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Genome-wide expression analysis reveals diverse effects of acute nicotine exposure on neuronal function-related genes and pathways

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Previous human and animal studies demonstrate that acute nicotine exposure has complicated influences on the function of the nervous system, which may lead to long-lasting effects on the behavior and physiology of the subject. To determine the genes and pathways that might account for long-term changes after acute nicotine exposure, a pathway-focused oligoarray specifically designed for drug addiction research was used to assess acute nicotine effect on gene expression in the neuron-like SH-SY5Y cells. Our results showed that 295 genes involved in various biological functions were differentially regulated by 1 h of nicotine treatment. Among these genes, the expression changes of 221 were blocked by mecamylamine, indicating that the majority of nicotine-modulated genes were altered through the nicotinic acetylcholine receptors (nAChRs)-mediated signaling process. We further identified 14 biochemical pathways enriched among the nicotine-modulated genes, among which were those involved in neural development/synaptic plasticity, neuronal survival/death, immune response, or cellular metabolism. In the genes significantly regulated by nicotine but blocked by mecamylamine, 13 enriched pathways were detected. Nine of these pathways were shared with those enriched in the genes regulated by nicotine, including neuronal function-related pathways such as glucocorticoid receptor signaling, p38 MAPK signaling, PI3K/AKT signaling, and PTEN signaling, implying that nAChRs play important roles in the regulation of these biological processes. Together, our results not only provide insights into the mechanism underlying the acute response of neuronal cells to nicotine but also provide clues to how acute nicotine exposure exerts long-term effects on the nervous system.

Keywords: nicotine, neurons, mecamylamine, gene expression, p38

INTRODUCTION

Nicotine is the dependence-inducing constituent underlying tobacco smoking. Through interactions with nicotinic acetylcholine receptors (nAChRs) in the central nervous system (CNS), nicotine exposure not only stimulates the mesocorticolimbic dopamine system in the outer shell of the nucleus accumbens (NAc) and other brain regions (Benwell and Balfour, 1992, 1997; Gaddnas et al., 2001; Tammimaki et al., 2006) but also modulates the release of other neurotransmitters such as norepinephrine, serotonin, and GABA (Kenny et al., 2001; Barik and Wonnacott, 2006, 2009). Prolonged nicotine treatment can regulate the expression of genes/proteins involved in various functions such as *ERK1/2* and *CREB* (Brunzell et al., 2003), as well as their downstream targets such as *c-FOS* and *FOSB* (Pagliusi et al., 1996; Nisell et al., 1997; Soderstrom et al., 2007). Further, biochemical pathways underlying various physiological processes; e.g., MAPK signaling, phosphatidylinositol phosphatase signaling, growth factor signaling, and ubiquitin–proteasome pathways, are modulated by nicotine (Tang et al., 1998; Konu et al., 2001; Li et al., 2004). Through its direct or indirect interaction with these genes and biological pathways, nicotine is involved in the regulation of various physiological processes, such as learning and memory, angiogenesis, energy metabolism, synaptic function, response to oxidative stress, and addiction (Harkness and Millar, 2002; Dajas-Bailador and Wonnacott, 2004; Kane et al., 2004; Robinson and Kolb, 2004; Dasgupta and Chellappan, 2006; Dasgupta et al., 2006; Hwang and Li, 2006).

Although nicotine dependence is attributed mainly to repeated and chronic nicotine exposure, acute administration also can evoke noticeable physiological effects. For example, in humans, acute nicotine exposure, while significantly increasing the plasma nicotine concentration (Argacha et al., 2008), modulates a series of physiological processes such as aortic wave reflection, arterial stiffness, and plasma asymmetrical dimethylarginine (ADMA) concentration (Adamopoulos et al., 2009). Experimentally, acute nicotine administration can significantly increase activation in multiple regions of mouse brain (Suarez et al., 2009) and induce spinal motor circuit output in embryonic zebrafish (Thomas et al., 2008). Previous studies have demonstrated the beneficial effect of acute nicotine exposure on cognitive performance (Foulds et al., 1996; Phillips and Fox, 1998; Ernst et al., 2001; Kumari et al., 2003), as well as its antidepressant effect (Tizabi et al., 2009).

Various genes and biochemical processes are involved in the biological response to acute nicotine exposure. These include immediate-early genes such as *c-FOS*, *c-JUN*, *NURR77*, and *EGR1* (Ichino et al., 1999, 2002; Salminen et al., 1999) and the dendritically targeted early response genes activity-regulated

cytoskeleton-associated protein (*ARC*) and dendrin in specific brain regions of rats (Schochet et al., 2005, 2008; Schmitt et al., 2008). Acute nicotine exposure not only increases the activity of dopaminergic neurons and promotes dopamine release (Levin and Rose, 1995), but also enhances dopamine turnover and metabolism (Grenhoff and Svensson, 1988). The administration of a single dose of nicotine enhances the synthesis and release of striatal dynorphin, a component of the circuit promoting negative motivational and affective states, possibly under the regulation of dopamine and glutamate (Isola et al., 2009). Acute nicotine exposure may impair nNOS-dependent dilation of cerebral arterioles via a mechanism related to the increase in oxidative stress (Arrick and Mayhan, 2007). Even a few minutes of nicotine exposure can significantly suppress arachidonic acid signaling in various brain regions in rats (Chang et al., 2009). In mice, acute administration of a low dose of nicotine increases phosphor-ERK1/2 immunoreactivity in the NAc, prefrontal cortex, and some other regions innervated by the mesolimbic dopamine pathway (Barik and Wonnacott, 2009).

Clearly, acute nicotine exposure can evoke multiple effects in the neuronal system, but its action mechanism is not completely understood. The neuron-like human neuroblastoma cell line SH-SY5Y endogenously expresses various nAChR subunits (e.g., α3, α5, α7, β2, and β4; Gould et al., 1992; Peng et al., 1994), which are assembled to form different types of nAChRs, such as α3*-nAChR or homomeric α7-nAChR receptors. The cells therefore provide a suitable *in vitro* model to investigate alterations in nAChRs in the presence of cholinergic ligands and have been widely used in nicotine-related study. In an earlier study, 17 genes with diverse biological functions were found to be significantly regulated in SH-SY5Y cells after exposed to 1 mM nicotine for 1 h (Dunckley and Lukas, 2003). The authors further showed that most of these genes were regulated via nAChRs-mediated mechanisms. However, because of the limitation of array technologies and bioinformatics tools at that time, a detailed analysis, especially at the biological pathway level, was impossible. With the rapid progress in the fields of genomics and bioinformatics, a detailed and comprehensive profiling of the genes and pathways responsive to acute nicotine treatment becomes feasible and necessary. In this study, by using an established and well-tested *in vitro* model in the field, we investigated the relation between acute nicotine exposure and neuronal function by examining the genes and biochemical pathways differentially regulated by nicotine in SH-SY5Y cells using a customized pathway-focused microarray (Cao et al., 2011; Wei et al., 2011).

MATERIALS AND METHODS

CELL CULTURE AND DRUG TREATMENT

The human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in a 1:1 mixture of ATCC-formulated Eagle's Minimal Essential Medium and F12 medium supplemented with 10% fetal bovine serum (GIBCO Invitrogen, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2. At about 80% confluence, cells were treated with 1 mM nicotine (calculated as free base), 3 μM mecamylamine (nicotinic receptor antagonist), 1 mM nicotine in combination with 3 μM mecamylamine, or no drug (controls) for 1 h. For each experimental group, six independent cultures were prepared ($N = 6$).

RNA ISOLATION

Total RNA was isolated separately from each of the drug-treated and control cultures using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To eliminate potential residual DNA contamination, the RNA samples were treated with RNase-free DNase I at 37°C for 30 min followed by inactivation at 65°C for 10 min. The RNA concentration of each sample was measured at an optical density of 260 nm, and the integrity of the RNA was checked by examination of 28S and 18S ribosomal bands using agarose–formaldehyde gel electrophoresis.

MICROARRAY PRODUCTION

A pathway-focused oligoarray designed specifically for the study of drug addiction and related disorders was used. Briefly, 3,565 genes essential to CNS activities for the maintenance of neuronal homeostasis, as well as those associated with the neuron response to addictive substances such as nicotine, alcohol, and cocaine, were selected on the basis of an earlier version of a pathway-focused cDNA microarray (Konu et al., 2004a) and an extensive literature survey. These genes covered most of the major pathways related to cell metabolism, genetic information processing, cellular signaling transduction, neuron-related disease, and cell communication. OligoWiz[1] was used to design the oligonucleotide for each gene. The average length of these oligonucleotides was 59.2 ± 3.8 bases (mean ± SD), with a GC content of 0.53 ± 0.05 and a T_m of 76.4 ± 1.7°C. The oligonucleotides and 10 control clones were synthesized by SIGMA Genosys (Sigma-Aldrich, St. Louis, MO, USA) and spotted at a concentration of 40 μM in 3× SSC and 1.5 M betaine buffer onto CMT-GAPS II slides (Corning Life Sciences, Lowell, MA, USA) using an OmniGrid MicroArrayer OGR-03 (GeneMachines, San Carlos, CA, USA).

cDNA PROBE LABELING, HYBRIDIZATION, AND SCANNING

To reduce the potential influence of any gene-specific dye effect, a universal reference design was used in the microarray analysis. For each experimental group, six independent cultures were prepared. Another 12 independent control cultures were used as "universal controls." For each sample, 10 μg of total RNA was extracted for cDNA synthesis. Following purification with phenol:chloroform extraction and isopropanol precipitation, total RNA was dissolved in 28 μl of water, mixed with 4 μl of 10× buffer, 4 μl of 10 mM dTTP-free dNTP, 1 μl of 10 mM dTTP, 2 μl of 1 mM cyanine 3-dUTP (for either nicotine-treated or control sample) or cyanine 5-dUTP (for universal control sample; Enzo, Farmingdale, NY, USA), and 1 μl of Klenow fragment (50 units/μl), then incubated at 37°C for 3 h prior to purification with a QIAquick PCR kit (Qiagen, Valencia, CA, USA). The cyanine 3-labeled sample (either nicotine-treated or control) cDNA probes were mixed with the cyanine 5-labeled universal control cDNA probe and added to 7.5 μl of 20× SSC, 3 μg of *Cot*I DNA, 3 μg of polyA, and 0.5 μl of 10% SDS adjusted to a final volume of 50 μl. The mixture was applied to the pathway-focused oligonucleotide microarray 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 each. Hybridized slides were scanned

[1]http://www.cbs.dtu.dk/services/OligoWiz/

using the ScanArray Gx microarray scanner, and the intensities of each probe were quantified with the ScanArray Express microarray analysis system (PerkinElmer, Waltham, CA, USA).

