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Microarray Technology and its Application on Nicotine Research

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Abstract

Since its development, microarray technique has revolutionized almost all fields of biomedical research by enabling high-throughput gene expression profiling. Using cDNA microarrays, thousands of genes from various organisms have been examined with respect to differentiation / development, disease diagnosis, and drug discovery. Nevertheless, research on nicotine using cDNA microarrays has been rather limited. Therefore, it is our intention in this article to report the findings of our cDNA microarray study on nicotine. We first present an overview of the microarray technology, particularly focusing on the factors related to microarray design and analysis. Second, we provide a detailed description of several newly identified biological pathways in our laboratory, such as phosphatidylinositol signaling and calcium homeostasis, which are involved in response to chronic nicotine administration. Additionally, we illustrate how comparisons between microarray studies help identify candidate genes that potentially may explain the observed inverse association between smoking and schizophrenia. Lastly, given the early stage of microarray research on nicotine, we elaborate on the need for an efficient analysis of genetic networks to further enhance our understanding of the mechanisms involved in nicotine abuse and addiction.

Index Entries: nicotine; drug abuse; microarray; expression; pathways; review.

Introduction

According to the 1996 National Household Survey on Drug Abuse, an estimated 68.8 million Americans used tobacco products. It has been estimated that over 400,000 lives per year are lost through cigarette smoking in the United States alone and perhaps as many as 3 million lives worldwide *(1)*. Those who continue to smoke throughout their lives may lose an average of 8 yr of life. Despite the well-publicized adverse health effects of tobacco and the declining prevalence of smoking in the United States, approx 25.4% adults continue to

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smoke cigarettes, and virtually no further reduction in smoking has occurred in this country during the 1990s. Since the mid-1990s, the prevalence of smoking in both adults and children has actually increased in some ethnic groups, such as in African-Americans. Therefore, tobacco represents one of the most widely abused substances.

Amongst the potential psychoactive agents in tobacco smoke, nicotine appears to be the one primarily responsible for maintaining and regulating tobacco use. The behavioral and neurobiological effects of nicotine are similar to other drugs that are known to be addictive *(2)*. Habit-forming actions of nicotine appear to be triggered primarily through nicotinic acetylcholine receptors (nAChRs) on the cell bodies of dopaminergic neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc). These dopamine neurons are key components of the central nervous system (CNS) pathways that mediate drug reward. Animal studies have indicated that nicotine stimulates dopamine secretion in the outer shell of the NAc, in a manner similar to that of cocaine, amphetamine, and morphine *(3)*. On the other hand, epidemiological studies suggest that nicotine may have protective effects against degenerative processes such as those found in Alzheimer's disease (AD), Parkinson's disease (PD), and Tourette's syndrome. A strong inverse association between cigarette smoking and major depression and schizophrenia has also been established *(4–9)*. However, the mechanisms underlying these diverse associations of nicotine addiction and diseases are largely unknown.

Many genes involved in the dopaminergic reward pathway have been identified and characterized, such as tyrosine hydroxylase, dopamine transporter(s), dopamine receptors, catechol-O-methyltransferase, and monoamine oxidases A and B. Due to the fact that some of these genes were identified recently, no comprehensive study has been conducted to examine their expression profiles during nicotine administration. Furthermore, it is believed that there is significantly more number of genes involved in the mechanisms related to these behavioral and neurobiological effects of nicotine. One approach to understanding these physiological mechanisms is to identify patterns of gene expression associated with exposure to nicotine among different regions of the brain. However, before the advent of microarray technology, it was impossible for any research laboratory to study those candidate genes simultaneously due to the limitations of conventional technologies used for measuring mRNA expression level. Advances in large-scale cDNA microarray technique has made it possible for researchers in the drug-abuse field to compare expression profiles of thousands of genes at a time *(10–12)*. In this review, we first give a brief overview of the technology and factors associated with the application of this technique and then discuss several newly identified biological pathways responsive to chronic nicotine administration in our laboratory.

Progression of Molecular Techniques on High-Throughput Gene Expression

Various methods are available for quantifying gene expression levels, including Northern blot, S1 nuclease protection, differential hybridization screening *(13)*, subtractive library construction *(14)*, representational difference analysis *(15)*, differential display *(16)*, serial analysis of gene expression (SAGE) *(17,18)*, and cDNA microarrays *(19,20)*. The techniques of differential display and the generation of expressed sequence tags (ESTs) were first used for the identification of genes exhibiting marked differential expression across tissues, developmental stages, or normal vs pathological conditions. The analysis of gene expression patterns derived from normal and pathological situations is a valuable tool in the discovery of therapeutics targets and diagnostic markers. The recognition of coordinated expression profiles

among characterized or anonymous genes also enables inferences about biological pathways and gene functions. At the moment, the measurement of gene expression using cDNA microarray appears to be the sole approach to gene characterization capable of matching the speed of sequencing and the scale required for functional genomics.

Since its development, microarray technology, which facilitates the measurement of the relative gene expression levels through a massively parallel approach, has begun to revolutionize biomedical research. The technology behind microarrays was developed over the last several years once it became apparent that new and more powerful analytical approaches were needed to utilize the flood of genomic data and resources being acquired through various genome projects. Two dominant platforms evolved from these pioneering studies; one revolves around *in situ* synthesis of oligonucleotide on support matrixes (for a review, *see* ref. *21*), and the other consists of physically stamping specific target DNAs onto solid support (for a review, *see* ref. *22*). For economic, flexibility, and sensitivity reasons, the second platform has been favored by the academic research community.

In an array experiment, gene-specific polynucleotides derived from the 3′ end of RNA transcripts are individually arrayed on a single matrix. This matrix is then simultaneously probed with fluorescently tagged cDNA representations of total RNA pooled from test and reference samples, allowing one to determine the relative amount of transcript present in the pool by the type of fluorescent signal generated. Relative message abundance is determined by a direct comparison between a "tester" and a "reference" as an internal control is thus provided for each measurement. Even though the scheme is similar when using radiolabeled probe, it is not possible to carry out simultaneous hybridization of both test and reference samples. In such cases, serial or parallel hybridizations are required, introducing the possibility of high variability in the comparison of expression level.

Factors Affecting Microarray Technology

Microarray technology is becoming increasingly important in all areas of biomedical research because it opens new avenues to reassess established models and hypotheses and provides a much broader view for developing models and experimental approaches. Thus, microarray technology has the potential to become an essential tool for biomedical researchers to remain competitive. Many factors may affect the outcome of a microarray experiment; the following is a summary of several of those that should be considered in designing an experiment.

