Systems Genetics Analysis of Molecular Pathways Underlying Ethanol-Induced Behavioral Phenotypes

Jesse D. Ziebarth, Melloni N. Cook, Biao Li, Robert W. Williams, Lu Lu*, and Yan Cui*

Abstract— **Acute behavioral responses to alcohol have been shown to be contributing factors to alcoholism, a disease that may be associated with high stress levels and brain injury. In this study, the LXS line of recombinant inbred mouse strains is used to develop understanding of the genetic basis of ethanol response, by finding links between genotype, gene expression data, and phenotypes associated with increased locomotor activity in mice treated with ethanol. The results of quantitative trait locus (QTL) mapping for phenotypes and gene expression data for LXS mice treated with alcohol are compared with a control group. A method in which Bayesian network modeling is used to discover casual pathways from genotype to gene expression to phenotype is also presented.**

I. Introduction

ALCOHOLISM is a subtle form of brain injury with unique maladaptive patterns of neuronal plasticity. It is associated with high stress levels, and relapse of individuals with a history of alcoholism may be related to post traumatic brain injury. The genetic factors that contribute to alcoholism have been routinely studied using animal models, particularly rodents. One of the important results of these studies is that alcoholism has been shown to correlate with behavioral responses by mice treated with ethanol. The Long-Sleep (LS) and Short-Sleep (SS) mice lines have been derived to differ in ethanol sensitivity, as measured by the difference in time it takes the mice to regain their righting reflex after an intoxicating dose of alcohol [1]. LS and SS mice have been used to determine quantitative trait loci (QTLs) for ethanol sensitivity [2]. More recently, the LXS recombinant inbred panel has been developed from inbred LS and inbred SS mice [3]. Recombinant inbred lines offer the advantages of increased map resolution and trait heritability as well as only requiring genotyping once [4]. LXS mice have been used to identify QTLs and candidate genes potentially responsible for low-dose ethanol activation as well as ethanol sensitivity [4]-[6].

Despite the success in finding QTLs linked to phenotypes associated with alcohol consumption using LXS mice or other animal models, there remains a problem in understanding ethanol response, as the identification of the specific genes controlled by QTLs is still a rare occurrence [7]. In this study, we attempt to bridge the gap between QTL, gene expression, and phenotype through analysis of two sets of data derived from LXS mice strains. First, QTL mapping is used to identify the locations of the chromosome that determine phenotypes. Second, expression QTLs (eQTLs) are discovered linking the gene transcripts to marker genotype. The results of our eQTL mapping are compared with a recent database of candidate genes that are thought to potentially impact ethanol response [8]. Finally, we propose a method in which Bayesian network modeling is used to determine the causal pathways that link genotype to gene expression to phenotype.

II. Materials And Methods

A. Animals and Testing

The analysis performed in this paper utilized three different types of data derived from LXS recombinant inbred mice. The LXS panel contains 77 fully inbred mice strains. The genotype data used contains 2659 error-checked SNPs and microsatellites [3]. Quantitative phenotype traits were derived from strains of LXS mice under two sets of experimental conditions which allowed for the effect of alcohol on the mice to be determined. In the no restraint stress saline treated (NOS) group, mice were injected with .01 ml of 0.9% saline per kg of mouse weight. In the no restraint stress ethanol treated (NOE) group, the mice were injected with 1.8 g/kg of ethanol. The same set of 32 LXS strains were present in both the NOS and NOE groups. Five minutes after injection with saline or ethanol, the mice were placed in an elevated zero-maze, which is commonly used to measure the anxiety level and activity of mice, for 10 minutes and their activity was monitored. The activity of the mice was measured by counting the average number of beam breaks of infrared sensors in closed quadrants per second over minutes 0-5 (ACTCNT0-5), minutes 5-10 (ACTCNT5-10), and minutes 0-10 (TOTAL ACTCNT) of the time spent in the maze. The data also includes the percentage of time spent in open quadrants over minutes 0-5 (OPEN0-5), minutes 5-10 (OPEN5-10), and minutes 0-10 (TOTAL OPEN). The latency to enter an open quadrant (LAT) was also measured.