DATA NORMALIZATION AND STATISTICAL ANALYSIS

After scanning the arrays, we obtained the raw hybridization intensity for each element printed on the array and then used the background-subtracted median intensity of each spot for further statistical analysis. The two replicates on each chip were initially processed separately. The weakest 5% of spots and the saturated spots in each replicate were discarded. An intensity-dependent method, locally weighed linear regress (*Lowess*), was used to normalize the data of each replicate (Yang et al., 2002). To minimize experimental error, genes with six or fewer valid measurements were removed from further analysis, and the two technical replicates per chip were averaged to obtain the measurement of each gene for a given sample. The normalized data from each drug treatment group were compared with the normalized data of controls, and significantly regulated genes were identified by unpaired two-tailed Student's *t*-test. To control the multiple comparison error, the false discovery rate (FDR) was calculated by the method of Benjamini and Hochberg (1995) via MATLAB (The Mathworks Inc., Natick, MA, USA).

The genes significantly regulated by each drug treatment were analyzed by ingenuity pathway analysis (IPA)[2] with the goal of revealing the enriched biochemical pathways. The core of IPA is the ingenuity pathways knowledge base (IPKB), which contains the biological function, interaction, and other related information of a curated gene set and more than 330 biochemical pathways. This pathway-based software is designed to identify global canonical pathways, dynamically generated biological networks, and global functions from a given list of genes. Basically, the genes with their symbols, corresponding GenBank Accession Numbers, or both were uploaded into the IPA and compared with the genes included in each canonical pathway using the whole gene set of IPKB as the background. All the pathways with one or more genes overlapping the candidate genes were extracted. In IPA, each of these pathways was assigned a *p*-value, which denoted the probability of overlap between the pathway and input genes, via Fisher's exact test. Because a relatively large number of pathways were examined, multiple comparison correction for the individually calculated *p*-values was necessary to make reliable statistical inferences. The FDR was again calculated with the method of Benjamini and Hochberg (1995).

Quantitative real-time RT-PCR

The quantitative real-time RT-PCR (qRT-PCR) was carried out for eight representative genes selected from the aforementioned differentially expressed genes: cyclin d3 (*CCND3*), cell division cycle 42 (*CDC42*), death domain-associated protein 6 (*DAXX*), mitogen-activated protein kinase 14 (*MAPK14*), mads box transcription enhancer factor 2, polypeptide D (*MEF2D*), nerve growth factor receptor (*NGFR*), phospholipase A2, group IVA (*PLA2G4A*), and transformation-related protein 53 (*TRP53*). All primers and *Taq*Man probes for the eight genes were purchased from Applied Biosystems (ABI, Foster City, CA, USA), and qRT-PCR analysis was done as described previously (Gutala et al., 2004; Konu et al., 2004a). Briefly, PCR was carried out in a volume of 25 μl using the *Taq*Man assay on the ABI 7000 Sequence Detection System. The 18S ribosomal RNA was used as an internal control to normalize the expression patterns of target genes. Data analysis was performed using a comparative C_t method (Winer et al., 1999). Five independent cell cultures were prepared under the same conditions used for the microarray experiments for both the nicotine treatment and control groups ($N = 5$).

Western blotting analysis

The protein concentration of *MAPK14* (*p38*) was measured in both nicotine-treated (1 mM for 1 h) and control samples. After appropriate treatment, cells were harvested using RIPA buffer followed by incubation on ice for 30 min. The cells were centrifuged at 12,000×*g* for 15 min to remove debris, and the total protein content of the lysates was determined using the Bio-Rad protein assay (Bio-Rad, Chicago, IL, USA). The samples were boiled at 90°C for 5 min and then cooled on ice. Twenty micrograms of protein was fractionated on 10% SDS-polyacrylamide gel (containing 30% acrylamide and 0.8% bisacrylamide) in Tris–glycine buffer containing SDS at 80 V for approximately 1.5 h. The separated proteins were then transferred to a PVDF membrane (Millipore, Bedford, MA, USA) overnight at 30 V at 4°C. The membrane was blocked for 1 h at room temperature with 5% non-fat dry milk diluted in TBST buffer and incubated overnight with primary antibody at 4°C. After being washed three times in TBST, the membranes were subjected to secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescence kit (Bio-Rad), and the preparations were exposed to X-ray film. The *MAPK14* antibody was from Calbiochem (San Diego, CA, USA), and anti-α-tubulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the housekeeping protein α-tubulin. The *MAPK14* immunoreactivity bands were quantified using densitometry. After the films were developed, they were scanned on a Microtek ScanMaker *i*800 (Microtek Lab Inc., Cerritos, CA, USA) with ScanWizard 5.5 at a resolution of 600 dpi for quantitative analysis with ImageQuant 5.1 (Molecular Dynamics, Sunnyvale, CA, USA). The relative *MAPK14* values were normalized to α-tubulin, and the significance of the difference between the nicotine-treated and control samples was determined by *t*-test via MATLAB (The Mathworks, Natick, MA, USA). Four independent cell cultures were prepared under the same conditions used for the microarray experiments for both nicotine treatment and control groups ($N = 4$).

RESULTS

GENE EXPRESSION CHANGES IN RESPONSE TO ACUTE NICOTINE EXPOSURE

In the nicotine-treated group, 295 genes were significantly modulated ($p < 0.05$; **Table A1** in Appendix), with FDR values ≤0.258. Among these genes, 98 had *p*-values <0.01, which corresponded to an FDR value of 0.155. Of the 295 differentially expressed genes, 221 showed no significant changes ($p \geq 0.05$) in the cells treated with nicotine and mecamylamine, suggesting these genes were regulated through the mecamylamine-modulated nicotinic receptors.

[2]https://analysis.ingenuity.com

Of the 295 genes, 128 were down-regulated by acute nicotine exposure. These included multiple transcription factors; e.g., activating transcription factor 2 (*ATF2*), general transcription factor IIH polypeptide 2 (*GTF2H2*), nuclear respiratory factor 1 (*NRF1*), and transcription factor EB (*TRFEB*). A few genes related to cell adhesion were also down-regulated by acute nicotine exposure; e.g., ninjurin 1 (*NINJ1*), kit ligand (*KITLG*), integrin beta 1 (*ITGB1*), neurexin 1 (*NRXN1*), and laminin gamma 1 (*LAMC2*). Some cell cycle-related genes were suppressed; e.g., cell division cycle 14 homolog B (*CDC14B*), cell division cycle 2 homolog A (*CDC2A*), cell division cycle 42 homolog (*CDC42*), G1 to S phase transition 1 (*GSPT1*), and meiotic recombination 11 homolog A (*MRE11A*).

The remaining 167 differentially expressed genes were up-regulated by nicotine treatment. Genes included in this category included those related to: (a) mitochondrial electron transport; e.g., cytochrome *c* oxidase, subunit VIIC (*COX7C*), polypeptide 1 of cytochrome p450 family 17 subfamily A (*CYP17A1*), rieske iron–sulfur protein (*RISP*), and NADH dehydrogenase (ubiquinone) flavoprotein 1 (*NDUFV1*); (b) ubiquitination; e.g., ubiquitin-activating enzyme E1 Chr X (*UBE1X*), ubiquilin 1 (*UBQLN1*), ubiquitin-specific protease 28 (*USP28*), and ubiquitin-conjugating enzyme e2a (*UBE2A*); and (c) transport; e.g., potassium channel TWIK (*KCNK1*), nucleotide-sensitive chloride channel 1A (*CLNS1A*), T type alpha 1G subunit of voltage-dependent calcium channel (*CACNA1G*), solute carrier family 8 (sodium/calcium exchanger) member 2 (*SLC8A2*), and solute carrier family 2 (facilitated glucose transporter) member 4 (*SLC2A4*).

Of the 295 genes significantly regulated by nicotine treatment, 74 were differentially expressed ($p < 0.05$) in the cells treated with nicotine + mecamylamine (**Table A1** in Appendix). Most of these genes showed the same trend of regulation as those in the nicotine treatment group, suggesting that mecamylamine may have either no significant effect or a synergistic effect with nicotine on the expression changes in these genes.

PATHWAYS ENRICHED IN THE GENES REGULATED BY NICOTINE

As shown above, genes and biological processes involved in different functions were regulated by nicotine exposure. To further identify the biological themes underlying these genes, the biochemical pathways enriched in the nicotine-regulated genes were investigated. Two sets of genes were analyzed by IPA; i.e., those regulated significantly by 1 h of nicotine exposure ($N = 295$), and those with expression significantly changed by 1 h of nicotine with the effect being blocked by mecamylamine ($N = 221$). The second gene set included only those with p-values ≥0.05 during co-treatment with nicotine and mecamylamine. Although in this set, some genes may have considerable fold changes, the selection was based on p-value only.

Results from IPA analysis showed that 14 pathways were significantly enriched among the genes regulated by nicotine ($p < 0.05$; FDR < 0.05; **Figure 1**; **Table 1**), and 13 pathways were enriched in the second gene set. Among these pathways, nine were identified for both gene sets; i.e., glucocorticoid receptor signaling, p38 MAPK signaling, PI3K/AKT signaling, PTEN signaling, acute-phase response signaling, ERK/MAPK signaling, Toll-like receptor signaling, VEGF signaling, and T-cell receptor signaling. For the gene set regulated by nicotine treatment, five pathways were uniquely identified; i.e., mitochondrial dysfunction, PPAR signaling, IL-6 signaling, hepatic fibrosis/hepatic stellate cell activation, and death receptor signaling. The pathways uniquely identified in the second gene set were CD28 signaling in T-helper cells, CD40 signaling, role of NFAT in regulation of the immune response, and IL-8 signaling.

