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The genetical genomic path to understanding why rats and humans consume too much alcohol

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Abstract

Background: At the invitation of the Journal, we are providing a summary of our published work that has followed the publication in 2009 of our manuscript entitled “Genetical Genomic Determinants of Alcohol Consumption in Rats and Humans”. Our initial premise, which has been maintained throughout, is that knowledge regarding gene transcription would greatly enhance GWAS of alcohol-related phenotypes. We chose to concentrate our studies on the quantitative phenotype of alcohol consumption since high levels of alcohol consumption are a prerequisite for the development of alcohol use disorder (AUD). We also structured our studies to focus on “predisposition” to higher levels of alcohol consumption. We defined predisposition as a genetic structure and transcriptional pattern that is inherent in an organism and present prior to exposure to an environmental stimulus that engenders a physiological/behavioral response. In studies using humans, this interest in predisposition usually requires prolonged periods of cohort follow-up. On the other hand, studies with animals can use resources such as panels of recombinant inbred (RI) animals (in our case, the HXB/BXH rat panel) to capture the transcriptional landscape of animals not exposed to alcohol and compare this transcriptional landscape to levels of alcohol consumption collected from a different cohort of animals that are the same age, have an identical genetic composition, and are raised in an identical environment. The other benefit is that the stable genetic structure of inbred strains allows for a chronological expansion of information on these animals. This characteristic of the HXB/BXH RI rats allowed us to add important information as technology and analytical methods developed over time.

Methods, findings, and conclusions: Our initial studies relied on hybridization arrays for RNA quantification in brain, an initial set of polymorphic markers for the rat genome, and a standard behavioral (b)QTL analysis for alcohol consumption. What we added to the conceptual

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Conflict of Interest

The authors list no competing interests.

basis for analysis and interpretation was the calculation of transcript expression (e)QTLs and the requirements that: 1. the eQTL overlapped the location of the bQTL; and 2. the transcript levels were significantly correlated with the quantitative levels of alcohol consumption across rat strains. These criteria were used to identify genes (transcripts) as "candidate" contributors to the alcohol consumption phenotype. We soon realized that the search for candidate genes as unique determinants of a complex trait is irrational, since these phenotypes are best characterized by differences in genetic networks. Therefore, we incorporated Weighted Gene Coexpression Network Analysis (WGCNA) in our further work. We also realized the limitations of hybridization arrays for breadth of transcriptome coverage and quantification, and in the more current work used total RNA-Seq-derived data for characterizing nearly all of the brain transcriptome. Finally, we participated in the efforts for whole genome sequencing of the strains of the HXB/BXH panel, generating an extensive new panel of markers for remapping of the QTLs. We also realized that the biological determinants of a behavioral phenotype do not have to reside in brain and, by examining the liver transcriptome, we found that the gut-liver-brain axis was, in part, involved in predisposition to higher levels of free-choice alcohol consumption. In all, from the first exploration of the genetical genomics of the alcohol consumption phenotype, to the current status of our work, the function of the brain immune system, with emphasis on microglia and astrocytes, even prior to the animal being offered alcohol, has emerged as a most significant genetic contributor to the amount of alcohol an animal will consume on a daily basis. Particularly prominent was a cluster of inflammasome (NLRP3)-modulating transcripts (*P2rx4, Ift81, Oas1b, Txnip*) and a long noncoding transcript*, "Lrap"* that repeatedly appeared within a gene coexpression module associated with alcohol consumption levels. Interestingly, data from post-mortem tissue from brain of humans suffering from AUD also indicates a hyperactive neuroimmune function. The data from studies with animals may indicate that neuroimmune hyperactivity may be a trait rather than a state marker for AUD.

Review

Alcohol Use Disorder (AUD) as defined by DSM-5 [1] affects a large segment of the global human population. AUD contributes to billions of U.S. dollars in health costs and destroys the health and quality of life of its sufferers [2]. The effects of AUD [3,4] extend well beyond the individuals consuming the alcohol, to include others who suffer from the actions of the individuals with AUD (traffic accidents, homicides, violence, etc.) [5]. The etiology of AUD involves both genetic and environmental factors with each contributing almost equally to the risk of development of AUD. However, AUD cannot occur without the consumption of alcohol (ethanol) and both successful prevention and effective treatment of AUD depend on biological and environmental factors that promote "heavy drinking." Interestingly, genome-wide association studies (GWAS) in humans have demonstrated that AUD and heavy drinking have different genetic loci that contribute to these behaviors [6]. This genetic distinction leads to the premise that AUD is a progressive phenomenon in which certain genetic and environmental factors predispose the levels of alcohol consumption, and different or additional factors are necessary to predispose the neuro(mal)adaptive events that lead to AUD.

With regard to AUD, GWAS and other genetic analyses have identified a locus on human chromosome 4 aligning to the location of genes coding for the alcohol dehydrogenase

(ADH) enzymes as being significantly associated with the AUD diagnosis [7,8]. However, there continues to be some difficulty linking this locus to a functional explanation for the development and maintenance of AUD in humans [7,8]. One issue is the fact that only one of the seven ADH isoforms is expressed in the brain (Class III, ADH5, chi-ADH) [9,10] and that isoform has low ability to metabolize ethanol (Km>1000 mM). Furthermore, the Class III ADH gene has, at best, only a modest association with the markers highly associated with AUD [8]. Another unanswered question regarding the association of the ADH locus with AUD is whether a non-coding transcript is generated within the vicinity of that locus to influence transcriptional events elsewhere in the genome that contribute to AUD. It is important to note that, although ADH enzymes as well as quantities of alcohol consumed by an individual are highly associated with AUD, a significant number of other loci differentiate these traits.

