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ACCELERATED COMMUNICATION

Expression Profiling of Neural Cells Reveals Specific Patterns of Ethanol-Responsive Gene Expression

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ABSTRACT

Adaptive changes in gene expression are thought to contribute to dependence, addiction and other behavioral responses to chronic ethanol abuse. DNA array studies provide a nonbiased detection of networks of gene expression changes, allowing insight into functional consequences and mechanisms of such molecular responses. We used oligonucleotide arrays to study nearly 6000 genes in human SH-SY5Y neuroblastoma cells exposed to chronic ethanol. A set of 42 genes had consistently increased or decreased mRNA abundance after 3 days of ethanol treatment. Groups of genes related to norepinephrine production, glutathione metabolism, and protection against apoptosis were identified. Genes involved in catecholamine metabolism are of special interest because of the role of this pathway in mediating ethanol withdrawal symptoms (physical dependence). Ethanol treatment elevated dopamine β-hydroxylase (DBH, EC 1.14.17.1) mRNA and protein levels and increased releasable norepinephrine in SH-SY5Y cultures. Acute ethanol also increased DBH mRNA levels in mouse adrenal gland, suggesting in vivo functional consequences for ethanol regulation of DBH. In SH-SY5Y cells, ethanol also decreased mRNA and secreted protein levels for monocyte chemotactic protein 1, an effect that could contribute to the protective role of moderate ethanol consumption in atherosclerotic vascular disease. Finally, we identified a subset of genes similarly regulated by both ethanol and dibutyryl-cAMP treatment in SH-SY5Y cells. This suggests that ethanol and cAMP signaling share mechanistic features in regulating a subset of ethanol-responsive genes. Our findings offer new insights regarding possible molecular mechanisms underlying behavioral responses or medical consequences of ethanol consumption and alcoholism.

Ethanol is one of the most commonly used and abused drugs. As seen in alcoholics, chronic exposure to ethanol produces long-lasting behavioral adaptations including tolerance, dependence, sensitization, and craving. Furthermore, alcoholics also suffer long-term dysfunction in multiple organ systems, including the liver, immune system and heart. Recent work suggests that changes in gene expression mediate long-lasting ethanol-induced complex behaviors, as seen with other abused drugs (Miles, 1995; Nestler and Aghajanian, 1997). Furthermore, alterations in gene expression are seen in multiple organ systems or cell types with ethanol exposure and may underlie ethanol-induced organ toxicity (Miles, 1995). Consistent with this possibility, we and others, using nonbiased subtractive cloning and differential display methods, have identified ethanol-regulated genes in cultured cells and animal models (Miles et al., 1993, 1994; Schafer et al., 1998). In some cases it has been shown that changes in gene expression can occur rapidly after ethanol exposure and persist for long periods of time or until ethanol is removed (Miles et al., 1991; Verma and Davidson, 1997). Despite these advances, and the definition of distinct membrane protein or signaling pathway targets for acute ethanol action (Diamond

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ABBREVIATIONS: DBH, dopamine β-hydroxylase; NE, norepinephrine; db-cAMP, dibutyryl-cAMP; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; NET, sodium-dependent norepinephrine transporter; DLK1, δ-like protein 1; MCP1, monocyte chemoattractant protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag.

and Gordon, 1997), a clear consensus is lacking for how ethanol exerts its major cellular and behavioral responses.

Recently, high-density cDNA or oligonucleotide arrays have made it possible to study changes in complex patterns of gene expression. Expression studies with DNA arrays are thus more informative than nonparallel studies on single candidate genes (Gray et al., 1998; Fambrough et al., 1999; Iyer et al., 1999; Ly et al., 2000). Finding pathways of related genes responding to ethanol might provide important clues about the consequences and mechanisms of ethanol action at a cellular and organ system level.

We have previously used cultured neuroblastoma cells as a model for studying "direct" ethanol-induced changes in gene expression important for development of tolerance to and dependence upon ethanol (Miles et al., 1991, 1994; Wilke et al., 2000). Here we have used high-density oligonucleotide arrays to characterize the effects of ethanol on gene expression levels in human SH-SY5Y neuroblastoma cells. SH-SY5Y cells resemble mature noradrenergic neurons and have been used previously to investigate cellular effects of ethanol (Luo and Miller, 1997). We show that ethanol increases expression of dopamine β-hydroxylase (DBH, EC 1.14.17.1) and several other genes involved in norepinephrine (NE) production and increases releasable NE in SH-SY5Y cells. Furthermore, we identify other gene targets for ethanol regulation, which suggest possible molecular mechanisms of ethanol action. These results contribute to our understanding of ethanol action at a cellular level and may have implications for the treatment of behaviors, such as dependence, seen with chronic ethanol exposure.

Materials and Methods

Cell Culture and Animals. The human neuroblastoma cell line SH-SY5Y-AH1861 (passage number 7) was obtained from Dr. Robert Messing (University of California, San Francisco). Cells were routinely grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% (v/v) fetal bovine serum in a humidified atmosphere of 10% CO_2 in air. For gene expression analysis, 80% confluent cells were treated for 72 h in the absence or presence of 50, 100, or 150 mM ethanol or 1 mM dibutyryl-cAMP (db-cAMP). Culture media were renewed every 24 h during treatment protocols or every 2 days otherwise.

