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Analyses of differentially expressed genes after exposure to acute stress, acute ethanol, or a combination of both in mice

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Abstract

Alcohol abuse is a complex disorder, which is confounded by other factors, including stress. In the present study, we examined gene expression in the hippocampus of BXD recombinant inbred mice after exposure to ethanol (NOE), stress (RSS), and the combination of both (RSE). Mice were given an intra-peritoneal (i.p.) injection of 1.8 g/kg ethanol or saline, and subsets of both groups were exposed to acute restraint stress for 15 min or controls. Gene expression in the hippocampus was examined using microarray analysis. Genes that were significantly ($p < 0.05$, $q < 0.1$) differentially expressed were further evaluated. Bioinformatic analyses were predominantly performed using tools available at GeneNetwork.org, and included gene ontology, presence of *cis*-regulation or polymorphisms, phenotype correlations, and principal component analyses. Comparisons of differential gene expression between groups showed little overlap. Gene Ontology demonstrated distinct biological processes in each group with the combined exposure (RSE) being unique from either the ethanol (NOE) or stress (RSS) group, suggesting that the interaction between these variables is mediated through diverse molecular pathways. This supports the hypothesis that exposure to stress alters ethanol-induced gene expression changes and that exposure to alcohol alters stress-induced gene expression changes. Behavior was profiled in all groups following treatment, and many of the differentially expressed genes are correlated with behavioral variation within experimental groups. Interestingly, in each group several genes were correlated with the same phenotype, suggesting that these genes are the potential origins of significant genetic networks. The distinct sets of differentially expressed genes within each group

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provide the basis for identifying molecular networks that may aid in understanding the complex interactions between stress and ethanol, and potentially provide relevant therapeutic targets. Using *Ptp4a1*, a candidate gene underlying the quantitative trait locus for several of these phenotypes, and network analyses, we show that a large group of differentially expressed genes in the NOE group are highly interrelated, some of which have previously been linked to alcohol addiction or alcohol-related phenotypes.

Keywords

Ethanol; Stress; Gene expression; Hippocampus; Bioinformatic analysis

1. Introduction

Alcohol is one of the most widely abused drugs in the world, making it critical to identify factors that contribute to alcohol abuse and alcoholism. Stress is a factor that can increase the risk for alcoholism. Exposure to stress and alcohol have been shown to have a number of interactive effects, including that alcohol consumption can ameliorate the effects of stress (Becker, Lopez, & Doremus-Fitzwater, 2011; Moonat & Pandey, 2012; Pohorecky, 1991). Circulating glucocorticoid levels caused by stress may also enhance the reinforcing effects of alcohol (Anthenelli & Grandison, 2012; Costin, Wolen, Fitting, Shelton, & Miles, 2013; Fahlke & Hansen, 1999; Rose, Shaw, Prendergast, & Little, 2014). Despite its anxiolytic properties, alcohol activates the hypothalamic-pituitary-adrenal (HPA) axis, causing release of corticosterone in rodents (Dogan, Lei, Beach, Brody, & Philibert, 2016; Yong et al., 2014). While advances have been made in understanding this complex relationship between stress and alcohol consumption, it has yet to be fully understood, particularly at the molecular level.

Behavioral and physiological responses to stress and ethanol exposure have strong genetic underpinnings mediated by differential gene expression (Goldowitz et al., 2006; Hitzemann et al., 2004; Sinha, 2001; Sokoloff, Parker, Lim, & Palmer, 2011; Yang et al., 2014). However, studies are just beginning to characterize how genetic differences modulate the interactions between stress and alcohol. For example, lines of mice and rats selectively bred for alcohol preference phenotypes have shown behavioral differences as well as expression differences of various molecules in the HPA axis (Chester, Blose, Zweifel, & Froehlich, 2004; Chester, Kirchhoff, & Barrenha, 2014; Yong et al., 2014). The strains used in the present study, the BXD recombinant inbred (RI) strains, exhibited differences in HPA activation that are also correlated with a number of ethanol- and anxiety-related phenotypes (Porcu et al., 2011). Taken together, these studies demonstrate that genetic factors have a significant impact on responses to combined stress and alcohol exposure.

Many brain regions have been nominated as mediators of stress- and ethanol-related effects (Liu et al., 2006; Melendez, McGinty, Kalivas, & Becker, 2012; Paz & Pare, 2013; Sala et al., 2004). In the present study, changes in gene expression were examined in the hippocampus for the following reasons: 1) the hippocampus has been shown to be one of the major brain areas in the stress axis (as reviewed in McEwen, 2002; McEwen & Milner,

2007); 2) the hippocampal formation is particularly sensitive to ethanol exposure, exhibiting morphological and neurochemical changes after ethanol exposure (Beresford et al., 2006; Durazzo et al., 2011; Staples, Kim, & Mandyam, 2015); and 3) studies have used imaging technologies to examine neuroanatomical changes in individuals with post-traumatic stress disorder (PTSD) with concomitant alcohol abuse problems and demonstrated that while stress causes hippocampal deficits, alcohol abuse enhances these effects (Hedges & Woon, 2010; Star evi et al., 2015).

We used the BXD family of RI mice, which are derived by crossing C57BL/6J and DBA/2J inbred strains, followed by over 20 generations of inbreeding of the resulting progeny. BXD RI mice have been used in quantitative trait locus (QTL) analyses and have been instrumental in identifying relevant chromosomal loci involved in ethanol and stress responses as well as potential candidate genes that underlie these QTLs (Mulligan et al., 2011, 2012, 2006; Belknap & Atkins, 2001; Buck, Metten, Belknap, & Crabbe, 1997; Cook et al., 2015; Hitzemann et al., 2004; Porcu et al., 2011; Wang et al., 2012; Weng, Symons, & Singh, 2009). Furthermore, BXD RI mice have been extensively evaluated for expression differences across a wide variety of brain regions and other organs (Boughter et al., 2012; Di Curzio & Goldowitz, 2011; Jellen et al., 2012; Lu et al., 2008, 2011; Mozhui, Lu, Armstrong, & Williams, 2012; Overall et al., 2009; Parker et al., 2014; Swaminathan, Lu, Williams, Lu, & Jablonski, 2013). The combination of all the data generated using BXD mice provides a powerful collection of resources that can be used to uncover genetic networks involved in complex phenotypes, making them an excellent model system.

