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Expression of Glutamatergic Genes in Healthy Humans across 16 Brain Regions; Altered Expression in the Hippocampus after Chronic Exposure to Alcohol or Cocaine

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Abstract

We analyzed global patterns of expression in genes related to glutamatergic neurotransmission (glutamatergic genes) in healthy human adult brain before determining the effects of chronic alcohol and cocaine exposure on gene expression in the hippocampus.

RNA-Seq data from ‘BrainSpan’ was obtained across 16 brain regions from nine control adults. We also generated RNA-Seq data from postmortem hippocampus from eight alcoholics, eight cocaine addicts and eight controls. Expression analyses were undertaken of 28 genes encoding glutamate ionotropic (AMPA, kainate, NMDA) and metabotropic receptor subunits, together with glutamate transporters.

The expression of each gene was fairly consistent across the brain with the exception of the cerebellum, the thalamic mediodorsal nucleus and the striatum. *GRIN1*, encoding the essential NMDA subunit, had the highest expression across all brain regions. Six factors accounted for 84% of the variance in global gene expression. *GRIN2B* (encoding GluN2B), was up-regulated in both alcoholics and cocaine addicts (FDR corrected $p = 0.008$). Alcoholics showed up-regulation of three genes relative to controls and cocaine addicts: *GRIA4* (encoding GluA4), *GRIK3* (GluR7) and *GRM4* (mGluR4). Expression of both *GRM3* (mGluR3) and *GRIN2D* (GluN2D) was up-regulated in alcoholics and down-regulated in cocaine addicts relative to controls.

Glutamatergic genes are moderately to highly expressed throughout the brain. Six factors explain nearly all the variance in global gene expression. At least in the hippocampus, chronic alcohol use largely up-regulates glutamatergic genes. The NMDA GluN2B receptor subunit might be implicated in a common pathway to addiction, possibly in conjunction with the GABAB1 receptor subunit.

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CONFLICT OF INTEREST

None of the authors have any potential conflicts of interest.

Keywords

Alcoholism; RNA-Seq; GRIN2B; GRIN2D; NMDA receptors; GABAB1 receptor subunit; GABBR1; GRIA4; GRIK3; GRM3; GRM4

INTRODUCTION

Although levels of expression in genes related to glutamatergic neurotransmission (glutamatergic genes) across different brain regions have largely been measured in rodents, no comprehensive studies have been performed in human brain. The purpose of this study was to identify global patterns of expression in healthy human adult brain of the 28 glutamatergic genes that encode glutamate receptor and transporter proteins, to identify whether there is correlation of expression between these genes that are located in different chromosomal regions and then to determine the effects of chronic alcohol and cocaine exposure on gene expression in the hippocampus. We selected the hippocampus because of its role in long- and short-term episodic memory including the processing of contextual cues within memory/conditioning neuronal circuits that are considered to be important in drug reinforcement behaviors in animals and craving and relapse in humans (Koob & Volkow 2010; Luo *et al.* 2011). Moreover, imaging studies in humans have shown that cue-elicited craving activates the hippocampus (Volkow *et al.* 2004).

Many of the symptoms of addiction result from the imbalance in neuronal excitation and inhibition, which is largely due to alterations in transmission of excitatory glutamate and inhibitory gamma-aminobutyric acid (GABA). Chronic exposure to heavy alcohol consumption or cocaine use is known to result in widespread neuronal adaptations in limbic reward circuitry, including impairment in glutamate homeostasis (Kalivas 2009). Altered glutamate-glutamine metabolism may either be a predictor for the development of alcoholism or may be a persistent effect of long-term consumption (Thoma *et al.* 2011). Chronic exposure to alcohol and cocaine affects glutamate transporters together with ionotropic and metabotropic glutamate receptor availability and function Chandrasekar 2013; Jin *et al.* 2014; Kalivas 2009; Mao *et al.* 2013; Moussawi & Kalivas 2010; Schmidt and Pierce 2009). In particular, the NMDA receptor subunit GluN2B plays a key role in acute and chronic ethanol sensitivity (Badanich *et al.* 2011, Wang *et al.* 2007; Wills *et al.* 2012). Some of these changes may be substance specific but other changes may be more general, reflecting shared pathways in addiction (Zhou *et al.* 2011).

In order to study global glutamatergic gene expression we obtained RNA-Seq data from BrainSpan, a publicly available resource. Whole transcriptome data was available for postmortem samples of 16 brain regions from nine healthy men and women who died suddenly. We previously identified the expression of 21 GABAergic pathway genes in the BrainSpan dataset and performed a factor analysis on global expression (Enoch *et al.* 2013). Factor specificity for response to chronic alcohol/cocaine exposure was subsequently determined from the analysis of RNA-Seq data from postmortem hippocampus of eight alcoholics, eight cocaine addicts and eight controls (Enoch *et al.* 2012). This study revealed that there was indeed specificity in response of GABAergic gene groups, defined by

covariation in expression (Enoch *et al.* 2013). In summary, our previous studies provided some understanding about hippocampal changes in inhibitory GABAergic pathway genes subsequent to chronic alcohol and cocaine exposure. The current study is a parallel and complementary analysis in the same two datasets of 28 neuronal excitatory genes encoding glutamate receptors and transporters.