We had decided, some 20 years ago, that the examination of the transcriptome, in addition to the genome, may be a more profitable approach for insights regarding the biological factors that promote heavy drinking and contribute to the etiology of AUD and/or differentiate the trait of heavy drinking from AUD. It has been proposed that most genetic variants associated with complex behavioral traits in humans likely involve gene transcription [11]. Additionally, we decided to use animal models providing quantitative measures of alcohol consumption [12] to focus on this necessary factor for development of AUD (high levels of alcohol consumption). In other words, we wanted to go back to the beginning of the chain of events that leads to AUD. We use the word “predisposition” to describe the biological/genetic factors that are present prior to an organism (including human) being exposed to alcohol that then promote behavior associated with high levels of alcohol consumption. Animals from an inbred strain can be considered identical (monozygotic) twins and within a recombinant inbred (RI) panel of animals derived from two progenitor strains the comparison between RI strains is equivalent to a comparison of dizygotic (fraternal) twins. The HXB/BXH RI panel [13] allows for investigation of genetic predisposition, i.e., we used non-alcohol-treated rats within a particular strain to characterize the brain transcriptome and their genetically identical relatives (rats of the same strain) to ascertain levels of alcohol consumption. The transcriptome analysis provides the state of the brain prior to the rats being offered alcohol, and the transcripts found to be related to alcohol consumption levels across the tested strains are considered to predispose to a particular level of alcohol intake.

The use of RI strains of rodents also allows for a quantitative genetic analysis of traits such as quantitative transcriptome information (levels of RNA expression) as well as the behavioral traits such as the amount of alcohol an animal will consume given a choice of an alcohol solution or water. The comparison (or mapping) of a trait with differences in DNA sequence generates a genetic locus (Quantitative Trait Locus, QTL) associated with the trait being examined, much like the genomic locus derived from GWAS. We have added an additional requirement for interpretation to our QTL analysis for relating transcriptome information to alcohol drinking levels. We require that two trait QTL locations, i.e., the behavioral QTL, bQTL, and a QTL associated with gene expression levels, eQTL, overlap to indicate that difference in DNA sequence, e.g., single nucleotide polymorphisms (SNPs) located in the overlapping interval may influence both traits.

In 2009 we published an initial attempt to utilize both an animal model consisting of a panel of RI rats (the HXB/BXH panel) [14] and human subjects phenotyped for their levels of alcohol consumption (WHO/ISBRA study) [15]. The title of the manuscript was “Genetical Genomic Determinants of Alcohol Consumption in Rats and Humans” [14]. The intent was to ascertain whether the quantitative genetic analysis of the rats’ drinking behavior provided behavioral QTL (bQTL) locations that we could link to significant expression QTLs (eQTLs) derived from data we gathered on the rat whole brain quantitative measures of RNA expression. For transcript identification and quantification, we used what were, at the time, newly developed rat RNA hybridization arrays [14]. We further postulated that overlapping loci of the bQTL and eQTL in rats may also align with one or more loci determined from our genetic association study of alcohol consumption in humans. For the study with humans, we were using two small populations of Canadians and Australians (890 total) and a “SNP chip” containing “alcoholism-related gene sequences” [16]. Clearly, both approaches and technologies were not adequate by current standards. However, the results were instructive and informative. We were able to reap the benefit of information on the bQTLs for levels of alcohol consumption by rats, which demonstrated that the HXB/BXH RI panel provided a broad range of alcohol drinking levels and QTL locations that we could exploit in this original and in our future studies. At this point, we were still considering that we could use the bQTL/eQTL overlap to identify the individual transcripts contributing to the alcohol consumption phenotype, but due to the large intervals covered by the bQTLs there were numerous cis and trans eQTLs aligned to those intervals. In all, 20 transcripts had eQTLs that overlapped one of the 2 bQTLs for alcohol consumption, and these transcripts also survived two additional filters: (1) their broad-sense heritability had to be greater than 0.5 and (2) their RNA expression levels across the 30 strains had to be significantly correlated with levels of alcohol consumption.

The question that quickly arose was whether there was a functional relationship among the identified transcripts or whether they represented multiple “independent” biological processes involved in modulating alcohol consumption. Additionally, none of the transcripts identified by these rat studies were identical to the products of genes identified on the “SNP chip” hybridization arrays used to analyze the human DNA. It should be noted that the “SNP chip” hybridization array was a custom designed array containing markers to extract information for haplotypes that included 130 genes that were previously (through literature search [16]) associated with alcoholism, other drug addictions, mood and anxiety disorders. The reliability of such a limited number of markers (1,350 SNPs) and a limited number of individuals (890) was modest, but an acceptable standard at the time. We performed a sophisticated multivariate analysis on our results; however, this is no substitute for having proper power to generate reliable conclusions. Nevertheless, the one clear result emanating from the study with humans, was that the DSM-5 diagnosis of alcoholism was not associated with the same markers as quantity of alcohol consumed by humans (as already mentioned). If one were to allow some speculation, the results with human subjects did indicate some association with the GABA neurotransmitter system, particularly glutamic acid decarboxylase (GAD1) and the GABA receptor β2 subunit (GABRB2). Interestingly, or incidentally, an in-depth literature review of the functions of the 20 candidate transcripts derived from the study of the HXB/BXH rats, also generated a common theme in which the

transcripts could be linked to one or another GABA-related functions. An extensive review of the involvement of the GABA receptor system with alcoholism [17] was published at approximately the same time as our paper [14] and gave some support to the involvement of GABA receptors in the etiology of alcoholism. Overall, our work produced a publishable result, but not an unequivocal answer.

