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Comorbidities, Confounders, and the White Matter Transcriptome in Chronic Alcoholism

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Background: Alcohol abuse is the world's third leading cause of disease and disability, and one potential sequel of chronic abuse is alcohol-related brain damage (ARBD). This clinically manifests as cognitive dysfunction and pathologically as atrophy of white matter (WM) in particular. The mechanism linking chronic alcohol intoxication with ARBD remains largely unknown but it is also complicated by common comorbidities such as liver damage and nutritional deficiencies. Liver cirrhosis, in particular, often leads to hepatic encephalopathy (HE), a primary glial disease.

Methods: In a novel transcriptomic study, we targeted the WM only of chronic alcoholics in an attempt to tease apart the pathogenesis of ARBD. Specifically, in alcoholics with and without HE, we explored both the prefrontal and primary motor cortices, 2 regions that experience differential levels of neuronal loss.

Results: Our results suggest that HE, along with 2 confounders, gray matter contamination, and low RNA quality are major drivers of gene expression in ARBD. All 3 exceeded the effects of alcohol itself. In particular, low-quality RNA samples were characterized by an up-regulation of translation machinery, while HE was associated with a down-regulation of mitochondrial energy metabolism pathways.

Conclusions: The findings in HE alcoholics are consistent with the metabolic acidosis seen in this condition. In contrast non-HE alcoholics had widespread but only subtle changes in gene expression in their WM. Notwithstanding the latter result, this study demonstrates that significant confounders in transcriptomic studies of human postmortem brain tissue can be identified, quantified, and "removed" to reveal disease-specific signals.

Key Words: Alcoholism, Transcriptome, Human Postmortem Brain Tissue, Hepatic Encephalopathy, RNA Quality.

THE NEURODEGENERATIVE EFFECTS of chronic alcohol consumption, or alcohol-related brain damage (ARBD), manifest clinically as frontal lobe dysfunction with impairment of sustained attention and working memory (Kril et al., 1997; Uekermann et al., 2003). ARBD is a complex phenotype and the direct contribution of alcohol can be difficult to establish given common comorbidities such as liver damage, nutritional deficiencies, and concomitant lifestyle choices such as smoking (Butterworth, 1995). In particular, hepatic encephalopathy (HE), an acute consequence of terminal liver failure or chronic manifestation of liver cirrhosis, can augment ARBD (Harper and Kril, 1989).

On postmortem examination, chronic alcoholics display brain atrophy that correlates with lifetime consumption (Harding et al., 1996) and is largely due to white matter (WM) loss (Harper et al., 1985; Jensen and Pakkenberg, 1993; de La Monte, 1988). The gray matter (GM) atrophy that does occur is mainly due to dendritic dearborization with only a few susceptible neuronal populations such as the pyramidal neurons of the superior frontal cortex (Kril and Harper, 1989; Kril et al., 1997). In contrast, neurons in the primary motor, anterior cingulate, and inferior temporal cortices are reasonably well preserved (Kril and Harper, 1989).

Previous transcriptomic studies of ARBD have assayed GM or whole-brain homogenates and typically compared the motor cortex and prefrontal cortex due to their differential neuronal loss (Liu et al., 2004; Mayfield et al., 2002). These studies uncovered relatively subtle differential gene expression (DEG) compared with other neurodegenerative diseases (Flatscher-Bader et al., 2006) although one relatively constant finding has been the dysregulation of genes involved in myelination (Iwamoto et al., 2004; Lewohl et al., 2000; Liu et al., 2004). Neuroimaging studies have similarly demonstrated the loss of WM integrity in ARBD (Pfefferbaum et al., 2000, 2009) and it has been postulated that myelin degradation might be driving axonal degeneration and neuronal loss rather than vice versa (Pfefferbaum et al., 2010).

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Given the possibility of a primary glial role in ARBD pathogenesis, we compared the transcriptome of duplicate WM samples from the superior frontal gyrus (prefrontal cortex; Brodmann area 8) and precentral gyrus (primary motor cortex; Brodmann area 4) of 7 male alcoholics (3 with HE) and 3 male controls.

MATERIALS AND METHODS

Case Selection

Prior approval for this project was obtained from the Human Ethics Research Committee of the University of Sydney (HREC #13027). Frozen and fixed brain tissue of 7 alcoholics and 3 controls was obtained from New South Wales Tissue Research Centre (NSW TRC), following approval from their Scientific Advisory Committee. The NSW TRC is part-funded by the National Institute on Alcohol Abuse and Alcoholism (R24AA012725) to provide brain tissue for alcoholism research and their banking procedure has been previously published (Sheedy et al., 2008). NSW TRC supplied clinical and pathological information including cerebellar brain pH, alcohol consumption (mean daily and total lifetime), smoking status, liver pathology, and macro- and microscopic neuropathology. Their neuropathological examination included a detailed assessment for metabolic (HE) encephalopathy (Harris et al., 2008) scored as none, mild (<1 Alzheimer type II astrocyte [At2a] per high-power [200×] field), moderate (2 to 3 At2a per field), or severe (majority of astrocytes show At2a change). Lifestyle factors were estimated from next of kin questionnaires and available medical records. A Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) diagnosis was similarly assigned to each case. Mean daily alcohol consumption rates were recorded as grams of alcohol (EtOH) per day with chronic alcoholics defined as those individuals who had consumed greater than 80 g of alcohol per day for the majority of their adult life (usually >30 years), while controls had consumed <20 g of alcohol per day. Cigarette use was described in mean pack years where 1 mean pack year equals 1 packet of (20) cigarettes per day for 1 year.

