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Human Astrocytes Exhibit Tumor Microenvironment-, Age-, and Sex-Related Transcriptomic Signatures

Abstract

Astrocytes are critical for the development and function of synapses. There are notable species differences between human astrocytes and commonly used animal models. Yet, it is unclear whether astrocytic genes involved in synaptic function are stable or exhibit dynamic changes associated with disease states and age in humans, which is a barrier in understanding human astrocyte biology and its potential involvement in neurological diseases. To better understand the properties of human astrocytes, we acutely purified astrocytes from the cerebral cortices of over 40 humans across various ages, sexes, and disease states. We performed RNA sequencing to generate transcriptomic profiles of these astrocytes and identified genes associated with these biological variables. We found that human astrocytes in tumor-surrounding regions downregulate genes involved in synaptic function and sensing of signals in the microenvironment, suggesting involvement of peri-tumor astrocytes in tumor-associated neural circuit dysfunction. In aging, we also found downregulation of synaptic regulators and upregulation of markers of cytokine signaling, while in maturation we identified changes in ionic transport with implications for calcium signaling. In addition, we identified subtle sexual dimorphism in human cortical astrocytes, which has implications for observed sex differences across many neurological disorders. Overall, genes involved in synaptic function exhibit dynamic changes in the peritumor microenvironment and aging. This data provides powerful new insights into human

astrocyte biology in several biologically relevant states, that will aid in generating novel testable hypotheses about homeostatic and reactive astrocytes in humans.

Significance Statement

Astrocytes are an abundant class of cells playing integral roles at synapses. Astrocyte dysfunction is implicated in a variety of human neurological diseases. Yet our knowledge of astrocytes is largely based on mouse studies. Direct knowledge of human astrocyte biology remains limited. Here, we present transcriptomic profiles of human cortical astrocytes, and we identified molecular differences associated with age, sex, and disease state. We found that peritumor and aging astrocytes downregulate genes involved in astrocyte-synapse interactions. These data provide necessary insight into human astrocyte biology that will improve our understanding of human disease.

Introduction

Astrocytes are a major component of the central nervous system. Though astrocytes were long regarded as passive support cells, studies of murine astrocytes found they have active functions that are critical for the development and function of synapses. For example, astrocyte-secreted factors powerfully induce the formation of functional synapses *in vivo* and *in vitro*, which otherwise largely fails to occur (Banker, 1980; Ullian et al., 2001; Christopherson et al., 2005; Kucukdereli et al., 2011; Allen et al., 2012; Singh et al., 2016; Farhy-Tselnicker et al., 2017; Krencik et al., 2017; Stogsdill et al., 2017; Blanco-Suarez et al., 2018). In addition to important roles in synapse formation, astrocytes contribute to engulfment and elimination of synapses in

development (Chung et al., 2013; Tasdemir-Yilmaz and Freeman, 2014; Vainchtein et al., 2018; Lee et al., 2020). Moreover, astrocytes maintain extracellular potassium levels (Kuffler et al., 1966; Olsen and Sontheimer, 2008; Kelley et al., 2018b) and participate in recycling neurotransmitters (Rothstein et al., 1996), thus maintaining homeostasis at synapses. There is now a variety of evidence showing that astrocytes help shape circuit functions and behavior (Nedergaard, 1994; Parpura et al., 1994; Halassa et al., 2009; Robel et al., 2015; Papouin et al., 2017; Dowling and Allen, 2018; Mu et al., 2019; Nagai et al., 2019; Huang et al., 2020). Various groups have demonstrated that altering intracellular astrocyte signaling *in vivo* can induce abnormal behavior or correct phenotypic behavior in disease models (Chen et al., 2016; Ma et al., 2016; Ng et al., 2016; Kelley et al., 2018b; Yu et al., 2018; Ung et al., 2020; Yu et al., 2020; Nagai et al., 2021). Astrocytes are molecularly and functionally heterogeneous, potentially adapting to diverse roles they play in different brain regions (Tsai et al., 2012; Glasgow et al., 2014; Molofsky et al., 2014; Chai et al., 2017; John Lin et al., 2017; Morel et al., 2017; Miller et al., 2019; Diaz-Castro et al., 2021).

Astrocyte biology faces an added layer of complexity considering their significant dynamism in response to insult or injury (Poskanzer and Molofsky, 2018). Astrocytes undergoing reactive astrogliosis in response to a challenge can display stark morphological changes, including hypertrophy and retraction of processes, in addition to a plethora of intracellular alterations (Sofroniew, 2020). Reactivity is observed in many neurological disorders including traumatic brain injury, stroke, epilepsy, and neurodegenerative diseases, and there appears to be disease-specific aspects to this

response (Beach et al., 1989; Panickar and Norenberg, 2005; Binder and Steinhauser, 2006; Burda et al., 2016; Liddelow et al., 2017; Yu et al., 2020).

Given their many and varied roles in the central nervous system (CNS), astrocytes are frequently implicated in neurological pathologies (Tian et al., 2005; Yamanaka et al., 2008; Ballas et al., 2009; Lioy et al., 2011; Molofsky et al., 2012; Krencik et al., 2015; Robel et al., 2015; Windrem et al., 2017; Laug et al., 2019). Recently, transcriptomic analysis of neuropsychiatric disease found astrocytic genes included in the gene signatures of autism spectrum disorder, bipolar disorder, and schizophrenia (Gandal et al., 2018a; Gandal et al., 2018b). Astrocyte reactivity is also prominent in several neurodegenerative diseases, including Alzheimer disease and Parkinson disease (Beach et al., 1989). In amyotrophic lateral sclerosis (ALS), reactive astrogliosis occurs around degenerating motor neurons, and this reactivity precedes motor neuron death in the rat SOD1 model of ALS (Howland et al., 2002). Further investigation found that overexpressing GLT1 in astrocytes improved neuronal survival and delayed disease onset in the mSOD1 mouse model of ALS (Guo et al., 2003).

Our understanding of human astrocytes significantly trails our knowledge of murine astrocytes (de Majo et al., 2020). Although the majority of major astrocyte functions appear to be shared between mice and humans, it is still imperative to narrow this gap in knowledge as researchers continue to identify important differences between these species in the CNS. Firstly, human astrocytes are notably larger with more elaborate branching than rodent astrocytes *in vivo* and *in vitro* (Oberheim et al., 2006; Oberheim et al., 2009; Zhang et al., 2016). At the molecular level, previous characterization of human and mouse astrocyte transcriptomes found many genes

specifically expressed by human astrocytes (Zhang et al., 2016; Li et al., 2021). At a functional level, behavioral differences were observed *in vivo* when human glial progenitors were transplanted into mice (Han et al., 2013). Animal studies have produced, and continue to produce, a remarkable body of knowledge concerning the many vital astrocytic functions in the brain (e.g. synapse formation, circuit functions). It is because animal models clearly demonstrate the importance of astrocyte biology that complementary analysis is also required in humans.

Given the importance of astrocytes in synaptic function, a key question that needs to be answered is whether genes involved in astrocyte-synapse interactions are stable or exhibit dynamic changes associated with disease states and age in humans. With the advent of improved astrocyte purification methodologies, we can now extract highly pure populations of human astrocytes by targeting the astrocytic cell surface protein HepaCAM using antibodies. By employing an immunopanning technique, we previously published human astrocyte transcriptomes of twelve human cortical samples between the ages of 8 and 63 years old (Zhang et al., 2016). In this study, we acutely purified samples from over 40 patients, which now include astrocytes from healthy and diseased brain regions. For the first time, we are also presenting samples under the age of 8, allowing for analysis of human astrocyte maturation, as well as other biological variables of interest. Here, we describe some of the first transcriptomic data of human astrocytes in the tumor microenvironment, as well as changes in astrocyte gene expression associated with maturation, aging, and sex. Among our findings, we see downregulation of synaptic genes in peritumor astrocytes as well as aging astrocytes.

Materials and Methods

HUMAN TISSUE

Human tissue was obtained with informed consent and the approval of the UCLA Institutional Review Board. We obtained tissue primarily from brain surgeries at UCLA to treat epilepsy and tumors, plus one postmortem sample with short postmortem interval (<18 hours). All samples were from the cerebral cortex, primarily from the temporal lobe (n = 31), but several samples came from the frontal (n = 9) or parietal lobes (n = 5), or the insula (n =2). Tissue was immersed in 4° C media (saline or Hibernate-A medium) before transfer to the lab for dissection and dissociation. Six samples were obtained from surgeries offsite, which were shipped overnight in 4°C media for dissection and dissociation in the lab. The final cohort includes 7 peritumor samples, 30 epilepsy samples, and 12 controls totaling 49 samples from 41 patients (see Figure 1-1). No affected samples came from the same patient that provided a control sample.

VERTEBRATE ANIMALS

All mouse experimental procedures were performed with approval from the UCLA Chancellor's Animal Research Committee in compliance with all federal and state laws and policies. For *in situ* hybridization of mouse brain tissue, we used mice at postnatal day 71 (2 females, 1 male) from a C57/BL6 FVB mixed background.

PURIFICATION OF HUMAN ASTROCYTES

Human astrocytes were purified using immunopanning, as described in (Zhang et al., 2016). Briefly, we dissected gray matter from cortical tissue and enzymatically digested the tissue with papain (20 units/mL) for 80 minutes at 34.5°C. We then rinsed

194 the tissue in a protease inhibitor solution. We gently triturated the tissue to generate a
195 single-cell suspension, and we passed the cells over a series of plastic petri dishes that
196 were precoated with antibody. The cell suspension was incubated at room temperature
197 for 10-15 minutes on each dish, which contained anti-CD45 antibody (BD Pharmingen
198 550539) to deplete microglia, O4 hybridoma to deplete oligodendrocyte precursor cells,
199 GalC hybridoma to deplete oligodendrocytes and myelin, or anti-Thy1 (BD Pharmingen
200 550402) to deplete neurons. Finally, the astrocyte-enriched cell suspension was
201 incubated for 20 minutes at room temperature on a dish coated with anti-HepaCAM
202 antibody (R&D Systems MAB4108) to bind astrocytes. We washed the bound
203 astrocytes with PBS to remove contaminants, and we immediately harvested RNA by
204 applying 700 μL of TRIzol solution (Thermo Fisher Scientific 15596018). TRIzol solution
205 was then flash frozen in liquid nitrogen and stored at -80°C to await RNA purification.
206 The total time from receiving tissue to storing astrocyte RNA took approximately 4
207 hours.

