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Astrocyte-Specific Transcriptome Responses to Chronic Ethanol Consumption

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Abstract

Astrocytes play critical roles in central nervous system (CNS) homeostasis and are implicated in the pathogenesis of neurological and psychiatric conditions, including drug dependence. Little is known about the effects of chronic ethanol consumption on astrocyte gene expression. To address this gap in knowledge, we performed transcriptome-wide RNA sequencing of astrocytes isolated from the prefrontal cortex (PFC) of mice following chronic ethanol consumption. Differential expression analysis revealed ethanol-induced changes unique to astrocytes that were not identified in total homogenate preparations. Astrocyte-specific gene expression revealed calcium-related signaling and regulation of extracellular matrix genes as responses to chronic ethanol use. These findings emphasize the importance of investigating expression changes in specific cellular populations to define molecular consequences of chronic ethanol consumption in mammalian brain.

Introduction

Proper functioning of astrocytes is critical for normal brain function[1]. Originally considered supportive cells for neurons, astrocytes are now recognized as crucial regulators of synapse formation, elimination, and maintenance[2]. They contribute to the prevention of neuronal excitotoxicity and regulate synaptic plasticity by clearing and metabolizing extracellular neurotransmitters[3,4]. Astrocytes can sense and actively participate in neuronal activity via activation of G-protein-coupled-receptor (GPCR) signaling cascades and elevations in intracellular calcium[5]. In addition, astrocytes can secrete proteins to modify the extracellular matrix, shaping intercellular interactions and altering synaptic plasticity[6].

Astrocyte function can affect ethanol self-administration. For example, stimulation of nucleus accumbens core astrocytes using Designer Receptors Exclusively Activated by

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Disclosure

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Designer Drugs (DREADDs) decreases intermittent access self-administration, indicating that changes in astrocyte-specific activity, specifically Gαq-coupled GPCR signaling, are sufficient to modulate behavioral responses to ethanol[7]. Astrocytes utilize gap junction channels for communication via calcium signaling[8]. Astrocyte-specific gap junction inhibition increases motivation for ethanol self-administration[7], and leads to a transient increase in ethanol preference[9]. These studies suggest that changes in astrocyte function play an important role in ethanol consumption and preference; however, astrocyte-specific molecular changes in response to chronic ethanol exposure are largely unknown.

Gene expression studies also suggest that astrocytes may be involved in functional adaptations in response to ethanol exposure. Microarray analysis of post-mortem alcoholic human brain identified ethanol-responsive gene categories associated with glial function[10,11], and gene network analyses have identified astrocyte-related changes in ethanol-exposed rodent brain[12–14]. However, these and other studies have inferred cell-type enrichment based on gene expression data from total homogenate preparations, which likely fail to detect important cell-type specific responses to ethanol. In cultured astrocytes, acute ethanol exposure alters transcription of genes in functional categories such as calcium signaling, cytoskeleton remodeling, and extracellular matrix[15,16]. Although cultured astrocyte studies have provided insights to response to alcohol exposure, there are abundant gene expression and likely functional differences between cultured astrocytes and astrocytes isolated from mature brain[17,18]; thus, transcriptome analysis of acutely isolated cell-types should provide a deeper understanding of brain function and pathological states[19–21].

We report the first transcriptome-wide analysis of astrocyte-specific gene expression changes in response to chronic ethanol consumption. Astrocytes were isolated from the prefrontal cortex (PFC) after every-other-day (EOD) drinking, a paradigm that promotes escalation of intake[22]. We selected the PFC because it is known to be involved in drug and alcohol addiction[23]. RNA-sequencing was used to profile EOD-induced gene expression changes in astrocytes and total homogenate preparations. EOD caused differential regulation for several molecular functions, including calmodulin binding and extracellular matrix binding in astrocytes. Little overlap was observed between astrocyte-specific and total homogenate differential expression in response to ethanol; emphasizing the importance of studying cell-type specific expression changes to more completely understand the molecular consequences of chronic ethanol exposure in the adult mammalian brain.

Materials and Methods

Mice

Adult (8 weeks) male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and allowed to habituate to individual housing for at least one week. Mice were housed in the Animal Resource Center at The University of Texas Austin with 12-hr light/dark cycles and kept on a standard laboratory diet and water *ad libitum*. All experiments were approved by The University of Texas at Austin Institute for Animal Care and Use Committee and conducted in accordance with NIH guidelines regarding use of animals in research.

Every-other-day (EOD) ethanol drinking paradigm

Mice (n=12/group) were subjected to an EOD 2-bottle choice paradigm for 72 days (at least 36 drinking days total). Sample sizes were determined based on previous alcohol-related gene expression studies performed by our laboratory and others[12,24,25]. The EOD model is a variation of the continuous 2-bottle choice paradigm that promotes higher ethanol intake[22]. The treatment group had access to 15% (v/v) ethanol every other day and water every day, and the non-ethanol drinking control group only had access to water. To control for position preferences, bottle positions were switched daily. To control for evaporation and spillage, two bottles in an empty cage were also measured daily; one bottle containing water, and one containing ethanol. Ethanol quantity consumed (g/kg body weight/24h) and preference (ethanol consumed divided by total fluid intake) for each mouse was calculated. Mice were sacrificed 24 hours following their last drinking day.

Astrocyte enrichment and RNA isolation

Mice were anaesthetized briefly with isoflurane and perfused with phosphate-buffered saline (PBS). The PFC was dissected by removing the olfactory bulbs, then cutting the foremost 2mm of the cortex from each side at an approximate 50-degree angle from the midline, as previously described[12]. Tissue was minced and separated for total homogenate (approximately 5%) and astrocyte specific preparations. Total homogenate tissue was flash frozen in liquid nitrogen and stored at -80°C until further use. For astrocyte preparation, tissue was dissociated into a single cell suspension using the Neural Tissue Dissociation Kit with Papain (Miltenyi Biotec, Bergisch Gladbach, Germany). Myelin was removed by centrifugation in a 25% Percoll gradient. Astrocyte enrichment was performed using astrocyte cell surface antigen-2 (ACSA2) magnetic MicroBeads (Miltenyi Biotec). This isolation technique was selected based on previous success in purifying astrocytes from adult rodent brain[26–28]. RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and examined on the Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the Agilent Total RNA 6000 Pico Kit for quality and quantity. Three astrocyte samples were excluded due to low RNA concentrations. RNA concentrations of samples used for RNA-sequencing ranged from 128 - 2355 pg/μl, with RNA integrity number (RIN) scores ranging from 8.0–9.4.

