ONLINE METHODS

Animal experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of KU Leuven and were performed under the relevant Ethical Permission. C57BL/6J male mice aged to post-natal day (P)56 were used throughout the study.

Preparation of a single cell suspension. Preparation of the single cell suspension is described in detail in the Supplementary Text. Briefly, regions of interest (cortex and hippocampus) were quickly and carefully dissected in cold HBSS buffer without Ca^{2+} and Mg^{2+} (Sigma), under a binocular microscope. Myelinated parts were discarded, to decrease the amount of debris in the final cell suspension. Cortical cell suspensions were prepared from two littermate animals in parallel using separate tubes. Two hippocampal cell suspensions were prepared, each using four littermate animals, in two independent reactions. Tissue dissociation was run as previously described[1]. Briefly, tissue was dissociated using the neural tissue dissociation kit (P) (Miltenyi Biotech). Tissue was digested at 37°C using papain, supplemented with DNAse I. Tissue was mechanically dissociated using three rounds of trituration with 5 ml serological pipettes. The resulting suspension was then filtered through a 20 μm Nitex mesh (SEFAR) to remove any remaining clumps. Myelin and cell debris contamination was removed by equilibrium density centrifugation. 90% Percoll PLUS (Life Sciences) in 1x HBSS with Ca^{2+} and Mg^{2+} (Sigma) was added to the suspension to produce a final concentration of 24% Percoll. Additional DNAse I (Worthington) was added (125 U per 1 ml), before the cell suspension was centrifuged at $300g_{Av}$ for 11 minutes at room temperature (with minimal centrifuge braking). The resulting cell pellet was re-suspended in PBS (without Ca^{2+}/Mg^{2+}) containing 0.5% BSA (Sigma). Supernatants were centrifuged again at $300g_{Av}$ for 10 mins at room temperature. Any pelleted cells were re-suspended in 0.5% BSA/PBS (without Ca^{2+}/Mg^{2+}). Cells were pooled and FACS sorted.

FACS isolation of astrocytes: All steps were performed at 4°C. Cells were incubated with FcR (Miltenyi Biotec) blocking reagent at a 1:9 dilution for 10 min to block non-specific binding of antibodies. This was followed by addition of antibodies specific to the cell isolation protocol. ACSA-2-PE antibody (Miltenyi Biotec, 130102365) (1:140 dilution) and O1-eFluor660 (eBioscience, 50-6506-80) (1:810 dilution) were added to the cell suspension and incubated for 10 min. 0.5% BSA/dPBS (without Ca^{2+}/Mg^{2+}) was then added to the cell suspension as a washing step. Cells were recovered by centrifugation at $300g_{Av}$ for 10 min. The resulting pellet was then re-suspended in 0.5% BSA/dPBS and filtered through a 20 μm Nitex mesh. The vital dye 7-AAD (eBioscience, 00-6993) (1:100 dilution) was added to exclude dead cells during FACS.

FACS was performed on a BD FACSAria III using a 100 µm nozzle. Compensations were done on single-color controls, and gates were set on unstained samples. Forward scatter (FSC)/Side scatter (SSC) gating was used to remove clumps of cells and debris. Single ACSA-2-PE-positive/O1-eFluor660-negative/7-AAD-negative astrocytes were sorted into separate wells of unskirted 96 well PCR plates (VWR). Each plate also contained 1 well without any cell(s) (negative control), 1 well with 40 astrocytes (positive control: astrocytes) and 1 well with 40 cells negative for 7-AAD (positive control: viable cells). Each well contained 4.3 µl of lysis buffer composed of 2.3 µl 0.2% Triton X-100 (Sigma) with 2 U μl^{-1} RNase inhibitor (Clontech), 1 µl of HPLC-purified 10 µM Oligo-dT30VN oligonucleotide (AAGCAGTGGTATCAACGCAGAGTACT_{30}VN) and 1 µl of dNTP mix (Fermentas). Plates were kept at 4°C during the sort, sealed immediately afterwards, vortexed and spun down at 300g_{Av} for 30s. Plates were stored at -80°C until library preparation.

