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Dual-Trait Selection for Ethanol Consumption and Withdrawal: Genetic and Transcriptional Network Effects

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Abstract

Background—Data from C57BL/6J (B6) × DBA/2J (D2) F2 intercrosses (B6×D2 F2), standard and recombinant inbred strains, and heterogeneous stock mice indicate that a reciprocal (or inverse) genetic relationship exists between alcohol consumption and withdrawal severity. Furthermore, some genetic studies have detected reciprocal quantitative trait loci (QTLs) for these traits. We used a novel mouse model developed by simultaneous selection for both high alcohol consumption/low withdrawal and low alcohol consumption/high withdrawal and analyzed the gene expression and genome-wide genotypic differences.

Methods—Randomly chosen third selected generation (S₃) mice (N=24/sex/line), bred from a B6×D2 F2, were genotyped using the Mouse Universal Genotyping Array, which provided 2,760 informative markers. QTL analysis used a marker-by-marker strategy with the threshold for a significant log of the odds (LOD) set at 10. Gene expression in the ventral striatum was measured using the Illumina Mouse 8.2 array. Differential gene expression and the weighted gene coexpression network analysis (WGCNA) were implemented.

Results—Significant QTLs for consumption/withdrawal were detected on Chromosomes (Chr) 2, 4, 9, and 12. A suggestive QTL mapped to Chr 6. Some of the QTLs overlapped with known QTLs mapped for one of the traits individually. 1745 transcripts were detected as being differentially expressed between the lines; there was some overlap with known withdrawal genes

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(e.g. *Mpdz*) located within QTL regions. WGCNA revealed several modules of co-expressed genes showing significant effects in both differential expression and intramodular connectivity; a module richly annotated with kinase-related annotations was most affected.

Discussion—Marked effects of selection on expression and network structure were detected. QTLs overlapping with differentially expressed genes on Chr 2 (distal) and 4 suggest that these are cis-eQTLs (Chr 2: *Kif3b*, *Kcnq2*; Chr 4: *Mpdz*, *Snape3*). Other QTLs identified were on Chr 2 (proximal), 9, and 12. Network results point to involvement of kinase-related mechanisms and outline the need for further efforts such as interrogation of non-coding RNAs.

Keywords

alcohol consumption and withdrawal; weighted gene coexpression network analysis; dual-trait selective breeding; mouse; microarray

INTRODUCTION

Mice genetically predisposed to a severe ethanol withdrawal response are also often predisposed to low ethanol consumption and *vice versa* (Metten et al., 1998a). This phenomenon has also been observed in rats (Chester et al. 2003). Most frequently, it is observed in populations derived from the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains, e.g. Metten et al. (1998a), where consumption is measured using a standard two-bottle choice paradigm (10% ethanol vs water, continuous access) and withdrawal is measured using handling-induced convulsions (HICs) following injection or vapor inhalation. The reciprocal relationship has been observed in B6×D2 F₂ intercrosses, in lines derived from the F₂ by selective breeding for either drinking or withdrawal, in BXD recombinant inbred (BXD RI) strains and in F₁ crosses between BXD RIs (RIX; Metten et al., 1998a). The strength of the correlation varies (−0.23 to −0.52), but has been consistently negative (Metten et al., 1998a). Data from panels of inbred strains also support this negative genetic relationship (Metten & Crabbe, 2005; Belknap et al., 1993; Metten et al., 1998a). Data from animals selected from genetically heterogeneous stock (HS) populations have been partially supportive. Using an HS4 founder stock (derived from crossing 4 inbred strains), selectively bred lines for high vs. low consumption showed the expected negative relationships to acute withdrawal; however, animals selected for high or low withdrawal showed equivalent preference drinking (Hitzemann et al., 2009). Ethanol Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) selected mouse lines (derived from an 8-way cross) showed significant drinking differences (WSR>WSP) using different procedures (Kosobud et al., 1988); however, using current procedures, we found that genetic effects on drinking depended upon the replicate line examined (i.e., WSR-2>WSP-2, but WSP-1>WSR-1; Crabbe et al., 2013).

In the current study and beginning with a balanced B6×D2 F₂ intercross founder population, animals were simultaneously selectively bred for both high consumption and low acute withdrawal in one line, or *vice versa* in the other line. The former were termed SOTs, from the Old English for habitual drunkard; the latter were simply termed NOTs.

