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Nicotine Modulates the Expression of a Diverse Set of Genes in the Neuronal SH-SY5Y Cell Line*

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Nicotine exposure can have long lasting effects on nervous system function, some of which must contribute to nicotine dependence. Up-regulation, an increase in numbers of radioligand-binding nicotinic acetylcholine receptors (nAChR), occurs on exposure to nicotine at high concentrations. To determine whether altered gene expression might account for long term changes and up-regulation following nicotine exposure, we assessed effects of 1 h of 1 mM nicotine exposure on alteration of gene expression in the neuron-like SH-SY5Y neuroblastoma clonal line. Repeat and cross-controlled microarray analyses yielded a list of 17 genes from the initially screened ~5,000 whose expression was consistently altered following nicotine treatment. Subsequent quantitative, real time reverse transcriptase PCR analyses confirmed altered expression in 14 of 16 genes tested. Further, the general nAChR antagonist, d-tubocurarine, blocked all but two of the observed changes in gene expression, indicating that these changes are dependent on nAChR activation. Use of other antagonists revealed that nAChR subtypes can differentially affect gene expression. The genes affected code for proteins that may be broadly categorized into four groups: transcription factors, protein processing factors, RNA-binding proteins, and plasma membrane-associated proteins. Our results suggest that nicotinic activation of nAChR may have a broad role in affecting cellular physiology through modulating gene expression.

Nicotinic acetylcholine receptors (nAChR)[1] are ligand-gated cation channels implicated in a variety of neuronal functions, including memory processing (1), neurotransmitter release (2), cell survival (3), and synaptic plasticity (4). Nicotine is a tobacco alkaloid that acts acutely to stimulate nAChR channel opening, as does the endogenous nAChR ligand, the neurotransmitter acetylcholine. This functional response is a transient event thought only to alter electrical activity in excitable cells. However, some nAChR are expressed in nonexcitable tissues. Moreover, longer term psychological and physiological effects of nicotine on the brain and body also must reflect interactions with nAChR, but only if there are lasting consequences of ligand binding and/or ion channel opening. An improved understanding of both the molecular signaling cascades initiated by short or longer term interactions of nicotine with nAChR and the specific targets of those signaling cascades is essential to enhanced perspectives on normal physiological roles of nAChR.

Nicotine is known to affect the expression of several genes. Among these is the gene coding for tyrosine hydroxylase, which is involved in a rate-limiting step in catecholamine synthesis (5, 6), as well as genes involved in the regulation of food intake and energy expenditure, such as neuropeptide Y, orexins, and their receptors (7, 8). In addition, nicotine, like other substances of abuse, such as cocaine and alcohol, induces the expression of immediate early genes such as c-*fos* and *junB* in various brain regions (9–11). Nicotine also up-regulates the mRNA levels of c-*fos* and c-*jun* in the neuronal SH-SY5Y cell line.[2] Because these immediate early genes function as transcription factors, their nicotine-mediated up-regulation suggests that nicotine may regulate the expression of additional genes in SH-SY5Y cells.

Nicotine activates the mitogen-activated protein kinase (MAPK) signaling pathway in a variety of tissues and cell types (12–15). Recent work indicates that nicotine also activates this signaling pathway in SH-SY5Y cells (16). Further, nicotine and MAPK signaling pathways affect many of the same cellular processes, such as cell survival and memory processing (1, 3, 17, 18).

Beyond the role of nicotine in activating the MAPK cascade and early immediate gene expression, little is known about the specific genes that nicotine may regulate. We therefore investigated the effects of nicotine exposure on gene expression in the SH-SY5Y cell line using a microarray-based approach to identify candidate nicotine-regulated genes. We show that nicotine, at a concentration that induces up-regulation of nAChR, a process that has been implicated in nicotine dependence and tolerance, affects the expression of a wide range of genes that code for proteins with seemingly diverse functions. Collectively, these results demonstrate that nicotine can modulate the gene expression profile of a neuron-like cell line and suggest that some of the cellular and physiological effects of nicotine may result from these nAChR-mediated effects.

[1] The abbreviations used are: nAChR, nicotinic acetylcholine receptor(s); MAPK, mitogen-activated protein kinase; RT, reverse transcriptase; d-TC, d-tubocurarine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTPα, protein-tyrosine phosphatase receptor α; EGR1, early growth response 1; PIG7, p53-induced gene 7; RBBP6, retinoblastoma binding protein 6; UBE3A, ubiquitin ligase E3A; C2orf2, chromosome 2 open reading frame 2; TFPI2, tissue factor pathway inhibitor 2; ZFR, zinc finger RNA-binding protein; MLL3, myeloid/lymphoid or mixed lineage leukemia 3; FEZ, fasciculation and elongation protein ζ, Zygin; DHFR, dihydrofolate reductase.

[2] L. Lucero and R. J. Lukas, unpublished observation.

MATERIALS AND METHODS

Drug Treatment and RNA Isolation—The SH-SY5Y human neuroblastoma-derived cell line was grown to 90% confluence and treated with 1 mM nicotine for 1 h at 37 °C under standard incubation conditions (95% humidity, 5% CO_2) (19). Messenger RNA was isolated using the Oligotex® Direct mRNA isolation kit (Qiagen) following the manufacturer's protocol. In a repeat experiment for RT-PCR analyses (see below), SH-SY5Y cells at 90% confluence were treated with 1 mM nicotine alone, 100 μM d-tubocurarine (d-TC) alone, or 1 mM nicotine plus 100 μM d-TC for 1 h at 37 °C. Total RNA was isolated using TRIzol® reagent (Invitrogen). To assess nAChR subtype-specific effects on gene expression, SH-SY5Y cells were grown to 90% confluence and 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 1 h at 37 °C. Total RNA was isolated using TRIzol® reagent (Invitrogen). RNA concentrations were measured by spectrophotometry and adjusted accordingly. Only RNA samples with an A_{260}/A_{280} ratio > 1.4 were used for microarray hybridizations and RT-PCR experiments.

Synthesis of Fluorescent cDNA and Hybridization to Microarray Slides—Microarray analyses were performed by the University of Arizona Cancer Center Microarray Core Facility on a fee-for-service basis. Briefly, for both the control sample and the nicotine-treated sample, 4 μg of mRNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase, incorporating Cy3-dUTP into the control sample cDNA and Cy5-dUTP into the nicotine-treated sample cDNA. In separate reverse transcription reactions for an independent hybridization, the control sample cDNA was labeled with Cy5-dUTP, and the nicotine-treated sample cDNA was labeled with Cy3-dUTP. These reciprocal labeling reactions were performed twice and hybridized to individual arrays, yielding four replicate sets of "dye switch" data designed to control for any bias caused by selectivity of dye staining intensity of specific cDNAs.

