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Microarray Analysis Identifies Cerebellar Genes Sensitive to Chronic Ethanol Treatment in PKC γ Mice

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Abstract

Neuroadaptive changes that occur in the development of ethanol tolerance may be the result of alterations in gene expression. We have shown that PKC γ wild-type mice develop tolerance to the sedative-hypnotic effects of ethanol after chronic ethanol treatment; whereas, mutant mice do not, making these genotypes a suitable model for identifying changes in gene expression related to tolerance development. Using a two-stage process, several genes were initially identified using microarray analyses of cerebellar tissue from ethanol-treated PKC γ mutant and wild-type mice. Subsequent confirmation of a subset of these genes using qRT-PCR was done to verify gene expression changes. A total of 109 genes from different functional classifications were identified in these groups on the microarrays. Eight genes were selected for verification: three, *Twik-1*, *Plp*, and *Adk2*, were chosen as genes related to tolerance; another three, *Hsp70.2*, *Bdnf*, and *Th*, were chosen as genes related to resistance to tolerance; and two genes, *JunB* and *Nur77*, were selected as candidate genes sensitive to chronic ethanol. The results from the verification experiments indicated that *Twik-1*, which codes for a potassium channel, was associated with tolerance and appeared to be dependent on the presence of PKC γ . No genes were confirmed to be related to resistance to tolerance; however, expression of two of these, *Hsp70.2* and *Th*, were found to be sensitive to chronic ethanol and were added to the transcription factors, *JunB* and *Nur77*, confirmed by qRT-PCR, as a subset of genes that respond to chronic ethanol.

Keywords

Microarray; chronic ethanol; qRT-PCR; cerebellum; mouse; PKC γ

INTRODUCTION

Chronic exposure to ethanol induces persistent behavioral changes such as tolerance, dependence, addiction, and relapse. These behavioral changes are likely the result of ethanol-induced neuroadaptation in response to repeated exposure to the drug (Hoffman et al., 2000; Nestler and Aghajanian, 1997). Evidence suggests that changes in gene expression underlie these neuroadaptive processes (Rahman and Miles, 2001). Recently, several studies have used DNA microarrays to profile gene expression changes in rodents to identify genes related to alcohol preference (Edenberg et al., 2005; McBride et al., 2005; Mulligan et al., 2006; Murphy et al., 2002) and genes that respond to acute ethanol administration (Kerns et al., 2005). Gene

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expression changes after chronic ethanol treatment have been observed in mouse and rat hippocampus (Daniels and Buck, 2002; Saito et al., 2002) and in striatum (Smith et al., 2006) as well as in brain regions from human alcoholics (Flatscher-Bader et al., 2005, 2006; Lewohl et al., 2000; Mayfield et al., 2002). The investigations of chronic ethanol-induced gene expression alterations have identified genes from many different functional groups that are associated with the long-lasting effects of chronic ethanol exposure that encompass tolerance, dependence, and addiction. It has been hypothesized that the development of tolerance leads to dependence, ultimately resulting in addiction (Kalant, 1996). Therefore, the identification of genes associated with tolerance would provide genetic information concerning the first step in this process. Kalivas (2005) suggests that experiments that are designed to isolate specific parts of this temporal sequence would go a long way in distinguishing neuroadaptations related to each phase.

Data from this laboratory have shown that chronic ethanol diet produces tolerance to the sedative-hypnotic effects of ethanol in protein kinase C γ (PKC γ) wild-type mice, but not in PKC γ null mutant littermates (Bowers et al., 1999). This difference in tolerance development provides a unique comparison for identifying genes associated specifically with ethanol-induced tolerance since the PKC γ genotypes are isogenic except for the lack of PKC γ in the mutant line. We have shown that ethanol potentiation of GABA_A receptor function (Harris et al., 1995; Proctor et al., 2003) and 5-HT_{2A/C} (Bowers et al., submitted) receptor function are different between the genotypes in naïve animals. In order to identify patterns of gene expression that might reveal important neural pathways as well as single gene changes related to tolerance, we used Affymetrix microarrays to compare gene expression in the cerebellum of PKC γ mutant and wild-type mice after control- and chronic ethanol-diet. Those genes whose expression changes in wild-type mice, but not mutants, after chronic ethanol might be expected to be associated with the development of tolerance. Gene expression changes seen in mutant, but not wild-type mice, might be expected to be associated with a resistance to tolerance development; and gene expression changes observed in both genotypes could be classified as ethanol-responsive genes associated with the effects of chronic treatment. A two stage strategy was used with the microarray analyses providing a list of putative genes followed by confirmation using quantitative real time reverse transcriptase polymerase chain reactions (qRT-PCR) of a subset of genes from each of these groups.

We chose to perform the analyses in cerebellum because of its role in the sedative-hypnotic effects of ethanol, specifically the genetic correlation between Purkinje cell depression and the duration of the loss of righting reflex (Sorensen et al., 1980; Spuhler et al., 1982). PKC γ is neuronal specific and it is located throughout the brain including the Purkinje cells of the cerebellum (Naik et al., 2000). The cerebellum has been shown to be a brain region particularly sensitive to the toxic effects of chronic alcohol. Specifically, Purkinje cells and granule cells are vulnerable to cell loss and may represent the cerebellar atrophy seen in alcoholics (Andersen, 2004; Karhune et al., 1994; Oberdoerster and Rabin, 1999; Phillips et al., 1987).

MATERIALS and METHODS

2.1 Animals

PKC γ null mutant mice were derived using gene-targeting and homologous recombination techniques (Abeliovich et al., 1993) and are currently bred on an F1 C57BL/6 X 129/S6 mixed genetic background at the Institute for Behavioral Genetics (Boulder, CO). The F1 generations are bred from heterozygous crosses from two congenic strains: C57BL/6.PKC γ and 129/S6.PKC γ . This breeding strategy produces homozygous mutant, heterozygous, and homozygous wild-type genotypes within a single litter, thereby providing wild-type littermate controls for each experiment. The PKC γ mutation is maintained in a heterozygous condition on C57BL/6 and 129/S6 inbred strains because homozygous null mutant mice do not survive

on the C57BL/6 background (Bowers and Wehner, 2001). Genotyping was done as described by Bowers et al. (1999). Mice were housed in like-sex groups of 2–5 composed of all genotypes and were maintained on a 12-hour light/dark cycle (lights on at 0700). Food and water were available *ad libitum*. All animal use procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Colorado IACUC.

2.2 Chronic Ethanol Treatment

The basic experimental design was similar to that used to compare striatal gene expression in PKC γ wild types with mutants (Smith et al., 2006). Wild-type and mutant littermates were derived from 28 different litters for these studies. Mice were singly housed for chronic ethanol treatment and were weighed every other day. Ethanol was administered in a protein-enriched liquid diet that had been shown previously to produce tolerance in PKC γ wild types as described by Bowers et al. (1999) according to the following schedule: Day 1: 0% ethanol derived calories (EDC), Days 2 to 4: 18.5% EDC, Days 5–11: 27% EDC. Ethanol was replaced with sucrose in the control diet to provide a caloric balance to the calories derived from ethanol. Mutants consume less ethanol diet (Bowers et al., 1999) than wild-type mice; therefore, to control for volume, wild-type ethanol diet and all control diet animals had their intake yoked to the average mutant ethanol diet intake from the previous day. Wild types and mutants do not differ in ethanol metabolism (Harris et al., 1995). The liquid diet is the sole source of nutrition; therefore, if any animal lost more than 15% of their body weight due to this regimen, single food pellets (4.0 g) were provided to all mice to facilitate weight gain.