208 RNA LIBRARY CONSTRUCTION AND SEQUENCING

209 RNA was extracted from frozen TRIzol using the miRNeasy kit (Qiagen 217004),
210 according to the manufacturer's protocol. We checked RNA quality with the 2200
211 TapeStation System (Agilent G2964AA) and the RNA high sensitivity assay (Agilent
212 5067-5579). All RNA integrity numbers were ≥ 6.5, except RNA samples that were not
213 concentrated enough for accurate measurement. We then used the Nugen Ovation
214 RNAseq System V2 (Nugen 7102-32) to generate cDNA libraries, and we fragmented
215 the cDNA using a Covaris S220 focused-ultrasonicator (Covaris 500217). We amplified
216 and prepared these libraries for sequencing with the NEB Next Ultra RNA Library Prep

Kit (New England Biolabs E7530S) and NEBNext multiplex oligos for Illumina (NEB E7335S). We performed end repair and adapter ligation, and we amplified the final libraries using 10 cycles of PCR. The sequencing libraries were verified using the TapeStation D1000 assay (Agilent 5067-5582). Indexed libraries were pooled and sequenced using the Illumina HighSeq 4000 sequencer and obtained 18.9 million ± 1.6 million (mean ± SEM) single end, 50 bp reads across four batches.

READ ALIGNMENT AND QUANTIFICATION

We mapped reads using the STAR package (Dobin et al., 2013) and genome assembly GRCh38 (Ensembl, release 91), and obtained 77.0% ± 5.8% (mean ± standard deviation) uniquely aligned reads in all samples. Reads were counted using the HTSeq package (Anders et al., 2015), and reads were subsequently quantified by Reads Per Kilobase of transcript per Million mapped reads (RPKM) using EdgeR-limma packages in R (Figure 1-2).

DIFFERENTIAL GENE EXPRESSION ANALYSIS OF DISEASE AND SEX

We analyzed differential gene expression of disease and sex using the DESeq2 package in R, see Figures 2-1, 4-1, & 6-1 (Love et al., 2014). In this analysis, we included all samples and used the following command to create our linear model: ~ factor(Diagnosis) + Age + factor(Sex) + MicroPC + Batch, where Diagnosis was a factor with values [Control, Peritumor, Epilepsy], Age was a numeric value in years, Sex was a factor with values [Male, Female], and MicroPC was numeric value measuring microglia contamination. To calculate the “microPC”, we first determined the gene expression of microglia-specific genes (>10x enriched in microglia vs. astrocytes) in all samples, using the data from (Zhang et al., 2016). Then, we performed principal component analysis

(PCA) using the prcomp function in R on the scaled microglia gene expression, and we took the first principal component (PC1) as a summary measure of microglial gene expression in each sample. Results were cross-checked with leave-one-out validation, where the analysis was reiterated with the removal of one sample in each round for a total of 49 iterations. To determine how robust the analysis is to the effect of brain region, we reran the analysis using only temporal lobe tissue, see results in Figure 2-2. We further assessed the effect of brain region by performing differential gene expression analysis of region in samples from temporal lobe and frontal lobe (peritumor samples excluded, see Figure 2-3). We used the linear model: ~ factor(Region) + Age + factor(Sex) + MicroPC + Batch.

ANALYSIS OF HUMAN AGING GENES

To identify genes associated with aging astrocytes, we began with genes significantly associated with Age in the DESeq2 analysis of disease and sex, as described above. In order to separate genes that change in aging (i.e. later life) from genes that change in development (early life), we categorized samples in 3 categories, excluding peritumor samples: 0-21 years old (n = 34), 21-50 years old (n = 3), and 50+ years old (n = 5). We compared younger adults (21-50) to older adults (50+), and we narrowed the gene list to those with an average expression > 0.01 RPKM and 1.5-fold differences in the average expression between groups. This yielded a list of 394 gene entries, 277 of which were protein-coding, see Figure 5-1.

ANALYSIS OF HUMAN ASTROCYTE MATURATION

We analyzed differential gene expression across astrocyte maturation using a two-step process. First, we performed DESeq2 on samples ≤ 21 years old, excluding

peritumor samples (n = 35). Model: ~ factor(Diagnosis) + Age + factor(Sex) + MicroPC + OligPC + factor(Batch). The “oligPC” was calculated in the same manner as the microPC using gene expression of oligodendrocyte-specific genes identified using data from (Zhang et al., 2016). While it is necessary to use cell-type specific filters to exclude contamination, excluding oligodendrocyte-enriched genes may obscure potential lineage relationships between astrocytes and oligodendrocytes (Weng et al., 2019). Thus, we only used these filters for differential expression analysis and included the unfiltered complete dataset in Figure 1-2. Finally, we filtered out genes that were 10x enriched in human neurons over human astrocytes, using data from (Zhang et al., 2016). This yielded 1,463 gene entries significantly associated with maturation. We also performed a leave-one-out reanalysis to assess the robustness of the maturation gene expression findings, see Figure 2-2.

The DESeq2 analysis included samples as young as 7 months old, but we could capture changes from earlier stages in development by using our recently published transcriptomic profiles of fetal human astrocytes. We compared 4 fetal samples with our near-adult human samples between 13-21 years old (excluding peritumor, n = 11). For each sample, gene expression was converted to a percentile, where the most highly expressed gene = 1 and the least expressed gene = 0. Next, we conducted parallel t-tests with a Benjamini-Hochberg correction for multiple tests, performed in GraphPad Prism v8. This generated over 10,000 hits. Finally, we combined the two gene lists using an equal number of genes from each list, (i.e. we filtered results from the second analysis to the 1,463 top hits by p-value to match the first analysis), producing a final list of 2,749 genes associated with human astrocyte maturation, see Figure 4-2.

286 ANALYSIS OF DISEASE-ASSOCIATED GENES

287 We tested whether peritumor or maturation gene signatures were enriched with
288 disease-associated genes. We obtained gene lists for various neurological diseases
289 from a curated database, Phenopedia (Yu et al., 2010). Using these gene lists, we
290 performed Fisher's exact tests on differentially expressed genes in peritumor astrocytes
291 and corrected for multiple comparisons using the Benjamini-Hochberg method. The
292 results are in Figure 2-4.

293 MOUSE AGING GENES AND HUMAN COMPARISON

294 We accessed mouse astrocyte RNAseq data from the BioProject database
295 (www.ncbi.nlm.nih.gov/bioproject), accession no. PRJNA417856 (Clarke et al., 2018).
296 We downloaded FASTQ files of sequenced cortical astrocytes at ages postnatal day 7
297 (n = 3), 10 weeks (n = 3), and 2 years (n = 2). Reads were aligned with STAR 2.6.0 and
298 genome assembly GRCm38 (Ensembl release 100). All samples had >70% uniquely
299 mapped reads. We used HTSeq to generate counts, and then we determined
300 differential gene expression between two ages (day 7 vs. 10 weeks & 10 weeks vs. 2
301 years) using DESeq2. Model = ~ factor(Age), where Age is a binary factor.

302 We used the STRING database (string-db.org) to find and visualize protein-
303 protein interactions in human and mouse aging-associated genes. For mouse aging
304 genes, we combined the results of three studies of mouse astrocytes (Boisvert et al.,
305 2018; Clarke et al., 2018; Ximerakis et al., 2019).

306 GENE ONTOLOGY (GO)

307 To identify patterns in our various differentially expressed gene lists, we used the
308 online platform Metascape (metascape.org, (Zhou et al., 2019)). We input all protein-

coding genes from our gene sets, and conducted an enrichment analysis with default settings, with the following adjustments: reference data sets were limited to GO datasets (Molecular Functions, Biological Processes, and Cellular Components), and we defined a list of background genes (i.e. the set of genes expressed in astrocytes that are included in this analysis) as follows: for human analyses, the background gene list consisted of all genes with expression ≥0.05 RPKM in 30+% of our samples. For mouse analyses, the background list consisted of genes with ≥0.05 RPKM in 30+% in the mouse samples from (Clarke et al., 2018).

We used Fisher's exact test to individually test differentially expressed genes in peritumor astrocytes for enrichment of GO gene sets that were reportedly found in tumor-core astrocytes (Heiland et al., 2019): Positive regulation of receptor signaling via JAK/STAT (GO:0046427/GO:2000366), Negative regulation of receptor signaling via JAK/STAT (GO:0046426, GO:2000365), Interleukin-6 mediated signaling pathway (GO:0070102), and Response to interferon gamma (GO:0034341).

Pro- and anti-inflammatory genes were identified using GO annotations for positive regulation of inflammatory response (GO:0050729) and negative regulation of inflammatory response (GO:0050728) (Ashburner et al., 2000; Carbon et al., 2009; Gene Ontology, 2021).

RNASCOPE IN SITU HYBRIDIZATION

RNAScope in situ hybridization was performed on fresh frozen human tissue collected from surgeries and fresh frozen mouse tissue harvested after a 10-minute transcardial perfusion of phosphate buffered saline. Tissues were embedded in OCT compound (Fisher Scientific 23-730-571) and cut into 20-30 μm-thick sections.

RNAScope Multiplex Fluorescent V2 Assay (ACDBio 323100) was performed per manufacturer's protocols. Probes were purchased from ACDBio for human and mouse *SLC1A3* and *TLR4*. Images were captured using the Zeiss LSM 800 confocal microscope using at equal power and exposure across all samples stained with the same set of probes. Photoshop v22.1 was used to enhance brightness for publication.

STATISTICAL ANALYSES

Differential gene expression was analyzed using DESeq2. Enrichment of GO terms was analyzed using Metascape. Enrichment of disease-associated genes and peritumor genes was analyzed with Fisher's exact test in R using fisher.text(). All analyses are detailed under the corresponding sections above.