Sensitivity Comparison of Fluorescent vs Radioactive Labeled Complex Probes

RNA quality is a critical factor in hybridization performance, particularly when using fluorescence-labeled complex probe, as cellular protein, lipid, and carbohydrate can mediate significant nonspecific binding of fluorescently labeled cDNAs to slide surfaces. For radioactive detection, ^{33}P-dCTP is preferred to more energetic emitters, as array elements are physically labeled close to each other and strong hybridization with a radioactive target can easily interfere with detection of weak hybridization in surrounding targets. As for fluorescent labeling, Cy3-dUTP and Cy5-dUTP are frequently used together with one flourophore being used to label the tester and the other to label the reference sample. These flourophores have relatively high incorporation efficiencies when labeling with reverse transcriptase, good photostability and yield, and are widely separated in their excitation and emission spectra, allowing highly discriminating optical filtration.

A comparison of sensitivity between P^{33}- and fluorescent-labeled probes has been reviewed by Bertucci et al. *(23)* and is summarized in Table 1. The major conclusion from Table 1 is that the combination of nylon

Table 1
Comparison of Performance for Glass and Nylon Microarrays

Platform	Glass microarrays	Nylon microarrays
Targets	cDNA clones (PCR products)	cDNA clones (PCR products)
Support and format	~10,000 spots in a 2.0 × 7.0 cm glass slide	~ 2304 spots on a 2.0 × 7.0 cm nylon membrane
Sample amount	2–5 μg mRNA	10 μg total RNA
Hybridization volume	10–40 μL	4 mL
Detection	Fluorescence	^{33}P
Image acquisition	Fluorescence reader	Phosphor Imager
Detection limits (mRNA abundance)	1 / 100,000	1 / 10,000
Minimum sample amount for detection	20×10^6 molecules	0.2×10^6 molecules
Reuse	No	3–5 times

Modified from ref. *(23)*.

microarrays with ^{33}P-labeled radioactive probes provides approx 10-fold better sensitivity than glass slides with fluorescence-labeled probes. Therefore, it represents a useful approach in studies where amount of tissues under investigation is relatively limited. Additionally, nylon microarrays with ^{33}P-labeled radioactive probes can be reused 3–5 times after probe stripping *(23)*. The third advantage is that most molecular biologists are more familiar with the protocol used for nylon membrane with ^{33}P-labeled probes than that for the glass slides with fluorescence-labeled probes. However, there exist a couple of drawbacks for this platform. First, the number of genes printed on each membrane is significantly less than on a glass slide, which may limit its application when a large number of genes need to be assayed at a time. Second, the hybridization for both test and reference samples cannot be conducted simultaneously.

Total RNA vs Poly(A) RNA

Both total RNA and poly(A) RNA have been used in the preparation of complex cDNA probes from either cultured cells or tissues. There was a concern that the use of total RNA may result in the loss of information on some rare RNA transcripts. However, by comparing the poly(A) and total RNA probe preparation methods, it was concluded that both methodologies were comparable and no significant information on transcripts was lost *(24)*. In contrast, it appears that the use of total RNA may be advantageous because: 1) it requires less starting material than the poly(A) RNA, therefore it is useful in performing analysis with limited amounts of sample, such as brain punches used in experimental animal studies or autopsy samples from clinics; and 2) it involves less manipulation, and hence less transcript degradation may take place during the preparation of total RNA.

RNA Amplification

A clear limitation to the application of microarray technology to brain research is the large amounts of RNA sample required per hybridization. In order to obtain adequate fluorescence-labeled probe to perform a successful microarray, the total RNA required per array, is 50–200 μg (or 2–5 μg required when using poly(A) RNA; for a review, *see* ref. *22*). Although radioactive targets may have a higher intrinsic detectability, they too

reach a level of dilution that prohibits effective detection, thus precluding experimentation on limited tissue or small number of cells. Various means for improving signal from limited RNA sample have been proposed. For example, efficient mixing of the hybridization buffer should bring more molecules into contact with their cognate probe, increasing the number of productive events. This entails, however, a large "mixing" volume, which might offset the potential gain. Another method for increasing cDNA is RNA amplification, in which labeled target is made directly from a cDNA pool, having a T7 RNA polymerase promoter site at one end via in vitro transcription *(25)*. However, it has been a concern in the literature that polymerase chain reaction (PCR) amplification may introduce the possibility of PCR amplification error *(24)*.

Microdissection

The mammalian nervous system is made up of a heterogenous composite of hundreds of nuclei, all of which can be combined to produce a complex RNA expression profile. Therefore, microdissection capability might become critical for microarray studies involving heterogeneous tissues, and is also useful for associated technologies such as comparative genomic hybridization. The newly developed laser-capture microdissection technique now gives researchers the ability to isolate specific cell types or neurons from a given tissue section, offering the means for rapidly obtaining pure material in comparison with conventional techniques *(26)*. A great advantage of this technology is that it allows single-cell (or neuron) resolution of the tissue being analyzed. However, because a relatively small number of neurons are usually captured and current protocols for RNA labeling demand large quantities of RNA, the use of microdissected RNA on cDNA arrays has been impeded. Other strategies for minimizing the amount of sample to be used include PCR-amplification of total RNA before labeling or the generation of ^{33}P-labeled nucleic acids for nylon membranes and glass slides.

Data Mining and Normalization

Array technologies have made it straightforward to simultaneously monitor the expression patterns of thousands of genes, but how to make sense of such massive data sets still remains as a challenge. Several clustering techniques have been applied to the analysis of gene expression such as hierarchical clustering, k-means clustering *(27)*, self-organizing maps *(28)* and quality clustering *(29)*. For most biologists, the question is: which clustering technique(s) is likely to be the most useful for interpreting their gene-expression data? One simple approach is to use direct inspection to group genes together with similar expression patterns. This approach was used by Cho et al. *(30)* to cluster genes whose expression levels correlated with particular phases of the cell cycle. This method is best suited for instances in which the expression patterns of genes of interest are clear in advance (such as a periodic fluctuation in phase with the cell cycle). Nevertheless, clustering by eye does not scale well to large data sets and is less appropriate for discovering unexpected expression patterns. The two most commonly used computational approaches are hierarchical clustering and k-means algorithm, which cluster genes with similar expression levels based on a present number of clusters without imposing hierarchy *(31–33)*. In addition, self-organizing maps (SOMs) are unsupervised neural learning algorithms that have been used for grouping genes with similar patterns of expression in yeast *(28)*. For generating an SOM, a preset number of vectors (herein called neurons) are randomly initalized. Iteratively, the neurons are proportionally moved towards the original data vectors (gene expression profiles) that they resemble. Even though SOMs are advantageous over hierarchical and k-means algorithms, they still suffer from the fact that

one has to predefine the geometry and number of clusters. An innovative clustering algorithm, called quality clustering introduced by Heyer et al. *(29)*, allows genes with high jackknife correlation coefficients to be grouped together while the cluster diameter is minimized based on a preset threshold value.