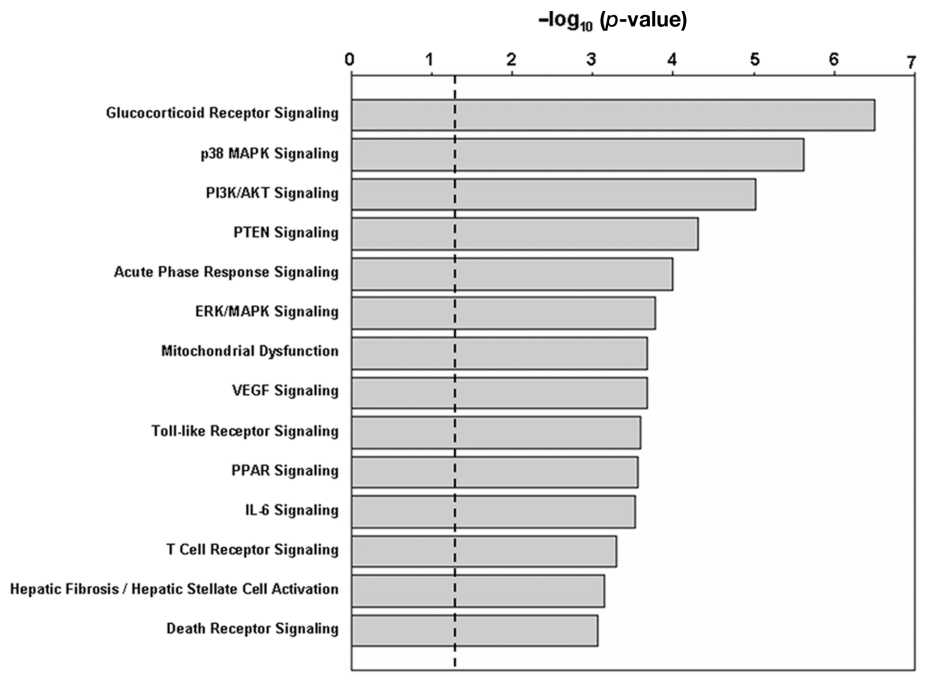

FIGURE 1 | Pathways significantly enriched in the genes differentially regulated by nicotine exposure. The p-values are shown in negative logarithm form at base 10. The dashed line indicates the threshold corresponding to $p = 0.05$. All 14 pathways shown have an FDR < 0.05.

Table 1 | Pathways enriched in genes significantly regulated by nicotine and genes significantly regulated by nicotine but not by nicotine + mecamylamine.

	Gene regulated by nicotine		Gene regulated by nicotine but blocked by NM*		
Pathway	***p*-Value**	**FDR**	***p*-Value**	**FDR**	**Genes significantly regulated by nicotine in the pathway****
Glucocorticoid receptor signaling	3.11×10^{-7}	1.77×10^{-4}	4.46×10^{-6}	7.00×10^{-4}	AGT, AR, BCL2, CD3G, DUSP1(MKP1), FGG, FOS, **GTF2H2**, IKBKG, JAK3, MAP3K7IP1(TAB1), MAPK14, MED14, NFATC3, PIK3CG, SMAD2, **STAT5A**, **TGFB2**, TGFBR2, TRAF6, YWHAH
p38 MAPK signaling	2.40×10^{-6}	6.82×10^{-4}	6.54×10^{-5}	2.57×10^{-3}	**ATF2**, DAXX, DUSP1(MKP1), HMGN1, IRAK3, MAP3K7IP1(TAB1), MAPK14, MEF2D, PLA2G4A, **TGFB2**, TGFBR2, TRAF6
PI3K/AKT signaling	9.55×10^{-6}	1.81×10^{-3}	3.29×10^{-5}	1.7×10^{-3}	BCL2, EIF4EBP1, FOXO1, IKBKG, ITGB1, JAK3, PIK3CG, PPM1L, **RPS6KB1**, SFN, **TRP53**, YWHAH
PTEN signaling	5.01×10^{-5}	7.13×10^{-3}	1.51×10^{-3}	0.030	BCL2, BCL2L11, CDC42, FOXO1, IKBKG, ITGB1, **NGFR**, PIK3CG, **RPS6KB1**, YWHAH
Acute-phase response signaling	1.02×10^{-4}	0.012	7.06×10^{-4}	0.018	AGT, APOA1, FGG, FOS, IKBKG, IL6R, IL6ST, MAP3K7IP1(TAB1), MAPK14, **NGFR**, PIK3CG, **TF**, TRAF6
ERK/MAPK signaling	1.70×10^{-4}	0.015	2.51×10^{-4}	0.016	**ARAF**, **ATF2**, DUSP1, EIF4EBP1, FOS, ITGB1, PIK3CG, PLA2G4A, PPM1L, **PPP1CA**, RAPGEF3, YWHAH
Mitochondrial dysfunction	2.09×10^{-4}	0.015	–	–	COX6B1, **COX7B**, COX7C, NDUFA4, **NDUFV1**, OGDH, SDHB, **SDHC**, SNCA, UQCRFS1
VEGF signaling	2.14×10^{-4}	0.015	6.17×10^{-4}	0.018	BCL2, EIF2B1**, EIF2B5**, FOXO1, PIK3CG, SFN, **VCL**, VEGFA
Toll-like receptor signaling	2.57×10^{-4}	0.015	2.94×10^{-3}	0.045	FOS, IKBKG, IRAK3, MAP3K7IP1(TAB1), **MAP4K4**, MAPK14, TRAF6
PPAR signaling	2.75×10^{-4}	0.015	–	–	FOS, IKBKG, MAP3K7IP1(TAB1), **MAP4K4**, **NGFR**, NR2F1, **STAT5A**, TRAF6
IL-6 signaling	2.95×10^{-4}	0.015	–	–	FOS, IKBKG, IL6R, IL6ST, MAP3K7IP1(TAB1), **MAP4K4**, MAPK14, **NGFR**, TRAF6
T-cell receptor signaling	5.13×10^{-4}	0.024	3.97×10^{-4}	0.013	CALM2, CD3G, CSK, FOS, IKBKG, NFATC3, PIK3CG, ZAP70
Hepatic fibrosis/hepatic stellate cell activation	7.24×10^{-4}	0.032	–	–	AGT, BCL2, IGF1R, IL6R, **NGFR**, SMAD2, **TGFB2**, TGFBR2, VEGFA
Death receptor signaling	8.71×10^{-4}	0.035	–	–	BCL2, CFLAR, DAXX, IKBKG, **MAP4K4**, TNFRSF10B
CD28 signaling in T-helper cells	–	–	2.90×10^{-5}	1.72×10^{-3}	ARPC1A, ARPC1B, CALM2, CD3G, CDC42, CSK, FOS, IKBKG, NFATC3, PIK3CG, ZAP70
CD40 signaling	–	–	1.38×10^{-3}	0.030	FOS, IKBKG, JAK3, MAP2K5, MAPK14, PIK3CG, TRAF6
Role of NFAT in regulation of the immune response	–	–	3.01×10^{-3}	0.045	CALM2, CD3G, FOS, GNA15, IKBKG, MEF2D, NFATC3, PIK3CG, ZAP70
IL-8 signaling	–	–	3.15×10^{-3}	0.045	BCL2, CCND3, EIF4EBP1, IKBKG, IRAK3, LIMK1, PIK3CG, TRAF6, VEGFA

**NM, nicotine + mecamylamine. **For each pathway, all the genes were significantly regulated by nicotine exposure ($p < 0.05$). The expression changes of the genes were blocked by the co-treatment of nicotine + mecamylamine except those shown in bold font, which means genes shown in bold font were differentially expressed in both the nicotine and nicotine + mecamylamine-treated samples.*

In the pathways identified for the two gene sets, the genes affected were not identical (**Table 1**). For the nicotine + mecamylamine-treated samples, the pathways included only genes whose expression was modulated by nicotine exposure but blocked by simultaneous treatment with the two chemicals. For the nicotine-treated samples, the pathways also included genes whose expression was modulated in both nicotine- and nicotine + mecamylamine-treated samples.

We also performed pathway analysis on the 74 genes significantly regulated in both nicotine-treated and the nicotine + mecamylamine-treated samples via IPA. Although a few pathways were assigned *p*-values <0.05, they are not reported here because of the relatively high FDR value after multiple comparison correction (with the minimum FDR being 0.262).

As shown in **Table 1**, multiple genes in each pathway were significantly regulated by nicotine exposure. Although the expression changes of some genes within each pathway were not statistically significant, they showed consistent patterns with those significantly modulated. One of the pathways, p38 MAPK signaling, is shown in **Figure 2**. Genes involved in this pathway were extensively regulated in response to nicotine exposure. The expression

of a few genes, such as *DAXX*, *MKP1/5*, *MAPK14*, and *MEF2*, was significantly modulated by acute nicotine exposure ($p < 0.05$). Although the expression of other genes; e.g., *ASK1*, *MKK4*, and *HPK1*, was not significantly regulated under the experimental conditions, they showed patterns consistent with those of significantly regulated genes.

Expression verification of representative genes

In an independent experiment involving the same treatments as in the microarray analysis, qPCR was performed on eight representative genes; i.e., *CCND3*, *CDC42*, *DAXX*, *MAPK14*, *MEF2D*, *NGFR*, *PLA2G4A*, and *TRP53*. All these genes were significantly regulated by nicotine exposure (**Table A1** in Appendix), and each gene was part of one or more enriched pathways associated with nicotine treatment (**Table 1**). For example, *CCND3* is part of the IL-8 signaling pathway; *CDC42* of CD28 signaling and PTEN signaling pathways; *DAXX* of the p38 MAPK signaling pathway; *MAPK14* of multiple pathways such as glucocorticoid receptor signaling, p38 MAPK signaling, acute-phase response signaling, and Toll-like receptor signaling; *MEF2D* of p38 MAPK signaling that plays a role of NFAT in regulation of the immune response; *NGFR* of PTEN signaling, acute-phase response signaling, PPAR signaling, and IL-6 signaling; *PLA2G4A* of p38 MAPK and ERK/MAPK signaling; and *TRP53* of the PI3K/AKT signaling pathway.

Figure 3 shows the fold change in activity along with the standard deviation of the eight genes in response to nicotine treatment relative to the controls, as detected by microarray and quantitative RT-PCR. These analyses revealed significant up-regulation of seven genes and down-regulation of *CDC42*, which is consistent with the result from the microarray analysis. Furthermore, a comparison of the fold changes of each gene detected by the two molecular techniques revealed a correlation coefficient of 0.87 ($p = 0.0068$), indicating overall measurement of gene expression from the two approaches was consistent. Although only eight representative genes were selected for verification by qRT-PCR, a comparison of the two techniques demonstrates that the results from our microarray analyses were highly reproducible and reliable.

Furthermore, we used Western blotting to determine the total amount of *MAPK14* (p38 MAPK) protein in lysates of cells treated with 1 mM nicotine and control samples. The results revealed significant induction (35.5%; $p = 0.03$) of *MAPK14* expression in nicotine-treated samples (**Figure 4**).