DATA DEPOSITION

All human RNAseq data is deposited on the Gene Expression Omnibus and will be made public before publication. Reviewers can access the data at GSE168375 using token *wfevaywutlgjzqt*.

Results

Purification of Human Cortical Astrocytes in Health and Disease

We obtained human cortical tissue from patients undergoing neurological surgery. Our final analysis includes 49 samples from 41 patients, with ages ranging from 7 months to 65 years old. Twelve of these samples were taken from normal regions of cortex that were resected *en route* to deep-seated pathologies. Nine of these normal specimens were obtained during surgery for deep epileptogenic foci. In these cases, we confirmed that normal specimens taken did not include abnormal-appearing

355 tissue using MRI-registered frameless stereotaxy, did not show abnormal interictal
356 activity on invasive electrode recordings, and did not demonstrate abnormal
357 histopathologic findings. Two other cases from which normal specimens were collected
358 were encapsulated, benign tumors, where normal-appearing brain tissue based on MRI-
359 registered frameless stereotaxy was collected outside a 1 cm margin from the tumor.
360 Finally, one normal tissue specimen was obtained from a patient at the time of death
361 from a cardiac condition, without other intracranial pathologies. A similar cohort of
362 control human astrocytes were sequenced and analyzed previously, where the authors
363 characterized the baseline characteristics of the human astrocyte transcriptome (Zhang
364 et al., 2016); of note, we used remaining RNA from 7 of these samples in this study.
365 From this point forward, we refer to normal brain tissue samples from these sources as
366 “controls”.

367 In the current study, we sought to compare normal astrocytes against disease-
368 affected astrocytes. Affected samples included in the analysis fall into two categories of
369 diagnosis: 1) 30 included brain tissue showing epileptiform activity on intra-operative
370 electrode recordings surrounding resected focal cortical dysplasia (FCD), a
371 developmental form of epilepsy; and 2) 7 included brain tissue immediately surrounding
372 a brain tumor (including glioblastoma, low grade glioma, and metastatic breast cancer)
373 based on MRI and visual inspection at the time of surgery (referred to here as
374 “peritumor”). Specific pathologic diagnoses are presented in Figure 1-1.

375 We purified human cortical astrocytes using immunopanning (Fig 1A). We
376 removed white matter and generated a single cell suspension with mechanical and
377 enzymatic digestion. The single cell suspension passes over antibody-coated Petri

dishes that bind contaminating cell types with cell type specific antigens. This immunopanning protocol uses anti-CD45 antibodies to pull down myeloid cells (i.e. microglia and macrophages), anti-GalC hybridoma cell supernatant to pull down oligodendrocytes and myelin debris, anti-O4 hybridoma cell supernatant to bind oligodendrocyte precursor cells (OPCs), and anti-Thy1 antibodies to bind neurons. Finally, the enriched suspension passes onto a dish coated in anti-HepaCAM antibodies, a cell-surface glycoprotein enriched in astrocytes. We harvested the astrocyte RNA from this dish and used it to perform RNA sequencing (RNAseq). The sequenced samples show high expression of astrocyte marker genes such as GFAP and SLC1A2, and extremely low expression of neuronal, myeloid, and endothelial genes. There are only slight traces of some oligodendrocyte-lineage marker genes (Fig 1B). Using immunopanning, we obtained RNA highly enriched for human cortical astrocytes in healthy and diseased states for bulk RNAseq (Figure 1-2).

Glioblastoma cells infiltrate surrounding brain tissue. To determine whether our purified astrocytes from the peritumor regions are bona fide astrocytes or infiltrating glioblastoma cells, we examined the expression of a glioblastoma marker gene AVIL (Xie et al., 2020) and did not detect expression in our peritumor astrocyte samples (Figure 1-2). Furthermore, we compared gene expression of astrocytes surrounding glioblastoma tissue (infiltrating) and astrocytes surrounding low grade glioma or metastatic tumors (non-infiltrating). Peritumor astrocyte signature genes described below are not more highly expressed by glioblastoma-surrounding astrocytes than non-infiltrating tumor-surrounding astrocytes (Figure 1-2). Although we cannot rule out contamination from a small number of infiltrating glioblastoma cells, the gene signatures

of peritumor astrocytes are likely from predominantly bona fide astrocytes instead of infiltrating cells.

Peritumor astrocytes downregulate genes involved in synaptic function and genes encoding cell surface receptors

After sequencing RNA from human astrocytes in FCD and peritumor regions, we employed differential gene expression analysis to examine their molecular signatures using the analysis package DESeq2 in R. We used a linear model that included variables for diagnosis, sequencing batch, age, and sex. To control for potential variance from low level microglial contamination, we included an additional variable that quantified microglial marker gene expression by performing principal components analysis (PCA) on microglial marker gene expression in our dataset. Including the first principal component in the linear model effectively eliminated significant differential expression of microglial genes.

First, we examined the effect of the brain tumor microenvironment on astrocyte gene expression. Brain tumors drive considerable changes in the surrounding microenvironment, and astrocytes themselves are known to readily change state in response to a variety of stimuli (Raore et al., 2011; Quail and Joyce, 2017). However, transcriptomic changes of peritumor astrocytes in humans have not been reported, to the best of our knowledge. Using our DESeq2 pipeline, we found 3,131 genes differentially expressed in peritumor astrocytes, providing a new resource for elucidating astrocytic changes in the brain tumor microenvironment (Figure 2-1). The vast majority of these findings were robust under leave-one-out validation (Figure 2-2) where the analysis was reiterated with the removal of one sample in each round.

Synaptic gene signatures in peritumor astrocytes

Many genes related to synaptic function are downregulated in the peritumor region (Fig. 2A-D), such as the glutamate transporters SLC1A2 and SLC1A3, which take up the excitatory neurotransmitter glutamate from the synaptic cleft and maintains excitation-inhibition balance in the brain (Yang et al., 2009). SLC1A2-knockout mice suffer from epileptic seizures and die prematurely (Tanaka et al., 1997). Also downregulated is the gene KCNJ10 encoding the inwardly rectifying potassium channel Kir4.1, which takes up potassium from the extracellular space after neuronal firing, buffers potassium levels in the astrocytic syncytium, and modulates neuronal excitability (Kofuji and Newman, 2004). Patients with mutations in the KCNJ10 gene suffer from seizure disorders (Reichold et al., 2010). A large proportion of human patients with brain tumors also suffer from epileptic seizures, which reduce their quality of life and sometimes cause death (Englot et al., 2016). Our observation of strong reductions of SLC1A2, SLC1A3, and KCNJ10 in peritumor astrocytes suggest potential involvement of astrocytes in tumor-associated seizures and reveal astrocytes as novel potential targets for treating these seizures. A study in a rat model of glioma supports the feasibility of this approach (Sattler et al., 2013). Furthermore, astrocyte secreted molecules that regulate synapse formation and maturation, SPARCL1, CHRDL1, and GPC5 are also downregulated in peritumor astrocytes (Fig. 2B, 2D). Together, these findings suggest decreased support of synapses in the tumor microenvironment that could contribute to dysregulation of synaptic activity and emergent clinical symptoms like seizures. Upregulated genes in peritumor astrocytes include the reactivity-

associated genes GFAP, TIMP1 and VIM, suggesting that astrocytes in the peritumor microenvironment are reactive in humans.

Cell surface receptors in peritumor astrocytes

Cell surface receptors mediate sensing of signals in the microenvironment. To examine the expression of genes encoding receptors located on the plasma membrane by peritumor astrocytes, we used a list of experimentally validated and in silico predicted genes encoding cell surface receptors (Bausch-Fluck et al., 2018). We determined the complete set of transmembrane receptors present in human astrocytes (Figure 2-5). We found 157 transmembrane receptor genes expressed by human astrocytes (average RPKM >1). Of these genes, peritumor astrocytes downregulate one fourth (40/157) and upregulate only 3/157, suggesting that peritumor astrocytes are impaired in their ability to receive and respond to external cues (Fig 2G).

Synaptic genes and cell surface receptors downregulated in peritumor astrocytes are associated with psychiatric disease risk

Having discovered genes up- and down-regulated in peritumor astrocytes, we next assessed whether these genes are involved in other diseases. We used the Phenopedia dataset for genes associated with various neurological diseases (Yu et al., 2010) and tested whether genes up and downregulated in peritumor astrocytes are significantly enriched in genes associated with the risk of each neurological disorder using Fisher's exact test followed by correction for multiple comparisons (Methods). Genes that were downregulated in peritumor astrocytes were enriched for genes with genetic links to several neurological diseases (Figure 2-4). Interestingly, these associations existed primarily among psychiatric disorders (bipolar disorder,

schizophrenia, mood disorders, obsessive-compulsive disorder, depressive disorder, and anxiety disorder). In addition, genes downregulated in peritumor astrocytes also significantly overlap with Alzheimer's disease risk genes, but not with other neurodegenerative disorders (Parkinson Disease, ALS, Frontotemporal Dementia, and Huntington Disease).

Next, we asked whether synaptic and receptor genes contributed to the association between peritumor genes and genes associated with psychiatric disease. We tested whether synaptic genes and receptor genes downregulated in peritumor astrocytes were enriched in genes associated with risks of the aforementioned six psychiatric disorders and Alzheimer's Disease. We found that both synaptic and receptor genes downregulated in peritumor astrocytes are enriched in genes associated with all six psychiatric disorders, whereas Alzheimer's Disease only associated with synaptic genes (Figure 2-4). We conclude that peritumor astrocytes downregulate receptor and synaptic genes that are associated with psychiatric disease risk, highlighting the potential importance of this core group of astrocytic genes in neural circuit development and function.

