Neurobiology of Disease

Ethanol-Responsive Brain Region Expression Networks: Implications for Behavioral Responses to Acute Ethanol in DBA/2J versus C57BL/6J Mice

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Activation of the mesolimbic dopamine reward pathway by acute ethanol produces reinforcement and changes in gene expression that appear to be crucial to the molecular basis for adaptive behaviors and addiction. The inbred mouse strains DBA/2J and C57BL/6J exhibit contrasting acute behavioral responses to ethanol. We used oligonucleotide microarrays and bioinformatics methods to characterize patterns of gene expression in three brain regions of the mesolimbic reward pathway of these strains. Expression profiling included examination of both differences in gene expression 4 h after saline injection or acute ethanol (2 g/kg). Using a rigorous stepwise method for microarray analysis, we identified 788 genes differentially expressed in control DBA/2J versus C57BL/6J mice and 307 ethanol-regulated genes in the nucleus accumbens, prefrontal cortex, and ventral tegmental area. There were strikingly divergent patterns of ethanol-responsive gene expression in the two strains. Ethanol-responsive genes also showed clustering at discrete chromosomal regions, suggesting local chromatin effects in regulation. Ethanol-regulated genes were generally related to neuroplasticity, but regulation of discrete functional groups and pathways was brain region specific: glucocorticoid signaling, neurogenesis, and myelination in the prefrontal cortex; neuropeptide signaling and developmental genes, including factor *Bdnf*, in the nucleus accumbens; and retinoic acid signaling in the ventral tegmental area. Bioinformatics analysis identified several potential candidate genes for quantitative trait loci linked to ethanol behaviors, further supporting a role for expression profiling in identifying genes for complex traits. Brain region-specific changes in signaling and neuronal plasticity may be critical components in development of lasting ethanol behavioral phenotypes such as dependence, sensitization, and craving.

Key words: **ethanol; neurobiology; microarray; behavior; bioinformatics; mouse genetics**

Introduction

Acute behavioral responses to ethanol have predictive value regarding risk for long-term ethanol drinking behavior in humans (Schuckit, 1994) and animal models (Metten et al., 1998). Acute ethanol-mediated brain signaling events may have a role in the genesis of long-lasting behaviors such as dependence, sensitization, and craving. Drug-induced changes in gene expression are proposed as critical molecular adaptations leading to addiction with repeated drug exposure (Nestler and Aghajanian, 1997). Characterization of acute ethanol effects on brain gene expression could thus provide insight into mechanisms of rewarding properties or other acute behavioral responses to ethanol, as well as the neurobiology of long-term behaviors such as addiction.

Rodent model systems differing in ethanol-related phenotypes have been used extensively to study the behavioral genetics of ethanol action (Crabbe et al., 1999). The inbred mouse strains DBA/2J (D2) and C57BL/6 (B6) differ markedly and inversely in a number of ethanol behaviors. D2 mice show larger locomotor responses to acute ethanol and drink less than B6 mice (Phillips et al., 1994; Metten et al., 1998). We hypothesize that differences in either specific basal gene expression or acute ethanol-evoked changes in gene expression could be important determinants of divergent behavioral responses to ethanol between D2 and B6 mice.

Expression profiling with DNA microarrays has been used to identify functionally relevant patterns of brain gene expression in complex behavioral traits (Mirnics et al., 2000). Microarrays have been used to study ethanol-responsive genes in neural cell cul-

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tures (Thibault et al., 2000) and genes differentially expressed in the prefrontal cortex and amygdala in response to chronic ethanol exposure in rats (Rimondini et al., 2002) and the prefrontal or motor cortex of human alcoholics (Lewohl et al., 2000). Expression profiling has also documented strain-specific differential expression in the D2 and B6 hippocampus during acute and chronic ethanol withdrawal (Daniels and Buck, 2002) or the whole brain after an anesthetic dose of ethanol (Treadwell and Singh, 2004). This latter study hinted at strain-specific differences in response to acute ethanol but did not actually report these. Xu et al. (2001) also reported on expression profiling comparison of untreated ILS and ISS mice, strains originally selected for differences in ethanol-induced sleep time.

The nucleus accumbens (NAC), prefrontal cortex (PFC), and ventral tegmental area (VTA) are major components of the mesolimbocortical dopamine pathway activated by acute ethanol or other drugs of abuse (Koob, 1992). To date, no comprehensive study of gene expression patterns across the mesolimbocortical system has been done to compare B6 and D2 mouse responses to acute ethanol. We therefore used oligonucleotide microarrays and bioinformatic tools to characterize expression networks in the NAC, VTA, and PFC of control or ethanol-treated D2 and B6 mice. Our results suggest several discrete mechanisms that may contribute to differences in ethanol behaviors between B6 and D2 mice.

Materials and Methods

Administration of acute ethanol and animal microdissection. All animals were treated according to protocols for animal care established by Virginia Commonwealth University and the National Institutes of Health. Adult male B6 or D2 mice (79–95 d old; The Jackson Laboratory, Bar Harbor, ME) were housed five to a cage with *ad libitum* access to standard rodent chow (catalog #7912; Harlan Teklad, Madison, WI) and water in a 12 h dark/light cycle. All injections were intraperitoneal. The Institutional Animal Care and Use Committees of Virginia Commonwealth University, the University of California, San Francisco, and the Ernest Gallo Clinic and Research Center approved all experimental procedures.

Mice were given a saline injection once daily for 5 d to habituate them to the injection process. On day 6, mice received either an injection of saline or 20% ethanol in saline at doses mentioned in the text. Experiments were performed in triplicate with five mice per treatment group, except for an ethanol dose–response study (see Fig. 5) done with duplicate ethanol or saline treatment groups of four mice per treatment. After 4 h, or as otherwise mentioned in Results, animals were killed by cervical dislocation. Mouse brains were extracted and chilled for 1 min in phosphate buffer on ice before microdissection. Dissections were completed 5–10 min from the time of death. The mouse brain micropunch dissection method training, dissecting block, and tools were provided by V. Gene Erwin (University of Colorado, Boulder, CO). Briefly, with the ventral surface of the brain up, a vertical slice was made just rostral to the optic chiasm. The rostral section was placed with the caudal face up, and the PFC was isolated by slicing a pie-shaped wedge overlapping the interhemispheric fissure from the dorsal cortical surface extending to the corpus callosum. The NAC was dissected with a 1.5 mm micropunch centered over each anterior commissure. The VTA was isolated from the remaining caudal brain section. After removal of the hypothalamus with tweezers, the caudal portion of the brain was placed dorsal side-up. A vertical slice was made rostral to the cerebellum, between the superior and inferior colliculi, and, after discarding the cerebellum, a slice was made at a 45° angle from the dorsal caudal end down toward the ventral rostral end. The cortical regions of the ventral portion were peeled away, and a transverse cut was made to separate the VTA from the dorsal midbrain. Brain regions were placed in individual tubes, frozen immediately with liquid nitrogen, and stored at −80°C until isolation of total RNA.

Tissue pooled from four or five animals was homogenized in STAT-60 reagent (Tel-Test, Friendswood, TX) using a Tekmar homogenizer, and total RNA was isolated according to the STAT-60 protocol. RNA concentration was determined by absorbance at 260 nm, and RNA quality was analyzed by agarose gel electrophoresis and 260:280 nm absorbance ratios. Total RNA (7 μg) derived from each pool was reverse transcribed into double-stranded cDNA using the Superscript II system (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA was synthesized from cDNA using a BioArray high-yield RNA-transcript labeling kit (ENZO Diagnostics, Farmingdale, NY) according to the instructions of the manufacturer, purified using an RNAeasy Mini kit (Qiagen, Mountain View, CA), and quantified by absorbance at 260 nm.

Microarray hybridization and scanning. Each treatment group or replicate was hybridized to an individual microarray for each of the three brain regions studied ($n = 36$ total microarrays for experiment 1). Labeled cRNA samples were analyzed on oligonucleotide microarrays (Murine GeneChip U74Av2; Affymetrix, Santa Clara, CA) that contain >12,000 named genes and expressed sequence tags. Array hybridization and scanning were performed exactly according to the protocol of the manufacturer and as described previously (Thibault et al., 2000). Arrays were then washed, stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR), and scanned according to standard protocols supplied by the manufacturer (Affymetrix).

Microarray data analysis. Microarray data were initially processed using Microarray Suite software (MAS; Affymetrix) version 4.0 or 5.0. Arrays were normalized to a median total hybridization intensity (target average intensity, 190). Array quality was assessed by accepting only arrays with a scaling factor of <2.3 and a 3′-5′-actin ratio of <2 and by examining chip validity and linearity of intensity values, according to MAS guidelines. Arrays determined to be acceptable were further analyzed in three steps to identify genes with altered expression patterns. First, the S-score algorithm, developed in this laboratory for analysis of Affymetrix oligonucleotide arrays (Zhang et al., 2002), was applied to compare hybridization signals between two arrays from different treatment samples. S-score results are independent of the initial analysis algorithm used (MAS 4.0 or MAS 5.0), have a normal distribution with mean of 0 and SD of 1, and are correlated with the fold change. An S-score of 2 corresponds to a $p = 0.0455$, uncorrected for biological variability or multiple comparisons. Three types of comparisons were made: (1) to study ethanol responses, S-scores were calculated for ethanol-treated samples versus saline control samples within each brain region and mouse strain; (2) to examine strain expression differences between D2 and B6 mice, S-scores were calculated for B6 saline control samples versus D2 saline control samples; and (3) control S-scores were calculated between biological replicates of the same saline control groups. S-scores were calculated within replicates.

