available at www.sciencedirect.com

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Nicotinic modulation of gene expression in SH-SY5Y neuroblastoma cells

Travis Dunckley[1], *Ronald J. Lukas**

Division of Neurobiology, Barrow, Neurological Institute, 350 West Thomas Road, Phoenix, AZ 85013, USA

ARTICLE INFO

Article history:
Accepted 29 July 2006
Available online 1 September 2006

Keywords:
Nicotine
Neuroblastoma cells
SH-SY5Y cells
Gene expression
Microarray
nAChR
Upregulation

Abbreviations:
dTC, D-tubocurarine
nAChR, nicotinic acetylcholine receptor(s)
RT-PCR, reverse transcription-polymerase chain reaction
qRT-PCR, quantitative real-time RT-PCR

ABSTRACT

Exposure to nicotine has a broad range of physiological and psychological effects that can be long lasting and contribute to nicotine dependence. On a time course longer than that needed to activate nicotinic acetylcholine receptor (nAChR) function, nicotine exposure induces functional inactivation of nAChR, upregulation of nAChR radioligand binding sites, and other alterations of cellular functions. To identify possible mechanisms underlying nicotine-induced changes in nAChR numbers and function, we defined changes in gene expression in neuron-like, SH-SY5Y human neuroblastoma cells following 24 h of continuous exposure to 1 mM nicotine. This treatment condition produces both functional inactivation and upregulation of nAChR. Repeat and cross-controlled microarray (~5000 genes queried) analyses revealed 163 genes whose expression was consistently altered at the $p<0.01$ level following nicotine treatment. Quantitative, real-time, reverse transcription-polymerase chain reaction analyses confirmed altered expression of thirteen out of fourteen of these genes chosen for further study, including contactin 1, myozenin 2, and ubiquitin-conjugating enzymes E2C and E2S. Inhibition or reversal of these effects by the general nAChR antagonist, D-tubocurarine, indicated that gene expression changes are dependent on nAChR activation. Studies using other nAChR subtype-selective antagonists identified gene expression changes that required activation of both α7- and α3*-nAChR, α7-nAChR alone, or either α7- or α3β4*-nAChR, suggesting some convergent and some divergent pathways of gene activation coupled to these nAChR subtypes. These results suggest that longer-term physiological and psychological effects of nicotine exposure and changes in nAChR expression may be due in part to effects on gene expression initiated by interactions with nAChR.

© 2006 Elsevier B.V. All rights reserved.

1. Introduction

Nicotinic acetylcholine receptors (nAChR) exist as a family of diverse subtypes and are prototypes of the ligand-gated ion channel superfamily of neurotransmitter receptors (Jensen et al., 2005; Lukas and Bencherif, 2006). nAChR have been implicated in a variety of processes in the nervous system including attention and cognition (Levin and Simon, 1998), mood and emotion (Shytle et al., 2002), synaptic plasticity (Ji et al., 2001), and cell survival (Donnely-Roberts and Brioni, 1999). Some of these effects simply reflect nAChR mediation of transmembrane ion flux altering cellular electrical excitability (Lukas and Bencherif, 2006). However, evidence is emerging that at least some nAChR subtypes are engaged in

* *Corresponding author*. Fax: +1 602 406 4172.
E-mail address: rlukas@chw.edu (R.J. Lukas).
[1] Current address, Translational Genomics Research Institute, Phoenix, AZ, USA.

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.07.111

intracellular signaling cascades with a broad range of consequences, such as changes in gene expression (Dajas-Bailador et al., 2002; Sharma and Vijayaraghavan, 2002; Brunzell et al., 2003; Dunckley and Lukas, 2003; Li et al., 2003, 2004; Sun et al., 2003).

Whereas nicotine exposure initially induces opening of nAChR ion channels, longer-term exposure leads to other changes. Seconds to hours of nicotine exposure induces nAChR functional desensitization and longer-lasting functional inactivation, whereas hours to days of nicotine exposure produce increases in numbers of nAChR-like radioliogand binding sites ("upregulation;" Gentry and Lukas, 2002). However, cell specificity of some of these effects suggests that non-nAChR proteins could be involved (Gentry and Lukas, 2002), and the fact that some of these effects occur on time-scales much longer than those involved in acute activation of receptor suggests engagement of other mechanisms in nicotine-induced changes in nAChR numbers and function.

We previously reported that 1 h of treatment with 1 mM nicotine acts through nAChR to alter expression of a select set of genes in the neuron-like, SH-SY5Y neuroblastoma cell line (Dunckley and Lukas, 2003). We postulated that some of these effects could contribute to the loss of nAChR function seen for exposure to nicotine under these conditions. In the current studies, we used cDNA microarray analyses to identify candidate genes in SH-SY5Y cells sensitive to 1 day of nicotine exposure and possibly involved in nicotine-induced upregulation of nAChR radioligand binding sites. We then used quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analyses to validate microarray findings and to extend the study to an examination of the pharmacology and nAChR subtype specificity/selectivity of gene expression effects. The results show reproducible and significant effects on gene expression that may underlie some of the effects of nicotine exposure on nAChR numbers and function and perhaps on other nervous system processes.

2. Results

2.1. *Twenty four hours of nicotine exposure affects gene expression*

To determine the extent to which nicotine exposure, under conditions that produce both functional inactivation of nAChR and upregulation of nAChR-like radioligand binding sites, affects gene expression in neuron-like SH-SY5Y cells naturally expressing human $\alpha3^*$- and $\alpha7$-nAChR, we treated cells under control conditions or in the continuous presence of 1 mM nicotine for 24 h and determined gene expression levels using a cDNA microarray representing ~5000 unique genes. Based on our previous results investigating the effects of 1-hour nicotine exposure on gene expression (Dunckley and Lukas, 2003; Ke et al., 1998), we anticipated that chronic nicotine treatment would have subtle effects on gene expression in the SH-SY5Y cell line. For this reason, we performed reciprocal fluorescent labeling of the control and nicotine-treated mRNA populations in duplicate, yielding a data set consisting of four independently hybridized, cross-controlled microarrays (see Experimental procedures).

Using a significance cut-off value of $p<0.01$, we observed expression changes for 82 genes and found 163 genes with

Table 1 – Microarray based analysis of nicotine-mediated gene expression changes

Gene name	Genbank #	Array 1 Ratio	Array 2 Ratio	Array 3 Ratio	Array 4 Ratio	Average Ratio	SD	*p* value
cadherin-like 23, *CDH23*	R91296	1.61	1.36	2.10	1.55	1.66	0.32	0.001
Complement component 7, *C7*	AA598478	1.31	1.17	1.29	1.34	1.30	0.09	0.001
Contactin 1, *CNTN1*	R25234	1.15	1.19	1.35	1.24	1.23	0.09	0.01
Eukaryotic translation initiation factor 4A isoform 2	W21081	1.34	1.45	1.34	1.42	1.39	0.06	0.0001
Golgi autoantigen, golgin subfamily a, 4, *golgA4*	AA460981	1.56	1.42	1.82	1.49	1.57	0.17	0.0001
Hypothetical zinc finger protein *MGC2396*	R08755	1.47	1.40	1.61	1.57	1.51	0.10	0.0001
Myeloid/lymphoid or mixed-lineage leukemia 3, *MLL3*	N91302	1.69	1.10	1.02	1.29	1.27	0.29	>0.01
myozenin 2 (calcineurin-binding protein calsarcin-1)	AA064973	1.75	1.76	1.33	1.90	1.69	0.25	0.001
Ninein (GSK3B interacting protein), *NIN*	R16456	1.24	1.20	1.65	1.59	1.42	0.23	0.01
RAD50 homolog, *RAD50*	H99196	1.29	1.30	1.29	1.34	1.30	0.03	0.001
Son of sevenless homolog 1, *SOS1*	H643241	1.31	1.46	1.37	1.40	1.39	0.06	0.0001
Stress-induced-phosphoprotein 1, *STIP1*	AA487635	0.78	0.82	0.68	0.81	0.76	0.06	0.01
Ubiquitin-conjugating enzyme E2C, *UBE2C*	AA430504	0.85	0.88	0.75	0.85	0.83	0.05	0.01
Ubiquitin-conjugating enzyme E2S, *UBE2S*	AA464729	0.85	0.87	0.82	0.81	0.83	0.03	0.01
Glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*	H16958	1.06	1.01	0.75	0.87	0.92	0.14	>0.01