Single cell cDNA and library preparation. We used a modified Smart-seq2 protocol with cDNA and sequencing libraries prepared as described[2], with necessary modifications. Briefly, samples were reverse transcribed according to the standard protocol with minor modifications. ERCC (External RNA Controls Consortium) control RNAs (ThermoFisher Scientific, 4456740) were added into the reverse transcription mix at a final dilution of 1:160x10^6. TSO (template switching oligonucleotide) (AAGCAGTGGTATCAACGCAGAGTACATrGrG+G in which the last guanosine is a locked nucleic acid: LNA) was used at 0.2 µM in the final reaction mix. Subsequent pre-amplification of cDNA used an ISPCR oligonucleotide (AAGCAGTGGTATCAACGCAGAGT) and 22 PCR cycles. cDNA was purified from the PCR mix using Agencourt Ampure XP beads (Beckman Coulter) with a modified bead:DNA ratio of 0.8 to 1. The quality of cDNA was checked by analyzing 11 single cell libraries from each 96 well plate using a NGS Fragment High Sensitivity Analysis Kit (Advanced Analytical) and a Fragment Analyzer (Advanced Analytical). Data were analyzed using PROSize 2.0 software. The cDNA concentration was measured in every well using a Quant-iT PicoGreen dsDNA Kit (Invitrogen), using a standard protocol. A Synergy 2 plate reader controlled by Gen5 software (BioTek) was used to measure fluorescence.

Libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina) with 4 sets of Nextera XT v2 index kits (sets A to D) (Illumina), using a standard protocol with minor modifications. Tagmentation was run on 0.125 ng cDNA (adjusted to a final volume of 1.25 µl) in a reaction mixture containing 2.5 µl Tagment DNA buffer and 1.25 µl of Amplicon Tagment Mix. This was followed by PCR amplification of adapter-ligated fragments, using a reaction mix consisting of 6.25 µl of Tagmentation product, 3.75 µl of Nextera PCR Master Mix and 1.25 µl of each Index primer (N7xx and N5xx). PCR was run using a standard program consisting of 12 cycles. Libraries prepared with 4 different sets of index kits were then pooled

and cleaned using Agencourt Ampure XP beads (Beckman Coulter). DNA was mixed with beads at a 1:0.6 ratio. Following an 8 min incubation, beads were recovered using a magnetic stand, supernatant was removed and beads were washed twice with 80% ethanol. Beads were then dried for 10 min before DNA was eluted in 50 µl of EB buffer (Qiagen). Bead purification was repeated a second time using a 1:1 DNA:bead ratio. Size distribution of library pools was checked using a Fragment Analyzer and a NGS Fragment High Sensitivity Analysis Kit, according to standard protocols (Supplementary Figure 6).

Library pools were sequenced (75 bp paired-end reads) using a NextSeq 500 system (Illumina) and a NextSeq 500/550 High Output Kit v2 (150 cycles) (Illumina). Libraries were sequenced on average to a depth of $2.1x10^6$ reads per library.

Analysis of RNA sequencing data. An initial quality check of sequenced libraries was undertaken using FASTQC 0.11.4 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). STAR 2.5.2b software[3] was used to map sequencing reads against Release M12 (GRCm38.p5) of the mouse reference genome (Gencode: https://www.gencodegenes.org), modified to take ERCC sequences (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_095047.txt) into account. Unique read maps were identified using STAR, after the removal of non-canonical unannotated junctions and non-canonical unannotated introns (using software specific parameters). Output alignment BAM files were then merged and sorted using Samtools version 1.4[4]. RNA quality metrics was collected with Picard Tools version 1.140 (http://broadinstitute.github.io/picard). Gene counts were generated using HTSeq version 0.6.1p1[5].

Clustering was done with Seurat[6] version 1.4.0.16, run on RStudio version 1.0.136, using R version 3.4.0. Data was normalized using the default Seurat method. High level cell type identification was performed with a starting base of 24,761 genes expressed across 2,031 samples. 16 cells were discarded, as they did not pass the Seurat default expression threshold, leaving 2,015 cells on which further analysis was performed. 5,455 highly variable genes ($\ln(\text{mean expression}) > 0.3$ and $\ln(\text{variance/mean}) > 0.1$) were identified and used for clustering with the default Seurat pipeline. Statistically significant Principle Components (PCs) 1-9 (having p-values $< 10^{-25}$ and showing a saturating standard deviation elbow) were chosen for high-level clustering. After discarding all other higher order cell types, astrocytes were reclustered using the default Seurat method. Analysis was performed based on the expression of 13,087 unique genes across 1,811 astrocytes. 886 highly variable genes ($\ln(\text{mean expression}) > 0.3$ and $\ln(\text{variance/mean}) > 0.2$) were identified and used for clustering. PCs 1-9 (having p-values < 0.075) and PC10 (having a p-value equal to 1 but being necessary for saturation of the standard deviation elbow) were chosen for astrocyte clustering. Subsequent