DISCUSSION

In this study, we profiled gene expression in the SH-SY5Y cell line in response to 1 h of 1 mM nicotine exposure using a pathway-focused oligonucleotide microarray. Treatment with 1 mM nicotine maximally induces the up-regulation of numbers of nAChR radioligand-binding sites in SH-SY5Y cells, and this up-regulation

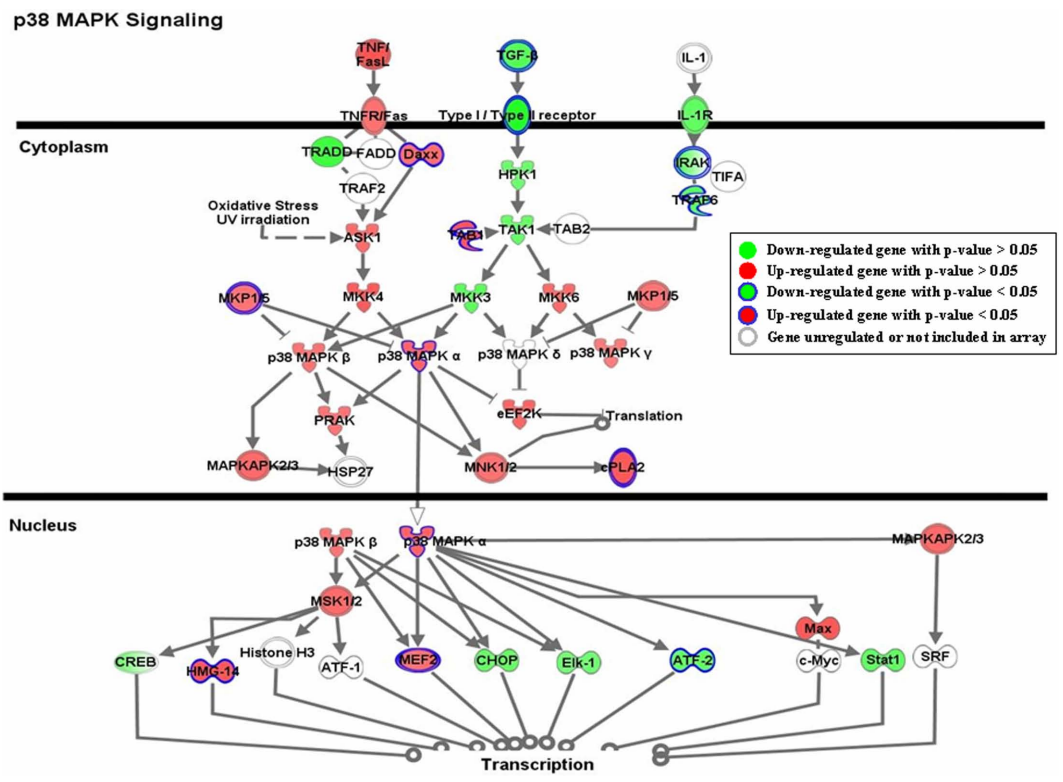

FIGURE 2 | Gene expression pattern of the P38 MAPK signaling pathway. Genes shown in green are down-regulated, whereas those shown in red are induced by acute nicotine treatment. The genes significantly regulated by nicotine are those with closed blue edges. To demonstrate the expression pattern of the pathway, all the genes with expression ratios >1.1 or <0.90 relative to the controls are also shown. The genes in white with light gray edges are either not regulated by nicotine (expression ratios between 0.9 and 1.1) or were not measured in our analysis. The pathway was drawn by the IPA program.

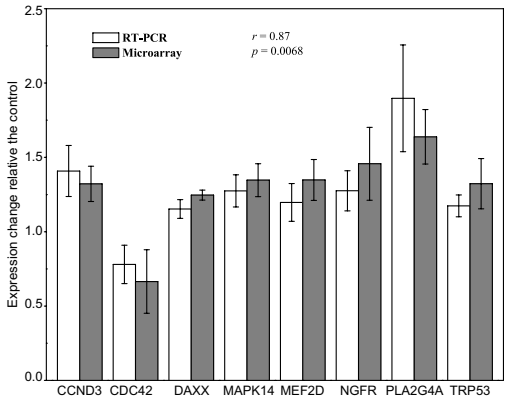

FIGURE 3 | Comparative plot of results from microarray and real-time RT-PCR verification for eight representative genes. *Y* axis represents the fold change of expression in 1-h nicotine-treated SH-SY5Y cells compared with the average expression in the control sample for that particular gene. For the qRT-PCR results, the expression of each gene was normalized to the average of control samples. Abbreviations: *CCND3*, cyclin D3; *CDC42*, cell division cycle 42 homolog; *DAXX*, Fas death domain-associated protein; *MAPK14*, mitogen-activated protein kinase 14; *MEF2D*, myocyte enhancer factor 2d; *NGFR*, nerve growth factor receptor; *PLA2G4A*, phospholipase A2, group IVA; *TRP53*, transformation-related protein 53.

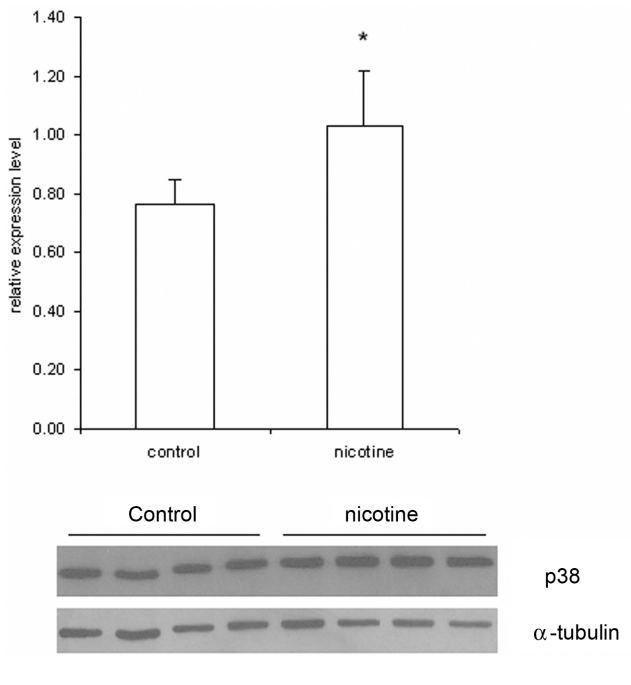

FIGURE 4 | Expression of *MAPK14* (p38 MAPK) protein in SH-SY5Y cells determined by Western blotting. The SH-SY5Y cells were treated for 1 h with or without (Controls) 1 mM nicotine. After treatment, protein was purified from the lysates and analyzed by Western blotting. In this figure, the *MAPK14* concentration was determined using a specific monoclonal antibody. The histogram shows the relative expression of *MAPK14* normalized to expression of α-tubulin, which demonstrated that the protein concentrations in nicotine-treated cells are significant higher than those in controls ($p < 0.03$). Data are presented as mean ± SEM ($N = 4$).

has been implicated in the long-lasting effects of nicotine exposure, such as dependence and tolerance (Ke et al., 1998; Dunckley and Lukas, 2003, 2006). Given that the purpose of this study was to determine the pharmacologic effects of nicotine using an *in vitro* system, we adopted a 1-mM concentration for nicotine, as used in a previous study (Dunckley and Lukas, 2003). However, the current study is neither a simple modeling of the physiological conditions of smokers, nor repeat earlier studies in the same model. Instead, it focuses on exploring the molecular response at the pathway level to acute nicotine exposure that may maximally activate the function of molecules such as nAChRs and their downstream genes in SH-SY5Y cells.

Nicotine evokes its physiological effects mainly by binding with nAChRs. As a broad-spectrum antagonist of nAChRs, mecamylamine at a concentration of 3 μM can block most of the responses of α3*-nAChR to nicotine, as well as a fraction of the α7-nAChR responses (Chavez-Noriega et al., 1997; Papke et al., 2001; Dunckley and Lukas, 2003, 2006). Of the 295 genes significantly regulated by nicotine, 221 were not differentially expressed during co-treatment of nicotine + mecamylamine, providing evidence of direct involvement of nAChR in the process of modulation of SH-SY5Y cells by nicotine. Thus, our results indicate that the changes in 75% (221/295) of the differentially expressed genes in response to nicotine are attributable to direct interaction with nAChRs, especially α3*-nAChR receptors, which is consistent with the conclusions drawn from previous studies (Dunckley and Lukas, 2003, 2006). However, it is difficult to confirm a clear relation between nicotinic receptor subtype and gene expression from our findings because of the existence of different subtypes of nAChRs on the membrane of SH-SY5Y cells. These receptors have different properties, such as sensitivity to nicotine, permeability to calcium, and propensity to desensitize. It is possible that the regulation of some genes is related to multiple subtypes of nAChRs, including those α3- or α7-containing receptors. Partially blocking the function of some types of receptors (e.g., α3*- and α7-containing) may reduce the overall activation of nAChRs, which may lead to more subtle regulation of some genes compared with the response when mecamylamine is not applied. On the other hand, our results do not imply that nAChRs play little or no role in the regulation of genes significantly regulated in both nicotine and nicotine + mecamylamine treatment. The expression changes of these genes may have multiple explanations. A small fraction of α3*-nAChR receptors remain functionally active under the influence of mecamylamine, which may be sufficient to regulate the expression of some genes. At the same time, α7 and other types of nAChR receptors may be responsible for the expression change of some other genes as well. It is also possible that the expression changes of some genes in response to nicotine are caused by mechanisms not directly involving nAChRs, as nicotine can readily cross the plasma membrane and affect intracellular processes through an nAChR-independent mechanism.

Our analysis showed that acute nicotine exposure has effects on the expression of a large number of genes with diverse functions (**Table A1** in Appendix). Among them are genes involved in the ubiquitin–proteasome pathway, such as proteasome subunit beta 4 (*PSMB4*), proteasome 26S subunit non-ATPase 2 (*PSMD2*), ubiquitin-activating enzyme E1 Chr X (*UBE1X*), ubiquitin-conjugating enzyme E2A

(*UBE2A*), ubiquilin 1 (*UBQLN1*), and ubiquitin-specific protease 28 (*USP28*). Earlier studies showed that genes involved in the ubiquitin–proteasome pathway were regulated in SH-SY5Y cells by 1 mM nicotine after both 1- and 24-h treatment (Dunckley and Lukas, 2003, 2006). In the current study, more genes related to this pathway were found to be modulated by nicotine exposure. The ubiquitin–proteasome pathway is regulated by nicotine in cell lines (Konu et al., 2004b; Ficklin et al., 2005; Wang et al., 2007), animal models (Kane et al., 2004; Li et al., 2004; Rezvani et al., 2007; Vadasz et al., 2007; Wang et al., 2009), and human smokers (Mexal et al., 2005). Thus, the changes in ubiquitin–proteasome-related genes observed in this study may not be specific for the relatively high nicotine concentration; instead, they may be caused by mechanisms similar to those discovered in previous studies.

