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

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*Research Articles: Neurobiology of Disease*

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

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2 Transcriptomic Signatures

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56

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

59

60 **Abstract**

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

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

82

### 83 **Significance Statement**

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

92

### 93 **Introduction**

94 Astrocytes are a major component of the central nervous system. Though  
95 astrocytes were long regarded as passive support cells, studies of murine astrocytes  
96 found they have active functions that are critical for the development and function of  
97 synapses. For example, astrocyte-secreted factors powerfully induce the formation of  
98 functional synapses *in vivo* and *in vitro*, which otherwise largely fails to occur (Banker,  
99 1980; Ullian et al., 2001; Christopherson et al., 2005; Kucukdereli et al., 2011; Allen et  
100 al., 2012; Singh et al., 2016; Farhy-Tselnicker et al., 2017; Krencik et al., 2017; Stogsdill  
101 et al., 2017; Blanco-Suarez et al., 2018). In addition to important roles in synapse  
102 formation, astrocytes contribute to engulfment and elimination of synapses in

103 development (Chung et al., 2013; Tasdemir-Yilmaz and Freeman, 2014; Vainchtein et  
104 al., 2018; Lee et al., 2020). Moreover, astrocytes maintain extracellular potassium  
105 levels (Kuffler et al., 1966; Olsen and Sontheimer, 2008; Kelley et al., 2018b) and  
106 participate in recycling neurotransmitters (Rothstein et al., 1996), thus maintaining  
107 homeostasis at synapses. There is now a variety of evidence showing that astrocytes  
108 help shape circuit functions and behavior (Nedergaard, 1994; Parpura et al., 1994;  
109 Halassa et al., 2009; Robel et al., 2015; Papouin et al., 2017; Dowling and Allen, 2018;  
110 Mu et al., 2019; Nagai et al., 2019; Huang et al., 2020). Various groups have  
111 demonstrated that altering intracellular astrocyte signaling *in vivo* can induce abnormal  
112 behavior or correct phenotypic behavior in disease models (Chen et al., 2016; Ma et al.,  
113 2016; Ng et al., 2016; Kelley et al., 2018b; Yu et al., 2018; Ung et al., 2020; Yu et al.,  
114 2020; Nagai et al., 2021). Astrocytes are molecularly and functionally heterogeneous,  
115 potentially adapting to diverse roles they play in different brain regions (Tsai et al., 2012;  
116 Glasgow et al., 2014; Molofsky et al., 2014; Chai et al., 2017; John Lin et al., 2017;  
117 Morel et al., 2017; Miller et al., 2019; Diaz-Castro et al., 2021).

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

125 response (Beach et al., 1989; Panickar and Norenberg, 2005; Binder and Steinhauser,  
126 2006; Burda et al., 2016; Liddelow et al., 2017; Yu et al., 2020).

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

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

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

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

170

171 **Materials and Methods**

## 172 HUMAN TISSUE

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

## 185 VERTEBRATE ANIMALS

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

## 190 PURIFICATION OF HUMAN ASTROCYTES

191 Human astrocytes were purified using immunopanning, as described in (Zhang et  
192 al., 2016). Briefly, we dissected gray matter from cortical tissue and enzymatically  
193 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  $\mu$ L of TRIzol solution (Thermo Fisher Scientific 15596018). TRIzol solution  
205 was then flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  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  $\geq 6.5$ , 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

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

#### 223 READ ALIGNMENT AND QUANTIFICATION

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

#### 230 DIFFERENTIAL GENE EXPRESSION ANALYSIS OF DISEASE AND SEX

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

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

#### 250 ANALYSIS OF HUMAN AGING GENES

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

#### 260 ANALYSIS OF HUMAN ASTROCYTE MATURATION

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

263 peritumor samples (n = 35). Model: ~ factor(Diagnosis) + Age + factor(Sex) + MicroPC  
264 + OligPC + factor(Batch). The “oligPC” was calculated in the same manner as the  
265 microPC using gene expression of oligodendrocyte-specific genes identified using data  
266 from (Zhang et al., 2016). While it is necessary to use cell-type specific filters to  
267 exclude contamination, excluding oligodendrocyte-enriched genes may obscure  
268 potential lineage relationships between astrocytes and oligodendrocytes (Weng et al.,  
269 2019). Thus, we only used these filters for differential expression analysis and included  
270 the unfiltered complete dataset in Figure 1-2. Finally, we filtered out genes that were  
271 10x enriched in human neurons over human astrocytes, using data from (Zhang et al.,  
272 2016). This yielded 1,463 gene entries significantly associated with maturation. We also  
273 performed a leave-one-out reanalysis to assess the robustness of the maturation gene  
274 expression findings, see Figure 2-2.