Prior to data analysis, however, normalization of microarray data represents an essential step to standardize variation resulting from printing and/or hybridization processes. Several methods, e.g., normalization based on housekeeping genes, global normalization, spiked controls *(34)*, or dilution series *(35)*, have been reported in the literature. Even though housekeeping genes such as β-actin and GAPDH have been extensively used to adjust for differences in the total mRNA concentrations and reverse transcriptuse (RT)-PCR procedures, expression levels of these genes may fluctuate under some experimental conditions *(36)*. Therefore, preliminary analysis is essential to establish a ubiquitously expressed set of genes whose expression levels stay relatively steady over the multiple conditions of each particular experiment. Global normalization may be preferred for microarrays that consist of a large number of randomly selected genes. Furthermore, spots on the microarray may be grouped within themselves in a pin-wise fashion before performing a global normalization (*12;* http://www.stat.berkeley.edu/users/terry/zarray/Html/normspie.html). Since global normalization requires the majority of the clones on the array to remain constant, pathway- or function-focused microarrays may not be applicable to this approach. For instance, microarrays printed with specific biochemical pathways or contain a majority of clones that have been previously shown to change upon the treatment in consideration may not be amendable to this type of normalization. In such cases, microarrays including spots with synthetic DNA sequences or sequences with no homology to the genome of the organism in use (spiked controls) may provide an unbiased and reliable way for array normalization *(34)*. A complex cDNA probe that contains labeled cDNA from the sample of interest combined with a known amount of labeled control cDNA, is then used for hybridization across multiple microarrays allowing for adjustment of the hybridization intensities to the control spot intensity. One has to ascertain, however, that the spiked controls have no detectable sequence homology to the genome under investigation.

Another challenge in microarray data analysis is the assessment of reliability (i.e., repeatability of measurements), because sequential procedures of a microarray experiment may introduce various random and/or systematic errors into the data (e.g., differential labeling efficiency, inconsistent pin geometry and relative pin height, and variable hybridization efficiency to name a few; *see* ref. *35* for details). Printing each clone in replicates (e.g., duplicate or triplicate) has been suggested as a way to assess the inherent error associated with the microarray procedure and consequently to filter out those clones measured in an unreliable fashion *(12,37,38)*. For example, based on the expression levels of 288 genes, Lee et al. *(37)* demonstrated that as the number of replicates increased, the reliability of a measurement could be better assessed. Kadota et al. *(38)* implemented a method called PRIM (preprocessing implementation for microarray) to preprocess cDNA microarray data, which was based on minimizing the cut-off value for calling a measurement unreliable while maximizing the number of observations retained for further analysis. Recently, we have developed an index called JRI (Jackknifed Reliability Index) in order to normalize the measurement error across multiple arrays *(12)* since microarrays may vary in their reliability among each other (Fig. 1). JRI tags each expression value per array with a reliability index that estimates a particular observation's influence on the sample kurtosis value of the pooled absolute duplicate differences using a jackknifing procedure. Pooling provides a more accurate estimate of

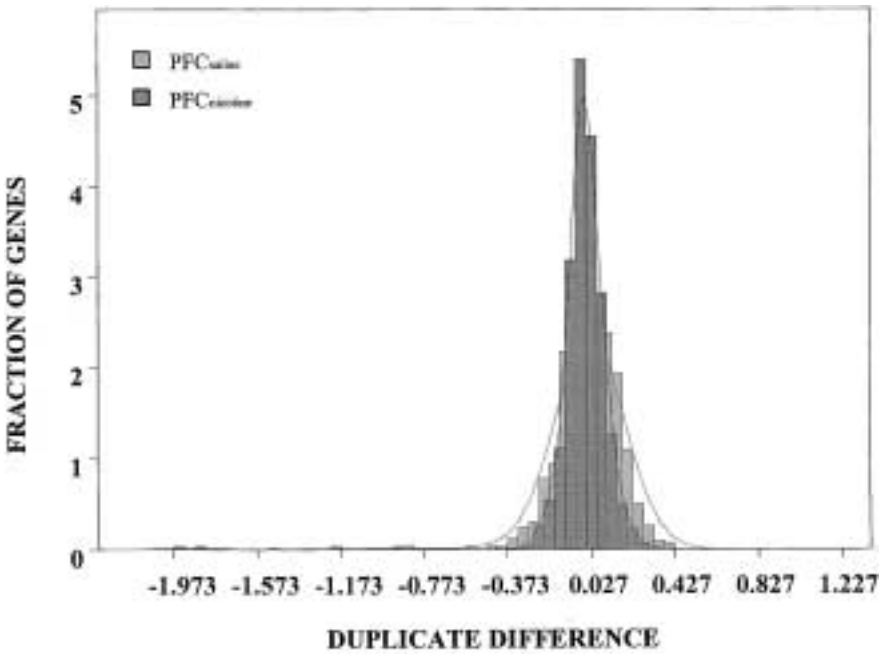

Fig. 1. Comparisons between different arrays in terms of duplicate differences of hybridization intensities. Hybridizations with prefrontal cortex samples from saline- and nicotine-treated animals (PFC_{saline}, $PFC_{nicotine}$, respectively) indicated that the spots in the PFC_{saline} were much more variably measured between duplicates than those in the $PFC_{nicotine}$ microarray.

the inherent error associated with microarray data due to increased sample size. Furthermore, a pooled cut-off value normalizes the variability with respect to the measurement error among different experiments (or arrays). The cut-off value to filter out the unreliable measurements is determined based on the distribution of the JRI values over the sorted absolute duplicate differences *(12)*. For example, in a study comprising of eight microarrays (4 brain regions and 2 treatments), the pooled cut-off value corresponded to an absolute duplicate difference greater than 0.14 at log10 scale (1.38 or 0.72-fold of control expression level), below which an observation was considered reliably measured. Accordingly, 13.5% of a total of 9216 observations was estimated to have a relatively large influence on the sample kurtosis value, and upon filtering the standard deviation of the mean correlation coefficient between duplicate measurements was greatly reduced (*see* Fig. 2 for an example).

