *Mt-nd1*, *Mt-nd2*, *Mt-nd4*, and *Mt-nd6*; (B) Complex III, *My-cyb* is included; (C) Complex IV, three genes; i.e., *Mt-co1*, *Mt-co2*, and *Mt-co3*; and (D) Complex V (ATP synthase), *Atp5b* and *Atp5j*. Abbreviations: *Atp5b* (ATP synthase, H⁺ transporting, F1 complex, beta subunit), *Atp5j* (ATP synthase, H⁺ transporting, F0 complex, subunit F6); *Mt-co1* (cytochrome c oxidase subunit I); *Mt-co2* (cytochrome c oxidase subunit II); *Mt-co3* (cytochrome c oxidase subunit III); *Mt-cyb* (cytochrome b); *Mt-nd1* (NADH dehydrogenase subunit I); *Mt-nd2* (NADH dehydrogenase subunit II); *Mt-nd4* (NADH dehydrogenase subunit IV); and *Mt-nd6* (NADH dehydrogenase subunit VI). Error bars represent SD for both figures 1 and 2.

Figure 2.

Comparative plot of data from microarray and real-time RT-PCR verification for four representative genes. The Y axis represents fold change of expression in brain regions of animal receiving nicotine treatment compared with average expression in brain tissue of control animal for that particular gene. The X axis represents the six brain regions analyzed; i.e., amygdala, hippocampus, NA, PFC, striatum, and VTA. For RT-PCR result, expression data of each gene were normalized to average of control samples. (A) *Atp5j*; (B) *Mt-co1*; (C) *Mt-nd6*; and (D) *Nurr1*. Abbreviations: *Atp5j* (ATP synthase, H⁺ transporting, F₀ complex, subunit F₆); *Mt-co1* (cytochrome c oxidase subunit I); *Mt-nd6* (NADH dehydrogenase subunit VI); *Nurr1* (nuclear receptor subfamily 4, group A, member 1).

Table 1

Genes significantly regulated by chronic nicotine treatment in each of the brain regions (FDR = 0.1)

Brain Region	Gene Symbol	Gene Name	Fold change $\pm$ SD
Amygdala	Ankrd12	Ankyrin repeat domain 12	0.85 $\pm$ 0.10
	Atp5i	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit F6	1.38 $\pm$ 0.25
	Cct5	Chaperonin containing Tcp1, subunit 5 (epsilon)	1.17 $\pm$ 0.09
	Chd6	Chromodomain helicase DNA binding protein 6	1.20 $\pm$ 0.07
	Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	1.16 $\pm$ 0.06
	LOC100046343	Similar to CCR4-NOT transcription complex, subunit 6	1.22 $\pm$ 0.12
	Mt-co1	Mitochondrially encoded cytochrome c oxidase I	1.32 $\pm$ 0.13
	Mt-co2	Mitochondrially encoded cytochrome c oxidase II	1.23 $\pm$ 0.08
	Mt-co3	Mitochondrially encoded cytochrome c oxidase III	1.26 $\pm$ 0.07
	Mt-cyb	Mitochondrially encoded cytochrome b	1.31 $\pm$ 0.12
	Mt-nd4	Mitochondrially encoded NADH dehydrogenase 4	1.19 $\pm$ 0.11
	Mt-nd6	Mitochondrially encoded NADH dehydrogenase 6	1.33 $\pm$ 0.21
	Psme4	Proteasome (prosome, macropain) activator subunit 4	0.76 $\pm$ 0.10
	Scg2	Secretogranin II (chromogranin C)	1.19 $\pm$ 0.03
	Zcchc12	Zinc finger, CCHC domain containing 12	0.84 $\pm$ 0.10
	Zik1	Zinc finger protein interacting with K protein 1	0.85 $\pm$ 0.09
Hippocampus	Arbp	Acidic ribosomal phosphoprotein P0	1.33 $\pm$ 0.14
	Atp5i	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit F6	1.23 $\pm$ 0.11
	Cd24a	CD24 antigen	1.21 $\pm$ 0.08
	Dnaib6	DnaI (Hsp40) homolog, subfamily B, member 6	0.85 $\pm$ 0.08
	Efnb3	Ephrin B3	0.74 $\pm$ 0.11
	Eif3s8	Eukaryotic translation initiation factor 3, subunit 8	1.16 $\pm$ 0.09
	Fhl1	Four and a half LIM domains 1	0.81 $\pm$ 0.07
	Ktn1	Kinectin 1	1.24 $\pm$ 0.14
	Matr3	Matrin 3	0.84 $\pm$ 0.07
	Mt-co1	Mitochondrially encoded cytochrome c oxidase I	1.31 $\pm$ 0.06
	Mt-co2	Mitochondrially encoded cytochrome c oxidase II	1.25 $\pm$ 0.23
	Mt-co3	Mitochondrially encoded cytochrome c oxidase III	1.19 $\pm$ 0.14
	Mt-nd2	Mitochondrially encoded NADH dehydrogenase 2	1.22 $\pm$ 0.09
	Mt-nd4	Mitochondrially encoded NADH dehydrogenase 4	1.17 $\pm$ 0.07
	Mt-rnr2	Mitochondrially encoded 16S RNA	1.16 $\pm$ 0.11
	Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1.24 $\pm$ 0.07
	Pbx1	Pre-B-cell leukemia transcription factor 1	1.27 $\pm$ 0.11
	Plk2	Polo-like kinase 2	1.34 $\pm$ 0.08
	Ptma	Prothymosin alpha	1.23 $\pm$ 0.10
	Rps6ka2	Ribosomal protein S6 kinase, polypeptide 2	0.84 $\pm$ 0.06
	Scg2	Secretogranin II	0.79 $\pm$ 0.07
	Scn3b	Sodium channel, voltage-gated, type III, beta	1.32 $\pm$ 0.06
	Sept7	Septin 7	1.24 $\pm$ 0.12
	Sez6	Seizure related gene 6	1.22 $\pm$ 0.15
	Sumo1	SMT3 suppressor of mif two 3 homolog 1	0.77 $\pm$ 0.15
	Tpt1	Tumor protein, translationally-controlled 1	1.22 $\pm$ 0.09
	Trim2	Tripartite motif protein 2	1.17 $\pm$ 0.11
	Zcchc12	Zinc finger, CCHC domain containing 12	0.73 $\pm$ 0.08
	Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	0.79 $\pm$ 0.07
	LOC100046343	Similar to CCR4-NOT transcription complex, subunit 6	0.80 $\pm$ 0.09
	Maged1	Melanoma antigen, family D, 1	0.81 $\pm$ 0.09
	Mt-co1	Mitochondrially encoded cytochrome c oxidase I	0.77 $\pm$ 0.07
	Mt-co2	Mitochondrially encoded cytochrome c oxidase II	0.80 $\pm$ 0.13
	Mt-co3	Mitochondrially encoded cytochrome c oxidase III	0.80 $\pm$ 0.09
	Mt-cyb	Mitochondrially encoded cytochrome b	0.79 $\pm$ 0.08
	Mt-nd2	Mitochondrially encoded NADH dehydrogenase 2	0.77 $\pm$ 0.13
	Mt-nd4	Mitochondrially encoded NADH dehydrogenase 4	0.72 $\pm$ 0.07