Animal studies were conducted on female DBA/2J mice (Simonsen Laboratories, Gilroy, CA) weighing 20 to 30 g at 8 weeks of age. All animals were housed individually under a 12:12 light/dark cycle at 22°C and given ad libitum access to food and water before and after injection procedures. Animals were injected intraperitoneally with 4 g/kg ethanol or saline at 10:00 AM, returned to their home cage, and euthanized 24 h later by cervical dislocation and decapitation. Adrenal glands were excised, immediately frozen in liquid nitrogen and stored at −80°C until needed. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and institutional guidelines.

Biotinylated cRNA Preparation for Array Hybridizations. Following ethanol treatment, cells were trypsinized and washed in ice-cold PBS. Poly A^+ RNA was directly extracted from cell pellets (30 to 40 $\times$ 10^6 cells) using Quick mRNA Prep kit (Amersham Pharmacia Biotech, Piscataway, NJ) or Oligotex direct mRNA kit (Qiagen, Santa Clarita, CA). Protocols for synthesis of cDNA and cRNA were performed according to Affymetrix (Santa Clara, CA) recommendations and have been previously described (Lockhart et al., 1996). Before hybridization, 10 μg of cRNA were fragmented randomly to an average size of 30 to 60 bases by incubation at 94°C for 35 min in 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate.

Array Hybridization and Scanning. Gene expression levels in response to ethanol or db-cAMP were monitored, using the Hu6000 or Hu6800 oligonucleotide arrays (Affymetrix), respectively. Both sets include four probe arrays (A, B, C, D) containing over 65,000 different oligonucleotides each. The Hu6800 arrays were used for the db-cAMP experiment since the Hu6000 arrays had been discontinued. The organization of these arrays has been previously described (Lockhart et al., 1996). Each RNA sample was hybridized once except for duplicate hybridizations that were done for the control and 100 mM ethanol-treated samples in one experiment. Hybridizations and scanning were completed as described previously (Lockhart et al., 1996). Briefly, aliquots of fragmented cRNA (10 μg in a 200 μl master mix) were hybridized to arrays at 40°C for 16 h in a rotisserie oven set at 60 rpm. Following hybridization, arrays were washed with 6$\times$ SSPE and 0.5$\times$ SSPE containing 0.005% Triton X-100, and stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). After washing, arrays were read with a dedicated confocal microscope scanner (Molecular Dynamics, Sunnyvale, CA or Hewlett Packard, Santa Clara, CA).

Data Analysis. Absolute and comparison analyses were conducted using GeneChip Software 3.1. The total fluorescence intensity for each array was scaled to a uniform value by normalizing the average intensity of all genes (total intensity/number of genes) to a fixed value of 74. Under these conditions, the scaling factor for all chips varied between 0.22 and 2.03. The protocols for analysis of Affymetrix arrays have been previously described in detail (Lockhart et al., 1996; Wodicka et al., 1997). The output of the GeneChip software consisted of a hybridization intensity ("average difference") and comparison between an ethanol-treated sample and control ("fold-change"). The average difference values represent the mean, excluding outliers, of probe pairs for a given gene. Confidence measures for the presence or absence of a given mRNA ("absolute call") and fold-change values ("difference call") were generated using a matrix-based decision algorithm (Wodicka et al., 1997). In all cases, the default values in the Affymetrix software were employed. Text file outputs of the GeneChip software can be viewed on our web site (htttp://www.egcrc.org/mmlab/SHSY_paper.htm).

Ethanol-responsive genes were selected by filtering data from seven comparison files from two independent experiments. We selected genes represented by at least 10 probe pairs on the arrays, whose expression levels differed from the control by more than 1.5-fold in at least two comparison files from each experiment. We also applied selection criteria derived by the Affymetrix GeneChip software. Genes consistently called "absent" or "not changed", respectively, by the absolute call or difference call algorithms were eliminated from the set of ethanol-responsive genes. The db-cAMP data was analyzed for correlation with the set of identified ethanol-responsive genes (Fig. 1B). Of the 42 ethanol-responsive genes, 31 were represented on the Hu6800 chips. Among this group, genes were displayed as db-cAMP-responsive (Fig. 5) if they differed from control more than 1.5-fold, and the changes were in the same direction as seen with ethanol.

Northern Blot and Reverse Transcriptase-PCR (RT-PCR) Analyses. Total RNA was extracted from control and ethanol-treated cells and analyzed by formaldehyde-agarose gel electrophoresis and Northern blot hybridization to complement oligonucleotide array results. RNA blots were probed with ^{32}P-labeled inserts of human DBH, sodium-dependent NE transporter (NET), δ-like protein (DLK1), and monocyte chemoattractant protein 1 (MCP1) cDNA. Probes were synthesized by RT-PCR using SH-SY5Y total RNA as template. PCR primers were: 5′-CCTCACTGGCTACTGCACGG-3′ and 5′-CTCTTCCAGTGTGGAGATG-3′ for DBH; 5′-AGAAGAATCACCAGCAGCAAGTG-3′ and 5′-GGTGCCTCAGTTTTCCCATTG-3′ for MCP1; 5′-GCATTGCGTTTGTCACACAGC-3′ and 5′-CTGTGGGTATCGTCTTCCC-3′ for DLK; and 5′-GGAGCTGGCCTAGTGTTC-3′ and 5′-CCATAGGCCAGTCTCTCCC-3′ for NET.

Equal loading between lanes was verified by hybridization with a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (CLONTECH, Palo Alto, CA). Hybridization to membranes was analyzed using a STORM 860 PhosphorImager and ImageQuant Software (Molecular Dynamics).