The rationale for using dual-trait selective breeding was that simultaneous selection for the two traits places significantly more selection pressure on the genes acting on the negative genetic correlation than either single trait selection does, and therefore, additional QTLs may be identified that were not previously detected. Genotyping of each of the single trait selected lines was limited to regions previously nominated by data from BXD RIs (Buck et al., 1997; Phillips et al., 1998). For the SOT/NOT selection, we used genome-wide single nucleotide polymorphism (SNP) genotyping to determine whether any new QTLs could be detected that were not previously detected in the single trait selections.

Gene expression data were collected for ventral striatum, a key region in ethanol reward circuitry (Koob and Volkow, 2010) and a region that previous studies had strongly implicated to have a role in ethanol drinking (Iancu et al., 2013b). The transcriptome data were interrogated using the Weighted Gene Coexpression Network Analysis (WGCNA; Zhang and Horvath, 2005), which we have successfully applied to the analysis of brain gene expression in complex murine crosses, and mouse lines selectively bred for other traits (Iancu et al., 2010; 2012a; 2013a;b). Coexpression analyses have provided insights into transcriptome organization in several species, including mouse (e.g. Fuller et al., 2007; Iancu et al. 2010; 2013a). Biological relevance of coexpression is supported by several lines of evidence: coexpressed genes can be independently detected across mouse strains and even species, often code for interacting proteins, display spatial co-localization, and share common transcription factor binding sites (Iancu et al., 2010).

METHODS AND MATERIALS

Animals

Testing was performed within the AAALAC-approved Portland Veterans Affairs Medical Center (PVAMC) Veterinary Medical Unit. Animals were housed in polycarbonate/polysulfone cages with corn cob bedding (Bed-o-Cob, The Andersons, Inc., Maumee, Ohio). Mice were weaned at 21 ± 1 days of age and separated by sex into groups of 2-5, with cages changed once weekly. Animals were fed standard rodent chow (Purina 5001), except during breeding (when dietary fat was increased to 9%, Purina 5008, PMI Nutrition International, Brentwood, MO, USA). Water was available *ad libitum*. Rooms were maintained at 22 ± 1 °C with a 12:12 hour light:dark cycle (lights on at 0600). Animal care and use were approved by the Institutional Animal Care and Use Committee at PVAMC and were in compliance with NIH and USDA guidelines.

Selection index and selective breeding

Tests were performed using well-established methods (Phillips et al., 1994; Metten et al., 1998b). A short two-bottle choice test was followed 1 week later by assessment of withdrawal severity derived from HIC scores following an acute, high-dose ethanol injection. Test order was determined in a separate study using B6×D2 F2 mice (not shown). Selection indices for the SOT/NOT lines used rank-ordering of drinking (g/kg) followed by withdrawal severity scores. For the SOT line, in each generation, animals with the greatest drinking values and lowest withdrawal scores were used to select breeders to perpetuate the

SOT line. Likewise, animals with the lowest drinking values and the greatest withdrawal severity scores were used to perpetuate the NOT line.

Behavioral Testing – Ethanol Consumption—Adult (7-14 weeks), naïve mice of both sexes were individually housed 7-10 days before testing for ethanol consumption began. Ethanol consumption was measured with a two-bottle free-choice procedure in which 3% then 10% ethanol (Decon Laboratories, Inc., King of Prussia, PA) was offered versus tap water over an 8-day period (Phillips et al., 1994). Each concentration was offered for four days, with the positions of the ethanol and water tubes switched every two days to control for drinking bottle position bias. Ethanol intake in g/kg per mouse was calculated for each day of drinking. The second and fourth days that 10% ethanol was offered (the second day in each case that ethanol was offered in each of the two positions) were averaged to calculate the drinking index. We elected to use this test because it allowed comparison with many published studies despite the fact that it is unlikely that blood ethanol concentrations achieved would be above modest levels (Dole and Gentry, 1984).

Behavioral Testing – Acute Withdrawal—One week after ethanol consumption was tested, mice were weighed and scored twice at a 20 minute interval for measurement of baseline handling-induced convulsion (HIC) severity (Metten et al., 1998b). The HIC is a measure of neuro-excitability (Goldstein and Pal, 1971). Withdrawal HICs have been shown to be sensitive to both ethanol dose and exposure duration (Goldstein, 1972), and acute ethanol withdrawal HIC severity is heritable (Metten et al., 1998b). Immediately after the second baseline measure, mice were injected with 4 g/kg ethanol i.p. (prepared as 20% v/v in physiological saline). Mice were scored for HICs beginning two hours after injection, and hourly through hour 12. After ethanol injection, HIC scores were initially suppressed for several hr, usually again exceeding baseline at 4 hr and then increasing for several hr thereafter. Therefore, the 9 HIC scores from 4 to 12 hr after injection were totaled. The primary index of withdrawal was the residual withdrawal severity with correction for baseline (RESIDUAL WD), calculated by regressing the total score on the average baseline. Each animal's RESIDUAL WD score was the residual of its total score from that regression.