In a separate experiment, we validated astrocyte specificity with whole brain isolation. RNA was isolated using the MagMAX-96 Total RNA Isolation Kit (Thermo Fisher Scientific, Rockford, IL), quantified with the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific) and analyzed for quality using a TapeStation instrument (Agilent Technologies). Reverse transcription was performed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was performed using TaqMan Universal PCR Master Mix and primer pairs and probes (Thermo Fisher Scientific) for astrocyte markers including *Gfap, Aldh1l1, Slc1a3*, as well as *Itgam* and *Rbfox3* to identify microglial and neuronal gene expression, respectively (Figure 2). 18S rRNA was used as an endogenous control gene.

RNA sequencing, read alignment, quality control, and quantification

A minimum of 1 ng RNA for each sample was submitted to the Genomic Sequencing and Analysis Facility at the University of Texas at Austin for mRNA selection with the MicroPoly(A) Purist Kit (Life Technologies, Carlsbad, CA) and library preparation with NEBNext Module Components (New England Biolabs, Ipswich, MA). Samples were sequenced on the Hi-Seq 4000 (Illumina) at a depth of ~20 million paired-end reads (150 base). Read quality was assessed using FASTQC (version 0.11.5)[29]. Adapter contamination was eliminated using Cutadapt (version 1.8), then reads were mapped to the mouse genome (UCSC mm10) with Bowtie2 (version 2.2.5)[30]. One sample was discarded due to poor mapping rate (< 50%). The final analyses included 20 ACSA2+ samples (n = 10/group) and 24 total homogenate samples (n = 12/group). Sorted BAM files were analyzed and duplicate reads filtered using Picard's MarkDuplicates tool (version 1.141). Raw counts were quantified using HTSeq (version 0.5.3p9)[31]. FPKM were counted from sorted BAM files using Cufflinks (version 2.2.1)[32]. To ensure no perturbation of the transcriptome occurred due to the isolation procedure, we compared ratios of the FPKM values for generally accepted astrocyte markers *Slc1a3* and *Glul*. Ratios of these markers were unchanged between ACSA2+ cells and total homogenate or between separate ACSA2+ samples, suggesting no marked transcriptome disruption occurs with astrocyte isolation. Raw and processed sequencing data from this study have been deposited to the Gene Expression Omnibus under accession number GSE92457.

Bioinformatics analysis

We utilized the *phyper* function of the *R* environment to determine whether there was significant overlap between our top 500 astrocyte-specific FPKM data and the top genes of a previously published astrocyte transcriptome[20]. Ethanol-mediated differential expression was determined using the Bioconductor package DESeq2[33]. Differentially expressed genes (nominal $p < 0.05$) were entered into Enrichr[34], an online tool that utilizes Fisher's exact test to identify biological functions and pathways that are enriched in a list of genes. Genes differentially expressed below a nominal p-value threshold of 0.05 were included in Enrichr analysis to minimize type II error and include enough genes for functional enrichment. We also used gene set enrichment analysis (GSEA; version 2.2.2, Broad Institute)[35], a ranked-based approach to identify enriched gene sets between control and ethanol-treated astrocytes. GSEA ranks all genes by expression level in both treatments and compares the pattern of expression in each treatment to established gene sets from the Molecular Signatures Database (version 5.1). Normalized counts obtained from DESeq2 were submitted to GSEA to calculate enrichment scores for gene sets. Specifically, Gene ontology (GO) molecular function gene sets (c5.mf.v5.1) were analyzed with GSEA to confirm Enrichr GO molecular function findings.

Immunohistochemistry

Mice were perfused with PBS and 4% paraformaldehyde (PFA), and brains were harvested. Brains were post-fixed in 4% PFA for 24 hours, then cryoprotected in 20% sucrose for 24 hours. Brains were frozen in optimal cutting temperature (OCT) and stored at -80C until sectioning. 20 μm sections were permeabilized in 0.05% Triton-X for 10 minutes and

blocked in 10% goat serum for 1 hour at RT. Sections were incubated overnight at 4°C in primary antibody (mouse anti-Glutamine synthetase [GS] 1:1000 [Millipore, Billerica, MA, Catalog #MAB302] and rabbit anti-CAMK2G 1:100 [Life Technologies, Carlsbad, CA, Catalog #PA514035]). Following three washes in PBS, sections were incubated in secondary antibody (goat anti-mouse 594, goat anti-rabbit 488, Thermo Fisher Scientific, Rockford, IL) for 2 hours at RT. Sections were mounted in 0.2% gelatin, dehydrated, and cover slipped with mounting medium (Vector Labs, Burlingame, CA). Sections were visualized using a Zeiss Axiovert 200 M fluorescent light microscope (Zeiss, Thornwood, NY). Bilateral images of the PFC (Bregma +2.8 to +2.24) were captured using a 20× objective. Images were analyzed using ImageJ (version 1.50i). GS, CAMK2G, and dual-labeled cells were quantified in medial regions of the PFC in two regions of interest per section (box, 500 × 500 μm). Cells were considered co-localized when they expressed both red (GS) and green (CAMK2G). Percentages of co-localized cells were averaged between two sections per animal (n = 3).

Results

Ethanol consumption

Mice subjected to EOD drinking consumed an average of 174 g/kg ethanol total over the course of the experiment. Average daily ethanol intake and preference are displayed in Figure 1. Daily ethanol intake significantly differed over time, as indicated by a repeated-measures ANOVA ($F(4.17, 55.90) = 11.34$, $p < 1.00e-04$). These results are consistent with previous work establishing the EOD paradigm as a relevant model for human excessive alcohol consumption and escalation of drug use[22].