Comparisons of Expression Profiling Among Brain Regions

Despite the extensive heterogeneity in brain tissue, different brain regions exhibit remarkable similarity in their expression profile for species ranging from mouse to human (Table 2). For example, we have shown that in rat, prefrontal cortex, nucleus accumbens, ventral tegmental area, and amygdala were highly correlated with each other in terms of the magnitude of gene expression (correlation coefficients: 0.96–0.98; 12); of the 539 clones examined, only 7 exhibited a differential expression greater than 1.8-fold among the four brain regions. Several other studies in mouse and human support our observation that the percentage of uniquely expressed

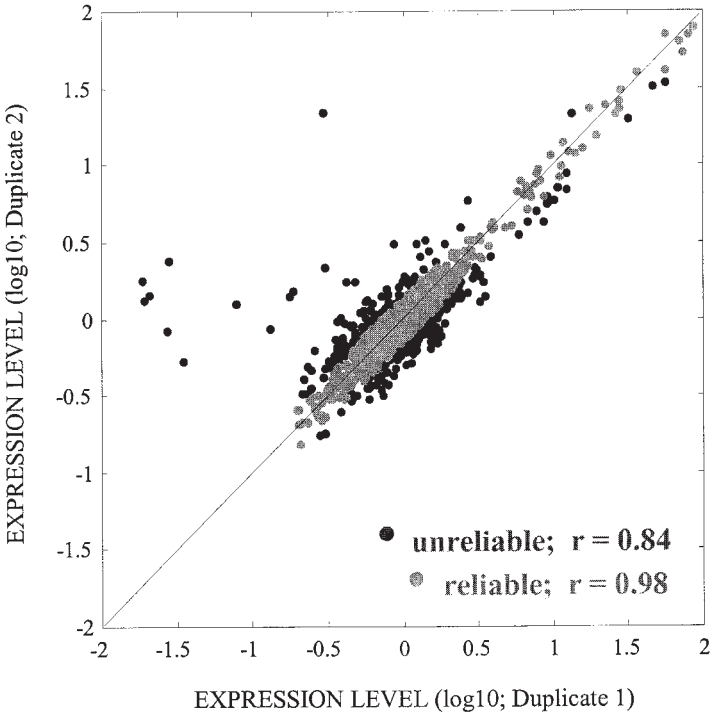

Fig. 2. Correlations between duplicate hybridization intensities before and after filtering out the unreliable measurements in a representative array, i.e., PFC_{saline}. Upon applying the cut-off value obtained by the JRI, the correlation coefficient between the duplicate measurements of the PFC_{saline} array has increased from 0.84 to 0.98.

Table 2
A List of Microarray studies assessing region-specific differences among Different Tissues and Strains[a]

Species	Compared tissues and strains	Total # clones	Differential expression		Reference
			#	%	
Mouse	Cortex, cerebellum, midbrain	13069	70	0.54	*(39)*
Mouse	129SvEv vs C57B1/6 brains	7169	73	1.02	*(39)*
Mouse	128SvEv vs C57B1/6 fibroblasts	7169	115	1.60	*(39)*
Mouse	Amygdala, cerebellum, hippocampus, olfactory bulb, periaqueductal gray	19022	455	2.39	*(41)*
Rat	Prefrontal cortex, nucleus accumbens, ventral tegmental area, amygdala	539	7	1.30	*(12)*
Human	Prefrontal cortex, motor cortex, visual cortex	1088	6	0.55	*(100)*
Human	Adult vs fetal brain	8300	100	1.20	*(101)*

[a] For each study, total number of clones used for the comparisons, and the number and percentage of differentially expressed genes among the regions of interest are indicated.

and/or enriched genes among different brain regions is relatively small (range: 0.54–2.39%; Table 2). Different mouse strains also are strikingly similar in terms of expression levels in brain (i.e., 1.02%) extending these regional similarities within an organism to genetically distinct sets of organisms *(39)*. Similarly, we compared two studies (*12*, http://bioinfo.amc.sara.nl/HTM-bin/index/cgi) that examined expression levels of 125 genes in rat and human, and found that expression levels were significantly correlated between species ($r = 0.22$, $p < 0.05$; Fig. 3), although different techniques were employed in these studies (cDNA microarrays in rat vs SAGE in human).

On the other hand, there is evidence suggesting that transcriptional differences between brain and other tissues are much more pronounced. For example, 13.6% of the genes were differentially expressed between brain and embryonic fibroblasts in mouse *(39)*. Accordingly, Geschwind *(40)* proposed that the complexity of a tissue, defined by its relative number of cell types, may be inversely correlated with the number of uniquely expressed genes in that tissue. For example, cerebellum with less than 10 specialized cell types was shown to have the most number of uniquely expressed genes relative to other brain regions, whereas cerebral cortex possessing more than a hundred cell types does not display as many differentially expressed genes *(39,40)*. However, transcriptional differences among various brain regions may be underestimated due to the inability to accurately detect very low expression levels and/or pooling mRNA from subnuclei or specialized neurons *(40)*. In fact, brain regions, e.g., amygdala, are highly heterogenous structures composed of multiple nuclei and neuronal subtypes that are distinct in their expression patterns *(41)*. Therefore, microarrays hybridized with heterogenous tissues seem to require a finer scale of regional assessment; and several recent advances including in vitro RNA amplification and microdissection techniques are likely to redefine the nature of region-specific differences in brain.

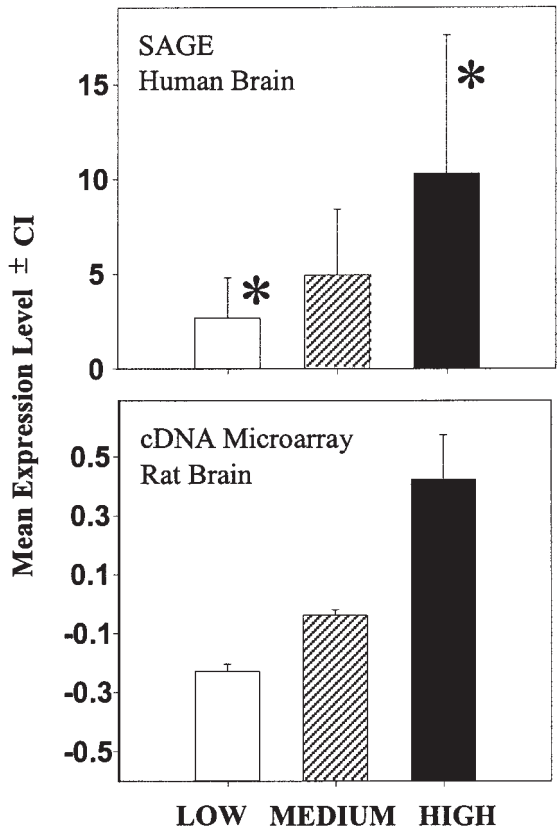

Fig. 3. Comparisons of gene expression levels ($n = 125$) between rat and human brain tissues. Rat and human datasets were compiled based on different methods, neural focused cDNA microarrays and Serial Analysis of Gene Expression (*17;* http://bioinfo.amc.sara.nl/HTM-bin/index.cgi), respectively. Expression levels are reported as normalized log-transformed hybridization intensities in rat brain, and SAGE tag counts normalized to a total of 100,000 transcripts/cell in human brain tissues. Genes were grouped as LOW (low 25th percentile), MEDIUM (middle 50th percentile), and HIGH (high 25th percentile) based on their expression levels in rat brain tissue (i.e., prefrontal cortex, nucleus accumbens, ventral tegmental area, and amygdala). Mean SAGE tag counts of groups LOW and HIGH were significantly different from each other with greater than 95% confidence, as indicated by * by respective columns.