Neuronal Counts

Paraffin-embedded formalin-fixed sections of the superior frontal gyrus (frontal cortex) and precentral gyrus (motor cortex) were stained with cresyl violet according to standard protocols. Neuronal counts were performed on an Olympus BH-2 microscope (Tokyo , Japan) at 200× magnification using a graticule (0.01 mm^2). For each case, the neurons in 3 cortical strips from the pial surface to the GM–WM junction of both regions were counted and the mean neurons per mm^2 calculated.

Statistical Analysis

An online power calculator (http://bioinformatics.mdanderson.org/MicroarraySampleSize/) suggested that 11 subjects per group would achieve 70% power at $\alpha = 0.05$, assuming a mean fold change = 1.5 and standard deviation of the gene expression (log2) = 0.6. In this study, we compared a total of 40 duplicate samples from 14 alcoholic and 6 control regions. The software program JMP 10.0.0 (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses with a p-value < 0.05 accepted as the level of significance and no corrections were made for multiple comparisons. Potential group differences in neuronal numbers, brain weight, mean daily and total lifetime alcohol consumption, brain pH, and RNA integrity number (RIN) were determined using analysis of variance, while potential differences in gender, liver pathology, and smoking status were determined by chi-square analysis.

Microarray

One hundred milligrams of frozen WM adjacent to the frontal and motor cortices was divided into two (biologic duplicates) and RNA extracted using a combination TRIzol/PureLink RNA Mini Kit protocol (Invitrogen, Carlsbad, CA) (excluding the optional DNase step). RNA was quantified using the Nanodrop spectrophotometer. RNA quality determination (Agilent Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA), cDNA preparation, microarray labeling, and scanning were performed at the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney). The GeneChip® 3′ IVT Express Kit (Affymetrix, Santa Clara, CA) was used to convert 100 ng of total RNA to cDNA with a T7 oligo(dT) primer, followed by second strand synthesis and IVT incubation for 16 hours to synthesize the biotin-labeled aRNA. The aRNA was then purified and fragmented and 6 μg hybridized to the HG-U219 array plate (Affymetrix). This 3′ gene expression analysis array utilizes a perfect-match-only design and has a relatively low number of probe sets (49,386), but all 19,094 protein-coding genes (HUGO; http://www.genenames.org) are represented. Hybridization and scanning were carried out using GeneTitan System (Affymetrix). Data analysis was carried out using Broad Institute's GenePattern (GP) analysis system hosted by the Peter Wills Bioinformatics Centre (PWBC) at the Garvan Institute of Medical Research, Sydney. All 40 samples passed quality control, and the CEL files were combined into a "gct" file using the GP "normalize affymetrix3" module. DEG was determined using Linear Models of Microarrays (Limma; Smyth, 2004) implemented in the GP tool LimmaGP (M. Cowley, unpublished data). The U219A array's 49,386 probes were collapsed down to 20,260 expressed genes based on the most differentially expressed probe. Here, p-values were adjusted for multiple comparisons using the false discovery rate (FDR) method with a q-value <0.25 accepted as significant. DEG summarization was carried out using gene set enrichment analysis (GSEA; Subramanian et al., 2005) with the molecular signature database c2.all.v3.0. In addition, we added alcohol-specific data sets from NCBI Gene Expression Omnibus: GDS3703, GDS1660, and GDS2841. GSEA gene lists were ranked on normalized enrichment scores (NES) with FDR q-value <0.05 considered significant.

Gray Matter Contamination

The variable and diffuse nature of the GM/WM boundary in the human brain means that GM could have inadvertently been included with WM samples. A previous study had defined 14 neuronal and 10 glial genes by the analysis of adjacent, but disparate WM and GM regions (Sibille et al., 2008). To explore the possibility of GM contamination, the mean expression of all these genes was calculated for each duplicate sample in our study and a mean neuronal/glial (N/G) ratio derived.

RIN Effects

The cohort was divided into low- and high-RIN groups around the median RIN value (7.6) and Limma and GSEA performed. For correlation analyses, RIN values were added to the "gct" file in Microsoft (MS) Excel (Redmond, WA) and "hierarchical clustering" repeated. RIN was selected as the "feature of interest" and the "nearest neighbors" algorithm applied. The transcripts were ranked by their respective R-values and these were exported to MS Excel. t-Statistics were generated using the following formula: $t = r\sqrt{((n-2)/(1-r^2))}$. p-Values were calculated in MS Excel using the TDIST function (TDIST [x, df, 2] or TDIST [$-x$, df, 2] where $x < 0$) and $p < 0.05$ used as an arbitrary level of significance. Correlations between RIN and selected genes were performed in JMP 10.0.0. The highest N/G ratios and lowest RIN samples (RIN <5)

were removed to achieve similar mean RINs and N/G ratios across the controls and 2 alcoholic groups. Analyses were then repeated in a final 32-sample subcohort.

RESULTS

Our aim in this study was to determine whether the WM transcriptome of chronic alcoholics was more informative for the pathogenesis of ARBD than previous microarray studies utilizing whole-brain samples. We further hypothesized that DEG would be greater in WM adjacent to the superior frontal gyrus (prefrontal cortex) than the precentral gyrus (motor cortex) consistent with the greater neuronal loss in the former (Kril et al., 1997).

Clinicopathological Comparisons

There was no difference in brain weight, brain pH, and the mean RIN between 7 male alcoholics and 3 male controls (Table 1). The groups were significantly different in alcohol consumption and smoking status although alcohol and smoking data were only available for 2 of the controls.