Astrocyte isolation

We first verified that the astrocyte isolation technique selectively targeted astrocytes, as observed in prior studies[26,36]. We used qRT-PCR to confirm that astrocyte-specific genes were enriched when astrocytes were isolated using ACSA2+ magnetic beads (Figure 2). We observed robust enrichment of astrocyte markers *Aldh1l1, Gfap, and Slc1a3* in ACSA2+ cells and negligible levels of the microglial marker *Itgam* and the neuronal marker *Rbfox3*, indicating an astrocyte-enriched population. In contrast, RNA from total homogenate tissue was enriched with *Rbfox3* and contained both astrocyte and microglia markers. In addition, the 500 highest expressed genes (based on FPKM expression level) in ACSA2+ cells from the PFC were compared to the top 500 highest expressed genes in astrocytes, microglia, neurons, and three oligodendrocyte cell-types from a previously published cell-type specific transcriptome resource[20]. We observed the most overlap between ACSA2+ cells and astrocytes, with 225 genes shared between the top 500 genes of each transcriptome ($p = 2.52e-214$). Genes that did not overlap could be due to factors that were distinct for each experiment, such as regional heterogeneity of astrocyte gene expression, differences in isolation protocol, or age-specific differences in gene expression. The 225 overlapping genes were submitted to Enrichr[34] for molecular function analysis and we identified categories including GTPase activity ($p = 2.58e-08$), guanyl nucleotide binding ($p = 5.67e-05$), and amino acid transmembrane transporter activity ($p = 5.47e-05$) as highly enriched in astrocytes (Supplementary Table 1).

Differential expression produced by chronic ethanol exposure

In response to EOD, a total of 800 genes were differentially expressed in PFC astrocytes ($p < 0.05$) (Figure 3A). In contrast, fewer genes (589) were differentially expressed in the total homogenate ($p < 0.05$) (Figure 3B). Differentially expressed genes are listed in Supplementary Tables 2 and 3. Only 24 genes were identified as differentially expressed in both preparations (Figure 3C); however, 15 of the overlapping genes (63%) changed in opposite directions, suggesting that in different cell-types, the same genes may have opposite ethanol-induced expression changes. In general, the magnitude of change in differentially expressed genes was greater in astrocytes compared to total homogenate (Figure 3D). The absolute value of $\log_2$-fold change in astrocytes was 0.27 (~20% change in expression, fold change=1.20), compared to 0.17 for total homogenate (~13% change, fold change=1.13). These results indicate that analyzing isolated astrocytes reveals unique genomic responses to ethanol that are not observed in total homogenate preparations.

Enrichr analysis of differentially expressed genes

Differentially expressed genes ($p < 0.05$) identified from astrocytes and total homogenate preparations were further characterized using the enrichment analysis tool Enrichr to identify biological functions and pathways regulated in response to ethanol exposure. Gene ontology (GO) molecular function terms associated with altered astrocyte expression after EOD are listed in Table 1 and Supplementary Table 4. The top-ranked GO term was calmodulin binding. Interestingly, calmodulin binding was also the top term identified in total homogenate preparations ($p = 1.64e-04$); however, the specific genes within the category were distinct from those in astrocytes. Results for total homogenate also included functionally distinct categories such as microfilament motor activity (GO:0000146, $p = 3.09e-04$) and coreceptor activity (GO:0015026, $p = 8.43e-04$) (Supplementary Table 5).

Enrichr was used to examine enrichment of biological pathways associated with astrocyte-specific differentially expressed genes, using multiple databases including Kegg, WikiPathways, and Reactome (Table 2). Consistent with the GO molecular function results described above for differential gene expression due to ethanol, pathway analysis identified the category “Calcium regulation in the cardiac cell”, further supporting a role for astrocyte-specific genes involved in calcium-related events occurring in response to ethanol. In addition, both molecular function and pathway analyses indicate changes in extracellular matrix genes in astrocytes (Table 1 and 2).

Gene Set Enrichment Analysis (GSEA)

Normalized counts for all identified genes were analyzed using GSEA[35] to identify gene sets associated with chronic ethanol-exposed astrocytes. The highest ranked altered molecular function was identified as “calmodulin binding” (Figure 4A; $p < 1.00e-03$). Additional gene sets enriched in ethanol-exposed astrocytes include specific transcriptional repressor protein ($p < 1.00e-03$), protein complex binding ($p = 0.02$), and extracellular matrix structural constituent (Figure 4B; $p = 0.03$). In total homogenate preparations, gene sets such as phospholipid binding, nucleoside triphosphatase activity, and endonuclease activity were differentially regulated. Complete lists of molecular function gene sets enriched in astrocytes and total homogenate tissue isolated from chronic ethanol-drinking mice are included in

Supplementary Tables 6 and 7. Overall, GSEA and Enrichr results demonstrate consistent evidence of an astrocyte-specific chronic ethanol response that includes altered calcium signaling and extracellular matrix remodeling.

Astrocytic protein expression of a differentially expressed calmodulin signaling gene

We chose a differentially expressed candidate gene, *Camk2g*, to confirm astrocyte-specific protein expression. *Camk2g* encodes a Ca2+/calmodulin dependent protein kinase isoform and was down-regulated in astrocytes following every-other-day drinking. As published data regarding CAMK2G's expression in astrocytes is lacking, we sought to verify that the protein was expressed in astrocytes in the mouse PFC. Co-localization analysis of CAMK2G and astrocyte-specific marker glutamine synthetase (GS) revealed that CAMK2G protein is expressed in about 30% of GS+ PFC astrocytes (Figure 5).

Comparison of previous studies differentially expressed genes

We compared our astrocyte-specific differential expression data to a previously published ethanol-related astrocyte-specific gene expression dataset, as well as several total homogenate datasets to validate the current study's ethanol-responsive gene expression changes (Table 3). The overlapping genes between our study and each compared previous study were submitted to Enrichr for GO molecular function analysis. The top two functional categories are listed for each in Table 3.