METHODS

BrainSpan samples

The publicly available data used in this study was derived from BrainSpan, an atlas of the developing human brain, accessed via the Allen Institute for Brain Science's hosting website at <http://www.brainspan.org/>. Based on RNA sequencing, BrainSpan provides unbiased coverage of the complete transcriptome in 16 targeted cortical and subcortical structures (Table 1) in 42 brain specimens to date, spanning pre-natal development to adulthood in both males and females. Details pertaining to selection criteria, sample and tissue acquisition and qualification, processing and dissection, RNA analysis, sequencing and alignment, quality control as well as expression quantification can be found in the BrainSpan Technical White Paper 2011 (link provided in References).

It should be noted that brain specimens were excluded if excessive drug or alcohol abuse was reported, if the individual had any known neurological or psychiatric disorders, or if any prolonged agonal conditions were reported including coma, hypoxia, prolonged pyrexia, seizures, prolonged dehydration, hypoglycemia and multiple organ failure. Other relevant exclusion factors included ingestion of neurotoxic substances at the time of death, suicide, brain damage and signs of neurodegeneration. For further details see BrainSpan Technical White Paper 2011 and Enoch *et al.* 2013.

Supplementary Table S1 lists details of the nine adult (aged 18 – 40 years) postmortem brain specimens (samples A – J) that are the focus of this study. Information about ethnicity was not available.

Miami Brain Bank hippocampal samples

A full description of the Methods has been previously published (Enoch *et al.* 2012; Zhou *et al.* 2011). Postmortem brain tissue was provided by the University of Miami Brain Endowment Bank™. Because we used publicly available pathological specimens, our study was exempt from NIH Institutional Review Board review. Samples were limited to sudden death without medical intervention or prolonged agonal periods. The postmortem interval (PMI) was less than 24 hours. Brain pH was measured as a quality control for each sample with values > 6.0. For further details see Mash *et al.* 2007.

The hippocampus was sampled bilaterally from coronal slices taken at the anterior level of the hippocampal body, including the dentate gyrus, the Cornu Ammonis fields CA1 – CA4 and the subiculum. The postmortem samples were taken from eight cocaine addicts, eight alcoholics and eight controls, all men. All subjects in the cocaine and alcohol groups met DSM-IV criteria for abuse or dependence. The cocaine addicts had long-standing histories of cocaine abuse and the deaths were attributed to cocaine intoxication. None of the cocaine

addicts had a history of other drug misuse/dependence or of alcohol misuse/dependence and had not been drinking prior to death. The alcoholics had histories of chronic heavy alcohol consumption and all had enlarged livers: four had fatty livers, one had hepatic fibrosis. None had cirrhosis or hepatic encephalopathy. None of the alcoholics had a history of drug misuse/dependence and a drug screen at the time of death was negative. The controls were age-matched and drug and alcohol free (negative urine screens, no history of licit or illicit drug use prior to death). Based on medical examiners' reports, next-of-kin informant reports, medical records and legal records, none of the subjects in this study had any other psychiatric disorders. The mean (SD) ages were: cocaine addicts: 39.9 (4.9) yrs; alcoholics: 36.9 (9.5) years; controls: 37.5 (6.1) years. The ethnicity ratio of Caucasian or Caucasian/Hispanics to African Americans was as follows: cocaine addicts: 5:3; alcoholics: 7:1; controls: 5:3.

High-throughput, massively parallel sequencing of Miami Brain Bank samples using an Illumina Genome Analyzer (GAIIx)

Details of the methods for construction of cDNA libraries have been described previously (Zhou *et al.* 2011). Sample preparation and sequencing on the Genome Analyzer (Illumina, San Diego, CA) were carried out according to the Illumina protocols with some modifications. Briefly, the double-stranded cDNA was treated with T4 DNA polymerase and the Klenow fragment for end repair. The 5' end of the DNA fragments were then phosphorylated by T4 polynucleotide kinase, and an adenosine base was added to the 3' end of the fragments by Klenow (3'-5' exo⁻). A universal adaptor was then added to both ends of the DNA fragments by A-T ligation. Following 18 cycles of PCR with the Phusion DNA polymerase, the DNA library was purified on a 2% agarose gel and fragments of 170 – 300 base-pair in size were recovered. Around 15 ng of the DNA library was then used for cluster generation on a grafted GAII Flow Cell, and sequenced on the Genome Analyzer for 36 cycles using the "Sequencing-by-synthesis" method.

Sequence base-calling, mapping to genome, data normalization and statistical analysis—Sequences were called from image files with the Illumina Genome Analyzer Pipeline (GAPipeline) and aligned to the reference genome (UCSC hg18) using Extended Eland in the GAPipeline. A total of 3 million uniquely mapped RNA-Seq reads for each sample were retrieved from export.txt files (output of Extended Eland). Based on their mapping locations, these selected reads were parsed with in-house Perl scripts to generate base coverage in WIG file format. After moving average smoothing, the chromosome locations of enrichment peaks were identified from pooled WIG files using in-house Perl scripts. The average sequencing reads of the most abundantly covered 50 bp in a single exon within an annotated Ref-Seq gene were counted for each sample. The read counts were then log2 transformed and quantile normalized (BioConductor limma package).