Microarray analyses reveal numerous, consistent alterations of gene expression following 24 h of chronic nicotine treatment. Microarray analyses were conducted as described in Experimental procedures to identify genes whose expression was changed relative to untreated control samples by 24 h of exposure to 1 mM nicotine. Indicated (columns left-to-right) are the names of nicotine-sensitive genes identified and subjected to further investigation, their Genbank accession numbers, expression ratios determined in each array study, the average ratio for gene changes across all four microarray hybridizations, the standard deviation of those averages (SD), and the significance value of the observed gene expression change. The expression ratio corresponds to the normalized value of fluorescence intensities of the nicotine-treated sample divided by that of the control sample. Therefore, a ratio greater than 1 indicates nicotine-induced upregulation of gene expression, whereas a ratio less than one indicates repression. The Genbank accession numbers correspond to those for the cDNAs printed on the microarray, not to the full-length mRNAs for each gene.

Table 2 – qRT-PCR validation of nicotine-induced changes in gene expression

Gene name	Nicotine	Range (one SD)	dTC	Range (one SD)	dTC+nicotine	Range (one SD)
CDH23	**−1.51**	−2.58/1.14	**−1.90**	−6.59/1.84	**−1.34**	−2.19/1.22
C7	**1.62**	1.28/2.06	**1.12**	1.03/1.21	**1.08**	1.04/1.12
CNTN1	**1.44***	1.37/1.53	**−1.05**	−1.24/1.03	**−1.04**	−1.39/1.36
eIF-4A	**3.61****	2.16/6.02	**1.57**	1.13/2.19	**−1.12**	−1.59/1.27
golgA4	**2.11****	1.49/2.87	**−1.03**	−1.48/1.40	**−1.02**	−1.23/1.18
MGC2396	**2.96****	2.47/3.50	**−1.65***	−1.97/−1.38	**1.22**	−1.04/1.56
MLL3	**3.45***	2.13/5.62	**−1.05**	−1.66/1.48	**−1.08**	−1.24/1.06
myozenin 2	**2.31***	1.95/2.75	**1.04**	−1.39/1.52	**−1.76**	−2.38/−1.29
NIN	**1.48****	1.39/1.59	**−1.35**	−2.65/1.45	**−1.02**	−2.19/2.13
Parkin	**−1.37***	−1.51/−1.23	**−1.35****	−1.42/−1.27	**−1.53**	−1.96/−1.19
RAD50	**1.31**	1.06/1.61	**1.31***	1.21/1.42	**1.25**	1.12/1.39
SOS1	**1.29**	1.13/1.40	**−1.39**	−1.64/−1.16	**−1.84***	−2.25/−1.50
STIP1	**−1.26***	−1.37/−1.14	**1.18**	1.05/1.33	**−1.10**	−1.32/1.15
UBE2C	**−1.49****	−1.83/−1.53	**−1.03**	−1.42/1.35	**−1.31**	−1.52/−1.13
UBE2S	**−1.49***	−1.67/−1.34	**−1.17**	−1.38/1.01	**1.17***	1.12/1.21
GAPDH	**1.11**	−1.23/1.51	**1.15**	−1.07/1.23	**−1.06**	−1.20/1.06

Quantitative real-time RT-PCR studies verify that prolonged nicotine treatment modulates the expression of numerous genes. qRT-PCR experiments were performed as described in Experimental procedures (see also Dunckley and Lukas, 2003) to extend, replicate, and cross-validate findings from microarray analyses. Samples were treated for 24 h in the absence of drug or for 24 h with 1 mM nicotine (nicotine), 100 μM D-tubocurarine (dTC), or both nicotine and dTC (dTC+nicotine). Numbers in boldface indicate the average fold change relative to untreated controls in expression of the indicated gene (n=4–5 reactions) based on differences in reaction cycles needed to achieve the same amount of reaction product. Values in the column to the immediate right of the corresponding, average fold change value (range as one SD) represent the variance in determined fold change.

* Indicates significance at the $p<0.05$ level as measured by paired t test.

** Indicates significance at the $p<0.01$ level.

$p<0.05$ (this gene list is provided as a supplement to this manuscript, and it and the entire data set can be found at ftp://srweb.chw.edu; a user ID and password for accessing and downloading the data set will be provided upon e-mail request). At the $p<0.01$ level of significance, approximately 50 false-positives are expected. At a significance level of $p<0.0001$ (no false-positives predicted), four genes were identified (Table 1)[2]. Analysis of the data using the expression analysis systematic explorer (EASE; http://david/niaid.nih.gov/david/ease.htm) and the GeneGo pathway analysis software did not reveal that any particular cadre of functionally relevant genes was selectively affected by nicotine exposure, in that there were no pathway or domain clusters of significance, although more than 5% of the genes on the filtered list fell into "physiological process" (12.7%), "cellular process" (9.6%), "binding" (9.6%), "metabolism" (8.4%), "catalytic activity" (7.2%), "cellular physiological process" (6.6%), or "cell growth and/or maintenance" (5.4%; sometimes overlapping) categories under the gene ontology analysis.

We chose 14 genes for further follow-up confirmation (Table 1). The genes were chosen either because they were among the four genes present at the highest level of significance ($p<0.0001$) or because they code for proteins that we considered to be of significant biological interest. Among these are genes involved in signal transduction, ubiquitin-mediated protein degradation, vesicle trafficking, and two genes that also were identified previously as showing altered expression following 1 h of 1 mM nicotine treatment (Dunckley and Lukas, 2003).

[2] Gene abbreviation names are indicated in the text or table or figure legends.

2.2. *Validation of microarray studies using quantitative real-time reverse transcription-polymerase chain reaction analyses*

An independent set of qRT-PCR analyses was conducted using total RNA isolated from three different preparations from cells treated in the absence or presence of 1 mM nicotine for 24 h to ascertain reliability and reproducibility of the microarray analyses. Vimentin mRNA was used as the control to assess fold changes in expression for the genes of interest because its expression levels were unaffected based on microarray analyses (data not shown). In addition, qRT-PCR experiments showed that both *vimentin* (data not shown) and *GAPDH* (Table 2, last row) genes were expressed at similar levels across all drug treated samples. Furthermore, we had previously observed that *vimentin* message levels are unaffected by 1 h of 1 mM nicotine exposure (Dunckley and Lukas, 2003). As a quality control measure, we also analyzed each PCR product by agarose gel electrophoresis (Fig. 1) and melting curve determinations (data not shown) to verify that a single product of the predicted size was produced. The qRT-PCR data validated the observed gene expression changes identified using microarray analyses for 13 of the 14 genes subjected to further study (Tables 2 and 3). The exception was cadherin-like 23 (*CDH23*), which was downregulated according to qRT-PCR results, counter to the effect reported by the microarray. Effects on complement component 7 (*C7*), *RAD50* homolog, and son of sevenless homolog (*SOS*) showed the same effect on qRT-PCR and microarray analyses, although they did not reach significance at 95% confidence for qRT-PCR analyses in this set of studies (however, see below). The bulk of the gene expression changes was consistent across replicate qRT-PCR analyses and