Manuscript received March 28, 2010. This work was supported in part by NIH grants DA021131 and AA014425.

J. D. Ziebarth (email: jziebart@uthsc.edu) and B. Li are with the Department of Molecular Sciences at the University of Tennessee Health Science Center (UTHSC), Memphis, TN 38163.

M. N. Cook is with the Department of Psychology at the University of Memphis, Memphis, TN 38152. (email: mcook1@memphis.edu).

R. W. Williams is with the Department of Anatomy and Neurobiology and the Center for Integrative and Translational Genomics at UTHSC (email: rwilliams@nb.uthsc.edu).

L. Lu is with the Department of Anatomy and Neurobiology at UTHSC (phone: 901-448-7557; email: llu@uthac.edu).

Y. Cui is with the Department of Molecular Sciences and the Center for Integrative and Translational Genomics at UTHSC (phone: 901-448-3240, e-mail: ycui2@uthsc.edu).

*Correspondence should be addressed to L.L. or Y.C.

[978-1-4244-6714-3/10/$26.00 ©2010 IEEE

The microarray data used in the experiment was generated with the Illumina Mouse 6.1 bead array from samples taken from the hippocampus of 60 to 74 day old LXS mice. 4 hours before the animals were sacrificed, the NOS set received a single IP injection of saline, while the NOE set received a single injection of ethanol.

B. QTL Mapping Methods

The QTL mapping of the phenotypes was performed by whole genome association mapping using the 32 LXS strains of the NOS and NOE groups independently using the WebQTL [9] feature of the GeneNetwork [10]. Expression QTL mapping was performed using QTL Reaper [11], [12]. For both WebQTL and QTL Reaper, a p-value was determined for each linkage between trait and genotype by performing 1000 or more permutations of the data.

III. RESULTS

A. Effect of Ethanol on Phenotype

LXS mice have been shown to have a strain dependent locomotor activating response to ethanol, which is believed to be analogous to the euphoric effect that alcohol produces in humans. Fig. 1 shows a plot of the TOTAL ACTCNT for each strain of LXS mouse used in the study in both NOS and NOE conditions. For all strains, the activity count is larger under the NOE conditions in comparison with the NOS condition. Except for latency, similar behavior was observed for all other phenotypes; ethanol resulted in an increase in activity and percent of time spent in open quadrants for most strains over all time periods. In order to quantify this trend, paired t-tests were performed for the phenotypes and are shown in Table I. With the exception of latency, the means of all phenotypes are statistically different for the different treatment environments, indicating that ethanol affects the behavior of the mice.

B. QTL Mapping

QTL mapping was performed on the 7 phenotypes discussed in the Methods section and shown in Table I for both the NOS and NOE treatment groups, and the results are summarized in Table II. Fig. 2 shows the QTL mapping results from the GeneNetwork [10] for the ACTCNT5-10 phenotype in the NOE data set. Only one significant QTL, located on chromosome 3, was found for this phenotype. In all phenotypes, a total of two significant QTLs (p-value < 0.05) were found for the NOE data, but there were not any significant QTLs in the NOS data set. The first significant NOE QTL was associated with activity count phenotypes and has a peak located near 135 Mb on chromosome 3. It has been previously identified in at least two previous studies of QTL mapping of motion or balance associated traits in LXS mice treated ethanol [4], [6]. The second significant QTL was associated with the time spent in open quadrants and had a peak near 19 Mb on chromosome 4.

TABLE I
PAIRED T-TESTS OF PHENOTYPES

Phenotype	NOS mean	NOE mean	P-value[a]
TOTAL ACTCNT	1.83	3.19	$<1x10^{-9}$
ACTCNT0-5	2.12	3.51	$<1x10^{-9}$
ACTCNT5-10	1.60	3.14	$<1x10^{-9}$
TOTAL OPEN	29.73	47.24	$<1x10^{-7}$
OPEN0-5	32.33	52.83	$<1x10^{-11}$
OPEN5-10	27.16	41.46	$<1x10^{-4}$
LATENCY	6.55	8.06	0.48

[a] Two-tailed p-value from a paired t-test.