identification of genes overexpressed in the astrocyte subtypes, including specific marker genes, was also performed using the default Seurat pipeline. Genes were identified using a number of criteria. First, only significantly up-regulated genes ($p < 0.01$) were considered. Second, genes had to be at least 1.28-fold overexpressed in the subtype of interest (when compared to other astrocytes). This number was empirically chosen to give the best compromise between the number of marker genes identified in each subtype that allowed functional annotation relative to background noise. Finally, markers had to be expressed in more than 25% of the cells identified as belonging to a particular subtype. These marker genes were further used for gene-enrichment and functional annotation analysis. Note that the AST2 marker *Unc13c* was found using the default PAGODA differential gene expression analysis pipeline[7]. It has a ln(mean expression) of 0.28, and a ln(variance/mean) of 0.41. Although it was excluded from our Seurat analysis, it remains the marker of choice for AST2, due to its remarkably high specificity.

Gene-enrichment and functional annotation analysis (GO[8], KEGG[9] and BioCarta[10]) of subtype overexpressed genes was performed using DAVID[11] version 6.8 (Supplementary Table 3; Supplementary File 3). All genes detected in astrocytes (13,087) were used as the background gene set. Only pathways with p-values < 0.1 (EASE score; modified Fischer's Exact Test) were taken into consideration. Additionally, only pathways with $p < 0.2$ (Benjamini-Hochberg test; false discovery rate (FDR) correction) were analyzed.

Genes identified as overexpressed in specific subtypes were also manually curated with the UniProt database (http://www.uniprot.org/)[12] for assignment of putative gene functions (Supplementary Tables 4-6).

Statistical information. Statistically significant PCs in the Seurat pipeline were found using 200 random samplings, each time randomly permuting 1% of genes for both the high and low level clusterings. For the identification of statistically significant marker genes (Supplementary File 1), Wilcoxon rank sum tests were run as suggested by the default Seurat pipeline[6]. The default pipeline of DAVID[11] was used to identify statistically significant functional categories and terms (Supplementary Table 3 and Supplementary File 3). Manual analysis by Uniprot[12] was performed on statistically significant genes identified by Seurat (Supplementary File 1).

Fluorescence *in situ* hybridization. The RNAscope *in situ* hybridization system (ACD) was used, according to standard instructions, with minor modifications. Briefly, brains of P56 C57BL/6J male littermates were quickly frozen in Optimum Cutting Temperature (OCT) compound (Tissue-Tek), using isopenthane chilled with liquid nitrogen. 10 µm thick brain slices were prepared using a NX70 cryostat (Prosan). Sections were subsequently fixed in ice-

cold 4% PFA for 30 min. Sections were then dehydrated using a series of ethanol solutions (50% - 100%), before drying and incubating with Protease IV for 20 min at room temperature. Slides were washed in PBS and hybridized with gene specific probes (Supplementary Table 7) for 2h at 40°C in a HybEZ Oven (ACD). Non-annealed probes were removed by washing sections in 1x proprietary wash buffer. Probe signal was then amplified via sequential hybridization of proprietary amplifiers and labelled probes (Amp 1 – Amp 4). Finally, sections were stained with DAPI and mounted using ProLong Diamond Antifade Mountant (Life technologies).

Imaging and data analysis. Sections were imaged using an Axio Scan Z1 microscope (Zeiss), operated by Zen 2.3 software (Zeiss). Images were acquired using standard excitation and emission filters. Images in Figure 2, Figure 3 and Supplementary Figure 19 were taken in the best focal plane using a 20x objective. Figures 4a and 4b are maximum projection images produced by collapsing images from 4 consecutive z-planes acquired using a 40x objective. Off-line analysis was performed using Zen 2.3 lite software (Zeiss). Individual astrocytes were identified based on colocalization of DAPI staining with the astrocyte specific probe, *Slc1a3*. Astrocytes subtypes were further identified based on the expression of specific marker genes.

Figure preparation. Figures were prepared using Inkscape 0.92.2, GIMP 2.8.22, Adobe Photoshop CS6 13.0.1 and Adobe Illustrator CS6 16.0.3.

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