For genomics methods, please see Supplementary Materials.

RESULTS

Dual-Trait Selection

Figure 1 (a) shows the response to selective breeding of SOT and NOT lines by generation S_3 . Data for S_3 mice during their selection testing for both traits are compared to the S_0 generation. S_3 SOT mice drank 1.2 g/kg/day more than S_0 , and NOT mice 2.2 g/kg/day less. By S_3 , withdrawal severity had decreased in SOT mice and increased in NOT mice to approximately the same extent (S_0 mean withdrawal was zero using this index). All values were significantly different from the S_0 ($|t| \geq 2.2$, $p \leq 0.05$) and SOT scores for each trait were significantly different from NOT scores at S_3 ($F_{1,131} = 13.7$, $p \leq 0.001$). Figure 1 (b) shows bivariate boxplots of drinking and withdrawal scores, further demonstrating the phenotypic segregation of SOT (red) and NOT (green) mice for both traits. Thus, the S_3

generation presented the optimal generation for detection and mapping of QTLs because random drift early in selection is relatively small compared to later generations.

Genetic distances between the SOT/NOT groups; QTL analysis

Genetic distances (based on allelic dissimilarity) among selected samples, as well as the inbred B6 and D2 ancestors, were calculated and plotted (Figure 1c). The figure suggests that the groups were well-separated genetically due to selection and between- versus within-group distances were significant (AMOVA, $p < 0.001$). This genetic analysis is distinct from and complementary to the marker- by-marker QTL detection procedure (below); inclusion of all genetic markers regardless of penetrance or significance level allows estimation of overall genetic divergence between selected lines.

Significant QTLs differentiating SOT from NOT lines were detected at LOD 10 on Chr 2, 4, 9 and 12 (Figure 2 & Table 1). A new, suggestive QTL (peak LOD = 8.7) on proximal Chr 6 was also detected. On Chr 2 there appear to be at least two QTLs: one (or possibly two) proximal QTL(s) for which B6 alleles are associated with the SOT phenotype and a distal QTL for which B6 alleles are associated with the NOT phenotype. QTLs detected on Chr 2 (proximal), 4 and 9 overlap with QTLs previously detected for ethanol consumption and withdrawal (Buck et al., 1997; Crabbe, 1998; Belknap & Atkins, 2001; Mulligan et al., 2006; Milner et al., 2013). The QTL detected on Chr 12 has been implicated previously, but only for chronic withdrawal severity (Crabbe, 1998).

Construction and validation of the consensus gene co-expression network

After filtering the gene expression data, 9,889 transcripts were retained for network construction; the transcripts were associated with 7,426 unique gene IDs. The WGCNA framework (Langfelder and Horvath, 2008; Iancu et al., 2012a) was used to construct a consensus network by combining SOT and NOT sample data. Clustering the adjacency matrix and combining close modules (see Supplementary Methods) revealed 21 modules, each randomly assigned a nominal color for easier graphical visualization; size of the modules varied between 42 (lavenderblush3) and 1122 (coral2) transcripts. 4,395 gene transcripts were not assigned to a distinct module by clustering and are denoted by “grey”. The composition of each module is found in Supplemental Table 1. Figure 3 visualizes the network as a cluster dendrogram. To validate the clustering results, we quantified (using Z scores) the “quality” of the modules (Langfelder et al., 2011); a bootstrapping procedure was used to compare average connectivity of modules to average connectivity of random transcript groups. Over half the modules (13) had high quality Z scores (>10) (Langfelder et al., 2011); the remainder of Z scores were > 2 , implying moderate quality. In contrast, Z score for the “grey module” (the aggregation of genes not belonging to actual modules) was -14 , indicating lack of coexpression among these transcripts.

GO annotation of consensus modules revealed association with distinct biological processes (Supplemental Table 2). Modules were also enriched in some cases with transcripts associated with specific cell types (Cahoy et al., 2008). Four modules were enriched with neuronal cell markers, two modules with oligodendrocyte markers and one module with astrocyte markers (Bonferroni corrected $p < 0.05$; Supplemental Table 3). The lightcyan

module was enriched in neuronal transcripts ($p < 0.00014$), and the lightpink4 module was enriched in oligodendrocyte transcripts ($p < 0.038$).

Additionally, we compared network edge weights between genes with known protein-protein interactions (PPI) versus random gene pairs. We found a ten-fold increase in average coexpression for genes with known PPI ($p < 10^{-15}$).