Discussion

There is growing evidence that astrocyte function is linked to neurological disorders[34,38]; however, the potential roles of astrocytes in the neurobiological mechanisms of alcohol use disorder are largely unknown. Here, we found that chronic ethanol consumption leads to unique changes to the astrocyte transcriptome in the mouse prefrontal cortex (PFC). Our results suggest astrocyte-specific gene expression changes involving calcium signaling and extracellular matrix regulation may contribute to the brain adaptations driven by chronic ethanol consumption.

Previous studies[11, 13] have attempted to parse out cell-type specific contributions to ethanol-induced gene expression changes observed in whole tissue, relying on the use of astrocyte-enriched gene lists that may not be accurate for astrocytes in each brain region or at every developmental stage. Our approach circumvents the need for predefined cell-type gene lists by isolating astrocytes from the adult mouse PFC and profiling ethanol-induced transcriptome alterations with RNA-sequencing. Our astrocyte transcriptome data shows significant overlap with that of a previously published astrocyte transcriptome study[20], and functional enrichment analysis of the overlapping genes adds confidence to the cellular identity of the isolated cells. In addition, as most prior astrocyte-specific transcriptome profiling has been performed on astrocytes isolated from immature mice or from broadly defined brain regions[17,20,39,40], our isolated astrocyte transcriptome data represent a new resource for researchers interested in biological functions of adult astrocytes in the mouse PFC.

Differential expression analysis revealed that most gene expression changes occurring in astrocytes isolated from mice with a history of chronic, voluntary ethanol consumption are not observable in total homogenate tissue. This is indicated by the small amount of overlap between differentially expressed genes in both astrocytes and total homogenate tissue. In addition, the differentially expressed genes common to both preparations do not consist of the most significantly differentially expressed genes in either. Therefore, not only are most ethanol-induced astrocyte gene expression changes not observed in total homogenate tissue, even the most significantly changing genes are not detected. A greater number of differentially expressed genes and slightly larger magnitude changes were observed in astrocytes compared to total homogenate tissue. This could be because gene expression changes observed in isolated astrocytes are not diluted by stably expressing transcripts in other cells that mask subtle differences in astrocytes. These results further emphasize the importance of examining isolated cellular populations to define changes in gene expression associated with alcohol abuse, and suggest that previous work examining total homogenate preparations likely underestimates astrocyte-specific effects.

We compared our astrocyte-specific expression data to published reports investigating ethanol-related gene expression changes, and found that 62 astrocyte-specific genes identified in the current study were also observed in response to acute ethanol in cultured astrocytes[16]. Importantly, these expression changes also overlapped significantly with genes identified in human alcoholic frontal cortex correlating to lifetime alcohol consumption (114 genes in common; $p = 2.66e\text{-}06$; Fisher's exact test)[41] and differentially expressed genes identified in a microarray study of human alcoholic frontal cortex[11]. This overlap could be explained by increased astrocyte representation in total homogenate human brain tissue, as human astrocytes have a much larger volume than mouse astrocytes[42] and the human cerebral cortex may contain more astrocytes relative to neurons compared to mouse cortex[43], although the ratio of astrocytes to neurons in both species has not been definitively established. There is also likely brain-region heterogeneity that has not been fully elucidated[44]. Recent evidence suggests the relative abundance of astrocytes in mouse cortex may be lower than previously thought[45], possibly explaining the lack of agreement between astrocyte-specific and mouse total homogenate findings. Comparison with differential expression identified in a previously published microarray study of every-other-day drinking mice[12] showed less overlap with astrocyte differentially expressed genes, consistent with the present study. Enrichr analysis identified calmodulin binding ($p = 2.68e\text{-}05$) and calmodulin-dependent protein kinase activity (GO:0004683; $p = 9.67e\text{-}04$) as common important functional categories shared between astrocyte and human alcohol differentially expressed genes, suggesting that additional astrocyte-specific studies will inform our understanding of molecular changes occurring in human alcoholics.

Enrichr analysis of differentially expressed genes as well as GSEA of normalized counts for all measured genes indicate changes in calmodulin binding in astrocytes after EOD exposure. Changes in calmodulin binding indicate altered Ca^{2+} activity, as calmodulin is activated with intracellular release of Ca^{2+} [46]. Pathway analysis with Enrichr also suggested altered Ca^{2+} signaling in astrocytes after EOD. The ability for astrocytes to respond to neuronal activity through G-protein coupled receptor (GPCR) activation, intracellular Ca^{2+} fluctuations, and subsequent release of gliotransmitters is a mechanism underlying astrocyte

modulation of synaptic transmission[5]. Previous studies have indicated an involvement for astrocyte Ca^{2+} signaling in a wide range of behaviors[5], including motivation for alcohol consumption[7]. Our study provides the first evidence of chronic ethanol-mediated Ca^{2+} signaling modulation specifically in PFC astrocytes. GSEA analysis indicated an overall up-regulation of calmodulin binding related genes in astrocytes isolated from ethanol drinking mice. This suggests an increase in Ca^{2+}-related signaling cascades in PFC astrocytes 24 hours after ethanol exposure, possibly mediated by GPCR activation. Indeed, genes coding for multiple members of the regulators of G-protein signaling (RGS) family had increased expression in astrocytes after ethanol consumption, including *Rgs4*, *Rgs5*, and *Rgs16*. Altered expression of RGS proteins, specifically *Rgs4*, has been implicated in neuropsychiatric disorders including drug addiction[47]. Other genes involved in calmodulin binding showed decreased expression in astrocytes after EOD, including the calcium/calmodulin dependent protein kinase *Camk2g*. Altered expression of *Camk2g* has been identified in other studies related to alcohol abuse[16,48] including an astrocyte-specific transcriptome study[16], however the role of *Camk2g* in astrocytes has not been characterized, nor has its protein expression pattern in mouse PFC. Double immunohistochemistry of CAMK2G and GS revealed astrocytic expression of the protein in a minority of astrocytes, indicating possible subpopulations of PFC astrocytes that may have functional heterogeneity, which warrants further investigation. In addition, while CAMK2G was expressed mainly in non-astrocyte cells, our study showed the RNA was differentially expressed in astrocytes but not total homogenate tissue. This illustrates the importance of how certain genes can be altered by alcohol only in specific cell populations; which may have been obscured in previous studies. Expression changes of a particular gene in one cell type may have different functional consequences than in another, and isolating cell populations is a way to isolate these cell-specific changes and interpret potential functional changes more accurately. In cultured cortical astrocytes, CaMKII inhibition can modulate glutamate uptake and lead to release of ATP, possibly altering synaptic activity through activation of adenosine receptors[49], processes that can affect ethanol reward[50,51]. Therefore, changes in astrocyte calmodulin signaling specifically affecting calcium/calmodulin dependent protein kinases in astrocytes may have behavioral effects relevant to ethanol consumption.

