PNAS

Supporting Information for

Circadian clock protein BMAL1 broadly influences autophagy and endolysosomal function in astrocytes

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SI Appendix

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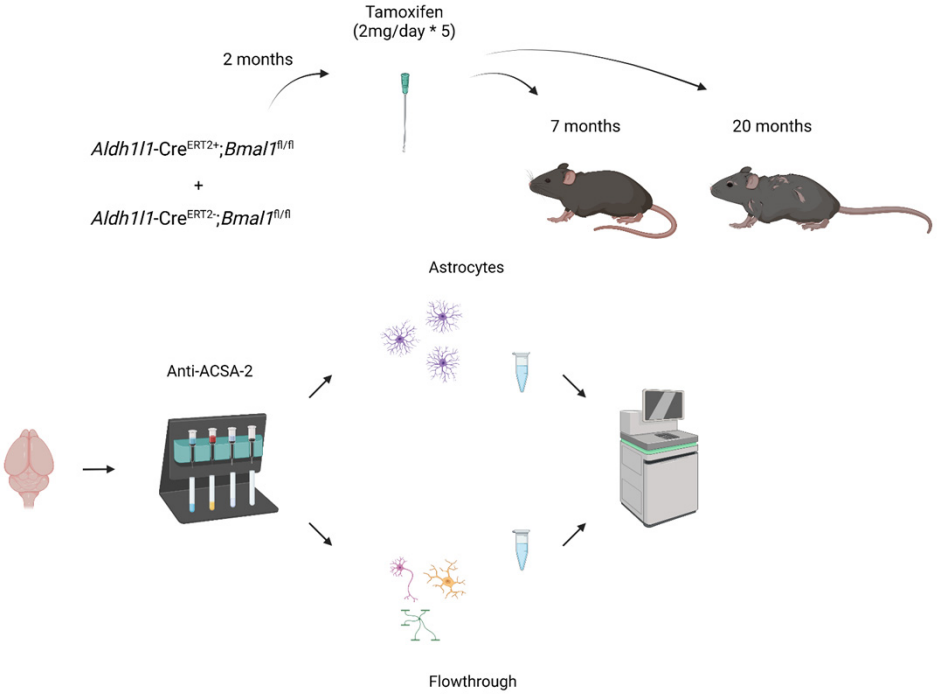