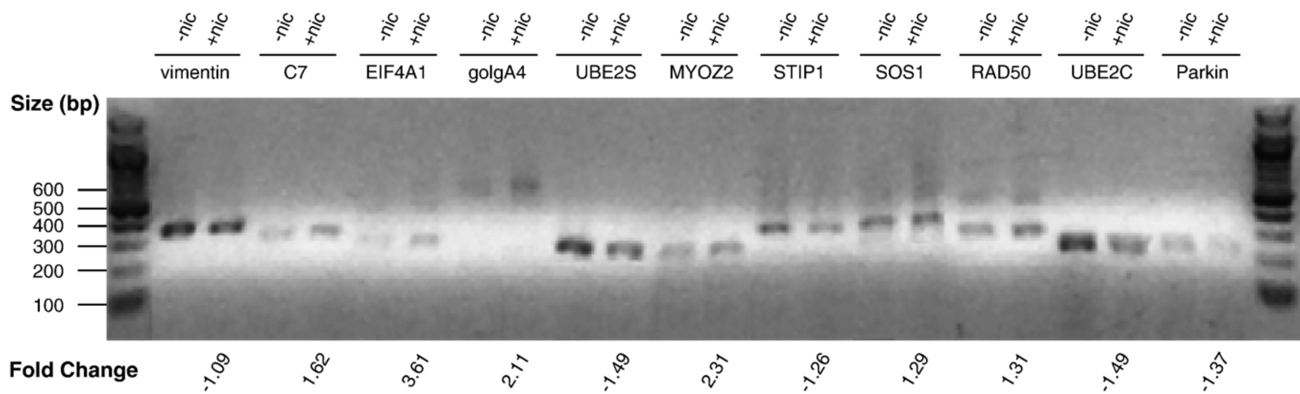

Fig. 1 – Demonstration of specificity of nicotine-induced changes in gene expression and uniformity of reaction product as assessed using RT-PCR analysis. Selected genes identified via microarray analysis as being sensitive to nicotine exposure were targeted for replicate studies and validation of findings based on quantitative real-time RT-PCR-based as described in Experimental procedures. Comparisons were made for RNA isolated from untreated control cells (lanes labeled "−nic") or for samples treated for 24 h in the presence of 1 mM nicotine (lanes labeled "+nic"). Portions of the samples were resolved on an agarose gel to confirm that each PCR reaction generated only one product of the predicted size, indicating specificity of the primers used (see size calibration ladders in leftmost and rightmost lanes). The specific genes targeted (labels above the relevant lanes) were (left to right) *vimentin*, complement component 7 (*C7*), eukaryotic translation initiation factor 1 (*eIF-4A1*), golgi autoantigen 4 (*golgA4*), ubiquitin-conjugating enzyme E2S (*UBE2S*), myozenin 2 (*MYOZ2*), stress-induced phosphoprotein 1 (*STIP1*), son of sevenless homolog 1 (*SOS1*), RAD50 homolog (*RAD50*), ubiquitin-conjugating enzyme E2C (*UBE2C*), and *Parkin*. Calculated expression ratios (fold change) averaged from at least four PCR reactions for the nicotine-treated sample relative to the control sample are indicated beneath the relevant lanes.

statistically significant even though they were subtle in absolute terms.

The *Parkin* gene was not represented on the microarray used in these studies. However, we included it in our qRT-PCR analyses for several reasons. First, the *Parkin* gene encodes a ubiquitin protein ligase, and several other genes implicated in ubiquitin-mediated protein degradation were altered in response to nicotine (see Table 1; ubiquitin-conjugating enzymes E2C and E2S; *UBE2C* and *UBE2S*). Second, we have shown previously that the expression of ubiquitin protein ligase E3A (UBE3A), another E3 ubiquitin protein ligase, is downregulated following 1 h of 1 mM nicotine treatment (Dunckley and Lukas, 2003). Since expression of UBE3A was unaffected following 24 h of nicotine treatment (data not shown), we sought to determine if any additional ubiquitin protein ligases could be affected during chronic nicotine exposure. Third, there is a tentative association between smoking and decreased risk of developing Parkinson's disease (for review, see Huang et al., 2003), suggesting that chronic nicotine exposure may affect the expression of genes involved in the development of this disorder. The qRT-PCR results for *Parkin* show a statistically significant downregulation of expression following 24 h of nicotine treatment (Table 2).

2.3. *Effects of nicotine on gene expression differ as a function of length of drug exposure*

We have previously reported that nicotine affects the expression of a select set of genes in SH-SY5Y cells following 1 h of exposure (Dunckley and Lukas, 2003). Not surprisingly, the genes identified here as being affected following 24 h of nicotine treatment largely differ from those identified previously following 1 h of treatment. Matrin 3, tissue factor pathway inhibitor 2, zinc finger RNA binding protein, dihydrofolate reductase, ubiquitin protein ligase E3A, chromosome 2 open reading frame, retinoblastoma binding protein 6, protein tyrosine phosphatase receptor type A, PIG7/LPS-induced TNF-α factor, and cDNA DKFZp564F112 genes, which were downregulated according to qRT-PCR studies of 1 h nicotine effects, and the early growth response 1 gene, which was upregulated under the same conditions (Dunckley and Lukas, 2003), were not significantly affected after 24 h of nicotine exposure. Only two genes, contactin 1 (*CNTN1*) and mixed lineage leukemia 3 (*MLL3*) show altered gene expression following both 1- and 24-hour nicotine exposures (see Table 2 and Dunckley and Lukas, 2003). However, and interestingly, both genes were repressed following a 1-hour treatment (−1.4 to −1.7-fold for *CNTN*; −1.4 to −1.5-fold for *MLL3*; qRT-PCR results), whereas both genes are significantly induced (1.4 to 2.2-fold for *CNTN*, 1.4 to 3.5-fold for *MLL3*) following 24 h of continuous nicotine exposure (Tables 2 and 3).

These results suggest that a simple mechanism involving nAChR activation and a persisting effect on gene expression does not explain the results. There are several alternative explanations that are not necessarily mutually exclusive. One is that an initial activation of nAChR sets into motion a cascade of gene expression effects, some of which are triggers for others. Another is a similar scenario but also includes ongoing modulation of gene regulatory events by sustained, low level activity of nAChR. Although nAChR undergo desensitization and other phases of longer-lasting functional inactivation during sustained nicotine exposure, the loss of function is not complete (Ke et al., 1998). Lastly, because different nAChR subtypes have different rates, extents, and nicotine concentration dependencies for functional inactivation processes, there also is the possibility that relative contributions of