275         The DESeq2 analysis included samples as young as 7 months old, but we could  
276 capture changes from earlier stages in development by using our recently published  
277 transcriptomic profiles of fetal human astrocytes. We compared 4 fetal samples with  
278 our near-adult human samples between 13-21 years old (excluding peritumor, n = 11).  
279 For each sample, gene expression was converted to a percentile, where the most highly  
280 expressed gene = 1 and the least expressed gene = 0. Next, we conducted parallel t-  
281 tests with a Benjamini-Hochberg correction for multiple tests, performed in GraphPad  
282 Prism v8. This generated over 10,000 hits. Finally, we combined the two gene lists  
283 using an equal number of genes from each list, (i.e. we filtered results from the second  
284 analysis to the 1,463 top hits by p-value to match the first analysis), producing a final list  
285 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](http://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](http://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](http://metascape.org), (Zhou et al., 2019)). We input all protein-

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

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

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

#### 327 RNASCOPE IN SITU HYBRIDIZATION

328 RNAScope in situ hybridization was performed on fresh frozen human tissue  
329 collected from surgeries and fresh frozen mouse tissue harvested after a 10-minute  
330 transcardial perfusion of phosphate buffered saline. Tissues were embedded in OCT  
331 compound (Fisher Scientific 23-730-571) and cut into 20-30  $\mu\text{m}$ -thick sections.

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

#### 337 STATISTICAL ANALYSES

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

#### 342 DATA DEPOSITION

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

346

## 347 Results

### 348 Purification of Human Cortical Astrocytes in Health and Disease

349 We obtained human cortical tissue from patients undergoing neurological  
350 surgery. Our final analysis includes 49 samples from 41 patients, with ages ranging  
351 from 7 months to 65 years old. Twelve of these samples were taken from normal  
352 regions of cortex that were resected *en route* to deep-seated pathologies. Nine of these  
353 normal specimens were obtained during surgery for deep epileptogenic foci. In these  
354 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

378 dishes that bind contaminating cell types with cell type specific antigens. This  
379 immunopanning protocol uses anti-CD45 antibodies to pull down myeloid cells (i.e.  
380 microglia and macrophages), anti-GalC hybridoma cell supernatant to pull down  
381 oligodendrocytes and myelin debris, anti-O4 hybridoma cell supernatant to bind  
382 oligodendrocyte precursor cells (OPCs), and anti-Thy1 antibodies to bind neurons.  
383 Finally, the enriched suspension passes onto a dish coated in anti-HepaCAM  
384 antibodies, a cell-surface glycoprotein enriched in astrocytes. We harvested the  
385 astrocyte RNA from this dish and used it to perform RNA sequencing (RNAseq). The  
386 sequenced samples show high expression of astrocyte marker genes such as GFAP  
387 and SLC1A2, and extremely low expression of neuronal, myeloid, and endothelial  
388 genes. There are only slight traces of some oligodendrocyte-lineage marker genes (Fig  
389 1B). Using immunopanning, we obtained RNA highly enriched for human cortical  
390 astrocytes in healthy and diseased states for bulk RNAseq (Figure 1-2).

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

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

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

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

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

424 *Synaptic gene signatures in peritumor astrocytes*

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

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

448 *Cell surface receptors in peritumor astrocytes*

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

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

460 Having discovered genes up- and down-regulated in peritumor astrocytes, we  
461 next assessed whether these genes are involved in other diseases. We used the  
462 Phenopedia dataset for genes associated with various neurological diseases (Yu et al.,  
463 2010) and tested whether genes up and downregulated in peritumor astrocytes are  
464 significantly enriched in genes associated with the risk of each neurological disorder  
465 using Fisher's exact test followed by correction for multiple comparisons (Methods).  
466 Genes that were downregulated in peritumor astrocytes were enriched for genes with  
467 genetic links to several neurological diseases (Figure 2-4). Interestingly, these  
468 associations existed primarily among psychiatric disorders (bipolar disorder,

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

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

#### 485 **Gene ontology of peritumor astrocyte signatures**

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

492 as cation transport (Fig 2C), further supporting the hypothesis that peritumor astrocytes  
493 are defective in supporting or participating in normal synaptic signaling. Interestingly,  
494 both up- and downregulated gene lists are enriched for extracellular matrix genes (Fig  
495 2E), which is notable considering the importance of extracellular remodeling in tumor  
496 expansion and migration as well as synaptic plasticity (Nguyen et al., 2020; Winkler et  
497 al., 2020). Broadly, we see peritumor astrocytes alter extracellular matrix gene  
498 expression while upregulating genes necessary for cell division and translation, and  
499 downregulating expression of genes related to synaptic transmission and ionic  
500 homeostasis, revealing potential contribution of astrocytes to neural circuit dysfunction  
501 associated with brain tumors.