Gene ontology of peritumor astrocyte signatures

To find larger patterns in the data, we performed pathway analysis with the online tool Metascape to identify gene ontology (GO) terms that are enriched in our gene lists (Figure 2-6). Among upregulated genes, we found highly significant enrichment of GO terms related to cell cycle and protein translation, consistent with the presence of proliferative reactive astrocytes in the peritumor region. Meanwhile, downregulated genes were enriched for an array of functional terms related to synaptic function as well

as cation transport (Fig 2C), further supporting the hypothesis that peritumor astrocytes are defective in supporting or participating in normal synaptic signaling. Interestingly, both up- and downregulated gene lists are enriched for extracellular matrix genes (Fig 2E), which is notable considering the importance of extracellular remodeling in tumor expansion and migration as well as synaptic plasticity (Nguyen et al., 2020; Winkler et al., 2020). Broadly, we see peritumor astrocytes alter extracellular matrix gene expression while upregulating genes necessary for cell division and translation, and downregulating expression of genes related to synaptic transmission and ionic homeostasis, revealing potential contribution of astrocytes to neural circuit dysfunction associated with brain tumors.

Peritumor astrocytes differ from tumor-core astrocytes

To assess whether peritumor astrocytes resemble tumor-core associated astrocytes, we compared our peritumor astrocyte dataset to a previously published tumor-core associated astrocyte dataset (Heiland et al., 2019). Heiland and colleagues reported that tumor-core astrocytes contribute to an immunosuppressive environment in part due to increased JAK/STAT pathway activation. Among peritumor astrocytes, however, we did not find significant enrichment of the JAK/STAT pathway among differentially expressed genes. We also failed to find enrichment of interferon gamma response genes and interleukin-6 response genes, which were also identified by Heiland et al. When we examined pro- and anti-inflammatory genes that are differentially expressed in peritumor astrocytes, we found that the majority of pro-inflammatory genes were upregulated (10/11, Fig 2F), and the majority of anti-inflammatory genes were downregulated (16/26, Fig 2F). Taken together, these findings

515 suggest a contrast between an anti-inflammatory signature of tumor-core astrocytes,
516 and an at least partly pro-inflammatory signature of peritumor astrocytes.

517 **Peritumor astrocytes attenuate core astrocytic genes**

518 To assess whether peritumor astrocytes may lose normal astrocyte function, we
519 examined genes highly expressed by astrocytes and found that peritumor astrocytes
520 downregulate several known markers of mature astrocytes (Fig. 2D). Furthermore, we
521 examined a list of the top 50 astrocyte markers identified in a meta-analysis of human
522 gene expression (Kelley et al., 2018a). Peritumor astrocytes significantly
523 downregulated 41/50 genes, with 50/50 trending downward (Fig 2H). These results are
524 consistent with a possible loss of normal astrocytic functions in the peritumor
525 microenvironment.

526 **TLR4 is expressed by human astrocytes and not mouse astrocytes**

527 To assess changes of peritumor astrocytes using an orthogonal approach, we
528 performed *in situ* hybridization with RNAscope. Based on RNAseq, we found that toll-
529 like receptor 4 (TLR4) was downregulated in peritumor astrocytes. TLR4 is a member
530 of the toll-like receptor family of pattern recognition receptors, which recognize
531 pathogen-associated molecular patterns and initiate innate immune responses (Park
532 and Lee, 2013). Specifically, TLR4 encodes a transmembrane protein that binds
533 bacterial lipopolysaccharides and triggers innate immune responses to bacterial
534 infection. Moreover, TLR4 also recognizes endogenous ligands, such as heat shock
535 proteins and lipoproteins from damaged cells (Vaure and Liu, 2014). Previous studies
536 in mice found that TLR4 was highly enriched in myeloid cells, such as microglia in the
537 brain, but we detected significant mRNA expression of TLR4 in astrocytes in humans

(this study and Zhang et al. 2016). To directly compare TLR4 expression between species, we performed RNAscope *in situ* hybridization on human and mouse cortical tissue. Whereas a small minority of mouse astrocytes expressed TLR4 (7%, 4/58 cells, n = 3), a majority of human astrocytes showed expression of TLR4 (62%, 18/29 cells, n = 2, Fig. 3A, B). Even more strikingly, $TLR4^+$ astrocytes in humans contain larger numbers of $TLR4^+$ mRNA puncta than did $TLR4^+$ astrocytes in mice (Fig. 3C). Though the small sample size does not provide a definitive conclusion, we find evidence of human-specific expression of TLR4 in astrocytes that corroborates findings from RNAseq, suggesting enhanced ability of human astrocytes to detect TLR4 ligands, such as signals from bacteria and damaged cells, compared with mouse astrocytes. Furthermore, this mode of signaling is notably reduced in peritumor astrocytes.

The transcriptomes of astrocytes in FCD and control do not differ

Next, we examined the transcriptional signature of FCD. FCD is characterized by abnormalities in neuronal migration during development. Patients with FCD display abnormal radial and/or tangential lamination in a local region of cerebral cortex. More severe cases include dysmorphic neurons, and others also develop large and often multi-nucleated cells called balloon cells (Gaitanis and Donahue, 2013). Our DESeq2 analysis found only 24 protein-coding genes significantly associated with epilepsy, and all but one of those genes (SCN4B) had low expression (an average expression <1 RPKM; see Figure 4-1). Samples from FCD patients did not separate from controls in PCA, hierarchical clustering, or expression of reactive astrocyte markers (data not shown). Therefore, astrocytes in FCD in humans do not exhibit robust gene expression changes. However, we cannot exclude the possibility that a small subpopulation of

561 astrocytes immediately adjacent to FCD lesions have gene expression changes that
562 were undetectable at the population level. Given the lack of robust differences between
563 control samples and epilepsy samples, they were used along with control samples in
564 subsequent analyses.

565 **Genes involved in ion transport and calcium signaling change with astrocyte**
566 **maturation**

567 Developing and mature brains have drastically different cognitive capacities,
568 learning potentials, and susceptibilities to disease. Astrocytes are critical for the
569 development of neural circuits, maintenance of homeostasis in adults, and responses
570 and repair in neurological diseases (Huang et al., 2004; Sofroniew and Vinters, 2010;
571 Chung et al., 2013). However, cellular and molecular changes of astrocytes during brain
572 development and maturation in humans are unclear. Previous studies have performed
573 transcriptome profiling of a small number of samples of human astrocytes from fetuses,
574 children ≥8 years old, and adults (Zhang et al., 2016). The gene expression profiles of
575 astrocytes during an important period of development, birth to 8 years, remain unknown.
576 Therefore, molecular knowledge of astrocyte development and maturation in humans is
577 incomplete. Here, we recruited patients throughout development and adulthood (n=16
578 samples between 0-5 years old; 6 samples between 6-10 years old; 12 samples
579 between 11-17 years old; and 8 adult samples, excluding peritumor; Figure 1-1),
580 purified astrocytes, and performed RNA-seq. We analyzed maturation-associated genes
581 based on our new RNAseq data using a linear model (DESeq2 R package, detailed in
582 Methods) and also included fetal data from our previous study (Li et al., 2021) after
583 normalizing data from two studies using percentiles (detailed in Methods). Our final

584 results find 1509 upregulated genes and 1240 downregulated genes associated with
585 astrocyte maturation across human development (Fig. 4C, Figure 4-2). Major astrocyte
586 markers were not highly expressed in fetal astrocytes, but even the youngest postnatal
587 samples (7 months) showed high expression of most markers. However, postnatal
588 gene expression continued to evolve. Notably, top genes changing in the postnatal
589 epoch displayed a shift in expression starting around 8 years old (Fig 4A).

590 To assess the changes associated with astrocyte maturation, we analyzed gene
591 ontology of up- and downregulated astrocyte maturation genes using Metascape (see
592 Figure 4-3). Upregulated genes showed significant enrichment for several GO terms
593 related to ion homeostasis, as well as lipid metabolism (Fig 4B). Many of the genes
594 pertaining to ion transport are specifically related to calcium transport and signaling (Fig
595 4D), which is intriguing given the importance of calcium as a signaling molecule,
596 particularly in astrocytes. Therefore, immature and mature astrocytes may differ in ion
597 transport and calcium signaling, thus altering many downstream signaling pathways that
598 affect both astrocytes and surrounding neurons in development. Downregulated GO
599 terms are almost entirely related to cell cycle and cell division, which is expected to
600 decline throughout development (Fig 4B). We also observe a general upward trend for
601 several astrocyte marker genes that we would also expect to increase across
602 maturation (Fig 4D).

603 Characterizing the maturation of human astrocytes directly contributes to our
604 understanding of human astrocyte biology and brain development, but most existing
605 knowledge is derived from animal studies. Therefore, it is vital to determine which
606 aspects of human biology are recapitulated by animal models and which are wholly

607 unique. We compared our analysis of human astrocyte maturation with a published
608 study that measured mouse astrocyte gene expression across several ages (Clarke et
609 al., 2018). We compared astrocyte gene expression data from mice at an early
610 developmental age, postnatal day 7 (P7), and a young adult timepoint, 10 weeks, and
611 identified 4,417 genes associated with mouse astrocyte maturation. Most of the mouse
612 and human astrocyte maturation genes we found were not direct orthologues (Fig 4C),
613 and yet both gene lists had remarkably similar patterns based on gene ontology (Fig
614 4B). Both species downregulate cell division and upregulate ionic transport and calcium
615 signaling genes across maturation. The conservation of these patterns in evolution
616 suggests the importance of these astrocytic developmental changes. Based on this
617 analysis, we find that human and mouse astrocytes share broad outcomes in maturation
618 but differences concerning the exact pattern of molecular changes. While mouse
619 models may not recapitulate every aspect of human biology, our data suggest that the
620 maturation of astrocytes in humans can be accurately modeled in mice.