Semiquantitative RT-PCR was used to quantify DBH mRNA levels in adrenal glands of mice acutely injected with saline or ethanol. Following extraction, total RNA was transcribed into single-stranded cDNA using Superscript Choice (Life Technologies, Inc.). Duplex reactions with cDNA aliquots were conducted using GAPDH as an endogenous amplification standard. PCR conditions were optimized so that amplification of both GAPDH and DBH cDNA was in the exponential phase. Each amplification cycle consisted of 30 s of denaturation at 94°C, 30 s of primer annealing at 58°C followed by a 30-s extension at 72°C. GAPDH PCR primers were obtained from CLONTECH and mouse DBH primers were 5′-CTTGGAAGAGC-

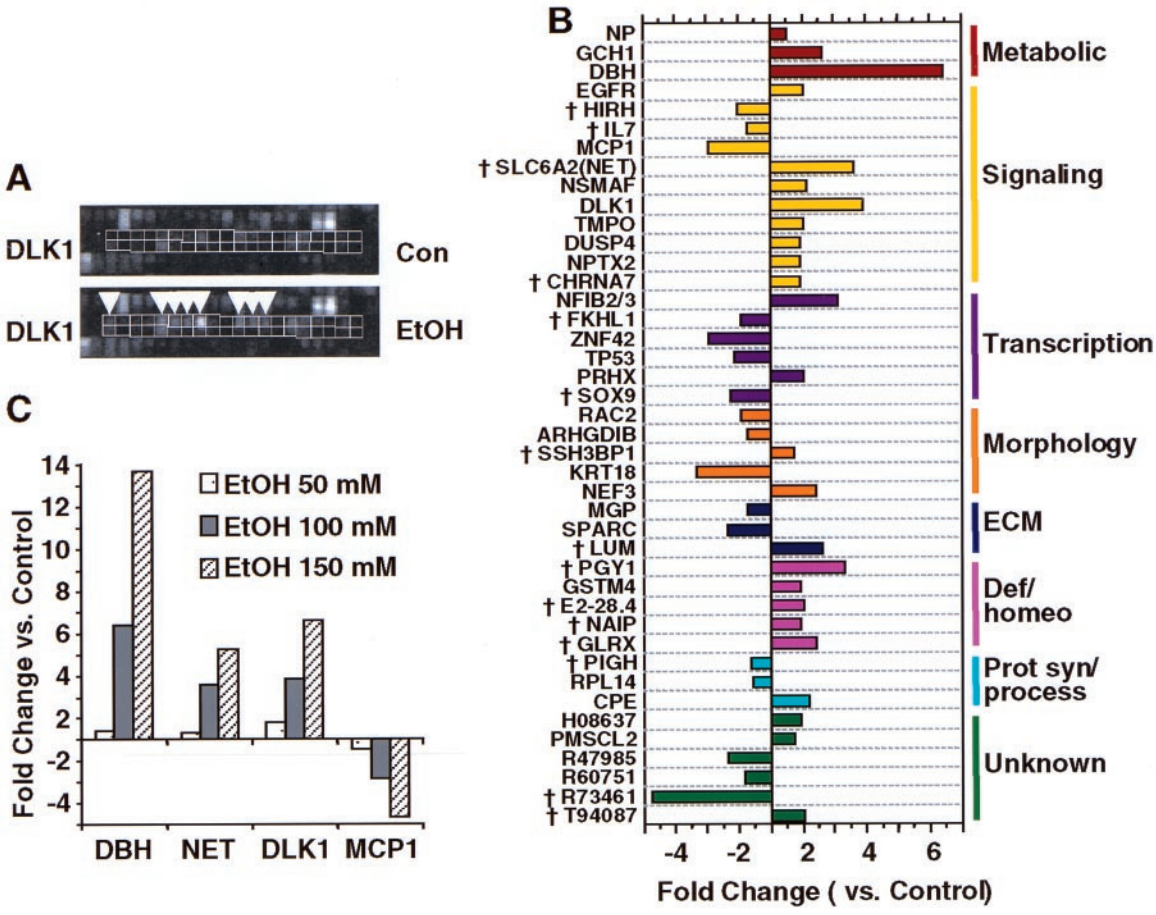

Fig. 1. Representative ethanol-induced changes in mRNA levels in SH-SY5Y cells detected by oligonucleotide array hybridization. A, representative oligonucleotide array hybridization patterns for δ-like protein (DLK1) mRNA. The 20 probe pairs corresponding to DLK1 are outlined by a grid pattern. The upper row in each case corresponds to perfect match (PM) oligonuceotides, which were designed to be complementary to the target sequence. The lower row of mismatched (MM) oligonucleotides are used for subtraction of nonspecific background and cross-hybridization signals. In the control, DLK1 mRNA was present at ~4 copies/cell, whereas in the ethanol-treated sample DLK1 mRNA was present at approximately 14 copies/cell. The set of oligonucleotide probes showing the most pronounced increases in hybridization with RNA from ethanol-treated cells (100 mM, 72 h) are indicated by arrows. The patterns of stronger and weaker hybridization signal across the probe sets are systematic and reproducible, and are the result of sequence-dependent differences in hybridization behavior. B, changes in mRNA abundance for all selected transcripts after 3 days of treatment with 100 mM ethanol. Results are representative of experiments performed twice and are expressed as a fold-change in normalized hybridization intensities between control and ethanol-treated samples. In some cases (†), fold-changes must be regarded as estimates due to low expression levels in either control or ethanol-treated samples. Transcripts are labeled according to gene symbols from UniGene (www.ncbi.nlm.gov/Unigene): ARHGDIB, rho GDP-dissociation inhibitor-2 (L20688); CHRNA7, nicotinic acetylcholine receptor, α7 subunit (X70297); CPE, carboxypeptidase E (X51405); DBH, dopamine β-hydroxylase (X13255); DLK1, δ-like protein (T49117); DUSP4, dual specific phosphatase (U21108); E2–28.4, similar to E2–28.4 ubiquitin-conjugating enzyme (R01227); EGFR, epidermal growth factor receptor (H02836); FKHL1, transcription factor BF1 (R60332); GCH1, GTP cyclohydrolase (U19523); GLRX, glutaredoxin (X76648); PIGH, phosphatidyl inositol glycan (L19783); GSTM4, glutathione *S*-transferase M4 (M99422); HIRH, intercrine-α (H14506); IL7, interleukin-7 (J04156); KRT18, keratin 18 (T53412); LUM, lumican (U21128); MCP1, monocyte chemotactic protein-1 (M26683); MGP, matrix Gla protein (H52207); NAIP, neuronal apoptosis inhibitory protein (U192521); NEF3, neurofilament-M (Y00067); NFIB2/3, nuclear factor I, B2/3 subunits (H91713); NP, purine nucleotide phosphorylase (T47964); NPTX2, neuronal pentraxin-2 (U29195); NSMAF, FAN protein (R41765); PGY1, P glycoprotein 1(M29447); PMSCL2, autoantigen PM-SCL (R40490); PRHX, proline-rich homeobox (X67235); RAC2, ρ-related small GTP-binding protein (H42477); RPL14, ribosomal protein LI4 (R82938); SLC6A2(NET), norepinephrine transporter (M65105); SOX9, sex-determining region Y-box 9 (Z46629); SPARC, secreted protein, acidic, cysteine-rich (T54767); SSH3BP1, spectrin SH3 domain binding protein 1 (R34245); TMPO, thymopoetin (U09086); TP53, p53 (X54156); ZNF42, zinc finger protein 42 (R83364). ESTs without homology to known genes are identified by GenBank accession numbers. Genes are grouped based on biological function: Metabolic, metabolic enzymes; Signaling, signaling molecules; Transcription, transcription factors; Morphology, cytoskeleton proteins or regulators of cytoskeleton organization; ECM, extracellular matrix glycoproteins; Def/Homeo, proteins involved in protection against oxidative stress or apoptosis; Pro syn/process, proteins involved in polypeptide synthesis or processing; or Unknown. C, oligonucleotide array analysis of ethanol concentration-response (72 h) for DBH, DLK1, NET, and MCP1 mRNA levels. Results are representative of experiments performed twice and are expressed as described above.