Three of the alcoholics also had a pathological diagnosis of moderate or severe HE. This group included 2 individuals who had abstained from alcohol for 15 years and 12 months, respectively prior to their death. In comparison, the non-HE alcoholics had either none or less than 1 At2a per high-power field on average across their basal ganglia and frontal cortex. There was no difference in mean daily alcohol consumption (HE = 100.0 ± 20.0 vs. 114.5 ± 48.9 g/d $p = 0.45$) or lifetime alcohol consumption between the 2 alcoholic groups.

Table 1. Demographic and Clinical Factors

Characteristics (±SD)	Alcoholics ($n = 7$)	Controls ($n = 3$)	p-Value
Mean age (years)	61.4 (10.7)	64.3 (17.6)	0.75
Mean PMI (hours)	37.1 (18.2)	25.7 (3.1)	0.33
Mean brain weight (g)	1,423 (106)	1,427 (65)	0.95
Mean brain pH	6.5 (0.4)	6.7 (0.1)	0.43
Mean RIN (4 samples each)	7.3 (1.3)	7.9 (0.8)	0.46
Mean agonal factor score[a]	4.6 (2.8)	3.0 (2.6)	0.43
Severity of liver pathology[b] (0, 1, 2, and 3)	0, 0, 2, and 4	1, 0, 1, and 0[c]	
DSM-IV diagnosis: unclassified, alcohol abuse, dependence, remission[d]	1, 2, 3, and 1		
Hepatic encephalopathy (HE) score (none, mild, moderate, and severe)	4, 0, 2, and 1	n.d.	
Mean daily alcohol consumption (g/d)[d]	108.3 (37.3)	11.0 (11.3)	**<0.01**
Lifetime alcohol consumption (kg)[e]	1,944 (1,033)	295 (205)	0.08
Smoking ever/never (current)[d]	6/0	1/1[c]	0.06[f]
Mean smoking pack years[d]	74.8 (86.6)	22.0 (31.1)	**0.45**

n.d., not detected; PMI, postmortem interval; RIN, RNA integrity number.

[a]Agonal factor score = # of agonal factors × rate of death score (1 = rapid, 2 = intermediate, and 3 = slow).

[b]Liver pathology: 0 = nil or congestion, 1 = mild steatosis, 2 = severe steatosis/mild fibrosis; 3 = cirrhosis or severe inflammation (acute).

[c]Missing data for 1 case.

[d]Estimated from medical history and next of kin questionnaires.

[e]Calculated by mean daily consumption × 365 × years drinking (excluding periods of abstinence).

[f]Chi-square test.

Significant p-values are in bold type ($p < 0.05$).

Neuronal Counts

Consistent with previous studies in our laboratory (Kril et al., 1997), the mean neuronal density in the frontal cortex of alcoholics (111.6 ± 13.5 mm^2) was significantly less than controls (159.0 ± 23.3 mm^2; $p = 0.003$), but there was no difference seen in the motor cortex (142.1 ± 30.5 vs. 147.6 ± 11.6 mm^2, $p = 0.78$). Furthermore, there was an inverse correlation between mean daily alcohol consumption and neurons in the frontal ($r^2 = 0.72$, $p = 0.004$) but not the motor cortex ($r^2 = 0.12$, $p = 0.36$).

There was no difference in neuronal density between alcoholics with or without HE in either the frontal or motor cortex although when stratified on HE status it was only the frontal cortex of the non-HE alcoholics that were significantly different to the controls (109.6 ± 15.4 mm^3; $p = 0.03$).

Transcriptomic Analyses

We carried out a transcriptome (microarray) analysis using the HG-U219A array, GeneTitan system (both Affymetrix), and GP program suite. We initially explored the extent of DEG in 28 alcoholic and 12 controls samples. Limma showed that 162 (DEGs) (FDR q-value < 0.25), whereas GSEA showed that translation-related pathways (NES of KEGG_Ribosome = 3.83) were up-regulated while cell cycle-related (Reactome_DNA_Replication = 2.52) and energy metabolism pathways (Mootha_Mitochondria = 2.41) were overrepresented with down-regulated genes (Table S2). Contrary to our hypothesis, these changes were more amplified in the motor cortex, while the mitochondrial energy metabolism pathways were only down-regulated among the HE alcoholics (Table S2).

Confounders

Hierarchical clustering of all 40 total samples revealed 3 major branches (Fig. 1). The first branch consisted of 4 samples from 1 HE alcoholic that coincided with the 4 lowest RIN values in the study (Table S1). The other 2 major branches (2 and 3) had equivalent numbers of samples in

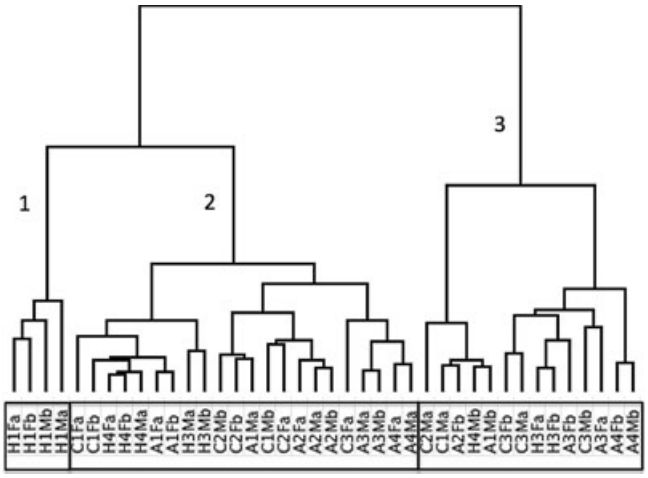

Fig. 1. Dendrogram from hierarchical clustering of all 40 samples. A dendrogram generated in GP "Hierarchical Clustering Viewer" shows the distribution of the 40 samples based on their genome-wide expression. There are 3 major branches (1, 2 and, 3). All 4 samples from 1 HE alcoholic (H1) cluster together (1) otherwise there are no obvious relationships in branches 2 and 3. Unexpectedly, only half of the duplicates cluster together. HE alcoholics (H), non-HE alcoholics (A), controls (C), frontal cortex (F), motor cortex(M), and duplicate samples (a and b). GP, gene pattern; HE, hepatic encephalopathy.

