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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 intraperitoneal (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 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.

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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;

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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; Starčević 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.

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