Table 3 – nAChR inhibitors' effects on expression of candidate genes

Gene name	Nicotine	Range (one SD)	α-cobratoxin	Range (one SD)	α-cobratoxin + nicotine	Range (one SD)	Mecamylamine	Range (one SD)	Mecamylamine + nicotine	Range (one SD)
C7	**2.08****	1.79/2.43	**1.78****	1.67/1.89	**−1.55***	−1.85/−1.29	**−1.01**	−1.03/1.01	**−1.06**	−1.12/1.00
CNTN1	**2.20***	1.61/3.01	**1.04**	−1.31/1.42	**−2.01***	−2.55/−1.59	**−1.04**	−1.22/1.13	**1.09**	−1.10/1.32
eIF-4A	**1.47****	1.35/1.61	**−1.04**	−1.07/−1.01	**−1.87****	−1.93/−1.83	**−1.18**	−1.33/−1.05	**−1.06**	−1.19/1.05
golgA4	**2.79****	2.48/3.18	**3.39****	2.89/3.97	**1.06**	−1.27/1.42	**2.92***	1.87/4.59	**1.82**	1.04/3.16
MGC2396	**2.87****	2.55/3.22	**2.91****	2.62/3.22	**2.57***	1.99/3.38	**2.57****	2.28/2.89	**2.85****	2.41/3.36
MLL3	**1.44****	1.35/1.52	**1.12**	1.04/1.20	**−1.94****	−1.97/−1.89	**1.25***	1.17/1.33	**1.19**	1.05/1.35
myozenin 2	**2.38****	2.01/2.81	**2.50****	2.33/2.67	**−1.21**	−1.33/−1.09	**1.29****	1.22/1.35	**−1.05**	−1.15/1.03
NIN	**2.33****	2.04/2.66	**1.00**	−1.10/1.13	**−1.64****	−1.73/−1.55	**1.06***	1.04/1.08	**1.54***	1.27/1.87
RAD50	**1.41****	1.32/1.52	**1.33****	1.24/1.42	**−1.91****	−2.03/−1.79	**1.05**	−1.01/1.12	**1.06**	1.02/1.11
SOS1	**1.60****	1.54/1.67	**1.01**	−1.10/1.14	**−2.93****	−3.27/−2.62	**1.08**	−1.03/1.22	**−1.05**	−1.11/−1.01
STIP1	**−1.53***	−1.71/−1.37	**−1.33***	−1.44/−1.22	**−1.27**	−1.46/−1.11	**−1.23****	−1.24/−1.21	**−1.37****	−1.45/−1.28
UBE2C	**−1.95****	−1.99/−1.91	**−1.61**	−2.07/−1.26	**−2.52****	−3.05/−2.07	**−1.20**	−1.36/−1.06	**−2.58****	−3.09/−2.16
UBE2S	**−1.72***	−2.01/−1.46	**−3.07****	−3.58/−2.64	**−2.33***	−2.87/−1.89	**1.24**	−1.37/2.10	**−2.69****	−3.10/−2.35
GAPDH	**−1.05**	−1.18/1.08	**1.04**	−1.29/1.38	**−1.15**	−1.35/−1.05	**−1.03**	−1.32/1.25	**1.03**	1.02/1.04

nAChR subtypes differentially affect gene expression in response to chronic nicotine treatment. qRT-PCR experiments were performed as described for Table 2. Samples were treated for 24 h with 1 mM nicotine alone, 1 μM α-cobratoxin, α-cobratoxin + nicotine, 3 μM mecamylamine, or mecamylamine + nicotine. Numbers in boldface represent the average fold change in expression (relative to untreated controls) of the indicated gene across multiple, independent PCR reactions ($n = 4$–5). Values in the columns to the immediate right of the fold change values (range as one SD) represent the variance in determined fold change.

* Indicates significance at the $p < 0.05$ level as measured by paired t test.

** Indicates significance at the $p < 0.01$ level.

different receptor subtypes to effects of 1 h of nicotine exposure could be different from those upon 24 h of nicotine exposure. In addition, intermediaries in the signaling cascade from nAChR activation to gene expression also are likely to differ depending on nAChR subtype activated and strength of that activity, and the intermediaries also would be subject to desensitization as a function of time after nicotine exposure and their initial stimulation. It is not surprising that gene expression changes taken as snapshots at different times of nicotine exposure will be distinctive given the complex interplay between activation/desensitization of nAChR subtypes, downstream signaling elements, and a temporally changing pattern of gene expression, whether for gene products significantly altered here or those more subtly altered and escaping detection in these studies.

2.4. *nAChR activation is required for nicotine's effects on gene expression*

Nicotine rapidly crosses the plasma membrane, making it possible that it could alter gene expression through nAChR-dependent or nAChR-independent signaling pathways. To determine whether nAChR activation is necessary to affect gene expression, we used qRT-PCR analysis to assess sensitivity of the effects of 24 h of nicotine treatment to blockade by the general nAChR antagonist dTC using the same preparations from cells used in the initial, validation phase, qRT-PCR studies of nicotine effects alone on gene expression (Table 2). Thus, total RNA was isolated from control cells, from nicotine-treated cells (1 mM), from dTC treated cells (100 μM), and from cells treated with the combination of 1 mM nicotine and 100 μM dTC. dTC inhibited or reversed the effects of nicotine on the thirteen genes showing sensitivity to nicotine exposure identified based on microarray studies and confirmed using qRT-PCR (Table 2). dTC did not block the effect of nicotine exposure on *Parkin*, although the effect of nicotine in the presence of dTC was no longer significant at the $p<0.05$ level, nor did dTC plus nicotine exposure significantly alter cadherin-like 23 gene expression. Nevertheless, these results indicate that the majority of gene expression changes in response to nicotine require the interaction of nicotine with nAChR and at least an initial phase of nAChR activation.

2.5. *Roles of distinct nAChR subtypes in nicotine's regulation of gene expression*

The SH-SY5Y cell line expresses α3, α5, α7, β2, and β4 nAChR subunits that assemble to form various α3*-nAChR subtypes or homomeric α7-nAChR (Lukas et al., 1993; Peng et al., 1997). To assess the extent to which these different nAChR subtypes contribute to the observed effects of nicotine on gene expression, we used subtype-selective antagonists to inhibit nicotine's actions. We treated the cells with 1 mM nicotine alone or with 1 mM nicotine in the presence of either 1 μM α-cobratoxin or 3 μM mecamylamine for 24 h. A 1 μM concentration of α-cobratoxin will specifically inhibit α7-nAChR responses to nicotine. Although interactions of mecamylamine (or of dTC) at non-nAChR targets cannot be entirely discounted, mecamylamine at low micromolar concentrations inhibits nAChR containing α3 subunits that dominate ion flux responses to nicotinic agonists in SH-SY5Y cells (Lukas et al., 1993). Mecamylamine at a concentration of 3 μM would be expected to inhibit about 80% of the nicotinic responses of α3*-nAChR (Lukas et al., 1993) and only 20% of α7-nAChR responses to nicotine (Papke et al., 2001). qRT-PCR analyses of these drug effects were done for an entirely different set of cells than those used for microarray or initial qRT-PCR studies.

In the presence of 1 mM nicotine alone, each of the thirteen genes whose expression level changed on such exposure to nicotine as assessed using initial microarray (Table 1) and validation qRT-PCR studies (Table 2) again was shown to have altered expression at the $p<0.005$ level (Table 3).

Other results showed that α-cobratoxin inhibited or reversed the effects of nicotine for both the Golgi autoantigen/golgin subfamily A4 (*golgA4*) and Ninein/GSK3B interacting protein (*NIN*) genes (Tables 3 and 4 for a summary). In contrast, mecamylamine showed a trend toward inhibition of nicotine's effects that did not reach statistical significance. Since the 3 μM dose of mecamylamine used would be predicted to inhibit about 20% of α7-nAChR, this result is consistent with the interpretation that regulation of these two genes by 24 h of nicotine treatment may result from the activation of α7-nAChR alone.

For *myozenin 2*, *C7*, *CNTN1*, eukaryotic translation initiation factor 4A isoform 2 (*eIF-4A*), *MLL3*, *RAD50*, and *SOS* (see Tables 3 and 4), mecamylamine inhibited and α-cobratoxin reversed nicotine's effects on gene expression. These results suggest that the combined nicotinic stimulation of both α7-nAChR and α3*-nAChR is required to regulate these genes because blockade of either nAChR subtype prevented the nicotine effect. However, we cannot rule out the possibility that inhibition of roughly 20% of α7-nAChR in the presence of mecamylamine may be sufficient to block nicotinic regulation of these 7 genes, especially given the more dramatic reversal of nicotine's effects by α-cobratoxin.