621 **Human astrocytes downregulate genes involved in synaptic function in aging**

622 Aging is associated with increased risk of cognitive decline and increased
623 susceptibility to neurodegeneration and stroke. Astrocytes are important for maintaining
624 homeostasis of the brain. Yet, aging-associated changes in human astrocytes are
625 largely unknown. Characterizing these changes is the first step in elucidating potential
626 involvement of astrocytes in aging-associated cognitive decline and neurodegeneration
627 and developing astrocyte-targeted treatments. To identify genes with aging-associated
628 expression, we began with genes significantly associated with age in the DESeq2
629 analysis of all samples that we also used to identify disease-related genes. To identify

genes specifically associated with aging (changes after completing maturation) rather than general age (changes across the entire lifespan), we grouped samples into groups by age: 0-20 years old; 21-50 years old; and 50+ years old. From our list of age-associated genes, we extracted genes with average expression >1.5x higher or lower in the 50+ group vs. the 21-50 group. We further filtered by minimum RPKM level of 0.01 to exclude lowly expressed genes. Thus, we identified 394 (277 protein-coding) genes significantly associated with aging (Figure 5-1).

As in peritumor astrocytes, we find decreased expression of genes mediating astrocyte-synaptic interactions in older astrocytes (Fig. 5A). Most notably, there is a reduction of SLC1A3, a glutamate transporter that clears glutamate from the extracellular space. Under normal conditions, SLC1A3 is a highly expressed core marker of adult astrocytes (Zhang et al., 2016). There is also a decline in CHRDL1, which codes for an astrocyte-secreted factor that drives synapse maturation (Blanco-Suarez et al., 2018). Aging astrocytes also have lower expression of two genes coding for glycoproteins found in the extracellular matrix. The first, CSPG5, shapes neurite growth and localizes around GABAergic and glutamatergic synaptic terminals (Pinter et al., 2020), while the second, OLMF1, binds synaptic proteins such as synaptophysin and AMPA receptors (Nakaya et al., 2013). Declining expression of synaptic genes raises important questions about astrocytic roles in age-related cognitive decline and neurological disease.

Subjects over 50 show additional decreases in genes associated with energy metabolism (Fig. 5A). These include genes involved in mitochondrial generation of ATP such as ATP5A1, an ATP-synthase subunit, MRPL35, a mitochondrial ribosomal

component, and SLC25A5, a transporter that carries ATP out of the mitochondria. We also observe lower expression of the glycolytic enzyme PGAM1, and SLC13A5, a citrate transporter. Astrocytes are known to secrete citrate into the extracellular space where citrate has the ability to chelate calcium and magnesium ions, which are important to neuronal NMDA signaling (Westergaard et al., 2017). Together, these gene expression changes are consistent with decreased production of ATP in aging human astrocytes.

Lastly, we also observe an increase of several genes involved in cytokine signaling and senescence. These include the cytokines LIF, IL6, and CCL2, as well as cytokine regulator SOCS3 (Fig. 5B), which are also found in reactive astrocytes in mice (Zamanian et al., 2012). Therefore, these changes suggest that aging astrocytes may exhibit differences in interactions with neuronal synapses, altered energy metabolism, and increased cytokine signaling.

To assess the similarity between human and mouse astrocyte aging, we returned to the mouse aging dataset from (Clarke et al., 2018). They reported a list of 58 age-associated genes in mouse cortical astrocytes. We wanted to determine whether mice recapitulated human changes related to metabolism, synapses, cytokines and senescence. Due to the short length of the mouse gene list, we sought to identify trends in the whole dataset. To do so, we first chose relevant GO terms that captured the trends we observed in humans (“ATP metabolic process”, “synapse”, “cytokine-mediated signaling pathway”, and “cellular senescence”). Using these gene lists, we plotted differences in gene expression of all genes between the ten-week-old and two-year-old mice (Fig. 5C). There is a prominent right skew in cytokine signaling genes,

and we observe a slight left shift in ATP metabolism genes, suggesting mouse astrocytes also show signs of upregulating cytokine signaling genes while downregulating metabolic genes in old age. The distributions for synaptic and senescence genes were highly symmetrical, suggesting no broad trends associated with age. However, mouse astrocytes could show important changes in smaller subsets of synaptic or senescence genes upon further analysis. In total, we see evidence that mouse astrocytes share metabolic and cytokine features of human astrocyte aging.

Next, we examined changes in protein-protein interactions in aging astrocytes from humans and mice using the online tool STRING (string-db.org). We combined differentially expressed genes from three different studies of aging mouse astrocytes (Boisvert et al., 2018; Clarke et al., 2018; Ximerakis et al., 2019). Again, we see downregulation of mitochondrial ATP metabolism genes in both species, and both species also upregulated genes involved in inflammation such as cytokine signaling, the complement pathway, and interferon response pathway (Fig. 5D). Furthermore, in aging human astrocytes, inositol triphosphate-calcium signaling pathway and senescence genes are upregulated whereas growth factor signaling genes are downregulated. In aging mouse astrocytes, mRNA splicing genes are upregulated and ribosomal translation genes are downregulated.

Region-specific gene signatures in astrocytes

Studies in mice have found regional differences in gene expression, but little is known about potential differences across the human brain. We compared expression in temporal lobe (n = 27) and frontal lobe samples (n = 8) and found 64 differentially expressed genes. Interestingly, Sema3a, a gene expressed at higher levels in ventral

than in dorsal spinal cord astrocytes and repels axons from ventral spinal cords in mice (Molofsky et al., 2014) is expressed at higher levels in the frontal lobe than in the temporal lobe in humans, suggesting potentially conserved roles of astrocytic Sema3a in region specific axon guidance.

Subtle sexual dimorphism in astrocyte gene signatures

Female and male brains differ in their susceptibility to neurological disorders. For example, intellectual disability, autism spectrum disorder and Parkinson disease are more prevalent in men than in women (Werling and Geschwind, 2013; Gillies et al., 2014), whereas multiple sclerosis, Alzheimer disease, and anxiety disorder are more prevalent in women than in men (Seshadri et al., 1997; McLean et al., 2011; Westerlind et al., 2014). The cellular and molecular mechanisms underlying sexually dimorphic susceptibility to neurological and psychiatric disorders are largely unknown. While understanding of sexually dimorphic properties of other brain cells, particularly microglia, is increasing, sexual dimorphism in human astrocytes has not yet been reported. In our differential gene expression analysis of all 49 samples, we found 105 genes (40 protein coding) with expression levels significantly associated with sex (Fig. 6, Figure 6-1). This gene list represents the first evidence of sexual dimorphism in human cortical astrocytes, to the best of our knowledge. Some of these genes are transcription factors (POU5F1B, HOXC10) or epigenetic factors, which may globally regulate gene expression. For example, the lysine demethylases KDM6A and KDM5C located on the X-chromosome are expressed at higher levels by female than male astrocytes. Females have two copies of X-chromosome genes and males only have one copy. Most X-chromosome genes are subjected to X-inactivation in females, where only

one copy of the gene is expressed, thus making expression levels comparable in males and females. Interestingly, KDM6A and KDM5C expression in female astrocytes are approximately twice as high as in male astrocytes, likely by escaping from X-inactivation, as has been reported in other cell types (Tricarico et al., 2020). KDM6A demethylates histone 3 lysine 27 trimethylation, a repressive mark found in promoters and enhancers, thus contributing to gene activation. KDM5C demethylates histone 3 lysine 4 methylation associated with active promoters and enhancers, thus contributing to gene repression. Both KDM6A and KDM5C are associated with risk of intellectual disability, a disease more common in males than in females (Zablotsky et al., 2017). Lower expression of these genes in male astrocytes may make them more susceptible to mutations that reduce demethylase activity, leading to astrocyte gene regulation defects, neural circuit dysfunction, and intellectual disability. We also observed a diverse array of differentially expressed genes whose protein products are located in the plasma membrane, though their functions in astrocytes remain mysterious (TMEM176B, TMEM143, CD99). Together, this data represents the first evidence that human astrocytes display a subtle sexual dimorphism at the molecular level.

Discussion

We generated transcriptomic data of over 40 samples of acutely purified human astrocytes. These samples vary in age, sex, and disease state, allowing us to analyze these features in humans for the first time with RNAseq. Overall, genes associated with synaptic function change in multiple conditions, highlighting dynamism in astrocyte-synapse interactions in humans. Together, this data elucidates several fundamental

aspects of human astrocyte biology in health and disease, as well as drawing important comparisons to murine astrocytes.

Molecular Profile of Astrocytes in Human Disease

Prior to the advent of the immunopanning technique, astrocyte purification mainly relied on serum-selection (McCarthy and de Vellis, 1980). In these methods, heterogenous collections of cells were cultured with serum-containing media that preferentially allowed survival and propagation of astrocytes. However, these conditions were not physiological, as serum is a component of the blood that does not cross the blood brain barrier in healthy brain tissue. Astrocytes placed under these conditions upregulate reactive markers and adopt fibroblast-like morphology in culture (Foo et al., 2011; Zamanian et al., 2012). Using this method, it was challenging to study *in vivo* reactive astrocyte states, as the signal was masked by the response to serum during *in vitro* purification. Immunopanning allows for acute purification without the use of cell culture or serum, maintaining astrocytes in a near-physiological state (Zhang et al., 2016). This technique allowed us to characterize the transcriptomic profile of human astrocytes from two *in vivo* neurological disorders, FCD, and brain tumor.

Despite previous evidence that some forms of epilepsy can induce astrocyte reactivity (Binder and Steinhauser, 2006), our analysis does not find notable changes in the astrocytes in FCD. This may reflect differences in disease progression across different kinds of epilepsy. A human study of patients with FCD only observed astrocyte reactivity in the center of the disorganized cortex, not in outer regions with milder neuronal phenotypes (Rossini et al., 2017). Therefore, it is conceivable that human astrocytes in this epileptic context would not necessarily demonstrate reactivity, and our

findings in FCD should not be generalized to all forms of epilepsy. Additionally, bulk RNA-seq may miss reactive changes of a small subset of astrocytes.