Table 2

Enriched Gene Ontology categories identified by GSEA algorithm for each brain region

Gene Ontology Categories	No. of Genes	NES*	Permutation <i>p</i> -value
<i>Amygdala</i>			
Electron transport	10	1.83	0.008
Microtubule-based movement	9	1.71	0.013
Cell proliferation	6	-1.68	0.016
Protein folding	11	1.75	0.027
Protein biosynthesis	37	-1.81	0.030
Zinc ion binding	38	1.76	0.035
Protein polymerization	8	1.73	0.037
Regulation of transcription, DNA-dependent	36	1.76	0.045
Mitochondrion	28	1.67	0.048
<i>Hippocampus</i>			
Mitochondrial respiratory chain complex I	5	1.88	0.003
Mitochondrion	30	1.75	0.011
Electron transport	11	1.80	0.014
Protein biosynthesis	43	-1.79	0.018
Protein modification	7	-1.72	0.019
Positive regulation of transcription from RNA polymerase II promoter	6	1.71	0.024
Signal transduction	15	1.68	0.036
Nervous system development	12	-1.72	0.048
<i>Nucleus accumbens</i>			
Mitochondrion	26	-1.71	0.005
Proteolysis	9	1.50	0.017
Calcium ion binding	17	-1.60	0.018
mRNA processing	13	-1.58	0.037
Organ morphogenesis	5	-1.44	0.038
Electron transport	10	-1.54	0.039
Protein biosynthesis	39	1.46	0.041
RNA splicing	12	-1.45	0.044
<i>Prefrontal cortex</i>			
Mitochondrion	26	1.53	0.001
Mitochondrial respiratory chain complex I	5	1.58	0.003
Electron transport	10	1.65	0.003
Neuron migration	5	1.71	0.006
Protein folding	10	1.51	0.008
Protein biosynthesis	39	-1.63	0.009
ATP synthesis coupled proton transport	3	1.49	0.030
Ion transport	11	1.47	0.035
Response to stress	8	1.54	0.045
<i>Striatum</i>			
Mitochondrial respiratory chain complex I	5	-1.85	0.001
Microtubule-based process	10	1.68	0.001
Protein polymerization	8	1.61	0.001
Axon guidance	6	-1.66	0.018
Mitochondrion	26	-1.73	0.019
Protein amino acid phosphorylation	16	1.57	0.033
Protein biosynthesis	38	-1.53	0.034
Cell proliferation	6	-1.51	0.042
<i>Ventral tegment area</i>			
Ribonucleoprotein complex	25	-1.80	<0.001
Structural constituent of ribosome	23	-1.71	0.001
Mitochondrial respiratory chain complex I	5	1.69	0.001
Ribosome biogenesis and assembly	15	-1.76	0.002
RNA binding	27	-1.78	0.004
Electron transport	9	1.61	0.005
Axon	8	1.48	0.007
Protein biosynthesis	37	-1.55	0.009
RNA splicing	10	-1.66	0.012
Mitochondrion	23	1.65	0.014
Small GTPase mediated signal transduction	9	1.55	0.017
Cell adhesion	7	1.73	0.018
Ion transport	9	1.54	0.020
Proton-transporting two-sector ATPase complex	4	1.63	0.028
Protein complex	14	-1.78	0.037

* NES, normalized enrichment score.