The labeled cDNA probes were purified using the QIAquick® PCR purification kit (Qiagen). The purified cDNA was then mixed with a hybridization buffer containing 15 μg of poly(dA), 6 μg of yeast tRNA, 15 μg of CoT 10 DNA, 2× Denhardt's, 2.7× SSC, and 0.2% SDS, denatured, and hybridized for 16 h at 62 °C on a 5K cDNA microarray. The cDNAs imprinted on these arrays are available from Research Genetics. For detailed information about the microarray used in these experiments and the genes represented on the array refer to azccmicroarray.arl.arizona.edu/index.php3. Following hybridization, the slides were washed for 15 min in 0.5× SSC, 0.01% SDS and for 15 min in 0.06× SSC, 0.01% SDS, followed by a final 5-min wash with 0.06× SSC. The slides were then scanned for fluorescence emission from each spot on the array at 570 and 670 nm for Cy3 and Cy5, respectively.

Normalization of Fluorescence Intensity and Analysis of Expression Levels—In microarray studies performed at the Arizona Cancer Center microarray facility, median intensity of a given spot of 1.4-fold above background intensity in both the Cy5 and Cy3 channels was required for calculation of expression ratios. Background intensity was assessed by measuring fluorescence from a spot containing no cDNA. The raw levels of fluorescence for each spot were corrected for this background fluorescence and analyzed. The fluorescence intensity values in the two fluorescence channels were normalized relative to a set of housekeeping genes. Lastly, the standard deviation of the mean of the $\log_{10}$ of the ratios for the housekeeping genes was used to calculate 40, 60, 80, 90, 95, and 99% confidence intervals, against which the ratios of signal intensities for all other spots on the arrays with fluorescence above background were compared. The statistical significance of the changes identified were calculated using a paired, two-tailed *t* test comparing the observed expression ratios for the four replicates to a reference value of 1.

Quantitative, Real Time Polymerase Chain Reaction—For this analysis, 5 μg of DNase I-treated total RNA from the untreated SH-SY5Y cells and from each of the drug-treated cells was reverse transcribed using SuperScript® II reverse transcriptase (Invitrogen). Following reverse transcription, each sample was diluted such that cDNA corresponding to that produced from 10 ng of total RNA was used in subsequent PCRs. PCRs were performed using the LightCycler® (Roche Molecular Biochemicals), which allows real time monitoring of the increase in PCR product concentration after every cycle based on the fluorescence of the double-stranded DNA specific dye SYBR green (20, 21). The number of cycles required to produce a detectable product above background was measured for each sample. These cycle numbers were then used to calculate fold differences in the starting mRNA level for each sample using the following method. First, the cycle number difference for vimentin, a housekeeping gene, was determined in the

control sample and the appropriate drug-treated sample. This difference was referred to as Δ*H*. Next, the cycle number difference for the gene of interest was determined in the control sample and the appropriate drug-treated sample, yielding another value, Δ*I*. The cycle number difference for the gene of interest was then corrected for slight differences in the amount of total RNA in control and drug-treated samples by subtracting Δ*H* from Δ*I*, yielding a new value, Δ*K*. The expression ratio for the gene of interest was then calculated as $2^{-(\Delta K)}$ for genes that were induced and as $-(2^{\Delta K})$ for genes that were repressed. The expression ratios reported are the averages of three to eight replicate PCRs. The statistical significance was calculated using a paired, two-tailed *t* test comparing the cycle number difference for the gene of interest (Δ*I*) to that of vimentin (Δ*H*) across all replicates. Specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis. The primers were designed using Primer3 software (bioinformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi) and subsequently checked for specificity using BLAST (www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html).

RESULTS

Nicotine Consistently Alters the Expression of a Diverse Set of Genes—To assess the extent to which nicotine modulates gene expression in neuronal cells, we have performed analyses using a microarray containing cDNAs corresponding to ~5,000 different genes. These results provide the foundation for more exhaustive whole genome screening. However, here we have focused on a smaller number to identify novel nicotine-regulated genes and firmly establish the extent to which nicotine alters the expression of these genes. For these analyses, the neuroblastoma-derived SH-SY5Y cell-line was treated with 1 mM nicotine for 1 h. Treatment with 1 mM nicotine maximally induces a long lasting up-regulation in numbers of nAChR radioligand-binding sites in SH-SY5Y cells, and this up-regulation has been implicated in long lasting effects of nicotine exposure, such as nicotine dependence and tolerance (22). Messenger RNA isolated from these cells or from control cells was used in the microarray analyses. Based on previous results investigating the effects of nicotine on gene expression (23), we anticipated that nicotine may 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 "Materials and Methods"). Multiple replicate hybridizations using reverse labeled samples are necessary to elucidate significant changes in gene expression in the 1.2–1.6-fold range (see Ref. 24 and references therein). These multiple hybridizations and reciprocal labeling experiments were then assessed to identify the most consistent and significant (reproducible changes in effects between experiments) changes in gene expression.

We compared all of the data at the 40% confidence interval (see "Materials and Methods") for genes whose expression was consistently up-regulated or down-regulated in all four replicate hybridizations. We selected the 40% confidence interval to avoid excluding subtle gene expression changes. Using this comparison we identified a list of 17 consistently altered genes (Table I). Statistical analyses using Student's *t* test showed these changes to be significant at the *p* < 0.05 level for all but one of these genes (RAB6A). The number of affected genes increased to 51 when comparing only three arrays and to 392 genes if only one reciprocal labeling experiment was used. Thus, multiple replicate arrays using reverse labeled samples were necessary to minimize the number of false positives from the large data set and to confirm identification of candidate genes whose expression was changed subtly but reproducibly. Importantly, because there was some variability in expression ratios between hybridizations, it is possible that significant but subtle changes in gene expression were lost as more replicates

TABLE I
Microarray analyses reveal numerous, consistent, nicotine-induced alterations of gene expression

Listed are the gene names, GenBank™ accession numbers, and expression ratios for each individual array, as well as the average ratio (±S.D.) across all four microarray hybridizations. The 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 1 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.