To reduce the contribution of biological or technical noise, S-scores were divided by the greater of 1 or the SD of control S-scores within brain regions. We found that this general approach, which has been applied previously to microarray data (Hughes et al., 2000), reduces variance across experimental replicates, although it does result in a more conservative estimate of expression changes. Genes with consistently low expression values were filtered to eliminate genes with MAS 4.0 average difference values of <50 in all samples. Statistical analysis of microarrays (SAM), a rank-based permutation method, was used to identify genes with S-scores significantly different from 0 (Tusher et al., 2001). This gene list was used for subsequent multivariate or bioinformatic analyses. Ethanol-regulated genes were identified by performing two-class SAM on ethanol versus saline S-scores (e.g., D2 NAC E1 vs D2 NAC S1) and saline versus saline S-scores (e.g., D2 NAC S1 vs D2 NAC S2). Genes differentially expressed (basal) between mice strains were identified by one- and multi-class SAM on B6 saline versus D2 saline S-scores (e.g., B6 PFC S1 vs D2 PFC S1). This basal gene list was further filtered for an average S-score of ≤1.5 or −1.5 or less (composite significance, $p <$ 0.01). All SAM analyses used a false discovery rate of ≤10% to avoid eliminating genes that may be biologically important and could assist in interpretation of expression patterns in multivariate studies. In virtually all cases, the S-scores for samples with altered expression had a composite significance of $p < 0.01$ (uncorrected for multiple testing). Qualitatively similar results were obtained with multiple other analysis methods as

input for the SAM analysis, including PDNN (position-dependent nearest neighbor) (Zhang et al., 2003). Genes that showed both significant and reproducible changes were further analyzed for correlated gene expression patterns by application of k-means clustering as described by Eisen et al. (1998). The number of nodes for k-means clustering was estimated by principle component analysis and visually adjusted to remove repetitive nodes.

Our experimental design of including only three biological replicates likely limited detection of more subtle expression changes, particularly of low-abundance genes. However, we found that the S-score analysis method is particularly useful for studies having limited numbers of Affymetrix microarrays because the method uses the statistical power of all oligonucleotide pairs for a given gene (Zhang et al., 2002). Our inclusion of a method for decreasing the contribution of technical or biological noise increases the yield of statistical filtering measures such as SAM by decreasing the overall variance of the data (data not shown). Finally, we used a somewhat liberal false discovery rate in the SAM analysis to increase our ability to populate functional networks of genes in subsequent bioinformatic studies. Such measures are unlikely to contribute misleading results because the bioinformatic studies will only detect genes with coherence in their biological function.

Bioinformatics analysis of microarray data. The Expression Analysis Systematic Explorer (EASE version 1.21) (Hosack et al., 2003) nonbiased annotation analysis tool was used to identify biological themes among gene expression profiles and to group genes into functional classifications developed by several public databases. The following annotation groupings were analyzed for overrepresentation in gene lists: chromosome, SwissProt key word, PIR (The Protein Information Resource) key word, GenMAPP (Gene Map Annotator and Pathway Profiler) pathway, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway, Pfam (Protein families database of alignments and HMMs) domain, SMART (Simple Modular Architecture Research Tool) domain, Gene Ontology Consortium Biological Process, molecular function, and cellular component. EASE results were filtered to remove categories with EASE scores of >0.05. Gene Ontology Consortium (2001) categories with >250 members on the U74Av2 array were considered too broad and were removed. Redundant categories with the same gene members were removed to yield a single representative category.

Additional bioinformatics analysis of genes showing altered basal expression or regulation by ethanol was done by identifying the chromosomal location of all such genes and superimposing this on support intervals for various behavioral quantitative trait loci (QTL) data as noted in Results. Chromosome locations for genes probed by Affymetrix U74Av2 arrays were obtained through the University of California, Santa Cruz, Mouse Genome Database (http://www.genome.ucsc.edu). Genes were also probed for linkage of basal expression to defined chromosome locations through the WebQTL resource (http://www.webqtl.org). The WebQTL resource includes MG_U74Av2 expression data of forebrain samples from >30 B6 $\times$ D2 recombinant inbred (BXD RI) lines and can be used to link gene expression to genetic markers and correlate with behavioral QTL data contained in the same database (Chesler et al., 2003). Expression QTLs at a position coinciding with that of the gene itself are referred to as "*cis*-QTLs," whereas those occurring at remote locations are "*trans*-QTLs." The latter imply that expression for a given gene is controlled by a specific *trans*-chromosomal region (e.g., where a transcription factor is located). The WebQTL scriptable interface was used to retrieve single-marker mapping data for lists of ethanol-responsive genes and genes with differences in basal expression between strains. The genetic marker with the highest likelihood ratio statistic (LRS) was selected for each gene. Genes with LRSs of ≥ 9.2 [logarithm of odds (LOD) ≥ 2] were used for additional analysis.

Functional or regulatory relationships between genes from k-means clusters were further studied using several biomedical literature association tools. Accession numbers or Affymetrix probe set identification numbers were used with the BiblioSphere (Genomatix Software, Munich, Germany) or Chilibot (www.chilibot.net) (Chen and Sharp, 2004) programs for discovery of biomedical literature interrelations, conserved promoter motifs, or association with ethanol in the biomedical literature.

Quantitative real-time reverse transcription-PCR. Total RNA isolated for microarray hybridizations was used for reverse transcription-PCR. cDNA was generated from 1 μg of total RNA by reverse transcription with an iScript cDNA kit (Bio-Rad, Hercules, CA) according to the instructions of the manufacturer. Briefly, total RNA was incubated with oligo-dT, random hexamer primers, and reverse transcriptase at 55°C for 5 min, 42°C for 30 min, and 85°C for 5 min. cDNA was used either immediately or stored at −20°C. Biological triplicate pools of RNA (see above) were used to make three cDNA preparations for each treatment group. Quantitative real-time PCR (Q-rtPCR) was performed using the iCycler iQ system (Bio-Rad) according to the instructions of the manufacturer for SYBR Green I (Molecular Probes)-based detection. Technical replicate Q-rtPCR reactions ($n = 3$) were done for each cDNA preparation. Technical replicates were averaged before statistical analysis. Background fluorescence was normalized by addition of fluorescein. All primers were designed to minimize secondary structure formation and cross intron–exon boundaries (when available) to reduce amplification of genomic DNA. After Q-rtPCR, all reactions were checked by gel electrophoresis to ensure that they produced a single PCR product of the appropriate size. Results were normalized to 18S ribosomal RNA. GenBank accession numbers, forward (F) and reverse (R) primers, and annealing temperatures were used for the following genes: brain-derived neurotrophic growth factor (*Bdnf*), accession number X55573: F, 5′-AGC CTC CTC TAC TCT TTC TGC TG-3′; R, 5′-GTG CCT TTT GTC TAT GCC CCT G-3′; FK506-binding protein 5 (*Fkbp5*), accession number U16959: F, 5′-TTT CAG TGT CCA GCA CAG ATC-3′; R, 5′-CTA AGC AAA TCT CAT CAC CCG C-3′; proteolipid protein (*Plp*), accession number M14674: F, 5′-GGG TTA CAG AGG CCA ACA TCA AG-3′; R, 5′-TGA CAG GTG GTC CAG GTA TTG AAG-3′; tyrosine hydroxylase (*Th*), accession number M69200: F, 5′-ACA GTA CAT CCG TCA TGC CTC C-3′; R, 5′-AGC CCA AAC TCC ACA GTG AAC C-3′; and 18S rRNA, accession number X00686: F, 5′-TAA AGG AAT TGA CGG AAG GGC AC-3′; R, 5′-CCA GAC AAA TCG CTC CAC CAA C-3′.

Western blot analysis. D2 mice ($n = 3$ per treatment group) were treated with saline or ethanol (2 g/kg) for 6 h before harvesting brain tissue as above. The D2 PFC from individual mice was sonicated in 1% SDS, and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Samples (5, 10, and 20 μg protein content) were loaded into 10% Tris-HCl polyacrylamide precast gels (GeneMate; ISC BioExpress, Kaysville, UT), separated by gel electrophoresis at 100 V for 1 h in 25 mM Tris, 192 mM glycine, and 0.1% SDS, and transferred to a polyvinylidene difluoride membrane (Bio-Rad) at 30 mA overnight at 4°C. All remaining steps were performed at room temperature. After transfer, the membrane was incubated in 10% bovine serum albumin in Tris-buffered saline and 0.1% Tween 20 (BSA/TBSt). The membrane was probed with rabbit anti-mouse Fkbp5 IgG (PA1-020; Affinity Bioreagents, Golden, CO), diluted 1:15,000 in 1% BSA/TBSt for 2 h, washed three times for 15 min with TBSt, probed with HRP-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) in 1% BSA/TBSt for 1 h, and finally washed three times for 10 min with TBSt. Membranes were incubated with ECL reagent (Amersham Biosciences, Piscataway, NJ) according to the instructions of the manufacturer and visualized with an Eastman Kodak (Rochester, NY) EDAS 290 camera and software. To further ensure reproducible protein loading, membranes were stripped by washing three times for 10 min in 20 mM Tris, 50 mM DTT, and 10% SDS at 60°C and reprobed with mouse anti-mouse actin IgM (CP-01; Oncogene Sciences, San Diego, CA) and goat anti-mouse HRP-conjugated IgM (Calbiochem, La Jolla, CA) as above.