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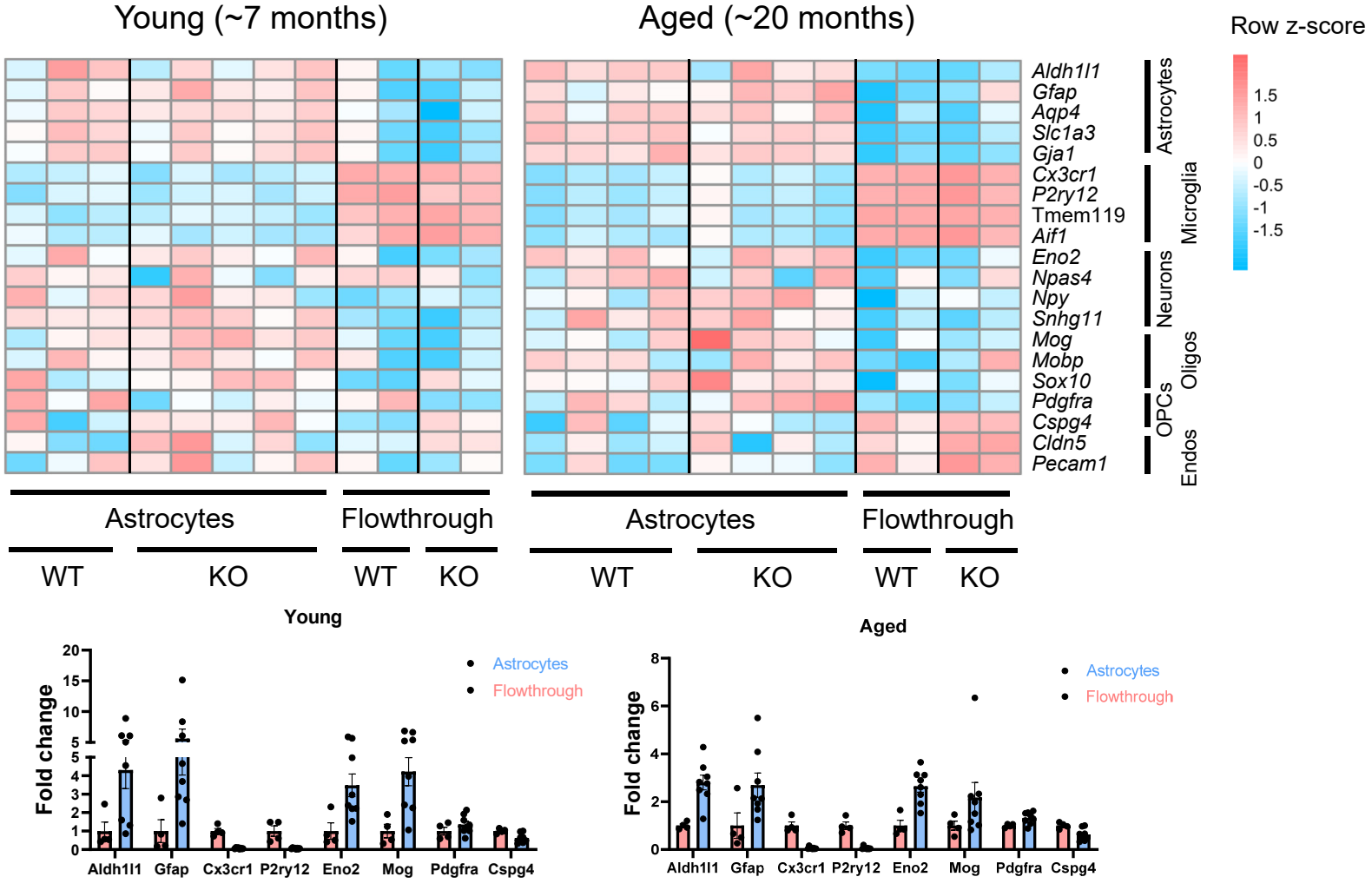

Figure S1. Magnetic microbead isolation of adult astrocytes

A) Schematic of Bmal1 aKO mouse generation and tamoxifen treatment for astrocyte isolation. Mice treated with tamoxifen at 2 months of age were aged to 7 or 20 months, after which whole brains were used for magnetic bead sorting of astrocytes and "flowthrough" samples for RNA sequencing. Created with Biorender.com. B) Top: heatmaps of cell-type specific markers expressed in astrocyte and "flowthrough" samples. Bottom: RNAseq individually plotted expression of cell-type purity markers, normalized to flowthrough samples.

