Elucidation of Ethanol Responsive Gene Networks in BXD Recombinant Inbred Strains

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Abstract

Initial responses to acute ethanol in humans have proven to be heritable predictors of future drinking behavior and long term risk for alcohol use disorders (AUD). Similar inverse relationships between acute ethanol sensitivity and ethanol preference also exist in mice. For example, C57BL/6J (B6) inbred mice consume significantly more ethanol than DBA/2J (D2) inbred mice, which generally avoid voluntary ethanol consumption but exhibit stronger responses following ethanol administration. Intriguingly, the divergent behavioral responses to ethanol that characterize B6 and D2 mice are accompanied by similar disparities in their neurogenomic response. Our laboratory previously reported that acute ethanol induces robust changes in gene expression across multiple brain regions within the mesolimbic dopamine pathway and the patterns of altered expression are markedly different between these two strains, particularly in the prefrontal cortex (PFC). The molecular networks comprised of these ethanol responsive genes may represent important functional pathways underlying the genetic component of individual variation in responses to acute ethanol and thus AUD susceptibility. In order to identify and better characterize acute ethanol responsive gene networks, we profiled the PFC transcriptome of 27 B6 × D2 (BXD) recombinant inbred mice 4 hours after receiving IP injections of either saline or ethanol (1.8 g/kg). We applied the S-score algorithm to measure the significance of each gene's change in expression between treatment groups and utilized a novel graph-theoretical approach to construct networks of genes exhibiting highly similar ethanol responses. A permutation based analysis of the S-score dataset revealed a subset of networks that are robustly affected by ethanol. These ethanol sensitive genes networks are enriched for genes involved in nervous system development and synaptic transmission, and significantly correlate with a variety of phenotypes, including behavioral responses to other drugs of abuse. Finally, we performed expression quantitative trait locus mapping with the S-score dataset and found the genetic response to acute ethanol exhibited by these networks is largely regulated by a small number of highly influential loci. Taken together, these results will provide valuable insight into the molecular mechanisms underlying acute ethanol sensitivity and may provide novel AUD susceptibility candidate genes.

Ethanol Responsive Genes

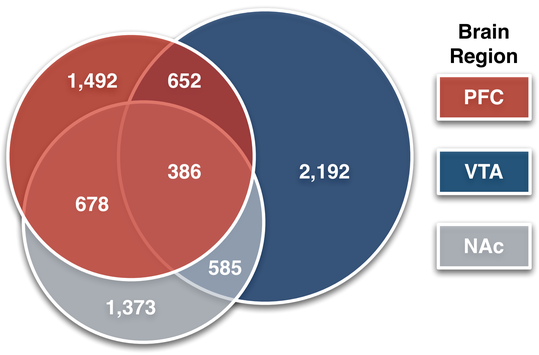

Figure 1 Using Fisher's method to analyze saline vs ethanol S-score data, we identified 1,492 probe-sets exhibiting significant ethanol responses in the prefrontal cortex (PFC), 1,373 in the nucleus accumbens (NAc) and 2,192 in the ventral tegmental area (VTA). Here we focus on the PFC but will examine the other regions in upcoming publications. The overlap between lists of ethanol responsive genes from each region is substantial and greatly exceeds what would be expected by chance.

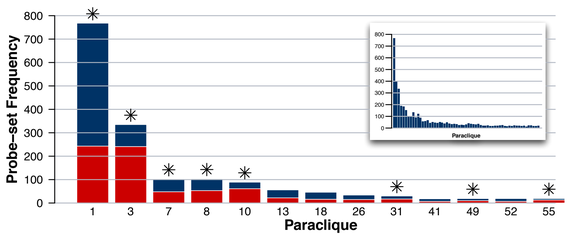

Figure 2 Applying the paraclique analysis method to the S-score data revealed 61 densely intercorrelated networks of genes, ranging in size from 768 probe-sets to 19 (inset graph). As this analysis was conducted with S-scores, rather than a typical expression summary measurement of steady state transcript levels, these networks represent genes that exhibit highly similar responses to ethanol. Figure 2 shows the number of probe-sets that are significantly ethanol responsive in a subset of the S-score paracliques.✳'s indicate those paracliques that are significantly enriched for ethanol responsive probe-sets.