Gene name	GenBank™ number	Array 1 ratio	Array 2 ratio	Array 3 ratio	Array 4 ratio	Average ratio	S.D.
Matrin3, Mat3	AA075307	1.29	1.52	1.33	1.39	1.38[a]	0.10
Contactin 1, CNTN1	H20566	0.73	0.69	0.48	0.83	0.68[b]	0.15
Fasciculation and elongation protein zeta, Zygin	H20759	1.26	1.16	1.55	1.52	1.37[b]	0.19
Tissue factor pathway inhibitor 2, TFPI2	AA399473	1.13	1.16	1.31	1.45	1.26[b]	0.15
Chromosome 2 open reading frame 2, C2orf2	N64731	0.85	0.68	0.62	0.82	0.74[b]	0.11
Zinc finger RNA binding protein, ZFR	AA232979	0.84	0.85	0.72	0.63	0.76[b]	0.10
Protein tyrosine phosphatase, receptor type A, PTPα	H82419	0.83	0.74	0.74	0.85	0.79[a]	0.06
RAB6A	H20138	0.81	0.79	0.48	0.77	0.71	0.16
PIG7/LPS-induced TNF-alpha factor, PIG7/LITAF1	AA625666	0.79	0.79	0.77	0.82	0.79[a]	0.02
Myeloid/lymphoid or mixed-lineage leukemia 3, MLL3	N91302	0.79	0.75	0.80	0.81	0.79[a]	0.02
Dihydrofolate reductase, DHFR	N52980	0.78	0.66	0.77	0.68	0.72[a]	0.06
Regulatory factor X-associated protein, RFXAP	AA057436	0.76	0.78	0.77	0.82	0.78[a]	0.03
EST	T69754	0.73	0.57	0.71	0.71	0.68[a]	0.07
Ubiquitin protein ligase E3A, UBE3A	R85213	0.70	0.75	0.76	0.81	0.75[a]	0.05
Retinoblastoma binding protein 6, RBBP6	R88741	0.65	0.64	0.68	0.79	0.69[a]	0.07
Homo sapiens mRNA; cDNA DKFZp564F112	N28268	0.49	0.52	0.75	0.45	0.54[b]	0.14
Early growth response 1, EGR1	AA486628	0.36	0.54	0.55	0.31	0.44[b]	0.12

[a] Significance at the $p < 0.05$ level as measured by paired t test.
[b] Significance at the $p < 0.01$ level.

were added to the analysis. The genes identified and the significance of their sensitivity to nicotine exposure will be discussed in greater detail below. However, it is notable that there were increases in expression of only 3 of the 17 genes, whereas expression of the other 14 genes was repressed by nicotine treatment. Also notable was the low magnitude of these changes resulting from nicotine treatment, including 26–38% increases in gene expression for the three up-regulated genes and a greater than 2-fold decrease in expression for only one of the 14 down-regulated genes.

Nicotine Alters Gene Expression through nAChR Activation—We used a pharmacological approach both as another way to test the significance and specificity of changes in gene expression and, because nicotine readily crosses the cell membrane, to assess whether nicotine altered gene expression through nAChR-dependent or nAChR-independent pathways. In the course of these studies, we also obtained sample replicates and analyzed them by real time RT-PCR to confirm the results of the microarray surveys. The SH-SY5Y cells were treated with 1 mM nicotine alone, 100 μM d-TC (a general nAChR antagonist) alone, or the combination of 100 μM d-TC plus 1 mM nicotine. We then utilized quantitative, real time RT-PCR using total RNA as the template to verify and replicate the gene expression changes observed in the microarray experiments (see "Materials and Methods"). Vimentin mRNA was chosen as the control for normalization because its expression level was unchanged in the microarray analyses (−1.03 ± 0.31). In addition, subsequent RT-PCR experiments wherein the levels of vimentin mRNA were compared with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA showed that both mRNAs were present at comparable levels in all drug-treated samples relative to the control sample (Fig. 1 and Table II (GAPDH row)). Further, when normalized to the amount of total RNA in each sample, none of the drug treatments altered the expression of either GAPDH or vimentin. This result is consistent with previous studies using human coronary artery endothelial cells where GAPDH expression was unchanged in response to nicotine (23). These combined results indicated that vimentin mRNA expression was unaltered in response to nicotine.

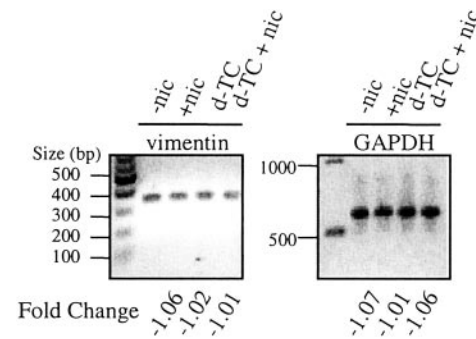The RT-PCR analyses confirmed statistically significant

FIG. 1. **Housekeeping gene expression is unaltered in response to both nicotine and d-tubocurarine.** Shown is an inverted image of an ethidium-stained agarose gel of RT-PCR samples corresponding to the product present after 22 cycles of PCR for both vimentin (*left*) and GAPDH (*right*). −*nic*, untreated control; +*nic*, samples treated with 1 mM nicotine for 1 h; *d-TC*, samples treated with 100 μM d-tubocurarine for 1 h; *d-TC + nic*, samples treated with 1 mM nicotine and 100 μM d-tubocurarine for 1 h. The fold change values were calculated as described under "Materials and Methods" and correspond to the fold change relative to the untreated (−*nic*) sample.

changes in expression of 14 of the 16 consistently altered genes that were tested (Table II; RAB6A and regulatory factor X-associated protein expression was not significantly changed; RT-PCR analysis of effects on expression of an expressed sequence tag was not performed). Each primer pair was checked for specificity by melting curve analysis and agarose gel electrophoresis to ensure that only specific product was quantitated (Fig. 2 and data not shown). In most cases, the magnitude of the change observed in the RT-PCR experiments was comparable with or greater than that seen on the microarrays. Importantly, pretreatment with d-TC blocked or reversed the nicotine-induced changes in mRNA levels for all of the genes identified (including a trend toward blockade of the effects of nicotine on ZFR expression) except for the FEZ1 variants 1 and 2 (Table II). These results implicate nAChR function in the observed nicotinic modulation of mRNA levels. Further, these results argue that the observed modulation of gene expression in the presence of nicotine is a specific effect of this drug acting at nAChR.