Blood ethanol concentrations were determined in an independent experiment using heterozygous PKC γ mice ($n = 8$) because of the possibility that stress related to removal of blood would influence gene expression. On Day 12, immediately after diets were removed, blood was collected from the retro-orbital sinus and ethanol concentrations determined as described by Smolen et al. (1986). Ethanol concentrations averaged 212.73 ± 81.7 mg% in this subset of mice.

2.3 Experimental Design of Gene Expression Studies

The analyses of cerebellar gene expression in PKC γ wild types and mutants were designed to be performed in two stages. In the first stage, Affymetrix arrays were used to provide a list of putative candidate genes that appeared to differ as a function of genotype and/or treatment. Because the number of samples was small in the microarray experiments, the power of the statistical analyses was limited. Therefore, changes in gene expression were verified in a subset of genes of interest using qRT-PCR techniques with RNA extracted from a group of mice treated independently from those used in the microarray experiments. An additional feature of the qRT-PCR confirmation process was that primer and probe sequences were designed from a different location on the gene than was used for the probe sequences on the arrays.

To prevent withdrawal, cerebella were removed immediately after removal of the diets at 0900 hr. For the microarray analyses, cerebellar tissues were collected from individual control- and ethanol-treated mice and RNA isolated from individual mice. Total cerebellar RNA was pooled from three mice for a total of 16 pools from 48 mice (12 mutant control-diet; 12 mutant ethanol-diet; 12 wild-type control diet; 12 wild-type ethanol diet). For the qRT-PCR confirmation studies, RNAs from individual mice from different litters and different ethanol treatment experiments were analyzed. Routinely, tissues from 9–15 mice from each group were analyzed in the qRT-PCR studies.

2.5 Tissue Preparation, RNA Isolation, cRNA Preparation

Procedures were identical to those described in Smith et al. (2006). Briefly, immediately after the diets were removed, mice were sacrificed by cervical dislocation. Cerebella were dissected on ice and frozen in liquid nitrogen, then stored at -70°C until use. Total RNA was isolated using the RNeasy Midi Kit (Qiagen, Valencia, CA) following polytron homogenization. RNA samples were stored individually in separate tubes at -70°C . RNA sample preparation and hybridization were carried out at the Gene Expression Core Facility at the University of Colorado Health Sciences Center (Denver, CO) according to the protocol outlined in the Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA).

Biotin-labeled cRNA was synthesized from the ds-cDNA template by in vitro transcription (IVT) using ENZO Bioarray High Yield RNA Transcript Labeling Kit (T7) (Enzo Diagnostics, Inc. Farmingdale, NY) in the presence of biotinylated ribonucleotides and was purified using the RNeasy Mini Kit (Qiagen) for RNA cleanup. cRNA was fragmented for 35 min at 94°C in fragmentation buffer (Affymetrix, Inc.) then added to hybridization cocktail (Affymetrix, Inc.). Biotinylated cRNA samples were hybridized to MG-U74Av2 GeneChip (Affymetrix, Inc.) and were incubated for 16 hours at 45°C in a GeneChip Hybridization Oven 460 (Affymetrix, Inc.). The hybridized samples were washed and stained with Streptavidin-phycoerythrin using a GeneChip Fluidics Station 400 (Affymetrix, Inc.).

2.6 Quantitative Real-Time RT-PCR (qRT-PCR)

Quantitative RT-PCRs were performed as described in Smith et al. (2006) using the ABI PRISMTM Sequence Detection System 7000 and the ABI Gene Amp PCR System 9700 (Applied Biosystems Inc., Foster City, CA) controlled by ABI PRISM 7000 SDS Software Version 1.0 and SDS 7900HT Software Version 2.2, respectively (Applied Biosystems, Inc.). BLAST analyses were conducted for all primer and probe sequences to insure that they were specific for their respective genes and were selected from a different region of the gene than those represented by the Affymetrix MG-U74Av2 probe sets (Liu et al., 2003). The gene used as the reference gene was hypoxanthine guanine phosphoribosyl transferase (HPRT). Cerebellar RNA was isolated as described above and stored in 1 $\mu\text{g}/\mu\text{l}$ aliquots at -70°C until used. qRT-PCR was conducted using the TaqMan[®] One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Inc.).

RNA samples were run in triplicate for the genes of interest and for the reference gene within the same experiment. Triplicate Cts were averaged for each sample. The choice of HPRT as a reference gene was supported by the observation that expression of HPRT did not differ among the experimental groups. Relative quantification of gene expression (relative amount of target RNA) was determined using an equation adapted from the equation $2^{-\Delta\Delta\text{Ct}}$ outlined in Applied Biosystem User Bulletin #2.

2.7 Statistical Analysis

Four U74Av2 microarrays were analyzed for each genotype/treatment combination with each individual sample consisting of RNA pooled from three mice (see above). Individual arrays were assessed for various quality control parameters as described in the Affymetrix GeneChip Expression Analysis Technical Manual (2004). Probe sets were converted to a single expression value using the Tukey's one-step biweight procedure (MAS5, Affymetrix). Global scaling was applied such that the average signal intensity of the array was set to a default target signal of 500. All subsequent analyses were conducted in GeneSpring GX (v. 7.2, Agilent Technologies) or MS Excel 2000. Probe sets called "absent" by the MAS5 algorithm (i.e., undetectable levels of the transcript) were removed under the condition that retained transcripts were required to be present in 3 out of 4 arrays in at least 1 of the 4 genotypes/treatment conditions. Higher level analyses are described below under Results.

Relative amounts of RNA calculated from the qRT-PCR experiments were analyzed by two-way ANOVA with diet-treatment and genotype as independent variables. Because the qRT-PCR assays were done to confirm results from the microarrays, planned comparisons were done *apriori* within genotype using one-tailed Student's t-tests to determine diet effects.