Our conceptual goal shifted to adopt an “unsupervised” approach to the acquisition of data for assessment of the genomic determinants of alcohol consumption. By unsupervised we mean the use of an analytical pipeline that does not use pre-categorized data (e.g., hybridization arrays based on genes previously reported to be related to alcoholism) to ascertain relationships between variables (e.g., gene expression and alcohol consumption). One of the real benefits of using a panel of RI animals is that they remain genetically stable, and if environment is also maintained in a stable fashion, one can expect genetic mediation of behavior to remain constant over generations [18]. This allows for continued additions to prior work using animals of a stable and known genetic composition. Two technological advances occurred during the time that we were performing our early studies. One was the development of high throughput affordable RNA sequencing methods and instruments, and the other was the generation of the concept and analytical tools for performing gene co-expression network analysis (WGCNA) [19]. WGCNA, through an unsupervised process, generates modules (collections of genes) and networks of modules based on analysis of coordinated (correlated) gene expression values (the caveats of using WGCNA are included in the glossary). The implication of coordinated gene expression is that quantitative coexpression of gene products is an indication of related gene function, and that coexpression modules were composed of functionally related gene products. This assumption allowed insight into the function of poorly annotated gene products through “guilt by association” with those gene products that had well defined roles in biology.

In our next attempt [20] at improving our understanding of the genetic influence on alcohol consumption, we used DNA and RNA sequencing, as well as WGCNA, to examine the rat brain transcriptome. The genomes of the progenitor strains of the HXB/BXH RI panel (SHR and BN-Lx) were sequenced and aligned to the rat reference genome to identify SNPs and indels that could then be imputed into a strain-specific genome map of the RI panel. RNA-Seq data were derived from whole brain samples from the two progenitor strains, and RNA expression data were also gathered on whole brains from strains of the RI panel by hybridization with the Affymetrix Rat Exon Array1.0 ST. In this study [20], probe sequences from the exon array that did not align perfectly/uniquely to our rat reference genomes for the progenitor strains (i.e., aligned to an undefined genome region) were eliminated from further analysis. The remaining probes were aligned with the rat brain transcriptome determined from the RNA-Seq data in order to allow aggregation/annotation of probe sets on the arrays based on the reconstructed brain transcriptome. Differences in DNA sequence, and therefore RNA sequence between the extracted material from the rat strains and the probe sequences on the exon array can and do have a significant impact on the quantitative results derived from the hybridization procedure [21]. Therefore, we decided to account for the mismatches on the Affymetrix RNA hybridization arrays, and to create a “mask” to eliminate these mismatches. This procedure resulted in a substantial improvement in the accuracy of the data, and the reproducibility of the results. The main aspects of the results from both papers

have survived several changes in technology and analysis (see below). We also modified the WGCNA analysis parameters to generate a smaller module size for a more granular examination of the resultant networks.

In this publication [20], we also analyzed and provided data on the brain transcriptomes of 6 pairs of rat lines selected for alcohol “preference” in laboratories around the world (HAD1/LAD1 and HAD2/LAD2 from Indiana University USA, and the P/NP lines from the same institution; AA/ANA lines from Helsinki, Finland; sP/sNP from Cagliari, Italy; and UchB/UchA from the University of Chile). The purpose of this study with the selected lines was to compare the effects of the independent selection experiments on brain RNA expression in alcohol naïve rats and to compare results using the selected lines with the “candidate genes” derived from the data using the HXB/BXH RI strains.

Our analysis of the lines of rats selected for high and low alcohol preference started with the identification of differentially expressed brain transcripts (DETs) within each pair of high versus low alcohol consuming strains. The results for each pair were merged through meta-analysis (meta-analysis false discovery rate (FDR) <0.05). Even among the 10 DETs with the lowest meta-analysis p-value, no one transcript was differentially expressed (unadjusted p-value<0.05) in the same direction in all 6 pairs of selected lines, although two of the ten transcripts did show significant differences in expression, in the same direction, between high and low drinking rats in four of the selected pairs. There were also instances in which the differences between high and low preference lines were in the same direction across several pairs but not statistically significant. Thus, on a transcript level, we were not able to identify a unique candidate transcript that was differentially expressed ($p < 0.05$) between high and low ethanol consuming lines in all 6 pairs.

The results of our improved approach and analysis of hybridization array data by WGCNA, however, generated a clearer view of an interactive process contributing to various levels of alcohol consumption in our RI animal model. The analysis identified a “candidate” gene coexpression module through WGCNA using the transcriptome results from the RI rats. The candidate module, comprising 17 gene products, was associated with alcohol consumption/preference based on a p-value that combined information from a correlation analysis of the module’s eigengene with alcohol consumption in the RI panel and an enrichment analysis for genes differentially expressed in the selected lines meta-analysis. In addition, the candidate had a module eigengene QTL that overlapped a bQTL for alcohol consumption on rat Chr 12.

The masked Affymetrix “chip” used in this set of experiments did afford us the ability to include the expression levels of isoforms of certain genes in our analysis, as well as unannotated genes, whose products were also included in our analysis. Interestingly, the hub gene (the most connected transcript in the candidate module) was the product of an unannotated gene. This transcript contained three exons when reconstructed (GENE_03745 AND 03746). We named this transcript *Lrap* (Long non-coding RNA for Alcohol Preference, see more later). The module also contained four other non-annotated transcripts (GENE_04887; 03396; 018197; 27603) and 3 genes from our meta-analysis of selected

lines (*Tmem116, Oas1b, and Pcdhb5)*. The remainder of the transcripts were *Maats1, Coq5, Txnip, P2rx4, Ift81, Anxa11, Slc24a6*, and *Parp3*.

We used GO and KEGG databases and leaned heavily on the literature to perform what is referred to as a Formal Concept Analysis [22] to identify function, cellular location and interacting partners for each of the transcripts in the module. The combined set of transcripts could be assigned to three functional categories: Generating and Responding to Immune Signals; Glial/Neuronal Communication; and Energy, Redox and Calcium Homeostasis, and these transcripts were primarily associated with microglia and astrocytes.

