Intra- and inter-regional ethanol responsive gene networks of the mesolimbocortical reward pathway

Aaron R. Wolen[1], Charles A. Phillips[4], Michael A. Langston[4], Alex H. Putman, Nathan A. Bruce, Paul J. Vorster, Robert W. Williams[5], Lu Lu[5] and Michael F. Miles[2,3]

[1]Departments of Human and Molecular Genetics, [2]Pharmacology and Toxicology, and [3]Neurology
Virginia Commonwealth University

[4]Department of Electrical Engineering and Computer Science
University of Tennessee

[5]Department of Anatomy and Neurobiology
University of Tennessee Health Sciences

VCU Medical Center
Virginia Commonwealth University

abstract

The prefrontal cortex (PFC), nucleus accumbens (NAc) and ventral midbrain (VMb) are key brain regions that comprise the mesolimbocortical reward pathway. Previously, we have demonstrated that the transcriptomes of these three regions are robustly altered by exposure to acute ethanol in naive BXD recombinant inbred (RI) strains. As initial level of response (LR) to ethanol is a heritable trait that predicts long term risk for alcohol use disorders (AUDs), acute ethanol induced gene expression changes within these regions may represent key intermediate phenotypes that stand between AUD susceptibility and the causal genetic variants. Here, we attempted to reconstruct the biological pathways underlying LR variability by identifying ethanol responsive gene (ERG) networks within and across the PFC, NAc and VMb. Each ERG network represents a group of genes that exhibit a tightly correlated transcriptional response to acute ethanol across all profiled RI strains. Intra-region ERG networks were significantly enriched for genes involved in nervous system development and synaptic transmission. While characterizing intra-region ERG networks provides valuable insights into region-specific consequences of acute ethanol exposure, many of the key neuroadaptations associated with the development of AUDs and addiction to other drugs of abuse are attributed to changes that alter communication between brain regions. We therefore constructed inter-region ERG networks, composed of genes whose response to acute ethanol is correlated across brain regions, in order to identify ethanol sensitive pathways that connect regions of the mesolimbocortical system. We found that the frequency of NAc/PFC connections were more common than NAc/VMb or PFC/VMb connections. These results will provide novel information about the molecular pathways that underlie acute ethanol sensitivity and may provide novel AUD susceptibility candidate genes.

materials & methods

Mice: Thirty five members of the BXD family and their progenitors, the C57BL6/J (B6) and DBA/2J (D2) strains, were euthanized 4 hours following an intraperitoneal injection of either saline or ethanol (1.8g/kg). Medial prefrontal cortex, nucleus accumbens and ventral midbrain tissue was isolated and subjected to RNA extraction as previously described (Kerns, 2005). Each BXD strain consisted of 5-8 mice per treatment and each progenitor strain consisted of 16 mice per treatment.

Microarray analysis: 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 summarize each probe-set's S-scores across all strains and comparing these values to 10,000 random permutations of the observed S-score matrix.

Gene network construction: The Weighted Gene Co-Expression Network (WGCNA) package for R (Langfelder, 2008) was used to identify co-expression networks within the 3,520 probe-sets that exhibited a significant ethanol response in at least one the three profiled brain regions (Fig. 1).

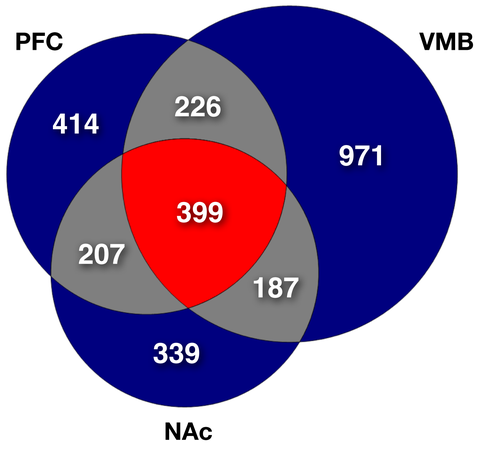

Figure 1 A large subset of the 3,520 ethanol responsive probe-sets identified in the S-score analysis were differentially expressed in multiple profiled brain regions.