RESULTS

3.1 Microarrays

Of the 12,488 probe sets on the U74Av2 microarray, 6315 reached the criterion of "present" (i.e., expressed at a detectable level) in at least 3 out of 4 samples from each group (wild-type ethanol and control, mutant ethanol and control). Using this basic set of genes, a one-way ANOVA was conducted to test for differences due to ethanol treatment within each genotype. Because the experimental design required that confirmation studies would be performed, a liberal statistical criteria of $p < 0.05$, and a fold change of greater than + 1.3, were applied to array results. In general, changes in gene expression rarely reached over two-fold (mutant: 1.39 ± 0.02 fold; wild-type: 1.38 ± 0.04 fold). This is consistent with results from microarray data comparing PKC γ mutant and wild-type gene expression in striatum (Smith et al, 2006) as well as results from other gene expression studies in human and mouse (Lewohl et al, 2000; Daniels and Buck, 2002). Genes from each genotype were subjected to functional group analysis using the DAVID Bioinformatics Resources Database (<http://niaid.abcc.ncifcrf.gov/home.jsp>). All genes were categorized into functional groups at GO Level 2. The parent GO levels were Biological Processes, Cellular Components, and Molecular Function, which were divided further into Level 2 GO terms. Each group was enriched by the genes listed within that group at a significance level of $p < 0.05$. There was considerable overlap among the genes for assignment to particular groups; therefore, the tables list the genes in the order in which they appeared first in the analysis, but are also identified with additional group assignments as they appeared later by the superscript classifications. Genes that changed in both genotypes were also analyzed; however, no genes were significantly assigned to any functional group. Table 1 lists 44 genes whose expression changed by chronic ethanol treatment in mutant mice only; 50 EST's were also significantly changed (data not shown). Of these 44 genes, 27 were up-regulated and 17 were down-regulated. Table 2 lists 43 genes whose expression changed as a result of chronic ethanol-treatment in wild-type mice only; 43 ESTs were also significantly changed (data not shown). Twenty-six genes were up-regulated and 17 were down-regulated. The functional group analyses resulted in more gene-enriched groups in the mutant mice compared to wild-type mice. In addition to the functional group analyses, genes from each genotype were subjected to KEGG pathway mapping using DAVID. In the mutant mice, no genes significantly mapped to a particular pathway; however, in the wild-type mice three genes significantly mapped to the inositol phosphate metabolism pathway at $p < 0.05$. These genes were dystrophin myotonic-protein kinase, cdc-like kinase, and inositol (myo)-1(or4)-monophosphatase 1. These three genes also mapped to the phosphatidylinositol signaling system pathway at $p = 0.075$. This suggests that in wild-type mice chronic ethanol may activate the phosphatidylinositol system; however, in mice that lack PKC γ this pathway may be disrupted.

Eleven genes were changed in both genotypes (Table 3) and 4 ESTs were changed in both mutant and wild-type mice (data not shown). Of these genes, 6 were up-regulated in both genotypes, 2 were down-regulated in both genotypes, and 3 genes demonstrated interactions between genotype and diet with up-regulation in one genotype and down-regulation in the other. A complete list of genes are available upon request.

The microarray data generated a putative list of genes whose expression may be related to 1) tolerance development, 2) resistance to tolerance, and 3) the effects of chronic ethanol treatment. Confirmation was done in the second stage of analysis using an independent method

of gene expression quantification, qRT-PCR. From the microarray results, three genes that changed in mutant mice only (*Hsp70.2*, *Bdnf*, *Th*), three genes that changed in wild-type mice only (*Twik-1*, *Plp*, and *Adk2*), one gene whose expression was altered in the same direction in both genotypes (*JunB*), and one gene that changed in opposite directions after chronic ethanol (*Nur77*) were chosen for further analysis. These genes were selected based on their possible significance in the neurobiology of addiction (Janak et al., 2006; Nakahara et al., 2002; Nestler, 2001, 2005). Some of the genes have been reported in gene expression studies of human alcoholics (Flatscher-Bader et al., 2005; Lewohl et al., 2000; Liu et al., 2004; Mayfield et al., 2002) and in gene expression studies of chronic ethanol in mice (Daniels and Buck, 2002).

3.2 qRT-PCR Verifications

Results from the microarray experiments indicated that gene expression levels for *Hsp70.2* and *Th* were significantly increased only in mutant cerebellum after chronic ethanol diet ($p < 0.003$; $p < 0.009$, respectively) (Figure 1a,c); whereas, *Bdnf* gene expression decreased in mutant cerebellum after ethanol diet ($p < 0.02$). However, qRT-PCR confirmations using 9–10 independent samples from wild-type and mutant cerebellum after control and ethanol diet revealed that for *Hsp70.2*, expression levels were increased in both mutant and wild-type mice after ethanol diet ($t_{18} = 2.68$, $p < 0.008$; $t_{16} = 3.17$, $p < 0.003$; one-tailed, respectively), indicating that changes in this gene were more likely the result of exposure to chronic ethanol diet in both genotypes ($F_{1,37} = 17.56$, $p < 0.0001$, diet effect) (Figure 1b). The increase in gene expression for *Th* in mutant cerebellum after ethanol diet was confirmed by qRT-PCR ($t_{18} = 2.37$, $p < 0.02$, one-tailed) (Figure 1d). The results of a two-way ANOVA (genotype $\times$ diet) also indicated significant genotype ($F_{1,37} = 16.92$, $p < 0.0001$) and diet ($F_{1,37} = 7.55$, $p < 0.02$) effects. The overall diet effect was due to a small, but significant increase in gene expression in wild-type ethanol-treated mice ($t_{16} = 2.49$, $p < 0.02$, one-tailed). Although the impact of the ethanol diet was greater in mutant mice, an overall effect of chronic ethanol cannot be ruled out. For *Bdnf*, the decrease in gene expression seen in the arrays for ethanol-treated mutant mice was not confirmed by qRT-PCR.

Of the three genes chosen from the microarrays for confirmation by qRT-PCR that were changed in wild-type mice after chronic ethanol diet, only one, the gene for the potassium channel, *Twik-1* (*Kcnk1*), was verified as a gene regulated by chronic diet in ethanol-treated wild-type mice, but not mutants (Figure 1e). The microarray results indicated that *Twik-1* gene expression was decreased in ethanol-treated wild-type mice ($p < 0.03$). This was confirmed using 14 independent samples for the qRT-PCR analyses ($t_{26} = 1.71$, $p < 0.05$, one-tailed) (Figure 1f). Data from the microarrays indicated that expression of the gene encoding the proteolipid protein, *Plp*, was significantly decreased in wild-type mice ($p < 0.03$), but not mutants ($p = 0.43$), after chronic ethanol diet; however, qRT-PCR results from 13–14 independent RNA samples demonstrated that *Plp* expression was decreased in both genotypes as a function of ethanol diet ($F_{1,54} = 16.59$, $p < 0.0001$, diet effect) (Figure 2a,b). The increase in expression found in the microarrays in wild-type ethanol-treated mice for *Adk2* ($p < 0.04$) was not confirmed by qRT-PCR ($n = 9$ –10 independent samples; data not shown).

One gene from the microarrays that changed in the same direction in both genotypes after chronic ethanol diet was chosen for confirmation as a gene associated with a response to chronic ethanol exposure. The transcription factor, *JunB*, was up-regulated in both genotypes ($p < 0.05$; $p < 0.01$; mutant and wild-type, respectively). qRT-PCR confirmation with 10 independent RNA samples verified the up-regulation due to chronic ethanol diet in both mutants and wild-type mice ($F_{1,39} = 14.06$, $p < 0.001$, diet effect) (Figure 2c,d).