Multiple genes involved in mitochondrial functions also were found to be regulated by nicotine; these include acyl-coenzyme A thioesterase 3 (*ACATE3*), ATP synthase subunit g (*ATP5L*), mitochondrial ribosomal protein S7 (*MRPS7*), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (*NDUFA4*), and *RISP*. Previous studies have demonstrated that nicotine exposure can modulate the morphology (Onal et al., 2004) and function of mitochondria, such as protein turnover (Katyare and Shallom, 1988), enzyme activity (Xie et al., 2005), and generation of reactive oxygen species (Cormier et al., 2001; Soto-Otero et al., 2002). Recently, we showed that nicotine exposure regulates the electron transport system in multiple brain regions of rats (Wang et al., 2009). The results from the current study indicate that acute nicotine exposure also can regulate the expression of mitochondrial genes, potentially leading to the modulation of mitochondrial functions. In addition, several genes involved in synaptic transmission were identified, such as neuron-specific gene family member 1 (*NSG1*), synuclein alpha (*SNCA*), synapsin 2 (*SYN2*), synaptotagmin 2 (*SYT2*), synaptotagmin 7 (*SYT7*), and tachykinin 2 (*TAC2*). The expression of *NSG1* in mouse cortical neurons is regulated by treatment with nicotine, alcohol, or both (Wang et al., 2007). *SNCA* is suggested as a candidate associated with alcohol, cocaine, and methamphetamine dependence (Kobayashi et al., 2004; Bonsch et al., 2005; Mash et al., 2008; Spence et al., 2009). The phosphorylation of synapsin is regulated by cocaine in a region-specific manner in rat brain (Edwards et al., 2007). The regulation of these synaptic transmission-related genes indicates that even a short nicotine exposure may have important effects on neuronal functions.

Among the 74 genes significantly regulated by both nicotine and nicotine + mecamylamine treatment, some have already been reported to be involved in the cellular response to nicotine exposure. Activating transcription factor 2 (*ATF2*), a DNA-binding protein that binds to cAMP response elements, was suppressed in both treatment conditions and is involved in mediating the activation of tyrosine hydroxylase transcription under nicotine treatment in PC12 cells (Gueorguiev et al., 2006). Nerve growth factor (*NGF*), a member of the neurotrophin family, is an essential mediator of neuronal activity and synaptic plasticity (Levi-Montalcini, 1987; Thoenen et al., 1987). Both *NGF* and its receptor, *NGFR*, were up-regulated in the two treatment groups. Nicotine has been reported to regulate the expression of *NGF* and *NGFR* in cell lines or tissues (Terry and Clarke, 1994; Jonnala et al., 2002; Garrido et al., 2003; Hernandez and Terry, 2005; French et al., 2006). Neurexin 1 (*NRXN1*) was down-regulated in both treatment groups. This and another member of the same family, *NRXN3*, were associated with smoking behaviors in several recent genetic studies (Bierut et al., 2007; Nussbaum et al., 2008; Novak et al., 2009). Although our data provide no further information on the mechanisms by which these genes are regulated, it is clear that acute nicotine exposure is responsible for their expression changes.

Consistently, the pathways enriched in nicotine-regulated genes were involved in many biological processes, such as neural development/synaptic plasticity, cell survival/death, immune response, and cellular metabolism. Of the identified pathways, nine were enriched in genes significantly regulated by nicotine, as well as those regulated by nicotine but blocked by mecamylamine, indicating these pathways were regulated mainly through nAChR-dependent mechanisms. Among these pathways, acute-phase response signaling, Toll-like receptor signaling, and T-cell receptor signaling play important roles in the immune response. Although the other pathways; i.e., glucocorticoid receptor signaling, p38 MAPK signaling, PI3K/AKT signaling, PTEN signaling, ERK/MAPK, and VEGF signaling, are involved in a large range of physiological processes, all of them represent key pathways for maintaining proper neuronal functions, such as cellular signaling and proliferation and differentiation of neurons. The modulation of the neuronal function-related pathways may have relatively long-lasting consequences. One example is that adolescent rats display different long-term neuroadaptive responses to acute nicotine than do adult animals, which is suggested to be related to the immature or still-developing plasticity mechanisms in the prefrontal cortex of young animals (Schochet et al., 2004). Among these pathways, particularly interesting are the glucocorticoid receptor and p38 MAPK signaling.

For the glucocorticoid receptor signaling pathway, 21 genes were significantly regulated by acute nicotine treatment (**Table 1**). The glucocorticoid receptor, *GCCR*α (also known as nuclear receptor subfamily 3, group c, member 1; *NR3C1*) was slightly suppressed by nicotine (fold change 0.83; p-value = 0.09). The regulation of these genes clearly indicates that the glucocorticoid receptor signaling pathway is modulated by acute nicotine exposure. Glucocorticoids, cortisol in humans, and corticosterone in the rodent, are secreted by the adrenal cortex and belong to the family of steroid hormones. Glucocorticoids and their receptors are essential for the development and survival of vertebrates (Cole et al., 1995). All the cellular responses to glucocorticoids are attributed to their binding to the intracellular glucocorticoid receptor (GCCR) and the translocation of the complexes to the nucleus, which then modulates gene expression through diverse mechanisms (Dittmar et al., 1997, 1998; Robinson-Rechavi et al., 2001). Although the activity of the GCCR is directly related to gene transactivation, considerable cross-talk occurs between the GCCR and a cohort of molecules to mediate their functions as transcriptional regulators. Studies on animals and humans have outlined the importance of glucocorticoids in mediating drug-seeking behavior and the rewarding properties of psychostimulant drugs (Piazza and Le Moal, 1997; Marinelli and Piazza, 2002). For example, in mice, activation of glucocorticoid receptors increases the propensity to self-administer cocaine by acting on the postsynaptic dopaminoceptive neurons of the dopaminergic system (Ambroggi et al., 2009). It was also found that learning and memory deficits in cocaine-dependent humans are associated with higher cortisol concentrations (Fox et al., 2009). The

neuronal circuitry adaptations induced by heroin self-administration were correlated with elevated glucocorticoid production in certain brain regions of rats (Weber et al., 2009). Corticosteroid receptor modulation of the mesoaccumbens dopamine neurotransmission is believed to be a key neurobiological mechanism mediating the effects of stress in addiction (Tye et al., 2009). Nicotine can also interact with glucocorticoid receptor signaling cascades directly or indirectly. Furthermore, the relations between nicotine and glucocorticoids are bidirectional; i.e., not only can nicotine affect the hypothalamic–pituitary–adrenal (HPA) axis and circulating glucocorticoids, but adrenal steroids can modulate nicotine's behavioral and physiological actions (Caggiula et al., 1998). Nicotine administration elevates plasma corticosterone in animals (Caggiula et al., 1998). Similarly, both cigaret smoking and nicotine infusion rapidly increase circulating cortisol concentrations in humans (Caggiula et al., 1998). In human smokers, the degree of nicotine addiction is reflected by changes in the sensitivity of central glucocorticoid receptors (Reuter et al., 2004). Nicotine administration to pregnant animals can lead to fetal overexposure to maternal glucocorticoid and result in fetal adrenocortical dysfunction and other diseases after birth (Chen et al., 2007). The significant regulation of the corticosteroid receptor signaling pathway by acute nicotine exposure provides additional evidence of the importance of this pathway in nicotine addiction, and the significantly regulated genes in this pathway are excellent candidates for further investigation. At the same time, the cross-talk between glucocorticoid receptor signaling and other pathways, such as MAPK signaling and PI3K/AKT signaling (Clark and Lasa, 2003; Gupta et al., 2007), implies that the regulation of this pathway indirectly induces modulations in other biological processes.

The MAPK signaling pathway is a major modulator of cell growth, division, and differentiation (Adams and Sweatt, 2002) and plays a key role in mediating neuronal activation induced by dopamine, glutamate, and drugs of abuse (Jiao et al., 2007). Nicotine activates the MAPK signaling pathway in a variety of tissues and cell types, including SH-SY5Y cells (Heusch and Maneckjee, 1998; Tang et al., 1998; Dajas-Nakayama et al., 2001; Wang et al., 2001; Bailador et al., 2002). Furthermore, many cellular processes are affected by both nicotine and MAPK signaling, such as cell survival and memory processing (Levin and Simon, 1998). Despite these known interactions between MAPK signaling and nicotine exposure, our work has shown a more detailed pattern of the effect of acute nicotine exposure on this pathway. A number of genes were significantly regulated by nicotine exposure (**Table 1**). There were also genes in this pathway that were regulated to a lesser extent (**Figure 2**). Whereas nicotine exposure decreases the amount of mRNA for TGF-beta 2 (*TGFB2*), *TGFBR2*, interleukin 1 receptor (*IL1R*), and interleukin 1 receptor-associated kinase 1 (*IRAK*), as well as a couple downstream genes, it also induces the expression of a number of genes, such as death-associated protein 6 (*DAXX*), *MAPK14* (p38 MAPK), and mads box transcription enhancer factor 2 polypeptide D (*MEF2D*). The regulation pattern of this pathway clearly indicates the cross-talk and interaction between different cascades. Earlier studies showed that nicotine treatment decreases the amount of TGF-beta mRNA in different tissues. For example, nicotine treatment significantly suppresses the release of TGF-beta 1 in bovine aortic endothelial cells (Cucina et al., 1999) and several other cell lines (Lane et al., 2005). This protein has a bimodal dose-dependent effect on the proliferation of various cell types, being stimulatory at low concentration and inhibitory at high concentration (Pollman et al., 1999; Ghosh et al., 2005). The observed mRNA decrease of *TGFB2* and its receptor, *TGFBR2*, may indicate an increase in cell proliferation, although this remains to be established. Our analysis further showed a moderate enhancement of *MAPK14* as judged by both mRNA and protein expression. *MEF2* is one of the many downstream targets regulated by *MAPK14*. In our analysis, the transcription of two members of the MEF2 family was induced in the nicotine-treated samples; i.e., *MEF2D* (fold change 1.35; $p = 0.008$) and *MEF2C* (fold change 1.27; $p = 0.177$). *MEF2* genes are highly expressed in neurons of the CNS (Heidenreich and Linseman, 2004; Shalizi and Bonni, 2005) and play important roles in neuronal differentiation and maintenance. Blocking the function of *MEF2* genes may result in the death of neurons, whereas their expression can promote neuronal survival (Mao et al., 1999; Li et al., 2001; Gaudilliere et al., 2002). Although the activity of *MEF2* is regulated mainly via its phosphorylation by *MAPK14* or other kinases, considering the expression induction of *MAPK14* and *MEF2* genes, it may be reasonable to assume the activity of *MEF2* is enhanced by acute nicotine exposure in SH-SY5Y cells.