#### 502 **Peritumor astrocytes differ from tumor-core astrocytes**

503 To assess whether peritumor astrocytes resemble tumor-core associated  
504 astrocytes, we compared our peritumor astrocyte dataset to a previously published  
505 tumor-core associated astrocyte dataset (Heiland et al., 2019). Heiland and colleagues  
506 reported that tumor-core astrocytes contribute to an immunosuppressive environment in  
507 part due to increased JAK/STAT pathway activation. Among peritumor astrocytes,  
508 however, we did not find significant enrichment of the JAK/STAT pathway among  
509 differentially expressed genes. We also failed to find enrichment of interferon gamma  
510 response genes and interleukin-6 response genes, which were also identified by  
511 Heiland et al. When we examined pro- and anti-inflammatory genes that are  
512 differentially expressed in peritumor astrocytes, we found that the majority of pro-  
513 inflammatory genes were upregulated (10/11, Fig 2F), and the majority of anti-  
514 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

538 (this study and Zhang et al. 2016). To directly compare TLR4 expression between  
539 species, we performed RNAscope *in situ* hybridization on human and mouse cortical  
540 tissue. Whereas a small minority of mouse astrocytes expressed TLR4 (7%, 4/58 cells,  
541  $n = 3$ ), a majority of human astrocytes showed expression of TLR4 (62%, 18/29 cells,  $n$   
542  $= 2$ , Fig. 3A, B). Even more strikingly, TLR4<sup>+</sup> astrocytes in humans contain larger  
543 numbers of TLR4<sup>+</sup> mRNA puncta than did TLR4<sup>+</sup> astrocytes in mice (Fig. 3C). Though  
544 the small sample size does not provide a definitive conclusion, we find evidence of  
545 human-specific expression of TLR4 in astrocytes that corroborates findings from  
546 RNAseq, suggesting enhanced ability of human astrocytes to detect TLR4 ligands, such  
547 as signals from bacteria and damaged cells, compared with mouse astrocytes.  
548 Furthermore, this mode of signaling is notably reduced in peritumor astrocytes.

#### 549 **The transcriptomes of astrocytes in FCD and control do not differ**

550       Next, we examined the transcriptional signature of FCD. FCD is characterized  
551 by abnormalities in neuronal migration during development. Patients with FCD display  
552 abnormal radial and/or tangential lamination in a local region of cerebral cortex. More  
553 severe cases include dysmorphic neurons, and others also develop large and often  
554 multi-nucleated cells called balloon cells (Gaitanis and Donahue, 2013). Our DESeq2  
555 analysis found only 24 protein-coding genes significantly associated with epilepsy, and  
556 all but one of those genes (SCN4B) had low expression (an average expression  $<1$   
557 RPKM; see Figure 4-1). Samples from FCD patients did not separate from controls in  
558 PCA, hierarchical clustering, or expression of reactive astrocyte markers (data not  
559 shown). Therefore, astrocytes in FCD in humans do not exhibit robust gene expression  
560 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  $\geq 8$  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

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

637 As in peritumor astrocytes, we find decreased expression of genes mediating  
638 astrocyte-synaptic interactions in older astrocytes (Fig. 5A). Most notably, there is a  
639 reduction of SLC1A3, a glutamate transporter that clears glutamate from the  
640 extracellular space. Under normal conditions, SLC1A3 is a highly expressed core  
641 marker of adult astrocytes (Zhang et al., 2016). There is also a decline in CHRD1,  
642 which codes for an astrocyte-secreted factor that drives synapse maturation (Blanco-  
643 Suarez et al., 2018). Aging astrocytes also have lower expression of two genes coding  
644 for glycoproteins found in the extracellular matrix. The first, CSPG5, shapes neurite  
645 growth and localizes around GABAergic and glutamatergic synaptic terminals (Pinter et  
646 al., 2020), while the second, OLMF1, binds synaptic proteins such as synaptophysin  
647 and AMPA receptors (Nakaya et al., 2013). Declining expression of synaptic genes  
648 raises important questions about astrocytic roles in age-related cognitive decline and  
649 neurological disease.

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

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

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

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

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

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

#### 694 **Region-specific gene signatures in astrocytes**

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

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

### 703 **Subtle sexual dimorphism in astrocyte gene signatures**

704 Female and male brains differ in their susceptibility to neurological disorders. For  
705 example, intellectual disability, autism spectrum disorder and Parkinson disease are  
706 more prevalent in men than in women (Werling and Geschwind, 2013; Gillies et al.,  
707 2014), whereas multiple sclerosis, Alzheimer disease, and anxiety disorder are more  
708 prevalent in women than in men (Seshadri et al., 1997; McLean et al., 2011; Westerlind  
709 et al., 2014). The cellular and molecular mechanisms underlying sexually dimorphic  
710 susceptibility to neurological and psychiatric disorders are largely unknown. While  
711 understanding of sexually dimorphic properties of other brain cells, particularly  
712 microglia, is increasing, sexual dimorphism in human astrocytes has not yet been  
713 reported. In our differential gene expression analysis of all 49 samples, we found 105  
714 genes (40 protein coding) with expression levels significantly associated with sex (Fig.  
715 6, Figure 6-1). This gene list represents the first evidence of sexual dimorphism in  
716 human cortical astrocytes, to the best of our knowledge. Some of these genes are  
717 transcription factors (POU5F1B, HOXC10) or epigenetic factors, which may globally  
718 regulate gene expression. For example, the lysine demethylases KDM6A and KDM5C  
719 located on the X-chromosome are expressed at higher levels by female than male  
720 astrocytes. Females have two copies of X-chromosome genes and males only have one  
721 copy. Most X-chromosome genes are subjected to X-inactivation in females, where only