Pathway analysis with Enrichr and GSEA identified a consistent overall up-regulation of extracellular matrix (ECM) genes in astrocytes following chronic ethanol exposure. The ECM and its associated proteins have been gaining attention as important factors in regulating plasticity-mediated addiction processes[52,53]. In addition, ECM genes were differentially expressed in cultured astrocytes in response to acute ethanol exposure[16]. One ECM gene with increased expression in astrocytes following chronic ethanol was *Plat* (tissue plasminogen activator [tPA]), a serine protease that regulates the structure of the ECM and can mediate many forms of synaptic plasticity[54]. tPA is elevated during ethanol withdrawal and its deletion in mice reduces chronic ethanol-induced withdrawal seizures[55]. The known roles of tPA in ethanol-related behaviors combined with our gene expression data hints at the intriguing possibility that PFC astrocytes may influence chronic ethanol consumption through the regulation of tPA. Many more ECM genes had increased expression in EOD-treated astrocytes, including laminin genes (*Lama4*, *Lamb1*, and *Lamc1*), collagen genes (*Col4a2* and *Col4a4*), and integrin genes (*Itga1, Itga7*). Overall, these changes indicate

complex alterations in the structure of the ECM and altered interactions between cells, which could influence synaptic plasticity and behavior[56]. While further research is needed to determine the possible consequences of these changes, our results point to an astrocyte-specific role in ECM remodeling after chronic ethanol exposure.

A major goal of transcriptome profiling is to elucidate gene pathways or networks that are perturbed by the disease state. We used several computational approaches to highlight potential new astrocytic targets that may be important for escalation of ethanol intake in an EOD paradigm, including Ca^{2+}-related signaling and ECM components. It should be emphasized that changes in astrocytes from chronic ethanol consumption are only weakly and partially represented in the total homogenate, demonstrating the importance of transcriptome profiling in specific cell populations to better understand the cellular mechanisms of addiction. In addition, differentially expressed genes in astrocytes overlapped with changes observed in human alcoholic brain, indicating the relevance of these findings to human alcohol abuse. There is emerging interest in using gene expression profiles to predict therapeutics[57], and the success of this approach for brain diseases will likely require expression profiles from astrocytes and other distinct brain cell populations. As our study is the first to profile the astrocyte-specific transcriptome in the adult mouse PFC, our data not only provides a framework for studying astrocyte responses in relation to chronic ethanol, it serves as a novel resource for future efforts in understanding the complexity of astrocyte function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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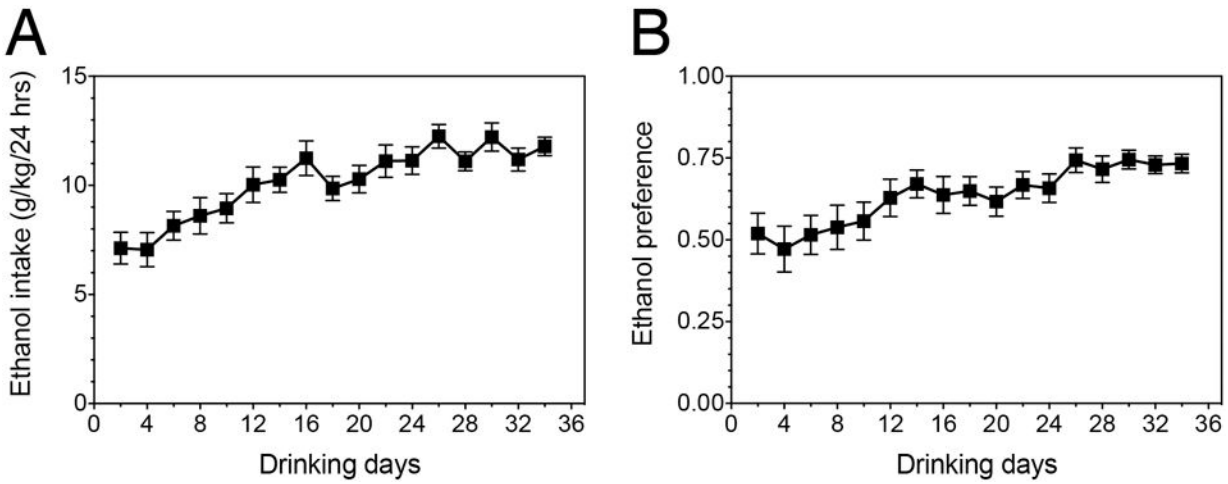

Figure 1.

Ethanol consumption and preference of EOD drinking mice. (A) Average daily ethanol intake throughout the course of the experiment. (B) Average ethanol preference throughout the course of the experiment (amount of ethanol consumed divided by the total amount of fluids consumed per day). Values are mean ± SEM, for n=12.