Results

Differential gene expression in B6 and D2 control mice

Differences in control expression of specific genes could contribute to divergent ethanol behavioral phenotypes in B6 and D2 mice. We detected 788 genes with differential expression in saline-treated D2 and B6 NAC, PFC, and VTA, with a large number differentially expressed in more than a single brain region (Fig. 1*A*). (A list of all genes is available at http://www.brainchip.vcu.edu/kerns_apptable1.pdf.) The number of genes differentially expressed was distributed unevenly with PFC > NAC > VTA. Mul-

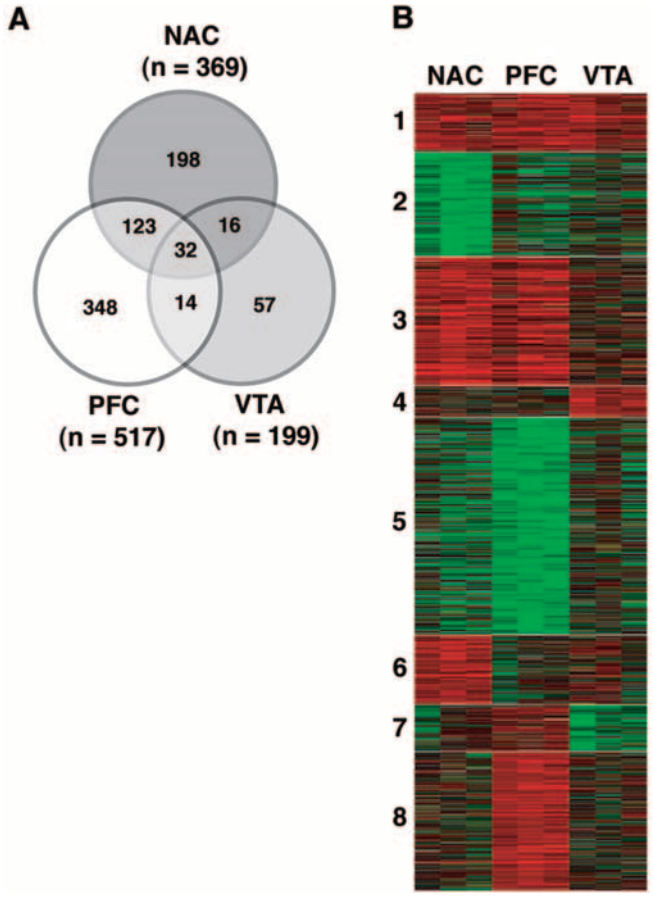

Figure 1. Genes differentially expressed in control B6 versus D2 mice. ***A***, Venn diagram of overlapping and nonoverlapping genes differentially expressed in saline-treated D2 and B6 NAC, PFC, and VTA. Region-specific expression patterns are represented as shaded circles (PFC, open; NAC, light; VTA, dark). Numbers in parentheses refer to the total number of genes differentially expressed in D2 and B6 mice in that brain region. ***B***, k-Means clustering analysis of genes differentially expressed in control D2 and B6 NAC, PFC, and VTA. S-scores of B6 versus D2 saline were processed and clustered as described in Materials and Methods. The tree view image displays genes more highly expressed in B6 mice (red), more highly expressed in D2 mice (green), or with no difference between the strains (black).

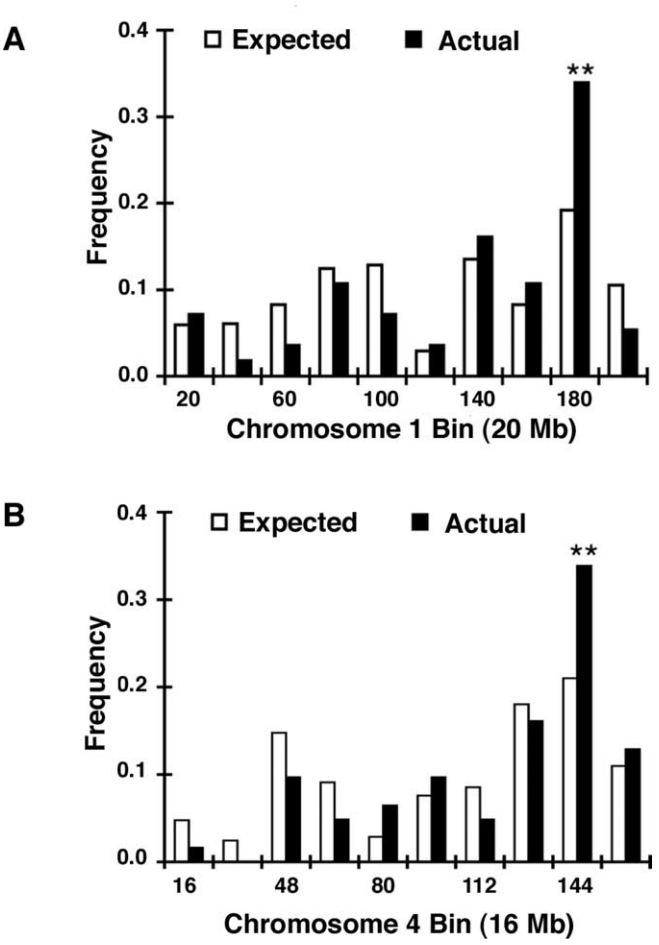

Figure 2. Distribution of genes differentially expressed in control D2 versus B6 mice on chromosomes 1 and 4. Histograms display the relative distribution frequency of genes on chromosomes 1 (***A***) and 4 (***B***). All genes contained on the U74Av2 microarrays (open boxes) or those differentially expressed between B6 and D2 in the NAC, PFC, or VTA (filled boxes) are shown. U74Av2 arrays contained 724 probe sets for genes on Chr 1 and 738 probe sets on Chr 4. A total of 56 genes on Chr 1 and 62 genes on Chr 4 were differentially expressed in control B6 versus D2 mice. Bin size is 20 Mb for Chr 1 and 16 Mb for Chr 4. The locations of bins with a larger than expected number of genes differentially expressed are indicated (**$p < 0.01$; χ^2 analysis).

tivariate analysis (k-means clustering) (Fig. 1*B*) confirmed that more genes were differentially expressed in the PFC than other brain regions, and most genes were differentially expressed in more than one brain region of a strain (Fig. 1*B*, clusters 1, 3).

Chromosomal locations of genes differentially expressed between B6 and D2 mice were aligned with regions containing ethanol behavioral QTLs to detect potential candidate genes. Surprisingly, many genes were tightly grouped in two areas on chromosomes (Chr) 1 and 4 (Chr 1, 170–190 Mb; Chr 4, 130–150 Mb) (Fig. 2). These groupings exceeded that expected by gene density (see legend to Fig. 2). The large group of genes located at the distal end of Chr 1 (Fig. 2*A*) is in the same region that has been linked to behavioral QTLs for ethanol traits, including acute locomotor activation, acute withdrawal, and preference drinking (see supplemental Table 3, available at www.jneurosci.org as supplemental material).

Identification of genes regulated by acute ethanol

Acute behavioral responses to ethanol might differ between B6 and D2 mice because of differences in signaling events evoked by acute ethanol exposure. Differences in acute ethanol signaling could also affect long-term behavioral responses such as drinking behavior. Using gene expression as a surrogate measure for signal transduction, we therefore performed microarray studies on brain regions from B6 or D2 mice exposed to acute ethanol. We used an activating dose (2 g/kg) of intraperitoneal ethanol for these studies because this protocol has been widely used to document differences in acute behavioral responses to ethanol in B6 and D2 mice (Phillips et al., 1995). Preliminary time course studies showed that a 4 h time point captured a spectrum of early, intermediate, and late gene expression responses to ethanol (data not shown).

Microarray analysis identified 307 genes upregulated or downregulated by ethanol in brain regions of either B6 or D2 mice. (A list of all genes is available at http://www.brainchip.vcu.edu/kerns_apptable2.pdf.) For the purposes of summarizing these changes, ethanol-regulated genes identified by SAM were filtered to count genes with an average S-score over three observations of ≥ 1.5 or -1.5 or less. Figure 3 shows that more genes were regulated by acute ethanol in D2 than B6 mice, and responses to ethanol occurred in NAC $\approx$ PFC $\gg$ VTA. Additionally, in D2 NAC and PFC, strikingly more genes were upregulated than downregulated by ethanol, whereas for B6 NAC and PFC, genes downregulated by ethanol were more common.

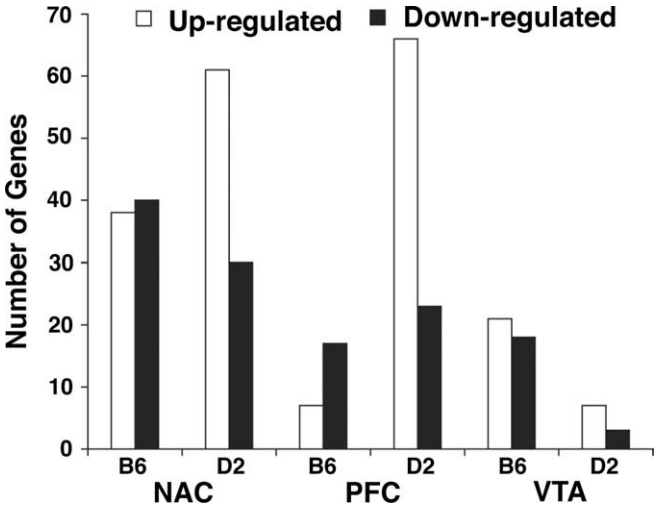

Figure 3. Number of ethanol-regulated genes by mouse strain and brain region. Ethanol-regulated genes identified by SAM were filtered to summarize the number of ethanol-regulated genes in specific brain regions. Genes were included if the mean S-score across three replicate experiments was ≥1.5 or −1.5 or less.