Previously, our lab found significant strain differences in anxiety-related behaviors in the elevated zero maze in BXD mice exposed to ethanol, stress, or a combination of both (Ziebarth et al., 2012). We were subsequently able to identify a QTL on murine chromosome 1 mediating these genetic differences (Cook et al., 2015). These results provide the impetus for examining changes in gene expression in animals that were behaviorally tested. Specifically, to begin to define genetic networks important in stress and ethanol interactions, we compared differences in gene expression after exposure to stress, ethanol, or the combination of both. The identification of gene networks is a more salient approach to understanding such interactions, as the identification of a single gene in these complex phenotypes has generally provided an incomplete picture (Wolen & Miles, 2012). The purpose of the present study was to identify differentially expressed genes and use an array of bioinformatic analyses to identify the most salient candidate genes that may be members of relevant genetic networks underlying stress and ethanol interactions.

2. Materials and methods

2.1. Acute stress and acute ethanol treatments, tissue dissection, and RNA isolation

Forty-five BXD strains and parental strains of adult male and female mice (an average of 2 mice per strain per group; total number of animals used = 241) were used to examine the changes in gene expression following either exposure to stress, ethanol, or the combination of both (Ziebarth et al., 2012). All animals were age- and sex-matched with littermates assigned to different groups but tested on the same day; the majority of animals were 65–90 days old at the time of testing. Within each strain, animals were separated into 4 groups:

acute stress (RSS), acute ethanol (NOE), combined acute stress with ethanol injection (RSE), and saline control (NOS). Ethanol-treated animals received a 1.8-g/kg i.p. injection of ethanol (12.5% v/v). Stress-exposed animals were immobilized in a tube for 15 min, and the RSS group was given an isovolumetric injection of saline as opposed to the RSE group who received ethanol. Control animals received saline injections (isovolumetric to the ethanol dose), but were not exposed to stress or ethanol injections. In animals receiving combined treatments, ethanol injections occurred immediately after the stress exposure. Five minutes post-injection, animals were tested in an elevated zero maze as previously reported (Cook, Crounse, & Flaherty, 2002; Cook et al., 2015; Ziebarth et al., 2012). Each animal was tested individually in an elevated zero maze for 10 min. The measures collected include time and activity in both the open and closed quadrants, and latency to enter the open quadrants. Four hours after the initial injection, mice were sacrificed by cervical dislocation and brains were removed (Wang et al., 2012). Hippocampal dissection was conducted and hippocampal RNA was isolated according to manufacturer's protocol using RNA STAT-60 (Lu, Airey, & Williams, 2001; Wang et al., 2012). All animal work was conducted in accordance with procedures approved by the Institutional Animal Care and Use Committees at The University of Tennessee Health Science Center and University of Memphis following NIH guidelines.

2.2. Microarray analysis of stress and/or ethanol-treatment groups

Gene expression in BXD strains was examined using microarray analysis as previously described (Mozhui et al., 2010; Wang et al., 2012; Ziebarth et al., 2012). Hippocampal gene expression was analyzed using Illumina v6.1 microarrays, according to the manufacturer's protocol (<http://www.illumina.com/>). All data were normalized using the rank invariant method and background subtraction protocols outlined by Illumina in the BeadStation software.

2.3. Analyzed groups and criteria

The following data sets for the three treatment groups (RSS, NOE, RSE) and control group (NOS) were used in the analyses and can be publicly accessed at GeneNetwork.org: UTHSC Hippocampus Illumina v6.1 NOS (Nov12) RankInv; UTHSC Hippocampus Illumina v6.1 RSS (Nov12) RankInv; UTHSC Hippocampus Illumina v6.1 NOE (Nov12) RankInv; and UTHSC Hippocampus Illumina v6.1 RSE (Nov12) RankInv. Each group contained at least 34 strains. One-way ANOVA and Tukey's *post hoc* tests were performed to determine significant differences in gene expression after stress and/or ethanol exposure. The majority of the analyses were performed between the NOS group versus the RSS or NOE or RSE groups, although additional comparisons were made between the three experimental groups, e.g., RSE vs. NOE. Local discovery rate estimation (*q* value) from a vector of *p* values for each contrast was calculated using the "*q* value" package in R (Storey & Tibshirani, 2003; Storey, 2011). Criteria for defining differentially expressed genes included a Tukey's adjusted *p* value < 0.05 and *q* value < 0.1.

2.4. Further analysis of genes

Multiple bioinformatic methods were used to further analyze the differentially expressed genes in each treatment group. Gene Ontology was performed on the significantly

differentially expressed genes in the three treatment groups and the RSE vs. NOE comparison to determine whether any biological and molecular processes were over-represented (Zhang, Kirov, & Snoddy, 2015). Expression quantitative trait locus (eQTL) analyses were also conducted using tools in GeneNetwork.org. Significant eQTLs are reported as $p < 0.05$ based on 2000 or more permutation tests with linkage reported in terms of likelihood ratio statistics (LRS). eQTLs were evaluated to determine whether differentially expressed genes in each group were *cis*-regulated. *Cis*-regulated genes are located within 5–10 Mb of the gene's location and are therefore more likely to control their own expression and have downstream effects on other genes' expression and/or phenotypes (Ciobanu et al., 2010; Overall et al., 2009; Ziebarth et al., 2012). *Trans*-regulated genes are located more than 10 Mb from the gene or on a different chromosome than the gene's location. We also determined whether differentially expressed genes in each treatment group contained polymorphisms, including nonsynonymous single nucleotide polymorphism (SNP), codon deletion, frameshift, etc.

Differentially expressed genes were correlated with all the phenotypes available on GeneNetwork.org using Pearson's Rank coefficient, with significant correlations at $p < 0.05$. In secondary analyses, particular emphasis was placed on the phenotypes of these mice used for expression analyses in the present study to assess whether multiple genes correlated with different experimental measures as described above. Upon identification of these gene sets, we performed a principal component analysis (PCA) and additional network analyses using GeneNetwork.org.

2.5. Comparison with INIA RNA-Seq data

Comparisons were made between the list of differentially expressed genes in the present study and those identified in the paper by Mulligan et al. in this current issue (complete Materials and Methods from Mulligan et al. used in this study can be found in the Supplementary Materials and Methods). This data set includes 23,250 transcript-level measurements. In the study by Mulligan et al., the parental C57BL/6J and DBA/2J strains ($n = 2$ per strain, per group) were examined.

The parameters for exposure used in this experiment have been previously described in Becker and Lopez (2004). Two different groups were examined: air control (AIR) and chronic intermittent ethanol (CIE) -exposed via vapor inhalation. CIE animals were exposed to 5 cycles of alcohol vapor for 16 h a day for 4 days, followed by a 72-h abstinence period and alcohol self-administration. Mice in the air-control treatment group were exposed to 5 cycles of air exposure for 16 h a day for 5 days. Tissue was collected at 72 h after the final ethanol exposure. Samples were collected from different brain regions. After quality control using PCA, the data set included nine tissue types (cortex, nucleus accumbens core, nucleus accumbens shell, dorsomedial striatum, bed nucleus stria terminalis, central nucleus of the amygdala, CA1 and CA3 of the hippocampus, and ventral tegmental area). Poly-A enriched mRNA from laser-capture microdissected brain regions was sequenced on two platforms, ABI SOLID 550XL Wildfire (167 samples) and Ion Proton (39 samples). Count data were generated, filtered, transformed, and batch-corrected (see Supplementary Materials and

Methods for more details). The full dataset is available at GeneNetwork.org (INIA LCM [11 Regions] RNA-seq Transcript Level [Dec15]).