For stress-induced phosphoprotein 1 (*STIP1*), ubiquitin-conjugating enzymes *UBE2C* and *UBE2S*, and hypothetical zinc finger protein *MGC2396*, neither α-cobratoxin nor mecamylamine blocked the effect that chronic nicotine treatment had on gene expression (Tables 3 and 4; although there was a trend toward inhibition by α-cobratoxin of nicotine's effect on *STIP1*). Importantly, the general nAChR antagonist dTC inhibited (or tended to inhibit –*UBE2C*) nicotine's effects on the expression of these genes (see Tables 2 and 4). These results suggest that nicotinic stimulation of either α7- or α3*-nAChR may be sufficient to affect the expression of these genes, but that blockade of both subtypes by dTC was required to prevent nicotine action.

These studies allowed us to group affected genes based on their responses to nicotine in the presence of antagonists (Table 4). Those genes whose expression appears to be regulated by the simultaneous action of α7- and α3*-nAChR are in group I, those whose expression appears to be altered by α7-nAChR activation alone are in group II, and those whose expression is affected by either α7-nAChR or α3*-nAChR activation are in group III. A completely α3*-nAChR-specific inhibitor would be useful to solidify these classifications.

2.6. *Effects of nAChR antagonist treatment alone on gene expression*

In the course of performing the above inhibition studies, we treated SH-SY5Y cells with dTC, α-cobratoxin, or mecamylamine

Table 4 – Functional grouping of candidate genes

Gene name	Nicotine	dTC	cbtx	meca	Nicotine			
					dTC	cbtx	mec	
C7	↑		↑		I	R	I	Group 1
CNTN1	↑				I	R	I	α7 and α3
eIF-4A	↑	(↑)			I	R	I	
MLL3	↑			↑	I	R	I	
myozenin 2	↑		↑	↑	R	(R)	I	
RAD50	↑	↑	↑		(I)	R	I	
SOS	↑	(↓)			R	R	I	
golgA4	↑		↑	↑	I	I	(I)	Group 2
NIN	↑			(↑)	I	R	(I)	α7
STIP1	↓		↓	↓	I	(I)		Group 3
UBE2C	↓	(↑)	(↓)	(↓)	(I)			α7 or α3
UBE2S	↓		↓		I			
MGC2396	↑	↓	↑	↑	I			
Parkin	↓	↓	ND	ND		ND	ND	

Summary of chronic nicotine treatment-induced gene expression changes and their sensitivity to blockade by nicotinic antagonists. qRT-PCR results from Tables 2 and 3 are grouped based on the effects of various antagonists and according to gene name (column 1); effect of 1 mM nicotine, 100 μM D-tubocurarine (dTC), 1 μM α-cobratoxin (cbtx), or 3 μM mecamylamine (meca) alone (columns 2–5, respectively); or effect of nicotine in the presence of dTC, cbtx, or meca (columns 6–8 under the horizontal bar labeled "nicotine" above). Group 1: gene expression changes in response to nicotine that are either inhibited or reversed by D-tubocurarine (dTC), α-cobratoxin (cbtx), or mecamylamine (mec). Group 2: gene expression changes that are inhibited by either dTC or cbtx. Group 3: gene expression changes that are inhibited or reversed by dTC only. Upward-pointing arrows (↑) indicate significant induction alone whereas downward-pointing arrows (↓) indicate significant repression alone. I indicates nicotinic effects that are significantly inhibited in the presence of the indicated antagonist. R indicates nicotinic effects that are reversed in the presence of the indicated antagonist. Any symbol in parentheses indicates a trend toward the indicated effect that did not reach statistical significance.

alone. Interestingly, the antagonists sometimes altered gene expression, yielding effects like those of nicotine alone. For example, both α-cobratoxin and mecamylamine regulated the expression of *myozenin 2*, *golgA4*, *MGC2396*, and *STIP1* and showed a trend toward regulation of *UBE2C* expression comparably to nicotine's effects. The *C7*, *RAD50*, and *UBE2S* genes were affected by α-cobratoxin or nicotine but not by mecamylamine. In contrast, *MLL3* gene expression was upregulated by either mecamylamine or nicotine but not by α-cobratoxin. Although neither α-cobratoxin nor mecamylamine had effects opposite to those of nicotine alone, dTC had an effect on *MGC2396* opposite to that of nicotine and showed a trend toward an opposite effect on *SOS* and *STIP1*. *RAD50* and *Parkin* gene expression was affected in the same way by dTC or nicotine alone, and a trend toward a nicotine-like effect was seen for dTC regulation of *eIF-4A*. Nevertheless, in almost every case where an antagonist alone had an effect on gene expression, antagonist inhibited or reversed the effect of nicotine. The exceptions were effects of mecamylamine on group 3 (affected by nicotine actions through either α7- or α3*-nAChR) genes for *STIP1*, *UBE2C*, and *MGC2396* and of α-cobratoxin on group 3 genes for *UBE2C*, *UBE2S*, and *MGC2396*. In the cases where both mecamylamine and α-cobratoxin had effects on gene expression, dTC had the opposite or no effect. Some effects of these antagonists also were observed upon a shorter, 1-hour treatment (Dunckley and Lukas, 2003).

Although we cannot formally rule out the possibility that the nAChR antagonists used could act at non-nAChR targets to affect gene expression, in cases where antagonists mimic effects of nicotine on gene expression, simple ligand occupancy of the receptor by either agonist or antagonist may trigger the relevant signals that affect expression of a small subset of genes without requiring nAChR channel opening. Perhaps gene expression studies could be used to discover and characterize any such novel signaling via nAChR. There is another formal possibility that an antagonist could mimic the effects of nicotine if nicotine was producing blockade of nAChR through desensitization. However, desensitization, while substantial, is incomplete in SH-SY5Y cells under the conditions used (Ke et al., 1998). Moreover, such an effect would imply that there is a basal level of nAChR stimulation in SH-SY5Y cells that is altered by nicotine-induced desensitization or functional block by an antagonist. This might occur in neurons subject to cholinergic input, but it has not been detected in SH-SY5Y cells. The potential roles of basal cholinergic signaling in control of expression of pathologically relevant genes such as *Parkin* might be interesting.

3. Discussion

3.1. *Principal findings and conclusions*

This study demonstrates that 1 day of 1 mM nicotine exposure significantly alters expression of 163 out of ~5000 genes queried by cDNA microarray. qRT-PCR studies confirm and validate these findings for thirteen out of fourteen genes chosen for further study based on their potential biological significance. These effects are relatively small in magnitude, but they are inhibited or reversed by the general nAChR

antagonist, dTC. Antagonists that are specific or selective for α7- or α3β4*-nAChR inhibit or reverse effects on selected sets of gene. The primary interpretations of these findings are that at least initial activation of nAChR channel activity is required for the effects of nicotine on gene expression but that effects on different sets of genes are mediated by nicotine's actions at α7-nAChR alone, through both α7- and α3*-nAChR, or via either α7- or α3*-nAChR. Temporal patterns of effects could be influenced by changes in levels of function of nAChR or downstream elements in nAChR-coupled signaling cascades, even if there was a complete loss of nAChR function upon sustained nicotine exposure. However, we favor the idea that differences in gene expression observed after 1 h compared to 24 h of nicotine exposure result from a combination of a cascade of changes induced by the initial process of nAChR activation and later modulated by sustained, low level nAChR signaling. Potential explanations as to why some effects of nicotine appear to be mimicked by antagonists have been described in the Results section. Here, we devote ourselves to higher order interpretation of the findings, their meaning, and their relationship to other reports in the literature.