Multivariate analysis (k-means clustering) detected groups of ethanol-regulated genes in D2 and B6 NAC, PFC, and VTA with common expression patterns (Fig. 4). Control expression S-scores were included in this multivariate analysis to identify ethanol-responsive genes with differential expression between B6 and D2 mice. In addition to general trends noted above, Figure 4 displays several features of the ethanol responses: (1) except for cluster 6, which was strongly induced by ethanol in B6 NAC and mildly decreased in B6 PFC, most ethanol-responsive genes were regulated in only one of the three brain regions analyzed, VTA (clusters 1, 5), NAC (clusters 3, 7, 8, 11), or PFC (clusters 2, 9, 10); (2) a considerable number of genes were regulated by ethanol in opposite directions in D2 and B6 mice (see clusters 1, 5, 6); (3) most clusters showed both altered control expression and differential responses to ethanol between B6 and D2 (see clusters 1, 5, 9); and (4) relatively few ethanol-responsive genes were similarly regulated by ethanol in both D2 and B6 mice (cluster 11).

Bioinformatic analysis of microarray expression patterns

To identify potential biological phenotypes associated with gene expression patterns contained in Figures 1*B* and 4, we used a multilayered strategy of bioinformatics analysis as described in Materials and Methods. All 788 genes with differential expression in control D2 and B6 mice from Figure 1 were analyzed by EASE for overrepresentation of functional categories compared with all genes present on the U74Av2 array (supplemental Table 1, available at www.jneurosci.org as supplemental material). Major significant groups included genes functioning in glutamate or carboxylic acid metabolism as well as several categories related to protein synthesis (translation, chaperones, and peptidases).

Biological function categories of ethanol-responsive expression patterns (supplemental Table 2, available at www.jneurosci.org as supplemental material) contained a number of categories relevant to neuronal function, development, and synaptic plasticity. This was more evident when genes from related brain region-specific clusters were analyzed. The VTA-related clusters (1, 5) were remarkable for overrepresentation of genes related to retinoic acid signaling. NAC clusters (3, 6–8, 11) showed a complex group of functions, including categories related to neuronal transmission (ion channels and synaptic proteins), neuropeptides, and signal transduction. The PFC had an overrepresentation of genes related to myelin and neurogenesis. High-throughput literature association analysis of brain region-selective clusters revealed more striking functional relationships between ethanol-responsive clusters from Figure 4.

Ventral tegmental area

Clusters 1 and 5 showed both altered control expression and divergent ethanol responsiveness in the VTA of D2 and B6 mice. These genes included a POU (Pit1-Oct1/2-Unc86) domain transcription factor (*Pou5fl* or *Oct-3/4*) and the homeobox protein gastrulation homeobox 2 (*Gbx2*), both of which function in differentiation of the hindbrain–midbrain boundary (Reim and Brand, 2002) (Fig. 4, cluster 1). Additionally, ethanol modulated expression of retinol-binding protein 4 (*Rbp4*), which functions in retinoic acid signaling. Retinoic acid signaling is important in differentiation and function of dopaminergic neuron pathways (Samad et al., 1997) and has been suggested as a mechanism underlying ethanol effects on the developing nervous system (Deltour et al., 1996). Retinoic acid signaling is known to regulate *Gbx2*, *Pou5fl*, Reticulon 1 (*Rtn1*), and *Rbp4*, among many other target genes. *Rtn1* (Fig. 4, cluster 5) is differentially regulated by ethanol withdrawal in the cerebellum of D2 versus B6 mice (Schafer et al., 2001). We showed previously that the human homolog of *Rtn1* is upregulated in prefrontal cortex tissue from alcoholics (Lewohl et al., 2000).

Nucleus accumbens

This brain region showed complex ethanol-responsive patterns with multiple clusters of genes (Fig. 4, clusters 3, 7, 6, 8, 11). Clusters 3 and 7 had opposing responses to ethanol in the NAC of D2 mice but responded minimally in B6 mice. EASE and direct literature network analysis identified multiple neuropeptide-signaling genes regulated by ethanol in the NAC, including cholecystikinin (*Cck1*), pituitary adenylate cyclase-activating polypetide 1 (*Adcyap1/Pacap*), preproenkephalin 1 (*Penk1*), tachykinin 1 (*Tac1*), and *Tac2*. All of these neuropeptides are known to interact with dopaminergic signaling. For example, *Cck1* modulates mesolimbic dopamine function in response to stress (Rotzinger et al., 2002). Acute ethanol exposure causes release of enkephalin in the nucleus accumbens (Olive et al., 2001); thus decreases in *Penk1* mRNA seen on microarrays may represent a compensatory response.

Several growth factors or developmental genes contained in NAC clusters are likely functional either in acute behavioral responses to ethanol or as part of signaling events leading to behaviors seen with repeated or long-term ethanol exposure (e.g., sensitization and craving). *Bdnf* has been strongly implicated in neuroplasticity resulting from multiple drugs of abuse (Horger et al., 1999; Akbarian et al., 2002). *Bdnf* modulates dopamine release in the striatum, is associated with expression of the dopamine D_3 receptor, and functions in development of behavioral sensitization (Horger et al., 1999; Guillin et al., 2004). The developmental genes T-box brain 1 (*Tbr1*), and forkhead box P1 (*Foxp1*) were coinduced with *Bdnf* in the NAC of D2 mice. Expression of the neuropeptide gene *Pacap* was also closely correlated with *Bdnf* in cluster 7. *Pacap* regulates *Bdnf* expression through enhancement of NMDA receptor function (Yaka et al., 2003). Mice lacking *Pacap* show marked increased locomotor responses, decreased anxiety (Hashimoto et al., 2001), and reduced levels of *Bdnf* (Zink et al., 2004). Mutation of the *Drosophila Pacap* homolog *amnesiac* increases sensitivity to sedative properties of ethanol (Moore et al., 1998). Additional NAC cluster genes that interact with *Bdnf* or the trkB/Akt-signaling cascade