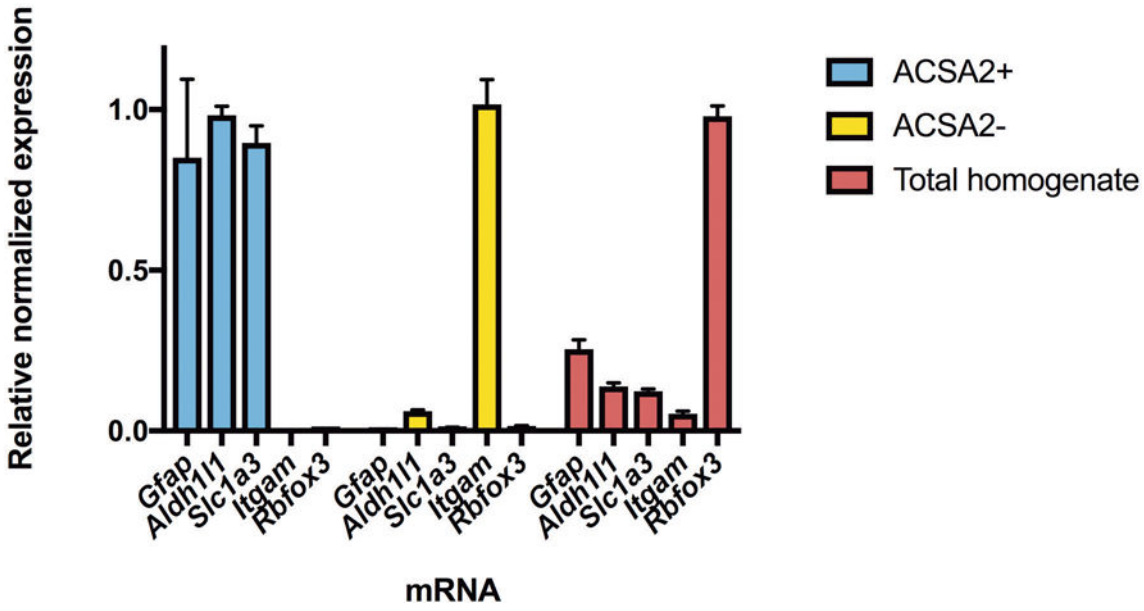

Figure 2.

Analysis of cell fractions following astrocyte enrichment. The ACSA2+ fraction was isolated using the ACSA2 microbeads, while the ACSA2- fraction represents unbound cells. The total homogenate was not fractionated and contains all cells. qRT-PCR for astrocyte markers including *Slc1a3, Aldh1l1* and *Gfap* confirm that the ACSA2+ fraction was enriched with astrocytes compared to the ACSA2- and total homogenate fractions. Markers for neurons (*Rbfox3*) and microglia (*Itgam*) show little neuronal or microglial gene expression in ACSA2+ cells. Expression levels are relative to 18S rRNA and normalized to the sample with highest expression for each marker. Values are mean ± SEM, for n=3.

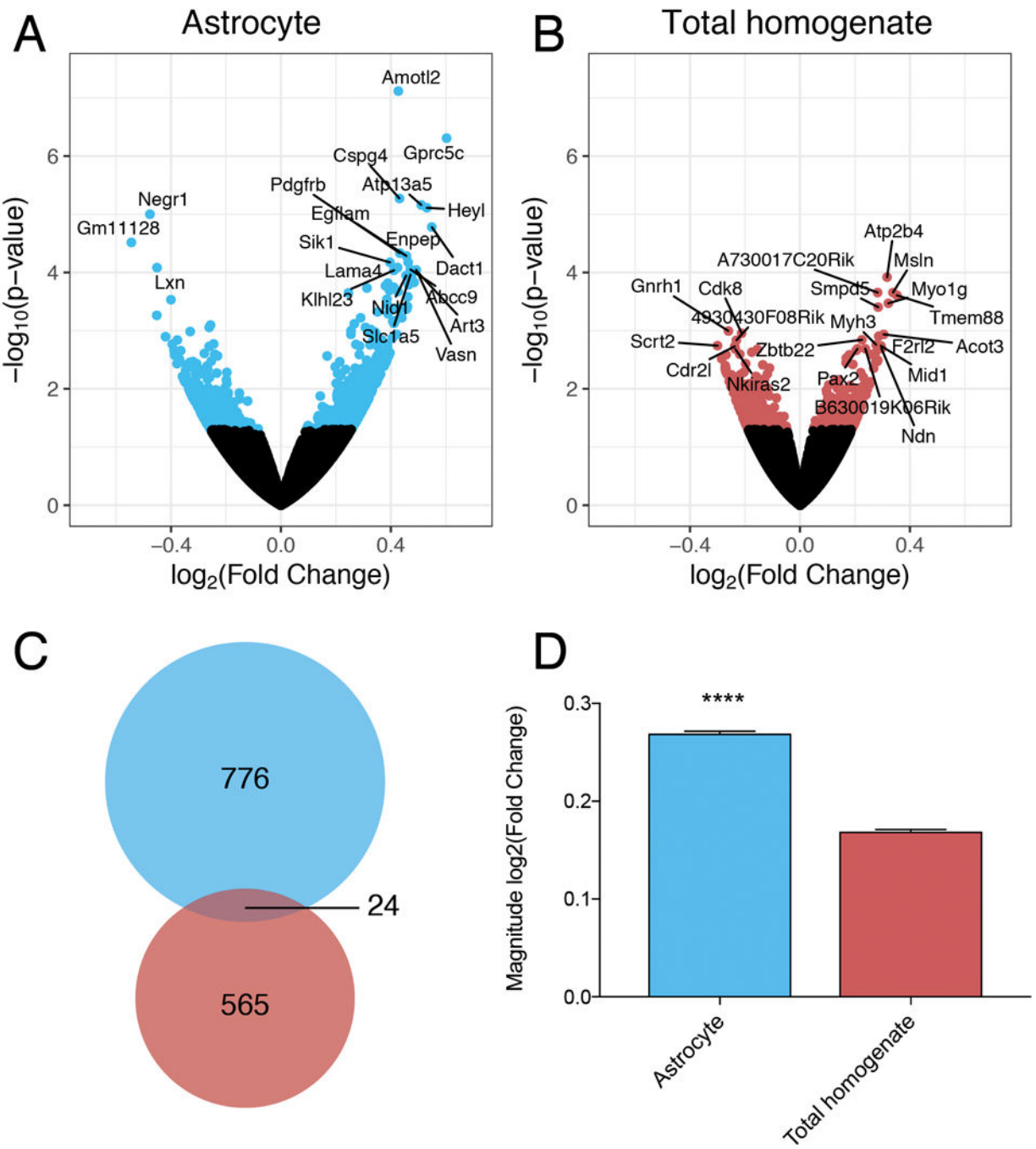

Figure 3.