Partitioning & Analysis of Ethanol Responsive Genes

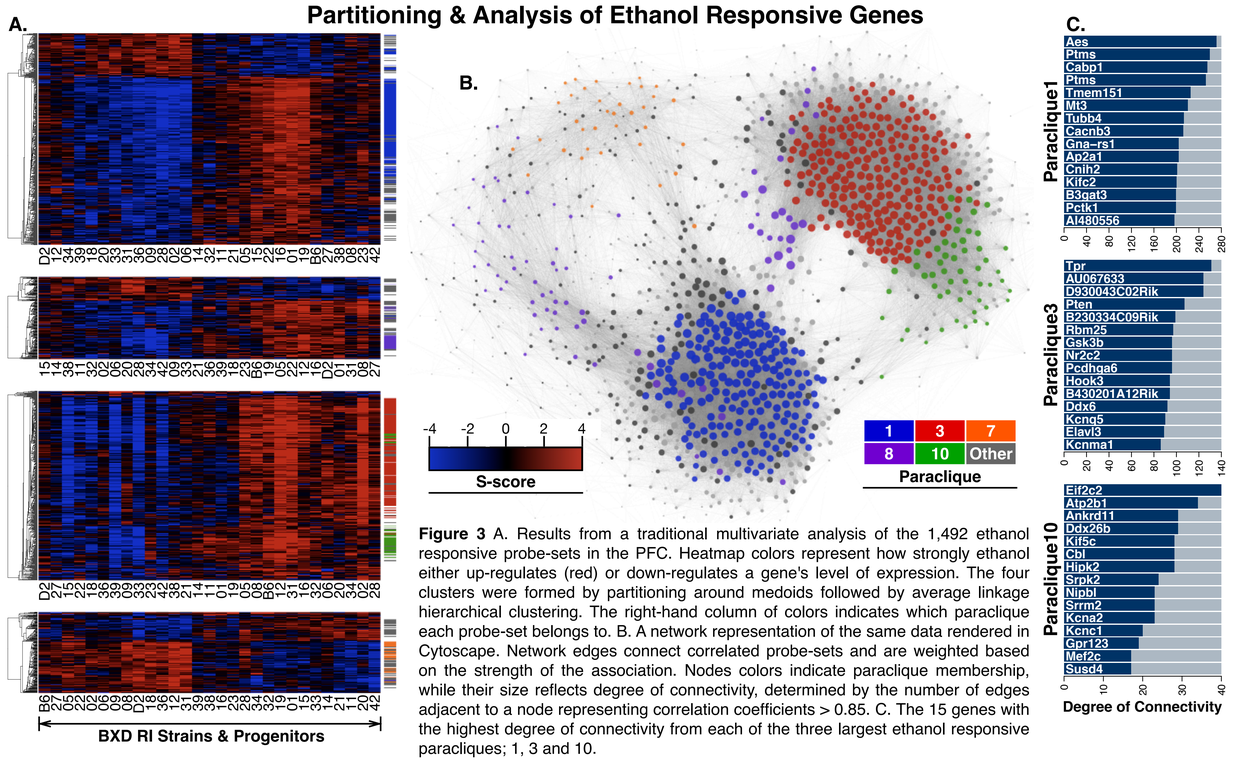

Figure 3 A. Results from a traditional multivariate analysis of the 1,492 ethanol responsive probe-sets in the PFC. Heatmap colors represent how strongly ethanol either up-regulates (red) or down-regulates a gene's level of expression. The four clusters were formed by partitioning around medoids followed by average linkage hierarchical clustering. The right-hand column of colors indicates which paraclique each probe-set belongs to. B. A network representation of the same data rendered in Cytoscape. Network edges connect correlated probe-sets and are weighted based on the strength of the association. Nodes colors indicate paraclique membership, while their size reflects degree of connectivity, determined by the number of edges adjacent to a node representing correlation coefficients > 0.85. C. The 15 genes with the highest degree of connectivity from each of the three largest ethanol responsive paracliques; 1, 3 and 10.

Functional Analysis

Paraclique 1

Ontology	p-value
GTPase activity	1.50E-07
Neurotransmitter secretion	2.31E-06
Regulation of synaptic plasticity	4.40E-05
Synapse part	3.08E-09
Dendrite	8.56E-09
Synaptosome	7.85E-07
Postsynaptic membrane	6.64E-05
Clathrin-coated vesicle	7.21E-05
Pten pathway	2.86E-06
Endometrial cancer	2.64E-05
Synaptic vesicle trafficking	3.42E-05
RING-type zinc fingers	1.20E-07
Coronins	1.26E-04