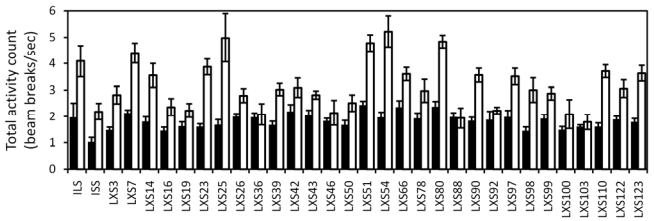

Fig. 1. Comparison of total mouse activity count (TOTAL ACTCNT) for each LXS mouse strain under NOS (black bars) and NOE (white bars) treatments.

C. eQTL Mapping

Expression QTL (eQTL) mapping was then performed to determine links between genotype and gene expression data for both the NOS and NOE data sets. Recently, Guo et al. [8] released the ERGR, ethanol-related gene resource (http://bioinfo.mc.vanderbilt.edu/ERGR/). The ERGR is a database of candidate genes that have been found from 30 linkage, association, and microarray expression studies that have involved both humans and animals. Of particular interest to this study, the ERGR included the candidate genes selected from 11 microarray experiments that were performed with RNA derived from the whole or portions of the brain, after a treatment testing sensitivity, tolerance, response or preference to ethanol. From these 11 data sets, 674 unique, annotated candidate genes were selected. Then, we investigated whether or not these candidate genes were, as a group, more likely to be involved in eQTL mapping of mice treated with ethanol.

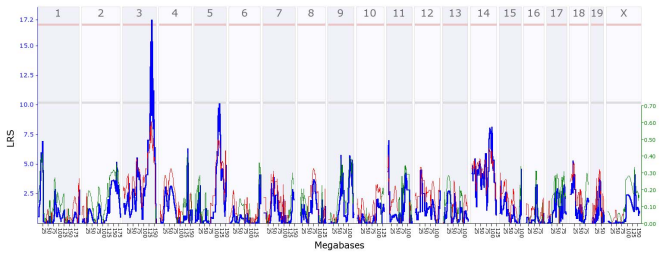

Fig. 2. QTL mapping of ACTCNT5-10 genotype from NOE data set. The suggestive QTL threshold (p-value < 0.63) is shown with a grey line, and the significant QTL threshold (p-value < 0.05) is shown with a red line.

TABLE II
QTL MAPPING

Chr	Mb[a]	LOD[a]	Phenotype	Data Set
3[b]	134.87	3.60	ACTCNT0-5, ACTCNT5-10, TOTAL ACT[c]	NOE
14[b]	19.14	4.41	OPEN0-5,OPEN5-10,[c] TOTAL OPEN	NOE
7[c]	144.25	3.37	LAT	NOE
13[c]	45.56	2.40	OPEN0-5, OPEN5-10, TOTALOPEN	NOE
19[c]	42.08	2.30	OPEN0-5, TOTALOPEN	NOE
4[c]	135.72	1.59	LAT	NOS
12[c]	101.52	2.58	ACTCNT5-10	NOS
15[c]	72.37	2.34	OPEN0-5, TOTACT	NOS

[a] Selected at maximum LOD for all mapped phenotypes.
[b] Significant (p-value ≤ 0.05) for phenotypes unless otherwise noted.
[c] Suggestive (p-value ≤ 0.63).

After performing the eQTL mapping, all significant marker and gene combinations (p-value ≤ 0.05) were selected for both the NOS and NOE data sets. This step produced 52,896 gene and SNP pairs in the NOS data set and 55,560 pairs in the NOE data set. Next, the pairs involving the previously selected candidate genes were counted. For the NOS data, 1708 pairs, or 3.2% of the significant eQTL interactions, involved a candidate gene for ethanol response. For the NOE data, 2250, or 4.0% of the significant eQTL interactions involved a candidate gene. However, a given gene would often have many significant eQTL interactions because nearby SNPs have similar profiles or the gene could interact with different unlinked SNPs. Therefore, the number of unique candidate genes involved in an eQTL was determined for both treatments. 83 of the 674 candidate genes were involved in significant eQTLs in both the NOS and NOE treatment groups, 64 genes were found in eQTLs in only the NOS treatment, and 72 genes were found from only the NOE data.