3.2. Mechanisms involved in nicotinic receptor mediation of altered gene expression

Our previous work indicated that 1 mM nicotine exposure for 1 h produces a loss in function of α3*-nAChR and a transient decrease in cell surface radioligand binding sites corresponding to α7-nAChR expressed by SH-SY5Y cells without having much of an effect on total numbers (intracellular and cell surface) of radioligand binding sites corresponding to α3*-nAChR or intracellular pools of α7-nAChR (Ke et al., 1998). Whereas the loss in α3*-nAChR function persists after 24 h of nicotine exposure, there is an increase in numbers of radioligand binding sites corresponding to α3*- and α7-nAChR that is dominated for the latter nAChR subtype by an increase in intracellular pools (Ke et al., 1998). However, there remains a finite amount of α3*-nAChR functional activity (Ke et al., 1998), and other studies suggest that some signaling through α7-nAChR persists (Dajas-Bailador and Wonnacott, 2004). Thus, because there may be some level of nAChR activity even in the presence of a functionally inactivating concentration of nicotine, changes in gene expression might not simply reflect a response to transient nAChR activation occurring in the first few moments of exposure to nicotine. Even if gene expression effects result only from transient activation of nAChR, the initial changes set in motion upon binding of nicotine and subsequent transient nAChR activation could manifest as later gene expression changes that reflect different functional readouts of complex signaling pathways at 1 or 24 h of continuous nicotine exposure.

It is possible that α7- and α3β4*-nAChR mediating effects of nicotine on gene expression in SH-SY5Y cells contribute to different effects seen after 1 h as opposed to 24 h of nicotine exposure. Firstly, the degree to which these nAChR subtypes become functionally inactivated may differ for the two exposure times, and the subtype more resistant to functional inactivation might dominate effects of 24 h of nicotine exposure. In our 1-hour and more clearly in the current 24-hour nicotine exposure studies, α-cobratoxin more commonly reversed rather than just inhibited effects of nicotine exposure, whereas mecamylamine inhibited or showed a trend to inhibition of effects. This suggests that signaling through α7-nAChR exerts a stronger influence on expression of the genes studied than α3*-nAChR, especially for 24 h of nicotine treatment. Secondly, given high calcium ion permeability of α7-nAChR (Fucile, 2004), signaling pathways triggered by nicotine interaction with α3β4*- or α7-nAChR are likely to be different (Dajas-Bailador and Wonnacott, 2004). Contributions of both, one, or either of these nAChR subtypes are indicated by the antagonism studies performed. The pathways engaged could either diverge or converge, thus having different or common endpoints as gene expression changes. For example, α7-nAChR stimulation leads to activation of the ERK 1/2 signaling pathway (Dajas-Bailador et al., 2002). It also is possible that initial activation could be followed by either sustained activation or later desensitization of signaling cascades put into motion by nAChR–nicotine interactions. At the supramolecular level, in neurons expressing both subtypes, α3β4*- and α7-nAChR are known to have different subcellular localizations (Shoop et al., 2002) and may have physically distinct coupling to intracellular signaling cascade molecules (Lioudyno et al., 2004). This may also occur in clonal SH-SY5Y line, although there is no evidence supporting or refuting differential sequestration of nAChR subtypes.

Given these general issues, some remarks are warranted about specific effects observed on expression of selected genes. For example, effects in the current and previous study on enzymes involved in the ubiquitin process could be explained if the decrease in ubiquitin protein ligase E3A expression observed after 1 h of nicotine exposure but not after 24 h of nicotine exposure is due to transient activity of nAChR or signaling pathways that later desensitize, if the gene is more of an early than late response gene in a cascade triggered by initial and transient nAChR activation, or some combination of these or other factors. Changes observed in the current study in *UBE2C* and *UBE2S* could reflect their sensitivity to sustained, low level activation of nAChR or downstream signaling pathways or their position farther down the response cascade triggered by transient nAChR activation. Similar lines of reasoning about nAChR and downstream pathway activation/desensitization and placement of genes of interest in the expression change hierarchy could account for the initial decrease after 1 h of nicotine exposure followed by an increase at 24 h of nicotine exposure on *MLL3* and contactin expression. However, because dTC inhibited or reversed all or virtually all of the effects of 24 or 1 hour nicotine exposure, nAChR function seems to be required on some level.

3.3. Nicotine- or smoking-sensitive gene expression changes across diverse studies

We previously described effects of 1 h of 1 mM nicotine treatment on gene expression in the SH-SY5Y neuroblastoma-derived cell line based on initial microarray analyses validated by qRT-PCR (Dunckley and Lukas, 2003). Several previous studies have noted effects of nicotine exposure acting on a variety of cell types or in the rodent brain on expression of selected genes (e.g., see Stachowiak et al., 1988; Sun et al., 2003; Li et al., 2003; Konu et al., 2001). Additionally, microarray

studies have been done examining effects of nicotine or smoking on gene expression in human or rodent brain or in selected cell lines (Konu et al., 2001, 2004; Kane et al., 2004; Li et al., 2004; Belluardo et al., 2005; Mexal et al., 2005). For example, several genes in the NMDA receptor-associated post synaptic density complex from the human hippocampus were sensitive to smoking status (Mexal et al., 2005). Effects of nicotine exposure depended on the rat brain region studied according to Li et al. (2004). Nicotine exposure had effects in PC12 rat pheochromocytoma cells on genes involved in protein metabolism and the Akt signaling pathway (Konu et al., 2004) implicated in nicotine's neuroprotective effects (Nakayama et al., 2002). The PC12 line shares neural crest origins and nAChR profiles with SH-SY5Y cells examined in this study and in Dunckley and Lukas (2003), suggesting usefulness in cross-study comparisons. Studies using human or rodent brain are relevant to effects of nicotine on neuronal gene expression or gene expression in other cell types, although interpretations are complicated because of the heterogeneity of cells contributing to effects observed.

Of the several dozen genes with changed expression in the PC12 cell line (Konu et al., 2004) or in human (Mexal et al., 2005) or rodent (Li et al., 2004) brain that also were queried in Dunckley and Lukas (2003) and the current study, very few were also altered by nicotine exposure in SH-SY5Y cells. Among the exceptions, it is interesting that Dunckley and Lukas (2003; UBE3A), Konu et al. (2004), Li et al. (2004; UCEL5), Mexal et al. (2005; UCEL3), and the current studies (*UBE2C*, *UBE2S*, *Parkin*) all identified genes encoding elements in the ubiquitin–proteasome pathway as targets, although no two studies identified the same gene. Protein processing genes also are targets [e.g., FK506 binding protein 1B in Mexal et al., 2005 and FK506 binding protein 1A in this study based on microarray results; see Supplementary data). There are some affected genes (e.g., golg4A, V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1) that are upregulated by nicotine exposure in SH-SY5Y cells but downregulated in human smoker hippocampus (Mexal et al., 2005). See Supplementary data for the filtered list of nicotine-sensitive genes in SH-SY5Y cells that were identified based on microarray results and are discussed here (yes-1) or below.

With regard to genes potentially involved in cell survival/apoptosis, early growth response-1 (EGR1) and fibroblast growth factor-2 (FGF-2) were affected by nicotine exposure in Belluardo et al. (2005), Li et al. (2004) also noted effects on FGF2, and Li et al. (2004); Mexal et al. (2005) showed effects on the fibroblast growth factor receptor-8 gene. However, none of these was affected by 24 h of 1 mM nicotine exposure in this study, although altered EGR-1 was seen for 1 h of nicotine exposure in SH-SY5Y cells (Dunckley and Lukas, 2003). Altered BDNF expression was not observed. However, there was a trend toward altered inositol 1,4,5-triphosphate receptor change in SH-SY5Y cells. Among other, functionally interesting gene effects, plecstrin homology, Sec7, and coiled coil domains 1 (cytohesin 1) were altered in Li et al. (2004) and showed a trend toward change in the current study. NADH dehydrogenase (ubiquinone) 1α subcomplex 5 (13 kDa, B13) was altered in Mexal et al. (2005) and in the current study. Spastic ataxia of Charlexoix-Saguenay (sacsin), *trans*-Golgi network protein 2, zinc finger protein 184 (Kruppel-like), and tousled-like kinase 1 were altered in Mexal et al. (2005) and showed a trend toward change in the current study. However, more comprehensive gene expression analyses are needed to facilitate more complete comparisons of nicotine effects on gene expression across experimental systems, and full-genome chips promise to facilitate such study.