Paraclique 3

Ontology	p-value
Synapse part	1.83E-06
Postsynaptic density	1.28E-05
Presynaptic membrane	2.07E-05
FHF complex	2.84E-05
Axon	4.27E-05
Sin3-type complex	2.28E-04
Postsynaptic membrane	2.44E-04
Dendrite	2.99E-04
Histone deacetylase complex	3.56E-04
Potassium channels	2.28E-05
Centaurins	9.76E-05
Enflurane	4.98E-06

Paraclique 10

Ontology	p-value
Bromodomain 2	1.79E-05
PRKA anchor protein family	4.20E-04
Potassium channels	5.82E-04
Kinesins	2.53E-03

Category

- Molecular Function
- Biological Process
- Cellular Component
- Pathway
- Gene Family
- Drug

Table 1 To determine the biological significance of these results, we tested the ethanol responsive paracliques for enrichment of gene ontology categories or genes that participate in known molecular pathways. These analyses were conducted using ToppGene, available at http://toppgene.cchmc.org. We limited the analysis to probe only categories that contained between 3 and 300 members and corrected for multiple testing using a FDR of 1%. Table 1 presents only the top results from this analysis, which have been curated to exclude redundant categories.

Trans Band Analysis

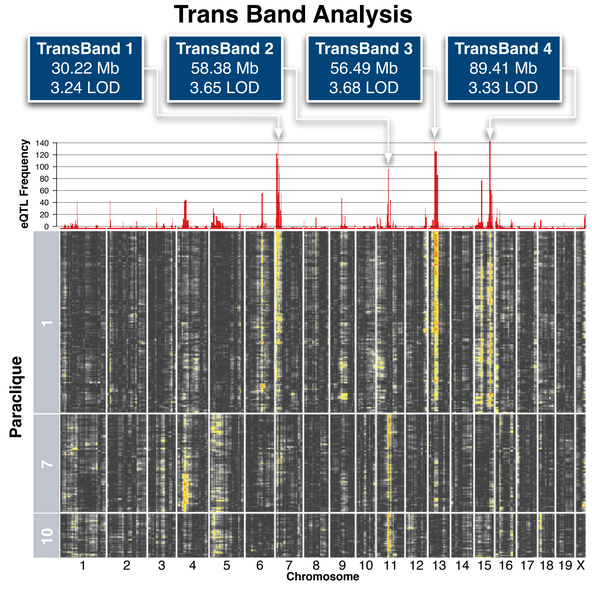

Figure 4 Results from expression QTL mapping of S-scores for all probe-sets from an ethanol responsive paraclique with at least one suggestive eQTL (genomewide corrected p-value < 0.63). The QTL heatmap (http://www.genenetwork.org/heatmap.html) indicates the association between a probe-set (rows) and each genetic marker (columns) across the genome, warmer colors represent stronger linkage. The eQTL frequency at each locus is plotted on the adjacent graph and reveals the existence of at least 4 trans bands. These trans bands on chromsomes 7, 11, 13 and 15 may represent the major regulators of neurogenomic response to acute ethanol.

Candidate Trans Band Regulator Genes

Trans Band	Gene	Mb	Para-clique	*EtOH Response*	*cis eQTL*	*p-value*	*Missense SNPs*
1	Zfp428	25.30	1	8.90E-03	30.14	2.55E-01	0
	Atp1a3	25.76	1	5.68E-03	15.62	1.64E-01	0
	Grik5	25.79	1	1.29E-03	--	--	0
	Cic	26.08	37	2.90E-02	**34.41**	**8.10E-02**	0
	Numbl	28.07	1	1.30E-02	30.14	4.10E-02	0
	2900074C18Rik	28.44	1	8.77E-03	--	--	0
	Eid2	29.05	1	1.23E-02	--	--	0
	Lrfn1	29.25	1	1.83E-02	30.14	5.60E-02	0
2	Atox1	55.26	5	7.36E-04	--	--	0
	Gria1	57.13	3	1.57E-03	56.55	2.6E-05*	4
	Usp22	60.97	8	6.21E-05	20.85	7.63E-01	0
	Ncor1	62.20	10	5.61E-05	**58.06**	**1.91E-01**	1
	2310047M10Rik	68.87	1	2.07E-03	--	--	1
	Vamp2	68.90	1	0.00E+00	--	--	0

Table 2 We performed a principal component analysis using S-scores from each ethanol responsive paraclique. The principal component (PC) trait accounting for the largest proportion of a paraclique's expression variance was used as a single synthetic trait, representative of the corresponding paraclique. All PC traits used in subsequent analyses accounted for > 70% of a paraclique's expression variance. QTL mapping of these traits allowed us to identify the loci that most strongly mediate the ethanol response of these networks and define support intervals that indicate the genomic regions most likely to harbor the underlying regulators. Candidate regulator genes were identified by screening for probe-sets within a trans band support interval and were prioritized based on paraclique membership, presence of cis eQTL or missense polymorphism between the B6 and D2 progenitor strains. The table lists the top candidate genes for trans band 1, which regulates all three ethanol responsive paracliques, and trans band 2, which regulates paracliques 3 and 10.