TABLE II

Real time RT-PCR studies verify that nicotine modulates the expression of numerous genes

Real time RT-PCR experiments were performed as described under "Materials and Methods." Matrin3 (mRNA) and Contactin 1 (mRNA) samples represent reactions performed using the same samples that were used in the microarray hybridizations. All of the other results are from a replicate experiment wherein total RNA was isolated and used as the template for the RT reactions. Column labels are as follows: Nicotine, samples treated with 1 mM nicotine for 1 h; d-TC, samples treated with 100 μM d-tubocurarine for 1 h; d-TC + nicotine, samples treated with 1 mM nicotine and 100 μM d-tubocurarine for 1 h. The numbers in bold represent the average fold changes across multiple independent PCRs (from three to eight reactions). The standard deviation of the ΔK values (see "Materials and Methods") was used to calculate a range of fold induction or repression for each sample to determine the reliability of the results. Each range column represents the range of values for the drug-treated sample to the left of that column.

Gene name	Nicotine	Range ± S.D.	d-TC	Range ± S.D.	d-TC + nicotine	Range ± S.D.
Matrin3 (mRNA)	**−1.82**[a]	−2.17/−1.52				
Contactin1 (mRNA)	**−2.76**[a]	−3.32/−2.28				
Matrin3	**−1.58**[a]	−1.89/−1.32	**1.52**[a]	1.36/1.72	**1.88**	1.40/2.51
Contactin1	**−1.48**[a]	−1.57/−1.37	**1.41**	1.15/1.74	**1.59**	1.29/1.96
FEZ1 variant1	**−1.34**[a]	−1.48/−1.21	**−1.34**	−1.58/−1.13	**−1.63**	−2.35/−1.14
FEZ1 variant2	**−1.69**[a]	−2.07/−1.39	**−1.75**	−2.60/−1.18	**−2.72**[b]	−2.99/−2.46
TFPI2	**−1.57**[b]	−1.85/−1.33	**−1.18**	−1.36/−1.03	**−1.30**	−1.66/−1.02
C2orf2	**−1.94**[b]	−2.58/−1.44	**−1.12**	−1.51/1.21	**−1.07**	−1.51/1.32
ZFR	**−2.61**[b]	−3.20/−2.11	**−1.33**	−1.51/−1.17	**−1.55**[a]	−1.75/−1.37
PTPα	**−1.67**[b]	−1.91/−1.46	**−1.08**	−1.26/1.08	**−1.03**	−1.31/1.22
RAB6A	**1.15**	−1.12/1.48	**−1.01**	−1.18/1.16	**−1.16**	−1.55/1.14
PIG7/LITAF1	**−1.56**[a]	−1.85/−1.31	**−1.04**	−1.23/1.15	**−1.18**	−1.60/1.15
MLL3	**−1.48**[a]	−1.75/−1.24	**1.16**[a]	1.13/1.19	**−1.01**	−1.44/1.40
DHFR	**−1.70**[b]	−2.00/−1.45	**−1.48**[b]	−1.79/−1.23	**−1.18**	−1.67/1.20
RFXAP	**−1.15**	−1.40/1.05	**−1.01**	−1.18/1.16	**1.09**	1.02/1.17
UBE3A	**−1.43**[a]	−1.71/−1.21	**1.28**[a]	1.20/1.38	**1.44**	−1.09/2.27
RBBP6	**−3.75**[b]	−4.79/−2.95	**−1.03**	−1.18/1.12	**1.09**	1.02/1.17
cDNA DKFZp564F112	**−1.86**[b]	−2.06/−1.69	**−1.05**	−1.14/1.04	**1.21**[a]	1.15/1.27
EGR1	**1.91**[b]	1.68/2.50	**−1.09**	−1.74/1.45	**1.10**	−1.27/1.53
GAPDH	**1.00**	−1.05/1.06	**−1.01**	−1.07/1.04	**−1.02**	−1.06/1.03

[a] Significance at the $p < 0.05$ level as measured by paired t test.
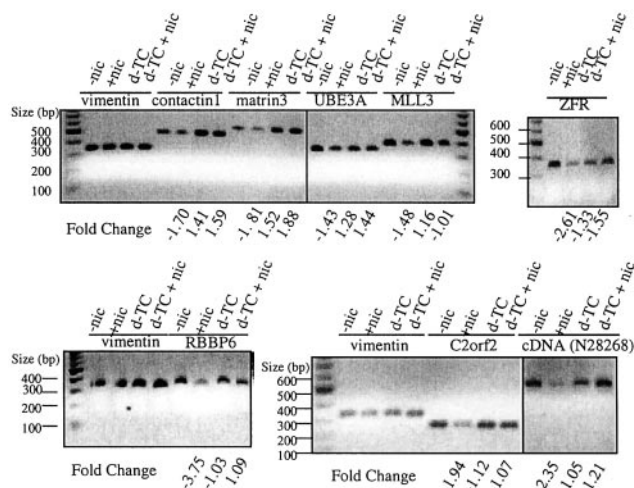[b] Significance at the $p < 0.01$ level.

FIG. 2. **The RT-PCR products are specific for each gene.** Each PCR generates only one product of the predicted size, indicating the specificity of the primers used. The specific genes targeted in the PCRs are indicated above the lanes. −*nic*, untreated control; +*nic*, samples treated with 1 mM nicotine for 1 h; *d-TC*, samples treated with 100 μM d-tubocurarine for 1 h; *d-TC + nic*, samples treated with 1 mM nic and 100 μM d-tubocurarine for 1 h. Quantitation of the amount of product was performed in real time as described under "Materials and Methods." Calculated expression ratios for each drug-treated sample relative to the control sample are indicated beneath each lane. These values represent the average values over at least three PCRs.