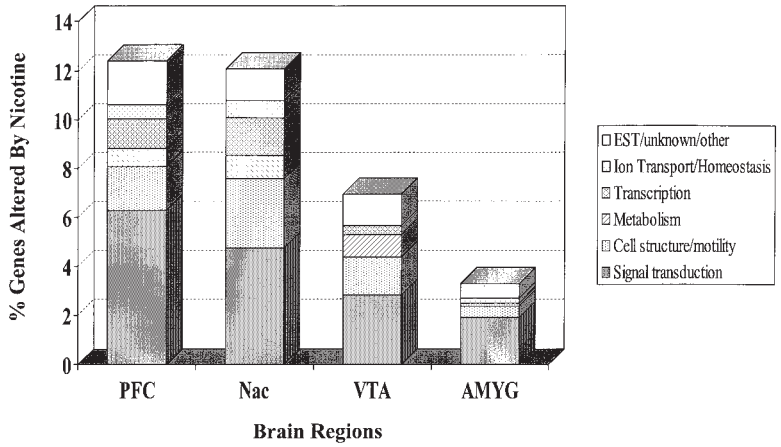

Fig. 4. Percentage of reliably measured genes that were altered by chronic nicotine administration (*see* ref. *12*), and their functional classification within each of the four brain regions under investigation, i.e., PFC, prefrontal cortex; NAc, nucleus accumbens; VTA, ventral tegmental area; AMYG, amygdala. Criteria for fold difference was set at greater than 1.38 or less than 0.72-fold.

Identification of Biological Pathways Involved in Nicotine Administration

Microarray technique provides a powerful tool for identifying functional clusters among genes under investigation. We performed a comprehensive mRNA expression profiling in regards to chronic nicotine treatment in rat brain to assess regional transcriptional differences among different brain regions, i.e., prefrontal cortex (PFC), nucleus accumbens (NAc), ventral tegmental area (VTA), and amygdala (AMYG). Our analyses indicated that only 3.3–12.4% of genes, depending on the brain region, showed any alteration in their gene expression levels due to chronic nicotine administration (based on 1.38- or 0.72-fold difference between nicotine and control experiments; Fig. 4). PFC and NAc were found to be the most responsive regions to chronic nicotine treatment, followed by VTA and AMYG regions. We also found that PFC and NAc shared a significantly greater degree of similarity to each other ($r_{PFC,NAc} = 0.71$; $p = 1.3 \times 10^{-15}$; $N = 95$ genes showing alteration in at least one brain region, and with reliable measurements across all arrays) than they did to either VTA or AMYG.

Moreover, based on the functional differences of genes included in our arrays, transcriptional response to chronic nicotine treatment could be classified into the following categories: cell signaling, cell structure/adhesion, transcriptional regulation, metabolism, ion transport/homeostasis (Fig. 4). In this report, we limited our focus on the region-specific effects of nicotine on the phosphatidylinositol signaling and neuroprotection "pathways."

Phosotidylinositol Signaling and Calcium Homeostasis

Previously, it has been demonstrated in the literature that nicotine may induce a dose-dependent increase in the intracellular calcium concentration $[Ca^{2+}]_i$ via increasing calcium influx from extracellular space, and activating inositol(1,4,5)-trisphosphate (Ins(1,4,5)P3) receptor-coupled intracellular calcium reserves. For example, Zhang and Melvin *(42)* have shown that nicotine first triggers the release of acetylcholine then leads to increased Ins(1,4,5)P3 con-

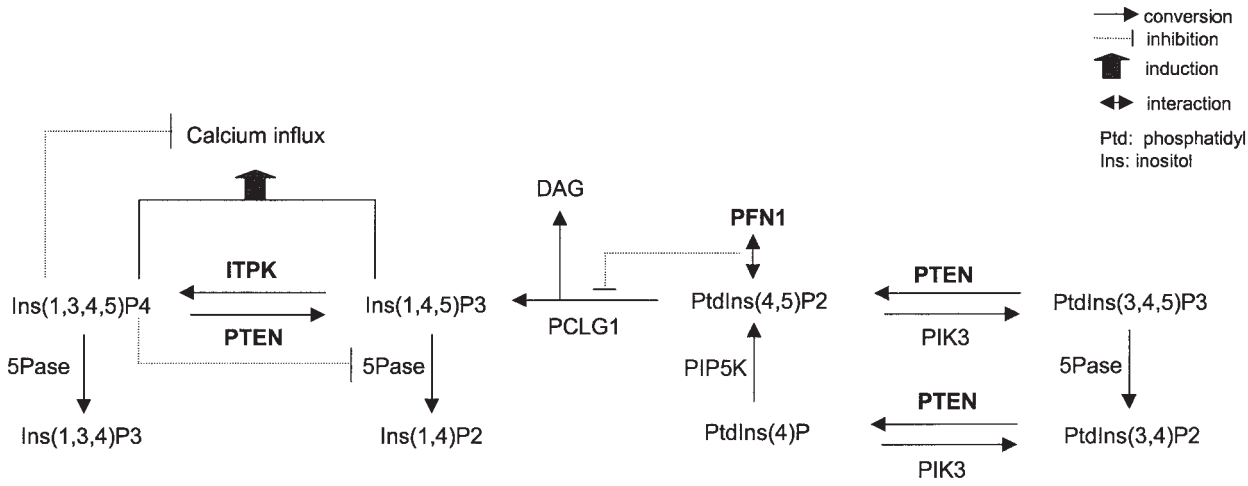

Fig. 5. Conversion of PtdIns(4,5)P2 into Ins(1,4,5)P3 and DAG by PCLG1 elevates intracellular calcium concentration $[Ca]_i$ via activation of Ins(1,4,5)P3 receptors on the endoplasmic reticulum. PFN1, an actin binding protein, plays a regulatory role in the phosphatidylinositol signaling by binding to PtdIns (4,5)P2 and inhibiting its hydrolysis by PCLG1. Upon PCLG1 activation, the increase in $[Ca]_i$ is further accentuated via activation of the store-operated calcium current (I_{CRAC}) in the presence of both Ins(1,3,4,5)P4 and Ins(1,4,5)P3 (50). Overexpression of ITPK producing excess amounts of Ins(1,3,4,5)P4 may deplete Ins(1,4,5)P3, thereby inhibiting the InsP3 receptor mediated calcium influx. Furthermore, PTEN may contribute to the regulation of $[Ca]_i$ by converting Ins(1,3,4,5)P4 back into Ins(1,4,5)P3. At high levels, PTEN antagonizes PIK3/PKB/AKT pathway whose constitutive activation is associated with a variety of cancers. Genes whose expression levels modulated upon chronic nicotine administration are shown in bold (see text for details).
PIK3: phosphatidylinositol 3-kinase; PTEN: phosphate and tensin homolog; 5Pase: inositol 5-phosphatase activity; PIP5K: phosphatidylinositol 4-phosphate 5-kinase; PCLG1: phospholipase C gamma 1; PFN1: profilin 1; DAG: 1,2-diacylglycerol; ITPK: inositol(1,4,5)-trisphosphate 3-kinase.