The present study used differential gene expression data from Mulligan et al. (current issue), generated using generalized linear model (*glm*) function in R where gene expression (Y) is dependent on treatment, strain, region, or treatment interactions with strain and region: $Y \sim \text{Treatment} + \text{Strain} + \text{Tissue} + \text{Treatment} * \text{Strain} + \text{Treatment} * \text{Region}$. Significant differential expression was identified as $p < 0.05$, and only relevant categories (i.e., treatment, treatment interactions) were examined.

3. Results

Initial analyses were performed to determine gene expression changes that were observed after exposure to stress (RSS compared to the NOS control group) or ethanol (NOE vs. NOS), using the criteria defined in the Materials and Methods. Exposure to stress resulted in 15 genes (Supplementary Table 1) that changed expression; the majority (10 out of 15) of genes increased expression. In contrast, exposure to ethanol resulted in 243 differentially expressed genes (Supplementary Table 1). Expression changes ranged from a 1.8-fold decrease to a 1.3-fold increase; three-fourths of the genes decreased expression while the remaining one-fourth increased expression.

Because our primary interest was in assessing how stress and ethanol interact, we sought to determine whether a comparably large number of significantly differentially expressed genes would be observed following exposure to both ethanol and stress in comparison to controls (RSE vs. NOS). Ninety-nine genes were differentially expressed at the aforementioned criteria (Supplementary Table 1). The majority of changes were increases in expression as high as a 1.8-fold increase. Less than one-fifth of the genes decreased expression following treatment, with changes as low as 1.5-fold. Twenty-nine genes were differentially expressed in more than one treatment group, with two genes differentially expressed across all three treatment groups. However, the majority of differentially expressed genes were distinct between the three groups (Fig. 1).

We used a second dataset generated using RNA-seq to examine whether these genes were differentially expressed using a different, chronic ethanol-exposure paradigm (see Supplementary Materials and Methods). Comparisons demonstrated that as many as 20% of the genes differentially expressed in our paradigms were confirmed, even though the RNA-seq data were generated from animals exposed to chronic ethanol via vapor inhalation. Genes from all three groups (RSS, RSE, and NOE) were found to be differentially expressed following chronic ethanol exposure, suggesting that the vapor inhalation procedure may be affecting mechanisms associated with stress responses. Of the genes that were the same between the two studies, approximately half change in the same direction (Table 1).

Gene ontology and enrichment analyses were performed in each treatment group to identify over-represented biological processes (Table 2). The RSE group had a number of significant categories, with the majority relating to cell signaling, including synaptic transmission and intracellular signaling. In contrast, the NOE group showed a number of significantly

enriched categories, with the majority being related to cellular metabolism and immune system activation. The RSS category had the smallest number of differentially expressed genes and, therefore, there were only a few significant gene ontology categories, including several related to mitosis and cell cycle progression, transcription, and RNA regulation. Thus, the genes that are differentially expressed following each treatment exposure paradigm are distinct with combined exposure to ethanol and stress, resulting in significantly different categories than exposure to each alone.

Our initial hypothesis was that changes in gene expression after exposure to stress were modulated by subsequent exposure to ethanol, and as shown in Fig. 1, our initial analysis supports this hypothesis. To examine this possibility further, we determined whether there were genes that were only significantly different in the stress-alone group (RSS), compared to the combined stress and ethanol group (RSE). However, there were no genes that met our criteria ($p < 0.05$, $q < 0.1$). Similar analyses were conducted between the ethanol-alone group (NOE) and the combined ethanol and stress group (RSE). In contrast to the first comparison, this comparison yielded 67 differentially expressed genes that met our criteria (Supplementary Table 1). When compared to the control group, the direction of the expression change in the NOE and RSE group were in opposite directions (Fig. 2). The relatively large number of unique genes identified in this analysis demonstrates the robust ability of ethanol to alter the response of specific genes to exposure to acute stress. Gene ontology was performed to assess the functional categories of these genes and, similar to what was shown above, a unique set of functional categories was obtained with the majority related to neuron development and differentiation, suggesting that the genes in this category may be involved in hippocampal neurogenesis (Table 2).

The genes were further analyzed to determine whether they contained a non-synonymous polymorphism and/or were *cis*-regulated. Depending upon the group, 11–20% of the differentially expressed genes have non-synonymous polymorphisms (Supplementary Table 1). There were only four significant *cis*-regulated genes in the treatment groups: *Znhit1*, *Serpinh1*, *A930017M01Rik*, and *Mcm5* (also see Supplementary Table 1). Only one of the genes, *Emcn*, contained both a polymorphism and was *cis*-regulated.

Additional analyses were conducted to assess the correlation of the differentially expressed genes with any of the anxiety- and/or ethanol-related phenotypes that are publicly available on GeneNetwork.org (Table 3). Only appropriate correlated phenotypes in each group were considered in these analyses. For example, only anxiety-related phenotypes were correlated with genes from the RSS group. Over three-fourths of the genes in the RSS group were significantly correlated with anxiety-related phenotypes from a host of studies, with one example being an anxiety assay in the elevated plus maze after 4 weeks of chronic variable stress. An even higher percentage, over 95%, of the genes in the NOE group were correlated with multiple ethanol-related phenotypes such as ethanol consumption in a two-bottle choice test, locomotor activity after an ethanol injection, and ethanol consumption in a drinking in the dark paradigm. Virtually all of the genes in the RSE group and the RSE vs. NOE group were correlated with ethanol-and/ or anxiety-related phenotypes.

Because the mice used for expression analysis in this study were tested for anxiety-related phenotypes in an elevated zero maze prior to tissue collection, we further analyzed the relationship between the differentially expressed genes and the relevant phenotypes within each group (Cook et al., 2002, 2015; see Table 4). In the RSS group, 40% of the genes were correlated with relevant RSS phenotypes, while 16% of the genes from the NOE group were correlated with relevant NOE phenotypes. Similar to the RSS group, 47% of the genes from the RSE group were correlated with relevant RSE phenotypes. Interestingly, 75% of the genes in the RSE vs. NOE group were correlated with phenotypes from the RSE or RSS groups. These results show that there were strong relationships between the differentially expressed genes and the specific relevant behavioral phenotypes.