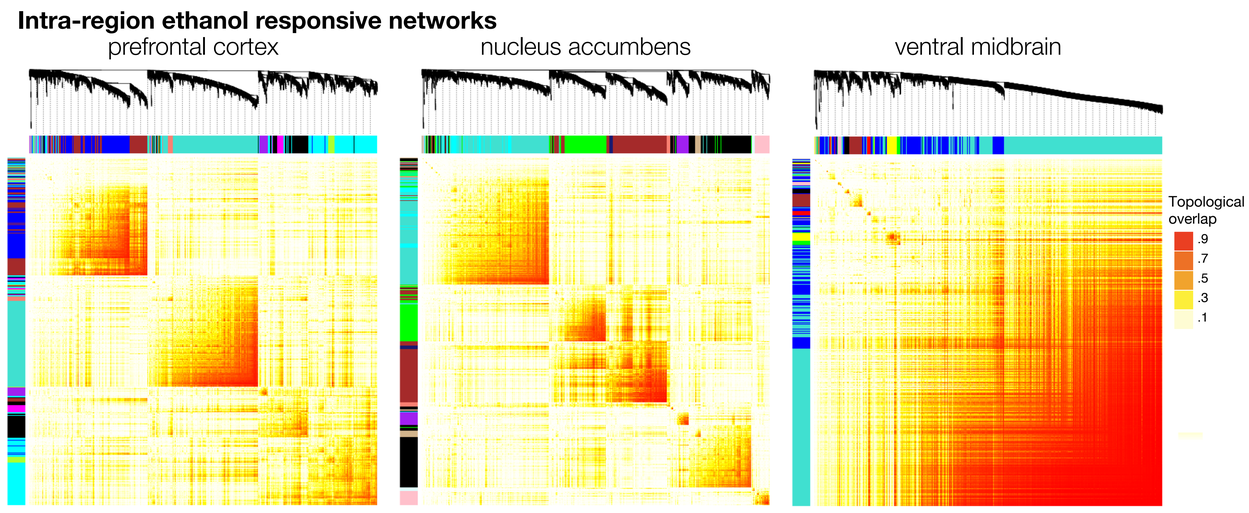

Figure 2 Each region's S-score expression data was transformed to a weighted adjacency matrix, providing a measurement of ethanol response similarity for all pairwise gene comparisons. This, in turn, was transformed to a topological overlap matrix (TOM), which indicates the degree of commonality between two genes network neighborhoods. The heatmaps above visualize each region's TOM, where warmer colors indicate increased overlap. Hierarchical clustering was performed so that groups of genes with high TOM could be easily identified. Individual modules were formed by applying a branch cutting algorithm to the resulting dendrograms (pictured above each heatmap). These modules are denoted by the strips of colors adjacent to each heatmap.

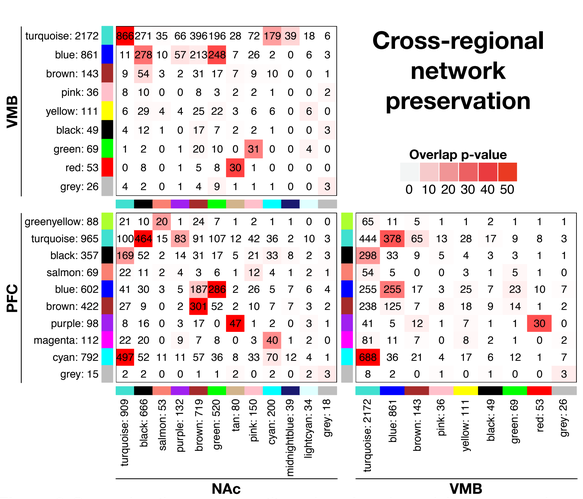

Figure 3 Comparing the gene constituencies of each module across regions revealed these networks are largely preserved across brain regions. The contingency tables above indicate the number of probesets in common between the corresponding row and column modules. The total probeset count for each module is provided in the axis labels. Fisher's exact test was used to to determine the statistical significance of overlapping modules, log(p-values) from this analysis were used to color code the table.