Materials & Methods

BXD RI Strains Our study includes 27 male BXD strains and their progenitors, the C57BL/6J (B6) and DBA/2J (D2) inbred strains. Each RI strain consisted of 5-8 mice per treatment and each progenitor strain consisted of 16 mice per treatment.

Microarray Analysis Mice were euthanized 4 hours post IP injection by cervical dislocation and dissected immediately thereafter. Medial prefrontal cortex, nucleus accumbens and ventral tegmental area tissue was isolated and subjected to RNA extraction as previously described (Kerns, 2005). Pooled samples of 4-5 mice were processed according to the Affymetrix GeneChip standard protocol and hybridized to Mouse Genome 430 2.0 arrays. We used the S-score algorithm (Zhang, 2002) to measure the ethanol induced change in transcript abundance by comparing expression levels between concordant BXD strains across the saline and ethanol treatment groups. The statistical significance of a probe-set's ethanol response was assessed using Fisher's Combined Probability Test to combine each probe-set's S-scores across all strains and comparing these values to 1,000 random permutations of the observed S-score matrix.

QTL Mapping was conducted using GeneNetwork (Chesler, 2004) and the qtl package for R (Broman, 2009). Confidence intervals for QTLs were calculated using 98% Bayes credible intervals as recommended by Manichaikul (2006).

Paracliques were constructed using A novel graph theoretical algorithm, originally described by Baldwin (2005), was applied to saline vs ethanol S-score expression data to construct paracliques of genes that are correlate with 70% of the other the other paraclique members at > 0.7.

Network-Phenotype Correlations

Phenotype Authors (PubMed ID)	Year	*r*	n	p-value
Protein kinase C (PKC) activity in hippocampus				
Wehner, J.M. *et al* (2400904)	1990	-0.89	9	4.29E-04
Morphine response, locomotion 165-180 min after injection				
Philip, V.M. *et al*	2009	0.62	24	9.01E-04
Naloxone-induced morphine withdrawal				
Philip, V.M. *et al*	2009	0.61	24	1.13E-03
Ethanol response, 2-bottle choice 2 hour access consumption				
Lopez, M.F. *et al*	2010	-0.79	12	1.30E-03
Learning and memory performance, Morris water maze				
Kempermann G. *et al* (12153537)	2002	0.85	9	1.94E-03
Cerebellum fissure number				
Neumann P.E. *et al* (8374795)	1993	0.61	21	2.60E-03
Zinc level in medial prefrontal cortex				
Jones, L.C. *et al* (16910173)	2006	0.75	12	3.17E-03
Iron level in ventral midbrain of males				
Jones B.C. *et al* (14744041)	2003	0.75	12	3.87E-03
Conditioned place preference (CPP) baseline control for cocaine				
Philip, V.M. *et al*	2009	-0.57	23	3.90E-03
Ethanol metabolism rate in response to 2 g/kg ip injection				
Grisel, J.E. *et al* (12045568)	2002	-0.58	22	3.97E-03

Table 3 In order to determine the functional impact of these ethanol responsive gene networks on a phenotypic level, we used GeneNetwork's rich database of BXD phenotypes to identify associations with the paraclique PC traits. Listed above are the top 10 phenotypic correlates of paraclique 3's PC trait.

Results Summary

- **We identified genes whose expression level is significantly altered by acute ethanol in the PFC, NAc and VTA. There is substantial overlap between the lists of ethanol responsive genes from these three brain regions.**
- **Applying a graph theoretical network based approach to saline vs ethanol microarray expression data uncovered 61 networks of genes exhibiting highly similar responses to acute ethanol in the prefrontal cortex.**
- **A subset of these gene networks were significantly enriched for genes identified as ethanol responsive by the analysis of S-scores.**
- **Genes encoding for synaptic proteins and responsible for regulating neurotransmitter systems are highly over represented in these ethanol responsive gene networks.**
- **Genetical genomic analysis of probe-sets from these paracliques revealed a small group of highly influential regulatory hotspots that are largely responsible for mediating the ethanol response of these networks.**
- **We have identified a list of high priority candidate genes as potential regulators underlying the major trans bands.**

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