CATTTCAGTCGCTG-3′ and 5′-CATTTTGGAGTCACAGGGTCCG-TTG-3′.

Western Blot Analysis and ELISA. Western blot analysis of whole cell protein homogenates (100 μg) from SH-SY5Y cells was done as described previously (Thibault et al., 1997) using a sheep polyclonal antibody against human DBH protein (324383, Calbiochem-Novabiochem, La Jolla, CA). MCP1 peptide release was monitored in the culture media from control and ethanol-treated SH-SY5Y cells using a Quantikine MCP1 immunoassay as described by the manufacturer (R&D Systems, Minneapolis, MN).

Norepinephrine Detection by HPLC. SH-SY5Y cells (10^6 cells) were cultured in 10 mm^2 petri dishes according to conditions described above. Culture media from cells treated in the absence or presence of ethanol were analyzed for NE content by reverse-phase HPLC with electrochemical detection according to standard procedures (Gamache et al., 1993). For experiments with potassium-evoked NE release, culture media were removed after 72 h treatment +/− ethanol and replaced with media containing 50 mM KCl +/− ethanol. After a 15-min incubation, media (5 ml) were removed and processed for NE determination. All HPLC equipment was from ESA, Inc. (Chelmsford, MA). Following precipitation with 0.1 M perchloric acid and centrifugation over a 3000 mol. wt. cut-off centrifugal filter, a 10-μl aliquot of culture media was injected onto an ESA HR-80 column (C-18, 4.6 mm × 8 cm, 3 μm particle size). Eluents were detected using a model 5011 analytical cell with a palladium reference electrode, a model 5020 guard cell, and a model 5200A Coulochem II electrochemical detector. Electrode settings were +350 mV for the guard cell, −100 mV for the preoxidation electrode, and +280 mV for the detection electrode. Samples were analyzed at 5 nA sensitivity and compared with a two-point monoamine standard calibration curve at 1 and 5 pg/μl using the model 501 analysis software package. NE levels were corrected for total protein content of each culture dish.

Results

Identification of Ethanol-Regulated Genes in SH-SY5Y Cells. To monitor gene expression responses to chronic ethanol exposure, SH-SY5Y cells were treated for 72 h with 50, 100, or 150 mM ethanol. Gene expression profiles were generated for each sample (treated and control) by hybridization to oligonucleotide arrays containing probes for about 6000 human genes and expressed sequence tags (ESTs). We generated data from a total of 10 hybridizations for two independent experiments. The full dataset for these ethanol experiments can be found on our web site (htttp://www.egcrc.org/mmlab/SHSY_paper.htm). The total number of mRNA species detected as clearly present in any given experiment varied between 2900 and 3600 for both the control and ethanol-treated samples (not shown). Pair-wise comparisons between control and ethanol-treated samples were made within each experiment, giving a total of seven comparison files. Results were filtered according to multiple criteria as described under *Materials and Methods*. Under these strict criteria, 17 genes were (≥1.5-fold change in at least four comparisons) down-regulated and 25 genes were up-regulated in response to ethanol treatment. In contrast, analyses comparing replicate pairs of identically treated cultures selected only three genes total when filtered using the same criteria (not shown). Representative hybridization results for one ethanol-responsive gene, DLK1, are shown in Fig. 1A.