In stark contrast, peritumor astrocytes demonstrate a robust change in gene expression. Peritumor astrocytes strongly decrease expression of glutamate transporters (SLC1A2 and SLC1A3) that are normally highly expressed in astrocytes and help maintain the excitation-inhibition balance of the brain. Seizure activity is common in individuals with brain tumors, and seizures are often the precipitating event that leads to medical treatment (Englot et al., 2016). Astrocytes may contribute to unregulated excitation in the brain by downregulating these important glutamate transporters. Excessive excitation not only causes harmful seizures; there is recent evidence that neurons form synaptic structures with tumor cells, and neuronal activity drives further proliferation and infiltration of tumor cells (Venkataramani et al., 2019; Venkatesh et al., 2019; Zeng et al., 2019). This raises the exciting possibility of therapeutically targeting glutamate uptake in astrocytes, in addition to existing anti-seizure medications. In future studies, it will be vital to determine whether these general patterns hold for the various classes and origins of brain tumors, and at what stage of disease progression they appear. Beyond glutamate transporters, peritumor astrocytes downregulate several other genes that normally support synaptic function, suggesting further impacts on circuit function.

Indeed, our findings portray a general loss of function in peritumor astrocytes. There is a strong upregulation of cellular proliferation markers in conjunction with near-universal downregulation of core astrocyte genes. We specifically identified a

widespread reduction of astrocyte receptor genes, which may limit their ability to sense and respond to their environment.

Interestingly, there may be one important gain-of-function in peritumor astrocytes. A study of human astrocytes from tumor cores concluded that astrocytes contributed to an immune-suppressive environment that permitted tumor proliferation and infiltration. Tumor-core astrocytes showed changes in the JAK/STAT and interferon gamma response as well as upregulation of the anti-inflammatory cytokine Il-10 (Heiland et al., 2019), which were not present in the peritumor astrocytes. On the contrary, peritumor astrocytes upregulate many pro-inflammatory genes and downregulate anti-inflammatory genes. Although loss of synaptic support from peritumor astrocytes may contribute to circuit disruption and tumor-associated seizure activity, an increase in pro-inflammatory signaling may play a vital role in containing tumor growth and migration by promoting an immune response.

Human Astrocyte Maturation

Human brain development proceeds through a cascade of complex and reciprocal interactions between several maturing cell types. For example, neurons largely fail to make functional synapses in the absence of astrocytes, and astrocytes lack morphological complexity without the presence of neurons (Banker, 1980; Stogsdill et al., 2017). The developmental trajectory of most major brain cells has been described by the presence of cell-specific transcription factors that drive cells toward a specific fate, such as NEUROD1 in neurons and OLIG2 in oligodendrocytes. Although some important regulators have been identified, astrocyte development and maturation remain less well understood (Kang et al., 2012; Glasgow et al., 2014; Chaboub et al.,

2016; Li et al., 2019). Previous work compared fetal astrocytes to postnatal astrocytes that helped identify markers of mature versus immature astrocytes, and here we extend that work by creating a developmental timeline across postnatal astrocyte maturation in humans. This provides some of the first insight into how human astrocytes mature past the early stages of development. We found a shift in astrocytic gene expression around 8 years old that persists into early adulthood. This time frame coincides with the onset of increased synaptic pruning in the cortex, as evidenced by a decline in cortical synaptic spine density beginning around puberty (Huttenlocher, 1979; Huttenlocher and Dabholkar, 1997; Petanjek et al., 2011). Based on this data, astrocytes adopt functions for the support and maintenance of synapses before birth, but they adopt new roles in synaptic remodeling during postnatal maturation. These data also provide new markers to use in untangling astrocytic gene networks and molecular mechanisms that related to increased synaptic pruning. Pathway analysis of astrocyte maturation genes identified downregulation of proliferative pathways and upregulation of pathways related to ion homeostasis and lipid metabolism. We also find these patterns of astrocyte maturation preserved in mice, even though mice and humans showed divergent sets of maturation-related genes. This suggests mice and human astrocytes share many aspects of their developmental arcs, though they may express different sets of genes to achieve the same functional goal. Future studies should aim to identify the signaling mechanisms that drive astrocyte maturation, as aberrations in astrocyte maturation could contribute to dysfunction in neural circuits and ultimately neurodevelopmental disorders.

Aging in Human Astrocytes

Astrocytes become reactive in age-related neurological diseases such as Alzheimer Disease (Beach et al., 1989). It is important to determine whether reactivity is induced purely by disease progression or whether astrocyte reactivity occurs in the course of normal aging, which may further contribute to aspects of disease progression. We observe declining expression of synaptic genes, including the glutamate transporter gene SLC1A3. As we noted in peritumor astrocytes, altered expression of this gene product would impact the balance of excitation-inhibition in the brain and impair circuit function. Two separate animal studies have identified increasing expression of reactive markers across age in the mouse brain, both in the cortex and subcortical regions (Boisvert et al., 2018; Clarke et al., 2018). We find corresponding evidence in our analysis of aging human astrocytes where several genes involved in cytokine signaling are upregulated, including CCL2, IL6, and SOCS3. We also observe a modest increase in senescence markers CDKN1A and CDKN2A. As our cohort only extends to age 65, further study of astrocytes at more advanced ages could uncover much larger changes in the astrocyte transcriptome throughout the aging process. While reactive and senescence markers increase, we observe a decrease in genes related to energy metabolism. Astrocytes typically provide metabolic support to aid in proper neuronal function and signaling, so these changes may contribute to age-related declines in cognition. An important question for further examination is whether a decrease in neuronal support is reflective of astrocytic dysfunction or declining demand from neurons. Our study does not have a large cohort of patients in the aging adult group. Given the higher variability of human data caused by greater genetic and environmental

diversity compared with laboratory mice, future studies with large sample sizes will further expand our understanding of astrocyte aging in humans.

Sexual Dimorphism of Human Astrocytes

Many neurological diseases have differences in incidence and prognosis depending on sex, but little is known about the mechanisms that underlie these differences. Recently, multiple findings identified sex differences in microglia, but sex differences in astrocytes remain elusive despite their extensive interactions with microglia. Slight differences in astrocyte number and morphology were reported in sub-cortical regions of the brain in rats, such as the amygdala (Mong and McCarthy, 2002; Johnson et al., 2008). Here, we report the first evidence of sexual dimorphism in human astrocytes, to the best of our knowledge. Female cortical astrocytes have higher transcription of several plasma membrane proteins, including somatostatin receptor SSTR2, a transcriptional target of p53, PERP, and transmembrane protein TMEM176B. We also observe differential expression of genes encoding epigenetic regulators located on sex chromosomes, such as demethylases KDM5C and KDM6A. Though healthy astrocytes demonstrate relatively few sex differences, further studies should investigate whether underlying differences in epigenetic state could contribute to sex-specific responses to insult or injury and ultimately underlie sex differences in neurological disease.

Naturally, limited access to fresh brain tissue limits many studies of the human brain, including this one, and our findings are not an exhaustive list of changes in human astrocytes. Further studies are needed to clarify context-dependent expression in human astrocytes in other diseases and patient populations to identify astrocytic roles

in human health and disease. Our discovery of changes in genes involved in synaptic function across multiple conditions in human astrocytes is an important step in that direction, and our dataset will provide valuable insight for further investigation of human biology and novel approaches for neurological disease.

Figure Legends

Figure 1. Acute purification of human astrocytes from cerebral cortex. A) Diagram of human astrocyte purification by immunopanning. Surgically resected tissue underwent enzymatic digestion and gentle mechanical digestion to generate a single cell suspension. These cells were passed over a series of plates coated with cell-type-specific antibodies to deplete microglia, oligodendrocyte-lineage cells, and neurons before finally passing to a plate that specifically binds astrocytes using an anti-HepaCAM antibody. B) Heatmaps showing the expression of cell type specific genes in RPKM after RNA sequencing of immunopanned astrocytes. All samples are highly enriched in astrocytic genes (red), with little to no expression of gene markers for neurons, myeloid cells (i.e. microglia or macrophages), oligodendrocyte-lineage cells, or endothelial cells. Detailed sample info and total gene expression are detailed in Figure 1-1 and 1-2.

Figure 2. Transcriptomic signature of human astrocytes in the peritumor microenvironment. A) Volcano plot showing differential gene expression in human peritumor astrocytes vs. controls; red = $p < 0.05$. Full DGE in Figure 2-1; cross-validation in Figure 2-2; effects of brain region in Figure 2-3. B) Bar plots of astrocyte genes with changing expression in the peritumor microenvironment. C) Selected gene

ontology terms that are significantly enriched in up- (left) and downregulated (right) genes in peritumor astrocytes; dashed lines: $p < 0.05$. Full GO results in Figure 2-6. Analysis of synaptic genes and disease-associated genes in Figure 2-4. D) Heatmaps of differential gene expression in peritumor astrocytes related to (top) synaptic function, (middle) astrocyte reactivity, and (bottom) mature astrocyte markers. E) Heatmaps of extracellular matrix genes with increased (red) or decreased (blue) expression in peritumor astrocytes, all significant at $p < 0.05$. F) Normalized gene expression of anti-inflammatory (top) and pro-inflammatory (bottom) genes that are differentially expressed in peritumor astrocytes ($p < 0.05$). G) Normalized gene expression of plasma membrane receptors that are differentially expressed in peritumor astrocytes ($p < .05$). Full gene list in Figure 2-5. H) Normalized gene expression of the top 50 astrocyte marker genes, identified by Kelley et al. (2018a), in peritumor and control astrocytes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. *In situ* hybridization validation of human astrocyte RNAseq. A) RNAscope *in situ* hybridization of TLR4 (yellow) in astrocytes (SLC1A3, red) in both human and mouse tissues. Scale bar = 50 μm. B) Quantification of $TLR4^+$ astrocytes in human and mouse cortical tissue. Error bars = standard error. C) Histogram depicting the number of $TLR4^+$ puncta in an astrocytic cell body labeled by SLC1A3 in human and mouse cortical tissue.