It was particularly interesting that our analysis with no presupposed outcomes (i.e., unsupervised) led us to insights that are currently extremely popular [23,24] in explaining excessive alcohol consumption and AUD. Blednov *et al.* [25] and Crews *et al.* [26] introduced the idea that immune function and inflammation contribute to high levels of alcohol consumption in mice and rats. Our studies [20] provided data for invoking a role of the NLRP3 inflammasome and gene products (*Txnip, P2rx4* and *Oas1b*) that modulate its function in production of the cytokines, IL-1β and IL-18 in microglia and astrocytes. These data provided insight on the genetic mediators of the link between immune system activation in brain and predisposition to high alcohol consumption.

The data gathered for the paper by Saba *et al.* [20], in addition to identifying the category “Generating and Responding to Immune Signals” as an important component of a predisposition to high levels of alcohol drinking also, again, pointed to the importance of GABAergic, purinergic and glutamatergic transmitter systems and energy and intracellular calcium homeostasis in this predisposition. The importance of the GABA system was noted in our earlier paper [14] but the transcripts leading to that conclusion were different from the later work. The most obvious reasons for the differences were the use of different hybridization arrays as well as the “mask” used in our later studies to obviate the confounding effect of polymorphisms on transcript quantification by hybridization. One transcript that was highly consistent between the two studies was *P2rx4* which was originally correlated with alcohol consumption across the HXB/BXH strains (-0.70, $p<0.0004$ [14]). In the later study [20], it was a highly connected member of the “candidate module” associated with alcohol consumption. *P2rx4* is one of the candidate module transcripts that impact the function of the inflammasome (NLRP3) and influence the production of IL-1β [27]. It is of interest, that the recent work of Varodyan *et al.* [24] has linked the production of IL-1β to control of GABAergic signaling in the prefrontal cortex of alcohol-consuming mice. The immune function phenotype and the presence of the *P2rx4* transcript have remained stable features of all our work.

The characterization of the unannotated novel transcript which resembled a lncRNA and was the hub gene for the “candidate module” associated with a predisposition for escalating levels of alcohol consumption (*Lrap*) was the subject of our next investigation of the genetical genomic determinants of alcohol consumption [28]. The reconstructed *Lrap* transcript derived from rat brain was mapped to Chr 12 in the rat genome (the location of one of the QTLs for alcohol consumption) and homologs were identified in mouse (Chr 5) and human (Chr 12). To ascertain the function of *Lrap* we created *Lrap* knockout (-/-)

rats using CRISPR/Cas9 technology [28]. The sgRNA was designed to remove the putative coding region of exon 3 of the rat Lrap gene. This region had homologous sequences in both mouse and human DNA. The $Lrap^{-/-}$ and heterozygous $Lrap^{+/-}$ rats were generated on a Wistar rat (WT) background. The WT, $Lrap^{+/-}$ and $Lrap^{-/-}$ rats did not demonstrate any easily observable physical differences, but when tested in a two-bottle choice paradigm of alcohol consumption the $Lrap^{-/-}$ rats drank significantly more alcohol solution than the WT rats, while the $Lrap^{+/-}$ rats demonstrated an intermediate level of alcohol consumption. The consumption of water did not differ among the WT and genetically modified rats. The direction of response in alcohol consumption was predictable based on the negative correlation we found between levels of Lrap RNA and alcohol consumption in our prior studies [20] using the HXB/BXH RI panel. We also found that the relationships of the transcripts in our "candidate module" were disrupted by the genetic manipulation of Lrap. The levels of *P2rx4* were significantly diminished in the $Lrap^{-/-}$ and $Lrap^{+/-}$ rats, compared to the WT animals, indicating that Lrap may be involved in transcriptional control of *P2rx4* expression [28]. Again, this could be predicted by the observed association of the levels of *Lrap* and *P2rx4* transcripts across the HXB/BXH RI panel [20].

The other notable observation made in the examination of transcript abundance in brains of WT and genetically modified rats was that disruption of the Lrap gene produced a major shift in expression of isoforms of the expressed protein coding transcripts. RNA-Seq analysis of whole brains from WT and $Lrap^{-/-}$ rats demonstrated that disruption of the *Lrap* gene produced a large number of differentially expressed transcripts/isoforms (at an FDR of 0.10 there were 750 differentially expressed transcripts of annotated protein coding genes). On further examination, it was found that a large number of the changes were in expression of particular isoforms of a gene, while other isoform(s) would remain unchanged [29]. One of the main functions of lncRNAs is modulation of alternative splicing [30] and thus we hypothesized that Lrap was involved in the process of creating alternative splice variants of particular genes. However, we also found that even though *P2rx4* had two measurable splice variants in brain as measured by hybridization, both isoforms were reduced proportionately such that the ratio of the *P2rx4* isoforms was not altered (however, see later RNA-Seq results).

We were sufficiently intrigued by the results of disrupting the *Lrap* gene structure on the selective expression of particular isoforms of a large number of genes, that we proceeded to use RNA-Seq for transcript identification and quantification in the RI rat panel [29]. We generated RNA-Seq data for the brain transcriptome for all transcripts >200bp for 3 male rats of each of the available 30 HXB/BXH strains, and used both the Ensembl reference rat transcriptome (v.99) and our new reconstruction pipeline that includes two computational approaches (StringTie and aptardi) to identify transcripts and their isoforms [29,31]. StringTie is particularly suited to identify the coding region of isoforms and aptardi was developed by our group to identify alternative polyadenylation sites of the 3' non-coding region (UTR) [32]. In general, the 3' UTR harbors sites for binding of microRNAs that modulate transcript stability and translation [33,34]. We applied stringent quality control and a heritability filter (the broad sense heritability had to exceed 0.4) to characterize expressed transcripts generated by RNA-Seq. After such steps, in tissue from brain, 18,543 reconstructed transcripts remained for further analysis. Within this group