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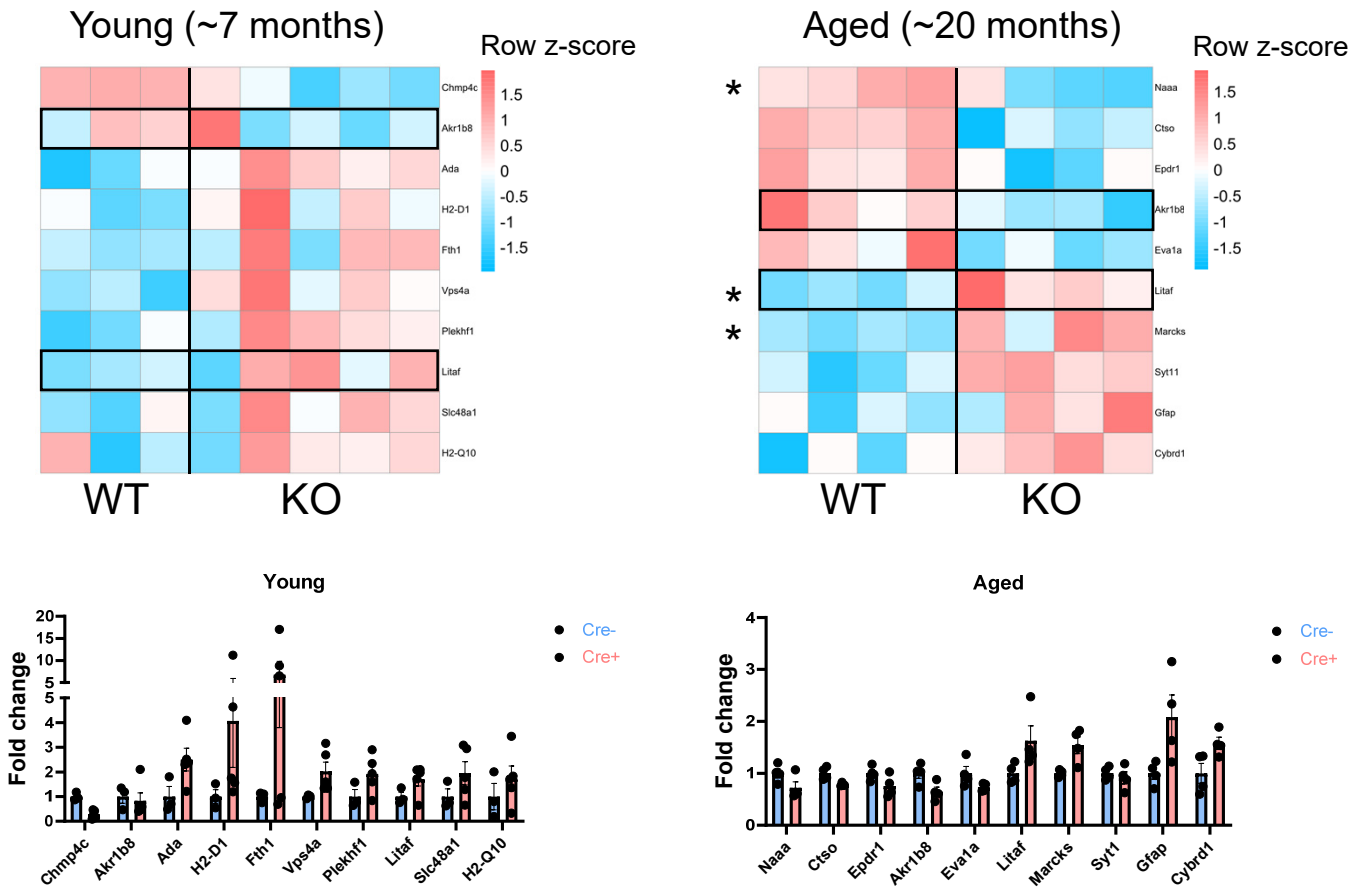

Figure S2. Top lysosome pathway genes from isolated astrocytes
A) Top: Heatmaps of the top 10 differentially expressed genes within the "lysosome" GO cellular component pathway. Boxes around *Akr1b8* and *Litaf* indicate their differential expression in both the young and aged astrocyte analyses. * = FDR < 0.15. Bottom: RNAseq individual plotted expression of lysosome genes, normalized to Cre- controls.