One additional gene encoding the transcription factor *Nur77* (*Nr4a1*), was chosen for confirmation because the results from the microarrays indicated that an interaction between genotype and diet had occurred. Data from the microarrays indicated that the gene encoding

the nuclear receptor subfamily 4, group A, member 1 (*Nr4a1*), referred to as *Nur77*, was significantly up-regulated 2.23 fold ($p = 0.02$) in wild-type mice, and was significantly down-regulated 2.52 fold in mutant mice ($p = 0.04$) after chronic ethanol treatment. Verification experiments using 10–15 independent RNA samples from PKC γ mutant and wild-type mice confirmed the up-regulation (1.92 fold) seen in the wild-type mice ($t_{27} = 1.87$, $p < 0.04$, one-tailed), however, the down-regulation in mutant mice was not confirmed (Figure 2e,f). Analysis by ANOVA indicated that there was a significant diet effect ($F_{1,46} = 4.82$, $p < 0.04$), but no genotype $\times$ diet interaction. This was due to a non-significant up-regulation in mutant mice in the qRT-PCR samples. Therefore *Nur77* may also be involved in the effects of chronic ethanol treatment, unrelated to PKC γ .

DISCUSSION

In the microarray experiments, the expression of several genes was altered due to chronic ethanol treatment that may be potentially associated with 1) the development of tolerance; i.e., those genes that were changed in wild-type mice only, 2) the resistance to tolerance; i.e. those genes that were changed in mutant mice only, or 3) the effects of chronic ethanol treatment; i.e., those genes that changed in both genotypes. Liberal criteria were used to identify genes that might be associated with these phenotypes. However, until the changes in expression were verified by an independent method, the genes identified in the microarrays were considered putative candidates only. In the present study, the expression of eight genes were selected for verification based on their relevance to ethanol responses or to addiction, in general. Of these eight genes, six were confirmed by qRT-PCR (*Twik-1*, *JunB*, *Nur77*, *Hsp70.2*, *Th* and *Plp*). Three of these (*Hsp70.2*, *Plp*, and *Th*) that had appeared to be associated with either tolerance development or resistance to the development of tolerance based on microarray results, were found later to be more generally associated with the effects of exposure to chronic ethanol based on qRT-PCR studies. Two genes that were altered on the microarrays, *Bdnf*, and *Adk2*, were not confirmed.

Alterations in gene expression produced by chronic treatment were examined at one time point taken immediately after removal of ethanol or control diet and prior to the development of withdrawal symptoms. It is likely that many changes related to the development of ethanol tolerance may occur within a very specific time window during ethanol treatment. Thus, transient changes or early changes in expression would be missed without detailed time course studies. The genes whose changes in expression were detected in the present study probably reflect rather stable or late changes in gene expression. In addition, because tissue was taken immediately after removal of the ethanol diet, the presence of ethanol cannot be ruled out as a factor in gene expression changes. As will be discussed, there is some overlap with previous studies of the short-term and long-term effects of ethanol treatment on gene expression in mice, cell cultures, and brain tissue from human alcoholics.

4.1 Expression Changes Associated with Tolerance Development

Twik-1—Potassium channels have been shown to be affected by ethanol exposure. Recently it has been reported that G-protein-coupled inwardly rectifying potassium channel function is increased in cerebellar granule cells after acute ethanol exposure (Lewohl et al., 1999). In a microarray analysis of gene expression in the frontal cortex of human alcoholics, Lewohl et al. (2000) found a down-regulation of an ATP-regulated inwardly rectifying potassium channel, suggesting that chronic alcohol may decrease potassium channel expression and may be a factor in tolerance development. In the present study, microarray analysis demonstrated that the gene encoding a weak inwardly rectifying potassium channel (*Twik-1* or *Kcnk1*) was found to be down-regulated in wild-type mice only. This effect was confirmed by qRT-PCR indicating that this type of potassium channel may also play a role in tolerance development.

In mouse, Twik-1 protein is found in abundance in the cerebellar granule cell layer and is activated by PKC (Lesage et al., 1997). Results from the present study indicate that PKC γ may play a significant role in Twik-1 activation. Twik-1 is structurally different than other inwardly rectifying potassium channels in that it belongs to a family of 2 pore-domain potassium channels and functions to set the resting membrane potential in many cell types, including cerebellar granule cells (Lesage and Lazdunski, 2000). Expression of Twik-1 appears to be sensitive to other brain perturbations. The results of two microarray analyses in rat have shown that Twik-1 is also down-regulated in response to brain injury (Rao et al., 2003) and after chronic nicotine exposure (Konu et al., 2001).

4.2 Genes Up-Regulated by Chronic Ethanol Treatment

JunB, Nur77, Hsp70.2, Th—It is not surprising that changes in the mRNA concentrations of some transcriptional factors occur in the cerebellum as a function of chronic ethanol treatment. Both *JunB* and *Nur77* expression were increased as a function of chronic treatment, but in contrast to the usual transient increases seen in immediate early gene expression, these two factors had increases that must have persisted, or had been induced late in treatment, in order to be detected here.

Both *JunB* and *Nur77* have been previously implicated in the actions of alcohol or neuroadaptations to alcohol treatment (Beckman et al., 1997; Ogilvie et al., 1998). Members of the immediate early gene *Fos* (*c-Fos*, *FosB*, *Fra-1* and *Fra-2*) and *Jun* (*c-Jun*, *JunB* and *JunD*) families code for proteins that dimerize to form the AP-1 transcription factor which recognizes the binding motif TGACTCA. Supershift assays of AP-1 DNA binding activity in rat cortex, hippocampus, and cerebellum assayed at various times after chronic ethanol vapor treatment demonstrated increased AP-1 binding in all of these regions. Immediately after cessation of ethanol treatment, all members of the Jun family including JunB as well as cFos and Fos-B proteins were elevated in all brain regions studied (Beckman et al., 1997). Elevated *JunB* expression in both wild-type and mutant PKC γ mice after chronic ethanol treatment reproduces these effects of ethanol treatment observed in rat cerebellum observed immediately after removal from ethanol vapor. Human epidermal keratinocytes also respond to ethanol exposure with increased expression of *JunB*, and this effect is associated with increased PKC activation (Kharbanda et al., 1993). In addition, carbachol-stimulated expression of *JunB* in SH-SY5Y cells is potentiated in response to ethanol. This effect is inhibited by blocking activation of PKC, suggesting a role for PKC in ethanol potentiation of *JunB* expression (Ding et al., 1998). However, results from the present study would indicate that the isotype of PKC involved in this regulation of *JunB* expression is not PKC γ . Nur77, also known as NR4A2 and NGFI-B, is a member of the nuclear receptor family of steroid/thyroid-like receptors (Paulsen et al., 1995). As a transcriptional factor, it regulates the expression of genes by binding as monomers or dimers to hormone response elements (HREs) with two canonical sequences, 5'-AGGTCA and 5'-AGAACA, or by binding at the NGFI-B response element (NBRE) at 5'-(A/T)AAAGGTCA as a monomer (Meinke and Sigler, 1999). *Nur77* is highly expressed in many brain regions including the Purkinje cell layer and the lateral deep nucleus of the cerebellum, in hippocampus, frontal cortex, striatal complex, and hypothalamus (Xiao et al., 1996). Recently, Daniels and Buck (2002) demonstrated increased *Nur77* expression in the hippocampus during withdrawal from chronic ethanol treatment suggesting that *Nur77* has some role in the hippocampus related to the hyperexcitability that causes handling induced seizures.