Representative results for all 42 genes after 72 h of treatment with 100 mM ethanol are shown in Fig. 1B. One gene, encoding the neuronal acetylcholine receptor α7 subunit (CHRNA7), was shown previously to be increased by ethanol in SH-SY5Y cells (Gorbounova et al., 1998). To our knowledge, all the other genes in this set are genes previously unidentified as ethanol-responsive in neuronal cells. These genes encode proteins of various functional classes including metabolic enzymes, transcription factors, or structural proteins. At the concentrations used here, ethanol did not induce many large changes in mRNA levels. However, our confidence in the observed changes was high because of their reproducibility. For example, 14 genes, including those encoding MCP1, DLK1, intermediate-sized filament cytokeratin 18 (KRT18), DBH, CHRNA7, matrix Gla protein, neuronal apoptosis inhibitory protein, and dual-specificity phosphatase-4 (DUSP4) differed consistently in expression levels between control and ethanol-treated samples in at least six of the seven independent pair-wise comparisons. The absolute value of the fold-change in a single experiment should be regarded as less informative than the fact that a gene consistently surpassed our selection threshold. Although the changes in expression levels for genes encoding NET and neurofilament M (NEF3) did not pass all our strict selection criteria, they were included in the list of ethanol-responsive genes. Changes in mRNA levels for these genes were confirmed on independent RNA preparations by Northern blot analysis (see below) or with RT-PCR (data not shown), suggesting that the filtering criteria for our array results were rather conservative.

Strikingly, ethanol treatment increased the mRNA levels of three genes involved in NE synthesis or re-uptake. These three genes encode DBH, the enzyme that catalyzes formation of NE from dopamine, NET, which is involved in NE re-uptake, and GTP cyclohydrolase (GCH1, EC 3.5.4.16), the rate limiting enzyme in biosynthesis of 3*R*-L-*erythro*-5,6,7,8-tetrahydrobiopterin, the essential cofactor for tyrosine hydroxylase (EC 1.14.16.2). Tyrosine hydroxylase catalyzes formation of dihydroxyphenylalanine from tyrosine, the initial step in catecholamine biosynthesis. Together, induction of DBH, NET, and GCH1 strongly suggest that ethanol modulates NE production in SH-SY5Y cells.

Ethanol treatment caused increased expression of glutathione *S*-transferase (GSTM4), glutaredoxin, and the multidrug resistance protein-1 (PGY1) (Fig. 1B), all of which are involved in glutathione metabolism or transport (Jedlitschky et al., 1994). Additionally, our initial low-stringency filtering indicated that ethanol increased mRNA levels of glutathione peroxidase and glutamate cysteinyl ligase (not shown). However, because of very low levels of expression, these genes were not included in our high-confidence list. Increases in expression of multiple genes affecting glutathione levels suggests that changes in oxidative stress occur with chronic ethanol exposure. Perhaps consistent with this, we also found that ethanol treatment caused increases in expression of neuronal apoptosis inhibitory protein and decreased expression of p53. Both of these changes in expression levels would be predicted to protect against apoptosis (Liston et al., 1996; Brown and Wouters, 1999).

Ethanol Regulation of DBH, NET, DLK1, and MCP1 Expression. The largest relative change in expression for any gene was observed for DBH. DBH mRNA levels increased in a dose-dependent manner in response to ethanol, with a 5- to 6-fold increase at 100 mM (Fig. 1C). NET mRNA levels showed a similar dose-responsive increase (Fig. 1C). Ethanol caused very consistent dose-responsive changes in

the expression of two other genes, DLK1 that codes for an epidermal growth factor-like protein suggested to have a role in adrenal differentiation (Lee et al., 1995) and MCP1 a chemokine that has a pivotal role in early stages of artherosclerosis (Boring et al., 1998). Because these four genes showed relatively large changes in expression and represent potentially important targets of ethanol regulation, they were chosen for further characterization.

Northern blot analyses confirmed that ethanol treatment increased DBH, DLK1, and NET mRNA levels and decreased MCP1 transcript levels (Fig. 2A). Ethanol also produced a dose-dependent decrease in the amount of secreted MCP1 peptide in SH-SY5Y cells (Fig. 2C) and in HUV-EC-C human endothelial cells (not shown). Similarly, increased DBH mRNA levels in ethanol-treated cells were correlated with increased amounts of DBH protein (Fig. 2B). We hypothesized that ethanol up-regulation of DBH would increase NE production. In SH-SY5Y cells we found that ethanol caused a dose-dependent increase in the cumulative spontaneous release of NE over a 24-h period (Fig. 3A). Furthermore, ethanol treatment increased NE release evoked by potassium depolarization over a 15-min period (Fig. 3B).