Because multiple genes correlated with a relevant phenotype, we examined the relationship among the multiple genes that correlated with each relevant phenotype. For each treatment group, a specific phenotype correlation is shown (Fig. 3). Many differentially expressed genes from the RSS group are correlated with activity in the closed quadrants, with 40% of the total variance explained by the first principal component (PC) in a PCA. Of the 29 genes that correlated with activity in the closed quadrants for the NOE group, approximately 45% of the total variance is explained by a single PC. In addition, time in the open quadrants is correlated with 29 differentially expressed genes from the RSE group, and 35% of the total variance can be explained by the first PC. Similarly, for the RSE vs. NOE group, 40% of the total variance can be explained by the first PC for the time in open quadrants phenotype.

Previous studies have evaluated whether any of the specific phenotypes mapped back to the location of the differentially expressed genes in their respective group (Bice et al., 2010, 2006; Carhuatanta, Shea, Herman, & Jankord, 2014; Cook et al., 2015; Overall et al., 2009; Palmer, Lessov-Schlaggar, Ponder, McKinnon, & Phillips, 2006; Porcu et al., 2011; Urquhart et al., 2016). However, in the present study, only one phenotype mapped back to the location of a gene that was correlated with it: *Cnot6l* from the RSE vs. NOE group. This suggests that the differentially expressed genes from our lists may be downstream from other intermediary genes that regulate these pathways.

Previously our lab identified a significant QTL on chromosome 1 at 23–31.5 Mb that mediates genetic differences in activity in the closed quadrants of the elevated zero maze in the NOE group (Cook et al., 2015). This region appears to be important in mediating ethanol- and anxiety-related phenotypes and has been identified as a QTL in a number of studies, including studies that examined the behavioral phenotypes of the mice used in this study, and thus provided the rationale for us to further examine this location (Buck et al., 1997; Cook et al., 2015; Philip et al., 2010; Ziebarth et al., 2012; Zou, Weng, Symons, & Singh, 2009). Over 130 differentially regulated genes from all four groups were significantly correlated with different ethanol- and/or anxiety-related phenotypes that map to this region; examples can be seen in Fig. 4. These correlated phenotypes used different paradigms including the elevated zero maze, light-dark box, and activity box (Cook et al., 2015; Philip et al., 2010).

Because the long-term goal of this research is to identify genetic networks involved in ethanol and stress interactions and because of the small number of *cis*-regulated genes, we

used a potential quantitative trait gene (QTG) from this region on chromosome 1, *Ptp4a1* (protein tyrosine phosphatase 4a1), and assessed the relationship among genes that were correlated with it in the NOE group (Cook et al., 2015). Over 20 highly interrelated genes were correlated with the expression of *Ptp4a1* (Fig. 5). Interestingly, previous research has shown that *Ptp4a1* and other genes in this network, such as *Zfp451*, have differential expression in alcoholics and various brain tissues following exposure to alcohol in animal models, thus reiterating the importance of this network (Bell et al., 2009; Zuo, Zhang, Deng, & Luo, 2013; Zuo et al., 2014, 2011). These results provide strong support for the relevance of this network.

4. Discussion

In the present study, we compared changes in gene expression in the hippocampus of mice exposed to either ethanol, stress, or a combination of both. The majority of differentially expressed genes were distinct between the groups, as confirmed by the diverse biological categories identified by Gene Ontology. Phenotype correlations were evaluated, and correlations were found between specific genes and related phenotypes from other studies. Of more interest, many of the differentially expressed genes were correlated with behavioral phenotypes from the relevant treatment groups. The ultimate long-term goal of these studies is to identify molecular networks mediating ethanol and stress interactions and thus, we used *Ptp4a1*, a gene we previously identified as a candidate gene underlying a QTL for several of these phenotypes, as an anchor gene to assess whether any of the differentially expressed genes were part of its network. We identified a large network of over 20 genes and as discussed above, the interrelatedness of these genes provides support that this network is likely to be important in mediating stress and ethanol interactions.

In this study, changes in gene expression observed after acute ethanol exposure were compared to changes observed following chronic ethanol exposure administered in a vapor inhalation chamber. Typically, ethanol-induced expression changes occur in a time-dependent manner (Bergeson et al., 2005; Morganstern et al., 2010; Most, Ferguson, & Harris, 2014; Robinson, Alexander, Bluett, Patel, & McCool, 2015). Therefore, it is interesting to note that approximately 12–20% of the genes change expression in both studies, suggesting that these pathways are particularly important in mediating responses to ethanol exposure. Moreover, several of the genes that change expression following exposure to stress or stress and ethanol combined were also found in the animals treated with alcohol via vapor inhalation, including *Astn1*, *Col5a2*, *Hdac2*, *Ifitm3*, *Jam2*, *Mapkpb1*, *Nfat5*, *Ntng2*, *Per3*, and *Serpinh1*, suggesting that the stress of the vapor inhalation procedure may be influencing gene expression, and that these genes could be important in mediating ethanol/stress interactions. The direction of the expression changes in both the acute exposure in the present study and the chronic exposure were the same in approximately half of the genes, suggesting that these genes are particularly responsive to these kinds of exposures. Previous studies have often found changes occurring in opposing directions following different types of alcohol exposure (Costin, Dever, & Miles, 2013; Osterndorff-Kahanek et al., 2015). Due to multiple cycles of exposure and withdrawal, the chronic intermittent ethanol protocol induces metabolic stress and physiological dependence. Dependence and withdrawal potentially invoke a negative state that is thought to drive

drinking (Cunningham, Fidler, Murphy, Mulgrew, & Smitasin, 2013). The fact that multiple types of stressors (metabolic, physiological, and psychological) converge on a shared set of genes is interesting.

The hippocampus is one of the brain regions that is typically highly affected by exposure to stress (Alfonso, Frasch, & Flugge, 2005; Hunter, Gagnidze, McEwen, & Pfaff, 2015; McEwen & Milner, 2007; Mychasiuk, Muhammad, & Kolb, 2016). Therefore, it is perhaps somewhat surprising that the smallest number of changes in gene expression were observed in the group that received exposure to stress and not ethanol (RSS). In the present study, mice were exposed to restraint stress for 15 min, and it is possible that longer exposure to restraint stress would have resulted in more extensive gene expression changes, as shown in other studies (Joëls & Baram, 2009; Liu, Serova, Kvetnanský, & Sabben, 2008; Sannino et al., 2016). Exposure to different types of stressors can also result in different patterns of gene expression changes, and the use of a single type of acute stress is a caveat in the interpretation of the present results (Hueston et al., 2011; Joëls & Baram, 2009). An additional caveat is that gene expression changes were measured at only a single time point following the stress exposure, because time has previously been shown to be an important variable in the determination of which genes are differentially expressed (Aujla et al., 2013; Du, Stasko, Costo, Davisson, & Gardiner, 2007; Lima et al., 2016; Smith et al., 2016).