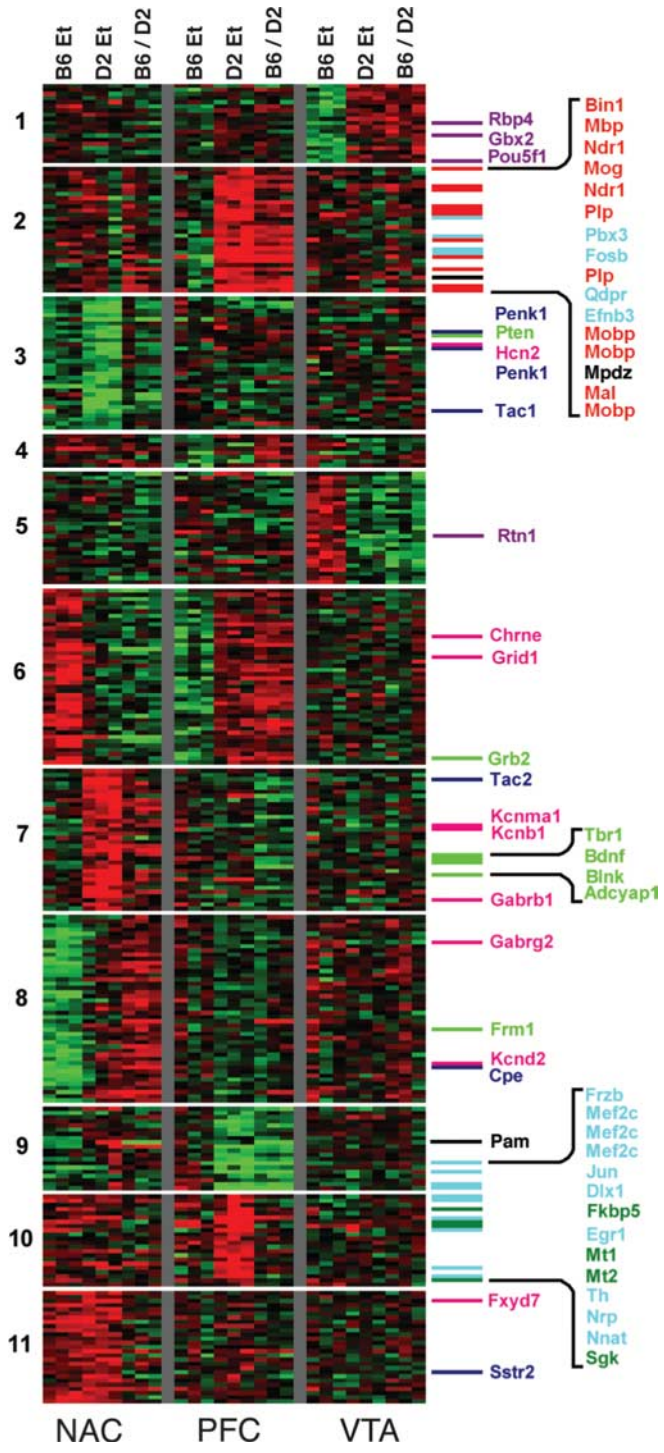

Figure 4. Multivariate analysis of ethanol-regulated genes in D2 and B6 NAC, PFC, and VTA. A tree-view image of k-means clustering for 307 genes regulated by ethanol in the NAC, PFC, and VTA of D2 and B6 mice is shown. Genes were selected by statistical filtering of ethanol versus saline (D2 Et, B6 Et) S-scores as described in Materials and Methods. Corresponding values for B6 saline versus D2 saline S-scores (B6/D2) are also shown. Red, Upregulated by ethanol or more highly expressed in B6 mice (B6/D2); green, downregulated by ethanol or more highly expressed in D2 mice; black, no difference. Relevant functional groups and genes mentioned in Results are indicated as follows: purple, retinoic acid-signaling, *Rbp4* (plasma), *Gbx2*, *Pou5f1*, *Rtn1*; blue, myelin-related, *Bin1*, *Mbp*, *Ndrg1*, *Mog* (myelin oligodendrocyte glycoprotein), *Mobp*, *Plp* (myelin), *Mal* (T-cell differentiation protein); light blue, neuronal development, *Pbx3*, *Fosb*, *Qdpr* (quininoid dihydropteridine reductase), *Efnb3*, *Frzb*, *Mef2c* (myocyte enhancer factor 2C), *Jun*, *Dlx1*, *Egr1*, *Th*, *Nrp*, *Nnat*; green, glucocorticoid-signaling, *Fkbp5*, *Mt1*, *Mt2*, *Sgk*; navy blue, neuropeptide signaling, *Penk1*, *Tac1*, *Tac2*, *Cpe* (carboxypeptidase E), *Sstr2* (somatostatin receptor 2); pink, ion channels, *Hcn2* (hyperpolarization-activated, cyclic nucleotide-gated K^+ 2), *Chrne* (cholinergic receptor, nicotinic, ϵ polypeptide), *Grid1*, *Kcnma1*, *Kcnb1* (K^+ voltage-gated channel, *Shab*-related subfamily, member 1), *Gabrb1*, *Gabrg2* ($GABA_A$ receptor, subunit γ2), *Kcnd2* (K^+ voltage-gated channel, *Shal*-related family, member 2), *Fxyd7* (FXYD domain-containing ion transport regulator 2); lime green, BDNF signaling, *Pten*, *Grb2*, *Tbr1*, *Bdnf*, *Blnk*, *Adcyap1*, *Frm1* (fragile X mental retardation syndrome 1 homolog); black, other, *Mpdz*, *Pam*.

included B-cell linker 1 (*Blnk1*), growth factor receptor-bound substrate 2 (*Grb2*), phosphatase and tension homolog (*Pten*), *Fmr1*, and *Foxp1*. All of these genes were regulated by ethanol in the NAC, although not all in the same direction or strain.

Ion channels regulated by ethanol in the NAC include the β1 subunit of $GABA_A$ receptors (*Gabrb1*) and the α1 subunit of the BK potassium channel K^+ large conductance Ca^{2+}-activated channel, subfamily M, α member (*mSLO1*, *Kcnma1*). Both of these have been implicated previously in ethanol responses or alcoholism. *Gabrb1* has been suggested as a candidate gene in alcohol dependence in human genetic studies (Song et al., 2003), but this is the first report of ethanol regulation of mRNA for this subunit. Similarly, several studies in rodent models show that ethanol activates the BK calcium-activated potassium channel (Dopico et al., 1998), thus decreasing neuronal excitation. Recent genetic studies in *Caenorhabditis elegans* implicate the worm BK channel (*Slo-1*) as a major mediator of acute effects of ethanol on locomotion in that species (Davies et al., 2003). Again, studies here are the first to show ethanol regulation of mRNA expression for BK channels. The presence of *Kcnma1*-, *Gabrb1*-, and *Bdnf*-related genes in cluster 7 suggests that genes of this cluster might collectively have a crucial role in acute responses to ethanol.

Prefrontal cortex

Acute ethanol responses in D2 PFC had two distinct patterns: genes upregulated by ethanol in D2 ≫ B6 PFC (Fig. 4, cluster 10) and genes also with altered control expression in B6 versus D2 PFC (Fig. 4, clusters 2, 9). EASE analysis indicated that these clusters contain an overrepresentation of genes involved in mainly three functions: neurogenesis and plasticity, metal ion homeostasis, and myelination (supplemental Table 2, available at www.jneurosci.org as supplemental material). Some of the most robust responses to ethanol were genes in cluster 10 that showed strong induction by ethanol in the PFC of D2 mice. These genes include serum/glucocorticoid-regulated kinase (*Sgk*), *Fkbp5*, and metallothioneins 1 and 2 (*Mt1* and *Mt2*). These genes are related by their strong response to ethanol in the PFC and dose-responsive steady increases with higher ethanol concentrations (see Fig. 5, results with *Mt2*, *Fkbp5*). All of these genes also have consensus glucocorticoid-binding sites in their promoters (Scherf et al., 2000) or are known to respond to glucocorticoids (Chilibot analysis). *Sgk* regulates multiple ion channels (Farman et al., 2002) and has been implicated in memory consolidation of spatial learning (Tsai et al., 2002).

A number of genes induced by ethanol in PFC function in neuronal development or plasticity. Immediate early genes, commonly regulated with neuronal activation, included FBJ osteosarcoma oncogene B (*Fosb*), early growth response 1 (*Egr1*) (Jones et al., 2001), and *Jun*. The extensive group of development-related genes regulated by ethanol in D2 PFC included the homeobox genes distal-less homeobox 1 (*Dlx1*), pre B-cell leukemia transcription factor 3 (*Pbx3*), ephrin B3 (*Efnb3*), frizzled-related protein (*Frzb*), neuronatin (*Nnat*), neuropilin (*Nrp*), *Th*, and multiple myelin-related genes (see below). *Dlx1* and *Pbx3* are colocalized in their brain expression patterns (Toresson et al., 2000). *Dlx* functions in forebrain development, governing

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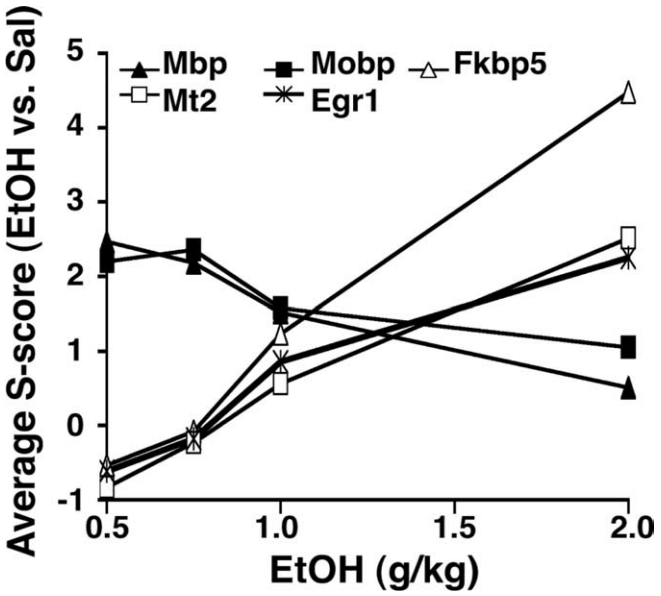

Figure 5. Ethanol dose–response results for selected genes. Average ethanol versus saline S-scores of gene expression in D2 PFC at 4 h in response to 0.5, 0.75, 1.0, and 2.0 g/kg ethanol are shown. S-scores are the average from two replicate experiments.

forebrain-specific combinations of transcription factor expression (Andrews et al., 2003). *Efnb3*, a ligand for ephrin receptor tyrosine kinases, functions in synaptic plasticity and development of spinal cord circuits underlying locomotor activity (Kullander et al., 2003) and was recently identified by microarray studies as a candidate gene for a QTL for acute functional tolerance to ethanol (Tabakoff et al., 2003). Coordinate induction of *Th*, which catalyzes the rate-limiting step in dopamine biosynthesis, and quininoid dihydropteridine reductase (*Qpdr*), which recycles a cofactor for *Th*, may reflect increased demand for dopamine biosynthesis after acute ethanol.

Cluster 2 included a number of myelin and myelin-related genes with higher control expression in B6 PFC and upregulated by ethanol in D2 PFC. The genes N-myc downstream regulated 1 (*Ndrg1*) and bridging integrator 1 (*Bin1*) were also tightly correlated with myelin gene expression in cluster 2. *Ndrg1* and *Bin1* are both known to interact with signaling by the *c-Myc* protooncogene (Butler et al., 1997; Shimono et al., 1999). Many of the myelin genes and the c-myc-related signaling molecules (*c-Myc*, *Ndrg1*, and *Bin1*) have known or predicted cAMP response element-binding protein (CREB) binding sites in their promoters (data not shown; data derived from BiblioSphere analysis). Other signaling molecules and transcription factors related to myelin and oligodendrocyte function were also induced by ethanol in the PFC. For example, endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 2 (*Edg2*), is a G-protein coupled receptor activated by lysophosphatidic acid (for review, see Yoshida and Ueda, 2001) and expressed in brain primarily on oligodendrocytes. *Edg2* was induced by ethanol but grouped in cluster 10, likely because of a lack of differential control expression in B6 versus D2 mice.

Dose–response studies subdivide ethanol responses

To further characterize ethanol expression responses, we performed additional microarray analyses on limited dose–response studies at 0.5–2 g/kg ethanol. A single time point (4 h) was again used because of technical limitations on the number of animals and microarrays that could be processed at one time. Detailed analysis of this data will be presented elsewhere (R. T. Kerns and M. F. Miles, unpublished data). However, analysis of select regions of clusters from Figure 4 identified several types of dose responses in DBA/2J mice. For example, Figure 5 shows that the myelin-related genes myelin-associated oligodendrocytic basic protein (*Mobp*) and myelin basic protein (*Mbp*) (Fig. 4, cluster 2) had decreasing inductions in the PFC of D2 animals from 0.5 to 2 g/kg ethanol. This suggests that maximal induction by ethanol for these genes actually occurs at doses lower than 0.5 g/kg. In contrast, *Fkbp5* and *Mt2*, members of another PFC cluster of genes induced by ethanol in D2 animals (Fig. 4, cluster 10), showed significant inductions only at the 2 g/kg ethanol dose.