Public access to RNA-Seq data from Miami Brain Bank hippocampal samples

—The RNA-Seq data has been deposited in the GenBank database (accession number SRA029279) and is publicly available as follows:

(<http://www.ncbi.nlm.nih.gov/Traces/sra/?study=SRP005408>)

Selection of candidate glutamatergic genes

We selected a total of 28 glutamatergic candidate genes for gene expression analyses in both samples (Table 2). The rationale for selection of these particular genes is that they encode all the ionotropic receptor subunits: AMPA, kainate, NMDA; the metabotropic receptor subunits and glial transporters and neuronal transporters. We did not include *SLC17A6* (encoding VGLUT2), *SLC17A8* (encoding VGLUT3), *SLC1A7* (encoding EAAT5) and *GRM6* (encoding mGluR6) because the expression levels of these genes in our hippocampal samples of controls, alcoholics and cocaine addicts were very low.

All 28 genes were available from the Miami Brain Bank RNA-Seq data. However, in the BrainSpan RNA-Seq data, expression data for *GRIN3B* and *SLC1A7* were missing 9 and 13 values respectively and was of overall poor quality. Therefore expression data for these two genes was not included in the BrainSpan analyses. However *GRIN3B* and *SLC1A7* data from the Miami Brain Bank were good quality and were included in the hippocampal analyses.

Statistical analyses

BrainSpan samples—This study utilized the RNA-Seq data obtained via the BrainSpan “RNA-Seq summarized to genes” downloadable archive file which contains normalized expression values and meta-data. The archive consists of RPKM (Reads Per Kilobase of transcript per Million mapped reads) values for each gene measured in each of the collected brain structures from each sample. After the archive was downloaded and uncompressed, the relevant information (genes and samples of interest) was extracted and prepared using simple Perl commands. The data was then imported into the R package for statistical computing which was used for all subsequent analysis. Box plots were used to visualize expression profiles both sample by sample and gene by gene. Scatter plots and linear regressions were used to visualize correlations in expression which was quantified using the correlation coefficient R^2 . With the exception of the box plots which consistently show $\log_2$ -transformed RPKM values, no data manipulation was undertaken.

A factor analysis was performed using the original BrainSpan gene expression values for the 26 glutamatergic genes that were expressed in the 16 brain regions. The fitting method was principal axis factoring and the rotation method was set to varimax (orthogonal rotation) since we did not expect the factors to be correlated. The factor analysis was executed with R version 2.15.3 using the psych (Procedures for Psychological, Psychometric, and Personality Research) package version 1.4.4.

We used two criteria for factor selection: (a) the communality estimate of each variable should be greater than 0.50 (i.e. the proportion of the variance of each variable that the factors account for is greater than 0.50) and (b) to include factors which explained 0.05 of the total variance. Five factors that each accounted for 0.05 of the variance were extracted however the communality estimate for *GRIN2B* was only 0.36. We were able to satisfy our primary criterion by adopting a six factor solution that accounted for 0.84 of the total variance with one factor accounting for 0.04 of the variance; the *GRIN2B* communality estimate rose to 0.55 and the mean (SD) total communality estimate was 0.83 (0.13).

Miami Brain Bank hippocampal samples—Scatter plots of log2 transformed, quantile normalized expression levels of all the gene transcripts in alcoholics and cocaine addicts relative to controls were derived. Linear regression analyses were performed using JMP v10 with quantile normalized gene expression values as the dependent variable and diagnosis (alcoholic, cocaine addict or control), PMI, age, and ethnicity (Caucasian/Hispanic or African American coded 1 or 2) as the independent variables. Age, ethnicity and PMI were included in the analyses if $p \leq 0.1$. P-values were corrected using the False Discovery Rate (FDR) (Benjamini *et al.* 2001) based on 28 candidate genes.

Since the aim of this study was to detect both specific and overlapping changes in gene expression in cocaine addicts and alcoholics, we performed secondary analyses on genes that showed nominally significant changes ($p < 0.05$) across the three groups (2 df). We based our analysis for each gene on visual inspection of box plots, for example, if a gene had higher expression in alcoholics compared with both cocaine addicts and controls, the relevant analysis would be: alcoholics vs (cocaine addicts + controls) (1 df).

Global P-values were calculated for the primary analyses and for the secondary analyses using the truncated product method (Zaykin *et al.* 2007), a modified Fisher's method (<ftp://statgen.ncsu.edu/pub/zaykin/tpm>). P-values from all 28 independent tests with $p < 0.05$ were combined and global significance was assessed by evaluating the distribution of their product.

Validation analysis in hippocampal samples from the two sources—We used RNA-Seq to quantify mRNA transcripts in postmortem total hippocampus from the eight male controls obtained from the University of Miami Brain Bank and compared the expression of the 26 glutamatergic genes (*GRIN3B* and *SLC1A7* excluded) in our eight hippocampal samples with gene expression in the nine BrainSpan hippocampal samples.