D. Bayesian Network Modeling

As mentioned previously, one of the challenges that has faced genomic studies of ethanol response in mice is identifying the specific genes that are responsible for phenotype differences. In particular, we are interested in determining the casual pathways that link genotype to gene expression to phenotype. Bayesian networks (BNs), which consist of directed acyclic graphs and probabilistic distributions over sets of variables, have shown to be a promising tool for inferring biological networks from high-throughput data. To construct the BNs, QTL mapping of phenotype and microarray gene expression data is performed. If a gene and a phenotype are found to map to the same QTL, they are grouped into a triplet. QTL mapping is able to find genes and phenotypes that are related to the same genetic loci, but is unable to determine how the three interact. Using the QTL as a casual anchor, three types of interaction pathways in a triplet are possible: 1) the QTL could influence the gene which then influences the phenotype, 2) the QTL could influence the phenotype, which then influences the gene expression, and 3) the QTL could influence the gene and phenotype independently. Of most interest is determination of triplets that best fit model 1, enabling the determination of casual pathways that link a QTL to a gene to a phenotype. To determine the triplets that fit model 1, we score each of the three possible models using the BDe scoring metric [13] and give each model a weight. The weight function is $W_i = e^{s_i} / \sum_{i=1}^{3} e^{s_i}$. Before scoring the models with this method, the gene expression and phenotype data are discretized into two bins, representing high or low values, using k-means clustering. While this does result in the loss of some data, it offers the advantages of increased computing speed and the ability to capture non-linear interactions that are not possible with other scoring methods. If the weight of model 1 is greater than 0.5 for a triplet, then the gene expression is considered casual to the phenotype and the QTL, gene, and phenotype are grouped into a BN.

This BN modeling method was used to investigate *trans*-regulated genes that potentially connect the QTL located on chromosome 3 with activity count phenotypes. An LOD cutoff of 2.0 was used to select associations between the QTL and both genes and phenotypes, and 17 genes linking the QTL on chromosome 3 to activity count phenotypes with a weight > 0.5 were identified. This list of genes was then filtered to select those genes that have been previously associated with alcohol in the ERGR [8] or through GO annotations [14], [15]. Fig. 3 shows three pathways from the rs1377430 SNP located at 135.95 Mb on chromosome 3 to *trans*-regulated genes to activity count phenotypes. The weights of the QTL to gene to phenotype pathways are 0.94, 0.72, and 0.64 in Models A, B, and C, respectively. *Cryab*, shown in Model A, is located on chromosome 9 of the mouse genome, and has been previously identified as a candidate ethanol related gene in experiments involving alcoholism in humans [16], and preference for ethanol in rats [17] and mice [18]. This gene was mapped to chromosome 3 in the eQTL mapping results of the NOE dataset with a significant p-value; however, there was not a significant interaction between *Cryab* and chromosome 3 in the eQTL mapping of the NOS dataset. The associations between *Miox* and *Rims1*, shown in Models B and C, and rs12477430 did not meet the threshold for significance in the eQTL studies, but satisfied the criteria used to group triplets here. *Miox*, on chromosome 15, is involved in the alcohol catabolic process (GO:0046164) [15]. *Rims1* is located on chromosome 1 and has been previously found to be differentially expressed in the nucleus accumbens of alcohol preferring rats [19]. It is also of interest because it contains a PDZ domain, and *Mpdz* is one of the few genes that has been clearly associated with an ethanol-related phenotype [20].