To further examine our initial hypothesis that gene expression changes after exposure to ethanol were modulated by previous exposure to stress, we looked at an additional group of genes that were only significantly different between the combined ethanol and stress group and the other two groups. There were no significantly differentially expressed genes from the RSE vs. RSS group that met our criteria. In contrast, over 60 genes were differentially expressed between the RSE vs. NOE group. Even though the stress was relatively weak, it appeared that the combination of stress and ethanol exposure magnified the stress response rather than the stress response being overridden by the ethanol exposure. It is possible that because we were looking for gene expression changes in the hippocampus, an area that is highly affected by stress, that this effect was more pronounced and different results could be observed if other brain regions were examined (Gerges, Alzoubi, Park, Diamond, & Alkadhi, 2004; Kim & Yoon, 1998; Smith, Makino, Kvetnansky, & Post, 1995).

Among the differentially expressed genes in each group, many genes have been previously linked to anxiety- and/or ethanol-related behaviors. Differentially expressed genes in the RSS group, such as *Plin4*, have been linked to differences in glucocorticoid receptor regulation in relation to anxiety-like behavior and social stress (Stankiewicz, Goscik, Majewska, Swiergiel, & Juszczak, 2015). Additionally, differentially expressed genes in the RSE group such as *Adcy8*, *BC006965*, *Luc7l2*, *Kcnh*, and *Tsc22d3* have been shown to be linked to changes in gene expression after exposure to multiple types of chronic or other kinds of stress and differential gene expression in response to stress across different genetic animal models (Li et al., 2013; Stankiewicz et al., 2014; Szklarczyk, Korostynski, Golda, Solecki, & Przewlocki, 2012; Tordera et al., 2011; Tsolakidou et al., 2010). Over 30 of the differentially expressed genes from the NOE, RSE, or RSE vs. NOE group have been reported as involved in alcohol-related behaviors or expression changes in relation to alcohol exposure. For example, multiple genes from all three groups have been found to have altered

gene expression in regions such as the hippocampus, nucleus accumbens, amygdala, and ventral tegmental area following exposure to ethanol in animals: *Actn1*, *Aif1*, *Dcx*, *Errfi1*, *Havcr2*, *Homer1*, *Ifitm3*, *Jam2*, *Kif15*, *Mfsd2*, *Mtfmt*, *Serpinh1*, *Synj1*, *Thrsp*, *Tsc22d3*, *Zfp451* (McBride et al., 2013, 2014, 2012; Bell et al., 2009; Kimpel et al., 2007; Lee et al., 2015; Piechota et al., 2010; Rodd et al., 2008). Other studies examining differences in alcohol preference and alcohol consumption in animal models identified *Cacnalg*, *Kcnh7*, *Fgf12*, *Gad1*, *Luc7l2*, *Smpd3*, and *Ttn* (Carr et al., 2007; Fehr, Rademacher, & Buck, 2003; McClintick et al., 2013; Mulligan et al., 2008; Wang et al., 2014, p. P12). Additionally, seven genes from the RSE vs. NOE or NOE group (*Ankib1*, *Astn1*, *Ccdc88a*, *Cpne3*, *Tnn*, *Tns1*, and *Synpo2*) have been shown to be either differentially expressed in human alcoholics versus controls, or identified in a human genome-wide association study on alcoholism (Beech et al., 2014; Gelernter et al., 2014; Kryger & Wilce, 2010; Zuo et al., 2012). Finally, six differentially expressed genes (*Adcy8*, *Myef2*, *Per3*, *Synpo2*, *Tbc1d9b*, and *Tsc22d3*) have been linked to both stress and ethanol responses, although separately. Examples of the responses include 1) increased anxiety after exposure to various chronic stressors, 2) increased alcohol preference and consumption, and 3) alterations in methylation after alcohol use in humans (Bell et al., 2009; Li et al., 2016; McClintick et al., 2013; Philibert, Plume, Gibbons, Brody, & Beach, 2012; Stankiewicz et al., 2015; Szkarczyk et al., 2012; Tordera et al., 2011; Wang et al., 2012).

Previously, our lab identified a significant QTL on murine chromosome 1 at 23–31.5 Mb underlying the phenotype activity in the closed arms of an elevated zero maze after an ethanol injection. Subsequent analysis identified *Ptp4a1* as one possible candidate gene based on the criteria that 1) it is *cis*-regulated, 2) it contains a nonsynonymous polymorphism, and 3) it is significantly correlated with the phenotype. Moreover, *Ptp4a1* has been identified as a candidate gene in a human genome-wide association study for alcohol dependence across three different human populations, and has been found to correlate with a number of other genes related to alcohol addiction (Zuo et al., 2011, 2013, 2014). Therefore, we used *Ptp4a1* as an ideal gene to begin to define networks of genes that could be mediating ethanol/stress interactions. In the present study, we evaluated whether any of the genes that were differentially expressed following alcohol exposure were correlated with expression of *Ptp4a1*, and found 23 highly interrelated genes within this network. Interestingly, genes in this network, such as *Zfp451*, have been previously found to be differentially expressed or been linked to alcohol use (Bell et al., 2009). Future studies will elevate the relationship of the significant differentially expressed genes and their relationship to *Ptp4a1*.

In this study, distinct patterns of gene expression were observed in the hippocampus in animals exposed to stress, ethanol, or the combination of both, demonstrating that these two variables have more than simply a synergistic relationship and underscoring the importance of identifying relevant genetic networks. Understanding this complex interaction remains the long-term goal of this line of research and will be the subject of future studies. Determining the critical networks has a strong potential to provide therapeutic targets important in treating individuals that have both stress/ anxiety and alcohol-abuse disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alcohol.2016.08.008>.

Fig. 1. Distinct genes were differentially expressed among the three treatment groups
Venn diagram of overlapping significantly differentially expressed genes with criteria as follows: p value < 0.05 , q value < 0.1 .