of transcripts, the transcript to gene ratio was 3.06 compared to the ratio of 1.25 in the data derived from the Affymetrix hybridization arrays. When we subjected this RNA-Seq-derived data to WGCNA, 215 modules were generated, but only one module eigengene correlated with alcohol consumption values across the HXB/BXH RI panel and had a module eigengene QTL that overlapped (fell within the 95% Bayesian credible interval for) the behavioral QTL for alcohol consumption on Chr 12 (bQTL peak, Chr 12: 40.5 Mb; module eigengene QTL peak Chr 12: 39.1 Mb). This module included six transcripts (*Lrap, Ift81, Pxrx4, Oas1b, Mapkapk5* and the unannotated *AABR07065438.1*). Four of these gene products were associated with levels of alcohol consumption in our prior work (*Lrap*, *Ift81*, *P2rx4*, *Oas1b*) [20]. The function of the module generated from RNA-Seq [29] was again related to immune system functions in the brain, inflammation and macrophage/microglial function. With regard to isoforms, the “candidate module” contained only one isoform of each transcript, even though three of the represented genes had more than one isoform (P2rx4, Ift81 and Mapkapk5). The other isoforms of these genes, including the second isoform of P2rx4, were components of other modules after WGCNA. The interpretation of this finding can be, that the expression of different transcript isoforms is coordinated with expression levels of particular other transcripts, i.e., coexpression modules, and a particular isoform may serve a different function than other isoforms of the same gene. This does not provide a conclusion for the ultimate function of Lrap in alcohol drinking behavior, but if Lrap can control the levels of a specific isoform it may selectively affect levels of an isoform in one defined module and affect that module’s biologic function. A current study by Li, *et al.* [35], with data from human subjects, provided significant evidence that RNA alternative splicing impacts the susceptibility to AUD. Even though this study [35] and a second study [36] on alternative splicing and AUD, did not consider the effect of alternative splicing on alcohol consumption levels as a prerequisite for AUD, it would be of benefit to thoroughly investigate the function of Lrap in humans.

Up to this point we had identified and characterized brain transcripts and coexpression modules whose expression (e)QTLs and module eigengene (m)QTLs overlapped the behavioral (b)QTL for alcohol consumption on Chr. 12. However, we had identified another bQTL for alcohol consumption on Chr 1 and yet found no module in brain whose mQTL overlapped this bQTL. We had always ascribed to a holistic view of mammalian physiology and the premise that metabolism and function of various organs is interrelated [37]. One of the more discussed organ/organ interactions in the field of alcohol research is the communication between liver and brain [38]. We therefore performed an analysis of the whole rat liver transcriptome in 21 alcohol-naive strains of HXB/BXH RI rats using the Affymetrix Rat Exon Array 1.0 ST and after application of WGCNA we identified 735 gene coexpression modules [38]. Again, only one coexpression module, containing 15 transcripts, met all the criteria for a “candidate module” (significant correlation of the module eigengene with alcohol consumption across the RI panel; mQTL LOD score of 7.4 and peak location overlapping with the interval of the Chr 1 bQTL, i.e., mQTL Chr 1: 167.5–177.0 Mb, bQTL Chr 1: 167.2–248.8 Mb). The hub gene of this module was *Cyp2r1* which converts cholecalciferol to vitamin D3, and its levels were positively correlated with alcohol consumption levels of the rat strains in the RI panel. Interestingly, the function of *Cyp2r*1 and the products of the other annotated transcripts in this module (*rnfl41, Lyve1,*

Tmem9b, Capn5, Serpinh1, Ripply1, Tmc3, Acer3, Prcp, Galnt18 and Aida) could all be related to immune function of the liver [39], including production and release of immune system modulators (e.g., bradykinin generated by *Prcp*). Bradykinin plays an important role in control of the permeability of the blood/brain barrier [40] and is also involved in chronic inflammation [41].

In analyzing our cumulative results on the relationship of gene transcription to levels of alcohol consumption, one finds that innate (inherited) characteristics of immune systems of the brain and communication of liver-derived immune modulators with brain immune systems account for a major part of the genetic variance controlling the predisposition to alcohol intake by rats. To properly extrapolate our results to humans, one must be careful to select the most appropriate measure of alcohol consumption in both humans and rats in order to rationally assume congruence of genetic influences between species. Dick *et al.* [42] assessed various measures of alcohol consumption in mono- and dizygotic twins, i.e. frequency, regular quantity (a measure combining frequency and quantity), frequency of intoxication and maximum number of drinks in a single day (all measures based on the definition of a standard drink). All of these measures showed significant heritability values, with monozygotic twin values exceeding those of dizygotic twins. However, the heritability values depended on the drinking variable being measured indicating different genetic architectures underlying each of the defined variables. One should also be cognizant of the reality that the same GENE does not need to be responsible for a closely matched phenotype across species. A similar molecular pathway which may contain dissimilar gene products may better explain the genetic/phenotypic correlations in a different species or even a different population such as our results from the selected line pairs described earlier. Finally, it is clear that GWAS do not provide organ information as provided by transcriptome analysis, and a holistic view of complex polygenic traits should always be considered.

In conclusion, our studies, as a whole, indicate that inherent differences in immune system responsiveness play a role in determining the amount of alcohol that a rat will voluntarily consume when alcohol is made available. It certainly remains unresolved whether similar pathways are important for levels of alcohol consumption in human, but recent work using single cell RNA-Seq [43] does substantiate significant differences in RNA expression for neuroinflammatory proteins in astrocytes, microglia, and oligodendrocytes of the human frontal cortex when comparing post-mortem tissue from controls and alcohol-dependent individuals. As mentioned earlier, high levels of alcohol consumption are a prerequisite for development of AUD, and studies of post-mortem tissue from various areas of brain from deceased alcoholics [44,45] have demonstrated increased levels of pro-inflammatory cytokines compared to tissue from non-alcoholic individuals. Furthermore, CSF levels of IL-6, which is considered a pro-inflammatory cytokine, were higher in individuals drinking alcohol at levels greater than one standard drink per day as compared to non-drinkers [46]. In examining the post-mortem brain tissue of alcoholics, Zou and Crews [44] noted that the increase in the levels of the pro-inflammatory cytokine IL-1β were coincident with increase in the inflammasome-related protein NLRP3 (NALP3). As mentioned in the abstract, and amplified throughout this manuscript, our studies have brought focus on a group of inflammasome (NLRP3)-modulating transcripts, *P2rx4*, *Ift81*, *Oas1b*, and *Txnip*, in rat brain as predisposing mediators of the level of free-choice alcohol consumption.