Differential network connectivity between SOT and NOT networks

Module disruption was assessed by comparing networks constructed using the SOT and NOT data, respectively; statistical significance was evaluated by comparing the difference between the SOT and NOT networks with chance differences, which arise by comparing two networks that are both comprised of a mixture of SOT and NOT samples (see Supplemental Methods). Additional details of this procedure are available elsewhere (Iancu et al., 2012b; 2013a).

Several modules were strongly affected by selection (identified by changes in corKME: the correlation of transcripts within a module to the module eigengene, similar to the first principal component; see Supplemental Table 4 for definitions of abbreviations and data). Several modules showed disruption Z scores below -10 : “darkturquoise” (-10.5), “lightcyan1” (-54.6), “lightpink4” (-55.6) and “plum2” (-22). Several other modules displayed moderate but significant disruption (Z scores between -2 and -10). The lightpink4 module showed the most pronounced corKME disruption, which was also reflected in all other measures of module disruption. Key GO categories enriched in the lightpink4 module include: 1) positive regulation of MAP kinase cascade by fibroblast growth factor receptor signaling pathway; 2) ubiquinone biosynthetic process; 3) negative regulation of activation of membrane attack complex; 4) clathrin adaptor complex; and 5) UDP-N-acetylmuramate dehydrogenase activity (Supplemental Table 2). We illustrate connectivity differences emerging in the lightpink4 module in Figure 4. The SOT group appears to have much stronger connectivity/coordination compared with the NOT group.

The complete collection of summary disruption Z scores is presented in Supplemental Table 4. Within the most strongly affected modules, individual genes with connectivity profiles strongly affected by selection were identified (Supplemental Table 5). In the lightpink4 module, 238 of 515 transcripts showed disruption Z scores of < -10 .

Differential expression between SOT and NOT selected lines

Differential gene expression (DE) between the selected lines was evaluated for all probes included in the network analysis. P-values were calculated using eBayes modified t-statistic and further adjusted using the False Discovery Rate (FDR; Benjamini and Hochberg, 1995). At an FDR of 0.01, we detected 1,745 differentially expressed transcripts; Ensembl annotation was available for 1,647 transcripts. Identities and module assignments of DE probes are provided in Supplemental Table 6 and GO annotations of DE transcripts are in Supplemental Table 7. For the GO categories biological process, cellular compartment and metabolic function, DE transcripts were markedly enriched for regulation of double-strand

break repair via homologous recombination, intracellular part and structural constituent of myelin sheath, respectively.

Several modules incorporated many DE transcripts, quantified by comparing the number of actual and expected number of DE transcripts within each module (Fisher's exact test); five modules were significantly enriched (Supplemental Table 8). The most significantly enriched was the lightpink4 module ($p < 2 \times 10^{-88}$). The darkolivegreen module also showed a highly significant enrichment ($p < 5 \times 10^{-52}$); interestingly, module disruption in this module was modest (Supplemental Table 4).

Alignment of QTL, gene network and differential expression data

The differentially expressed genes were cross-referenced with the detected reciprocal QTLs. Table 2 presents the differentially expressed genes under the QTL intervals. Although all QTL intervals were scanned for DE genes, of particular interest are the highly significant QTLs on Chr 2 (proximal), which had been previously detected for consumption (Belknap and Atkins, 2001) and, for withdrawal, QTLs on Chr 2 (proximal and distal; both suggestive) and on Chr 4 (significant) (e.g. Buck et al., 1997). To increase sensitivity and considering the likelihood of strong linkage disequilibrium in this cross, we chose to interpret broadly QTL intervals to include 25 to 100 Mbp and 150 to 185 Mbp on Chr 2 and 75 to 125 Mbp on Chr 4. Transcripts in these intervals that were differentially expressed (DE) are highlighted in Supplemental Table 6. To provide independent validation, we leveraged a B6×D2 F₂ dataset derived from striatal tissue that has been used previously to detect eQTLs (Iancu et al., 2013a). Genes that are 1) under the broadly defined significant QTL intervals, 2) differentially expressed and 3) previously reported as cis-eQTLs in the F₂ cross (Iancu et al., 2013a) are considered the strongest candidates for further investigation. For the proximal Chr 2 interval, none of the DE transcripts were associated with cis-eQTLs. However, in the distal Chr 2 interval, cis-eQTLs aligned with four DE transcripts. Alignment was most significant for *Kif3b* ($p < 2.4 \times 10^{-20}$) and *Kcnq2* (6.3×10^{-23}). For the Chr 4 interval, two DE transcripts were associated with cis-eQTLs (*Mpdz* and *Snapc3*).