Fig. 2. Examples of differentially expressed genes where ethanol exposure increases gene expression and combined exposure to ethanol and stress decreases expression, similar to stress alone

The graphs show mean ($\pm$ SEM) differences in expression after treatment from the control, NOS, group with increased expression above the zero line and decreased expression below the zero line. In each graph, red (left) bars show difference in expression after stress alone (RSS), green (middle) bars show difference in expression after ethanol alone (NOE), and blue (right) bars show difference in expression after combined stress and ethanol (RSE). A) Differential expression of *Cyfip1* (Probe ID: ILM5690082), difference from NOS group: RSS (M = -0.08, SEM = 0.23), NOE (M = 0.41, SEM = 0.27), RSE (M = -0.22, SEM = 0.33). B) Differential expression of *Hdac2* (Probe ID: ILM4050433), difference from NOS group: RSS (M = -0.09, SEM = 0.21), NOE (M = 0.42, SEM = 0.19), RSE (M = -0.21, SEM = 0.21). C) Differential expression of *Serinc1* (Probe ID: ILM6650020), difference from NOS group: RSS (M = -0.01, SEM = 0.38), NOE (M = 0.62, SEM = 0.27), RSE (M = -0.14, SEM = 0.36). An * represents a significance difference at $p < 0.05$ and $q < 0.1$ and

shows the significant difference between the NOE and RSE groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. The relationship among differentially expressed genes correlated with the same phenotypes and examples of significant correlations between a differentially expressed gene and its correlated phenotype

A) Principal component analysis (PCA) examining the relationship among five genes correlated with activity in the closed quadrants of the elevated zero maze for the RSS group. B) Correlation scatterplot between *Defb22* (Probe ID: ILM101050408) from the RSS group and activity in closed quadrants of an elevated zero maze, defined as number of beam breaks per second, after restraint stress for 15 min during the first 5 min for females (Trait ID: 12460) ($r = 0.489$, $p = 3.39E-03$). C) PCA examining the relationship among 29 genes correlated with activity in the closed quadrants of the elevated zero maze for the NOE group. D) Correlation scatterplot between *Kihl12* (Probe ID: ILM6040520) from the NOE group and activity in closed quadrants of an elevated zero maze, defined as number of beam breaks per second, after ethanol injection of 1.8 g/kg during last 5 min in females (Trait ID: 12373) ($r = 0.579$, $p = 7.23E-05$). E) PCA examining the relationship among 29 genes correlated with activity in the closed quadrants of the elevated zero maze for the RSE group. F) Correlation scatterplot between *Zfp867* (Probe ID: ILM101050408) from the RSE group and activity in closed quadrants of an elevated zero maze, defined as number of beam breaks per second, after ethanol injection of 1.8 g/kg during last 5 min in females (Trait ID: 12426) ($r = -0.630$, $p = 3.72E-05$). G) PCA examining the relationship among 29 genes correlated with activity in the closed quadrants of the elevated zero maze for the RSE vs. NOE group. H) Correlation scatterplot between *9330168M11Rik* (Probe ID: ILM101050408) from the RSE vs. NOE group and activity in closed quadrants of an elevated zero maze, defined as number of beam breaks per second, after ethanol injection of 1.8 g/kg during last 5 min in females (Trait ID: 12428) ($r = -0.621$, $p = 5.31E-05$).

($r = 0.579$, $p = 7.23\text{E-}05$). E) PCA examining the relationship among 29 genes correlated with time in the open quadrants of the elevated zero maze for the RSE group. F) Correlation scatterplot between *Zfp867* (Probe ID: ILM4610341) and percent time spent in open quadrants of the elevated zero maze after restraint stress for 15 min and an ethanol injection of 1.8 g/kg during the first 5 min in females (Trait ID: 12426) ($r = -0.630$, $p = 3.72\text{E-}05$). G) PCA examining the relationship among 27 genes correlated with time in open quadrants of the elevated zero maze for the RSE vs. NOE group. H) Correlation scatterplot between *9330168M11Rik* (Probe ID: ILM106110280) from the RSE vs. NOE group and percent time spent in open quadrants of the elevated zero maze after restraint stress of 15 min and an ethanol injection of 1.8 g/kg during the 10 min for females (Trait ID: 12428) ($r = -0.621$, $p = 5.31\text{E-}05$).

Fig. 4. Multiple stress- and/or alcohol-related phenotypes that are significantly correlated with differentially expressed genes from the RSS, NOE, RSE, and RSE vs. NOE groups have significant QTLs on chromosome 1 between 20–40 Mb

11699 (fifth, red line) phenotype: locomotor activity after ethanol injection of 2.25 g/kg in 10-min sessions for females. 12390 (second, orange line) phenotype: activity in closed quadrants of an elevated zero maze after ethanol injection of 1.8 g/kg during last 5 min for males and females. 12449 (top, light-green line) phenotype: activity in closed quadrants of an elevated zero maze after restraint stress for 15 min and an ethanol injection of 1.8 g/kg during the first 5 min for males and females. 12635 (third, light-blue line) phenotype: number of rears in the light side of a light-dark box in 5-min sessions with an ethanol injection of 1.8 g/kg for males (exact same line as dark blue line). 10970 (third, dark blue line) phenotype: rearing events in the light side of a light-dark box in 5 min sessions with an ethanol injection of 1.8 g/kg for males. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. *Ptp4a1* (protein tyrosine phosphatase 4a1) genetic pathway showing the relationship among its correlated genes that are differentially expressed after exposure to ethanol Twenty-three differentially expressed genes are significantly correlated with *Ptp4a1* and each other. Each box contains the gene symbol, probe ID, and Pearson's correlation p value. Bold red lines denote Pearson's correlations coefficients of 0.7–1. Solid orange lines denote Pearson's correlations coefficients of 0.5–0.7. Dashed pink lines denote Pearson's correlations coefficients of 0–0.5. Dashed black lines denote Pearson's correlations coefficients of –0.5 to 0. Solid green lines denote Pearson's correlations coefficients of –0.7 to –0.5. Solid blue lines denote Pearson's correlations coefficients of –1.0 to –0.7. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Relationship between differentially expressed genes after acute treatment and chronic treatment.

Direction of Change		Gene Symbol
Acute^a	Chronic^a	
↑	↑	<i>Armex3, Atp10a, Jam2, Mapkbp1, Mtfmt, Sult1a1, Vps50</i>
↑	↓	<i>Brox, Cpne3, Edem1, Eme1, Hfin1, Map3k7, Mdm1, Nfat5, Slc25a33, Tsc22d3</i>
↓	↑	<i>0610009O20Rik, Asb7, Astn1, Exosc9, Gulp1, Ifitm3, Klf3, Mrps16, Nedd8, Pogk, Serpinh1, Thrsp</i>
↓	↓	<i>A930024N18Rik, Alg8, Ankrd11, Brd4, Col5a2, Hdac2, Ntng2, Olfm13, Per3, Rapgef5, Tns1, Ttc3, Tll7, Tubgcp5, Vps29</i>

^a Acute treatment from the present study. Chronic treatment from Mulligan et al., in this current journal edition.

Table 2

Top Gene Ontology Categories from each treatment group.