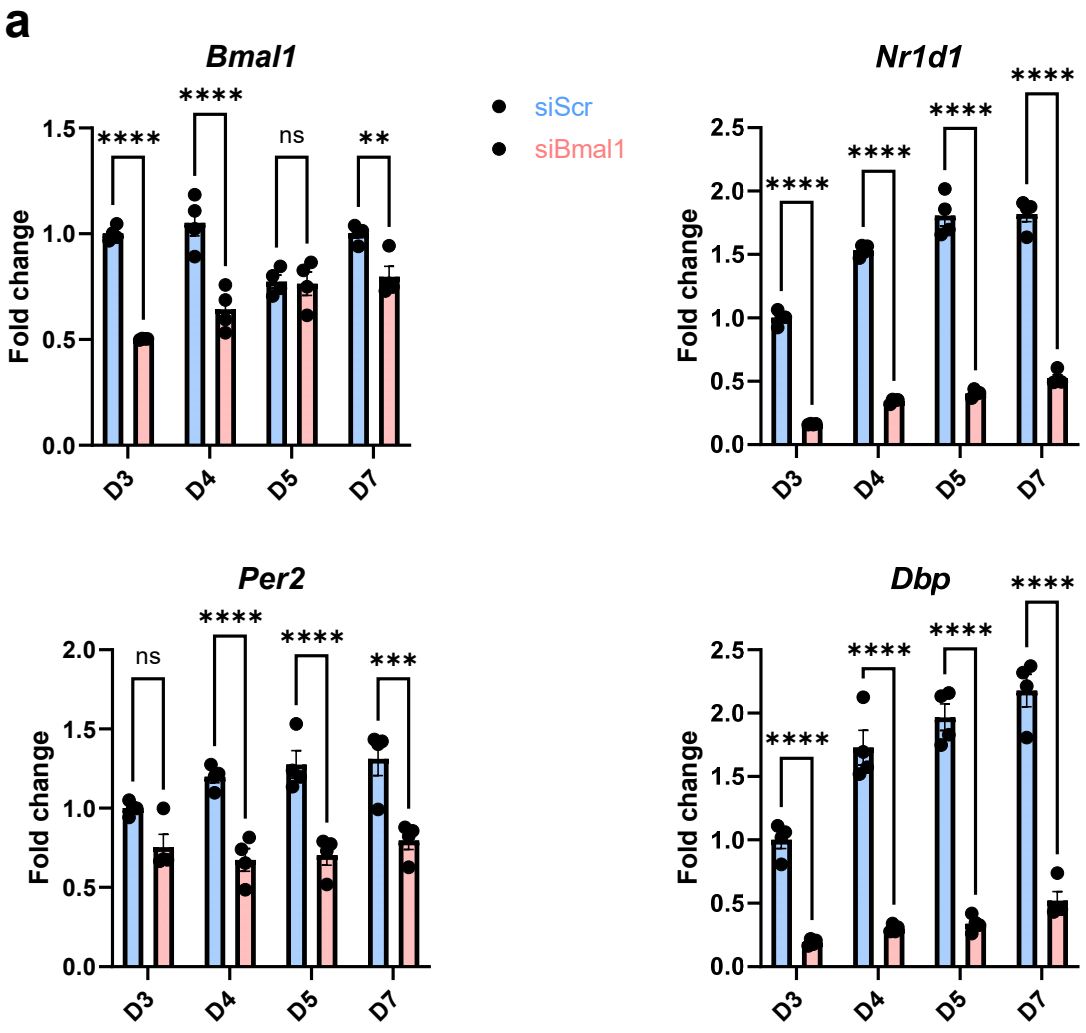

Figure S3. Bmal1 deletion in astrocytes perturbs clock gene expression *in vitro*
A) Fluidigm qPCR analysis of circadian clock gene expression for samples of primary cultured astrocytes at 3-7 days post-transfection with siScramble or siBmal1. N= 4 wells per condition, ** =p<0.005, *** =p<0.0005, **** =p<0.0001 by two-way ANOVA with Sidak multiple comparisons test.