In order to determine the *cis*-genes that may potentially be involved in these pathways, eQTL mapping and GO

annotations were examined for the 181 genes located between 125-147 Mb on chromosome 3. In particular, we were interested in the cluster of alcohol dehydrogenase (ADH) genes located near 138 Mb. Of the 7 ADH genes in the cluster, only *Adh5* had a significant *cis*-eQTL. 25 of the 181 genes located near 138 Mb had a significant eQTL; however, of these, only *Adh5* had an alcohol related GO annotation. Additionally, *Adh5* has been previously shown to be the only ADH gene that was significantly expressed in the brains of both mice [21] and humans [22]. The involvement of the *Adh5* gene in the pathways shown in Fig. 3 are also of interest because, while ethanol has been shown to be metabolized in the brain, the exact enzymatic pathways involved have not been well established. One study determined that, while catalase and CYP2E1 are the primary enzymes involved in ethanol oxidation in the brain, ADH may be responsible for up to 20% of the ethanol-derived acetaldehyde in the brain [23]. Furthermore, some behavioral reactions to ethanol have been shown to be influenced by products of ethanol oxidation, such as acetaldehyde [24]. Genotypic differences in the QTL near the *Adh5* gene could, therefore, play an important role in the ethanol metabolism in the brain, with the pathways in Fig. 3 showing potential downstream effects in gene expression and phenotype resulting from variation in the expression of ADH.

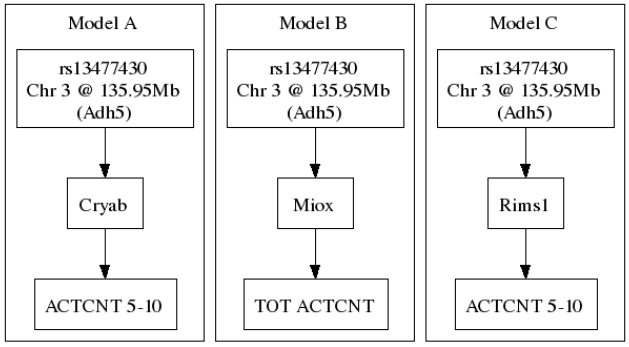

Fig. 3. Casual pathway models linking an ethanol related QTL on chromosome 3 to *trans*-regulated genes to the activity count phenotypes for LXS mice treated with ethanol.

IV. References

[1] G. E. McClearn and R. Kakihana, "Selective breeding for ethanol sensitivity: short-sleep and long-sleep mice," in *Development of Animal Models as Pharmacogenetic Tools,* G. E. McClearn, R. A. Deitrich, and V. G. Erwin, Eds.Washington, D.C.: U.S. Government Printing Office, 1981, pp. 81-113.

[2] P. D. Markel, B. Bennett, M. Beeson, L. Gordon, and T. E. Johnson. "Confirmation of quantitative trait loci for ethanol sensitivity in long-sleep and short-sleep mice," *Genome Res.*, vol. 7, no. 2, pp. 92-99, Feb. 1997.

[3] R. W. Williams, et al. "Genetic structure of the LXS panel of recombinant inbred mouse strains: a powerful resource for complex trait analysis," *Mamm. Genome*, vol. 15, no. 8, pp. 637-647, Aug. 2004.

[4] B. Bennett, P. Carsone-Link, N. R. Zahniser, and T. E. Johnson, "Confirmation and fine-mapping of ethanol sensitivity quantitative trait loci, and candidate gene testing in the LXS recombinant inbred mice," *J. Pharmacol. Exp. Ther.*, vol. 319, no. 1, pp. 299-307, Oct. 2006.

[5] B. Bennett, C. Downing, P. Carsone-Link, H. Ponicsan, C. Ruf, and T. E. Johnson, "Quantitative trait locus mapping for acute functional tolerance to ethanol in the LXS recombinant inbred panel," *Alcohol Clin. Exp. Res.*, vol. 31, no. 2, pp. 200-208, Feb. 2007.

[6] C. Downing, P. Carsone-Link, B. Bennett, and T. E. Johnson, "QTL mapping for low-dose ethanol activation in the LXS recombinant inbred strains," *Alcohol Clin. Exp. Res.*, vol. 30, no. 7, pp. 1111-1120, July 2006.