3.4. *Gene expression changes that could contribute to nicotine-mediated nAChR upregulation and functional inactivation*

We previously reported that the expression of UBE3A, a ubiquitin protein ligase, is repressed following 1 h of nicotine treatment. In this study, we identify altered regulation of three additional components of the protein ubiquitination machinery, *UBE2C* (ubiquitin conjugating enzyme E2C), *UBE2S* (ubiquitin conjugating enzyme E2S), and *Parkin* (E3 ubiquitin protein ligase) following 24 h of nicotine treatment. The finding that nicotine affects the expression of four members of the ubiquitination system suggests that there may be post-translational effects that could underlie cellular responses to nicotine. Aside from involvement of products of ubiquitin–proteasome pathway genes in eliminating defective or unassembled proteins, they also have roles in trafficking of signaling molecules such as receptors (DiAntonio et al., 2001) including nAChR (Keller et al., 1998). Given that changes in nAChR radioligand binding site levels, intracellular disposition, and function seem to have post-transcriptional bases (Bencherif et al., 1995; Ke et al., 1998), the ubiquitin system emerges as a potentially important target for studies of regulation of nAChR expression by nicotine exposure. Furthermore, this implies that proteomic screening may be useful to identify molecular bases for nicotine's effects. Additionally, the identification of *Parkin*, mutation of which is associated with some autosomal recessive forms of Parkinson's disease, as a gene whose expression appears to be responsive to nicotine is intriguing given that some studies have associated cigarette smoking with decreased risk of developing Parkinson's disease (Allam et al., 2003; Checkoway et al., 2002).

Our previous studies also indicated that 1-hour treatment with nicotine repressed expression of contactin (Dunckley and Lukas, 2003) under conditions where cell surface expression of radioligand binding sites corresponding to α7-nAChR was transiently decreased (Ke et al., 1998). By contrast, we now show that 24 h of treatment with nicotine, which induces an increase in cell surface α7-nAChR expression (Ke et al., 1998), also induces contactin expression. This suggests the hypothesis that contactin, which is known to be involved in trafficking and cell surface expression of Na^+ channels, may also be involved in cell surface trafficking of α7-nAChR and may function as a mediator of nicotine's effects on α7-nAChR expression (Kazarinova-Noyes et al., 2001).

4. Experimental procedures

4.1. *Drug treatment and RNA isolation*

Cells of the SH-SY5Y human neuroblastoma line, maintained as previously described (Lukas et al., 1993), were grown to

90% confluence and treated with 1 mM nicotine for 24 h at 37 °C in standard, serum-supplemented medium or were subjected to mock nicotine exposure. This condition for treatment was chosen because it produces both functional inactivation of nAChR containing α3 and other subunits (α3*-nAChR) and upregulation in numbers of radioligand binding sites corresponding to α3*- or α7-nAChR (Ke et al., 1998) and because it complements our previous studies done using a 1-hour incubation period (Dunckley and Lukas, 2003) that produces functional inactivation without upregulation (Ke et al., 1998). Total RNA was isolated using TRIzol® reagent (Invitrogen) from SH-SY5Y cells plated at 90% confluence and treated with 1 mM nicotine alone, 100 μM D-tubocurarine (dTC) alone, or 1 mM nicotine plus 100 μM dTC for 24 h at 37 °C (Dunckley and Lukas, 2003). To assess nAChR subtype specificity of effects, RNA was isolated from cells treated with 1 mM nicotine, 1 μM α-cobratoxin, 1 μM α-cobratoxin plus 1 mM nicotine, 3 μM mecamylamine, or 3 μM mecamylamine plus 1 mM nicotine for 24 h at 37 °C. RNA concentrations were measured by spectrophotometry and adjusted accordingly. Only RNA samples with an OD260/OD280 ratio >1.6 were used for microarray hybridizations and qRT-PCR experiments.

4.2. *Synthesis of fluorescent cDNA and hybridization to microarray slides*

Microarray analyses were performed as previously described (Dunckley and Lukas, 2003) at the University of Arizona Cancer Center Microarray Core Facility on a fee-for-service basis. Control and nicotine-treated samples were alternately labeled with either Cy5 or Cy3 to eliminate any possible bias due to higher dye staining of specific cDNAs, and a total of four separate chips querying four different cell preparations were processed to identify gene candidates sensitive to nicotine exposure. For detailed information about the microarrays used in these experiments and the genes represented on the array, please refer to http://azcc-microarray.arl.arizona.edu/index.php3.

4.3. *Normalization of fluorescence intensity and analysis of expression levels*

Microarray results were processed as described (Dunckley and Lukas, 2003) except that raw microarray data were normalized relative to all genes represented on the array using GeneSpring 5.0 software. The statistical significance of the changes identified was calculated within the GeneSpring software using a paired, two-tailed *t* test comparing the observed expression ratios averaged across the results of the four replicate microarray studies to a reference value of one. GeneGo and EASE pathway analysis software was used to determine whether functionally related genes were similarly affected by nicotine exposure.

4.4. *Quantitative real-time polymerase chain reaction*

Reverse transcription and qRT-PCR using a LightCycler (Roche Molecular Biochemicals) and SYBR green labeling of double-stranded DNA products were performed as previously described (Dunckley and Lukas, 2003). Specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis, which also was used to confirm singularity of PCR products (Fig. 1). Primers were designed using Primer3 software: http://bioinformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi and subsequently checked for specificity using BLAST: http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html. The expression ratios reported are the average of 4 to 5 replicate qRT-PCR reactions on RNA isolated from three independent cell treatments. Statistical significance was calculated using a paired, two-tailed *t* test.

Acknowledgments

This work was supported by endowment and capitalization funds from the Men's and Women's Boards of the Barrow Neurological Foundation and Epi-Hab Phoenix, Inc. by grants from the Arizona Disease Control Research Commission (9730 and 9615), the National Institutes of Health (NS40417 and DA15389), and the Council for Tobacco Research—U.S.A., Inc. (4366), and by a postdoctoral fellowship from Philip Morris USA Inc. and Philip Morris International. The contents of this report are solely the responsibility of the authors and do not necessarily represent the views of the aforementioned awarding agencies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.brainres.2006.07.111.

REFERENCES

Allam, M.F., Del Castillo, A.S., Navajas, R.F., 2003. Parkinson's disease, smoking and family history: meta-analysis. Eur. J. Neurol. 10, 59–62.

Belluardo, N., Olsson, P.A., Mudo', G., Summer, W.H., Amato, G., Fuxe, K., 2005. Transcription factor gene expression profiling after acute intermittent nicotine treatment in the rat cerebral cortex. Neuroscience 133, 787–796.

Bencherif, M., Fowler, K., Lukas, R.J., Lippiello, P.M., 1995. Mechanisms of upregulation of neuronal nicotinic acetylcholine receptors in clonal cell lines and primary cultures of fetal rat brain. J. Pharmacol. Exp. Ther. 275, 987–994.

Brunzell, D.H., Russell, D.S., Picciotto, M.R., 2003. In vivo nicotine treatment regulates mesocorticolimbic CREB and ERK signaling in C57Bl/6J mice. J. Neurochem. 84, 1431–1441.