centrations in isolated rat sublingual mucous acini. In a similar study using PC-12 cells, Gueorguiev et al. *(43)* showed that upon ligand binding, nicotine induces a rapid increase in $[Ca^{2+}]_i$ via voltage-gated calcium channels. This initial transient increase is followed by a more steady rise in $[Ca^{2+}]_i$ via activation of store- operated calcium channels and accumulation of Ins(1,4,5)P3. Nicotine also was shown to result in increases in inositol(1,3,4)-trisphosphate, inositol(1,3,4,5)-tetrakisphosphate (Ins(1,3,4,5)P4), and inositol(1,3,4,5,6)-pentakisphosphate in cultured adrenal chromaffin cells *(44,45)*. An increase in $[Ca^{2+}]_i$ is associated with activation of many signaling pathways ranging from CRE-dependent gene expression to stress activated protein kinases, JNK/SAPK *(46)*. Therefore, modulation of intracellular calcium and Ins(1,4,5)P3/Ins(1,3,4,5)P4 levels by nicotine makes it a highly modulatory substance in controlling cellular activities such as transcription and signaling.

In this study, we identified a number of genes, i.e., PFN1, ITPKB, and PTEN, involved in Ins(1,4,5)P3 turnover as well as intracellular calcium homeostasis, as potential modulators/targets of nicotine in rat brain (*see* Fig. 5; Table 3). We observed that the expression level of PFN1 was reduced by 0.52- and 0.56-fold, in PFC and NAc of nicotine-treated rats, respectively. On the other hand, ITPKB expression was specifically increased only in NAc of rats treated with nicotine (2.45-fold; Table 3). Even though nicotine has been shown to be capable of increasing $[Ca^{2+}]_i$ via Ins(1,4,5)P3 accumulation, chronic nicotine also may deplete Ins(1,4,5)P3-sensitive

Table 3
Modulation of Expression Levels by Chronic Nicotine Treatment for Genes that are Involved in Phosphatidylinositol Signaling Pathway[a]

UNIGENE ID	Gene description	Gene	Region			
	phosphatidylinositol signaling		**P**	**N**	**V**	**A**
Hs.78877	Inositol 1,4,5-trisphosphate 3-kinase B	ITPKB	nd	↑	nd	n/a
Hs.75721	Profilin 1	PFN1	↑	↓	nd	nd
Hs.10712	Phosphatase and tensin homolog	PTEN	nd	nd	nd	↑
Hs.77432	Epidermal growth factor receptor	EGFR	nd	nd	nd	↓

[a] Unigene ID number, gene description, gene name, and affected brain regions are given. Arrows indicate the direction of the alteration in gene expression.

Note: Fold difference (D): D>1.8 (↑); D<0.57 (↓). nd, no difference; n/a; unreliable measurement; P, prefrontal cortex; N, nucleus accumbens; V, ventral tegmental area; A, amygdala.

Ca^{2+} stores, for example, in T-cells *(47)*. Hermasura et al. *(48)* have recently shown that Ins(1,3,4,5)P4 is a natural Ins(1,4,5)P3 5-phosphatase inhibitor that enhances Ca^{2+} influx by reducing the conversion of Ins(1,4,5)P3 to Ins(1,4)P2, thereby helping sustain relatively high level of Ins(1,4,5)P3 in the presence of Ins(1,3,4,5)P4 (Fig. 5). However, the presence of excess ITPKB may lead to an overproduction of Ins(1,3,4,5)P4, which in turn may deplete Ins(1,4,5)P3, inhibiting Ins(1,4,5)P3 receptor-induced intracellular calcium accumulation. Our microarray data suggests that phosphatidylinositol signaling pathway was modulated in PFC and NAc upon chronic nicotine administration leading to a potential increase in the production of Ins(1,4,5)P3 in PFC and NAc, and Ins(1,3,4,5)P4 only in NAc.

On the other hand, we found that PTEN expression was increased only in amygdala upon chronic nicotine administration, implicating the presence of a negative feedback on the phosphatidylinositol pathway in this brain region. PTEN exhibits 3-phosphatase activity towards Ins(1,3,4,5)P4, and thus reduces the availability of Ins(1,3,4,5)P4 (Fig. 5). Epidermal growth factor receptor (EGFR) also was down-regulated by nicotine in amygdala (i.e., 0.57-fold reduction) supporting the reported finding that an increase in the expression of PTEN leads to down-regulation of EGFR *(49)*. Since PTEN was shown to be a tumor-suppressor gene, whose inactivation constitutively activates PI3/AKT pathway *(50)*, nicotine's involvement in the modulation of PTEN expression may help understand the link between nicotine and cancer *(51,52)*.

Based on these findings, it is tempting to suggest that modulation of ITPK and PTEN expression by nicotine may act as an important switch/tuner in the regulation of intracellular calcium concentration. Moreover, the extent of this activation/inactivation in the phosphatidylinositol pathway is likely to depend on the dose of nicotine administration and cellular characteristics of the tissues (e.g., type and number of receptors) under investigation.

Neuroprotective Effects

An increasingly large number of studies indicate that nicotine, an agonist of nicotinic acetylcholine receptors (nAChRs), is highly neuroprotective against exposure to agents that cause exicitotoxicity and neural degeneration. Chronic nicotine administration produces a significant dose-related protection against 6-hydroxydopamine-induced Parkinsonian-like neurodegeneration in the striatal dopaminergic nerve terminals via activation of α4 and perhaps other nAChR subunits *(53)*. Similarly,

Table 4
Modulation of Expression Levels by Nicotine for Genes that are Involved in Neuroprotection[a]

UNIGENE ID	Gene Description	Gene	Region			
	Growth factors, receptors, cytokines		**P**	**N**	**V**	**A**
Hs.101766	TGF-β receptor associated protein-1	TRAP-1	↑	↑	nd	nd
Hs.74615	Platelet-derived growth factor receptor, alpha	PDGFRA	nd	↑	n/a	nd
Hs.748	Fibroblast growth factor receptor 1	FGFR1	nd	↑	nd	nd
Hs.239176	Insulin-like growth factor 1 receptor	IGF1R	n/a	↑	nd	nd
Hs.2250	Leukemia inhibitory factor	LIF	nd	↑	↓	nd
Hs.76144	Platelet-derived growth factor receptor, beta	PDGFRB	↓	↓	nd	nd
Hs.82173	TGF-β inducible early protein	TIEG1	↓	↓↓	nd	nd
Hs.82028	Transforming growth factor, beta receptor II	TGFBR2	n/a	↓	nd	n/a
	Signaling via NF-κB and JNK					
Hs.47007	Serine/threonine protein-kinase	NIK	nd	↑	nd	nd
Hs.82979	MAP kinase kinase kinase kinase 2 (GCK)	MAP4K2	n/a	↑	nd	nd
Hs.54589	NCK adaptor protein 1	NCK1	n/a	↑	nd	nd
Hs.30223	MAP kinase kinase kinase 10 (MLK2)	MAP3K10	n/a	↑	nd	nd
	Cell adhesion, axonal growth					
Hs.9004	Chondroitin sulfate proteoglycan 4 (NG2)	CSPG4	↑↑	↑↑	nd	nd
Hs.204133	Hexabrachion (tenascin C, cytotactin)	HXB	n/a	↑	nd	n/a
Hs.151250	Telencephalin	ICAM5	n/a	↑	↑	n/a
Hs.171921	Semaphorin 3C	SEMA3C	nd	↓	n/a	nd

[a] Unigene ID number, gene description, gene name, and affected brain regions are listed. Arrows indicate the direction of the alteration in gene expression.