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

738

### 739 **Discussion**

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

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

#### 747 **Molecular Profile of Astrocytes in Human Disease**

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

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

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

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

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

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

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

### 803 **Human Astrocyte Maturation**

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

813 2016; Li et al., 2019). Previous work compared fetal astrocytes to postnatal astrocytes  
814 that helped identify markers of mature versus immature astrocytes, and here we extend  
815 that work by creating a developmental timeline across postnatal astrocyte maturation in  
816 humans. This provides some of the first insight into how human astrocytes mature past  
817 the early stages of development. We found a shift in astrocytic gene expression around  
818 8 years old that persists into early adulthood. This time frame coincides with the onset  
819 of increased synaptic pruning in the cortex, as evidenced by a decline in cortical  
820 synaptic spine density beginning around puberty (Huttenlocher, 1979; Huttenlocher and  
821 Dabholkar, 1997; Petanjek et al., 2011). Based on this data, astrocytes adopt functions  
822 for the support and maintenance of synapses before birth, but they adopt new roles in  
823 synaptic remodeling during postnatal maturation. These data also provide new markers  
824 to use in untangling astrocytic gene networks and molecular mechanisms that related to  
825 increased synaptic pruning. Pathway analysis of astrocyte maturation genes identified  
826 downregulation of proliferative pathways and upregulation of pathways related to ion  
827 homeostasis and lipid metabolism. We also find these patterns of astrocyte maturation  
828 preserved in mice, even though mice and humans showed divergent sets of maturation-  
829 related genes. This suggests mice and human astrocytes share many aspects of their  
830 developmental arcs, though they may express different sets of genes to achieve the  
831 same functional goal. Future studies should aim to identify the signaling mechanisms  
832 that drive astrocyte maturation, as aberrations in astrocyte maturation could contribute  
833 to dysfunction in neural circuits and ultimately neurodevelopmental disorders.

#### 834 **Aging in Human Astrocytes**

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

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

### 859 **Sexual Dimorphism of Human Astrocytes**

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

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

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

884

#### 885 **Figure Legends**

886 **Figure 1.** Acute purification of human astrocytes from cerebral cortex. A) Diagram of  
887 human astrocyte purification by immunopanning. Surgically resected tissue underwent  
888 enzymatic digestion and gentle mechanical digestion to generate a single cell  
889 suspension. These cells were passed over a series of plates coated with cell-type-  
890 specific antibodies to deplete microglia, oligodendrocyte-lineage cells, and neurons  
891 before finally passing to a plate that specifically binds astrocytes using an anti-  
892 HepaCAM antibody. B) Heatmaps showing the expression of cell type specific genes  
893 in RPKM after RNA sequencing of immunopanned astrocytes. All samples are highly  
894 enriched in astrocytic genes (red), with little to no expression of gene markers for  
895 neurons, myeloid cells (i.e. microglia or macrophages), oligodendrocyte-lineage cells, or  
896 endothelial cells. Detailed sample info and total gene expression are detailed in Figure  
897 1-1 and 1-2.

898

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

904 ontology terms that are significantly enriched in up- (left) and downregulated (right)  
905 genes in peritumor astrocytes; dashed lines:  $p < 0.05$ . Full GO results in Figure 2-6.  
906 Analysis of synaptic genes and disease-associated genes in Figure 2-4. D) Heatmaps  
907 of differential gene expression in peritumor astrocytes related to (top) synaptic function,  
908 (middle) astrocyte reactivity, and (bottom) mature astrocyte markers. E) Heatmaps of  
909 extracellular matrix genes with increased (red) or decreased (blue) expression in  
910 peritumor astrocytes, all significant at  $p < 0.05$ . F) Normalized gene expression of anti-  
911 inflammatory (top) and pro-inflammatory (bottom) genes that are differentially expressed  
912 in peritumor astrocytes ( $p < 0.05$ ). G) Normalized gene expression of plasma membrane  
913 receptors that are differentially expressed in peritumor astrocytes ( $p < .05$ ). Full gene list  
914 in Figure 2-5. H) Normalized gene expression of the top 50 astrocyte marker genes,  
915 identified by Kelley et al. (2018a), in peritumor and control astrocytes. \*  $p < 0.05$ , \*\*  $p <$   
916  $0.01$ , \*\*\*  $p < 0.001$ .

917

918 **Figure 3.** *In situ* hybridization validation of human astrocyte RNAseq. A) RNAscope *in*  
919 *situ* hybridization of TLR4 (yellow) in astrocytes (SLC1A3, red) in both human and  
920 mouse tissues. Scale bar = 50  $\mu\text{m}$ . B) Quantification of TLR4<sup>+</sup> astrocytes in human and  
921 mouse cortical tissue. Error bars = standard error. C) Histogram depicting the number  
922 of TLR4<sup>+</sup> puncta in an astrocytic cell body labeled by SLC1A3 in human and mouse  
923 cortical tissue.