It should be noted that the read counts were independently normalized within the two datasets and therefore gene expression levels can only be compared within each dataset and not between the two datasets.

RESULTS

BrainSpan: global patterns of glutamatergic gene expression

The global RPKM expression levels across all 26 glutamatergic genes varied across individuals: sample A (male, 18yrs) had the lowest and samples B, C and F (respectively: female 19 yrs, female 21 yrs male 36 years) had the highest global expression values (Figure S1). Since sample A had data available for only 13/16 brain regions, box plots excluding these 3 regions were generated for all 9 samples and yielded the same order of expression level as observed in Figure S1.

Figure 1 shows the expression of each gene averaged across all 16 brain regions. *GRIN1*, encoding the essential NMDA GluN1 subunit, is the most highly expressed gene across the brain. The expression of each gene is fairly consistent across all brain regions with the exception of the cerebellum (CBC), the mediodorsal nucleus of the thalamus (MD) and the

striatum (STR) (Figure S2 – S9). A total of 23 of the 26 glutamatergic genes have differential expression in the CBC, MD and STR relative to other brain regions (Figure S2 – S9). Only *GRIN1*, *GRIK5* and *SLC1A1* are each expressed to the same degree across all 16 brain regions.

BrainSpan: Factor analysis of correlation of expression of glutamatergic genes across the whole brain

Figure 2 presents the correlation in expressions of the 26 glutamatergic genes across the whole brain. The factor analysis identified six factors that together accounted for 0.84 of the variance in total glutamatergic gene expression (Table 3). Out of the total of 26 genes, 19 loaded 0.5 onto only one factor. The remaining 7 genes loaded 0.5 onto two factors. A total of 14 genes loaded 0.5 onto the most abundant Factor 1 (0.29 variance), including all AMPA genes and 4/6 NMDA receptor genes (Table 3). The 7 genes loading onto factor 2 (20% of the variance) showed considerably higher expression in the cerebellum relative to all other regions (Figure S2, S3, S5, S7–S9). There was no obvious pattern to genes loading onto the other factors.

Results from the Miami Brain Bank human hippocampal samples

As we have previously reported, expression of 16,008 gene transcripts was detected in the human hippocampus (Zhou *et al.* 2011). Figures 3 and Figure S10 show scatter plots of log₂ transformed, quantile normalized expression levels of the 28 candidate glutamatergic genes superimposed on the total number of gene transcripts in alcoholics and cocaine addicts respectively relative to controls. It can be seen from these figures that the 28 glutamatergic genes are moderately to highly expressed in the hippocampus. The highest expression values were detected for two glutamate transporters, *SLC17A7* and *SLC1A2*, and for *GRIN1* that encodes the ubiquitous subunit GluN1.

Primary analyses—The selected glutamatergic genes were strong candidates for involvement in addiction. Indeed, 9/28 of the genes showed nominally significant differences in gene expression between alcoholics, cocaine addicts and controls (2df) (Table 4). The global p-value, calculated using the truncated product method (Zaykin *et al.* 2007) for overall differences in expression of the 28 genes across alcoholics, cocaine addicts and controls (Table 4, primary analysis) was $p = 1.0 \times 10^{-6}$. After FDR correction, only the results for *GRIN2B* ($p = 0.008$) and *GRIN2D* ($p = 0.028$) were significant.

Secondary analyses to detect specific effects of alcohol or cocaine exposure

—Box plots for the nine genes that had nominally significant differences in gene expression across alcoholics, cocaine addicts and controls (2 df) are shown in Figure 4. From this figure it can be seen that *GRIA4*, *GRIK3* and *GRM4* have higher expression in alcoholics compared with both cocaine addicts and controls. Therefore for these three genes we deemed that the relevant analysis should be: alcoholics vs (cocaine addicts + controls) (1df) (Table 4). The global p-value for the secondary analyses shown in Table 4 was $p = 1.4 \times 10^{-7}$.

Loading of genes showing hippocampal expression changes after chronic alcohol / cocaine exposure onto the six gene expression factors

The 6 genes that showed FDR-corrected expression changes in the hippocampus in response to chronic exposure to alcohol / cocaine had their primary loadings on factor 2 (*GRIA4*, *GRM4*), factor 4 (*GRM3*), factor 5 (*GRIN2D*) and factor 6 (*GRIK3*, *GRIN2B*).

Validation analysis in hippocampal samples from two sources—As shown in Figure S11, we compared the mean (S.E.) RPKM expression values of the 26 glutamatergic genes in the BrainSpan hippocampal samples with the mean (S.E.) RPKM expression values in the hippocampal samples from the controls in our own RNA-Seq study. As can be seen, the pattern of relative gene expression was virtually the same in the two datasets irrespective of differences in sequencing technology and analytical pipelines used for the RNA-Seq.

DISCUSSION

Our study using RNA-Seq BrainSpan data from healthy humans has shown that genes encoding glutamate receptors and transporters are moderately expressed throughout the brain with the exception of the CBC, the MD and STR. Within these three regions, 23 of the 26 genes have differential expression relative to other brain regions.