DISCUSSION

To our knowledge, the data presented here are the first to utilize a dual-trait selection for alcohol-associated phenotypes. However, the high consumption/low withdrawal (SOT) and low consumption/high withdrawal (NOT) phenotypes build upon previous data demonstrating that at least for B6×D2 crosses, consumption and withdrawal have a significant negative genetic correlation and thus, some common genetic basis. Our working hypothesis was that the dual-trait procedure would increase selection pressure on these common loci and perhaps find new common loci not detected in single trait selection.

The current study again confirms the presence of QTLs on Chr 2, 4, and 9. The significant QTL on Chr 12, found here by genome-wide genotyping, is new and must be considered provisional until replicated (a replicate SOT/NOT selection is in progress). The suggestive QTL on Chr 6 was also detected in the HS4-derived selection (Hitzemann et al., 2009). Preference or withdrawal QTLs previously detected on Chr 1, 3, 4 (distal), 9 (distal), 15 and 19 were not confirmed here suggesting that these loci are either unlikely to be associated

with common genetic mechanisms between the two traits and/or have small effect sizes. The question naturally arises as to whether the Chr 2 (proximal and distal), 4 (mid) and 9 (proximal) QTLs are simply a composite of separate preference and withdrawal loci or are true pleiotropic loci. The single trait selection data would favor the former argument; for example, the Chr 4 (mid) QTL has been repeatedly detected for acute and chronic withdrawal but not preference consumption. However, these data should be interpreted cautiously as most of the early mapping studies were significantly underpowered and often depended on guidance to detect QTLs from a small panel of BXD RIs.

The Chr 4 QTL is instructive in this regard since the associated withdrawal quantitative trait gene (QTG), *Mpdz*, has been identified (Shirley et al., 2004; Milner et al., 2013). *Mpdz* is differentially expressed between the SOT and NOT lines in the predicted direction and shows strong cis-regulation. Importantly, knockout heterozygote animals implicate *Mpdz* in regulation of ethanol consumption (Milner et al., 2013). We interpret these data to show that *Mpdz* does have a role in the negative genetic correlation between withdrawal and preference.

In this study, identification of chromosomal regions where QTLs have effects on the two traits in an inverse direction could be due to two causes. The first is pleiotropy, where a single gene is responsible causing inverse or reciprocal effects on the two phenotypes. The second is that two (or more) distinct closely-linked genes could be responsible. In this study, the map resolution is too low to distinguish from among these two possibilities, but the present data have identified target chromosomal regions for further research with KOs, knock-ins, transgenics, expression and other studies better suited to directly test pleiotropy vs closely-linked genes at the single gene level. A good example is the gene *Mpdz* noted above. In other words, this study points to hypotheses about pleiotropy suitable for testing using other methods that are more gene specific. We cannot directly test these hypotheses at the present time from the data presented here.

Analysis of the Chr 2 data is complicated by the likely presence of two proximal and one distal QTLs. As noted in Belknap and Atkins (2001), a proximal Chr 2 QTL for ethanol consumption has been detected in multiple studies. Buck and colleagues, using the BXD recombinant inbred strain panel, a B6D2F2 intercross, and the High Alcohol Withdrawal (HAW) and Low Alcohol Withdrawal (LAW) selected lines, detected 2 QTLs on Chr 2 that were associated with acute withdrawal. For the proximal QTL, the B6 allele was associated with reduced withdrawal while for the distal QTL, the B6 allele increased withdrawal (Buck et al., 1997). The proximal QTL data were corroborated using the WSP and WSR selected lines (Crabbe et al., 1994). The directions of the allele effects in both projects were in the predicted direction for influencing the negative genetic correlation. Data in Figure 2 are consistent with these observations. Of the differentially expressed transcripts within the proximal QTL, none were found to be cis-regulated. However, two differentially expressed and strongly cis-regulated candidates, *Kif3b* and *Kcnq2*, aligned with the distal QTL. *Kcnq2* (M-type potassium channel) is an appealing candidate given that *Kcnq2* mutations are associated with a variety of seizure disorders and given that there are specific channel agonists, e.g., retigabine (ezogabine).