The studies with humans diagnosed with AUD or those drinking high quantities of alcohol cannot provide an answer as to whether differences in the modulation of NLRP3 function are inherent, genetically determined characteristics of those who drink at high levels and transition to AUD, or whether differences in inflammasome function in those who drink alcohol at high levels is the result of the alcohol consumption. Our studies with rats would suggest that inherent differences in the modulation of inflammasome (NLRP3) function in brain drives higher levels of alcohol consumption rather than alcohol causing differences in NLRP3 function by a yet undefined mechanism. Whether conclusions generated in studies with rats extrapolate to humans still needs to be resolved.

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Glossary

AUD
Alcohol Use Disorder. As described by the Diagnostic and Statistical Manual of Mental Disorders, v.5 (DSM-5) [1].

CSF
Cerebrospinal fluid.

Cytokine
Substance secreted by cells of the immune system.

Eigengene
In gene coexpression networks (WGCNA) the first principal component analysis of each module is used as a representative of their respective gene-expression profile and is called the eigengene [47].

Genetical Genomics
Combined study of gene expression and marker genotypes to detect genetic loci that control gene expression [48].

GWAS
Genome-wide association study. Compares DNA of a large population to identify genetic variants (markers) associated with a trait.

Haplotype
Group of DNA variants or genetic markers that are inherited together on a chromosome.

Heavy drinking
The National Institute of Alcohol Abuse and Alcoholism sets the threshold at more than 14 drinks per week for men (or >4 drinks per occasion); more than 7 drinks per week for women (or >3 drinks per occasion); and more than 7 drinks per week for all adults 65 years and above [49].

HXB/BXH RI panel
Derived from gender-reciprocal cross between spontaneously hypertensive rat SHR/Ola and Brown Norway congenic, BN-Lx carrying polydactyly-luxate syndrome [13].

Hybridization Array
Technique where a collection of nucleic acid probes (DNA or RNA) are fixed on a solid surface and then a labeled sample of nucleic acid is allowed to bind (hybridize) to its complementary probe sequence on the array.

Indel
Indel mutation refers to insertion-deletion of bases (nucleotides) in the genome of an organism [50].

Inflammasome
Protein complex that is part of the innate immune system. NLRP3 (also called NALP3) is a member of the inflammasome family.

lncRNA
Long non-coding RNA. Class of RNA molecules longer than 200 nucleotides and not translated into proteins.

QTL, (behavioral, bQTL; expression, eQTL)
Statistical method that links phenotypic (trait) and genotypic (molecular marker) data. Cis eQTL: genetic variation that acts on local genes; trans eQTL: genetic variation that acts on distant genes or genes on different chromosomes [51].

Recombinant Inbred (RI)
Recombinant inbred strains have chromosomes that incorporate an essentially permanent set of recombination events between chromosomes inherited from two or more inbred strains [52]

RNA-Seq
Analyzes the order of nucleotides in RNA to study the transcriptome (complete set of an organism’s transcribed genes).

SgRNA
Single guide RNA. A key component of the CRISPR-Cas9 gene-editing system.

SNP

Single nucleotide polymorphism. Substitution of a single nucleotide at a specific position in the genome [53].

Trait and State Markers

Markers that distinguish between the biological status of a subject (state marker) and the properties of biological and behavioral processes that are present before any clinical manifestation (trait marker; e.g., DNA).

WGCNA

Weighted Gene Coexpression Network Analysis. Systems biology method that builds gene networks (find modules of highly correlated gene expression) [19]. Limitations of using this analytical method: While it is a powerful approach, many parameters in WGCNA can present problems to the user if not applied correctly. For instance, before generating the correlation networks, users have a choice of network types (signed, unsigned), correlation methods (Pearson, Spearman), power values for weighting the correlation, and correlation cut-offs, among many other options. The wide swathe of options and parameters needed to conduct an end-to-end WGCNA could make the analyses highly error-prone. In fact, selecting an inappropriate method, parameter, or threshold for the type or spread of your data could lead to misinterpretation of correlations where outliers are not treated correctly, networks that are not biologically realistic, and ultimately inaccurate conclusions that could hinder future research [54].

References

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5. Washington, DC: American Psychiatric Association; 2013 May 22.
2. NIAAA: National Institute on Alcohol Abuse and Alcoholism. “Overview of Alcohol Consumption.” In: Alcohol’s Effects on Health. 2024.
3. Glantz MD, Bharat C, Degenhardt L, Sampson NA, Scott KM, Lim CCW, et al. WHO World Mental Health Survey Collaborators. The epidemiology of alcohol use disorders cross-nationally: Findings from the World Mental Health Surveys. Addict Behav 2020 Mar;102:106128. [PubMed: 31865172]
4. Center for Behavioral Health Statistics and Quality. National Survey on Drug Use and Health (NSDUH): Detailed tables. 2021.
5. Alpert HR, Slater ME, Yoon YH, Chen CM, Winstanley N, Esser MB. Alcohol Consumption and 15 Causes of Fatal Injuries: A Systematic Review and Meta-Analysis. Am J Prev Med 2022 Aug;63(2):286–300. [PubMed: 35581102]
6. Kranzler HR, Zhou H, Kember RL, Vickers Smith R, Justice AC, Damrauer S, et al. Genome-wide association study of alcohol consumption and use disorder in 274,424 individuals from multiple populations. Nat Commun 2019 Apr 2;10(1):1499. [PubMed: 30940813]
7. Edenberg HJ, Foroud T. Genetics and alcoholism. Nature reviews Gastroenterology & hepatology. 2013 Aug;10(8):487–94. [PubMed: 23712313]
8. Edenberg HJ, McClintick JN. Alcohol dehydrogenases, aldehyde dehydrogenases, and alcohol use disorders: a critical review. Alcoholism: Clinical and Experimental Research. 2018 Dec;42(12):2281–97. [PubMed: 30320893]
9. Beisswenger TB, Holmquist B, Vallee BL. chi-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: implications and inferences. Proceedings of the National Academy of Sciences. 1985 Dec;82(24):8369–73.