The factor analysis revealed few patterns in gene expression other than for factor 2 (0.20 variance). The seven genes loading onto this factor had exceptionally high expression in the cerebellum. Fourteen of the 26 genes loaded 0.5 onto factor 1 that accounted for 0.29 of the total variance. These genes encoded all 4 AMPA receptors, 4/6 NMDA receptors, 4/7 metabotropic receptors and 1/4 transporters. Genes encoding GABAergic ionotropic receptors are clustered in chromosomal regions and in our earlier study we showed that these gene clusters loaded onto specific factors (Enoch *et al.* 2013). However, genes encoding ionotropic glutamate receptors are located in different chromosomal regions and this may explain the fact that none of the factors were specific for particular types of glutamate receptors. In the earlier study of GABAergic genes the factor analysis revealed that genes loading onto four of the six factors were sensitive to the effects of alcohol/cocaine. These distinct groups of genes included the chromosome 4 cluster, previously associated with alcohol and drug dependence in humans, and genes involved in GABA synthesis and synaptic transport (Enoch *et al.* 2013). However, in the current study the glutamatergic genes that were sensitive to chronic alcohol / cocaine exposure did not load onto specific factors.

In the second part of our study comparing RNA-Seq data from postmortem hippocampus of alcoholics, cocaine addicts, and controls, the strongest finding was for *GRIN2B* (encoding the NMDA receptor subunit GluN2B), that was up-regulated in both alcoholics and cocaine addicts, suggesting a shared pathway for addiction. Expression of both *GRM3* (encoding mGluR3) and *GRIN2D* (encoding the NMDA receptor subunit GluN2D) was up-regulated in alcoholics and down-regulated in cocaine addicts relative to controls. After performing secondary analyses we found specific effects of alcohol exposure: the alcoholics had FDR corrected up-regulation of three genes relative to controls and cocaine addicts: *GRIA4* (encoding the AMPA receptor subunit GluA4), *GRIK3* (encoding the kainate receptor

subunit GluR7) and *GRM4* (encoding the metabotropic receptor subunit mGluR4). In contrast, in an earlier parallel study in this same dataset (Enoch *et al.* 2012) the predominant finding in alcoholics and cocaine addicts was down-regulation of GABAergic gene expression in the hippocampus. Moreover, GABAergic genes that showed expression changes were grouped in specific factors that had biological relevance (Enoch *et al.* 2013). This was not the case with the glutamatergic genes; as mentioned above, the factor analysis of global gene expression did not identify specificity in response to chronic alcohol and cocaine exposure.

Lovinger *et al.* (1989) first showed that acute intake of ethanol in the intoxication range of 5 – 50 mM inhibits ion currents in NMDA receptors (NMDARs) on hippocampal neurons. Subsequently it has been shown in both in vitro and in vivo models, that chronic exposure to ethanol increases GluN1 and GluN2B mRNA and receptor protein gene levels (Chandrasekar 2013). Studies in GluN2B knockout mice have demonstrated that GluN2B plays a key role in both the acute and chronic actions of ethanol (Wills *et al.* 2012) and this subunit is involved in regulating low-dose stimulant effects as well as the depressant / hypnotic effects of ethanol (Badanich *et al.* 2011). In addition, up-regulation of GluN2B has been associated with cocaine seeking behavior (Xie *et al.* 2013) and behavioral sensitization to the locomotor-activating effects of cocaine (Schmidt *et al.* 2010).

NMDARs are tetrameric non-selective cation channels that are formed by two GluN1 subunits and any one of four GluN2 (A-D) subunit isoforms. Glutamate binds at GluN2 subunits and glycine binds at GluN1 subunits. The GluN2 subunits control the electrophysiological properties of the NMDAR and confer distinct functional properties (Niciu *et al.* 2012). For example, NMDARs including GluN2B have much slower deactivation kinetics than GluN2A, thereby remaining open longer and allowing much greater charge transfer and calcium signaling (Traynelis *et al.* 2010). GluN2D has even slower deactivation kinetics than GluN2B (Vance *et al.* 2011). Therefore up-regulation of both these NMDARs may be critical to allostatic adaptation to chronic alcohol exposure.

The activation of NMDARs requires both a ligand (glutamate) and voltage. In essence, glutamate acts on AMPA receptors (AMPA) which then depolarize the membrane, thereby permitting glutamate to act on NMDARs. AMPARs are grouped into two functionally distinct tetrameric assemblies based on the presence of GluA2 that blocks the flow of calcium ions (Bowie 2012). GluA2-lacking AMPARs exhibit large single channel currents (Bowie 2012). *GRIA4* has the lowest expression of the four AMPA receptors in the hippocampus (Figure S2). Up-regulation of *GRIA4*, encoding GluA4, after chronic alcohol exposure may result in increased depolarization of the membrane and subsequent up-regulation of *GRIN2B* and *GRIN2D*.

In our study, chronic exposure to ethanol also resulted in up-regulation of genes encoding two metabotropic receptors: *GRM3* encoding the Group II mGluR3 and *GRM4*, encoding the Group III mGluR4. mGluR3 receptors are localized pre- and post-synaptically as well as on glia and negatively regulate transmitter release (Moussawi & Kalivas 2010). Both group II and Group III receptors are coupled to G proteins which when activated inhibit adenylyl cyclase and cAMP formation, thereby limiting downstream protein kinase A activation.