them but their membership was unrelated to their group status. Surprisingly, the biologic duplicates (a and b) from each region only clustered together in approximately 50% of cases. As a lack of duplicate pairing is often indicative of systematic errors in microarray experiments, we explored this issue further by carrying out a regional analysis (frontal vs. motor cortex of alcoholics and controls separately). This analysis showed that DEGs were almost totally confined to the controls (6,827 vs. 3 at FDR q-value <0.25; Table S3). Furthermore, GSEA showed that pathways up-regulated in controls motor cortex (vs. frontal cortex) were the same as those down-regulated in the alcoholic motor cortex, while the most differentially expressed genes in the former were all neuronal in nature (Table S3). Together, these data suggested that GM had contaminated some of the control motor cortex samples.

Gray Matter Contamination

As a means of quantifying putative GM contamination, we turned to a previous study that had characterized GM- (n = 14) and WM-specific transcripts (n = 10) in human postmortem brain tissue (Sibille et al., 2008). In the Sibille and colleagues (2008) study, *MOG* (encoding myelin oligodendrocyte glycoprotein) had the highest WM/GM expression ratio. We calculated the ratio of mean expression of neuronal transcripts to glial transcripts for each duplicate sample. The N/G ratios ranged from 0.25 to 8.83 (mean = 1.29 ± SD = 1.66; Table S1) with 7 samples exceeding a ratio of 2.0 and 2 exceeding 4.0 (both were control motor cortex samples; Table S1). In particular, the control sample with the highest N/G ratio (C2 Ma) also had the lowest *MOG* expression, while its duplicate's (C2 Mb) ratio was 1.09 (Fig. 2).

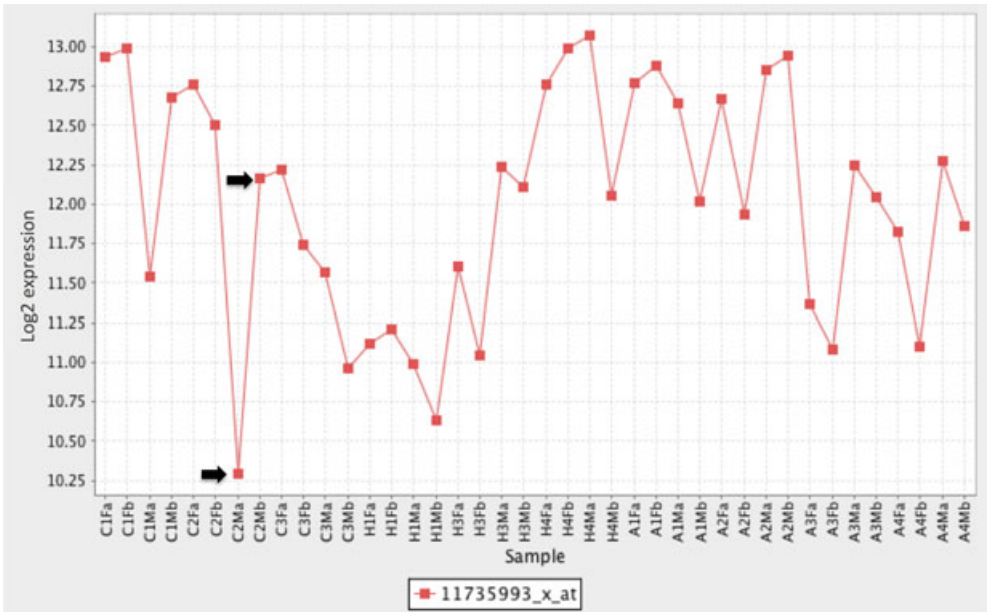

Fig. 2. The effect of gray matter (GM) contamination on gene expression. A GenePattern-generated profile of the myelin oligodendrocyte glycoprotein (*MOG*) transcript (Affymetrix probe ID = 11735993_x_at) demonstrates variable GM contamination among the 40 samples. *MOG* had previously been shown to have the greatest differential expression between white matter and GM samples (Sibille et al., 2008). In our cohort, the sample with the lowest MOG expression, C2 Ma (arrow, bottom), had the highest neuronal/glial gene (N/G) ratio (8.83), while its duplicate sample, C2 Mb (arrow, top), had a ratio of only 1.09 (see Table S1 for N/G on all samples). The GM contamination of C2 Ma was largely responsible for the differential expression seen between the motor and frontal cortices of the controls only (Table S3).