Characterization of select genes

We used quantitative Q-rtPCR and Western blot analysis to further confirm select microarray results. *Plp* and *Fkbp5* were chosen for Q-rtPCR studies because they represent members of a large group of genes induced by ethanol in the PFC of D2 animals (Fig. 4, clusters 2, 10). *Plp* also represents a member of the myelin-related gene group in cluster 2 (Fig. 4). *Th* is the rate-limiting enzyme in catecholamine biosynthesis, and microarray studies showed induction by ethanol in the PFC of D2 mice. Figure 6*A* shows expression levels of *Bdnf*, *Fkbp5*, *Plp*, and *Th* in saline- and ethanol-treated D2 PFC normalized to 18S rRNA levels in the same samples. For comparison, Figure 6*B* plots mean MAS 5.0 intensity values from array hybridizations. Western blot analysis of *Fkbp5* showed the protein coded by this gene with qualitatively increased abundance in the prefrontal cortex by 6 h after 2 g/kg ethanol treatment in DBA/2J animals (Fig. 6*C*).

Correlation of expression profiling with QTL studies on ethanol phenotypes

We predicted that genes identified on microarray studies above might be candidate genes contributing to behavioral QTLs identified for acute responses to ethanol or ethanol drinking behavior (for review, see Crabbe et al., 1999). We first mapped expression changes identified in our studies versus chromosomal regions assigned to confirmed QTLs for acute locomotor responses to ethanol, acute ethanol withdrawal, or ethanol preference drinking (for review, see Belknap et al., 1993). Other ethanol behavioral QTLs might also involve genes identified in these studies, but we focused on these behaviors for reasons noted above. From a total of 996 genes either differentially expressed between B6 and D2 mice after saline injection or differentially regulated by ethanol in these lines, 151 were mapped to chromosomal regions implicated in QTLs. Supplemental Table 3 (available at www.jneurosci.org as supplemental material) summarizes results obtained for the subgroup of genes ($n = 36$) regulated by ethanol. (For a list of all genes with expression differences between control B6 and D2 mice mapped to confirmed QTLs, see supplemental Table 5, available at www.jneurosci.org as supplemental material.) This analysis identified the multiple pdz (postsynaptic density-95/Discs large/zona occludens-1) domain protein (*Mpdz*) gene as a strong candidate for the alcohol withdrawal 2 (*Alcw2*) acute withdrawal QTL on Chr 4, thus supporting the classic genetic approach reported by Shirley et al. (2004). *Mpdz* was both differentially expressed between B6 and D2 mice and was ethanol responsive (induced in the PFC of D2 mice) (Fig. 4, cluster 2). In support of bioinformatic studies on *Bdnf* signaling described above, *Bdnf* was located within the support interval for the acute locomotor responses to ethanol 3 (*Actre3*) ethanol locomotor activation QTL.

As an additional approach to link expression profiling results

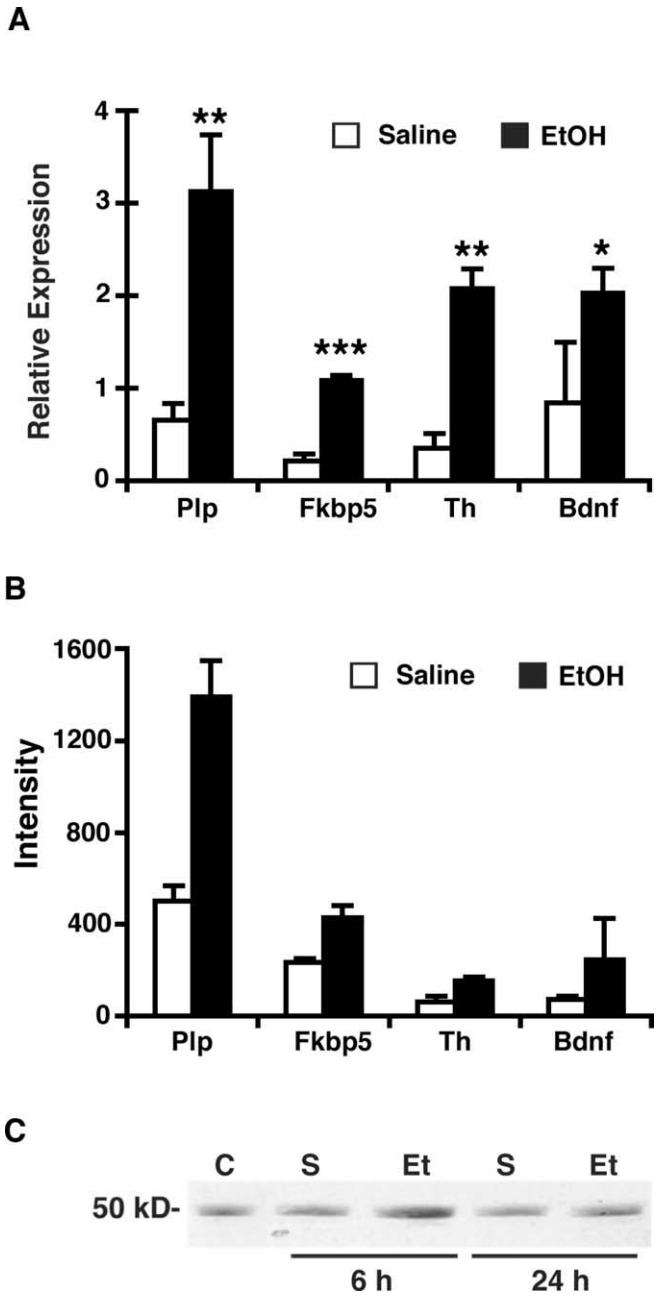

Figure 6. Verification of select microarray data by quantitative real-time PCR and Western blot analysis. ***A***, Q-rtPCR quantitation of *Plp*, *Fkbp5*, and *Th* mRNA levels in ethanol (2 g/kg, 4 h)- and saline-treated D2 PFC or *Bdnf* in ethanol- and saline-treated D2 NAC. Primer sequences are provided in Materials and Methods. Expression levels were normalized relative to 18S rRNA. Significance was determined by two-sample Student's *t* test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). ***B***, MAS 5.0 intensity values from microarray hybridizations for *Plp*, *Fkbp5*, and *Th* in D2 PFC or *Bdnf* in D2 NAC from the same experiment as in ***A***. Corresponding mean S-score values for D2 EtOH versus saline from three experiments, calculated as described in Materials and Methods, were as follows: *Plp*, 6.8; *Fkbp5*, 2.6; *Th*, 3.2; and *Bdnf*, 3.0. ***C***, Western blot analysis of Fkbp5 in protein isolates from saline (S)- and ethanol (Et)-treated D2 PFC at the indicated times after 2 g/kg ethanol. Results are representative of experiments repeated three times. C, Control.

to ethanol behaviors, we used the WebQTL Project (http:/www.webqtl.org/) resources as described in Materials and Methods. By correlating expression patterns with genotype data and previous behavioral QTL analyses across the same BXD lines, it is possible to link expression patterns to specific chromosomal loci (Chesler et al., 2003). As shown in supplemental Table 4 (available at www.jneurosci.org as supplemental material), using WebQTL, we identified multiple ethanol-responsive genes as having *cis*- or *trans*-QTLs in regions previously linked to the ethanol behaviors mentioned above. Peptidylglycine α-amidating monoxygenase (*Pam*) showed both *cis*- and *trans*-QTLs in the region of several ethanol behavioral QTLs contained on chromosome 1. A large number of genes with differences in expression between control B6 and D2 mice also had significant expression QTLs overlapping with behavioral QTLs (see supplemental Table 6, available at www.jneurosci.org as supplemental material).

We also identified the distribution of linkage peaks (peak LRS scores) from WebQTL (5/03 U74Av2 database) for all ethanol-responsive genes included in Figure 4. The premise of this analysis was that common chromosomal regions (*trans*-QTLs) not studied above (supplemental Table 4, available at www.jneurosci.org as supplemental material) might modulate basal expression of these genes across BXD RI lines. Figure 7*A* shows a histogram for LRS scores of >9.2 (LOD >2) for the 307 ethanol-responsive genes. The list of genes and their linkage regions are also included (see supplemental Table 7, available at www.jneurosci.org as supplemental material). Strikingly, there were several regions (proximal Chr 2, mid Chr 4, and distal Chr 19) linked to the expression for groups of ethanol-regulated genes. Chromosome 19 had the largest such group, with >17 genes having *trans*-QTLs with LRS of >9.2 within a 20 Mb region (Chr 19, 31–51 Mb). These genes also showed correlated responses to ethanol in our data (Fig. 7*B*), with the majority being in NAC clusters 6–8 from Figure 4. These clusters all predominantly contain genes with increased or decreased expression in NAC after ethanol treatment. Of the genes in these NAC clusters discussed above, *Cck1* and *Gabrb1* both had strong linkage to the 31–51 Mb region of Chr 19.