924

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

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

941

942 **Figure 5.** Age-associated genes in human astrocytes. Full expression data presented in  
943 Figure 5-1. A) Expression of age-associated human astrocyte genes with decreased  
944 expression in older adults (50+ years old) compared to younger adults (21-50 years  
945 old). B) Age-associated human astrocyte genes with increased expression in older  
946 adults compared to younger adults. All genes shown are significantly associated with  
947 age, and at least 1.5-fold enriched in younger or older adults. C) Change in RNA  
948 expression of mouse astrocytes (10 weeks old vs. 2 years old, from (Clarke et al.,  
949 2018)) in various gene ontology categories. Gene lists derived from the following GO

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

953

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

958

#### 959 **Extended Data Legends**

960 **Figure 1-1.** Human astrocyte metadata

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

962 **Figure 2-1.** Differential Gene Expression in Peritumor Astrocytes

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

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

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

967 **Figure 2-5.** Astrocyte Receptor Genes

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

969 **Figure 4-1.** Differential Gene Expression in FCD Astrocytes

970 **Figure 4-2.** Genes *Upregulated* Across Maturation

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

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

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

974

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Figure 1

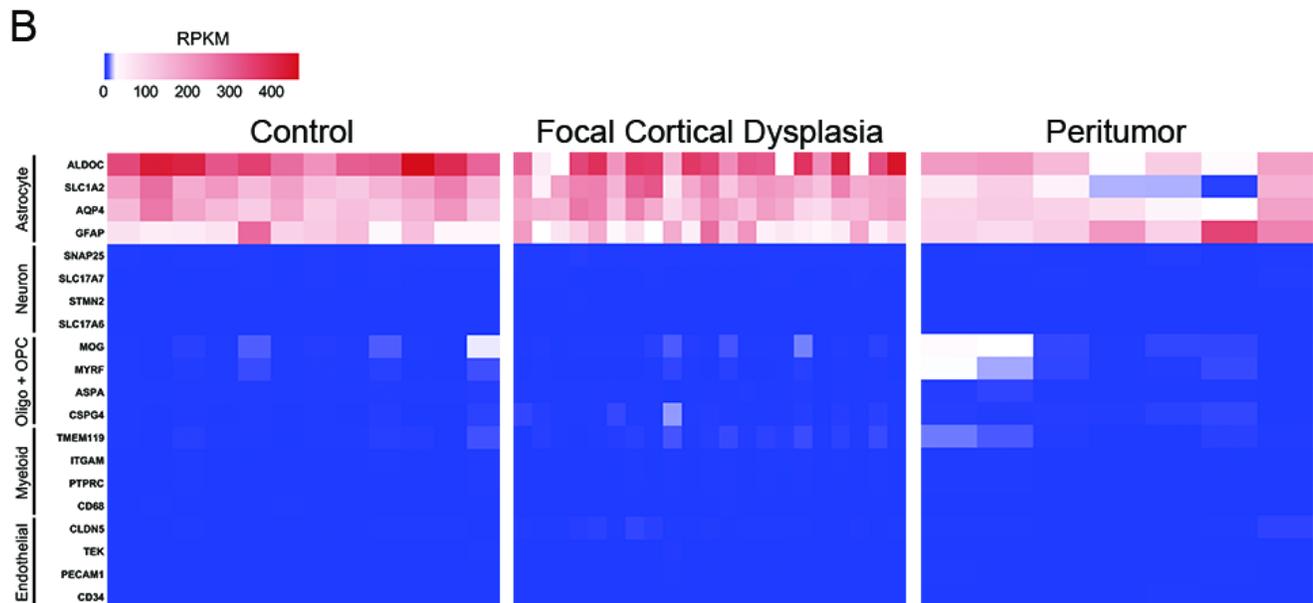
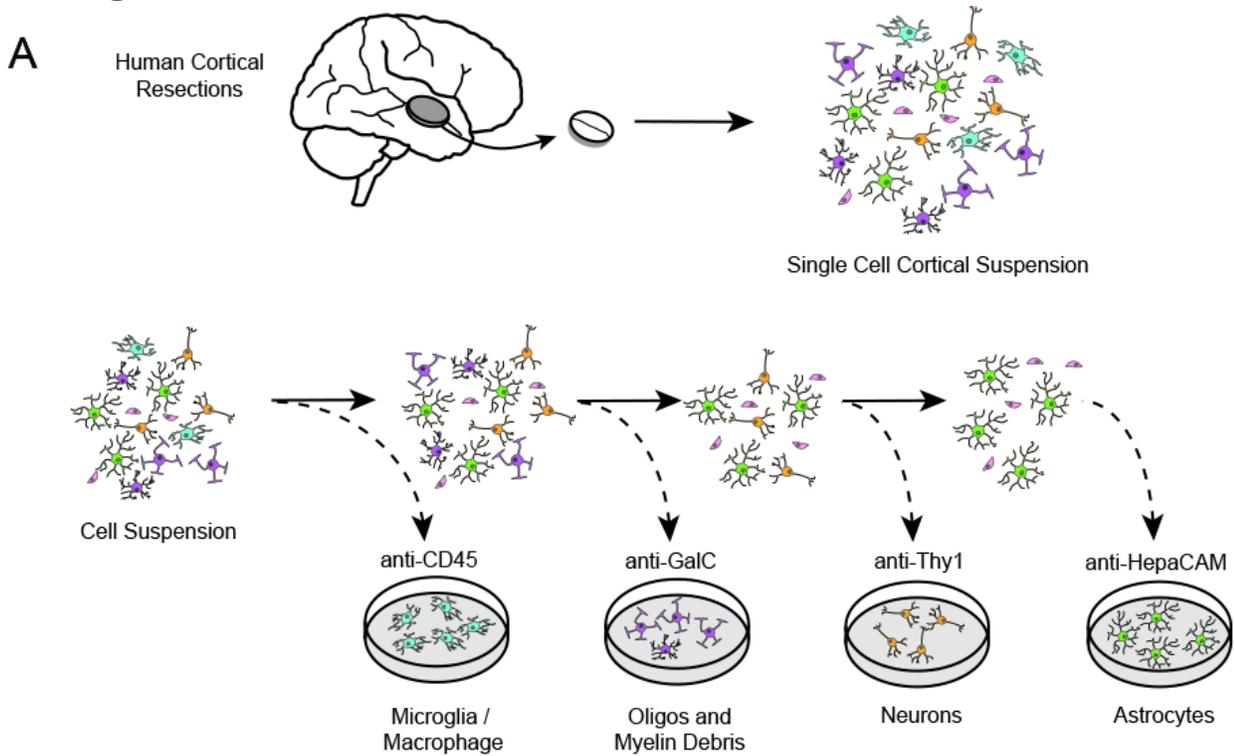