RIN Effects

Many neurodegenerative diseases result in low brain pH and RIN relative to control individuals and the effects on DEG can exceed those associated with the disease itself (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006). Therefore, a low RIN not only indicates greater RNA degradation in a sample but also the high likelihood of gene expression patterns unrelated to phenotype. Although there was no difference in mean RIN between the alcoholics and controls (Table 1), 3 HE alcoholics did have a lower mean brain pH (6.2 ± 0.4 vs. 6.7 ± 0.2; $p < 0.0001$) and mean RIN (6.7 ± 1.7 vs. 7.7 ± 0.7; $p = 0.04$) than the non-HE alcoholics. As we had already noted greater differential expression among the HE alcoholics (Table S2), we explored whether RIN might also be a confounder here by splitting the cohort into low-RIN (range = 4.2–7.8) and high-RIN (7.8–9.0) groups and repeating both Limma and GSEA. This revealed an extraordinarily high 17,794 DEGs of a total 20,260 (FDR q-value <0.25) (Table S4). In particular, "up-regulated" translation-related pathways, such as KEGG_Ribosome (4.93) and Reactome_Peptide_Chain_ Elongation (4.90), were far higher in magnitude than seen in the initial alcoholic analysis, suggesting that this effect in particular was probably due to the lower mean RIN among the HE alcoholics. (Table S4). Interestingly, if these same analyses were carried out with the high ($n = 20$) or low-RIN ($n = 20$) samples only, the down-regulation of energy metabolism falls away when comparing high-RIN HE alcoholics and controls (Table S4). Although this result is based on a single individual (H4), it does suggest that low-RIN samples are driving this effect as well.

Final Analysis

To minimize the effects of both these confounders, we omitted samples until the mean N/G ratios and RINs were similar between the 3 subgroups leaving us with a 32-sample cohort (23 alcoholic (including 8 HE alcoholics) and 9 control samples; Table S5). The revised analysis of all alcoholics versus controls now revealed only 28 DEGs (FDR q-value <0.25; see Table S6 for analysis summary; a full gene list is available from the author) compared with 162 DEGs seen with the full cohort. GSEA showed that protein translation machinery was now less prominent among the "up-regulated" pathways, while mitochondrial energy metabolism pathways were no longer "down-regulated" (top 5 up- and down-regulated pathways shown in Table 2).

When the 32-sample cohort was stratified by HE status, there were now more DEGs in the non-HE group (600 vs. 31, FDR q-value <0.25; see Table S6 for summary; full gene lists are available from the author). However, in contrast to relatively high numbers of DEGs, GSEA revealed few down-regulated pathways among the non-HE alcoholics with the suspected RIN effect, protein translation, still prominently seen in "up-regulated" pathways (Table 3 and Table S6).

In contrast, mitochondrial energy metabolism pathways were prominently down-regulated in HE alcoholics (Table 4). However, the effect size was larger in HE alcoholics' frontal samples that did have a lower mean RIN compared with the controls. These stratified analyses suggest that both RIN and HE have deleterious effects on energy metabolism (Table S6).

Table 2. Summary of the GSEA of RIN-matched alcoholics and controls

	NES	q-Value
Up		
REACTOME_SMOOTH_MUSCLE_CONTRACTION	2.47	<0.01
REACTOME_TRANSLATION	2.45	<0.01
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	2.43	<0.01
REACTOME_PEPTIDE_CHAIN_ELONGATION	2.43	<0.01
MILI_PSEUDOPODIA_CHEMOTAXIS_UP	2.41	<0.01
Down		
PYEON_HPV_POSITIVE_TCMORS_UP	−2.36	0.01
FINETTI_BREAST_CANCER_BASAL_VS_LCMINAL	−2.27	<0.01
REACTOME_G2_M_CHECKPOINTS	−2.23	0.01
REACTOME_DNA_STRAND_ELONGATION	−2.21	<0.01
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	−2.21	<0.01

GSEA, gene set enrichment analysis; NES, normalized enrichment score; RIN, RNA integrity number.

Table 3. Summary of the GSEA of RIN-matched non-HE Alcoholics

	NES	q-Value
Up		
REACTOME_VIRAL_MRNA_TRANSLATION	2.93	<0.01
REACTOME_PEPTIDE_CHAIN_ELONGATION	2.84	<0.01
REACTOME_FORMATION_OF_A_POOL_OF_FREE_40S_SUBUNITS	2.78	<0.01
REACTOME_TRANSLATION	2.74	<0.01
REACTOME_GTP_HYDROLYSIS_AND_JOINING_OF_THE_60S_RIBOSOMAL_SUBUNIT	2.71	<0.01
Down		
PYEON_HPV_POSITIVE_TCMORS_UP	−2.58	<0.01
DAZARD_RESPONSE_TO_UV_NHEK_DN	−2.36	<0.01
PYEON_CANCER_HEAD_AND_NECK_VS_CERVICAL_UP	−2.21	0.01
CHEN_HOXA5_TARGETS_9HR_UP	−2.12	0.02
PUJANA_XPRSS_INT_NETWORK	−2.11	0.01

GSEA, gene set enrichment analysis; HE, hepatic encephalopathy; NES, normalized enrichment score; RIN, RNA integrity number.

Table 4. Summary of the GSEA of RIN-matched HE Alcoholics

	NES	q-Value
Up		
BIOCARTA_WNT_PATHWAY	2.10	0.21
BARRIER_CANCER_RELAPSE_NORMAL_SAMPLE_DN	2.07	0.13
BASSO_CD40_SIGNALING_UP	2.07	0.09
NIKOLSKY_BREAST_CANCER_7P22_AMPLICON	2.05	0.08
FAELT_B_CLL_WITH_VH3_21_UP	2.02	0.09
Down		
KEGG_OXIDATIVE_PHOSPHORYLATION	−2.82	<0.01
MOOTHA_VOXPHOS	−2.78	<0.01
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	−2.72	<0.01
REACTOME_REGULATION_OF_INSULIN_SECRETION	−2.70	<0.01
REACTOME_ELECTRON_TRANSPORT_CHAIN	−2.59	<0.01

GSEA, gene set enrichment analysis; HE, hepatic encephalopathy; NES, normalized enrichment score; RIN, RNA integrity number.