Alignment of differential transcript expression and the proximal Chr 9 QTL was featured in the Mulligan et al. (2006) meta analysis that focused on relating ethanol consumption (2-bottle choice) and whole brain RNA expression data from selected lines, inbred mouse strains and a Chr 9 congenic strain derived from a B6×D2 intercross. A key candidate QTG that emerged from this analysis was *Scn4b*, which was subsequently also found to be associated with a different alcohol drinking phenotype, drinking in the dark (DID; Mulligan et al., 2011). However, there was no significant difference in *Scn4b* expression between the SOT and NOT lines (Supplemental Table 6). As we only sampled one brain region, this may be an example of where whole brain studies detected an effect not seen in a discrete brain region. However, it seems unlikely that not detecting a difference was due to inadequate sample power as N=48/line were used.

Three generations of SOT/NOT selection was associated with marked changes in coexpression network structure, especially in the lightpink4 module. Not only were there significant differences in intramodular connectivity between SOT and NOT lines, more than half of the module transcripts were differentially expressed. We hypothesized that such marked differences in expression could be caused by differential expression of miRNAs preferentially targeting the module, which in turn could potentially affect all their target genes. However, we did not detect selection effects on the expression levels of the surveyed microRNAs (data not shown). Similarly, transcription factors preferentially targeting specific modules could also account for changes in expression or modular connectivity within the modules; however, no differentially expressed transcription factors were detected. This suggests that other factor(s), not measured in the current study (e.g., non-coding RNAs), could play a significant role; evaluation of this hypothesis will require an alternative strategy of transcriptome analysis such as RNA-Seq (Hitzemann et al., 2013).

While specific causal links resulting in disruption of the lightpink4 module are not fully clear, the large transcriptional effects suggest a role in the behavioral differences. GO annotation of the lightpink4 module revealed enrichment in MAP kinase signaling-associated transcripts (e.g., *MAP2k1* [Chr 9] and *MAP4k4* [Chr 1]). These observations are consistent with other studies associating MAP kinase signaling pathways with ethanol consumption, reward and physical dependence. For example, inhibition of EGFR/ERK signaling decreases ethanol but not sucrose consumption in rats (Corl et al., 2009). Inhibition of ERK1/2 signaling alters operant self-administration of ethanol in B6 mice (Faccidomo et al., 2009). Rewarding effects could be achieved by activated ERK in the mesolimbic pathway. Modulatory action of compounds such as BDNF and GDNF are dependent on MAP kinases (Jeanblanc et al., 2013; Carnicella et al., 2008). In ethanol dependent rats, neuroadaptations encompassing MEK/ERK signaling occur in a region-specific manner (Hansson et al., 2008). Additionally, relapse-inducing cues activated ERK1/2 signaling in the basolateral amygdala of rats previously trained to self-administer ethanol (Radwanska et al., 2008).

A role for MAP kinases in ethanol related phenotypes has also been identified using less biased genetic approaches. In cultured B6 cortical neurons, several MAP kinase genes, including those in the lightpink4 module, are upregulated following exposure to ethanol (Wang et al., 2007). Comparison of gene expression across multiple regions between alcohol

preferring (AA) and alcohol non-preferring (ANA) rat selected lines identified a cluster of signal transduction genes associated with MAPK signaling, with increased expression in the nucleus accumbens of AA rats (Sommer et al., 2006). MAP kinase regulation was over-represented in a group of genes differentially expressed in the nucleus accumbens between chronic alcohol-exposed vs. alcohol naïve AA rats (Bell et al., 2009). In a meta-analysis of microarray studies comparing high and low alcohol preference animals, *MAP4k4*, a lightpink4 member, showed differential expression across several lines and was within the top 75 transcripts with regard to absolute effect size (Mulligan et al., 2006). However, in BXD RI strains alone, there were no significant correlations between consumption or withdrawal and striatal expression of either of the MAP kinases present in the lightpink4 module (GeneNetwork databases [www.genenetwork.org]). This may illustrate the increased signal available from selected lines relative to the BXD RIs.