Note: Fold difference (D): D>1.8 (↑↑); 1.35<D<1.8 (↑); D<0.57 (↓↓); 0.57>D>0.74 (↓). nd, no difference; n/a, unreliable measurement; P, prefrontal cortex; N, nucleus accumbens; V, ventral tegmental area; A, amygdala.

nAChR α7 subunit is implicated in the neuroprotection against beta-amyloid (Aβ) enhanced glutamate neurotoxicity, which is suspected to play a role in the pathogenesis of AD *(54)*. Nicotine also protects against NMDA and glutamate excitotoxicity in primary hippocampal cultures and in cortical neurons via nAChR α7 subunit activation in a calcium-dependent manner *(55,56)*. Pretreatment with nicotine protects the spinal cord neurons against arachidonic acid-induced apoptosis, further supporting the anti-apoptotic effects of nicotine on neurons *(57)*.

Our microarray data suggests that modulation of growth factors and cytokines (e.g., a decrease in transforming growth factor-β (TGF-β) type II receptor and an increase in FGFR1, PDGFRA, IGFR1, LIF, and possibly others), as well as activation of signaling pathways (e.g., nuclear factor-kappaB (NF-κB) and c-Jun N-terminal kinase (JNK) cascades via upregulation of NIK, GCK, MLK2, and NCK1), potentially underlies the neuroprotective effects of nicotine in a region specific manner, i.e., NAc and to a lesser degree in PFC (Table 4 and see below for detail). Furthermore, several genes involved in remyelination and axonal growth, i.e., CSPG4, HXB, ICAM5/telencephalin, and SEMA3C, also are modulated by nicotine, pointing to an ongoing process of neural regeneration, perhaps as a response to nicotine-induced damage to neurons. Remarkably, this hypothesis is well-complemented by previous findings from the literature confirming that microarray studies are useful tools for revealing the intricate communication among different signaling pathways.

Growth Factors/Cytokines

Growth factor receptors, PDGFRA, IGF1R, and FGFR1, and LIF were upregulated in the NAc of animals exposed to chronic nicotine (Table 4). Previous studies indicate that signaling via PDGFRA, IGF-I, and FGF-2 may play an important role in protection against cellular transformation and apoptosis *(58–62)*. Similarly, LIF, a cytokine functionally similar to ciliary neurotrophic factor (CNTF), promotes survival in nervous tissue mainly through the activation of NF-κB signaling pathway *(63,64)*.

Upon nicotine treatment, the expression of TGF-β receptor type II and TIEG1 (i.e., a downstream component of TGF-β and Smad signaling pathway; *65*) were reduced, whereas expression of TRAP-1 (i.e., an inhibitor / modulator of activated TGF-β receptors; *66*) was induced in NAc and possibly in PFC region. TGF-β signaling was shown to exhibit either neuroprotective or neurotoxic effects in a manner that is isoform-, dose-, and tissue-specific *(67–69)*. Interestingly, TGF-β2 has been reported to enhance NMDA-mediated neurotoxicity, at least in neuronal / astrocyte *(30)*, and in neuronal / glial and pure neuronal cultures *(70)*. Taken together, these findings suggest that nicotine downregulates TGF-β signaling in a brain region-specific manner, and this inhibition may partly contribute to nicotine's neuroprotective effects against glutamate-induced damage in nervous tissue.

NF-kB and JNK Signaling Cascades

Our results point to the activation of NF-κB and JNK signaling pathways in NAc (but not in VTA and AMYG) upon chronic nicotine administration. We have found that NF-κB inducing kinase, NIK, which leads to phosphorylation of IκB kinase that in turn phosphorylates the inhibitory regulator IκB, was upregulated in response to nicotine in NAc (Table 4). Furthermore, overexpression of GCK in the NAc of nicotine-treated rats suggested that both JNK and NF-κB pathways might be modulated by nicotine. Indeed, GCK was shown to increase JNK and NF-κB activity, coincidentally preventing apoptosis in early-stage melanoma cells *(71)*. Furthermore, there is favorable evidence for the presence of a cross-talk between NF-κB and TGF-β signaling pathways. NF-κB suppresses TGF-β mediated signaling via interaction with Smad signaling complexes *(72,73)*. In addition, a recent microarray study demonstrated that TGF-β2 also represses NF-κB activity in granule neurons in vitro *(74)*. Observed downregulation of TGF-β signaling upon exposure to nicotine corresponds well with the activation of NF-κB transcriptional response, which may underlie some of the neuroprotective effects of nicotine. For example, NF-κB suppresses apoptosis and protects neurons against oxidative stress *(75)* Cytokines such as CNTF and LIF activating NF-κB also increase survival in developing sensory neurons *(64)*. Furthermore, involvement of JNK in response to nicotine is intriguing because JNK cascade responds to the ossilations in the $[Ca^{2+}]_i$. An increase in or a depletion of intracellular calcium stores may invoke the activation of JNK, making it a potent stress-response modulator *(76)*. Since nicotine seems to modulate $[Ca^{2+}]_i$, JNK may mediate some of the transcriptional responses to nicotine in connection with NF-κB. Further support for the activation of JNK in NAc comes from NAc-specific upregulation of MLK2 and NCK1 (Table 4), both of which preferentially activate JNK when compared with other MAPK pathways *(77,78)*.