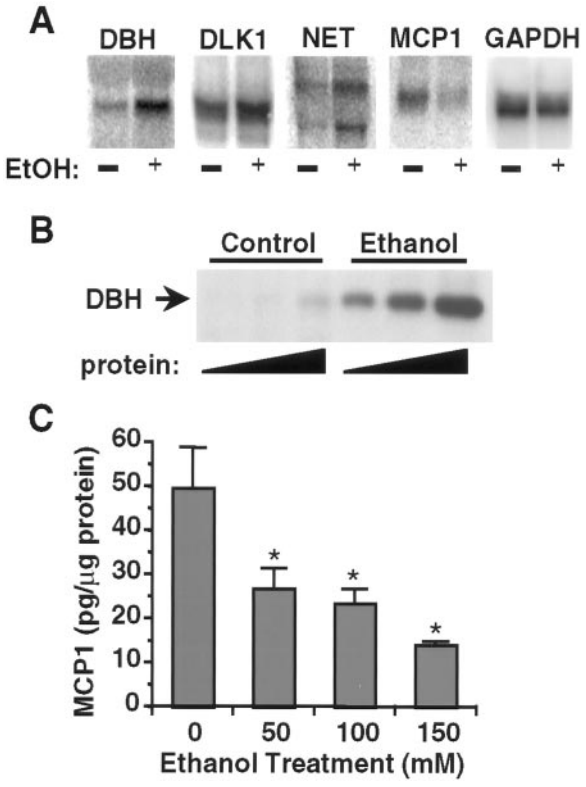

Fig. 2. Ethanol regulation of DBH, DLK1, NET, and MCP1 expression in SH-SY5Y cells. A, confirmation of ethanol-induced changes in mRNA levels by Northern blot analysis. Total RNA was isolated from cells treated with 150 mM ethanol for 3 days. Independent Northern blots hybridized with probes for DBH, DLK1, NET, and MCP1 are indicated. Equal loading of RNA was verified by rehybridization of blots with a GAPDH cDNA probe. A representative GAPDH hybridization of a blot previously probed with NET is shown. B, Western blot analysis of DBH protein levels. Whole cell extracts (75, 100, and 150 μg/lane) were prepared from cells treated for 3 days with 150 mM ethanol. C, ELISA analysis of MCP1 peptide release. Cells were treated for 3 days with increasing concentrations of ethanol as indicated. Culture media from the last 24-h incubation were collected and analyzed for MCP1 content. Results are expressed as picograms of peptide released per microgram of cellular protein and are the mean ± S.D. of determinations from four separate wells. Results are representative of experiments performed three times. *P = .01 versus control cells, ANOVA with Scheffé post hoc analysis.

To determine whether ethanol regulated DBH mRNA abundance in vivo, we studied DBH gene expression in adrenal glands of ethanol-treated DBA/2J mice. Since preliminary time course studies indicated that short-term ethanol exposure (<24 h) could increase DBH and DLK1 mRNA levels in SH-SY5Y cells (S. Rahman and M. F. Miles, manuscript in preparation), DBH transcript levels were monitored in mice by quantitative RT-PCR 24 h after injection of a single dose of 4 g/kg ethanol or saline. No difference in expression was observed in the brain at this time point (data not shown). However, a significant increase in DBH mRNA levels was detected in adrenal glands of ethanol-treated mice compared with control mice 24 h after injection (Fig. 4).

Coregulation of Ethanol-Responsive Gene Expression by Dibutyryl-cAMP. Several of the ethanol-responsive genes identified, including DBH, DLK1, and MCP1, are known to be regulated by analogs of cAMP in other model systems (Gaetano et al., 1992; Satriano et al., 1993; Kim et al., 1994). Since ethanol has several prominent actions on cAMP signaling (Diamond and Gordon, 1997), we determined how many ethanol-responsive genes were also regulated by db-cAMP in SH-SY5Y cells. As outlined under *Materials and Methods*, we used a different set of arrays for this experiment since the original Hu6000 arrays were no longer available. The newer arrays contained probes for 31 of the 42 ethanol-responsive genes identified on the Hu6000 arrays (Fig. 1B). Strikingly, 11 of these 31 genes were regulated in the same manner following db-cAMP treatment (Fig. 5) as seen with ethanol treatment (Fig. 1B). None of the other 20 ethanol-responsive genes on these arrays showed any response (≥1.5-fold change) to db-cAMP. Northern blot studies further confirmed that DBH, DLK1, and MCP1 were similarly regulated by both db-cAMP and ethanol in SH-SY5Y cells (not shown).

Discussion

These studies using high-density arrays provide a broad and quantitative characterization of the molecular responses to ethanol in neural cells. In a nonbiased fashion, we identified patterns of gene regulation that may allow a better understanding of overall phenotypic changes in cellular or neuronal function consequent to chronic ethanol exposure. Our array studies had four major findings: 1) DBH and other genes involved in norepinephrine metabolism are regulated by ethanol, implicating norepinephrine in ethanol-related behaviors; 2) MCP1 is down-regulated by ethanol, predicting a mechanism for at least a portion of the protective effects of ethanol in atherosclerotic vascular disease; 3) ethanol regulates genes involved in apoptosis and oxidative stress (glutathione metabolism), suggesting a prominent role for oxidative stress in the cellular toxicity of ethanol; and 4) cAMP signaling and ethanol coregulate a subset of genes, implicating a role for cAMP in a significant portion of ethanol's cellular actions.