In an earlier study, Dunckley and Lukas (2003) analyzed the gene expression patterns in SH-SY5Y cells treated with nicotine under similar experimental conditions (i.e., 1 mM nicotine for 1 h) with different microarray platforms. Among the 17 genes identified, eight had valid expression measurements in our analysis, and their expression patterns were largely consistent in the two studies. While Dunckley and Lukas (2003) performed a more detailed investigation of the relation between the nicotine-regulated genes and nAChRs, we here provide a much more broad and comprehensive analysis and description of the biological processes/pathways underlying responses to acute nicotine exposure. Results from the two studies also indicate that more detailed and comprehensive information about the interaction between acute nicotine and SH-SY5Y cell line could be obtained.

CONCLUSION

By profiling the gene expression pattern in SH-SY5Y cell lines treated acutely with nicotine, we have identified a set of significantly modulated genes. Further, we showed that the majority of these differently expressed genes were regulated through the nAChR-mediated mechanism. From these significantly regulated genes, we have detected the enriched biochemical pathways, which included those potentially involved in the neuronal response to addictive drugs such as nicotine, including glucocorticoid receptor and p38 MAPK signaling. These findings can help us to understand the molecular mechanisms underlying the development of nicotine addiction. On the other hand, the identification of these genes and pathways further indicates that the physiological consequences of acute nicotine exposure are complicated. Thus, further investigation is necessary in order to obtain a more detailed and comprehensive understanding of the interaction between acute nicotine treatment and the nervous system.

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APPENDIX

Table A1 | Genes significantly expressed in nicotine-treated group.

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
ABCD3	ATP-binding cassette, subfamily D (ALD), member 3	0.047	1.17	0.129	1.24
ACAT2	Acetyl-coenzyme A acyltransferase 2	0.046	1.22	7.03×10^{-3}	1.34
ACATE3	Acyl-coenzyme A thioesterase 3	9.09×10^{-3}	0.78	0.052	0.86
ADCY8	Adenylyl cyclase 8	0.034	0.80	0.045	0.82
ADRA1B	Adrenergic receptor, alpha 1B	4.88×10^{-3}	1.31	0.361	1.17
AGT	Angiotensinogen	0.039	1.50	0.593	1.10
ALG2	Asparagine-linked glycosylation 2 homolog (yeast, alpha-1,3-mannosyltransferase)	2.75×10^{-3}	1.38	0.036	1.37
APC	Adenomatosis polyposis coli	0.046	0.83	0.780	0.96
APG4C	APG4 (ATG4) autophagy-related homolog C (*S. cerevisiae*)	0.045	0.83	0.702	1.08
APOA1	Apolipoprotein A-I	0.044	1.68	0.087	1.20
AR	Androgen receptor	0.016	0.76	0.939	1.01
ARAF1	V-raf murine sarcoma 3611 viral oncogene homolog 1	0.030	1.24	0.016	1.15
ARPC1A	Suppressor of profilin/p41 of actin-related complex 2/3	0.033	1.58	0.051	1.34
ARPC1B	Actin-related protein 2/3 complex, subunit 1B	0.042	1.26	0.282	1.11
ASPA	Aspartoacylase	0.041	0.75	0.131	1.57
ATF2	Activating transcription factor 2	0.016	0.80	0.026	0.93
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g; F1F0-ATP	0.048	0.74	0.218	1.41
ATP6V1A1	ATPase, H+ transporting, V1 subunit A, isoform 1; ATPase, H+ transporting	0.012	1.28	0.209	1.29
B3GALT3	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3	0.034	0.70	0.017	0.50
BCL2	B-cell leukemia/lymphoma 2	0.033	1.24	0.473	1.15
BCL2L11	Bcl-12-like 11 (apoptosis facilitator)	0.023	0.65	0.381	0.91
BEGAIN	Brain-enriched guanylate kinase-associated protein 1 mRNA, complete cds	0.030	1.26	0.111	1.15
BRCA1	Breast cancer 1	0.033	0.71	0.150	0.79
CACNA1G	Calcium channel, voltage-dependent, T type, alpha 1g subunit	0.019	1.38	0.842	1.03
CALCR	Calcitonin receptor	0.041	1.25	0.926	1.01
CALCRL	Calcitonin receptor-like receptor	0.026	1.63	0.033	1.87
CALD1	Caldesmon 1	0.020	1.27	0.068	1.34
CALM2	Calmodulin 2	0.043	1.39	0.187	1.11
CALR	Calreticulin	0.039	1.25	0.769	1.02
CAMK1	CaM-like protein kinase	3.08×10^{-3}	0.84	0.235	0.95
CATNA1	Catenin alpha 1	0.043	1.25	0.074	1.19
CATNB	Catenin beta	0.011	1.46	0.021	1.20
CCNA2	Cyclin A2	0.022	1.22	0.482	1.05
CCND3	Cyclin D3	8.99×10^{-3}	1.32	0.696	0.96
CCNE1	Cyclin E1	0.030	1.64	0.122	1.34
CD3G	CD3 antigen, gamma polypeptide	0.029	1.19	0.489	1.13
CDC14B	CDC14 cell division cycle 14 homolog B (*S. cerevisiae*)	0.024	0.56	0.110	0.68
CDC25A	Cell division cycle 25 homolog A (*S. cerevisiae*)	6.02×10^{-3}	0.75	7.82×10^{-4}	0.74
CDC2A	Cell division cycle 2 homolog A (*S. pombe*)	0.031	0.79	0.812	0.96
CDC42	Cell division cycle 42 homolog	9.85×10^{-3}	0.67	0.798	1.03
CDIPT	Phosphatidylinositol synthase	0.030	1.50	0.104	1.31
CDKL2	Cyclin-dependent kinase-like 2 (CDC2-related kinase)	0.046	1.33	0.198	1.44
CDRT1	CMT1A duplicated region transcript 1	0.012	1.40	0.784	0.95
CFLAR	CASP8 and FADD-like apoptosis regulator	0.010	1.35	0.313	0.95
CIAO1	WD40 protein Ciao1	0.015	0.64	0.666	0.94
CLCN5	Chloride channel 5	0.011	0.64	9.13×10^{-4}	0.80
CLNS1A	Chloride channel, nucleotide-sensitive, 1A	0.023	1.31	0.583	1.08
CLSTN3	Calsyntenin 3	0.033	1.33	0.093	1.22
COPS3	COP9 (constitutive photomorphogenic) homolog, subunit 3 (*Arabidopsis thaliana*)	0.046	1.45	0.734	1.03
COX6B	Cytochrome *c* oxidase, subunit VIb	6.98×10^{-3}	1.56	0.344	1.10

(Continued)

Table A1 | Continued

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
COX7B	Cytochrome *c* oxidase, subunit VIIb	0.032	1.22	0.028	1.21
COX7C	Cytochrome *c* oxidase, subunit VIIc	1.71×10^{-3}	1.31	0.150	1.12
COX8B	Cytochrome *c* oxidase, subunit VIIIb	3.37×10^{-3}	1.93	0.019	1.84
CREG	Cellular repressor of E1A-stimulated genes 1	0.023	1.25	0.621	1.06
CRSP2	Cofactor required for Sp1 transcriptional activation subunit 2 (150 kDa)	4.19×10^{-3}	0.67	0.108	0.82
CRYZ	Crystallin, zeta	0.035	1.14	0.078	1.17
CSK	C-src tyrosine kinase	0.044	1.45	0.110	1.29
CTCF	CCCTC-binding factor	0.029	1.18	0.014	1.31
CTNF	Ciliary neurotrophic factor receptor	4.85×10^{-3}	0.74	0.169	1.34
CYP17A1	Cytochrome P450, family 17, subfamily a, polypeptide 1	0.043	1.33	0.283	1.23
CYP2F1	Cytochrome P450, subfamily IIF, polypeptide 1	0.022	0.68	0.082	0.77
CYP2R1	Cytochrome P450, family 2, subfamily r, polypeptide 1	0.030	0.84	0.517	1.09
DAB1	Disabled homolog 1 (*Drosophila*)	0.036	0.55	0.245	0.76
DAXX	Death domain-associated protein 6	9.34×10^{-3}	1.25	0.501	1.11
DFFA	DNA fragmentation factor, 40 kD, beta polypeptide (caspase-activated DNase)	9.62×10^{-3}	0.88	0.522	1.03
DIO2	Deiodinase, iodothyronine, type II	0.040	1.27	0.583	1.14
DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	4.48×10^{-3}	0.66	0.481	1.11
DNCIC2	Dynein, cytoplasmic, intermediate polypeptide 2	0.024	1.38	0.294	0.96
DOCK7	Dedicator of cytokinesis 7	3.57×10^{-3}	1.39	0.256	1.26
DRAP1	Dr1 associated protein 1 (negative cofactor 2 alpha)	6.61×10^{-3}	0.75	0.025	0.79
DRD4	Dopamine receptor D4	0.013	1.23	0.714	1.02
DRPLA	Dentatorubral-pallidoluysian atrophy	5.89×10^{-6}	0.70	0.043	1.08
DSTN	Destrin	0.012	1.26	0.543	1.04
DUSP1	Dual specificity phosphatase 1 (or protein-tyrosine phosphatase, non-receptor-type, 10)	0.016	1.17	0.138	1.10
EDG4	Similar to endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 4	2.53×10^{-3}	1.34	0.225	0.85
EIF2B	Eukaryotic translation initiation factor 2B	8.54×10^{-5}	1.31	0.856	0.99
EIF2B5	Eukaryotic translation initiation factor 2B, subunit 5 epsilon	7.80×10^{-3}	1.20	0.029	1.09
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	0.014	0.65	0.501	0.83
EPHA1	Eph receptor a1	0.033	0.56	0.046	0.61
FASN	Fatty acid synthase	0.017	1.21	0.695	0.97
FGF13	Fibroblast growth factor 13	0.034	1.37	0.028	1.26
FGG	Fibrinogen, gamma polypeptide	0.014	1.22	0.583	1.04
FKRP	Fukutin related protein	0.040	0.70	0.737	0.95
FOS	Fos oncogene	0.031	1.28	0.414	1.14
FOXO1	Forkhead box 1	8.70×10^{-4}	0.52	0.627	1.12
FXC1	Similar to Mitochondrial import inner membrane translocase subunit TIM9 B (fracture callus protein 1)	0.043	1.23	0.852	0.97
GABARAP	Gamma-aminobutyric acid receptor-associated protein	0.012	0.79	0.906	1.01
GABRA3	Gamma-aminobutyric acid (GABA_A) receptor, subunit alpha 3	6.52×10^{-4}	0.77	0.113	0.83
GABRD	GABA(A) receptor delta subunit	0.026	0.63	0.861	1.03
GAL	Galanin	0.021	1.39	0.329	1.07
GALNT10	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 10	1.37×10^{-3}	1.33	0.662	1.05
GEMIN5	Gem (nuclear organelle) associated protein 5	0.015	0.66	0.511	0.95
GLCCI1	Glucocorticoid induced transcript 1	0.014	0.71	0.039	0.55
GLI1	GLI-Kruppel family member GLI1	0.010	1.33	0.869	0.99
GLI3	GLI-Kruppel family member Gli 3	0.037	0.87	0.012	0.67
GLUD1	Glutamate dehydrogenase	0.047	1.28	1.73×10^{-3}	1.36
GNA15	Guanine nucleotide binding protein, alpha 15	0.012	1.41	0.276	1.29
GPAA1	GPI anchor attachment protein 1	0.018	0.83	0.602	0.89
GRIK1	Glutamate receptor, ionotropic, kainate 1	0.034	0.67	0.074	0.79
GRM2	Glutamate receptor, metabotropic 2	5.59×10^{-3}	0.68	0.152	0.76