SOT/NOT selection was associated with marked changes in the transcriptome that were easily detected at the level of gene module disruption and transcript differential expression. For the lightpink4 module, differences in connectivity were particularly strong. Compared to similar studies, the extent of differential transcript expression is much greater as is the number of differentially expressed transcripts that also exhibit intra-modular connectivity changes (Iancu et al., 2013b). We detected several strong QTL intervals concordant with previous genetic studies. Many transcriptional effects were concentrated in the lightpink4 module, suggesting a clear path for further pursuit of these results. We would now like to identify regulatory factors that are related in a cis-manner to the detected QTLs, preferentially target the lightpink4 genes, and also examine differential expression and network intra-modular connectivity in other brain regions linked to withdrawal severity, such as hippocampus or amygdala.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Differences in phenotype and genotype emerging due to selection. (a) Ethanol drinking and residual withdrawal scores in the S3 generation of the SOT and NOT selected lines relative to the S0 generation means (solid line). Left Y-axis: Consumption is expressed as g/kg of 10% ethanol consumed/day. Light grey bars depict drinking increases (SOT line) and decreases (NOT line). Right Y-axis: Withdrawal is expressed as the residual from linear regression of post-injection HIC scores on baseline HIC (see Supplemental Methods). Black bars depict withdrawal increases (NOT line) and decreases (SOT line). All values were significantly different from the mean values in the S0 generation ($|t_s| \geq 2.17$, $p_s < 0.05$). (b) Bivariate boxplots of the drinking and withdrawal scores. The inner ellipses contain 50% of the data, while the outer ellipse is obtained by enlarging the inner ellipse by a factor of 3. The figure illustrates the phenotypic segregation of the SOT (red) and NOT (green) animals. (c) Multidimensional scaling (MDS) plots of genome-wide differences in genetic background between the SOT and NOT selected lines; colors as in (b). Total genetic distance between samples is computed by summation, over markers, of the number of different alleles. The collection of distances is projected on two dimensions for visualization. The figure illustrates the genetic segregation of the SOT (red) and NOT (green) animals. As expected, SOT animals are genetically close to B6 while NOTs are closer to D2; the distance between B6 and D2 is the largest possible distance given the design of our F2 intercross and selective breeding.

Figure 2. QTL analysis of the SOT and NOT selected lines on chromosomes 2, 4, 9 and 12
 Data are plotted as positive LOD scores. Given the multiple markers used in the QTL analysis (> 2900) and the genetic drift associated with short-term selective breeding, the threshold for a significant QTL was set at 10 (see Hitzemann et al. 2009). Alignment of QTL, gene network and differential expression data identified four cis-eQTLs strongly associated with differential expression: *Kif3b* and *Kcnq2* on Chr 2 (distal) and *Mpdz* and *Snapc3* on Chr 4 are shown along the x-axis. See Results.

Figure 3. Representation of the consensus network and resulting modules as a cluster dendrogram

Identification of modules is achieved by cutting the dendrogram tree (see text). Modules are represented by the colors below the cluster tree. Genes un-assigned to a specific module are denoted by grey. While colors are arbitrary, the width of the module color bar approximates the number of transcripts in the module.

Figure 4. Illustration of genetic selection effects on the lightpink4 module

a: Comparison of lightpink4 kME values. A majority of the genes become highly aligned with the module eigengene (kME values near 1). b: Illustration of the NOT module genes with edges weights > 0.2 . c: Illustration of SOT lightpink4 genes with edge weights > 0.83 . Even though for visual clarity the threshold is much higher than in NOT network, more genes and edges are selected.

Table 1

Quantitative Trait Loci (QTL) for the negative genetic correlation between drinking and withdrawal.

QTL (Chr)	Location (Mbp)	Allele associated with SOT phenotype *	Candidate Gene(s) for cis-eQTLs	Peak LOD	Previously Nominated? (Mbp; allele effect)	Original Reference
2	18.59-40.60	B6		23.86	Drinking (38.2; B6 ↑)	Phillips et al., 1998
	64.98-71.90	B6		18.83	Chronic WD (66.7; B6 ↓)	Buck et al., 2002
3					Acute WD (60-66; B6 ↓)	Buck et al., 1997
					Drinking (85.9; B6 ↑)	Phillips et al., 1998
	171.38-180.54	D2	<i>Kin3b, Kcnq2</i>	15.86	Acute WD (137-164 ^{**} ; B6 ↑)	Buck et al., 1997
3	10.31-32.73	B6		8.90	Acute WD (101.1; B6 ↓)	Hitzemann et al., 2009
					Drinking (101.1; B6 ↑)	Hitzemann et al., 2009
4	82.18-103.72	B6	<i>Mpdz, Snapc3</i>	15.81	Acute WD (<i>Mpdz</i> 81.2; B6 ↓)	Shirley et al., 2004
					Chronic WD (67-91; B6 ↓)	Buck et al., 2002
					Drinking (124.3; B6 ↑)	Phillips et al; 1998
6	98.45-99.54	B6		8.67	Acute WD (86-115; B6 ↓)	Buck et al; 1997
					Acute WD (101.1; B6 ↓)	Hitzemann et al., 2009
					Drinking (101.1; B6 ↑)	Hitzemann et al., 2009
9	30.66-31.16	B6		10.36	Drinking (32-76; B6 ↑)	Phillips et al; 1998
					Chronic WD (37-52; B6 ↓)	Buck et al., 2002
					Chronic WD (85 ^{**} ; B6 ↑)	Crabbe, 1998
12	9.85-15.86	D2		13.00	Chronic WD (120 ^{**} ; B6 ↓)	Crabbe, 1998

* SOT phenotype: high drinking & low withdrawal

** marker used is now identified as syntenic, meaning it could be anywhere on the Chr. The Mbp locations of these QTLs were identified using other markers or genes in the region of the original location of the marker.