GRM3 is the most highly expressed gene encoding metabotropic receptors in the hippocampus (Figure S7, S8). mGluR 2/3 receptors have been implicated in the reinforcing and dependence-inducing actions of ethanol (Kufahl *et al.* 2011). So far, there is little evidence to link mGluR4 with ethanol consumption (Mao *et al.* 2013).

Our study also found up-regulation of *GRIK3*, encoding the kainate receptor subunit GluR7. Less seems to be known about kainate receptors and their possible impact on addiction. A couple of studies showed that a *GRIK1* SNP was associated with alcoholism (Kranzler *et al.* 2009) and also moderated the effects of topiramate treatment in heavy drinkers (Kranzler *et al.* 2014). One study has shown that a *GRIK3* functional polymorphism predicted delirium tremens in alcoholics (Preuss *et al.* 2006).

A recent study by Jin and colleagues (2014) examined expression levels of the 16 genes that encode ionotropic receptor subunits in postmortem hippocampal dentate gyrus from 13 male alcoholics and 15 male controls. Total RNA was assayed using quantitative RT-PCR. The normalized mRNA expression of 15 /16 genes in the controls was similar to what we found in the Miami Brain Bank and the BrainSpan controls, the one exception being *GRIA2*. In the Jin *et al.* study (2014), the expression level of *GRIA2* was approximately 1.7 times greater than that of *GRIN1*, their second most highly expressed gene which had the highest expression of the ionotropic receptor genes in our two datasets. In contrast to our study, their predominant finding that survived correction for multiple comparisons was that *GRIN1* was significantly up-regulated in alcoholics relative to controls. There were only nominally significant results for *GRIN2D* and *GRIN2B*. Differing methodology might account for the different results in the two studies.

Many of the symptoms of addiction are due to the imbalance in neuronal excitation and inhibition, largely as a result of alterations in transmission of GABA and glutamate respectively. There is evidence that neuroadaptations in response to chronic alcohol exposure result in elevated extracellular glutamate levels (Holmes *et al.* 2013). In our earlier study of GABAergic pathway genes (Enoch *et al.* 2012), one of the principal findings was down-regulation of *GABBR1* in both alcoholics and cocaine addicts. *GABBR1* encodes the GABAB1 receptor subunit of metabotropic GABAB receptors (GABABRs) that are abundantly expressed at inhibitory and excitatory synapses throughout the brain. The GABAB1 subunit is necessary for activation by agonists (Chalifoux & Carter 2011). The GABABRs modulate glutamate and GABA neurotransmitters by their effects on calcium channels presynaptically and potassium channels postsynaptically. It is known that presynaptic GABABRs inhibit multivesicular release through their effects on calcium channels thereby decreasing synaptic glutamate concentrations (Chalifoux & Carter 2010). Therefore down-regulation of *GABBR1* is likely to result in elevated extracellular glutamate levels. In the present study we found robust up-regulation of *GRIN2B* and *GRIN2D* that encode two NMDAR subunits. It has been shown that there is postsynaptic interplay between NMDA and GABAB conductances resulting in bistability: at hyperpolarized potentials, GABAB conductance is high and NMDA conductance is low; in the depolarized state, GABAB conductance is low and NMDA conductance is high (Guetg *et al.* 2010; Maier *et al.* 2010; Sanders *et al.* 2013). The combined results from our earlier study (Enoch *et al.* 2012) and the current study suggest that homeostasis in GABABR and NMDAR

activity may be altered in alcoholics and cocaine addicts resulting in a hyperglutamatergic state.

At first glance it might be considered surprising that chronic exposure to alcohol and cocaine did not alter the expression of glutamate transporter genes. These high affinity glial and neuronal transporters modulate extracellular glutamate levels. Studies in rodents have shown that in the brain the majority of glutamate uptake is via EAAT2 encoded by *SLC1A2* and alterations in glutamate homeostasis following prolonged exposure to drugs of abuse are associated with changes in several key components including EAAT2 (Reissner & Kalivas 2010). However, our study in the human hippocampus showed only a nominally significant result for *SLC1A3* that encodes the glial transporter EAAT1 i.e. down-regulation of *SLC1A3* in both alcoholics and cocaine addicts relative to controls (data not shown). On the other hand, as mentioned above we have demonstrated down-regulation of *GABBR1* encoding the GABAB1 receptor subunit (Enoch *et al.* 2012) that is likely to result in a hyperglutamatergic state, the allostatic maintenance of which may not require alteration in levels of glutamate transporters.