Gene Ontology ID	Biological Process Category	Number of Genes	p value
RSS			
GO:0006310	DNA recombination	3	0.005
GO:0006355	regulation of transcription, DNA-dependent	12	0.0055
GO:2001141	regulation of RNA biosynthetic process	12	0.0056
GO:0051252	regulation of RNA metabolic process	12	0.0066
NOE			
GO:0051249	regulation of lymphocyte activation	26	4.35E-06
GO:0050863	regulation of T cell activation	20	2.67E-05
GO:0019538	protein metabolic process	171	0.0002
GO:0044267	cellular protein metabolic process	146	0.0002
GO:0046649	lymphocyte activation	32	0.0003
GO:0002682	regulation of immune system process	43	0.0007
GO:0046631	alpha-beta T cell activation	11	0.0007
GO:0044265	cellular macromolecule catabolic process	32	0.0007
GO:0051250	negative regulation of lymphocyte activation	11	0.001
RSE			
GO:0050896	response to stimulus	123	0.0004
GO:0032230	positive regulation of synaptic transmission, GABAergic	3	0.0004
GO:0035556	intracellular signal transduction	41	0.0007
GO:0035418	protein localization to synapse	3	0.0012
GO:0006950	response to stress	47	0.0013
GO:0031058	positive regulation of histone modification	4	0.0014
GO:0031571	mitotic cell cycle G1/S transition DNA damage checkpoint	3	0.0021
RSE-NOE			
GO:0048666	neuron development	7	0.0002
GO:0048468	cell development	10	0.0003
GO:0048699	generation of neurons	8	0.0004
GO:0022008	neurogenesis	8	0.0006
GO:0030182	neuron differentiation	7	0.001
GO:0050767	regulation of neurogenesis	5	0.0018
GO:0021766	hippocampus development	2	0.0035

Table 3

Examples of phenotypes correlated with differentially regulated genes after exposure to stress and/or ethanol.

ID	Phenotype	Authors	N#	Max LRS ^a	Max LRS Location	p value ^b (r value)	Correlated Genes
RSS							
11905	Transitions between light and dark sides of a light-dark box	Philip et al., 2010	27	13.9	Chr 9: 105.98	0.015–0.029 (0.41–0.45)	<i>Opa5</i> , <i>1700018P08Rik</i>
11982	Time in open arms of an elevated plus maze	Philip et al., 2010	28	15.7	Chr 4: 11.14	0.006–0.030 (–0.49–0.40)	<i>Ucp1</i> , <i>Till7</i> , <i>1700018P08Rik</i>
18061	Effects of chronic variable stress, singly-housed, time in closed arms of elevated plus maze	Carhuatanta et al., 2014	30	12.2	Chr 1: 194.70	0.014–0.038 (–0.43 to –0.37)	<i>Plin4</i> , <i>Vnn1</i> , <i>Ir225</i>
18064	Effects of chronic variable stress, singly-housed, time in open arms of elevated plus maze	Carhuatanta et al., 2014	30	12.3	Chr 1: 194.70	0.004–0.0316 (0.39–0.49)	<i>Plin4</i> , <i>Vnn1</i> , <i>Ir225</i>
NOE							
11003	Ethanol response, loss of righting reflex after ethanol injection, sleep time	Brigman, Mathur, Lu, Williams, & Holmes, 2009	17	17.4	Chr 14: 118.86	0.003–0.039 (–0.63–0.64)	<i>Msn</i> , <i>2510009E07rik</i> , <i>Tbxas1</i> , <i>Thbd</i> , <i>Fgf12</i> , <i>Tnfr1</i> , <i>Gm4888</i> , <i>Skint3</i> , <i>Plekha6</i>
11709	Ethanol response, locomotor activity, difference in distance traveled after ethanol injection between first and last 5 min	Philip et al., 2010	31	22.5	Chr 9: 74.38	0.018–0.036 (–0.41–0.40)	<i>Nkd2</i> , <i>49304327M23Rik</i> , <i>Klhl2</i> , <i>Cdk1</i> , <i>Wdr45b</i> , <i>Dnajc19</i> , <i>Mdm1</i>
11818	Ethanol response, motor coordination, time on dowel after ethanol injection	Philip et al., 2010	29	16.1	Chr 6: 76.13	0.001–0.043 (–0.55–0.38)	<i>Cbx1</i> , <i>Olf1320</i> , <i>Zfr</i> , <i>Brd1</i> , <i>Hspb1</i> , <i>Serinc3</i>
12817	Ethanol response, ethanol consumption using a 2-bottle choice after the fourth cycle of treatment in a chronic intermittent ethanol vapor chamber	Lopez et al. (Unpublished) ^c	16	24.4	Chr 11: 108.57	0.004–0.047 (–0.54–0.65)	<i>Tubgcp6</i> , <i>Rpl7</i> , <i>Fgf11</i> , <i>B94l</i> , <i>Mcm5</i> , <i>Sult1a1</i> , <i>Mrps11</i> , <i>Adgrd1</i> , <i>Ppp6r3</i> , <i>Serpinh1</i> , <i>Nine6</i> , <i>Spr2k</i> , <i>Slc6a1</i> , <i>Mdm1</i> , <i>Plin4</i> , <i>Dped</i> , <i>Klhl2</i>
12963	Ethanol response, ethanol intake using a 2-bottle choice test, after the second cycle of air exposure	Jones et al. (Unpublished) ^c	22	18.3	Chr 8: 16.85	0.003–0.049 (–0.44–0.58)	<i>Mcm5</i> , <i>Sult1a1</i> , <i>Bicd1</i> , <i>Cdk1</i> , <i>Plin4</i> , <i>Slc6a1</i>
RSE							
11440	Ethanol response, locomotor activity after ethanol injection, first 5 min	Philip et al., 2010	31	19.5	Chr 2: 147.88	0.009–0.048 (–0.45 to –0.35)	<i>Glt691</i> , <i>1700018P08Rik</i> , <i>Gpr156</i>
11982	Time in open arms of elevated plus maze	Philip et al., 2010	31	15.7	Chr 4: 11.14	0.007–0.041 (–0.46–0.36)	<i>Eat2</i> , <i>Igk</i> , <i>Syp</i> , <i>Ifitm3</i> , <i>Fat3</i>
12742	Ethanol response, consumption of ethanol using 2-bottle choice test after chronic intermittent ethanol vapor chamber	Lopez et al. (Unpublished) ^c	19	17.6	Chr 7: 99.79	0.006–0.035 (–0.59–0.55)	<i>Kcnh7</i> , <i>Zfp867</i> , <i>Ifitm1</i> , <i>Emcn</i> , <i>Syp</i>