Quantitative RT-PCR experiments in the present study identified two additional genes that were up-regulated in response to chronic ethanol diet, *Hsp70.2* and *Th*. Therefore, it was of interest to see if the up-regulation of the transcription factors, *JunB* and *Nur77* could be associated with the up-regulation of *Hsp70.2* and *Th*. A search of 5' untranslated regions (UTR)

of these genes for *JunB* and *Nur77* binding sites identified two potential sites for *Nur77* in 506 bp of the 5' UTR of the *Th* gene. This suggests a potential relationship between the up-regulation of these two genes. Further studies would need to be done to confirm whether *Th* is regulated by *Nur77*. No binding sites for either transcription factor were found in 630 bp of the 5' UTR of the *Hsp70.2* gene. This does not preclude unidentified binding sites further upstream of *Hsp70.2*, however.

Heat shock proteins (HSP) are considered molecular chaperones that regulate a variety of cellular functions and act as neuroprotective factors against environmental stressors such as heat shock, some chemical compounds, and brain injury (Allen and Chase, 2001; Ohtsuka et al., 2005). Alterations in expression of several members of the diverse heat shock gene family were observed in rat brain after chronic treatment with morphine (Ammon et al., 2003), after acute ethanol treatment of *C. elegans* (Kwon et al., 2004), and mouse hippocampus during withdrawal from chronic ethanol treatment (Daniels and Buck, 2002). Data from the microarray experiments presented here indicated that gene expression of one member of the 70 k Da HSP family, *Hsp70.2*, was up-regulated in mutant mice only after ethanol diet, suggesting that it may play a role in the resistance to tolerance. However, confirmation with independent samples showed that *Hsp70.2* was up-regulated in both genotypes after ethanol exposure. This implies that *Hsp70.2* responded to the deleterious effects of the chronic ethanol diet, unrelated to the development of tolerance or to regulation by PKC γ .

From previous studies, it might be expected that the catecholamine biosynthesis pathway would be impacted by exposure to ethanol. Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of dopamine (Kumer and Vrana, 1996). An up-regulation of *Th* was observed in prefrontal cortex in DBA/2J mice after acute ethanol treatment (Kerns et al., 2005). However, it was somewhat unexpected to find an increase in *Th* gene expression in the cerebellum, a brain region not usually associated with a dopamine response to reward. Studies have shown that dopamine innervation, tyrosine hydroxylase immunoreactivity, and mRNA are found in the cerebellum of humans, primates and rodents (Fujii et al., 1994; Hurley et al., 2003; Melchitzky and Lewis, 2000). In humans, it has been suggested that changes in dopaminergic markers in the cerebellum of Parkinson's patients may explain the poor motor coordination found in this disease (Hurley et al., 2003). Further evidence that tyrosine hydroxylase is involved in abnormal motor activity is the observation that abnormal expression of *Th* immunoreactivity is found ectopically in the cerebellum of several ataxic mouse mutants, including weaver mutants, rolling mouse Nagoya, *dilute-lethal* mice, and *pogo* mutant mice (Abbott and Sotelo, 2000; Jeong et al., 2001; Sawada et al., 1999). This may be relevant to the PKC γ mutant mice, since they demonstrate ataxia, due to abnormal development of the innervation of multiple climbing fibers onto Purkinje cells in the cerebellum (Chen et al., 1995). In the qRT-PCR experiments, increased expression of *Th* was found in mutant control-diet mice compared to wild-type control-diet mice (see Figure 1c,d) suggesting that endogenous *Th* expression is increased in PKC γ mutant cerebellum. Gayer et al. (1991) have shown that *Th* gene expression is increased in neuroblastoma cells after ethanol exposure. Although expression was increased in both genotypes, suggesting a general effect of chronic ethanol exposure, the effect was greater in mutant mice compared to wild-types. This may reflect a greater sensitivity in mutant mice to the interaction of ethanol and the stress of the chronic ethanol paradigm. Tyrosine hydroxylase mRNA has been shown to increase in response to stress, drug administration, and food restriction (Kumer and Vrana, 1996; Lindblom et al., 2006). Because the mutant mice do not become tolerant to the effects of ethanol (Bowers et al., 1999), they are physiologically stressed throughout the exposure and they consume less ethanol diet than wild-type mice; therefore, experiencing self-imposed food restriction.

4.3 One Gene Down-Regulated by Chronic Ethanol Treatment

Plp—Acute ethanol treatment induced an increase in expression of a cluster of myelin and myelin-related genes including proteolipid protein (*Plp*) in mouse prefrontal cortex (Kerns et al., 2005). Long-term exposure to ethanol produces the opposite effect in humans. As reported here, chronic treatment in the mouse also down-regulates *Plp* expression. Microarray analyses of brain tissue from human alcoholics have consistently revealed down-regulation of myelin-related genes (Flatscher-Bader et al., 2005; Lewohl et al., 2000; Liu et al., 2004; Mayfield et al., 2002; however, see Iwamoto et al., 2004). This implies that chronic alcohol abuse decreases the transcription of these genes resulting in a decrease in myelin-related proteins.

Neuroimaging studies of alcoholic men and women have shown reduced white and gray matter volumes in several brain regions compared to non-drinking controls that may be the result of the down-regulation of these genes (Fein et al., 2002; Hommer et al., 2001; Pfefferbaum et al., 1992). Specifically, cerebellar atrophy is one of the hallmarks of alcohol abuse (Estrin, 1987; Karhune et al., 1994; Torvik and Torp, 1986). Proteolipid protein (*Plp*) is one of the most abundant proteins in central nervous system myelin and accounts for nearly 50% of myelin-related proteins. Regulation of expression of the *Plp* gene is very important for normal brain structure and plays a role in the interaction between oligodendrocytes and axons (Wight and Dobretsova, 2004). Results from the microarray experiments in the present study initially indicated that *Plp* expression was significantly down-regulated in wild-type cerebellum after ethanol exposure; however, a nonsignificant decrease was also observed in PKC γ mutant mice suggesting that *Plp* expression is more likely related to chronic ethanol diet exposure than to tolerance development. Confirmation using independent RNA samples and qRT-PCR supported this observation since significant down-regulation was seen in both genotypes after ethanol diet. Levels of expression in mutant and wild-type control mice were similar indicating that the regulation of transcription of *Plp* is not dependent on PKC γ .

In conclusion, the data presented here are the results of a two-stage process for identifying genes that may be associated with the development of tolerance to the sedative-hypnotic effects of ethanol in mice. The premise of these experiments was to find genes differentially expressed between PKC γ wild-type mice that do develop tolerance and PKC γ mutant mice that do not. The preliminary screen of gene expression using microarray technology identified several candidate genes. Using relatively liberal criteria, a subset of genes was selected for confirmation by qRT-PCR in the second stage. This two stage process resulted in the following findings: 1) a relationship between PKC γ and tolerance development was confirmed for one gene, *Twik-1*; whereas, 2) the expression of five of the remaining genes were found to be associated with the effects of exposure to chronic ethanol independent of any regulation by PKC γ or tolerance development. Alterations in the transcriptional regulation of several of these genes including: *Twik-1*, *JunB*, *Nur77*, and *Plp* have been observed in a number of studies and are likely candidates for further study regarding acute and chronic effects of ethanol.

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FIGURE 1.