DISCUSSION

This study was designed to test whether gene expression and myelination deficits in ARBD are amplified in the WM transcriptome. The analysis proved complex with both putative and unexpected confounders disguising the direct effects of chronic alcoholism on the brain.

Of the known confounders, our previous work had suggested that a pathological diagnosis of HE was not only common in ARBD (20% of all chronic alcoholics; Sutherland et al., 2013), but HE alcoholics were likely to have lower brain pH and RIN (Sheedy et al., 2012). Similarly, studies of mood disorders had shown that low-pH brain samples are associated with a down-regulation of mitochondrial energy metabolism pathways and increased stress-induced or cell survival pathways (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006). As RIN and brain pH are reasonably well correlated, we were keen to explore "RIN" as the independent variable before investigating disease-specific effects. Low RIN did have an extensive effect on gene expression, with protein translation pathways in particular being highly up-regulated.

In contrast, GM contamination was a completely unexpected confounder. In retrospect, the arbitrary decision to split the tissue samples before, rather than subsequent to, RNA extraction allowed this issue to be detected. However, the omission of 12 samples to equilibrate the 3 groups for mean RIN and mean N/G ratio reduced the power of our subsequent analyses to approximately 50%.

These subcohort analyses showed that non-HE alcoholics had 10-fold more DEGs but substantially fewer dysregulated pathways (or gene sets) than HE alcoholics. In fact, non-HE alcoholics had virtually no down-regulated pathways at all. This apparent paradox might be due to the widespread actions of alcohol across the brain with DEGs not segregating sufficiently with any particular pathways to reach statistical significance. Another possible mechanism is that individual genes are affected by alcohol according to their quantity of guanine and cytosine bases, not by their pathway affiliations (Ponomarev et al., 2012). Ponomarev and colleagues (2012) showed that genes with a high "GC" content were more likely to have their expression modulated by alcohol-mediated epigenetic processes such as hypomethylation.

Our findings of subtle effects in the WM of patients with ARBD are consistent with previous transcriptomic studies (Flatscher-Bader et al., 2006; Liu et al., 2004), but contrary to our original hypothesis, myelin-related pathways were not significantly dysregulated here. We also hypothesized more severe WM changes adjacent to the prefrontal cortex, but our region-stratified analyses suggested that there were no differences in gene expression between the 2 cortices. These findings require replication in larger cohorts, but it appears that WM pathology is not driving differential neuronal loss in ARBD, a finding consistent with the long-held belief that axonal degeneration triggers WM loss rather than vice versa (Harper et al., 1985).

In contrast, the WM transcriptome of HE alcoholics was characterized by the down-regulation of mitochondrial energy metabolism pathways. The down-regulation of mitochondrial metabolism is consistent with lactic acidosis in HE, a feature attributed to inhibition of the tricarboxylic acid cycle enzyme a-ketoglutarate (Butterworth, 2010). Importantly, this effect remained after potential low-RIN/pH effects were removed, showing that HE and premortem hypoxia share some of their effects on brain metabolism. It was unclear why there was no down-regulation of mitochondrial energy metabolism pathways in low-RIN samples here, but this may reflect that the RIN and brain pH correlation is a moderate one, $r^2 = 0.47$ across the 70 alcoholics and controls screened in our previous study (Sheedy et al., 2012).

HE was generally thought to result from raised brain ammonia levels (Butterworth, 2010), but more recent in vitro work suggests that pro-inflammatory cytokines and oxidative stress combine with ammonia to produce astrocytic swelling (Rama Rao et al., 2010; Rao et al., 2012; Shawcross and Jalan, 2005; Shawcross et al., 2010). Indeed a recent transcriptomic study of parietal/occipital cortex samples from cirrhotic patients with or without HE confirmed that oxidative stress and microglial activation played major roles in the pathophysiology of HE (Gorg et al., 2013). Of the 67 dysregulated genes (p-value < 0.050) in HE alcoholics reported by Gorg and colleagues (2013), only 1 (*HLA-DQA1*) was also dysregulated here. *HLA-DQA1* encodes the alpha chain of the major histocompatibility complex class II heterodimer found on antigen-presenting cells and its up-regulation is consistent with microglial activation. However, it is not clear why there was not greater concordance between our WM and their cortical samples as both the HE alcoholics in the final analysis here also had hepatic cirrhosis. We have recently found that a subset of HE alcoholics display microglial proliferation throughout their brains, while other nonproliferative cases displayed dystrophic microglia in their

WM (Dennis et al., under review). We propose that microglia proliferation is part of an early neuroprotective response that ultimately fails because the underlying excessive cerebral ammonia levels and oxidative stress remains.

The major aim of this study was to investigate the WM transcriptome of alcoholics, but we are also able to provide additional insights into how gene expression patterns are related to RIN. It is now widely accepted that the severity and longevity of agonal events rather than postmortem effects are largely responsible for low pH and RIN in postmortem brain samples (Atz et al., 2007; Durrenberger et al., 2010; Weis et al., 2007). We have previously confirmed these same relationships in alcoholics with and without HE (Sheedy et al., 2012). It is also generally assumed that RIN values are inversely correlated with the extent of mRNA degradation and that different transcripts degrade at similar rates. Given that our 40 samples displayed a wide range of RINs, we tested these assumptions. Of the total 49,386 probes (representing 20,260 genes) on the Affymetrix HG-U219A array, 25,104 (51%) were significantly correlated with RIN. However, contrary to expectations, 14,326 or 57% of these transcripts were inversely correlated. In other words, as RNA levels fell the majority of mRNA species in our study actually increased (see Table S7 for data and correlations with RIN for commonly used reference genes).