One limitation of this study is that we only selected genes encoding the glutamate receptors and transporters that are sufficiently expressed in the hippocampus and did not take a more global approach by, for example including genes implicated in glutamate synthesis and metabolism as well as genes encoding glutamate receptor associated proteins. However we had to weigh up the positive aspect of including a much larger group of candidate genes against the negative aspect of much more stringent corrections for multiple testing. Therefore we opted for a conservative approach. Nevertheless, information about three other glutamatergic genes is available in our two previous publications about GABAergic gene expression in the same two datasets (Enoch *et al.* 2012, 2013). The expression of *GLS*, encoding the enzyme glutaminase that converts glutamine to glutamate, did not differ between alcoholics, cocaine addicts and controls. However, *GAD1* and *GAD2*, the genes encoding glutamic acid decarboxylase, the enzyme that converts glutamate to GABA, were both down-regulated in cocaine addicts relative to alcoholics and controls.

In conclusion, in this first comprehensive study of glutamatergic gene expression in healthy humans, we have shown that genes encoding glutamate ionotropic receptors, metabotropic receptors and transporters are moderately to highly expressed throughout the brain except in the cerebellum, the mediodorsal nucleus of thalamus and the striatum. Six factors explain nearly all the variance in global gene expression; one factor included seven genes with exceptionally high expression in the cerebellum. At least in the hippocampus, the effect of chronic alcohol use is largely to up-regulate glutamatergic genes. The effects of cocaine exposure are less evident. It is possible that the NMDA GluN2B receptor subunit might be implicated in a common pathway to addiction, possibly in conjunction with the GABAB1 receptor subunit. Finally, our findings in human samples validate the clinical relevance of similar findings in rodent models of chronic alcohol / cocaine exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

BrainSpan data: expression of glutamatergic genes averaged across all 16 brain regions The brain regions are listed in Table 1.

The ends of the whiskers represent the maximum and minimum values however when outliers are present the whisker on the appropriate side is taken to 1.5xIQR (interquartile range) from the quartile rather than the maximum or minimum value, and individual outlying data points are displayed as filled circles.

The following genes: *GRIK1*, *GRIK2*, *GRIN2C*, *GRM1*, *GRM4*, *SLC1A6*, have outliers that represent much higher expression in the CBC relative to other brain regions

FIGURE 2.
BrainSpan data: correlation matrix of gene-gene expression for each of the 26 glutamatergic genes
The heat map is shaded according to the Pearson Product-Moment correlation coefficient R.
In addition, the values printed in each cell are the R values.

FIGURE 3.

Expression of glutamatergic genes in the human hippocampus: alcoholics vs controls The genome-wide expression levels of 16,008 transcripts, including the 28 glutamatergic genes, are shown

FIGURE 4.

Expression of the nine genes that had nominally significant differences in gene expression across alcoholics, cocaine addicts and controls.

The ends of the box plot whiskers represent the maximum and minimum values.

TABLE 1

BrainSpan brain regions with gene expression data

	Brain Regions	Abbreviation
1	Hippocampus	HIP
2	Striatum	STR
3	Anterior (rostral) cingulate (medial prefrontal) cortex	MFC
4	Dorsolateral prefrontal cortex	DFC
5	Ventrolateral prefrontal cortex	VFC
6	Orbital frontal cortex	OFC
7	Amygdaloid complex	AMY
8	Cerebellar cortex	CBC
9	Inferolateral temporal cortex (area TEv, area 20)	ITC
10	Posterior (caudal) superior temporal cortex (area TAc)	STC
11	Mediodorsal nucleus of thalamus	MD
12	Posteroinferior (ventral) parietal cortex	IPC
13	Primary somatosensory cortex (area S1, areas 3,1,2)	S1C
14	Primary motor cortex (area M1, area 4)	M1C
15	Primary visual cortex (striate cortex, area V1/17)	V1C
16	Primary auditory cortex (core)	A1C

TABLE 2

Candidate glutamatergic genes

Gene ID	Gene	Chr	Protein	
AMPA Receptor Subunits				
1	GRIA1	5		GluA1
2	GRIA2	4		GluA2
3	GRIA3	X		GluA3
4	GRIA4	11		GluA4
Kainate Receptor Subunits				
5	GRIK1	21		GluR5
6	GRIK2	6		GluR6
7	GRIK3	1		GluR7
8	GRIK4	11		KA1
9	GRIK5	19		KA2
NMDA Receptor Subunits				
10	GRIN1	9		GluN1
11	GRIN2A	16		GluN2A
12	GRIN2B	12		GluN2B
13	GRIN2C	17		GluN2C
14	GRIN2D	19		GluN2D
15	GRIN3A	9		GluN3A
16	GRIN3B	19		GluN3B
Metabotropic Receptor Subunits				
17	GRM1	6	Group I	mGluR1
18	GRM5	11	Group I	mGluR5
19	GRM2	3	Group II	mGluR2
20	GRM3	7	Group II	mGluR3
21	GRM4	6	Group III	mGluR4
22	GRM7	3	Group III	mGluR7
23	GRM8	7	Group III	mGluR8
Glutamate Transporters				
24	SLC1A3	5	Glia	EAAT1
25	SLC1A2	11	Glia	EAAT2
26	SLC1A1	9	Glia	EAAT3
27	SLC1A6	19	neuron	EAAT4
28	SLC17A7	19	neuron	VGLUT1