[7] J. C. Crabbe, "Neurogenetic studies of alcohol addiction," *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, vol. 363, no. 1507, pp. 3201-3211, Oct. 2008.

[8] A. Y. Guo, B. T. Webb, M. F. Miles, M. P. Zimmerman, K. S. Kendler, and Z. Zhao, "ERGR: an ethanol related gene resource," *Nucleic Acids Res.*, vol. 37, pp. 1-6, Jan. 2009.

[9] E. J. Chesler, L. Lu, J. Wang, R. W. Williams, K. F. Manly, "WebQTL: rapid exploratory analysis of gene expression and genetic networks for brain and behavior," *Nat. Neurosci.*, vol. 7, no. 5, pp. 485-486, May 2004.

[10] J. Wang, et al. "Gene Network," June 15, 2001. [Online]. Available: http://www.genenetwork.org. [Accessed: March 27, 2010].

[11] H. Hubner, et al. "Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease," *Nat. Genet.*, vol. 37, no. 3, pp. 243-253, March 2005.

[12] "What is the QTL Reaper?" July 2005. [Online]. Available: www.genenetwork.org/qtlreaper.html.. [Accessed: March 27, 2010].

[13] D. Heckerman, D. Geiger, and D. M. Chickering, "Learning Bayesian networks: the combination of knowledge and statistical data," *Machine Learning*, vol. 20, no. 3, pp. 197-243, Sept. 1995.

[14] D. W. Huang, B. T. Sherman, and R. A. Lempicki, "Systematic and integrative analysis of large gene lists using DAVID Bioinformatics resources," *Nature Protoc.*, vol. 4, no. 1, pp. 44-57, Jan. 2009.

[15] G. Dennis Jr., et al., "DAVID: database for annotation, visualization, and integrated discovery," *Genome Biol*., vol. 4, no. 5, pp. P3, April 2003.

[16] R. D. Mayfield, J. M. Lewohl, P. R. Dodd, A. Herlihy, J. Liu, and R. A. Harris, "Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics," *J. Neurochem.*, vol. 81, no. 4, pp. 802-813, May 2002.

[17] M. W. Kimpel, et al., "Functional gene expression differences between inbred alcohol-preferring and –non-preferring rats in five brain regions," *Alcohol*, vol. 41, no. 2, pp. 95-132, March 2007.

[18] R. Hitzemann, et al. "On the integration of alcohol-related quantitative trait loci and gene expression analyses," *Alcohol Clin.Exp. Res.*, vol. 28, no. 10, pp. 1437-1448, Oct. 2004.

[19] Z. A. Rodd, et al. "Differential gene gene expression in the nucleus accumbens with ethanol self-administration in inbred alcohol-preferring rats", *Pharmacol. Biochem. Behav.*, vol. 89, no. 4, pp. 481-498, Feb. 2008.

[20] R. L. Shirley, N. A. Walter, M. T. Reilly, C. Fehr, and K. J. Buck, "*Mpdz* is a quantitative trait gene for drug withdrawal seizures," *Nat. Neurosci.*, vol. 7, no. 7, pp. 699-700, July 2004.

[21] S. V. Bhave, et al. "Gene array profiles of alcohol and aldehyde metabolizing enzymes in brains of C57BL/6 and DBA/2 mice," *Alcohol Clin. Exp. Res.*, vol. 30, no. 10, pp. 1659-1669, Oct. 2006.

[22] O. Mori, et al. "Histological distribution of class III alcohol dehydrogenase in human brain," *Brain Res.*, vol. 852, no. 1, pp. 186-190, Jan. 2000.

[23] S. M. Zimatkin, S. P. Pronko, V. Vasiliou, F. J. Gonzalez, and R. A. Deitrich. "Enzymatic mechanisms of ethanol oxidation in the brain," *Alcohol Clin. Exp. Res.*, vol. 30, no. 9, pp. 1500-1505, Sept. 2006.

[24] Deng, X. and Deitrich, R. A. "Putative role of acetaldehyde in ethanol addiction," vol. 1, no. 1, pp. 3-8, Jan. 2008.