In some cases, the modulation of gene expression observed using quantitative, real time RT-PCR was in the opposite direction to that observed in the microarray analysis (compare matrin3, FEZ1, TFPI2, and EGR1 in Tables I and II). Importantly, this apparent discrepancy does not reflect variation between the mRNA samples used for the microarrays and the replicate total RNA samples used for RT-PCR studies, because matrin3, which showed slight induction on nicotine exposure in the microarray experiments, was found in RT-PCR analyses of both mRNA and total RNA samples to be comparably repressed by nicotine treatment (Table II). Expression ratios were low for these four genes but still in the range for the 10 other genes for which RT-PCR and microarray studies showed concordance. Absolute expression levels were low (data not shown) for FEZ1, TFPI2, and EGR1 but not for matrin3. However, the 1.9-fold increase in EGR1 expression that is seen here in the quantitative, real time RT-PCR experiments with SH-SY5Y cells following 1 h of 1 mM nicotine treatment (Table II) is in concordance with results of another study showing ~2.8-fold induction of EGR1 in PC12 cells following 1 h of 200 μM nicotine treatment (25). Although we cannot fully explain the differences between the microarray and RT-PCR results, we consider that findings from quantitative, real time RT-PCR analyses, especially when corroborated by antagonist sensitivity of the effects of nicotine, are more reliable than more raw microarray results. Therefore, we used RT-PCR findings for our interpretations if there was ambiguity in results from the two types of analyses. Nevertheless, our observations underline the importance of secondary verification of microarray results and indicate potential complications in deriving gene expression profiling conclusions based solely on microarray analyses.

Nicotine Modulates the Levels of a Variety of mRNAs Coding for Plasma Membrane-associated Proteins—The RT-PCR analyses confirmed that nicotine treatment subtly but significantly altered the levels of a variety of classes of mRNAs. Nicotine repressed the expression of mRNAs that code for plasma membrane associated proteins, including contactin 1 and protein-tyrosine phosphatase receptor α (PTPα). Interestingly, the contactin 1 protein physically interacts with PTPα (26) as well as with voltage-gated Na$^+$ channels (27, 28). These observations suggest interesting possibilities for roles of contactin and PTPα in nAChR up-regulation (see "Discussion"). Another interesting observation is that whereas nicotine exposure repressed expression, d-TC significantly induced UBE3A expression and showed a distinct trend to induction of contactin 1 expression (Table II). This induction was not reversed when nicotine and d-TC were used in conjunction, suggesting at least two possible

explanations. First, there may be a low basal rate of spontaneous nAChR channel opening that helps to sustain the expression levels for these genes in the untreated case. Application of the nAChR antagonist may prevent this spontaneous channel opening, thereby resulting in increased expression. Nicotine-mediated channel opening would have the opposite effect, resulting in reduced expression. A second possibility is that binding of either agonist or antagonist to the nAChR may induce alternative conformational changes in the receptor that either activate or inhibit a regulatory pathway. Distinguishing between these possibilities will be a goal of future work.

Interestingly, nicotine also repressed the expression of another membrane-associated protein TFPI2. TFPI2 codes for a serine protease inhibitor that functions as a tumor suppressor and is repressed in invasive cells of many tumor types (29–32). Thus, 1 h of continuous nicotine treatment represses the expression of a known tumor suppressor gene in the SH-SY5Y cell line.

Nicotinic Receptor Stimulation Alters Transcription Factor Expression—Nicotine affected the expression of multiple mRNAs coding for proteins that either are known to be involved in transcription or that are implicated in transcription based on homology. The early growth response 1 (EGR1) mRNA is up-regulated (Table II). Results from the microarray analysis suggested that nicotinic stimulation down-regulated EGR1 expression (Table I). However, the expression levels of EGR1 are only slightly above background in the microarray analysis, decreasing the reliability of the observed expression ratios using this technique (see "Discussion"). The induction of EGR1 seen in the RT-PCR experiments (Table II) is consistent with prior observations showing induction of immediate early genes, such as *c-fos*, *c-jun*, and *junB* in SH-SY5Y cells and in various brain regions in response to nicotine (9–11, 33). Further, the 1.9-fold increase in EGR1 mRNA level observed here is consistent with the 2.8-fold induction seen in PC12 cells following 1 h of 200 μM nicotine treatment (25). Both cocaine and amphetamine have been shown to induce EGR1 expression in multiple brain regions. The identification of nicotine as an additional EGR1-activating drug in a neuronal cell line provides further evidence for a common regulatory mechanism for these drugs of abuse and suggests that the SY-SY5Y cell line may be useful for elucidating the common signaling pathways that these drugs activate.

The expression of several additional genes implicated in transcription was changed in response to nicotine. In contrast to EGR1, nicotine repressed the MLL3, p53-induced gene 7 (PIG7/LITAF1), and retinoblastoma binding protein 6 (RBBP6) genes (Table II). Alteration of these genes that are implicated directly or indirectly in transcription raises the possibility that either these genes could be involved in regulating the expression of the other genes identified here or that there may be additional genes whose expression may be affected in response to nicotine.

Nicotine Affects the Expression of Genes Coding for Protein Processing Factors and RNA-binding Proteins—Nicotine also altered the expression of two genes involved in protein processing, ubiquitin ligase E3A (UBE3A) and chromosome 2 open reading frame 2 (C2orf2). UBE3A exhibits brain specific maternal imprinting. Loss of function mutations in this gene are associated with Angelman syndrome (for review see Ref. 34), a disease in which seizures are common. Repression of this gene suggests an additional possible underlying mechanism for the observation that high doses of nicotine and various nAChR antagonists can induce seizures in animal models (35–40). C2orf2 encodes a protein of unknown function. However, this protein contains numerous conserved domains including four

WD repeats and a highly conserved serine protease domain suggestive of a role in protein processing (41).

Nicotine also alters the expression of two RNA-binding proteins, matrin3 and ZFR, potentially implicating nAChR in RNA processing events. The ZFR protein is essential for murine embryonic development (42). However, the human ZFR mRNA is highly expressed in adult brain (43), suggesting that this gene has important functions beyond development. Matrin3 is a member of a large family of RNA-binding proteins and is a nuclear matrix protein known to be involved in the nuclear retention of A-to-I edited mRNA (44). The potential role of these genes in nAChR function and in the physiological effects of nicotine is currently unclear. Regardless, repression of these mRNAs in response to nicotine suggests that nicotine may affect RNA metabolism.