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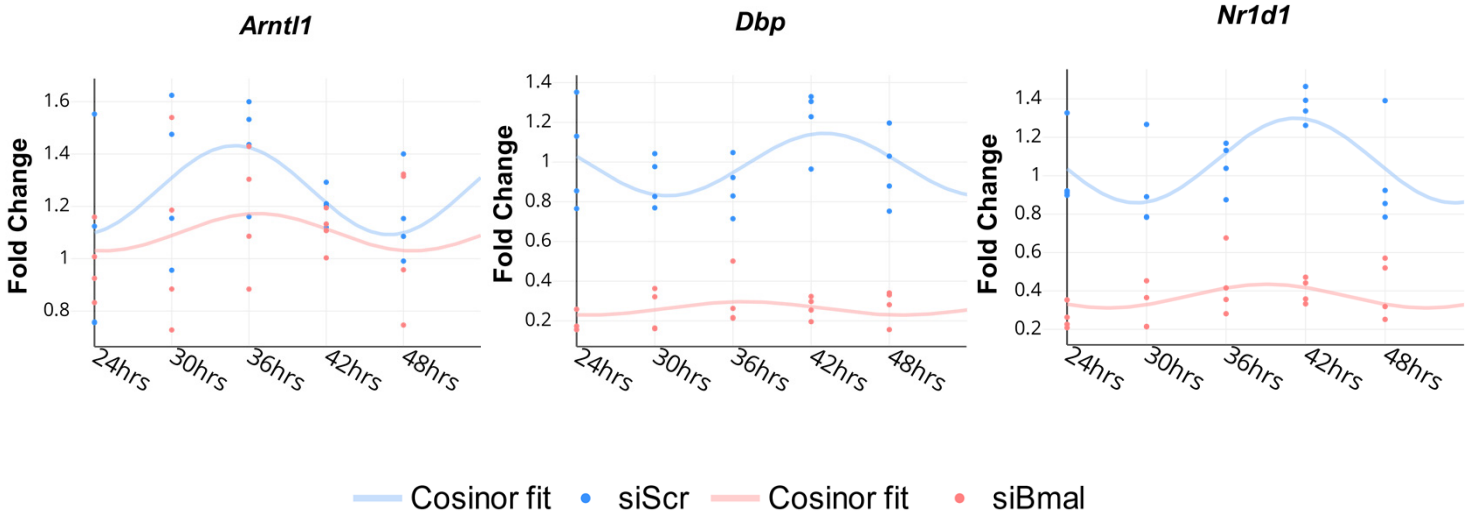

Figure S4. Cultured astrocytes show rhythmic transcription
A) qPCR quantification of clock gene expression in primary astrocytes for the same experiments as Figure 2c. Astrocyte RNA was collected at listed time points after synchronization by media change . N= 3-4 pups over 2 independent experiments (some overlapping points not visible), expression values normalized to siScramble 24-hour group. Nitecap expression analysis: siScramble Arntl1 cosinor p=0.087. siScramble Dbp cosinor p=0.081. siScramble Nr1d1 cosinor p=0.018.