10. Galter D, Carmine A, Buervenich S, Duester G, Olson L. Distribution of class I, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain. European Journal of Biochemistry. 2003 Mar;270(6):1316–26. [PubMed: 12631290]
11. Schaub MA, Boyle AP, Kundaje A, Batzoglou S, Snyder M. Linking disease associations with regulatory information in the human genome. Genome Research. 2012 Sep 1;22(9):1748–59. [PubMed: 22955986]
12. Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, McBride WJ, et al. Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. Behavior Genetics. 2002 Sep;32:363–88. [PubMed: 12405517]
13. Printz MP, Jirout M, Jaworski R, Alemayehu A, Kren V. Genetic Models in Applied Physiology. HXB/BXH rat recombinant inbred strain platform: a newly enhanced tool for cardiovascular, behavioral, and developmental genetics and genomics. Journal of Applied Physiology (Bethesda, Md.: 1985). 2003 Jun 1;94(6):2510–22. [PubMed: 12736193]
14. Tabakoff B, Saba L, Printz M, Flodman P, Hodgkinson C, Goldman D, et al. Genetical genomic determinants of alcohol consumption in rats and humans. BMC Biology. 2009 Dec;7:70. [PubMed: 19874574]
15. Glanz J, Grant B, Monteiro M, Tabakoff B. WHO/ISBRA study on state and trait markers of alcohol use and dependence: analysis of demographic, behavioral, physiologic, and drinking variables that contribute to dependence and seeking treatment. Alcoholism: Clinical and Experimental Research. 2002 Jul;26(7):1047–61. [PubMed: 12170115]
16. Hodgkinson CA, Yuan Q, Xu K, Shen PH, Heinz E, Lobos EA, et al. Addictions biology: haplotype-based analysis for 130 candidate genes on a single array. Alcohol & Alcoholism. 2008 Sep 1;43(5):505–15. [PubMed: 18477577]
17. Enoch MA. The role of GABAA receptors in the development of alcoholism. Pharmacology Biochemistry and Behavior. 2008 Jul 1;90(1):95–104. [PubMed: 18440057]
18. Philip VM, Duvvuru S, Gomero B, Ansah TA, Blaha CD, Cook MN, et al. High-throughput behavioral phenotyping in the expanded panel of BXD recombinant inbred strains. Genes, Brain and Behavior. 2010 Mar;9(2):129–59. [PubMed: 19958391]
19. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008 Dec;9:559. [PubMed: 19114008]
20. Saba LM, Flink SC, Vanderlinden LA, Israel Y, Tampier L, Colombo G, et al. The sequenced rat brain transcriptome–its use in identifying networks predisposing alcohol consumption. The FEBS Journal. 2015 Sep;282(18):3556–78. [PubMed: 26183165]
21. Jakubek YA, Cutler DJ. A model of binding on DNA microarrays: understanding the combined effect of probe synthesis failure, cross-hybridization, DNA fragmentation and other experimental details of affymetrix arrays. BMC Genomics. 2012 Dec;13:737. [PubMed: 23270536]
22. Alam M, Coulet A, Napoli A, Smaïl-Tabbone M. Formal concept analysis applied to transcriptomic data. InWhat can FCA do for Artificial Intelligence (FCA4AI)(ECAI 2012) 2012 Aug 27.
23. Lékó AH, Ray LA, Leggio L. The vicious cycle between (neuro) inflammation and alcohol use disorder: An opportunity to develop new medications?. Alcohol (Hanover, York County, Pa.). 2023 May;47(5):843–7.
24. Varodayan FP, Pahng AR, Davis TD, Gandhi P, Bajo M, Steinman MQ, et al. Chronic ethanol induces a pro-inflammatory switch in interleukin-1β regulation of GABAergic signaling in the medial prefrontal cortex of male mice. Brain, Behavior, and Immunity. 2023 May 1;110:125–39. [PubMed: 36863493]
25. Blednov YA, Benavidez JM, Geil C, Perra S, Morikawa H, Harris R. Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. Brain, Behavior, and Immunity. 2011 Jun 1;25:S92–105. [PubMed: 21266194]
26. Crews FT, Zou J, Qin L. Induction of innate immune genes in brain create the neurobiology of addiction. Brain, Behavior, and Immunity. 2011 Jun 1;25:S4–12. [PubMed: 21402143]
27. de Rivero Vaccari JP, Bastien D, Yurcisin G, Pineau I, Dietrich WD, De Koninck Y, et al. P2X4 receptors influence inflammasome activation after spinal cord injury. Journal of Neuroscience. 2012 Feb 29;32(9):3058–66. [PubMed: 22378878]