Different nAChR Subtypes Have Varying Effects on 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 (19, 45). To determine which receptor subtypes mediate the observed gene expression changes, we treated SH-SY5Y cells with nicotine in the presence of either 1 μM α-cobratoxin or 3 μM mecamylamine. α-Cobratoxin is thought to be a specific ligand for nAChR containing α1 or α7 subunits. A 1 μM dose of α-cobratoxin will specifically inhibit responses to nicotine of α7-nAChR expressed by SH-SY5Y cells. Although interactions of mecamylamine (or of d-tubocurarine) at non-nAChR targets cannot be entirely discounted, mecamylamine at low micromolar doses selectively inhibits nAChR containing α3 or α4 subunits. The mecamylamine inhibition profile for SH-SY5Y cells suggests that α3β4*-nAChR represent the primary α3*-nAChR subtype present (19). Mecamylamine at a concentration of 3 μM would be expected to inhibit about 80% of the nicotinic responses of α3β4*-nAChR and only 20% of α7-nAChR responses to nicotine (46).

Results showed that α-cobratoxin blocked nicotine-mediated repression of C2orf2 and RBBP6 (Table III). This result suggests that specific activation of α7-nAChR is both necessary and sufficient to down-regulate both of these mRNAs. In contrast, none of the observed changes in C2orf2 or RBBP6 gene expression were specifically blocked by mecamylamine at a 3 μM concentration. However, either α-cobratoxin or mecamylamine affected the nicotine-mediated repression of multiple genes. For contactin1, MLL3, UBE3A, ZFR, and DHFR, both α-cobratoxin and mecamylamine prevented nicotine-dependent repression of gene expression. These results implied that simultaneous signaling through both α7-nAChR and α3*-nAChR subtypes was required for nicotine-dependent repression of these mRNAs. In addition, MLL3 showed a trend toward induction in response to the combination of mecamylamine plus nicotine.

Antagonist effects on nicotine repression or induction of other genes were more complex. Repression of PTPα, PIG7, and cDNA DKFZp564F112 (represented by GenBank™ accession number N28268) were not blocked by either α-cobratoxin or mecamylamine. The combined set of observations that PTPα, PIG7, and N28268 repression are blocked when all nAChR subtypes are inhibited by d-TC (Table II) but not when α7- or α3β4*-nAChR are inhibited alone suggests that nicotine-mediated signaling through either receptor subtype is sufficient to modulate expression of these genes. However, it should be noted that we cannot rule out the possibility that the remaining 20% of functional α3β4*-nAChR in the presence of 3 μM mecamylamine may be sufficient to repress these genes in response to nicotine. Alternatively, repression of these genes could occur through additional α3* subtypes that are not inhibited by mecamylamine at a 3 μM concentration.

TABLE III

nAChR subtypes differentially affect gene expression

RT-PCR experiments were performed as described for Table II. Column labels are as follows: Nicotine, samples treated with 1 mM nicotine for 1 h; α-Cobratoxin, samples treated with 1 μM α-cobratoxin for 1 h; α-Cobratoxin + nicotine, samples treated with 1 μM α-cobratoxin and 1 mM nicotine for 1 h; Mecamylamine, samples treated with 3 μM mecamylamine for 1 h; Mecamylamine + nicotine, samples treated with 3 μM mecamylamine and 1 mM nicotine for 1 hr. The numbers in bold represent the average fold changes across multiple independent PCR reactions (from three to five reactions). The Range columns were calculated as for Table II. Several samples wherein the observed fold changes were over 3-fold have accompanying relatively large standard deviations. Serial dilution experiments in which the GAPDH mRNA was amplified indicated that the standard deviation increases as the difference in starting mRNA level between the control and test samples increases (data not shown), thereby possibly explaining this observation.

Gene name	Nicotine	Range ± S.D.	α-Cobratoxin	Range ± S.D.	α-Cobratoxin + nicotine	Range ± S.D.	Mecamylamine	Range ± S.D.	Mecamylamine + nicotine	Range ± S.D.
Matrin3	-1.54[a]	-1.68/-1.41	-1.06	-1.26/1.13	3.61[b]	1.75/7.41	1.01	-1.17/1.21	1.89[b]	1.33/2.69
Contactin1	-1.69[a]	-1.84/-1.56	-1.63[b]	-1.89/-1.41	1.07	1.01/1.14	1.23	-1.05/1.59	1.33	1.11/1.61
FEZ1 variant1	-1.51	-2.07/-1.10	-1.12	-1.52/1.21	-1.37	-1.83/-1.02	-1.23	-1.49/-1.01	-1.18	-1.34/-1.04
FEZ1 variant2	-1.65[a]	-1.93/-1.42	-1.87[a]	-2.38/-1.46	-2.12[a]	-2.23/-2.00	-1.58[a]	-1.88/-1.33	-1.65[a]	-1.80/-1.53
TFPI2	-1.43[b]	-1.69/-1.20	1.08	-1.09/1.27	1.02	-1.18/1.23	-1.15	-1.32/1.00	1.39[a]	1.31/1.48
C2orf2	-2.27[a]	-3.03/-1.69	-1.11	-1.82/1.47	-1.12	-1.75/1.40	-2.06[a]	-2.36/-1.82	-3.67[b]	-6.02/-2.25
ZFR	-1.73[b]	-2.03/-1.47	-1.02	-1.22/1.19	-1.04	-1.32/1.23	-1.41	-1.76/-1.12	-1.16	-1.62/1.20
PTPα	-2.10[a]	-2.53/-1.74	-1.80[a]	-1.97/-1.65	-1.59[a]	-1.87/-1.35	-2.20[a]	-2.69/-1.80	-1.59	-2.62/1.04
PIG7/LITAF1	-3.43[b]	-4.69/-2.51	1.20	1.02/1.48	-2.28[b]	-3.18/-1.63	-4.02	-8.34/-1.94	-4.37[a]	-7.16/-2.68
MLL3	-1.41[b]	-1.62/-1.23	-1.55[b]	-1.99/-1.21	-1.11	-1.40/1.14	1.13	-1.46/-1.14	2.07	-1.03/4.40
DHFR	-1.63[b]	-1.87/-1.44	-2.00[a]	-2.39/-1.67	-1.31	-1.65/-1.06	-1.38[b]	-1.62/-1.16	1.07	-1.11/1.27
UBE3A	-1.38[b]	-1.62/-1.16	-3.06[b]	-5.89/-1.60	-1.11	-1.40/1.15	-1.82[a]	-1.97/-1.67	1.01	-1.16/1.18
RBBP6	-2.12[b]	-2.64/-1.72	-1.11	-1.36/1.10	-1.11	-1.29/1.09	-1.17	-1.20/-1.07	-1.68[a]	-1.99/-1.42
cDNA DKFZp564F112	-1.42[a]	-1.61/-1.24	1.21	-1.45/2.14	-2.61[b]	-2.87/-2.36	-1.97	-3.68/-1.06	-2.45[b]	-3.25/-1.84
EGR1	1.97[b]	1.60/2.43	2.39[b]	1.87/3.07	2.45	1.07/5.58	2.71	1.11/6.63	3.44	1.23/9.58
GAPDH	-1.11	-1.31/1.06	-1.01	-1.38/1.34	-1.06	-1.27/1.13	1.03	-1.31/1.39	1.01	-1.17/1.19

[a] Significance at the $p < 0.01$ level.
[b] Significance at the $p < 0.05$ level as measured by paired t test.