Li and colleagues (2004) in their microarray analysis of mood disorders were the first to note the association between low-pH samples and gene expression in the human brain. They suggested that, in addition to increased RNA degradation, low-pH samples incorporate a "coordinated biologic response" in their transcriptome to the agonal period and this exceeded disease-specific effects (Li et al., 2004). The magnitude of this response reflects the quantity, severity of agonal events, and the duration of the agonal phase. A major issue in case–control experiments is that many neurological diseases have longer and more adverse premortem periods than controls (Monoranu et al., 2009). Putative RIN effects have previously been handled by exclusion of samples at a researcher-determined RIN cut off and potentially further normalization procedures based on RIN itself (Gebhardt et al., 2010; Ponomarev et al., 2012). However, RIN is a not linear scale, and the degradation profiles and expression patterns of individual genes seem to vary widely in their relationship to RIN. Therefore, in addition to RIN cut offs, it seems prudent to deliberatively interrogate data using RIN as the independent variable before considering disease-specific effects. If RIN effects are substantial, then disease-specific analyses can be undertaken in a RIN-matched subcohort.

In summary, this exploration of the WM transcriptome of patients with ARBD has exposed significant confounders that can be identified, quantified, and "removed" to reveal disease-specific signals. Unfortunately, the WM transcriptome of non-HE alcoholics seems to display the same subtle gene expression changes that have characterized studies utilizing whole-brain homogenates. Deciphering the direct effects of chronic alcohol intoxication per se will require larger cohorts that are matched for RIN and carefully screened for other pathologies such as HE. In contrast our findings on HE are consistent with the known or suspected pathogenic mechanisms in this condition.

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REFERENCES

Atz M, Walsh D, Cartagena P, Li J, Evans S, Choudary P, Overman K, Stein R, Tomita H, Potkin S, Myers R, Watson SJ, Jones EG, Akil H, Bunney WE Jr, Vawter MP (2007) Methodological considerations for gene expression profiling of human brain. J Neurosci Methods 163:295–309.

Butterworth RF (1995) Pathophysiology of alcoholic brain damage: synergistic effects of ethanol, thiamine deficiency and alcoholic liver disease. Metab Brain Dis 10:1–8.

Butterworth RF (2010) Altered glial-neuronal crosstalk: cornerstone in the pathogenesis of hepatic encephalopathy. Neurochem Int 57:383–388.

Dennis CV, Sheahan PJ, Graeber MB, Sheedy DL, Kril JJ (2013) Sutherland GT (2013) Microglial proliferation in the brain of chronic alcoholics with hepatic encephalopathy. Metab Brain Dis 2013 [Epub ahead of print].

Durrenberger PF, Fernando S, Kashefi SN, Ferrer I, Hauw JJ, Seilhean D, Smith C, Walker R, Al-Sarraj S, Troakes C, Palkovits M, Kasztner M, Huitinga I, Arzberger T, Dexter DT, Kretzschmar H, Reynolds R (2010) Effects of antemortem and postmortem variables on human brain mRNA quality: a BrainNet Europe study. J Neuropathol Exp Neurol 69:70–81.

Flatscher-Bader T, Van Der Brug MP, Landis N, Hwang JW, Harrison E, Wilce PA (2006) Comparative gene expression in brain regions of human alcoholics. Genes Brain Behav 5(Suppl 1):78–84.

Gebhardt FM, Scott HA, Dodd PR (2010) Housekeepers for accurate transcript expression analysis in Alzheimer's disease autopsy brain tissue. Alzheimers Dement 6:465–474.

Gorg B, Bidmon HJ, Haussinger D (2013). Gene expression profiling in the cerebral cortex of patients with cirrhosis with and without hepatic encephalopathy. Hepatology 2013;57:2436–2447.

Harding AJ, Halliday GM, Ng JL, Harper CG, Kril JJ (1996) Loss of vasopressin-immunoreactive neurons in alcoholics is dose-related and time-dependent. Neuroscience 72:699–708.

Harper C, Kril J (1989) Patterns of neuronal loss in the cerebral cortex in chronic alcoholic patients. J Neurol Sci 92:81–89.

Harper CG, Kril JJ, Holloway RL (1985) Brain shrinkage in chronic alcoholics: a pathological study. Br Med J (Clin Res Ed) 290:501–504.

Harris J, Chimelli L, Kril J, Ray D (2008) Nutritional deficiencies, metabolic disorders and toxins affecting the nervous system, in *Greenfield's Neuropathology* (Love S, Louis D, Ellison DW eds) pp 675–731.

Iwamoto K, Bundo M, Yamamoto M, Ozawa H, Saito T, Kato T (2004) Decreased expression of NEFH and PCP4/PEP19 in the prefrontal cortex of alcoholics. Neurosci Res 49:379–385.

Jensen GB, Pakkenberg B (1993) Do alcoholics drink their neurons away? Lancet 342:1201–1204.

Kril JJ, Halliday GM, Svoboda MD, Cartwright H (1997) The cerebral cortex is damaged in chronic alcoholics. Neuroscience 79:983–998.

Kril JJ, Harper CG (1989) Neuronal counts from four cortical regions of alcoholic brains. Acta Neuropathol 79:200–204.

de La Monte SM (1988) Disproportionate atrophy of cerebral white matter in chronic alcoholics. Arch Neurol 45:990–992.