Figure 4. Molecular characterization of human astrocyte maturation. Full maturation DGE results in Figure 4-2, including FCD samples as they were not substantially

different from controls (Figure 4-1). A) Heatmap of representative genes with changing expression across maturation (7 months – 21 years old, n = 26). Astrocytic gene expression approaches the mature pattern around 8 years of age. Plotted as Z-score of gene expression (RPKM); top bar: rainbow index of sample ages. B) Selected gene ontology terms enriched in genes that are up- (left) or downregulated (right) across maturation in both human astrocytes (black bars) and mouse astrocytes (grey bars, from (Clarke et al., 2018)). Dashed lines: $p < 0.05$. Full results reported in Figure 4-3. C) Venn diagrams quantifying astrocyte maturation-associated genes that are up- (left) and downregulated (right) in humans (red) and mice (blue). D) Top: heatmap of selected astrocyte maturation markers colored by percentile change of RNA expression (e.g. Δ percentile = +100 demonstrates a gene went from the least expressed gene to the most expressed gene) from fetal human astrocytes (Li et al., 2021) to mature human astrocytes (13-21 years old). Bottom: heatmap of selected calcium signaling genes, same quantification as the heatmap above.

Figure 5. Age-associated genes in human astrocytes. Full expression data presented in Figure 5-1. A) Expression of age-associated human astrocyte genes with decreased expression in older adults (50+ years old) compared to younger adults (21-50 years old). B) Age-associated human astrocyte genes with increased expression in older adults compared to younger adults. All genes shown are significantly associated with age, and at least 1.5-fold enriched in younger or older adults. C) Change in RNA expression of mouse astrocytes (10 weeks old vs. 2 years old, from (Clarke et al., 2018)) in various gene ontology categories. Gene lists derived from the following GO

annotations, from left to right: GO:0046034, GO:0045202, GO:0019221 and GO:0090398. D) Protein-protein interaction networks among human (top) and mouse (bottom) age-associated genes.

Figure 6. Sexually dimorphic genes in human astrocytes. Selected genes that are significantly associated with sex, including genes encoding transcription factors, epigenetic modifying enzymes, and proteins localized to the plasma membrane. Full DGE results presented in Figure 6-1.

Extended Data Legends

Figure 1-1. Human astrocyte metadata

Figure 1-2. Human Astrocyte Gene Expression (RPKM)

Figure 2-1. Differential Gene Expression in Peritumor Astrocytes

Figure 2-2. Cross-Validation of Differential Gene Expression; *Peritumor DGE leave-one-out*

Figure 2-3. Differential Gene Expression by Brain Region (Temporal vs. Frontal Cortex)