Gene expression changes in PKC γ mutant and wild-type mice after chronic ethanol diet as measured by microarray chip analyses (panels a., c., e.) with confirmation by qRT-PCR analyses of independent samples (panels b., d., f.). WTC: wild type control diet; WTE: wild type ethanol diet; MTC: mutant control diet; MTE: mutant ethanol diet. a.) Heat shock protein 70.2 microarray; * $p < 0.05$ increase after ethanol diet; b.) Heat shock protein 70.2 qRT-PCR; * $p < 0.001$ increase after ethanol diet; c.) Tyrosine hydroxylase microarray, * $p < 0.05$ increase after ethanol diet; d.) Tyrosine hydroxylase qRT-PCR, * $p < 0.02$ increase after ethanol diet; e.) Twik-1 microarray, * $p < 0.05$ decrease after ethanol diet; f.) Twik-1 qRT-PCR, * $p < 0.03$ decrease after ethanol diet.

FIGURE 2.

Gene expression changes in PKC γ mutant and wild-type mice after chronic ethanol diet as measured by microarray chip analyses (panels a., c., e.) with confirmation by qRT-PCR analyses of independent samples (panels b., d., f.). WTC: wild type control diet; WTE: wild type ethanol diet; MTC: mutant control diet; MTE: mutant ethanol diet. a.) Phospholipid protein microarray, * $p < 0.05$ decrease after ethanol diet; b.) Phospholipid protein qRT-PCR, * $p < 0.001$ decrease after ethanol diet; c.) JunB microarray, * $p < 0.05$ increase after ethanol diet; d.) JunB qRT-PCR, * $p < 0.001$ increase after ethanol diet; e.) Nur77 microarray, * $p < 0.05$ increase after ethanol diet in wild-type mice and decrease in mutant mice after ethanol diet; f.) Nur77 qRT-PCR, * $p < 0.05$ increase in both genotypes after ethanol diet.

TABLE 1

PKC γ mutant expression changes due to chronic ethanol diet. Cellular process with fold change $\geq \pm 1.3$; $p < 0.05$. Genes are divided into functional groups; $p < 0.05$.

Second Level GO Term	Gene Name	Gene Description	Fold Change	Sixth Level GO Term	Genbank Accession	Locuslink Accession	Affymetrix Accession
Cellular Physiological Processes	<i>Lyn</i>	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog ^{1,3,13}	-1.46	ATP binding; protein kinase activity	M57696	17096	103349_at
	<i>Igflr</i>	Insulin-like growth factor I receptor ^{1,3,12,13}	1.39	Insulin-like growth factor receptor signaling pathway	AF056187	16001	102224_at
	<i>Ar14</i>	ADP-ribosylation factor-like 4 ^{1,9,10,11,12}	1.71	GTP binding; GTPase activity	Y12577	11861	92805_s_at
	<i>Tle4</i>	Transducin-like enhancer of split 4, homolog of Drosophila E(spl) ^{1,2,3,9,10,11,13}	1.81	Wnt receptor signaling pathway	U61363	21888	102959_at
	<i>Th</i>	Tyrosine hydroxylase ^{1,3,8,9,11}	2.23	Catecholamine biosynthesis	M69200	21823	100690_at
	<i>Col4a1</i>	Procollagen, type IV, alpha 1 ^{1,10,11}	1.51	Extracellular matrix structural constituent	M15832	12826	101093_at
	<i>Clqr1</i>	Complement component 1 Q subcomponent, receptor ^{1,4,9,10,11,12}	1.73	Calcium binding; defense response	AF081789	17064	93694_at
	<i>Spp1</i>	Secreted phosphoprotein 1 ^{1,2,4,5,8,12,13}	1.71	Cell adhesion; cytokine activity	X13986	20750	97519_at
	<i>Grik2</i>	Glutamate receptor, ionotropic, kainate 2 (beta 2) ^{1,12,13}	1.57	Glutamate-gated ion channel; ion transport	X66117	14806	98863_at
	<i>Bdnf</i>	Brain derived neurotrophic factor ^{1,2,3,5,8,9,10,11,12,13}	-2.07	Growth factor activity; feeding behavior	X55573	12064	102727_at
	<i>Rad50</i>	RAD50 homolog (S. cerevisiae) ^{1,3,4,7,9,10,11,14}	-1.35	ATP binding; DNA repair	U66887	19360	100459_at
	<i>Casp3</i>	Caspase3, apoptosis related cysteine protease ^{1,3,4,5,10,14}	1.42	Caspase activity; apoptosis; B-cell homeostasis	U63720	12367	98437_at
	<i>Bat8</i>	HLA-B associated transcript 8 ^{1,3,9,10,11,12}	1.43	DNA binding; chromatin modification	AF109906	110147	100946_at
	<i>Hist 1 H2ab</i>	Histone 1, H2ab ^{1,3,9,10,11}	1.33	DNA binding; nucleosome assembly	M33988	319172	94805_f_at
	<i>Rbp1</i>	Retinol binding protein 1, cellular ^{1,3}	1.41	Retinoid metabolism	X60367	19659	104716_at
	<i>Gas1</i>	Growth arrest specific 1 ^{1,2,5,13}	1.31	Cell cycle arrest	X65128	14451	94813_at
	<i>Gadd45 a</i>	Growth arrest and DNA-damage-inducible 45 alpha ^{1,2,4,7,9,10,11,13}	1.59	Cell cycle arrest; response to DNA damage	U00937	13197	102292_at
	<i>Dnm12</i>	DNA methyltransferase 2 ^{1,3,9,10,11}	-1.68	DNA binding; proteolysis	AF012129	13434	93470_at
	<i>Hmgp2</i>	High mobility group nucleosomal binding domain 2 ^{1,3,9,10,11}	1.52	DNA binding; DNA packaging	X12944	15331	101589_at
	<i>Zfp61</i>	Zinc finger protein 61 ^{1,3,9,10,11,14}	-1.34	Regulation of transcription; DNA-dependent	L28167	22719	102303_i_at
	<i>Irx3</i>	Iroquois related homeobox 3 ^{1,3,9,10,11}	-1.41	DNA binding; regulation of transcription	Y15001	16373	99034_at
	<i>Cisx</i>	Cathepsin Z ^{1,3,9,10,11,12,14}	1.39	Lysosome; proteolysis	AJ242663	64138	92633_at
	<i>Ddc</i>	Dopa decarboxylase ^{1,3}	-1.75	Catecholamine biosynthesis	AF071068	13195	160074_at
	<i>Usp9x</i>	Ubiquitin specific protease 9, X chromosome ^{1,3,14}	-1.33	Ubiquitin-dependent protein catabolism	U67874	22284	99102_at
	<i>Bcat2</i>	Branched chain aminotransferase 2, mitochondrial ^{1,3,9,10,11,12}	1.44	Transferase activity; metabolism	AF031467	12036	100443_at