TABLE 3

Factor Analysis of correlations in expression between glutamatergic genes

Protein	Gene	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	h2
AMPA	GRIA1	0.61	0.29	0.44	0.15	-0.05	-0.02	0.68
	GRIA2	0.96	-0.01	0.24	0.07	-0.07	0.07	0.99
	GRIA3	0.87	-0.08	0.33	-0.05	-0.07	0.16	0.90
	GRIA4	0.56	0.64	0.06	-0.01	0.26	0.12	0.82
Kainate	GRIK1	0.10	0.69	0.18	-0.09	0.23	-0.01	0.58
	GRIK2	0.26	0.90	0.01	-0.03	-0.09	0.08	0.89
	GRIK3	0.61	0.13	0.34	-0.05	0.21	0.63	0.95
	GRIK4	0.42	0.19	0.62	0.28	0.00	0.03	0.68
	GRIK5	0.31	0.18	0.81	0.04	0.14	-0.08	0.81
NMDA	GRIN1	0.49	-0.04	0.70	-0.15	0.24	0.14	0.83
	GRIN2A	0.87	0.03	0.18	-0.06	0.05	0.36	0.92
	GRIN2B	0.47	-0.04	-0.01	-0.03	0.00	0.55	0.53
	GRIN2C	-0.33	0.87	-0.08	0.17	-0.15	-0.06	0.92
	GRIN2D	0.02	0.14	0.08	-0.04	0.94	0.06	0.92
	GRIN3A	0.77	-0.08	0.23	0.06	0.20	0.17	0.72
Metab Gp I	GRM1	0.11	0.91	0.07	-0.06	0.35	0.01	0.96
	GRM5	0.85	-0.09	0.17	0.10	0.32	0.11	0.88
	GRM2	0.46	0.02	0.74	-0.18	0.13	0.28	0.88
	GRM3	0.19	-0.15	0.37	0.67	0.02	0.00	0.64
	GRM4	-0.19	0.96	-0.01	-0.03	-0.05	-0.01	0.97
	GRM7	0.66	0.08	0.41	-0.11	0.59	0.00	0.97
	GRM8	0.71	-0.08	0.39	-0.16	0.04	-0.06	0.70
	SLC1A1	0.68	0.03	0.28	-0.01	0.64	0.02	0.96
Transporter	SLC1A2	-0.01	-0.13	-0.08	0.89	-0.01	-0.01	0.82
	SLC1A3	-0.13	0.14	-0.13	0.88	-0.08	-0.04	0.83

Protein	Gene	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	h2
Transporter	SLC1A6	-0.17	0.88	0.10	-0.05	-0.02	-0.03	0.82
	Variance	0.29	0.20	0.13	0.09	0.09	0.04	

Gene loadings 0.5 for each factor are identified by bold font h2 = the communality estimate i.e. the proportion of that variable that the factor accounts for.

TABLE 4
Analysis of gene expression changes in postmortem hippocampus from alcoholics, cocaine addicts and controls

PRIMARY ANALYSIS				SECONDARY ANALYSIS			
ID	GENE	Nominal P value 2df	FDR P value	Max Effect	Nominal P value	FDR P value	
1	GRIA1	0.162	0.324		0.162	0.324	
2	GRIA2	0.882	0.915		0.882	0.915	
3	GRIA3	0.398	0.557		0.398	0.557	
4	GRIA4	0.030	0.116	AD vs CT+CO	0.008	0.037	
5	GRIK1	0.656	0.799		0.656	0.799	
6	GRIK2	0.068	0.178		0.068	0.178	
7	GRIK3	0.026	0.116	AD vs CT+CO	0.006	0.034	
8	GRIK4	0.858	0.915		0.858	0.915	
9	GRIK5	0.342	0.504		0.342	0.504	
10	GRIN1	0.455	0.607		0.455	0.607	
11	GRIN2A	0.758	0.884		0.758	0.884	
12	GRIN2B	0.0003	0.008		0.0003	0.008	
13	GRIN2C	0.089	0.208		0.089	0.208	
14	GRIN2D	0.002	0.028		0.002	0.028	
15	GRIN3A	0.033	0.116		0.033	0.116	
16	GRIN3B	0.927	0.927		0.927	0.927	
17	GRM1	0.04	0.123		0.040	0.124	
18	GRM5	0.315	0.49		0.315	0.490	
19	GRM2	0.07	0.178		0.07	0.178	
20	GRM3	0.006	0.053		0.006	0.034	
21	GRM4	0.018	0.116	AD vs CT+CO	0.006	0.034	
22	GRM7	0.856	0.915		0.856	0.915	
23	GRM8	0.201	0.358		0.201	0.359	
24	SLC1A3	0.033	0.116		0.033	0.116	
25	SLC1A2	0.11	0.236		0.11	0.237	
26	SLC1A1	0.27	0.444		0.27	0.445	

PRIMARY ANALYSIS				SECONDARY ANALYSIS			
ID	GENE	Nominal P value 2df	FDR P value	Max Effect	Nominal P value	FDR P value	
27	SLC1A6	0.648	0.799		0.648	0.799	
28	SLC17A7	0.205	0.358		0.205	0.359	

AD = alcoholic; CO = cocaine addict; CT = control