Analysis of expression QTLs linked to regions containing ethanol QTLs of interest (supplemental Table 4, available at www.jneurosci.org as supplemental material) identified the *Pam* gene as having a strikingly strong *cis*-QTL peak. *Pam* had an expression QTL with an LRS of >40 centered at 100.4 Mb (D1Mit11) on Chr 1 (Fig. 8*A*), and the *Pam* gene is physically located at 98.36 Mb. The robustness on this QTL and the important role that *Pam* has in activating many peptide hormones or neuropeptides via amidation (Eipper et al., 1993) encouraged us to pursue further genetic correlations to *Pam* expression. When basal *Pam* expression in the BXD RI lines was correlated with behavioral QTL studies in WebQTL, the strongest correlation was to locomotor activity in ethanol sensitization (Phillips et al., 1996) (Fig. 8*B*). On interval mapping, this trait showed an LRS peak in the same location as *Pam* (Fig. 8*C*). These data suggest that the basal difference in *Pam* expression may contribute to genetic differences in locomotor sensitization after repeated ethanol exposure in the BXD recombinant inbred lines.

As described above, bioinformatic analysis indicated that a number of myelin-related genes were induced by ethanol in the PFC of D2 mice (Fig. 4, cluster 2). Such coordinate regulation of related genes suggests a common underlying mechanism. Because genes in cluster 2 also showed control expression differences between B6 and D2 animals, we used WebQTL to determine whether basal expression of myelin-related genes was linked to common chromosomal regions and whether such had correlations with other phenotypes. A series of myelin-related genes in cluster 2 [myelin and lymphocyte protein (*Mal*), *Mbp*, *Mobp*, and *Bin1*] had strong *trans*-QTLs in the 63–73 Mb region of Chr 1 (data not shown). Using principal components analysis of these myelin genes through WebQTL on-line resources, we derived a major factor [principal component 1 (PC1)] from these

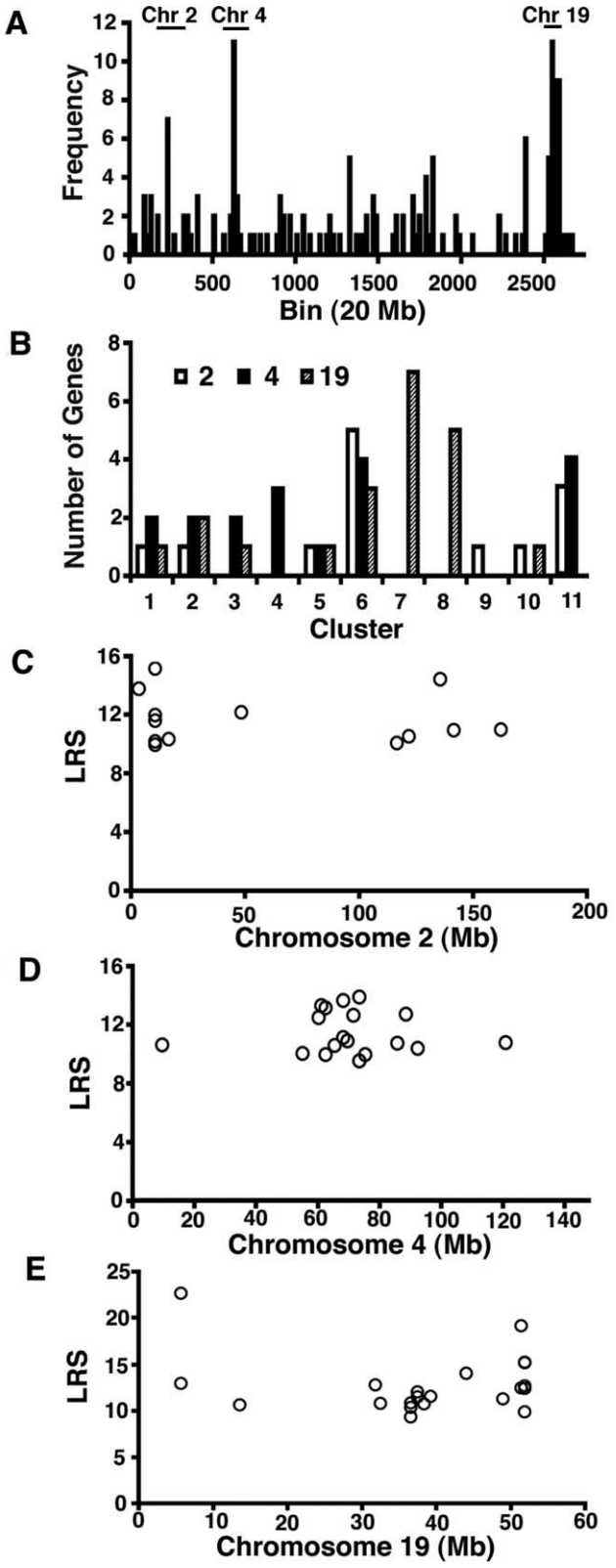

Figure 7. WebQTL linkage analysis of *trans*-QTLs for ethanol-responsive genes. ***A***, Histogram of chromosomal position for QTLs with LRS scores of ≥9.2 ($n = 159$) derived from ethanol-responsive genes. Bin size is 20 Mb. The largest groups of scores are linked to loci on Chrs 2, 4, and 19. ***B***, Graph of the number of QTLs from Chrs 2, 4, and 19 (***A***) for ethanol-responsive gene k-means clusters (from Fig. 4). Most Chr 19-linked genes are contained in clusters 6–8. ***C***, LRS values by chromosomal position for probe sets that are linked to Chr 2 ($n = 13$). ***D***, LRS values by chromosomal position of probe sets that link to Chr 4 ($n = 18$). ***E***, LRS values by chromosomal position of probe sets that link to Chr 19 ($n = 21$).

myelin genes driving the Chr 1 QTL (Fig. 8*D*). This PC1 factor was then used to search for correlated gene expression patterns from the WebQTL expression databases, looking for potential regulatory molecules residing in the Chr 1:63–73 Mb region. The transcription factor *Creb1*, located at 66.13 Mb, had a significant correlation (Spearman's $r = 0.73$; $p < 0.001$) with the PC1 pattern across the BXD RI lines (Fig. 8*E*). Finally, when the PC1 factor was used to search for correlated biochemical and behavioral phenotypes in the WebQTL phenotype database, we found that this myelin gene-derived component was highly correlated (Fig. 8*F*) with a study of basal dopamine transporter (*Dat1*, *Slc6a3*) abundance in the PFC of female mice (Jones et al., 1999). These findings, together with other bioinformatic studies mentioned above, suggest that ethanol may acutely directly or indirectly regulate multiple myelin-related genes in the PFC through a mechanism involving *Myc/Ndrg1*, *Dat1*, and *Creb1*.

Discussion

We investigated control and acute ethanol-evoked gene expression patterns in brain regions involving the mesolimbic dopamine pathway of B6 and D2 mice, two inbred mouse strains with divergent ethanol-related phenotypes. These studies identified brain region-specific patterns of gene expression with functional relevance to ethanol-related behaviors. Although additional work is needed to definitively link these genes with behavioral responses to ethanol in B6 and D2 mice, the identified expression patterns clearly contribute novel insight into brain mechanisms of acute ethanol action.

Expression patterns in control D2 and B6 mice

Approximately half of the genes differentially expressed in B6 versus D2 mice were altered across multiple brain regions, consistent with previous reports on mouse brain regional gene expression (Sandberg et al., 2000). A large number of these genes were clustered in regions of Chrs 1 and 4 (Fig. 2). Similarly, a recent report described Chr 11 as having a disproportionate number of expression differences between B6 and D2 hematopoetic stem cells (de Haan et al., 2002). These investigators suggested that a cell cycle QTL mapped to Chr 11 might result from a variation of a group of highly linked genes. The occurrence of linked expression patterns across such a large portion of a chromosome (e.g., Chr 1) suggests possible involvement of mechanisms affecting higher chromosomal order. Interestingly, Chr 1 also has several QTLs that have been identified for ethanol-related behaviors (Crabbe et al., 1999).

Our expression studies on B6 versus D2 control mice identified a large number of genes either residing within (supplemental Table 5, available at www.jneurosci.org as supplemental material) or having their basal expression linked to (supplemental Table 6, available at www.jneurosci.org as supplemental material) chromosomal regions harboring several ethanol QTLs. In the case of a very narrow QTL support interval (*Alcw2*), expression profiling identified a single gene (*Mpdz*) residing in the *Alcw2* support interval and both differentially expressed between B6 and D2 mice (supplemental Table 5, available at www.jneurosci.org as supplemental material) and regulated by ethanol (supplemental Table 3, available at www.jneurosci.org as supplemental material). Recent studies have confirmed *Mpdz* as a quantitative trait gene associated with the *Alcw2* QTL (Shirley et al., 2004), thus validating hypotheses from our microarray results. Together, these findings on *Mpdz* strengthen recent suggestions that expression profiling can accelerate identification of genes underlying QTLs for complex traits (Hitzemann et al., 2003).

Region-specific, ethanol-responsive gene expression patterns

Many ethanol-responsive genes were differentially regulated in D2 versus B6 mice (Fig. 4), suggesting fundamental genetic differences in signaling evoked by acute ethanol in these mice. k-Means analysis (Fig. 4) indicated that ethanol responses were primarily confined to single brain regions. Although there were genes that did respond to ethanol in multiple regions, these changes were sometimes of different directions in D2 versus B6 mice (Fig. 4, cluster 6). Bioinformatic analysis confirmed regional differences in functional patterns evoked by ethanol (Fig. 4; supplemental Table 2, available at www.jneurosci.org as supplemental material). These findings suggest that ethanol-evoked, brain region-specific signaling events predominate over more global responses to ethanol. This could be consistent with neural pathway activation of gene expression after acute ethanol stimulation. Strikingly, when basal expression of ethanol-responsive genes was assessed through WebQTL, a large group of genes from NAC clusters of Figure 4 were found to have *trans*-QTL links to a common region of Chr 19 (Fig. 7). This strongly suggests that these genes may have an common underlying mechanism(s) of regulation.