Second Level GO Term	Gene Name	Gene Description	Fold Change	Sixth Level GO Term	Genbank Accession	Locuslink Accession	Affymetrix Accession
	<i>Psmbl10</i>	Proteasome(prosome, macropain) subunit, beta type ^{10,13,10,14}	-1.32	Endopeptidase activity	Y10875	19171	101486_at
	<i>Tpi</i>	Triosephosphate isomerase ^{1,3}	-1.31	Fatty acid biosynthesis	L31777	21991	99566_at
	<i>Hsd17b12</i>	Hydroxysteroid (17-beta) dehydrogenase ^{12,13}	-1.45	Oxidoreductase activity; steroid biosynthesis	AF064635	56348	94276_at
	<i>Pja1</i>	Praja1, RING-H2 motif containing ^{1,3,13}	1.64	Ligase activity; protein catabolism	U06944	18744	101461_f_at
	<i>Nab2</i>	Ngfi-A binding protein ^{2,12,3,9,10,11}	-1.35	Regulation of transcription	U47543	17937	100962_at
	<i>Agt</i>	Angiotensin ^{1,2,3,4,6,8,12,13}	1.33	Regulation of blood pressure	AF045887	11606	101887_at
	<i>Hspa2</i>	Heat shock protein 2 ^{1,3,4,9,10,11,13}	1.63	Chaperone activity; response to heat	M20567	15512	99816_at
	<i>Hspa1b</i>	Heat shock protein 1B ^{1,2,3,4,5,7,13}	1.43	Response to heat; anti- apoptosis	AF109906	15511	97809_at
	<i>Per2</i>	Period homolog 2 (Drosophila) ^{1,3,6,10,11,13}	-2.40	Circadian rhythm; signal transduction	AF036893	18627	93694_at
Rhythmic Processes	<i>Cry1</i>	Cryptochrome 1 (photolyase-like) ⁶	-1.33	Circadian rhythm; lyase activity	AB000777	12952	94420_f_at
Membrane Bound Organelle	<i>Cops7a</i>	COP9 (constitutive photo-morphogenic) homolog, subunit 7a (Arabidopsis thaliana) ^{9,10,11}	-1.31	Cellular and developmental processes	AF071316	26894	92874_at
	<i>Arxa11</i>	Annexin A11 ^{9,10,11,13}	1.45	Binds to calyculin	U65986	11744	102815_at
Extracellular Space	<i>Ogn</i>	Osteoglycan ^{12,13}	1.82	Growth factor activity	D31951	18295	99549_at
Intracellular	<i>Mtcp1</i>	Mature T-cell proliferation ¹¹⁰	1.36	Mitochondrion	Z35294	26894	103043_at
	<i>Copa</i>	Coatomer protein complex subunit alpha ^{1,9,10,11,12}	-1.31	Mediates protein transport	AJ010391	12847	96854_at
	<i>Ggh</i>	Gamma-glutamyl hydrolase ^{1,3,9,10,11,12,14}	1.41	Lysosome; hydrolase activity	AF051102	14590	93575_at
No Assignment to Functional Group at GO Level 2 at p < 0.05	<i>Nefh</i> <i>A33010-</i> <i>N21Rik</i>	Neurofilament, heavy polypeptide ^{1,9,10,11} RIKEN cDNA A330103N2 gene	-1.30 1.39	Intermediate filament	M35131 AF056187	18026	103234_at 102224_at
	<i>Myadm</i>	Myeloid-associated differentiation marker	1.33	Integral to membranes	AJ001616	50918	96285_at

Functional Groups as determined by DAVID Bioinformatics Functional Group Analysis (<http://maid.abcc.ncifcrf.gov/home.jsp>):

¹ Cellular Physiological Processes;

² Negative Regulation of Biological Processes;

³ Metabolism;

⁴ Response to Stress;

⁵ Death;

⁶ Rhythmic Processes;

- ⁷ Response to Endogenous Stimuli;
- ⁸ Behavior;
- ⁹ Membrane-Bound Organelle;
- ¹⁰ Intracellular;
- ¹¹ Intracellular Organelle;
- ¹² Extracellular Space;
- ¹³ Protein Binding;
- ¹⁴ Hydrolase Activity.

TABLE 2

PKC γ wild-type expression changes due to chronic ethanol diet with fold change $\geq \pm 1.3$; $p < 0.05$. Genes are divided in functional groups; $p < 0.05$.

Second Level GO Term	Gene Name	Gene Description	Fold Change	Sixth Level GO Term	Genbank Accession	Locuslink Accession	Affymetrix Accession
Cellular Physiological Processes	<i>Prkcd</i>	Protein kinase C delta ^{1,3,7}	1.37	Protein serine/threonine kinase activity; amino acid phosphorylation	X60304	18753	104531_at
	<i>Ankl</i>	Ankyrin 1 erythroid ^{1,4,6}	1.35	Transcription factor activity; cytoskeleton	X69064	11733	10441_s_at
	<i>Edn1</i>	Endothelin 1 ^{1,2,3,6}	1.89	G-protein coupled receptor protein signaling pathway	U35233	13614	102737_at
	<i>Tle1</i>	Transducin-like enhancer of split 1, homolog of Drosophila E(sp1) ^{1,4,5,6}	-1.32	Wnt receptor signaling pathway; regulation of transcription	U61362	21885	102425_at
	<i>Egr1</i>	Early growth response 1 ^{1,4}	1.42	Regulation of transcription	M28845	13653	98579_at
	<i>Chd1</i>	Chromodomain helicase DNA binding protein 1 ^{1,4,5,7}	1.4	Chromatin assembly/disassembly; ATP-dependent helicase activity	L10410	12648	101459_at
	<i>Apaf1</i>	Apoptotic protease activating factor 1, 2,3,4,5,6,7	-1.41	Apoptosis; brain development; ATP binding	AF064071	11783	103796_at
	<i>Sept6</i>	Septin 6 ^{1,7}	1.33	GTP binding; cytokinesis	AB023622	56526	92462_at
	<i>Tfr</i>	Transferrin receptor ^{1,4,5}	-3.44	Endocytosis; iron ion transporter activity; proteolysis/peptidolysis	X57349	22042	X57349_5_at
	<i>Kcnk1</i>	Potassium channel, subfamily K, member 1 ^{1,4,5,6}	-1.73	Potassium ion transport; integral to membrane	AF033017	16525	102335_at
	<i>Dm15</i>	Dystrophia myotonia kinase, B15 ^{1,7}	1.42	Protein serine/threonine kinase activity; meiosis	Z38015	13401	93431_at
	<i>Mt3</i>	Metallothionein 3 ^{1,3,4,5}	1.37	Metal ion binding; negative regulation of neurogenesis	M93310	17751	95340_at
	<i>Alas2</i>	Aminolevulinic acid synthase 2, erythroid ^{1,5}	-2.00	5-aminolevulinate synthase activity; biosynthesis	M15268	11656	92768_at
	<i>Dao1</i>	D-amino acid oxidase ^{1,4,5,7}	1.38	Electron transport; tRNA aminoacylation for protein translation	D10210	13142	103602_at
	<i>Lamp2</i>	Lysosomal membrane glycoprotein 2 ^{1,4,5,7}	1.35	tRNA aminoacylation for protein translation; lysosome	M32017	16784	100136_at
	<i>Cik</i>	CDC-like kinase ^{1,4,5,7}	-1.31	Protein serine/threonine kinase activity	M38381	12747	93274_at
	<i>Klf4</i>	Kruppel-like factor 4 (gut) ^{1,4,5}	1.53	Regulation of transcription	U20344	16600	99622_at
	<i>Max</i>	Max protein ^{1,4,5,6}	-1.32	Regulation of transcription	M63903	17187	99095_at
	<i>Tcfap2b</i>	Transcription factor AP-2 beta ^{1,4,5,6}	1.33	Transcription factor activity; DNA binding	X78197	21419	92903_at
	<i>Rad23b</i>	RAD23b homolog (S. cerevisiae) ^{1,3,4,5}	1.43	DNA repair; response to DNA damage	X92411	19359	96102_at
	<i>Srrpd1</i>	Small nuclear ribonucleoprotein D1 ^{1,4}	-1.64	mRNA processing; spliceosome complex	M58558	20641	10057_at
	<i>Bpgm</i>	2,3-bisphosphoglycerate mutase ¹	-1.34	Catalytic activity; phosphotransferases	X13586	12183	94815_at
	<i>Suca2</i>	Succinate-Coenzyme A ligase, ADP-forming, beta subunit ^{1,4}	-1.34	Catalytic activity; glycolysis	AF058955	20916	93501_f_at
	<i>Impa1</i>	Inositol (myo)-1(or4)-monophosphatase 1 ¹	-1.33	Myo-inositol metabolism; magnesium ion binding	AF042730	55980	101498_at
	<i>Ptp4a2</i>	Protein tyrosine phosphatase 4a2 ¹	1.38	Protein amino acid dephosphorylation	AF064071	11783	103796_at