Differential gene expression in response to chronic ethanol exposure. Volcano plots show differentially expressed genes ($p < 0.05$) in (A) astrocytes (blue) and (B) total homogenate (red) after EOD. The top 20 differentially expressed genes for each preparation are labeled with gene symbols. (C) 24 genes (overlapping area) were differentially expressed in both the astrocytes (blue) and the total homogenate (red) after EOD. (D) Magnitude of fold change of differentially expressed genes in astrocyte and total homogenate preparations (*** indicates $p < 0.0001$, unpaired t-test)

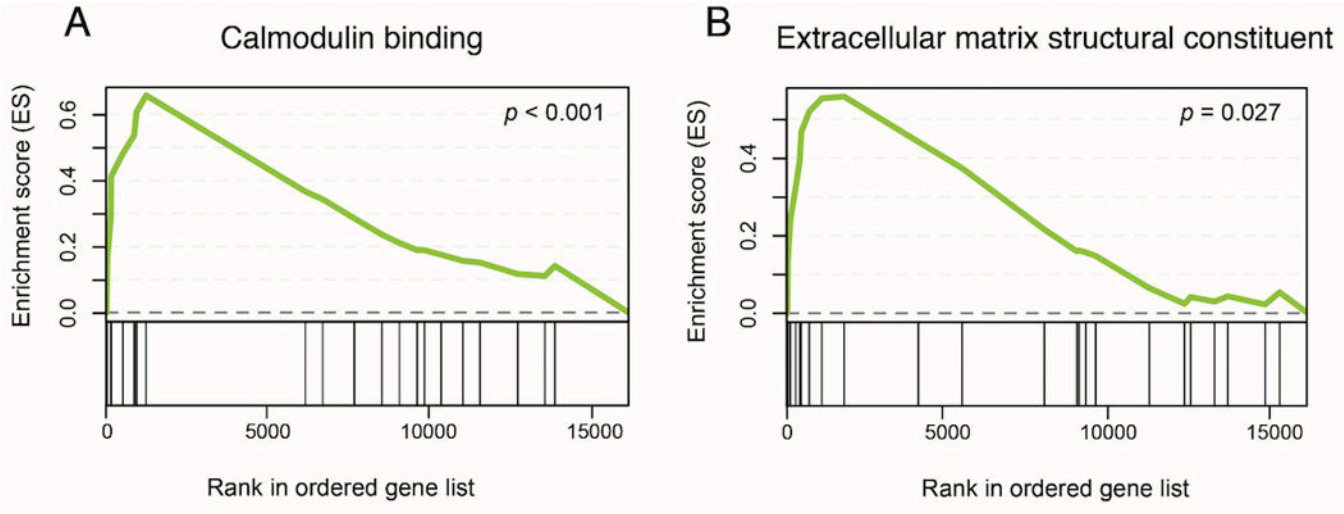

Figure 4.

Enrichment plots from GSEA. GSEA ranks genes by correlating gene expression with a specific phenotype. In panels A and B, genes that are more highly correlated with ethanol treatment are ranked closer to 0, while those correlated with the control phenotype rank closer to 15 000 (x-axis). Enrichment score (ES; y-axis) for a given gene set increases as a gene within the set is present while walking down the rank ordered list. The greatest deviation from 0 on the plot represents the ES for a given gene set.