The array studies described here showed only a relatively small number of marked changes in gene expression following ethanol exposure. The use of strict criteria with replicated experiments and the "pattern recognition" algorithms of the Affymetrix GeneChip software allowed detection of real but subtle changes in gene expression (Fig. 1B). How-

ever, it should be stressed that the set of 42 genes identified (Fig. 1B) probably represents a minimal gene list due to our conservative screening algorithm, designed to accept only the highest confidence changes.

The ethanol withdrawal syndrome seen in alcoholics is a profound clinical syndrome that causes significant morbidity and mortality. A major feature of the ethanol withdrawal syndrome is an extraordinary increase in circulating levels of epinephrine and norepinephrine (Sellers et al., 1976). Intriguingly, our most striking finding in these studies was that ethanol induced several genes involved in NE production. DBH, NET, and GCH1 were all induced by ethanol, with DBH being the largest responder of any gene monitored. Our studies documenting increased DBH protein (Fig. 2B) and releasable NE (Fig. 3) in SH-SY5Y cells and increased DBH mRNA in ethanol-treated mice (Fig. 4) strongly suggest that ethanol induction of DBH has physiological significance. Ethanol induced increases in DBH expression, as we document here in SH-SY5Y cells and mouse adrenal gland, might increase synthesis of catecholamines in the sympathetic nervous system or adrenal gland and thus contribute to the adrenergic discharge seen with alcohol withdrawal. Thus, the changes in gene expression induced by ethanol exposure could exacerbate physiological responses elicited by ethanol withdrawal.

Ethanol also produces beneficial effects on organ systems. Low levels of regular ethanol consumption are protective against atherosclerotic cardiovascular and cerebrovascular disease (Klatsky et al., 1990). Recent studies document that ethanol-induced decreases in atherogenesis are accompanied by decreased MCP1 expression in animal models of angioplasty-induced atherosclerosis (Merritt et al., 1997). MCP1 is a chemokine that appears to have a pivotal role in the early stages of atherogenesis. Apoliprotein E null mice lacking the CCR2 receptor for MCP1 show marked decreases in atherosclerosis (Boring et al., 1998). Our studies showed that ethanol treatment led to markedly decreased expression of MCP1 in SH-SY5Y cells (Figs. 1 and 2, A and C) or human HUV-EC-C endothelial cells (not shown). A recent report showing decreased MCP1 expression after red wine consumption suggested that antioxidant properties of the wine led to decreased MCP1 and decreased neointimal hyperplasia after vascular injury (Feng et al., 1999). Importantly, our studies suggest that ethanol itself regulates MCP1 expression. Defining the mechanism for ethanol regulation of MCP1 could generate novel insights for the treatment of atherosclerotic vascular disease.

Long-term ethanol use can cause end-organ damage to the liver, heart, immune system, skeletal muscle, and the peripheral and central nervous systems. Damage to the developing nervous system produces fetal alcohol syndrome (Becker et al., 1996). Although some of these actions may be secondary to ethanol metabolism or concurrent metabolic deficiency states suffered by alcoholics, direct ethanol toxicity is thought to play a role. Ethanol-induced oxidative stress has recently received notice as a possible mechanism underlying some forms of end-organ toxicity with ethanol. Ethanol exposure causes oxidative stress in hepatocytes (Higuchi et al., 1996), astrocytes (Montoliu et al., 1995), and peripheral nerves (Bosch-Morell et al., 1998). Metabolism of ethanol by alcohol dehydrogenase or the inducible microsomal ethanol-oxidizing system can produce oxidative stress (Bondy, 1992). Ethanol also decreases glutathione transport into mitochondria, a primary source of reactive oxygen species (Fernandez-Checa et al., 1997). Nonoxidative metabolism of ethanol to fatty acid ethyl esters can also increase production of reactive oxygen species (Bondy and Marwah, 1995). Our finding here that ethanol regulated several genes involved in glutathione