Second Level GO Term	Gene Name	Gene Description	Fold Change	Sixth Level GO Term	Genbank Accession	Locuslink Accession	Affymetrix Accession
Response to Stress	<i>Clqa</i>	Complement component 1,q subcomponent, alpha polypeptide ^{1,3,5}	1.42	Complement activation, classical pathway; extracellular space	X58861	12259	98562_at
	<i>Chtr1</i>	Corticotrophin releasing hormone receptor 1 ³	-1.41	G-protein coupled receptor activity; membrane	X72305	12921	95290_at
	<i>Banp</i>	Btg3 associated nuclear protein ⁴	-1.34	Putative transcription factor	AF091234	53325	98512_at
Intracellular/ Intracellular Organelle	<i>Mybpc3</i>	Myosin binding protein C, cardiac ^{4,6}	1.42	Actin binding; structural constituent of cytoskeleton	AF059576	17868	160582_at
	<i>Ddx3y</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked ^{4,5,7}	-2.75	ATP-dependent RNA helicase activity; nucleic acid binding	AJ007376	26900	103842_at
	<i>Psmc3</i>	Proteasome (prosome, macropain) 28 subunit, 3 ⁴	1.38	Proteasome activator complex	AB007139	19192	93803_at
Protein Binding	<i>Grb2</i>	Growth factor receptor bound protein 2 ^{4,5,6}	1.38	RAS protein signal transduction	U07617	14784	10134_at
	<i>Suca</i>	Synuclein, alpha ^{1,4,6}	-1.33	Cytoplasm	AF044672	20617	93273_at
	<i>Lmna</i>	Lamin A ^{1,4,6}	1.35	Intermediate filament	D49733	16905	98159_s_at
No Assignment to Functional Group at GO Level 2 at p < 0.05	<i>Cacypbp</i>	Calcyclin binding protein ^{1,4,5,6}	-1.34	Molecular bridge	U97327	12301	92596_at
	<i>Adk2</i>	Adenylate kinase 2 ^{1,4,5,7}	1.64	Catalytic activity	AB020202	11637	95148_at
	<i>Whp2</i>	WW domain binding protein 2 ⁶	1.35	Binding protein	U40826	22378	92683_at
Protein Binding	<i>Cldn5</i>	Claudin 5 ⁶	1.46	Integral to membrane	U82758	12741	104516_at
	<i>Btg2</i>	B-cell translocation gene 2, antiproliferative ^{1,3,6}	1.52	Antiproliferative protein	M64292	12277	101583_at
	<i>Nell2</i>	Nel-like 2 homolog (chicken)	1.55	Calcium ion binding	U59230	54003	92358_at
Response to Stress	<i>Plp</i>	Proteolipid protein (myelin)	-1.59	Myelination; synaptic transmission	M16472	18823	92802_s_at
	<i>Istr</i>	Immunoglobulin superfamily containing leucine-rich repeat	1.45	Extracellular space	AB024538	26968	99010_at
	<i>C920001</i> <i>C06Rik</i>	RIKEN cDNA C920001C06 gene	1.33		AB023622		92462_at

Functional groups as determined by DAVID Bioinformatics Functional Group Analysis (<http://niad.abcc.ncifcrf.gov/home.jsp>):

¹ Cellular Physiological Processes;

² Tube Development;

³ Response to Stress;

⁴ Intracellular/Intracellular Organelle;

⁵ Membrane-Bound Organelle;

⁶ Protein Binding;

⁷ Nucleotide Binding.

TABLE 3

Gene expression changes in both PKC γ mutant and wild-type mice due to chronic ethanol diet. Cellular process with fold change $\geq \pm 1.3$ in at least one group; both changes $p < 0.05$. Fold changes with positive values indicate up-regulation of expression; negative values indicate down-regulation of expression.

Second Level GO Term	Gene Name	Gene Description	MUT Fold Change	WT Fold Change	Sixth Level GO Term	Genbank Accession	Locuslink Accession	Affymetrix Accession
Cell Communication/Differentiation	<i>Ptpn</i>	Protein tyrosine phosphatase, receptor type, N	1.36	1.26	Integral to membrane; transmembrane receptor	U11812	19275	104422_at
	<i>Myo10</i>	Myosin X	1.32	1.62	protein tyrosine phosphatase signaling pathway	AJ249706	17909	100932_at
Cellular Physiological Process/Cell Death	<i>Junb</i>	Jun-B oncogene	4.16	2.22	Actin binding; cytoskeleton organization and biogenesis	U20735	16477	102362_i_at
	<i>Nr4a1</i>	Nuclear receptor subfamily 4, group A, member 1 (Nur77)	2.23	-2.52	Transcription factor activity; regulation of cell cycle; DNA binding	X16995	15370	102371_at
Metabolism	<i>Pnkdc</i>	Protein kinase, DNA activated, catalytic polypeptide	-1.54	-1.3	Transcription factor activity; inhibition of caspase activation; steroid hormone receptor activity	AB011543	19090	93476_at
	<i>Igf2</i>	Insulin-like growth factor binding protein 2	1.24	1.46	DNA repair; apoptosis; protein serine/threonine kinase activity	X81580	16008	98627_at
	<i>Hk2</i>	Hexokinase 2	-1.58	-1.76	Growth factor binding; regulation of cell growth	Y11666	15277	94375_at
	<i>Ptp4a2</i>	Protein tyrosine phosphatase 4a2	1.38	1.24	ATP binding; glycolysis; transferase activity	AF035644	19244	100595_at
Morphogenesis	<i>Efnb2</i>	Ephrin B2	-1.14	1.37	Protein amino acid dephosphorylation	U30244	13642	160857-at
Unclassified	<i>Nip-snap1</i>	4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1 (C.elegans)	1.4	-1.26	Development; integral to membrane; neurogenesis	AJ001260	18082	93251_at
	<i>Tex261</i>	Testis expressed gene 261	1.29	1.37	Biological process unknown	X81580	21766	98627_at