Table 2

Differentially expressed genes under the QTL regions nominated by dual-trait selection for drinking and withdrawal.

entrezgene	External gene id	Chr	Start position (bp)	End position (bp)	Gene name
241303	<i>Fam78a</i>	2	32050055	32084857	family with sequence similarity 78, member A
107771	<i>Bmyc</i>	2	25706879	25707717	brain expressed myelocytomatosis oncogene
70266	<i>Ccbl1</i>	2	30185124	30205847	cysteine conjugate-beta lyase 1
12908	<i>Crat</i>	2	30400471	30415813	carnitine acetyltransferase
98952	<i>Fam102a</i>	2	32535332	32569756	family with sequence similarity 102, member A
30839	<i>Fbxw5</i>	2	25500750	25505471	F-box and WD-40 domain protein 5
66691	<i>Gapvd1</i>	2	34676184	34755232	GTPase activating protein and VPS9 domains 1
241263	<i>Gpr158</i>	2	21367542	21830547	G protein-coupled receptor 158
14810	<i>Grin1</i>	2	25291181	25319187	glutamate receptor, ionotropic, NMDA1 (zeta 1)
14828	<i>Hspa5</i>	2	34771970	34776531	heat shock protein 5
68975	<i>Med27</i>	2	29346819	29524793	mediator complex subunit 27
67871	<i>Mrrf</i>	2	36136389	36190647	mitochondrial ribosome recycling factor
56876	<i>Nsmf</i>	2	25054355	25062881	NMDA receptor synaptonuclear signaling and neuronal migration factor
96979	<i>Piges2</i>	2	32395896	32402739	prostaglandin E synthase 2
269254	<i>Setx</i>	2	29124181	29182471	senataxin
227731	<i>Slc25a25</i>	2	32414487	32451445	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25
227613	<i>Tubb4b</i>	2	25222160	25224702	tubulin, beta 4B class IVB
98766	<i>Ubac1</i>	2	25998543	26021747	ubiquitin associated domain containing 1
14415	<i>Gad1</i>	2	70553072	70602014	glutamate decarboxylase 1
20823	<i>Ssb</i>	2	69861562	69871846	Sjogren syndrome antigen B
53416	<i>Stk39</i>	2	68210445	68472268	serine/threonine kinase 39
67126	<i>Atp5e</i>	2	174461072	174464105	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit
14683	<i>Gnas</i>	2	174284320	174346744	GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus
329575	<i>Gm14325</i>	2	177831791	177840336	predicted gene 14325
241846	<i>Lsm14b</i>	2	180024987	180035465	LSM14 homolog B (SCD6, S. cerevisiae)
15569	<i>Elav12</i>	4	91250763	91400785	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)
67733	<i>Igfb3bp</i>	4	99765402	99929813	integrin beta 3 binding protein (beta3-endonexin)

entrezgene	External gene_id	Chr	Start position (bp)	End position (bp)	Gene name
16451	<i>Jak1</i>	4	101152367	101265282	Janus kinase 1
242521	<i>Klhl9</i>	4	88718292	88722465	kelch-like 9
70122	<i>Mllt3</i>	4	87769925	88033364	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3
17475	<i>Mpdz</i>	4	81278500	81442815	multiple PDZ domain protein
320713	<i>Mysm1</i>	4	94942040	94979100	myb-like, SWIRM and MPN domains 1
67013	<i>Oma1</i>	4	103313812	103371868	OMA1 homolog, zinc metallopeptidase (S. cerevisiae)
72157	<i>Pgm2</i>	4	99929414	99987294	phosphoglucosyltransferase 2
68441	<i>Rraga</i>	4	86575673	86577281	Ras-related GTP binding A
77634	<i>Snopc3</i>	4	83417724	83467676	small nuclear RNA activating complex, polypeptide 3
242584	<i>Wdr78</i>	4	103038065	103114555	WD repeat domain 78
105005	<i>Fam84a</i>	12	14147599	14152038	family with sequence similarity 84, member A
105014	<i>Rdh14</i>	12	10390780	10395562	retinol dehydrogenase 14 (all-trans and 9-cis)