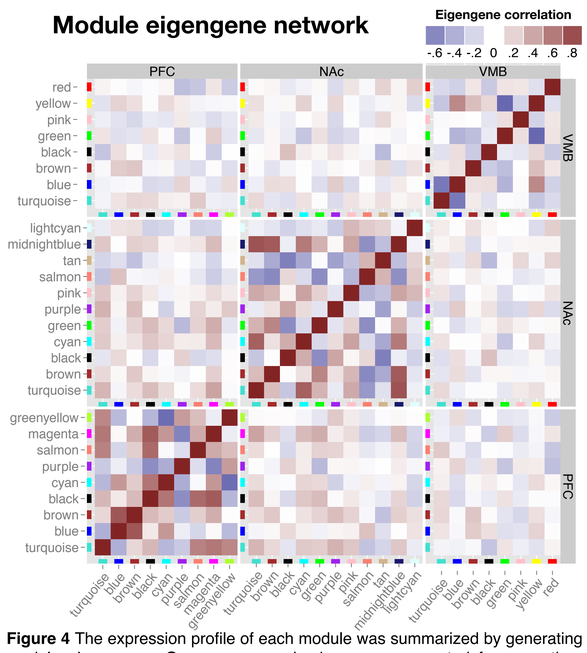

Figure 4 The expression profile of each module was summarized by generating module eigengenes. On average, each eigengene accounted for more than 50% of the expression variance in its constituent module. In order to examine the relationships between intra- and inter-regional modules we constructed an eigengene network (visualized in the above heatmap). This analysis provides evidence for strong interactions among intra-region modules. As expected, cross-region interactions are rarer. Fig. 4b although several PFC modules appeared to have relationships with modules in the NAC. For example the magenta PFC eigengene was significantly correlated with the turquoise NAc eigengen (r = 0.41, p-value = 0.02).

	Module	Hub gene	Module membership	Ethanol response
PFC	Turquoise	Aes amino-terminal enhancer of split	0.97	5.93E-05
	Blue	Grm3 metabotropic glutamate receptor	0.98	0.00E+00
	Cyan	Mtap2 microtubule-associated protein 2	0.96	1.67E-04
	Magenta	Nptxr neuronal pentraxin receptor	0.94	5.02E-03
	Brown	Mtap1b methyltransferase domain containing 1	0.96	0.00E+00
NAc	Turquoise	Pcmtd1 metabotropic glutamate receptor	0.96	1.97E-03
	Brown	Evi5 ecotropic viral integration site 5	0.97	0.00E+00
	Black	Ptprn protein tyrosine phosphatase receptor	0.96	1.35E-01
	Green	Klf12 Kruppel-like factor 12	0.97	8.79E-04
	Cyan	Kif5a kinesin family member 5A	0.91	2.94E-05
VMB	Turquoise	Rnf14 ring finger protein 14	0.99	0.00E+00
	Blue	Ptov1 prostate tumor over expressed gene 1	0.94	1.94E-02
	Brown	Icam5 intercellular adhesion molecule 5	0.93	6.55E-03
	Yellow	Camsap1 calmodulin regulated spectrin protein	0.94	1.40E-02
	Green	Arhgef9 rho/rac guanine exchange factor 2	0.96	4.30E-05
	Pink	Fkbp5 FK506 binding protein 5	0.87	1.86E-04

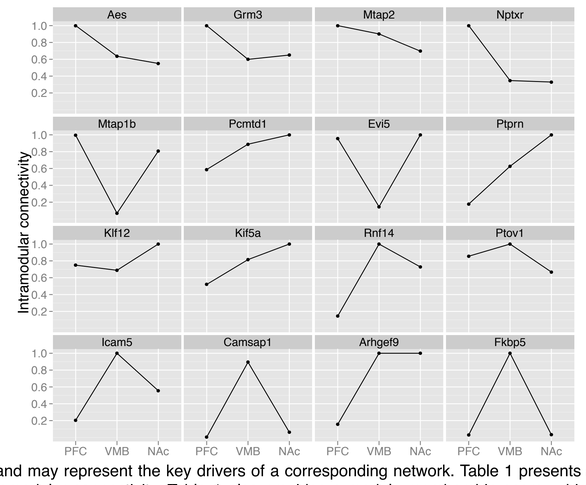

Table 1 Hub genes are the most highly interconnected members of a gene network and may represent the key drivers of a corresponding network. Table 1 presents a subset of top module hub genes that were identified by calculating each gene's intramodular connectivity. Table 1 also provides a module membership score, which indicates how strongly a gene relates to a module based on its correlation with the module eigengene, as well as ethanol responsive p-values from the analysis of S-scores. **Figure 5** In the adjacent grid of line graphs, each hub gene's intramodular connectivity (scaled to adjust for differences in module size) is plotted across all three brain regions. While genes such as Grm3 and Mtap2 appear to be highly connected across regions, the hub status for many genes appears highly region specific.