Figure 2

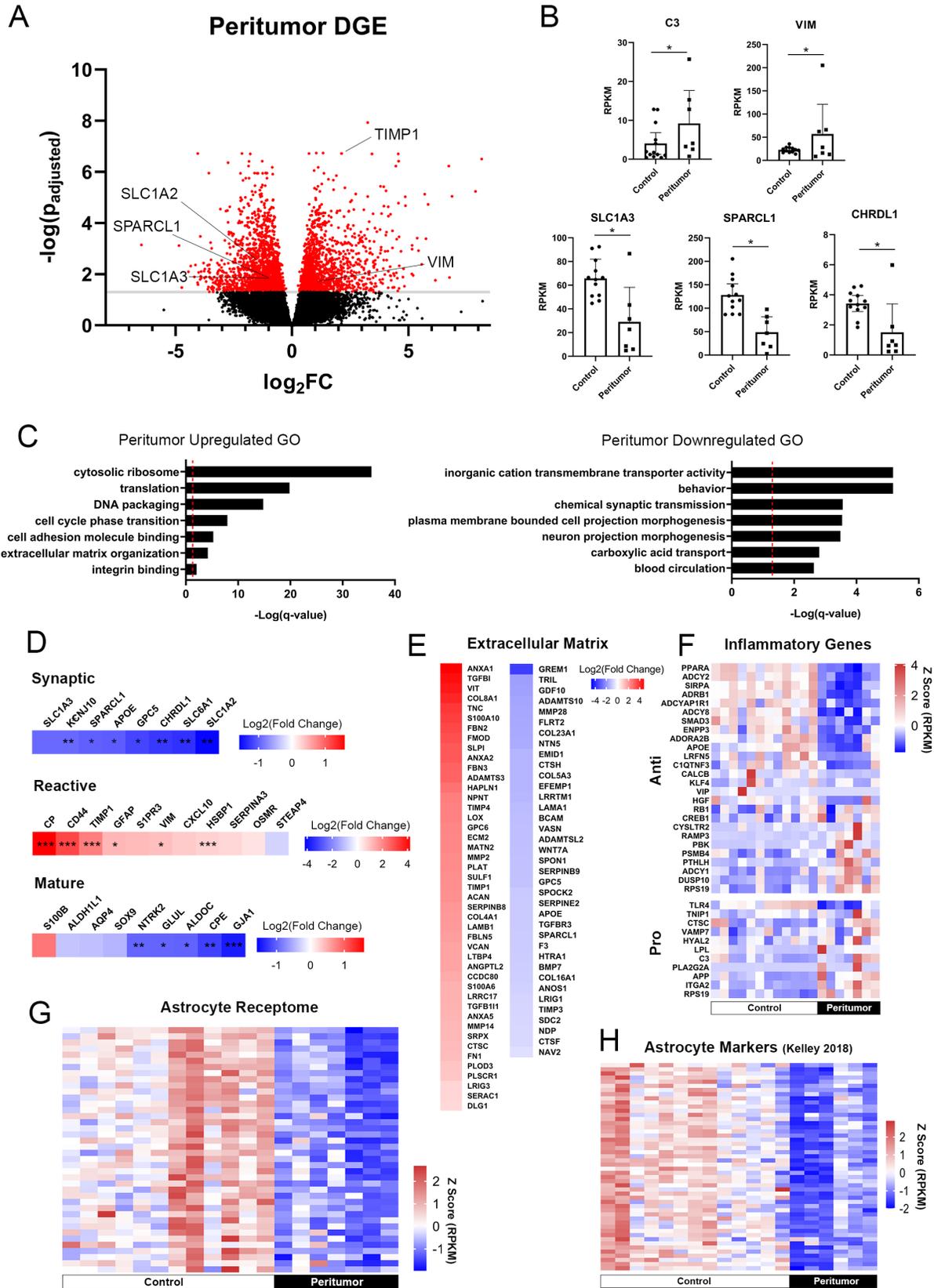


Figure 3