There were instances where α-cobratoxin exposure alone affected mRNA expression comparably to the effects of nicotine (Table III). This effect of α-cobratoxin alone also complicates the interpretation of findings for contactin 1, MLL3, DHFR, and UBE3A, where a block of the effects of nicotine by α-cobratoxin was seen. Similarly, there were instances where mecamylamine exposure alone affected mRNA expression comparably to nicotine, thereby possibly explaining why mecamylamine did not block the effects of nicotine on C2orf2. Additionally, the effect of mecamylamine alone complicates the interpretation of findings for DHFR and UBE3A, where a block of the effects of nicotine by mecamylamine was seen. These results imply that the combined effects of agonist and antagonist acting at nAChR can have differing effects on gene expression than either agonist or antagonist acting alone. Further, assuming that there are no effects of these antagonists on other targets, these results suggest that changes in the conformation of the nAChR that result from antagonist or agonist binding may play a role in activating signaling pathways that ultimately result in alterations of gene expression.

DISCUSSION

Microarray Analyses Can Elucidate Nicotine-dependent Changes in Gene Expression—The current findings indicate that exposure of neuronal SH-SY5Y cells to nicotine at a concentration that produces maximal nAChR up-regulation has relatively subtle effects on the expression of a range of genes coding for proteins with diverse functions. These effects can be identified using a microarray-based approach provided that sufficient replicate, reciprocal labeling experiments are performed to separate the true responses from the background noise. The microarray approach is useful for generating hypothesis about what genes are affected by a given treatment. However, subsequent RT-PCR experiments are required to independently validate the microarray results, especially when there is a low test/control expression ratio or when mRNA levels are only slightly above background. Further validation of results using pharmacological studies also is suggested when possible. In this study, quantitative, real time RT-PCR experiments confirmed altered expression following nicotine exposure for 14 of the 16 genes tested base on microarray findings (Table II). Ten of these genes are altered in a manner consistent with the observed expression ratios from the microarray analyses. In contrast, RT-PCR experiments to detect the mRNAs for matrin3, FEZ1, TFPI2, and EGR1 showed that the expression levels of these genes were altered in a direction opposite to that observed in the microarray analyses. This finding could reflect low expression ratios (Table I) and, except for matrin3, low absolute levels of mRNA expression for these genes in SH-SY5Y cells (data not shown). Nevertheless, pharmacological studies indicated nicotinic receptor antagonist sensitivity of effects of nicotine on expression of 13 of the 14 genes confirmed by RT-PCR analyses. Collectively, the results of this study illustrate the utility of microarrays as screening devices. Moreover, the results also underscore the need for more comprehensive RT-PCR studies to validate and extend the results of microarray analyses. In addition, pharmacological approaches can be valuable to provide further verification and illumination of observations.

Effects of Nicotine on Gene Expression Involve nAChR Activation—Nicotine rapidly crosses the plasma membrane and therefore could affect gene expression either through a nAChR-dependent signaling pathway or through a nAChR-independent pathway. To distinguish between these possibilities, we determined whether nicotine could modulate gene expression when nAChR activity was blocked by the general nAChR antagonist d-TC. The critical observation suggesting that nAChR

TABLE IV

Summary of nicotine treatment-induced gene expression changes and their sensitivity to blockade by nicotinic antagonists

RT-PCR results from Tables II and III are grouped based on the effects of various antagonists and according to gene name (column 1); effect of nicotine, d-TC, α-cobratoxin (cbtx), or mecamylamine (meca) alone (columns 2–5, respectively); or effect of nicotine in the presence of d-TC, α-cobratoxin, or mecamylamine (columns 6–8 under the horizontal bar labeled Nicotine above). Group 1: Gene expression changes in response to nicotine that are either blocked or reversed by d-TC, α-cobratoxin, or mecamylamine. Group 2: Gene expression changes that are blocked by either d-TC or cbtx. Group 3: Gene expression changes that are blocked or reversed by d-TC only. The ↑ indicates significant induction alone. The ↓ indicates significant repression alone. θ indicates nicotinic effects that are significantly blocked in the presence of the indicated antagonist. ∀ 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.

Gene Name	Nicotine	dTC	cbtx	mec	Nicotine dTC	cbtx	mec	
Matrin3	↓	↑			∀	∀	∀	
Contactin1	↓	(↑)	↓	(↑)	∀	Θ	Θ	
TFPI2	↓				Θ	Θ	∀	
ZFR	↓	(↓)		(↓)	(Θ)	Θ	Θ	Group 1
MLL3	↓	↓	↓		Θ	Θ	(∀)	
DHFR	↓	↓	↓	↓	Θ	Θ	Θ	
UBE3A	↓	↓	↓	↓	∀	Θ	Θ	
C2orf2	↓			↓	Θ	Θ		Group 2
RBBP6	↓				Θ	Θ		
PTPα	↓		↓	↓	Θ			
PIG7/LITAF1	↓			(↓)	Θ			Group 3
cDNA DKFZ	↓			(↓)	∀			
EGR1	↑		↑	(↑)	Θ			

activation is involved in the nicotine-dependent modulation of gene expression is that d-TC blocks most of the observed nicotine-induced changes in gene expression. This finding verifies that the observed subtle changes in gene expression resulting from nicotine exposure are a specific effect of this drug acting at nAChR. Further, this finding implies that there may be signaling pathways leading from the nAChR to the nucleus to affect gene expression (see below). However, it is possible that some of the observed effects on mRNA levels could result from posttranscriptional mechanisms.

