

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

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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.

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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 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 but one strain, 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 all 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

TABLE I
PAIRED T-TESTS OF PHENOTYPES

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

^a Two-tailed p-value from a paired t-test.

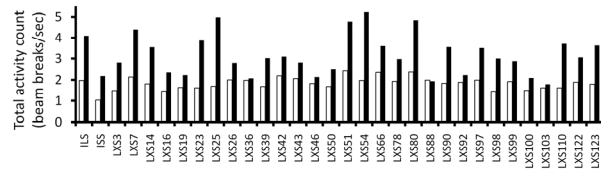


Fig. 1. Comparison of total mouse activity for each LXS mouse strain under NOS (white bars) and NOE (black bars) treatments.

summarized in Table II. Fig. 2 shows the QTL mapping results for the ACTCNT0-5 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 QTL's 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.

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,

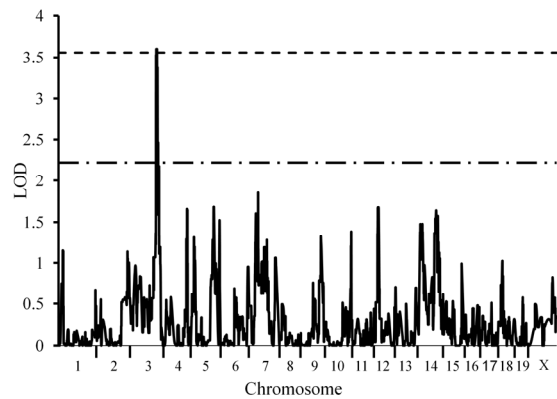


Fig. 2. QTL mapping of ACTCNT0-5 genotype from NOE data set. The suggestive QTL threshold (p-value < 0.63) is shown with a dotted-dashed line, and the significant QTL threshold (p-value < 0.05) is shown with a dashed line.

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).

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.

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. Leave-one-out cross validation is used to assess the networks, where the left out case is predicted to be high or low. Two metrics are used to validate the pathways. First, the network is assessed by determining the area under Receiver Operating Characteristic curve (AUROC). In a ROC plot, the horizontal axis represents the false positive rate and the vertical axis represents the true positive rate. For a perfect model, the AUROC curve is 1, while the AUROC of a random predictor is 0.5. The accuracy of the network is also determined by calculating the fraction of cases that are correctly predicted. A p-value from this accuracy can be determined through comparison with a random predictor and the binomial distribution.

Model A in Fig. 3 shows one of BNs that were found using the NOE data. In this model, an SNP located in the interval of the significant QTL found on chromosome 3 influences the expression of *Cryab* which influences the ACTCNT0-5 phenotype. The *Cryab* gene, which is located on chromosome 9 of the mouse genome, has been previously identified as a candidate ethanol related gene in experiments involving alcoholism in humans [14], and preference for ethanol in rats [15] and mice [16]. This gene was included in the list of candidate genes discussed previously, and was mapped to chromosome 3 in the eQTL mapping results of the NOE dataset. However, there was not a significant interaction between *Cryab* and chromosome 3 in the eQTL mapping of the NOS dataset. The weight of the marker to gene to phenotype pathway shown in Model A was 0.94, indicating that this type of interaction pathway best explained the data. The accuracy from the cross-validation was 0.72, corresponding to a p-value of 0.01 from a

binomial distribution. However, the AUROC was only 0.49, a performance similar to a random predictor.

In addition to 3 node BNs, larger BNs including multiple genes can be created. After the selection of triplets, genes that are linked to the same combination of QTL and phenotype are grouped. Then, a search is performed of all the possible BNs structures involving the QTL, phenotype, and group of genes, and each structure is scored with the BDe metric. Some constraints, such as limiting the number of genes in the networks to 3 or fewer and requiring that the QTL be the direct parent of *cis*-regulated genes, are placed on the structures to reduce the number of possible networks.

Model B in Fig. 3 is an example of one of the larger BNs. This BN is an expansion of Model A, and includes the same SNP and phenotype, as well as *Cryab*. Two other genes, *Metap1* and *Ddah1*, were also linked to this SNP and phenotype and were included in the network. *Metap1*, which is located on chromosome 3 at 138.1 Mb, has been previously identified as an ethanol-related candidate gene in a study of human alcoholism [17]. *DDah1*, located on chromosome 3 at 145.5 Mb, has also been previously linked to ethanol preference in rats [18]. The inclusion of these genes in the BN improved the results of the leave-one-out cross-validation, as the accuracy of the network increased to 0.84 (p-value = 0.001) and the AUROC was 0.71.

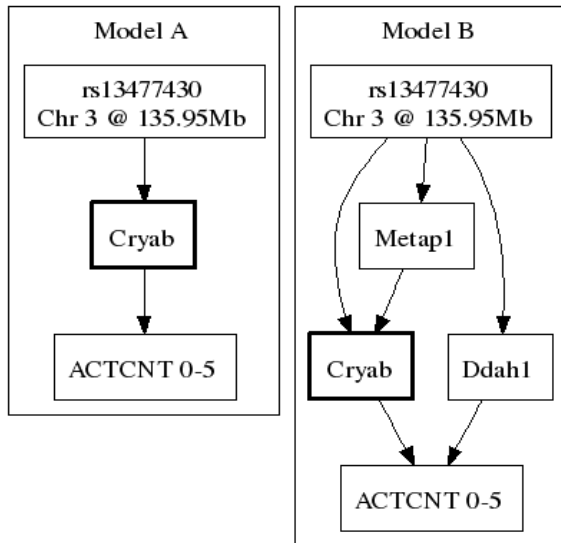


Fig. 3. Casual pathway models linking an ethanol related QTL on chromosome 3 to gene expression to the phenotype ACTCNT 0-5, a measure of the activity of LXS mice treated with ethanol. *Cryab*, a gene regulated by a *trans*-QTL, is shown in a box outlined with a bold line.

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