Phenotype Authors (PubMed ID)	*r*	p-value	Region	Module
Naloxone Bmax concentration (binding maximum)				
Wehner, J.M. *et al* (2400904)	-0.89	4.29E-04	PFC	Turquoise
Morphine response, locomotion 165-180 min after injection				
Philip, V.M. *et al*	-0.68	1.10E-04	PFC	Brown
Cocaine response (30 mg/kg), open field behavior				
Jone, B.C. *et al* (10591541)	0.71	5.20E-04	PFC	Magenta
Ethanol response (4 g/kg), handling induced convulsions				
Roberts, A.J. *et al* (8748968)	-0.65	9.90E-04	NAc	Turquoise
Ethanol response (1.8 g/kg), total locomotor activity				
Putman, A.H. *et al*	0.52	9.90E-04	NAc	Brown

Table 2 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 drug response phenotypes to identify associations with the paraclique PC traits. Listed above are subset of drug-response phenotypes that are strongly related to these modules.

Functional enrichment analysis

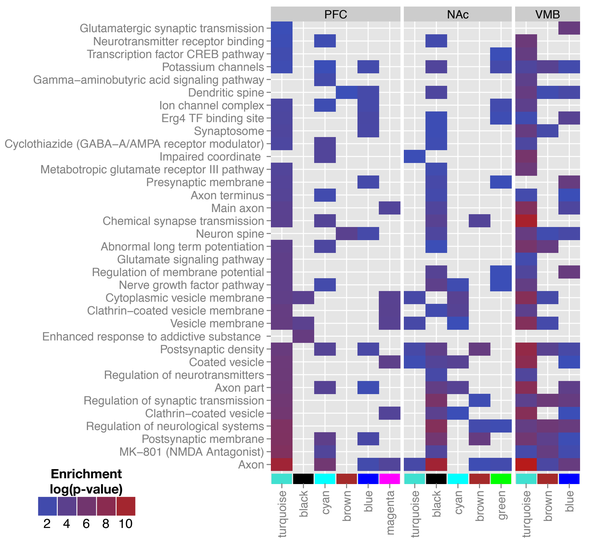

Figure 6 The biological significance of these modules was determined by searching for enrichment of gene ontology categories or genes that participate in known molecular pathways. These analyses were conducted using ToppGene, available at http://toppgene.cchmc.org. We limited the analysis to probe only categories that contained between 3 and 300 members and corrected for multiple testing using a FDR of 5%.

results summary

- **Using weighted gene co-expression analysis, we identified co-regulated modules of ethanol responsive genes within PFC, NAc and VMB.**
- **While the gene networks represented by these modules are largely preserved across the mesolimbocortical reward pathway, the transcriptional response of these networks to ethanol is largely region specific.**
- **Inter-modular connections are stronger between the PFC and NAc than any combination of connections including the VMB.**
- **Genes encoding for synaptic proteins and responsible for regulating neurotransmitter systems are highly over represented in these ethanol responsive gene networks.**
- **Many of the strongest phenotypic correlations with these gene modules were related to drug response phenotypes.**
- **Genetic manipulation of hub genes may represent the best strategy for validating these networks. However, the regional specificity of hub genes indicates that targeted brain regions must be chosen very carefully.**

tools

R (http://www.r-project.org) I **ggplot2** (http://had.co.nz/ggplot2)
GeneNetwork (webqtl.org) I **ToppGene** (http://toppgene.cchmc.org)

WGCNA
Langfelder, P., & Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis *BMC bioinformatics*, *9*, 559. doi: 10.1186/1471-2105-9-559

S-score algorithm
Zhang, L., Wang, L., Ravindranathan, A., & Miles, M. (2002). A new algorithm for analysis of oligonucleotide arrays: application to expression profiling in mouse brain regions. *Journal of molecular biology*, *317*(2), 225–235. doi:10.1006/jmbi.2001.5350

Supported by NIAAA INIA grants AA016667 and AA016662, and NIMH grant MH-20030.