Figure 2-4. Associations of Disease-Linked Genes and Peritumor Astrocyte Genes

Figure 2-5. Astrocyte Receptor Genes

Figure 2-6. GO Pathways *Upregulated* in Peritumor Astrocytes

Figure 4-1. Differential Gene Expression in FCD Astrocytes

Figure 4-2. Genes *Upregulated* Across Maturation

Figure 4-3. GO Analysis of Maturation Genes; *Human Up*

Figure 5-1. Age-Associated Genes in Human Astrocytes

973 **Figure 6-1.** Differential Gene Expression by Sex

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Figure 1

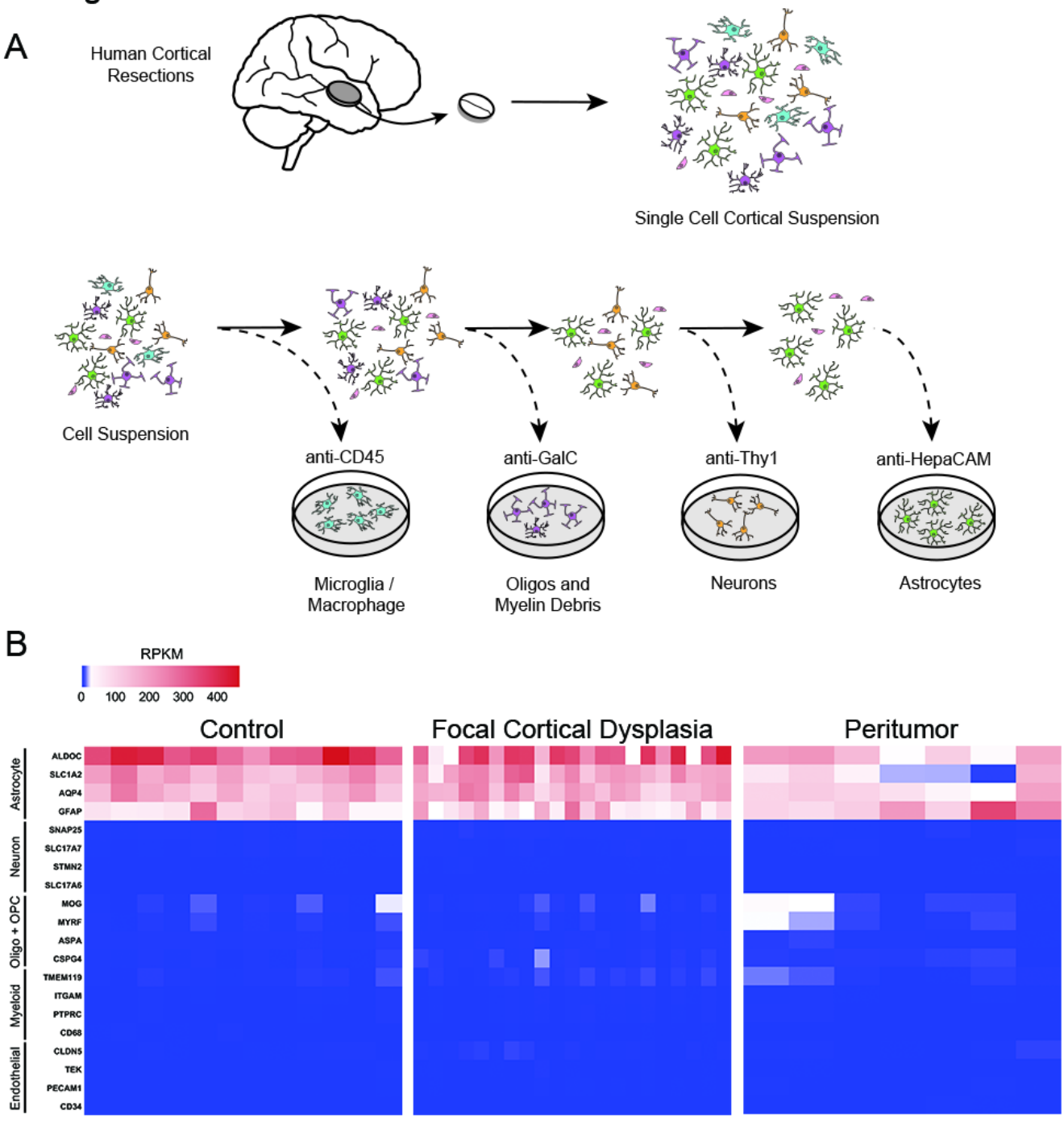


Figure 2

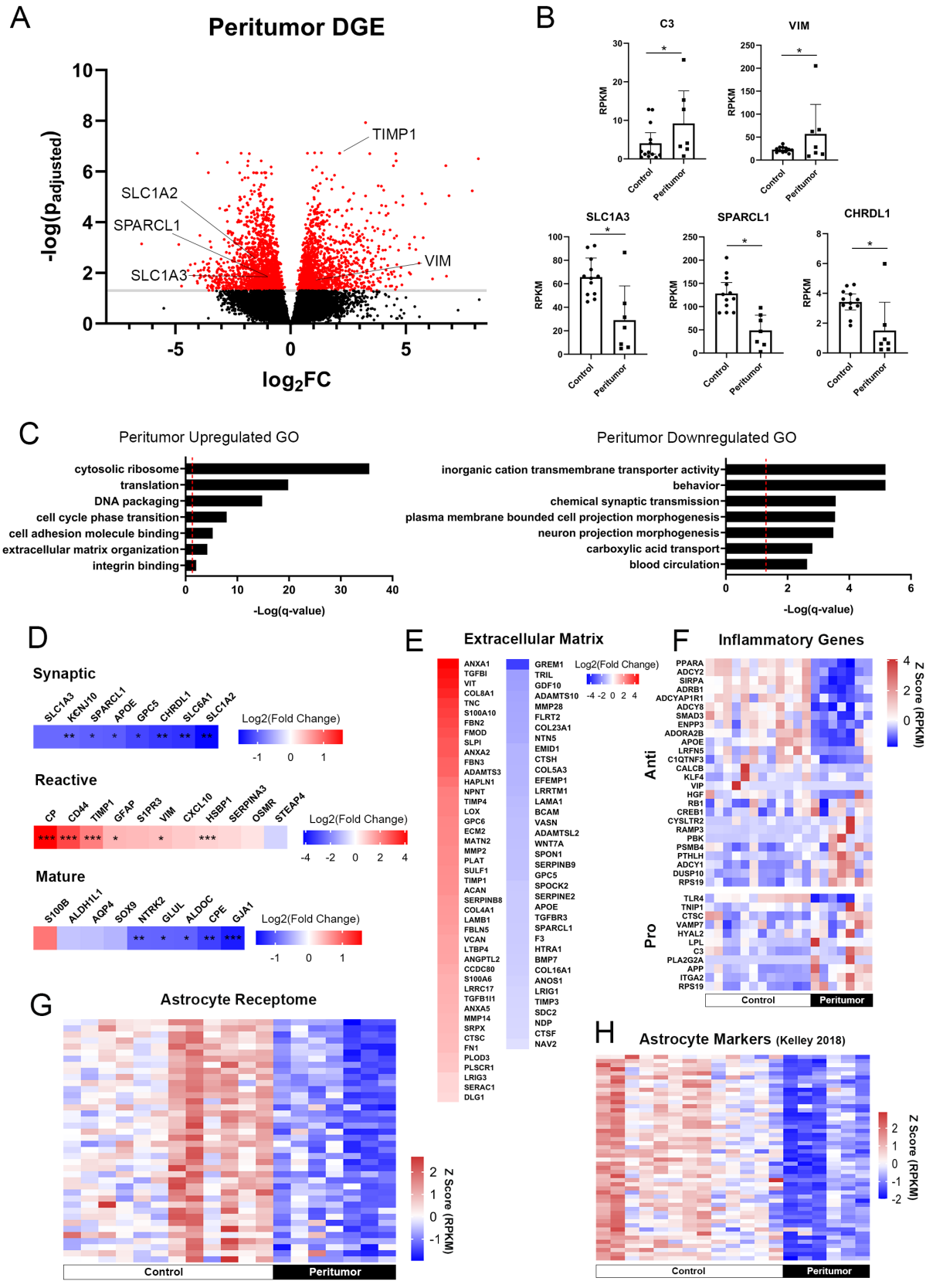
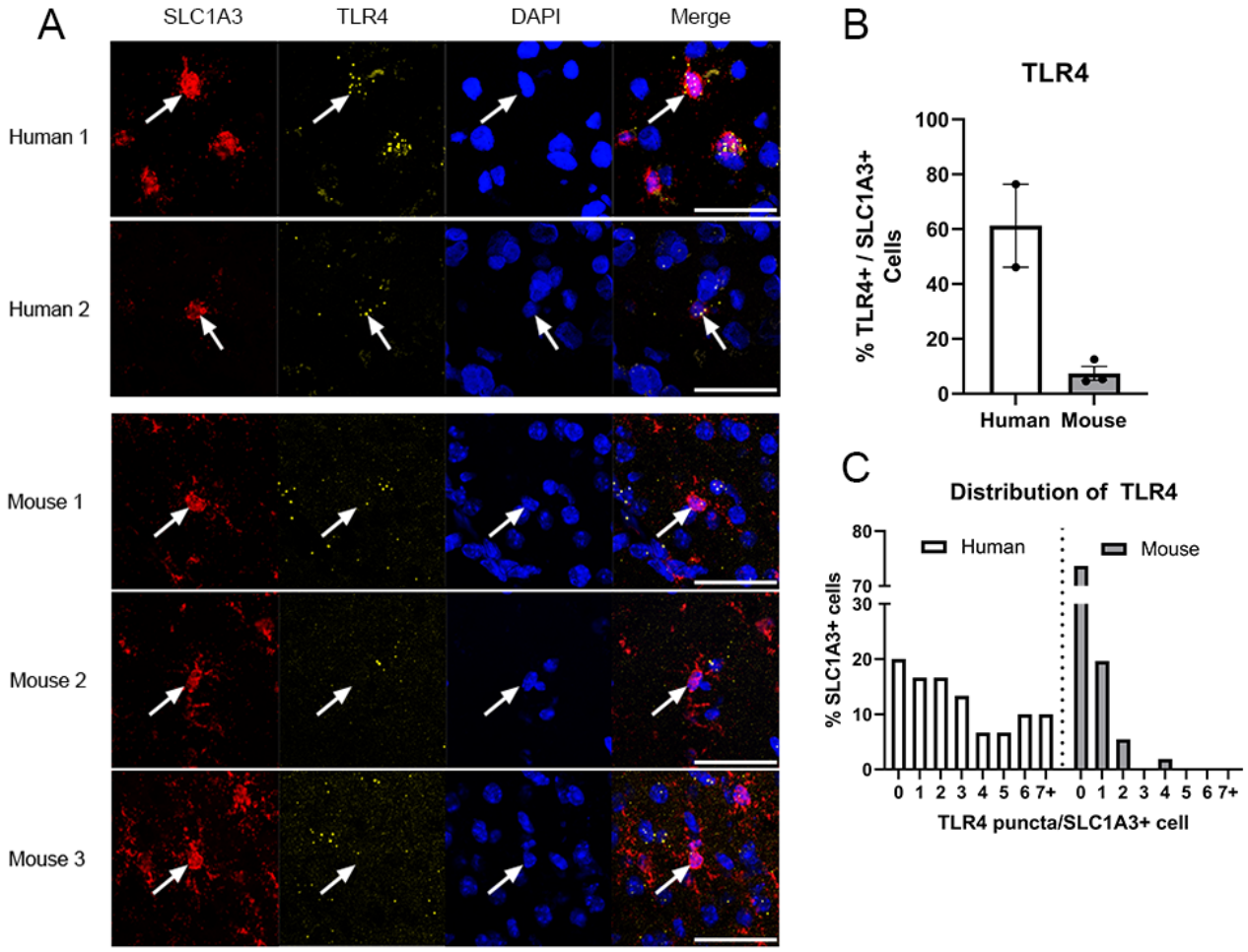


Figure 3



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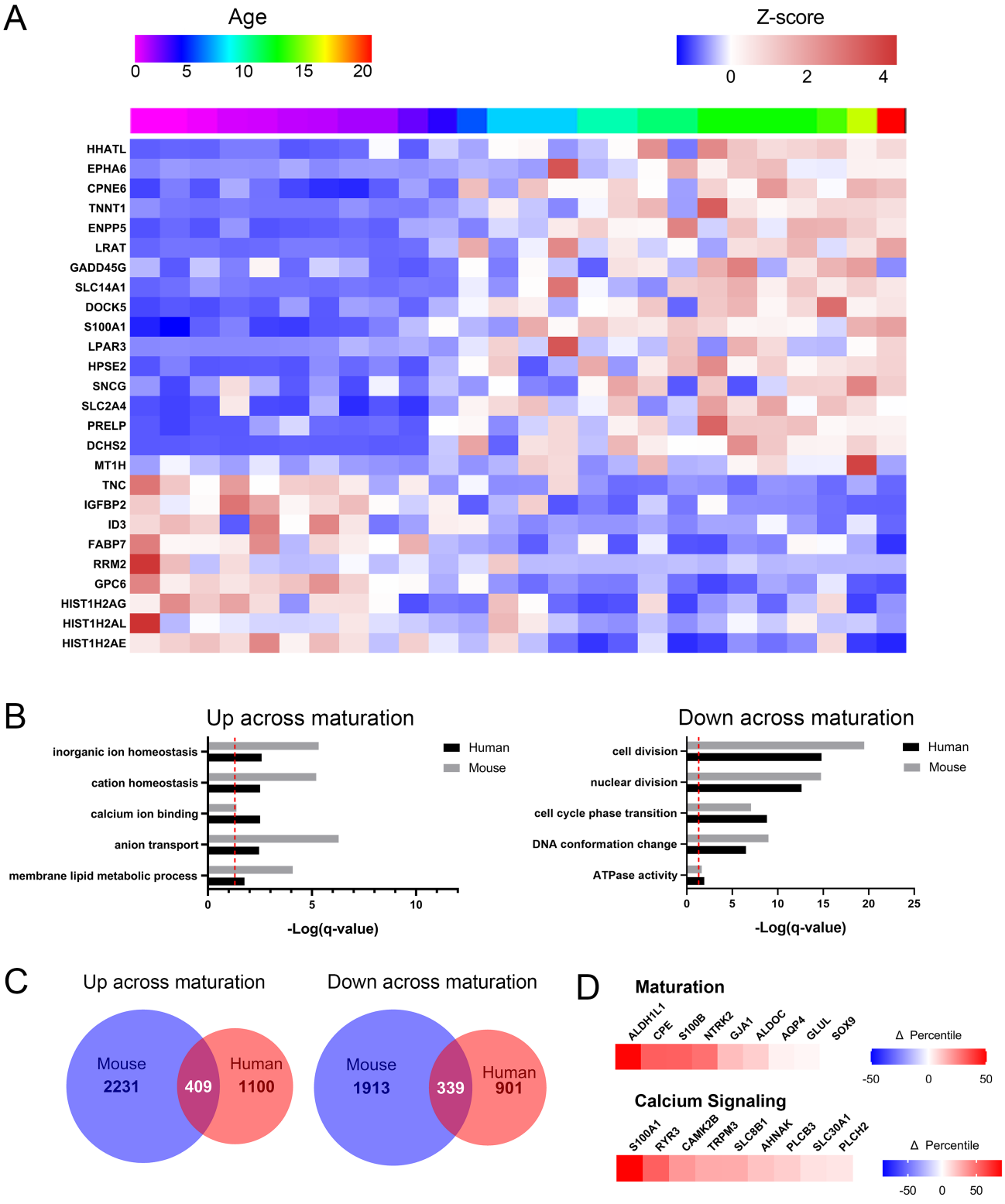

Figure 5

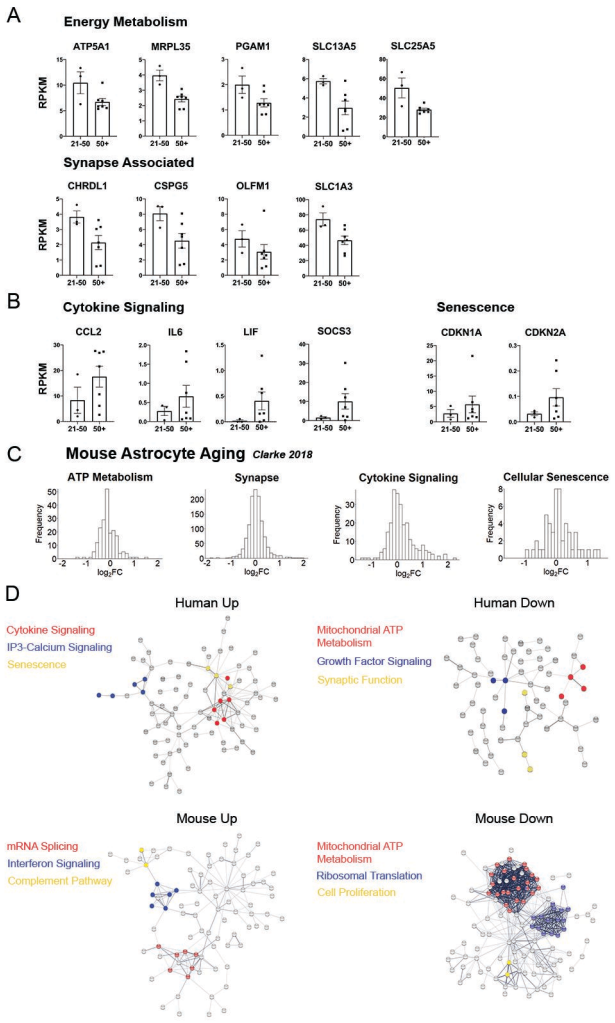

Figure 6