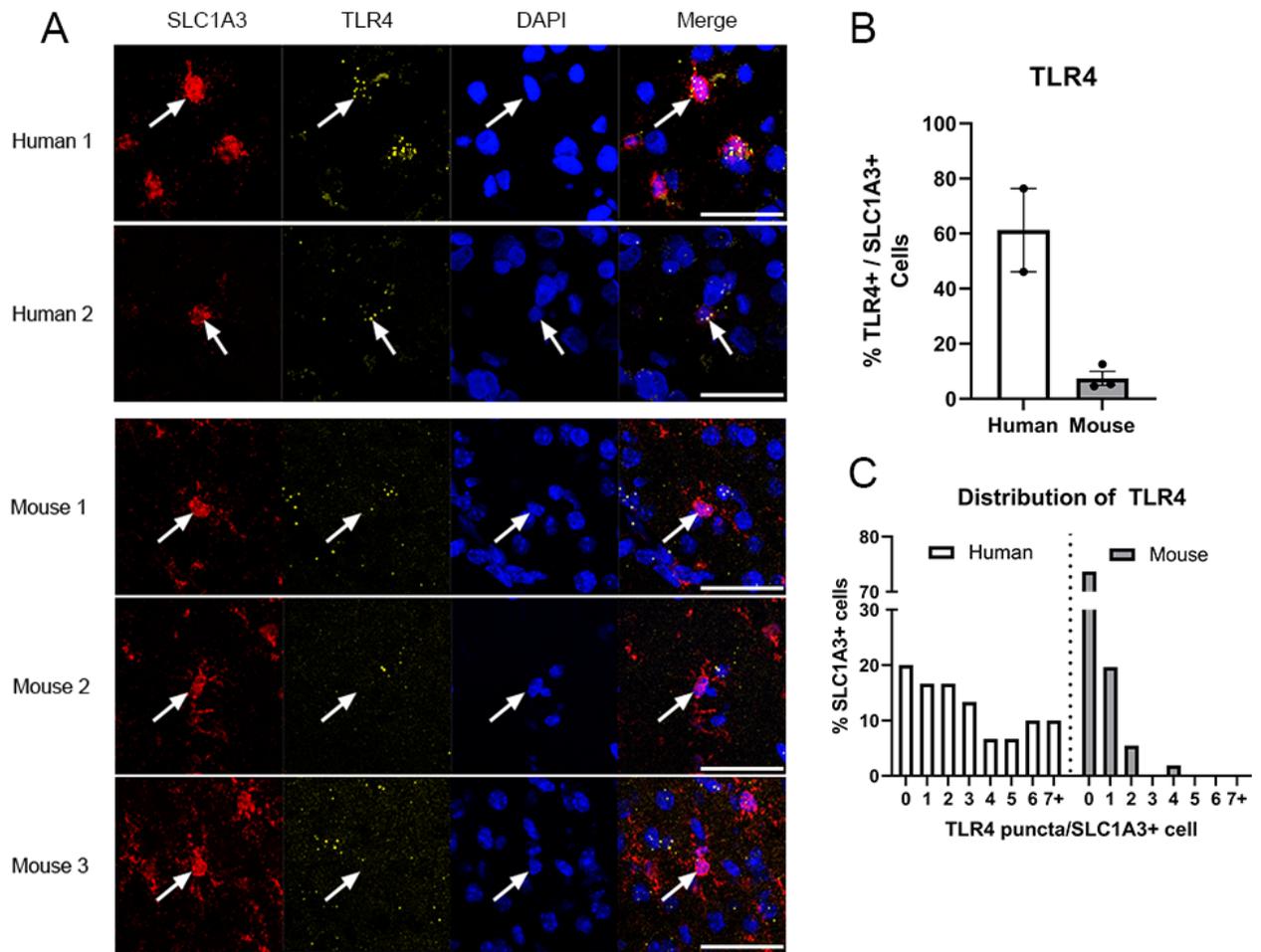


Figure 4

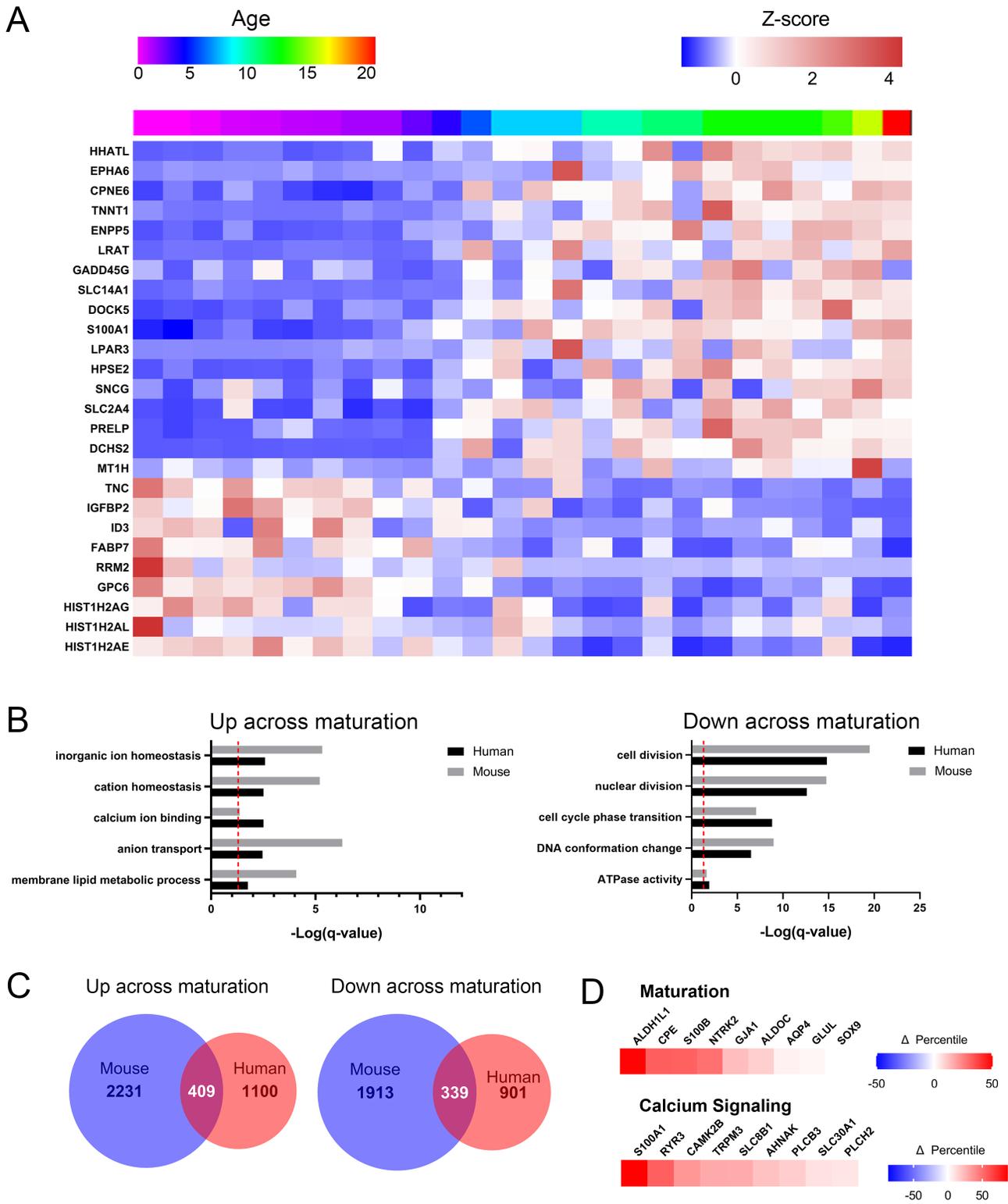