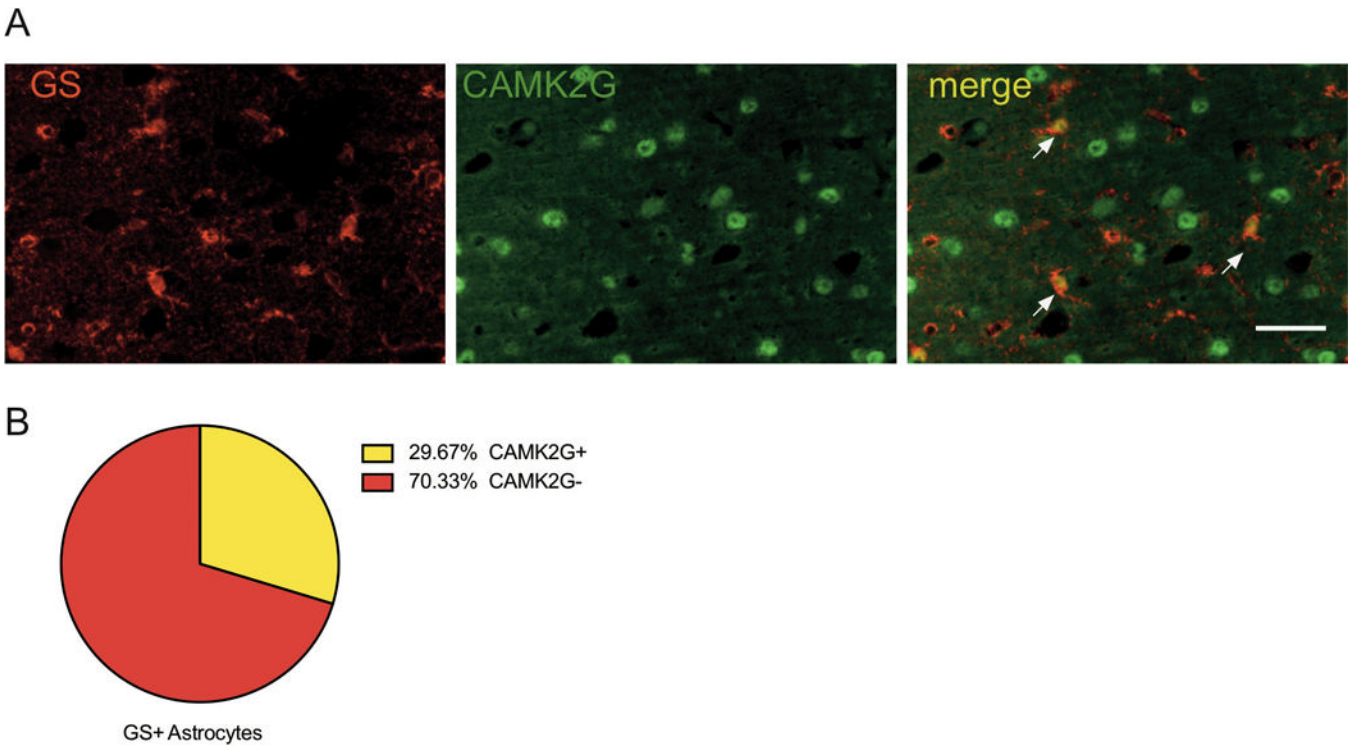

Figure 5.

CAMK2G expression in astrocytes. (A) Representative image shows co-localization of CAMK2G (green) and GS astrocytes (red). Arrowheads point to examples of co-localization. Scale bar corresponds to 50μm. (B) 30% of GS+ astrocytes expressed CAMK2G. Cell counts were averaged between regions of interest from two sections per animal (n = 3).

Table 1

Molecular functions differentially regulated in astrocytes after EOD drinking.

ID	Name	# DE/# in category	*p*-value
GO:0005516	*Calmodulin binding*	18/170	1.64e-04
GO:0017075	*Syntaxin-1 binding*	5/17	4.19e-04
GO:0005535	*Phosphatidylinositol phospholipase C activity*	6/28	7.12e-04
GO:0005178	*Integrin binding*	12/102	7.69e-04
GO:0050840	*Extracellular matrix binding*	8/51	8.77e-04
GO:0004629	*Phospholipase C activity*	6/30	1.05e-03
GO:0019838	*Growth factor binding*	13/123	1.30e-03
GO:0045125	*Bioactive lipid receptor activity*	3/8	3.07e-03
GO:0050839	*Cell adhesion molecule binding*	15/168	3.12e-03
GO:0016298	*Lipase activity*	11/105	3.20e-03
GO:0004879	*Ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity*	7/50	3.56e-03
GO:0070679	*Inositol 1,4,5 trisphosphate binding*	3/9	4.47e-03
GO:0001618	*Virus receptor activity*	5/29	5.40e-03
GO:0001540	*Beta-amyloid binding*	5/29	5.40e-03
GO:0022890	*Inorganic cation transmembrane transporter activity*	32/497	5.78e-03
GO:0008373	*Sialyltransferase activity*	4/20	7.38e-03
GO:0003707	*Steroid hormone receptor activity*	7/57	7.41e-03
GO:0008081	*Phosphoric diester hydrolase activity*	9/90	9.85e-03
GO:0015077	*Monovalent inorganic cation transmembrane transporter activity*	23/343	1.12e-02
GO:0005518	*Collagen binding*	7/62	1.16e-02

Table 2

Pathways differentially regulated in astrocytes after EOD drinking.

Database	Name	ID	#DE/# in category	*p*-value
KEGG	*ECM-receptor interaction*	Hsa04512	14/82	4.21e-06
KEGG	*cGMP-PKG signaling pathway*	Hsa04022	19/167	4.11e-05
KEGG	*Focal adhesion*	Hsa04510	20/202	1.83e-04
KEGG	*Vascular smooth muscle contraction*	Hsa04270	13/120	1.03e-03
KEGG	*PI3-Akt signaling pathway*	Hsa04151	26/341	1.32e-03
WikiPathways	*Calcium regulation in the cardiac cell*	WP553	20/150	2.34e-06
WikiPathways	*Myometrial relaxation and contraction pathways*	WP289	18/156	5.40e-05
WikiPathways	*Focal adhesion-Pi3K-Akt-mTOR signaling pathway*	WP2841	26/303	2.26e-04
WikiPathways	*Focal adhesion*	WP85	18/185	4.66e-04
WikiPathways	*Non-odorant GPCRs*	WP1396	22/256	6.54e-04
Reactome	*Laminin organization*	R-HSA-3000157	9/23	1.24e-07
Reactome	*Extracellular matrix organization*	R-HSA-1474244	28/283	1.05e-05
Reactome	*Non-integrin membrane-ECM interactions*	R-HSA-3000171	8/42	2.23e-04
Reactome	*ECM proteoglycans*	R-HSA-3000178	9/55	3.05e-04
Reactome	*Integrin cell surface interactions*	R-HSA-216083	10/62	3.12e-04

Table 3

Comparison of differentially expressed genes in astrocytes with previous literature

Tissue	Alcohol paradigm	Transcriptome profiling method	Number of overlapping differentially expressed genes	Top GO molecular function categories of overlapping genes	Study
Primary cultured mouse cortical astrocytes	Acute ethanol exposure	microarray	62	Peptidase regulator activity (GO:0061134), GTPase activity (GO:0003924)	Pignataro *et al.* 2013
Human frontal cortex	Lifetime alcohol consumption	RNA-sequencing	114	calmodulin binding (GO:0005516), calmodulin-dependent protein kinase activity (GO:0004683)	Farris *et al.* 2015
Human frontal cortex	Human alcoholic	microarray	106	Actin binding (GO:0003779), calcium ion binding (GO:0005509)	Ponomarev *et al.* 2012
Mouse frontal cortex	Chronic intermittent drinking	microarray	27	Transcription corepressor activity (GO: 0003714)	Osterndorff-Kahanek *et al.* 2013