(Continued)

Table A1 | Continued

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
GSPT1	G1 to phase transition 1	0.035	0.89	0.754	1.02
GSTM6	Glutathione S-transferase, mu6	7.76×10^{-3}	1.41	1.17×10^{-3}	1.42
GTF2H2	General transcription factor IIH, polypeptide 2 (44 kDa subunit)	0.011	0.54	0.023	0.64
GTF3C5	General transcription factor IIIC, polypeptide 5	0.036	1.36	0.018	1.36
GUK1	Guanylate kinase 1	0.019	0.57	0.821	1.06
H1F0	H1 histone family, member 0	0.016	1.22	0.561	1.07
HBA	Hemoglobin X, alpha-like embryonic chain in Hba complex	0.029	0.72	0.030	1.15
HBB	Hemoglobin beta chain complex	7.12×10^{-4}	0.73	0.153	0.61
HBG1	Hemoglobin, gamma A	4.84×10^{-3}	1.45	3.32×10^{-4}	1.71
HHL	RAB GTPase activating protein 1-like	0.042	1.27	0.034	1.27
HIATL2	Hippocampus abundant gene transcript-like 2	0.033	0.69	0.507	0.88
HIS4	Histone 4 protein	0.045	1.19	0.084	1.25
HMGN1	High mobility group nucleosomal binding domain 1	0.014	1.42	0.851	1.01
HRMT1L2	Heterogeneous nuclear ribonucleoproteins methyltransferase-like 2 (*S. cerevisiae*)	0.019	0.79	0.457	0.91
HTR3A	5-Hydroxytryptamine receptor 3a (Htr3a)	1.77×10^{-3}	1.65	0.783	1.07
IDG3G	Isocitrate dehydrogenase 3 (NAD +), gamma	0.038	1.41	0.020	1.31
IGF1R	Insulin like growth factor receptor 1	0.035	0.70	0.340	1.26
IGSF4A	Cell adhesion molecule 1	6.48×10^{-4}	1.21	0.957	1.00
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	0.031	1.27	0.255	1.15
IL6R	Interleukin 6 receptor	5.48×10^{-3}	1.17	0.265	0.94
IL6RA	Interleukin 6 receptor, alpha	0.044	0.62	0.721	1.05
IL6ST	Interleukin 6 signal transducer	9.39×10^{-3}	0.74	0.182	0.89
IL7R	Interleukin 7 receptor	0.047	1.37	0.026	1.36
IMPA1	Lithium-sensitive myo-inositol monophosphatase A1	0.015	1.25	0.388	1.22
ING4	Inhibitor of growth family, member 4	0.024	1.25	0.602	0.96
IRAK3	Interleukin-1 receptor-associated kinase 3	0.041	0.67	0.490	1.10
ITGB1	Integrin beta 1	0.043	0.86	0.776	0.97
ITPKA	Inositol 1,4,5-trisphosphate 3-kinase A	7.66×10^{-3}	1.14	1.91×10^{-4}	1.50
ITPR5	Inositol 1,4,5-triphosphate receptor 5	2.67×10^{-3}	0.81	0.015	0.83
JAK3	Janus kinase 3	0.024	0.80	0.206	0.84
KCMF1	Potassium channel modulatory factor 1	0.034	0.76	0.347	0.86
KCNB2	Potassium voltage-gated channel, Shab-related subfamily, member 2	0.039	0.80	3.60×10^{-3}	0.62
KCNC2	Potassium voltage-gated channel, Shaw-related subfamily, member 3	0.017	0.86	0.164	0.91
KCNK1	Putative potassium channel TWIK	0.012	1.20	0.095	1.15
KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1	1.93×10^{-3}	1.45	0.443	1.28
KITL	Kit ligand	1.05×10^{-3}	0.77	0.108	0.83
KPNB1	Karyopherin (importin) beta 1	5.08×10^{-3}	1.20	0.086	1.19
LAG3	Lymphocyte-activation gene 3	0.012	0.78	0.038	0.81
LAMB2	Laminin beta	0.043	1.54	0.539	1.06
LAMC1	Lamnin gamma	5.02×10^{-3}	0.76	0.205	0.87
LAMR1	Laminin receptor 1	0.044	1.21	0.045	1.20
LIMK1	Lim kinase LIM motif-containing protein kinase 1	0.040	0.71	0.147	0.69
LMO1	LIM domain only 3	0.023	0.82	0.451	0.86
LPL	Lipoprotein lipase	0.020	0.74	0.990	1.00
LRP1	Low density lipoprotein receptor-related protein 1	8.86×10^{-3}	0.74	0.800	0.98
LRP4	Low density lipoprotein receptor-related protein 4	2.93×10^{-3}	1.41	0.748	1.03
LTBP3	Latent transforming growth factor beta binding protein 3	0.030	1.27	0.428	1.06
MAF	Avian musculoaponeurotic fibrosarcoma (v-maf) AS42 oncogene homolog	0.012	1.34	0.041	0.90
MAP2K5	Mitogen-activated protein kinase kinase 5	2.96×10^{-3}	0.86	0.660	0.96
MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	7.63×10^{-3}	1.45	0.950	0.99
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	3.33×10^{-4}	0.69	1.48×10^{-3}	0.64

(Continued)

Table A1 | Continued

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
MAPK14	Mitogen-activated protein kinase 14	8.65×10^{-3}	1.35	0.445	1.12
MAS1	MAS1 oncogene	0.014	0.79	0.944	0.99
MATR3	Matrin 3	0.011	0.73	0.512	0.90
MCM5	Minichromosome maintenance deficient 5, cell division cycle 46 (*S. cerevisiae*)	0.041	1.10	0.181	1.12
MEF2D	Myocyte enhancer factor 2d	7.73×10^{-3}	1.35	0.152	1.31
MKLN1	Muskelin 1, intracellular mediator containing kelch motifs	4.12×10^{-5}	0.68	0.056	0.85
MLYCD	Malonyl-CoA decarboxylase	8.54×10^{-3}	1.66	0.023	1.46
MME	Membrane metallo endopeptidase	0.024	1.21	0.473	1.02
MRE11A	Meiotic recombination 11 homolog A (*S. cerevisiae*)	0.036	0.70	0.300	0.85
MRPL39	Mitochondrial ribosomal protein L39	1.22×10^{-3}	0.78	1.20×10^{-3}	0.69
MRPS14	Mitochondrial ribosomal protein S14	5.45×10^{-3}	0.71	0.945	1.01
MRPS17	Mitochondrial ribosomal protein S17	0.019	0.82	0.695	0.91
MRPS7	Mitochondrial ribosomal protein S7	7.10×10^{-3}	1.62	0.852	0.98
MTC	MT-protocadherin (KIAA 1775)	3.55×10^{-4}	1.29	0.984	1.00
MTEFR	Transcription termination factor, mitochondrial-like	0.025	0.66	0.542	0.87
MYO7A	Myosin VIIA	7.50×10^{-3}	0.64	0.361	0.88
NCBP1	Nuclear cap binding protein subunit 1, 80 kDa	2.15×10^{-3}	1.36	0.750	1.06
NDUFA4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	3.41×10^{-3}	1.40	0.909	1.01
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	0.022	1.65	0.043	1.37
NEU3	Neurotrophic tyrosine kinase, receptor, type 1	0.034	1.25	0.040	1.36
NEUD4	Neuronal d4 domain family member	0.018	1.70	0.029	1.36
NFATC3	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	0.010	0.74	0.084	0.77
NFE2L2	Nuclear factor, erythroid derived 2, like 2	1.38×10^{-4}	1.12	0.315	0.85
NGFA	Nerve growth factor, alpha	0.029	1.20	0.036	1.15
NGFR	Nerve growth factor receptor	4.51×10^{-3}	1.46	0.018	1.30
NINJ1	Ninjurin 1	0.031	0.72	0.111	0.73
NLK1	Nemo like kinase	3.33×10^{-3}	0.55	0.022	0.70
NOL5	Nucleolar protein 5	3.10×10^{-3}	0.80	0.071	0.82
NR2F1	Nuclear receptor subfamily 2, group F, member 1	1.74×10^{-3}	1.33	0.211	1.10
NRF1	Nuclear respiratory factor 1	0.040	0.83	0.166	0.66
NRXN1	Neurexin1	7.69×10^{-3}	0.78	1.73×10^{-4}	0.74
NSG1	Neuron-specific gene family member 1	0.039	0.84	0.309	0.95
NUCB	Calcium-binding protein	0.023	1.35	0.126	1.12
NXPH4	Neurexophilin4	3.87×10^{-3}	1.37	0.048	1.46
OGDH	Oxoglutarate dehydrogenase	0.031	1.27	0.947	0.99
OGN	Osteoglycin	1.48×10^{-3}	0.57	0.261	0.84
PBX4	Pre-B-cell leukemia transcription factor 4	7.99×10^{-4}	1.59	8.04×10^{-3}	1.38
PCP4	Purkinje cell protein 4	0.011	0.75	0.030	0.67
PDLIM2	PDZ and LIM domain 2	4.91×10^{-3}	1.99	0.014	1.55
PHYHIP	Phytanoyl-CoA hydroxylase interacting protein	5.61×10^{-3}	0.88	0.023	1.04
PIGQ	Glycosylphosphatidylinositol 1 homolog	0.013	1.36	0.035	1.18
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	0.049	1.30	0.657	1.08
PIR51	RAD51 associated protein 1	0.050	1.28	0.631	0.96
PLA2G4A	Phospholipase A2, group IVA (cytosolic, calcium-dependent)	3.26×10^{-3}	1.42	0.221	1.25
PMP22	Peripheral myelin protein 22	5.96×10^{-3}	1.17	0.118	1.07
POLE3	Polymerase (DNA directed), epsilon 3 (p17 subunit)	9.98×10^{-3}	1.39	0.124	1.18
PPIA	Peptidylprolyl isomerase A	0.040	1.38	0.130	1.17
PPM1L	Protein phosphatase 1 (formerly 2C)-like	0.032	1.35	0.212	1.31
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha isoform	0.020	1.21	9.25×10^{-3}	1.21
PPT	Palmitoyl-protein thioesterase	0.050	1.56	0.166	1.10
PRDX6	Peroxiredoxin 6	0.023	1.22	0.341	1.13