Figure 5

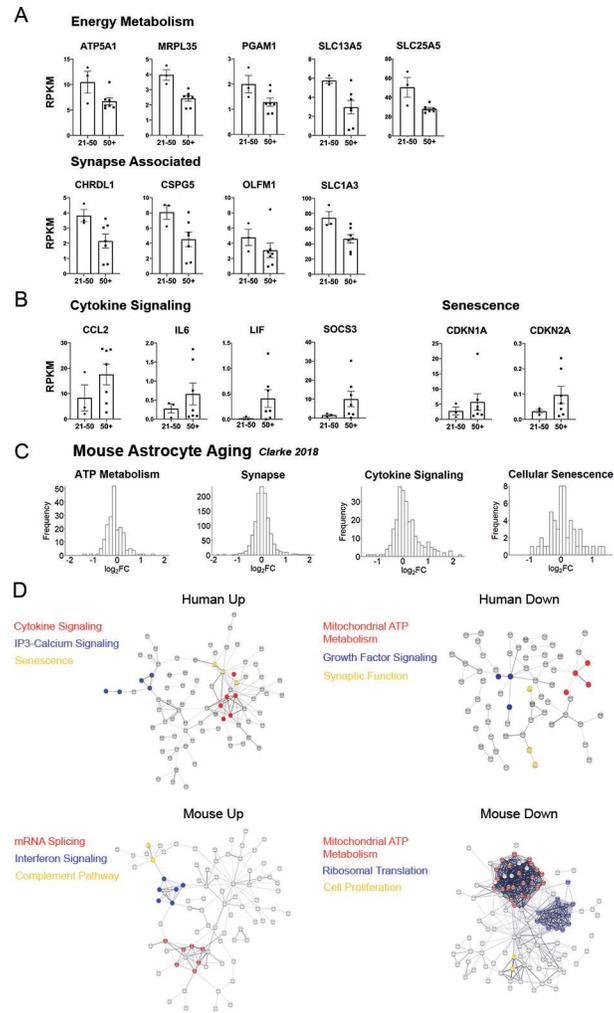


Figure 6