Individual nAChR Subtypes Differentially Affect Gene Expression—The SH-SY5Y cells express homomeric α7-nAChR and heteromeric α3*-nAChR subtypes. Experiments wherein α7-nAChR and α3*-nAChR subtypes were differentially inhibited using either 1 μM α-cobratoxin or 3 μM mecamylamine yielded four important observations (see Table IV for a summary of antagonist studies in RT-PCR analyses). First, α-cobratoxin prevented the nicotine-dependent down-regulation of both C2orf2 and RBBP6 mRNAs. This result indicates that down-regulation of these mRNAs results exclusively from activation of the α7-nAChR. Second, both α-cobratoxin and mecamylamine blocked, reduced, or reversed the repression of matrin3, contactin 1, TFPI2, MLL3, UBE3A, DHFR, and ZFR mRNAs (Table IV). This finding suggests that simultaneous nicotinic activation of both α7-nAChR and α3*-nAChR is required to repress these mRNAs. Third, neither antagonist prevented nicotine-mediated repression of the N28268 (cDNA DKFZ in Table IV) and PIG7 genes. However, d-TC, a general nAChR antagonist, prevented nicotinic effects on expression of these genes, suggesting that signaling through either α7- or α3*-nAChR is sufficient to affect expression of these messages. Nevertheless, we cannot currently rule out either of the possibilities that the 20% of active α3β4-nAChR that remain functional in the presence of 3 μM mecamylamine are sufficient to regulate these genes or that additional α3* subtypes may reg-

ulate these genes. Fourth, the three nAChR antagonists alone were able to alter the expression of some of the genes identified (see Tables II–IV for a summary of results). Although there is a formal possibility that antagonists could be affecting ongoing non-nAChR signaling that modulates gene expression, this result implies that there exists a nAChR-dependent pathway to repress gene expression that is independent of nAChR channel opening.

The assessment in this study of effects of nAChR antagonists alone should be, but is not, routine practice. Many studies showing the effects of antagonist plus agonist treatments, such as blockade of or failure to block nicotinic agonist effects and synergy with nicotinic agonist effects, need to be replicated with concomitant assessments of antagonist effects alone to help elucidate the bases for ligand actions. Had our studies not examined the effects of antagonists alone, interpretation of the results would have been misleadingly simplified.

What Are the Signaling Pathways Leading from nAChR Activation to Altered Gene Expression?—Our data suggest that at least two initial nAChR-mediated signals can modulate gene expression. Although d-TC blocked the majority of the effects of nicotine on gene expression, the levels of several mRNAs were affected by d-TC alone, and this effect was not reversed when nicotine and d-TC were used in conjunction. For DHFR, d-TC repressed expression comparably to nicotine. For contactin 1, matrin3, and UBE3A, d-TC elicited an increase in mRNA levels, whereas nicotine reduced mRNA levels. Additionally, α-cobratoxin and mecamylamine alone affected the expression of multiple genes. These observations suggest several possibilities. First, there may be at least two nAChR-dependent signaling pathways. One pathway may be dependent on channel opening and subsequent ion flow, and a second pathway may be activated by changes in nAChR conformation that result from either agonist or antagonist binding. In support of the first possibility, α7-nAChR are highly permeable to calcium, and in the SH-SY5Y cell, activation of α7-nAChR has been shown to activate the extracellular signal-regulated kinase 1/2 through a calcium-dependent mechanism (16). Nicotine has been shown to activate this pathway, which ultimately affects gene expression. In contrast, the observation that antagonists alone can significantly repress expression of some genes supports the view that alternative conformational states of the nAChR can affect the expression of some genes. Previous studies have not determined the effects of nAChR antagonists on activation of the MAPK pathway. It is therefore unclear whether mecamylamine, d-TC, or α-cobratoxin could activate the MAPK signaling pathway, thereby possibly explaining their effects on gene expression through a common signaling pathway.

Could Changes in Contactin 1 mRNA Levels Be Involved in Nicotine-induced nAChR Up-regulation?—One of the interesting aspects of nAChR function is the phenomenon of up-regulation. When SH-SY5Y cells are continuously exposed to nicotine, there is an early transient decrease in the total number of assembled, cell surface nAChR that display radioligand binding. However, numbers of total radioligand-binding sites increase immediately, reflecting an increase in intracellular pools (22). Over time, the decline in surface receptor numbers reverses, perhaps reflecting renewal and then later up-regulation of cell surface pools replenished from the increased intracellular pool of precursors. The mechanisms underlying this response are poorly understood.

Nicotine treatment significantly reduced the expression of the contactin 1 mRNA in as little as 1 h (Tables I and II and Fig. 2). Contactin physically interacts with many different proteins on the cell surface, including voltage-gated Na⁺ channels (26, 27), protein-tyrosine phosphatase receptors (25, 47–49),

contactin associated protein (50), and Fyn receptor tyrosine kinase (51). Interaction of contactin with voltage-gated Na^+ channels has been shown to increase the cell surface expression of fully assembled and functional channels (26, 27). This observation suggests an intriguing possible explanation for nAChR cell surface regulation. If contactin regulates the surface expression of nAChR in a manner similar to that of the voltage-gated Na^+ channels, then one would predict that repression of contactin 1 mRNA would result in reduced surface expression of nAChR. Consistent with this model, contactin mRNA is down-regulated following 1 h of nicotine exposure, a time in which surface expression of nAChR is also significantly reduced. Interestingly, after 24 h of nicotine exposure surface nAChR have returned nearly to pretreatment levels (22). This correlates with a 1.4-fold up-regulation of contactin 1 mRNA following 24 h of continuous exposure to 1 mM nicotine (data not shown). Future work will be aimed at elucidating the potential role of contactin 1 in the regulation of nAChR function.

Summary—Nicotine exposure has reproducible, but sometimes relatively subtle, effects on gene expression in a neuron-like cell line. These gene expression changes can be classified into three general groups based on the effects of nAChR antagonists. Further, many of these effects are pharmacologically specific and appear to be mediated by traditional nAChR channel function. Other effects of nicotine on gene expression may result from alternative, yet nAChR-dependent, mechanisms. Our results demonstrate the utility of microarrays in this type of analysis to identify candidate genes where subtle changes in gene expression, as would be predicted to result from drug exposure, occur. Our results also highlight some of the caveats in interpreting the results from such an approach, emphasizing the importance of secondarily verifying consistent changes in gene expression. From these studies come tangible suggestions and targets for future investigation as to how nicotine affects gene expression in the nervous system, potentially adding to ways in which this drug exerts its physiologically relevant effects.

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