(Continued)

Table A1 | Continued

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
PRRX1	Paired related homeobox 1	0.050	1.16	0.805	1.03
PSCDBP	Pleckstrin homology, Sec7, and coiled-coil domains, binding protein	9.26×10^{-3}	0.89	0.052	0.81
PSMB4	Proteasome (prosome, macropain) subunit, beta type, 4	2.14×10^{-3}	1.23	0.235	1.09
PSMD10	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 10	0.025	1.17	0.875	1.01
PSMD14	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	0.029	0.70	0.154	0.79
PSMD2	26S proteasome, non-ATPase, 2	2.37×10^{-3}	1.54	0.033	1.25
PTH	Parathyroid hormone	0.040	1.19	0.608	0.96
PTP4A2	Protein–tyrosine phosphatase type 4a, member 2	0.027	1.24	0.011	1.24
PTPRZ1	Protein–tyrosine phosphatase, receptor type, Z1	0.041	0.90	0.597	1.05
RAB1	Ras-related protein	0.036	1.23	0.029	1.36
RAB7	Ras 7member of RAS oncogene family	0.042	0.80	0.048	0.85
RAB9	Ras 9 member of RAS oncogene family	0.036	1.32	0.377	1.15
RAD1	RAD1 homolog (*S. pombe*)	6.01×10^{-4}	0.69	0.046	0.78
RAD51	RAD51 homolog (*S. cerevisiae*)	0.013	1.45	0.131	1.48
RALA	V-ral simian leukemia viral oncogene homolog A (Ras-related)	8.17×10^{-3}	1.74	0.096	1.27
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	0.032	0.84	0.158	0.89
RASA2	RAS p21 protein activator 2	0.034	0.73	0.537	1.18
RBL1	Retinoblastoma-like 1 (p107)	0.046	0.73	0.111	0.73
RIS2	Retroviral integration site 2	0.024	0.74	0.244	0.88
RISP	Ubiquinol–cytochrome *c* reductase, Rieske iron–sulfur polypeptide 1	0.048	0.76	0.159	0.84
RNF20	Ring finger protein 20	0.017	0.69	0.058	0.76
RPL18	Ribosomal protein L18	0.031	0.75	0.033	0.80
RPL29	Ribosomal protein L29	1.43×10^{-3}	1.50	0.110	1.19
RPL36	Ribosomal protein L36	0.031	0.85	0.979	1.00
RPS26	Ribosomal protein S26	0.032	1.57	0.192	1.14
RPS27L	Ribosomal protein S27	0.032	1.44	0.201	1.13
RPS6KA2	Ribosomal protein S6 kinase, 90kD, polypeptide 2	5.70×10^{-4}	0.82	0.708	0.95
RPS6KB1	Ribosomal protein S6 kinase, 70kD, polypeptide 1	0.037	1.28	0.017	1.25
SCN11A	Sodium channel, voltage-gated, type11, alpha polypeptide	0.024	1.45	0.346	0.86
SCO1	Similar to SCO cytochrome oxidase deficient homolog 1 (yeast)	0.033	0.84	0.641	1.05
SCUBE2	Signal peptide, CUB domain, EGF-like 2	2.96×10^{-3}	0.67	0.010	0.70
SCYA20	Small inducible cytokine subfamily A20	4.02×10^{-3}	0.75	0.288	0.91
SDHB	Succinate dehydrogenase complex, subunit A, flavoprotein	6.23×10^{-3}	1.32	0.181	0.87
SDHC	Succinate dehydrogenase complex, subunit C	0.017	1.18	0.035	1.13
SECISBP2	Selenocysteine insertion sequence-binding protein 2	8.27×10^{-3}	0.76	0.367	1.03
SERPINC1	Serine (or cysteine) peptidase inhibitor, clade C (antithrombin), member 1	0.040	1.35	0.152	1.37
SFN	Stratifin	0.022	1.14	0.635	1.04
SKIL	Ski like	8.52×10^{-3}	1.31	2.44×10^{-4}	1.17
SLC18A2	Solute carrier family 18 member 2	0.023	1.46	0.144	0.91
SLC29A1	Solute carrier family 29 (nucleoside transporters), member 1	0.021	0.73	0.164	1.41
SLC2A14	Solute carrier family 2 (facilitated glucose transporter), member 14	4.37×10^{-3}	0.68	0.032	0.73
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	0.030	1.58	0.221	1.32
SLC35D2	Solute carrier family 35, member D2	0.024	1.39	0.016	1.61
SLC35F5	Solute carrier family 35, member F5	0.018	0.73	0.890	0.98
SLC5A3	Solute carrier family 5 (inositol transporters), member 3	4.51×10^{-4}	0.63	4.60×10^{-3}	0.76
SLC6A4	Solute carrier family 6, member 4 (serotonin transporter)	3.78×10^{-6}	0.62	0.134	1.08
SLC8A2	Solute carrier family 8 (sodium/calcium exchanger), member 2	0.032	1.30	0.194	0.93
SMAD2	MAD homolog 2 (*Drosophila*)	0.035	0.83	0.323	1.09
SNCA	Synuclein, alpha	5.13×10^{-3}	0.71	0.523	0.87
SOX6	SoxLZ/Sox6 leucine zipper binding protein in testis	0.027	1.17	0.937	0.99
SPEN	SMART/HDAC1 associated repressor protein	0.024	0.79	0.013	0.68

(Continued)

Table A1 | Continued

		Nicotine		Nicotine and mecamylamine	
Gene symbol	**Gene name**	***p*-Value**	**Ratio**	***p*-Value**	**Ratio**
STAT5A	Signal transducer and activator of transcription 5a	0.021	1.25	0.044	1.24
SWAP70	Switch-associated protein 70	0.032	0.61	0.646	0.87
SYN2	Synapsin 2	0.014	1.25	0.988	1.00
SYT2	Synaptotagmin 2	1.36×10^{-3}	1.24	0.192	1.17
SYT7	Synaptotagmin 7	5.30×10^{-3}	1.69	0.492	1.08
TAC2	Tachykinin 2	3.45×10^{-3}	0.72	0.988	1.00
TBC1D17	TBC1 domain family, member 17	0.046	1.43	0.051	1.21
TBP1	Tat-binding protein-1	0.041	0.77	0.108	0.80
TCFEB	Transcription factor EB	6.61×10^{-5}	0.76	0.518	0.97
TDG	Thymine DNA glycosylase	0.032	0.57	0.108	0.70
TF	Transferrin	0.028	0.73	0.045	0.70
TFR	Transferrin receptor	5.66×10^{-3}	1.32	0.893	1.02
TGFB2	Transforming growth factor, beta 2	5.58×10^{-5}	0.74	0.019	0.71
TGFBR2	Transforming growth factor, beta receptor 2	6.22×10^{-3}	0.57	0.913	0.98
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	0.042	1.43	0.134	1.22
TRAF6	Tnf receptor-associated factor 6	0.035	0.75	0.654	0.94
TRG	Thyroid regulating gene	0.019	0.69	0.090	0.71
TRNT1	TRNA nucleotidyl transferase, CCA-adding1	0.022	0.76	0.538	0.97
TRP53	Transformation-related protein 53	6.94×10^{-3}	1.32	0.252	1.08
TSGA13	Testis specific gene A13	2.29×10^{-3}	1.07	5.98×10^{-3}	1.41
TUBB5	Beta-tubulin, 5	0.017	1.19	0.079	1.08
UAP1	Similar to UDP-*N*-acteylglucosamine pyrophosphorylase 1 homolog	0.021	1.24	0.526	0.93
UBE1X	Ubiquitin-activating enzyme e1, Chr X	0.019	1.14	0.761	0.99
UBE2A	Ubiquitin-conjugating enzyme E2A	0.018	1.54	0.250	1.10
UBE2G1	Ubiquitin-conjugating enzyme E2G 1 (UBC7 homolog, C. elegans)	0.023	0.88	0.425	1.22
UBQLN1	Ubiquilin 1	0.030	1.18	0.763	0.96
UBQLN3	Ubiquilin 3	0.016	0.58	0.036	0.60
UQCRFS1	Ubiquinol–cytochrome *c* reductase, Rieske iron–sulfur polypeptide 1	3.11×10^{-3}	1.53	0.832	0.98
USP28	Ubiquitin-specific protease 28	8.60×10^{-3}	1.29	0.062	1.29
VAMP3	Vesicle-associated membrane protein 3	0.036	1.21	9.68×10^{-3}	1.12
VCL	Vinculin	2.10×10^{-3}	1.39	3.10×10^{-3}	1.30
VEGFA	Vascular endothelial growth factor A	0.038	0.84	0.513	0.92
VGLL2	Vestigial like 2 homolog (*Drosophila*)	0.014	1.21	0.501	1.02
VIL1	Villin1	0.016	0.65	9.82×10^{-3}	0.79
WNT1	Wingless-related MMTV integration site 1	0.042	1.17	0.493	1.07
XCL2	Chemokine (C motif) ligand 2	0.023	1.38	0.073	1.33
XTP2	HBxAg transactivated protein 2	0.031	0.88	0.308	0.90
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	0.037	1.21	0.069	0.87
ZAP70	Zeta-chain (TCR) associated protein kinase	2.07×10^{-4}	1.36	0.139	1.09
ZFP98	Zinc finger protein 98	8.22×10^{-4}	1.21	0.616	0.97
ZNF189	Zinc finger protein 189	0.035	0.79	0.264	0.80
ZNF286	Zinc finger protein 286	0.012	1.18	0.381	1.09