Remyelination and Axonal Growth

Several genes involved in myelination, cell adhesion, axonal guidance, and neurite outgrowth also were modulated by nicotine in the NAc, but not in VTA or AMYG. In particular, ICAM5 / telencephalin, CSPG4, and HXB, were upregulated, whereas semaphorin 3C, a closely related member of SEMA3A, was downregulated in response to nicotine in NAc (CSPG4 also in PFC; Table 4). Telencephalin has been demonstrated as an inducer of neurite outgrowth *(79)*. Similarly, CSPG4 was shown to be involved in

remyelination *(80)*, cell migration, and axonal growth/maturation *(81)*. CSPG4 and HXB also enhanced the embryonic dopamine-cell attachment in a striatal wound model system in mouse *(82)*. Furthermore, a recent study demonstrated that demyelinated sites of injury in rat ascending dorsal columns accumulated CSPG4 and HXB proteins, and attracted axonal regrowth, whereas presence of semaphorin 3A repelled the axons *(83)*. Finally, PDGFRA, FGFR1, and IGF1R, all of which were shown to differentially regulate the number and maturation of oligodendrocytes, were upregulated by nicotine, further supporting nicotine's involvement in neuroprotection, and perhaps myelination (Table 4; *84*). A plausible explanation for the observed pattern of expression, especially regarding the wound/injury-associated transcriptional response, is that nicotine might have led to oxidative stress lesions in nucleus accumbens and possibly in prefrontal cortex, which in turn triggers the signaling pathways activated by stress (NF-κB and JNK), finally leading to axonal regeneration and remyelination.

Candidate Genes Involved in the Reported Inverse Association Between Nicotine Use and Schizophrenia

Nicotine's ability to bind to nAChRs, which are known to be functional in learning, memory, synaptic plasticity, and cognition, provides a possible link between nicotine use and the pathogenesis of neurodegenerative/psychiatric disorders such as AD and schizophrenia *(85,86)*. In fact, cholinergic deficits involving different subunits of nAChRs have been reported in these brain disorders *(87)*. Furthermore, the observed high incidence of smoking among schizophrenics has implicated nicotine as a self-medication that counteracts a potential deficit related to schizophrenia, thus leading to its abuse *(5)*.

Mirnics et al. *(88)* recently proposed a hypothesis for the pathogenesis of schizophrenia, which stated that alterations in the gene products that polygenically control mechanics of synaptic transmission may underlie schizophrenia. Their microarray study revealed that several presynaptic genes such as N-ethylmaleimide sensitive factor (NSF), synapsin II (SYN2), a vacuolar ATPase (ATP6D), synaptotagmin V (SYT5), synaptojanin 1 (SJAN1), and synaptogyrin 1 (SGYN1) were decreased to varying degrees in the prefrontal cortex of schizophrenic brains. Similarly, Vawter et al. *(89)* reported that several genes involved in synaptic transmission and plasticity were differentially expressed between schizophrenic and control samples. On the other hand, synaptotagmin mRNA increases during spontaneous waking and/or sleep deprivation (SYTIV; *90*). We found that exposure to chronic nicotine has led to upregulation of SYT5 mRNA by 66% in NAc. Our results also indicate that a vacuolar ATPases, ATP6D, downregulated in schizophrenia, was upregulated by 60% in response to chronic nicotine administration. Interestingly, a recent microarray study by Zhang et al. *(91)* also have shown that expression of ATP6E and ATP6S1, were downregulated in endothelial cells exposed to nicotine for 24 h. Previous studies have demonstrated that synaptotagmins participate in calcium-dependent synaptic vesicle exocytosis and neurotransmitter release *(92)* whereas vacuolar ATPases are responsible for acidification of the intracellular compartments, and also regulate endocytosis and intracellular membrane traffic *(93)*. Accordingly, modulation of the expression levels of these two families of proteins by nicotine may provide an important link between nicotine use and schizophrenia in regards to the regulation of neurotransmission.

Hakak et al. *(94)* uncovered a different set of genes involved in processes such as myelination, synaptic plasticity, neurotransmission, and phosphatidylinositol signaling, upon comparison of postmortem dorsolateral prefrontal cortex of schizophrenic and control patients. For example, they reported that expression level of cannabinoid receptor 1 (CB1) was

increased in schizophrenics. In contrast, we have found that nicotine has led to downregulation of CB1 by 37%. Cannabis is known to induce schizophrenia-like symptoms, and its downregulation by nicotine supports the self-medication hypothesis. Previous studies have documented an inverse relation between cholinergic transmission and activation of cannabinoid receptor in different strains of mice *(95)*. In the hypocampal neurons of the CB1-deficient mice, acetylcholine release was selectively enhanced, hence suggesting a role for CB1 in the cholinergic transmission *(96)*. Hakak et al. *(94)* also reported that expression levels of profilin and gelsolin, actin-binding proteins that interact with PtdIns(3,4)P2, were modulated in schizophrenics. The direction of the changes in the expression of these genes in schizophrenia is opposite to those we observed in the prefrontal cortex and nucleus accumbens of nicotine-treated rats. Previously, dysregulation of secondary messenger system involving diacylglycerol and InsP3 turnover has been hypothesized to occur in schizophrenics *(97)*. For example, inositol levels were reduced in the postmortem brain regions (frontal and occipital cortex, and cerebellum) of schizophrenic patients when compared to those of controls *(98)*. These findings imply that modulation of cholinergic transmission and phosphatidlyinositol signaling pathway may be important in the pathogenesis of schizophrenia, and provide new insights for understanding the high incidence of smoking among schizophrenics.

Prospects

There is no doubt that microarray technique will revolutionize our understanding of the extent of transcriptional response to abusive drugs by revealing the coordinated expression profiles, which previously had been impossible to obtain by using conventional molecular approaches such as Northern blot hybridization, RNase protection assay, or quantitative RT-PCR. Genomic information from the recently completed human draft genome sequence in combination with high-throughput expression data also is likely to provide the long-awaited answers to questions such as: how do individuals become drug addicted, why are some individuals more prone to addiction than others, and what kinds of genes/pathways are involved in drug addiction/withdrawal?

Major challenges that still remain to be addressed by the users of microarray technology, however, include issues related to experimental design and to the development of bioinformatic tools for storing and analyzing such massive data sets. Carefully designed and replicated microarray studies have the potential to provide testable hypotheses on the neurobiology of drug abuse and addiction. Moreover, analyzing expression profiles in response to a particular drug under variable conditions will enable us to determine the participating signaling pathways/candidate genes and to develop novel therapies.

Use and further development of bioinformatics tools, which allow storage and mining of such massive data, also will accelerate the discovery process in the drug abuse field. For example, *Drug & Alcohol Abuse Microarray Data Consortium* is one such effort that will provide the researchers in this field with the ability to explore high-throughput gene expression data made available for a variety of addictive drugs (http://www.wfubmc.edu/microarray/). Additionally, novel approaches such as automated extraction of biomedical knowledge from publicly available gene and text databases (http://www.pubgene.org; *99*) present an immense potential to help interpret the results of microarray experiments by determining functional and physical interactions among genes with similar expression patterns. In summary, we believe that microarray studies in the nicotine and other drugs of abuse field would highly benefit from a comprehensive approach, therefore future experiments should be designed as to allow for the assessment of dose-, time-, and tissue-specific expression profiles of a particular drug(s) under investigation.

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