ID	Phenotype	Authors	N#	Max LRS ^a	Max LRS Location	p value ^b (r value)	Correlated Genes
18058	Effects of chronic variable stress, singly-housed, freezing to tone after fear conditioning	Carhuatanta et al., 2014	29	22.2	Chr 13: 83.87	0.024-0.040 (-0.41-0.38)	<i>Fat3, D120062110Rik, Ins16</i>
11006	Ethanol response, ataxia measured by rotarod performance after injection	Brigman et al., 2009	19	17.9	Chr 14: 117.43	0.003-0.026 (0.50-0.62)	<i>Ccdc88a, Figla</i>
11703	Ethanol response, locomotor activity after injection last 5 min	Philip et al., 2010	30	21.8	Chr 9: 73.36	0.012-0.018 (-0.42 to -0.44)	<i>1810044K17Rik, Gm5130, Cpne3</i>
11723	Time in closed arms of elevated plus maze	Philip et al., 2010	30	17.9	Chr 3: 155.06	0.008-0.043 (-0.40-0.48)	<i>Cdk5r1, Haglr, Klf3, Jam2, Vmn2r106, A630009H07Rik, Igh-V</i>
18067	Effects of chronic variable stress, singly-housed, freezing to context after fear conditioning	Carhuatanta et al., 2014	25	15.2	Chr 13: 83.87	0.014-0.047 (-0.48 to -0.39)	<i>Espl1, 7420701103Rik, Cdk5r1, A230006K03Rik, 4930589L23Rik, 4930503E14Rik</i>

N# = number of strains.

^aLRS = likelihood ratio statistics.

^bThe range of p values and r values (Pearson's Correlation) of the genes to the right. Gene symbols are in order of significance from lowest to highest p value.

^cData available on GeneNetwork.org.

Table 4

Genes that are significantly correlated with behavioral phenotypes from mice tested in the present study.

Group	Phenotype	<i>p</i> value*	Gene Symbols
RSS	RSS group, activity in closed quadrants	0.0007–0.049	<i>Defb22, Gulp1, Plin4, Ssc5d, Vom1r1</i>
RSS	RSS group, time in open quadrants	0.0103–0.037	<i>Ssc5d, Ucp1</i>
NOE	NOE group, activity in closed quadrants	7.23E-05 – 0.018	<i>1700030P01Rik, 2900092C05Rik, 493041G21Rik, AU022751, B3galt5, Brd4, Dclre1a, Dnal1, Dock5, Elf2, Gopc, Ifitm3, Klhl12, Klhl2, L1Md-Tf30, Mdm1, Mef2a, Mrps11, Nkd2, Nme6, Phtf2, Ptrh1, Ranbp10, Thrsp, Tll11, Uqcr2, Wdr45b, Wldc5, Zfp451</i>
NOE	NOE group, time in open quadrants	0.0025–0.027	<i>2810454H06Rik, 4930573G07Rik, Cdh26, Cdk1, Dlgap5, Plin4</i>
NOE	NOE group, latency to enter an open quadrant	0.0009–0.003	<i>Mfsd2a, Plin4</i>
NOE	NOE group, time in open quadrants	0.0057–0.012	<i>2810454H06Rik, 4930573G07Rik, Cdk1, Serpinh1</i>
RSE	RSE group, time in open quadrants	3.72E-05 – 0.047	<i>1700003C15Rik, 2310035C23Rik, 640605C03Rik, 9430076D03Rik, Acbd5, Ankfn1, Cd40lg, Cyp3a25, D130062J10Rik, Ear10, Foxn3, Glt6d1, Igk, Kcnh7, Krtap28-13, Limk2, Lrrc2, Nat2, Nbas, Nkd2, Opn5, Otud7b, Slc35a33, Ssc5d, St8sia3os, Syp, Tra2b, Trim4a, Zfp867</i>
RSE	RSE group, activity in closed quadrants	0.0002–0.044	<i>1700018P08Rik, 2310035C23Rik, 6430605C03Rik, 9230019H11Rik, Afap112, Ankfn1, Dnajc2, Errf1, Havcr2, Hspbl, Ifitm1, Ifitm3, Igfbp7, Insl6, Lrrc2, Nat2, Nkd2, Otud7b, Prr5, Smpd3, Ssc5d, Sult1a1</i>
RSE	RSE group, latency to enter an open quadrant	0.0015–0.039	<i>Cmtm3, Errf1, Havcr2, Limk2, Sfmbt2, Slc37a1</i>
RSE vs. NOE	RSS group, activity in closed quadrants	0.0011–0.048	<i>3110005L21Rik, 4921511C20Rik, 4930503E14Rik, 4930589L23Rik, 9330168M11Rik, 9330209N08Rik, A230006K03Rik, Aldh8a1, Cyfip1, Fbxl5, Haglr, Klif3, Nr, Olfir173, Pecam1, Psg28, Rpl711-ps1, Tbc1d30, Vmn2r106, Zfp353-ps</i>
RSE vs. NOE	RSS group, time in open quadrants	3.17E-05 – 0.046	<i>4930503E14Rik, 4930589L23Rik, 9330209N08Rik, A230006K03Rik, A630009H07Rik, Cdk5r1, Cnot6l, Crb2, Cypt3, Hdac2, Jam2, Klif3, Lipt1, Nr, Pecam1, Sema4f, Serinc1, Synj1, Tbc1d9b, Tfcp2, Zfp353-ps, Zfp763</i>
RSE vs. NOE	RSS group, latency to enter an open quadrant	0.005–0.038	<i>9330168M11Rik, Psg28, Rpl711-ps1, Serinc1</i>
RSE vs. NOE	RSE group, activity in closed quadrants	0.0089–0.047	<i>1810044K17Rik, 3110005L21Rik, 4930503E14Rik, 4930589L23Rik, Cpne3, Cyfip1, Haglr, Nek1, Serpinb9c, Tfcp2, Tnn, Vmn2r106, Zfp689, Zpbp2</i>
RSE vs. NOE	RSE group, latency to enter an open quadrant	0.0016–0.0488	<i>Fbxl5, Jam2, Pecam1, Psg28, Zfp763, Zpbp2</i>
RSE vs. NOE	RSE group, time in open quadrant	5.31E-05 – 0.047	<i>1810044K17Rik, 3110005L21Rik, 4921511C20Rik, 4930503E14Rik, 4930589L23Rik, 7420701I03Rik, 9330168M11Rik, Aldh8a1, Cpne3, Esp1, Hdac2, Igh-V, Lipt1, Mfsd6l, Nr, Olfir1196, Pecam1, Rpl11, Serinc1, Serpinb9c, Tbc1d9b, Tfcp2, Tnn, Tube1, Unc119b, Zfp353-ps, Zfp689</i>

* The range of *p* values (Pearson's Correlation) of the genes to the right. Gene symbols are in alphabetical order.