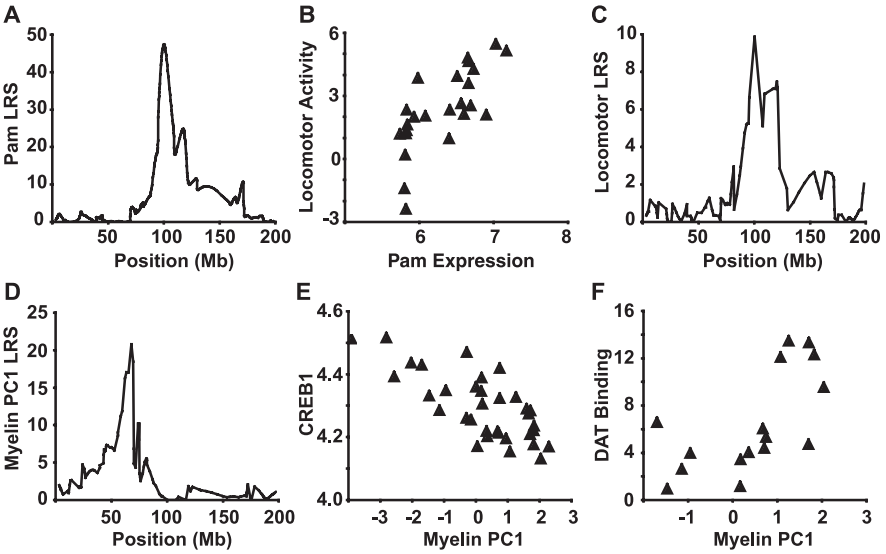

Figure 8. WebQTL analysis of *Pam* and myelin-related genes. ***A***, Interval mapping of Pam expression pattern across BXD recombinant inbred lines for loci on mouse Chr 1. Permutation and bootstrap testing ($n = 2000$) were done for significance of interval mapping. ***B***, Correlation of *Pam* gene expression across BXD lines with locomotor activity (centimeters per 10 min $\times 10^{-2}$; day 11 − day 3) after ethanol sensitization (Phillips et al., 1996). Spearman's rank-order correlation, $r = 0.81$ ($p < 0.000001$). ***C***, Interval mapping of locomotor sensitization phenotype across BXD lines. Trait data shown in ***B*** were analyzed by interval mapping with bootstrap and permutation testing. The Chr 1 linkage peak maps to the same position as the major *Pam* QTL. ***D***, Interval mapping for PC1 of myelin-related genes. Principal component analysis done within WebQTL was used to derive components from expression patterns of *Bin1* (probe set 92220_at), *Mal* (probe set 99089_at), *Mbp* (probe set 96311_at), *Mobp* (probe set 99048_g_at), and *Plp* (probe set 92802_at). A single principle component (PC1) accounted for >70% of the variance. Shown is the Chr 1 interval map for the derived PC1. All genes used for principal component analysis also had linkage peaks in same area of Chr 1. ***E***, Correlation of myelin PC1 with expression of *Creb1* (probe set 100496_at) across BXD lines. Pearson's correlation analysis, $r = -0.7443$. ***F***, Correlation of myelin PC1 with dopamine transporter binding activity ($\times 10^{-2}$) in the PFC (Jones et al., 1999). Spearman's rank-order correlation, $r = 0.72$ ($p < 0.001$). All WebQTL expression data were from the 3/04 robust multi-array average analysis (www.webqtl.org). BXD line 29 was excluded from all analyses because of anomalous behavior.

Bioinformatic analysis of ethanol-responsive clusters revealed regional functional changes that can primarily be summarized as follows: VTA, retinoic acid signaling and developmental; NAC, *Bdnf* signaling and neuropeptide expression; and PFC, glucocorticoid-responsive genes, development, and myelin related. However, the VTA, NAC, and PFC did express patterns all potentially related to a larger functional group, namely, neuroplasticity. For example, retinoic acid signaling, *Pou5fl*, and *Gbx2* (VTA) are all known to be crucial to development of the hindbrain–midbrain region, *Bdnf* signaling (NAC) is known to function in a number of different models of experience-dependent plasticity (Guillin et al., 2004), and *Sgk* (PFC) has been shown to modulate spatial learning and long-term potentiation in the hippocampus (Tsai et al., 2002). Furthermore, myelin (PFC)-associated molecules have recently been suggested to be a dynamic part of axonal remodeling (Josephson et al., 2003). Our results might thus implicate widespread ethanol-evoked neuroplasticity events across the mesolimbocortical dopamine pathway but with differing mechanisms within regions.

Our expression data suggests that *Pacap-Bdnf* signaling is a particularly important target of acute ethanol action in the NAC. In support of this premise, *Bdnf* is located within a QTL region (supplemental Table 3, available at www.jneurosci.org as supplemental material) linked to acute locomotor responses to ethanol (*Actre3*; Chr 2, 108–112 Mb; LOD >9) (Demarest et al., 2001). Thus, *Bdnf* may be a viable candidate gene for the *Actre3* QTL. In a very recent study, McGough et al. (2004) reported that *Bdnf*, via a mechanism requiring RACK1 (receptor for activated C kinase) protein, is induced by acute ethanol treatment or voluntary ethanol drinking in B6 mice. These investigators did not study responses in D2 mice. *Bdnf* underexpressing mice ($Bdnf^{+/-}$) also showed increased ethanol-conditioned place preference, increased sensitization after repeated ethanol exposure, and increased ethanol drinking after abstinence. These biochemical and behavioral data, together with our expression data and bioinformatic analysis, strongly implicate *Bdnf* signaling as an important factor in acute ethanol behavioral responses and drinking behavior.

Region-selective patterns evoked by ethanol can also be considered as being related to acute neurophysiological responses to ethanol. For example, $GABA_A$ receptor subunits (*Gabarb1* and *Gabarg2*), the glutamate receptor ionotropic δ1 (*Grid1*) glutamate receptor subunit, and the mSLO1 (*Kcnma1*) potassium channel subunit were all regulated by ethanol in the NAC. All of these receptors are known to be sites of acute ethanol action as mentioned in Results. Whether gene expression changes evoked in these receptor systems might therefore be related to more short-term adaptive responses, such as tolerance, remains to be determined.

Identifying expression differences for genes residing within ethanol QTL intervals is one method for relating genomics results to ethanol behaviors (supplemental Tables 3 and 5, available at www.jneurosci.org as supplemental material). However, overlaying our expression results with genetic data on both gene expression and behavior (WebQTL) increased our ability to correlate expression with behavior. This approach also derived new mechanistic information by linking basal expression levels for groups of genes to specific chromosomal regions. For example, our studies showed that ethanol coordinately regulated a group of myelin-

related genes. We found that these genes also had basal expression across the BXD RI lines strongly linked to a Chr 1 site near the transcription factor *Creb1* gene (Fig. 8). Numerous facts support a role for *Creb1* in the myelin response: (1) basal myelin gene expression is strongly linked to an interval on Chr 1 that contains the *Creb1* transcription factor; (2) *Creb1*, in turn, showed an expression pattern across the BXD RI lines that was significantly correlated with the PC1 factor derived from myelin expression profiles (Fig. 8*E*); (3) many of the myelin genes and *c-Myc*-related signaling molecules (*c-Myc*, *Nrd1*, and *Bin1*) have known or predicted CREB-binding sites in their promoters (data derived from BiblioSphere analysis); and (4) acute ethanol is known to activate cAMP signaling and CREB in multiple cell types and rodent brain, whereas chronic ethanol exposure has been shown to downregulate CREB signaling (Diamond and Gordon, 1997; Yang et al., 1998; Asher et al., 2002).

Coordinate upregulation of myelin-related genes in D2 PFC with acute ethanol contrasts with downregulation of multiple myelin-related genes observed in our previous microarray studies on postmortem frontal cortex from human alcoholics (Lewohl et al., 2000). This suggests that acute ethanol may cause direct effects on myelin-related signaling mechanisms subsequently downregulated with chronic exposure in alcoholism. CREB may be an excellent candidate for a proximal mechanism mediating ethanol effects on myelin-related gene expression, with activation by acute ethanol and downregulation by chronic exposure. Correlation of myelin gene-derived PC1 with dopamine transporter expression in the frontal cortex (Fig. 8) is further suggestive of a role for dopamine signaling in the myelin responses. It is unknown whether ethanol-induced alterations in myelin gene expression may have short- or long-term behavior consequences. However, clearly, dysfunction of myelin occurs in alcoholism (Kril and Harper, 1989).

WebQTL analysis also indicated a strong Chr 1 *cis*-QTL for *Pam* expression across the BXD lines (Fig. 8*A*). Pam is known to modulate functional activity of multiple neuropeptides, including NPY and PACAP, both of which influence ethanol behaviors (Moore et al., 1998; Thiele et al., 1998). The significant correlation of basal *Pam* expression with ethanol sensitization (Fig. 8*B*) further supports the potential importance of *Pam* in determining differential behavioral responses to ethanol in B6 versus D2 mice.

The results described above illustrate striking divergent regulation of gene expression by ethanol in D2 and B6 mice. Brain region-specific changes in signaling and neuronal plasticity may be critical components in the development of lasting behavioral phenotypes, such as dependence, sensitization, and craving. Superimposing our results on biochemical and genetic data has identified a testable set of hypotheses regarding genes related to ethanol behavioral QTLs and signaling events evoked by acute ethanol.

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