Lewohl JM, Wang L, Miles MF, Zhang L, Dodd PR, Harris RA (2000) Gene expression in human alcoholism: microarray analysis of frontal cortex. Alcohol Clin Exp Res 24:1873–1882.

Li JZ, Vawter MP, Walsh DM, Tomita H, Evans SJ, Choudary PV, Lopez JF, Avelar A, Shokoohi V, Chung T, Mesarwi O, Jones EG, Watson SJ, Akil H, Bunney WE Jr, Myers RM (2004) Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. Hum Mol Genet 13:609–616.

Liu J, Lewohl JM, Dodd PR, Randall PK, Harris RA, Mayfield RD (2004) Gene expression profiling of individual cases reveals consistent transcriptional changes in alcoholic human brain. J Neurochem 90:1050–1058.

Mayfield RD, Lewohl JM, Dodd PR, Herlihy A, Liu J, Harris RA (2002) Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. J Neurochem 81:802–813.

Mexal S, Berger R, Adams CE, Ross RG, Freedman R, Leonard S (2006) Brain pH has a significant impact on human postmortem hippocampal gene expression profiles. Brain Res 1106:1–11.

Monoranu CM, Apfelbacher M, Grunblatt E, Puppe B, Alafuzoff I, Ferrer I, Al-Saraj S, Keyvani K, Schmitt A, Falkai P, Schittenhelm J, Halliday G, Kril J, Harper C, Mclean C, Riederer P, Roggendorf W (2009) pH measurement as quality control on human post mortem brain tissue: a study of the BrainNet Europe consortium. Neuropathol Appl Neurobiol 35:329–337.

Pfefferbaum A, Rosenbloom MJ, Fama R, Sassoon SA, Sullivan EV (2010) Transcallosal white matter degradation detected with quantitative fiber tracking in alcoholic men and women: selective relations to dissociable functions. Alcohol Clin Exp Res 34:1201–1211.

Pfefferbaum A, Rosenbloom M, Rohlfing T, Sullivan EV (2009) Degradation of association and projection white matter systems in alcoholism detected with quantitative fiber tracking. Biol Psychiatry 65:680–690.

Pfefferbaum A, Sullivan EV, Hedehus M, Adalsteinsson E, Lim KO, Moseley M (2000) In vivo detection and functional correlates of white matter microstructural disruption in chronic alcoholism. Alcohol Clin Exp Res 24:1214–1221.

Ponomarev I, Wang S, Zhang L, Harris RA, Mayfield RD (2012) Gene coexpression networks in human brain identify epigenetic modifications in alcohol dependence. J Neurosci 32:1884–1897.

Rama Rao KV, Jayakumar AR, Tong X, Alvarez VM, Norenberg MD (2010) Marked potentiation of cell swelling by cytokines in ammonia-sensitized cultured astrocytes. J Neuroinflammation 7:66.

Rao KV, Brahmbhatt M, Norenberg MD (2012) Microglia contribute to ammonia-induced astrocyte swelling in culture. Metab Brain Dis 28:139–143.

Shawcross D, Jalan R (2005) The pathophysiologic basis of hepatic encephalopathy: central role for ammonia and inflammation. Cell Mol Life Sci 62:2295–2304.

Shawcross DL, Shabbir SS, Taylor NJ, Hughes RD (2010) Ammonia and the neutrophil in the pathogenesis of hepatic encephalopathy in cirrhosis. Hepatology 51:1062–1069.

Sheedy D, Garrick T, Dedova I, Hunt C, Miller R, Sundqvist N, Harper C (2008) An Australian Brain Bank: a critical investment with a high return! Cell Tissue Bank 9:205–216.

Sheedy D, Say M, Stevens J, Harper CG, Kril JJ (2012) Influence of liver pathology on markers of postmortem brain tissue quality. Alcohol Clin Exp Res 36:55–60.

Sibille E, Arango V, Joeyen-Waldorf J, Wang Y, Leman S, Surget A, Belzung C, Mann JJ, Lewis DA (2008) Large-scale estimates of cellular origins of mRNAs: enhancing the yield of transcriptome analyses. J Neurosci Methods 167:198–206.

Smyth GK (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3:Article3.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 102:15545–15550.

Sutherland GT, Sheedy D, Kril JJ (2013). Using autopsy brain tissue to study alcohol-related brain damage in the genomic age. Alcohol Clin Exp Res doi: 10.1111/acer.12243 [Epub ahead of print].

Uekermann J, Daum I, Schlebusch P, Wiebel B, Trenckmann U (2003) Depression and cognitive functioning in alcoholism. Addiction 98:1521–1529.

Vawter MP, Tomita H, Meng F, Bolstad B, Li J, Evans S, Choudary P, Atz M, Shao L, Neal C, Walsh DM, Burmeister M, Speed T, Myers R, Jones EG, Watson SJ, Akil H, Bunney WE (2006) Mitochondrial-related gene expression changes are sensitive to agonal-pH state: implications for brain disorders. Mol Psychiatry 11:615, 663–679.

Weis S, Llenos IC, Dulay JR, Elashoff M, Martinez-Murillo F, Miller CL (2007) Quality control for microarray analysis of human brain samples: the impact of postmortem factors, RNA characteristics, and histopathology. J Neurosci Methods 165:198–209.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Sample details of the entire cohort.

Table S2. Analysis of the entire cohort.

Table S3. Regional comparisons.

Table S4. RIN analyses in the full cohort.

Table S5. Sample details of the 32-sample subcohort.

Table S6. Alcohol analyses in the 32-sample subcohort.

Table S7. RIN and reference gene expression in the entire cohort.