Checkoway, H., Powers, K., Smith-Weller, T., Franklin, G.M., Longstreth Jr., W.T., Swanson, P.D., 2002. Parkinson's disease risks associated with cigarette smoking, alcohol consumption, and caffeine intake. Am. J. Epidemiol. 155, 732–738.

Dajas-Bailador, F., Wonnacott, S., 2004. Nicotinic acetylcholine receptors and the regulation of neuronal signaling. Trends Pharmacol. Sci. 25, 317–324.

Dajas-Bailador, F.A., Soliakov, L., Wonnacott, S., 2002. Nicotine activates the extracellular signal-regulated kinase 1/2 via the alpha7 nicotinic acetylcholine receptor and protein kinase A in SH-SY5Y cells and hippocampal neurones. J. Neurochem. 80, 520–530.

DiAntonio, A., Haghighi, A.P., Portman, S.L., Lee, J.D., Amaranto, A.M., Goodman, C.S., 2001. Ubiquitination-dependent mechanisms regulate synaptic growth and function. Nature 412, 449–452.
Donnely-Roberts, D.L., Brioni, J.D., 1999. Preclinical evidence on the neuroprotective effects of nicotinic ligands. In: Arneric, S.P., Brioni, J.D. (Eds.), Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities. Wiley-Liss, Inc., New York, pp. 337–348.
Dunckley, T., Lukas, R.J., 2003. Nicotine modulates the expression of a diverse set of genes in the neuronal SH-SY5Y cell line. J. Biol. Chem. 278, 15633–15640.
Fucile, S., 2004. Ca^{2+} permeability of nicotinic acetylcholine receptors. Cell Calcium 35, 1–8.
Gentry, C.L., Lukas, R.J., 2002. Regulation of nicotinic acetylcholine receptor numbers and function by chronic nicotine exposure. Curr. Drug Targets—CNS Neurol. Disord. 1, 359–385.
Huang, Z., de la Fuente-Fernandez, R., Stoessl, A.J., 2003. Etiology of Parkinson's disease. Can. J. Neurol. Sci. 30, S10–S18.
Jensen, A.A., Frolund, B., Liljefors, T., Krogsgaard-Larsen, P., 2005. Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. J. Med. Chem. 48, 4705–4745.
Ji, D., Lape, R., Dani, J.A., 2001. Timing and location of nicotinic activity enhances or depresses hippocampal synaptic plasticity. Neuron 31, 31–141.
Kane, J.K., Konu, O., Ma, J.Z., Li, M.D., 2004. Nicotine coregulates multiple pathways involved in protein modification/degradation in the rat brain. Mol. Brain Res. 132, 181–191.
Kazarinova-Noyes, K., Malhotra, J.D., McEwen, D.P., Mattei, L.N., Berglund, E.O., Ranscht, B., Levinson, S.R., Schachner, M., Shrager, P., Isom, L.L., Xiao, Z.C., 2001. Contactin associates with Na^{+} channels and increases their functional expression. J. Neurosci. 21, 7517–7525.
Ke, L., Eisenhour, C.E., Bencherif, M., Lukas, R.J., 1998. Effects of chronic nicotine treatment on expression and function of diverse nicotinic receptor subtypes. I. Dose- and time dependent effects of nicotine treatment. J. Pharmacol. Exp. Ther. 286, 825–840.
Keller, S.H., Lindstrom, J., Taylor, P., 1998. Inhibition of glucose trimming with castanospermine reduces calnexin association and promotes proteasome degradation of the alpha-subunit of the nicotinic acetylcholine receptor. J. Biol. Chem. 273, 17064–17072.
Konu, O., Kane, J.K., Barrett, T., Vawter, M.P., Chang, R., Ma, J.Z., Donovan, D.M., Sharp, B., Becker, K.G., Li, M.D., 2001. Region-specific transcriptional response to chronic nicotine in rat brain. Brain Res. 909, 194–203.
Konu, O., Xu, X., Ma, J.Z., Kane, J., Wang, J., Shi, S.J., Li, M.D., 2004. Application of a customized pathway-focused microarray for gene expression profiling of cellular homeostasis upon exposure to nicotine in PC12 cells. Mol. Brain Res. 121, 102–113.
Levin, E.D., Simon, B.B., 1998. Nicotinic acetylcholine involvement in cognitive function in animals. Psychopharmacology 138, 217–230.
Li, S.P., Park, M.S., Bahk, J.Y., Kim, M.O., 2003. Chronic nicotine and smoking exposure decreases GABA(B1) receptor expression in the rat hippocampus. Neurosci. Lett. 334, 135–139.
Li, M.D., Kane, J.K., Wang, J., Ma, J.Z., 2004. Time-dependent changes in transcriptional profiles within five rat brain regions in response to nicotine treatment. Mol. Brain Res. 132, 168–180.
Lioudyno, M., Hiel, H., Kong, J.H., Katz, E., Waldman, E., Parameshwaran-Iyer, S., Glowatzki, E., Fuchs, PA., 2004. A "synaptoplasmic cistern" mediates rapid inhibition of cochlear hair cells. J. Neurosci. 24, 11160–11164.
Lukas, R.J., Bencherif, M., 2006. Recent developments in nicotinic acetylcholine receptor biology. In: Arias, H. (Ed.), Biological and Biophysical Aspects of Ligand-Gated Ion Channel Receptor Superfamilies. Research Signpost, Trivandrum, pp. 27–59.
Lukas, R.J., Norman, S.A., Lucero, L., 1993. Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clonal line. Mol. Cell. Neurosci. 4, 1–12.
Mexal, S., Frank, M., Berger, R., Adams, C.E., Ross, R.G., Freedman, R., Leonard, S., 2005. Differential modulation of gene expression in the NMDA postsynaptic density of schizophrenic and control smokers. Mol. Brain Res. 139, 317–332.
Nakayama, H., Numakawa, T., Ikeuchi, T., 2002. Nicotine-induced phosphorylation of Akt through epidermal growth factor receptor and Src in PC12h cells. J. Neurochem. 83, 1372–1379.
Papke, R.L., Sanberg, P.R., Shytle, R.D., 2001. Analysis of mecamylamine stereoisomers on human nicotinic receptor subtypes. J. Pharmacol. Exp. Ther. 297, 646–656.
Peng, X., Gerzanich, V., Anand, R., Wang, F., Lindstrom, J., 1997. Chronic nicotine treatment upregulates alpha3 and alpha7 acetylcholine receptor subtypes expressed by the human neuroblastoma cell line SH-SY5Y. Mol. Pharmacol. 51, 776–784.
Sharma, G., Vijayaraghavan, S., 2002. Nicotinic receptor signaling in nonexcitable cells. J. Neurobiol. 53, 524–534.
Shoop, R.D., Esquenazi, E., Yamada, N., Ellisman, M.H., Berg, D.K., 2002. Ultrastructure of a somatic spine mat for nicotinic signaling in neurons. J. Neurosci. 22, 748–756.
Shytle, R.D., Silver, A.A., Lukas, R.J., Newman, M.B., Sheehan, D.V., Sanberg, P.R., 2002. Nicotinic acetylcholine receptors as targets for antidepressants. Mol. Psychiatry 7, 525–535.
Stachowiak, M.K., Goc, A., Hong, J.-S., Kaplan, B., Stachowiak, E.K., 1988. Neural and hormonal regulation of the tyrosine hydroxylase gene in adrenal medullary cells: participation of c- *fos* and AP1 factors. Mol. Cell. Neurosci. 1, 202–213.
Sun, B., Sterling, C.R., Tank, A.W., 2003. Chronic nicotine treatment leads to sustained stimulation of tyrosine hydroxylase gene transcription rate in rat adrenal medulla. J. Pharmacol. Exp. Ther. 304, 575–588.