28. Saba LM, Hoffman PL, Homanics GE, Mahaffey S, Daulatabad SV, Janga SC, et al. A long non-coding RNA (Lrap) modulates brain gene expression and levels of alcohol consumption in rats. Genes, Brain and Behavior. 2021 Feb;20(2):e12698. [PubMed: 32893479]
29. Lusk R, Hoffman PL, Mahaffey S, Rosean S, Smith H, Silhavy J, et al. Beyond genes: inclusion of alternative splicing and alternative polyadenylation to assess the genetic architecture of predisposition to voluntary alcohol consumption in brain of the HXB/BXH recombinant inbred rat panel. Frontiers in Genetics. 2022 Mar 15;13:821026. [PubMed: 35368676]
30. Porto FW, Daulatabad SV, Janga SC. Long non-coding RNA expression levels modulate cell-type-specific splicing patterns by altering their interaction landscape with RNA-binding proteins. Genes. 2019 Aug 6;10(8):593. [PubMed: 31390792]
31. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature Biotechnology. 2015 Mar;33(3):290–5.
32. Lusk R, Stene E, Banaei-Kashani F, Tabakoff B, Kechris K, Saba LM. Aptardi predicts polyadenylation sites in sample-specific transcriptomes using high-throughput RNA sequencing and DNA sequence. Nature Communications. 2021 Mar 12;12(1):1652.
33. Gu S, Jin L, Zhang F, Sarnow P, Kay MA. Biological basis for restriction of microRNA targets to the 3' untranslated region in mammalian mRNAs. Nature Structural & Molecular Biology. 2009 Feb;16(2):144–50.
34. Lau AG, Irier HA, Gu J, Tian D, Ku L, Liu G, et al. Distinct 3' UTRs differentially regulate activity-dependent translation of brain-derived neurotrophic factor (BDNF). Proceedings of the National Academy of Sciences. 2010 Sep 7;107(36):15945–50.
35. Li R, Reiter JL, Chen AB, Chen SX, Foroud T, Edenberg HJ, et al. RNA alternative splicing impacts the risk for alcohol use disorder. Molecular Psychiatry. 2023 Jul;28(7):2922–33. [PubMed: 37217680]
36. Huggett SB, Ikeda AS, Yuan Q, Benca-Bachman CE, Palmer RH. Genome-and transcriptome-wide splicing associations with alcohol use disorder. Scientific Reports. 2023 Mar 9;13(1):3950. [PubMed: 36894673]
37. Yan M, Man S, Sun B, Ma L, Guo L, Huang L, et al. Gut liver brain axis in diseases: the implications for therapeutic interventions. Signal Transduction and Targeted Therapy. 2023 Dec 6;8(1):443. [PubMed: 38057297]
38. Lee JS, O'Connell EM, Pacher P, Lohoff FW. PCSK9 and the gut-liver-brain axis: a novel therapeutic target for immune regulation in alcohol use disorder. Journal of Clinical Medicine. 2021 Apr 18;10(8):1758. [PubMed: 33919550]
39. Hoffman PL, Saba LM, Vanderlinden LA, Tabakoff B. Voluntary exposure to a toxin: the genetic influence on ethanol consumption. Mammalian Genome. 2018 Feb;29:128–40. [PubMed: 29196862]
40. Emerich DF, Dean RL, Osborn C, Bartus RT. The development of the bradykinin agonist labradimil as a means to increase the permeability of the blood-brain barrier: from concept to clinical evaluation. Clinical Pharmacokinetics. 2001 Feb;40:105–23. [PubMed: 11286321]
41. Rex DA, Deepak K, Vaid N, Dagamajalu S, Kandasamy RK, Flo TH, et al. A modular map of Bradykinin-mediated inflammatory signaling network. Journal of Cell Communication and Signaling. 2022 Jun;16(2):301–10. [PubMed: 34714516]
42. Dick DM, Meyers JL, Rose RJ, Kaprio J, Kendler KS. Measures of current alcohol consumption and problems: two independent twin studies suggest a complex genetic architecture. Alcoholism: Clinical and Experimental Research. 2011 Dec;35(12):2152–61. [PubMed: 21689117]
43. Brenner E, Tiwari GR, Kapoor M, Liu Y, Brock A, Mayfield RD. Single cell transcriptome profiling of the human alcohol-dependent brain. Human Molecular Genetics. 2020 Apr 1;29(7):1144–53. [PubMed: 32142123]
44. Zou J, Crews FT. Inflammasome-IL-1β signaling mediates ethanol inhibition of hippocampal neurogenesis. Frontiers in Neuroscience. 2012 May 30;6:77. [PubMed: 22661925]
45. Coleman LG Jr, Zou J, Qin L, Crews FT. HMGB1/IL-1β complexes regulate neuroimmune responses in alcoholism. Brain, Behavior, and Immunity. 2018 Aug 1;72:61–77. [PubMed: 29102800]

46. Guest J, Grant R, Mori TA, Croft KD. Changes in oxidative damage, inflammation and [NAD (H)] with age in cerebrospinal fluid. PLoS One. 2014 Jan 14;9(1):e85335. [PubMed: 24454842]

47. Lemoine GG, Scott-Boyer MP, Ambroise B, Périn O, Droit A. GWENA: gene co-expression networks analysis and extended modules characterization in a single Bioconductor package. BMC Bioinformatics. 2021 May 25;22(1):267. [PubMed: 34034647]

48. de Koning DJ, Haley CS. Genetical genomics in humans and model organisms. Trends Genet 2005 Jul;21(7):377–81. [PubMed: 15908034]

49. Reid MC, Fiellin DA, O'Connor PG. Hazardous and harmful alcohol consumption in primary care. Arch Intern Med 1999 Aug 9–23;159(15):1681–9. [PubMed: 10448769]

50. https://en.wikipedia.org/wiki/Indel

51. Miles C, Wayne M. Quantitative trait locus (QTL) analysis. Nature Education. 2008;1:208.

52. https://en.wikipedia.org/wiki/Recombinant_inbred_strain

53. https://en.wikipedia.org/wiki/Single-nucleotide_polymorphism

54. https://bigomics.ch/