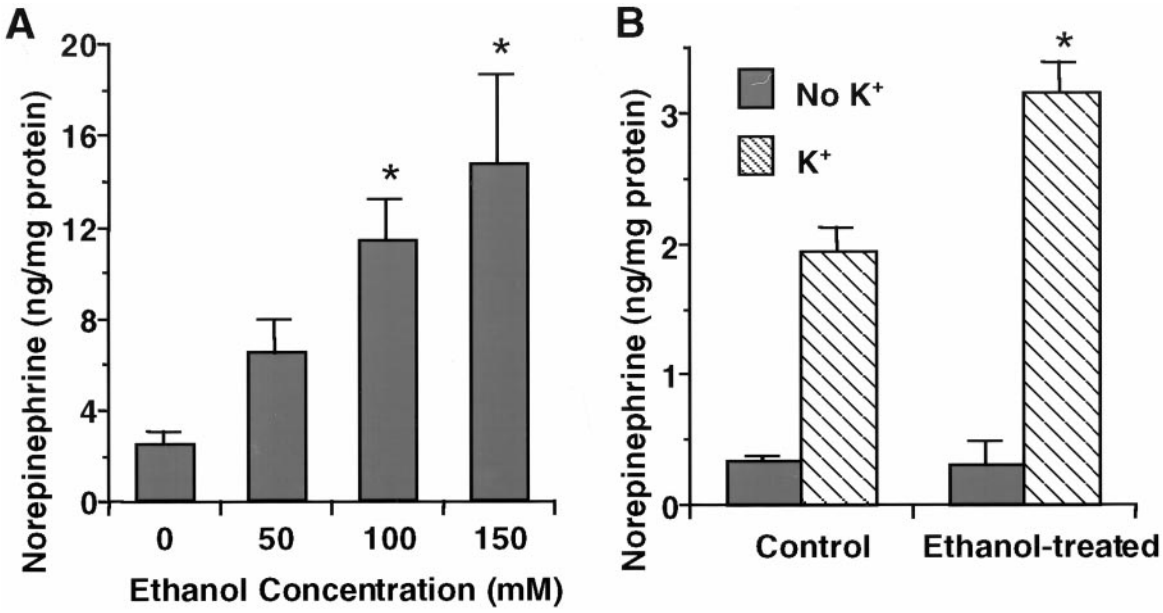

Fig. 3. Ethanol increases NE release from SH-SY5Y cells. A, SH-SY5Y cells were treated with ethanol for 48 h at the concentrations indicated. Spontaneous NE release was analyzed by HPLC as described under *Materials and Methods* and represents accumulation in culture media over a 24-h period. Results are the average ± S.D. of determinations from four separate wells. Data are expressed as nanograms of NE per milligram of cellular protein and are representative of experiments performed three times. *P = .001 versus control cells, ANOVA with Scheffé post hoc analysis. B, SH-SY5Y cells were grown for 72 h in the presence of 150 mM ethanol, and NE release was evoked by potassium stimulation as described under *Materials and Methods*. Culture media were collected after 15 min and processed for NE determinations. Data are expressed as above and are representative of experiments performed three times. *P = .001 versus control cells treated with potassium, ANOVA with Scheffé post hoc analysis.

metabolism and apoptosis is consistent with a compensatory response to ethanol-induced oxidative stress (Fig. 1). The nonbiased nature of expression profiling greatly strengthens existing arguments regarding oxidative stress as a mechanism for some ethanol-induced cellular toxicity. This action of ethanol could be important for end-organ toxicity in a variety of tissues, including the nervous system.

A large number of reports in animals and cultured cells have documented effects of acute or chronic ethanol on cAMP signaling (Diamond and Gordon, 1997). Furthermore, prior studies suggest a role for cAMP in ethanol intoxication in *Drosophila* (Moore et al., 1998) and preference drinking or acute behavioral sensitivity in mice (Thiele et al., 1998). We noted that expression of DBH and DLK1 can be induced and MCP1 repressed by agents that elevate cAMP levels (Gaetano et al., 1992; Satriano et al., 1993). Array hybridization studies showed that 11 of 31 ethanol-responsive genes responded in a similar fashion to db-cAMP treatment (Fig. 5). Our studies thus provide supportive evidence for the importance of cAMP in a subset of ethanol-regulated gene expression. Alternatively, ethanol and cAMP signaling may converge on a pathway that regulates a common set of genes (see Fig. 5). The lack of db-cAMP regulation for a number of ethanol-responsive genes (Fig. 1B versus Fig. 5) indicates that ethanol also regulates gene expression through mechanisms that are not in common with db-cAMP.

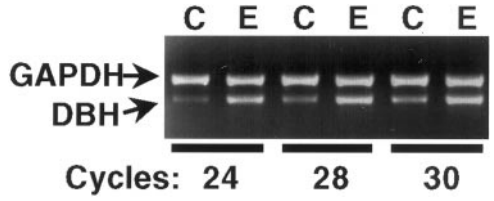

Fig. 4. Ethanol increases DBH mRNA levels in adrenal glands of DBA/2J mice. Mice were sacrificed 24 h after a single i.p. injection of saline (C) or 4 g/kg ethanol (E). DBH and GAPDH mRNA levels in adrenal glands were quantitated by duplex RT-PCR. Ethidium bromide-stained PCR products from 24, 28, or 30 amplification cycles are displayed. Results are representative of experiments performed four times. Similar results were obtained by Northern blot analysis (data not shown).

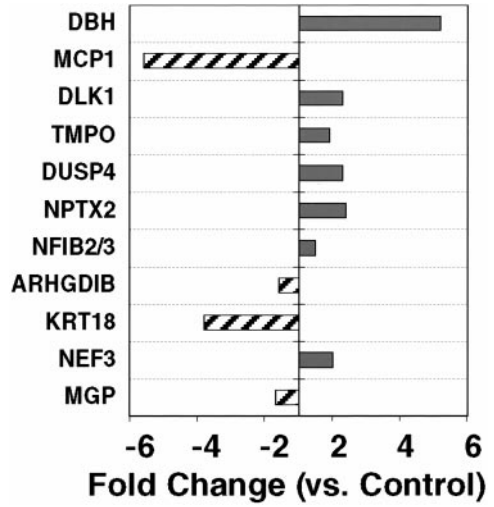

Fig. 5. A subset of ethanol-responsive genes responds similarly to db-cAMP treatment. Genes identified as ethanol-responsive in SH-SY5Y cells (Fig. 1B) were screened for their response to db-cAMP (72 h, 1 mM) by analysis with oligonucleotide arrays. Genes having at least a 1.5-fold change in the same direction as seen with ethanol are shown.

The studies described here have identified individual genes, metabolic pathways, and possible signaling components affected by chronic ethanol treatment in cultured neuronal cells. Our results may have important implications for understanding cellular or behavioral responses to chronic ethanol exposure as well as the molecular mechanism underlying such events. Array studies such as these may help identify candidate genes for investigating the genetics of alcoholism or ethanol-related behaviors. Furthermore, the pattern of ethanol-regulated genes identified in these studies might provide useful surrogate markers for evaluating responses to ethanol or potential therapeutic agents for alcoholism.

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