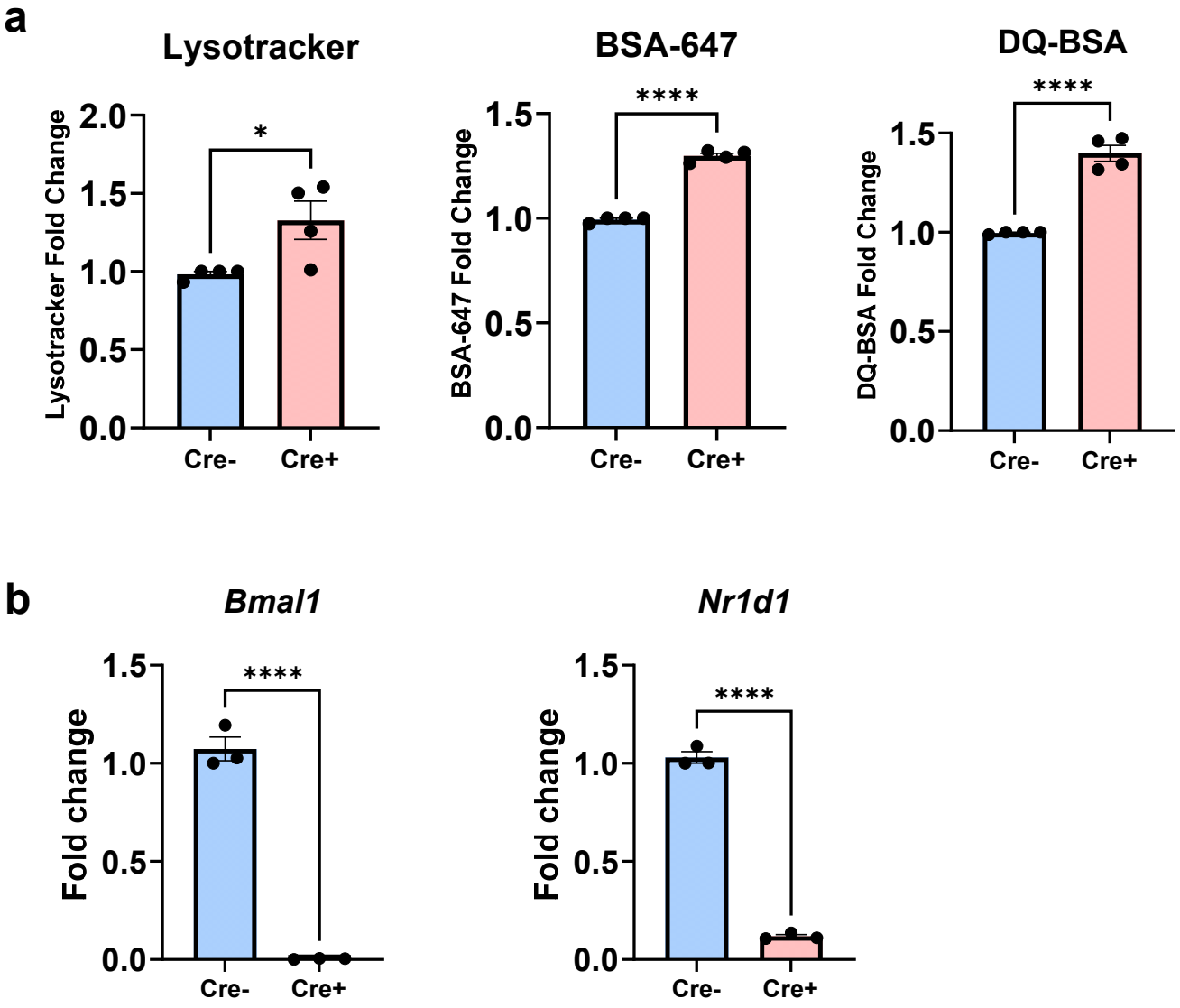

Figure S5. Bmal1 deletion via tamoxifen-induced Cre recombination elevates endolysosomal function

A) Flow cytometry quantification of Lysotracker, BSA-647 and DQ-BSA signal in tamoxifen-treated treated CAG-Cre^{ERT2-} Bmal1$^{fl/fl}$ and CAG-Cre^{ERT2+} Bmal1$^{fl/fl}$ astrocytes. N=4 independent experiments, MFI normalized per experiment to Cre- controls. B) qPCR validation of clock gene disruption in tamoxifen-treated CAG-Cre^{ERT2-} Bmal1$^{fl/fl}$ and CAG-Cre^{ERT2+} Bmal1$^{fl/fl}$ astrocytes. N=3 independent experiments, fold change normalized to Cre- controls. A-B) * =p<0.05, ** =p<0.005, *** =p<0.0005, **** =p<0.0001 by t-test.

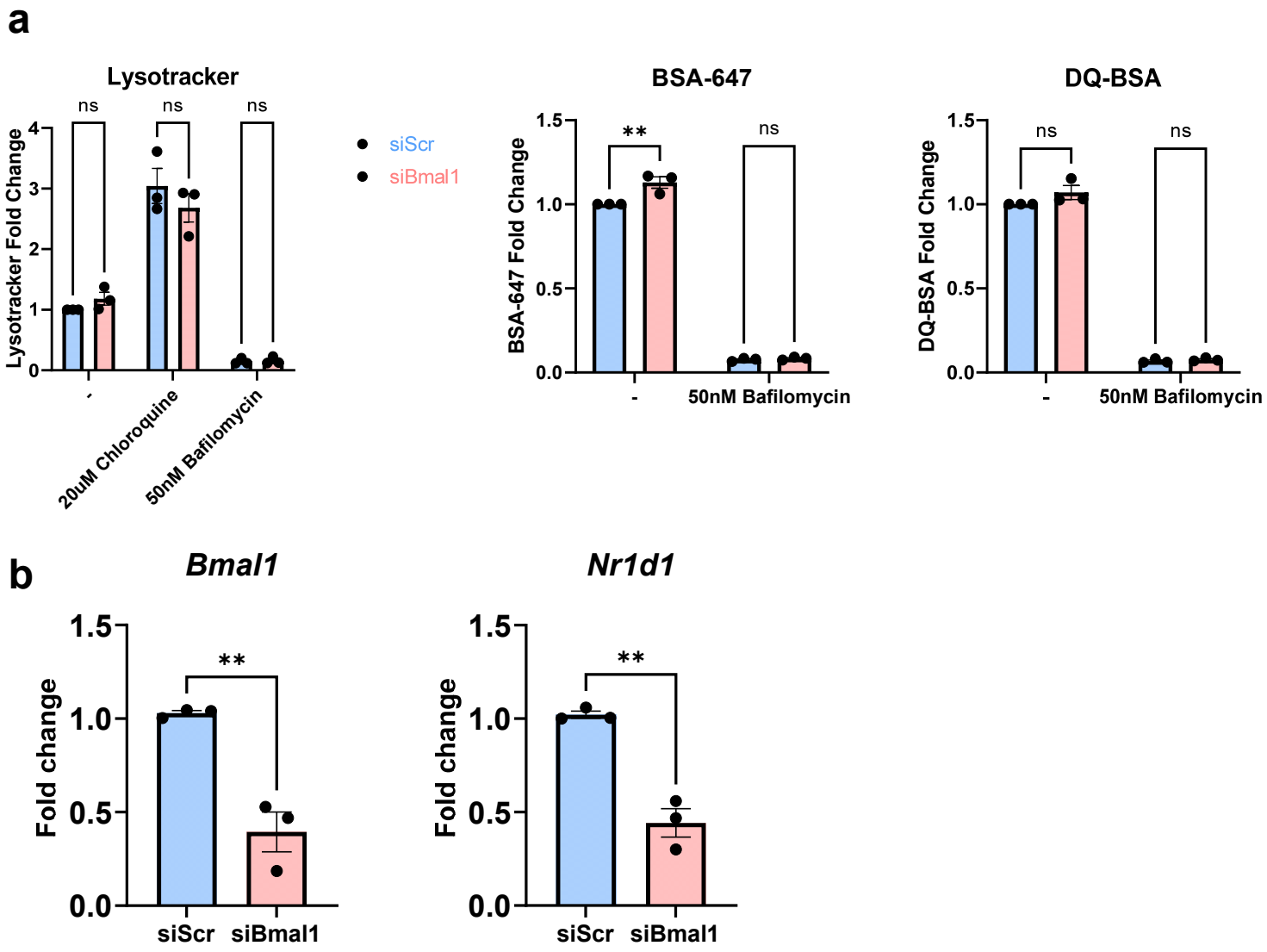

Figure S6. Bmal1 deletion in microglia mildly increases protein uptake, but not lysosomal function
A) Flow cytometry quantification of Lysotracker, BSA-647 and DQ-BSA signal in siScramble and siBmal1 microglia treated with serum-free medium, 20μM Chloroquine, or 50nM Bafilomycin for 3 hours. N=3 independent experiments, MFI normalized per experiment to serum-free media-treated siScramble controls. ** =p<0.005 by two-way ANOVA with Sidak multiple comparisons test. B) qPCR validation of clock gene disruption with siBmal1 in microglia. N=3 independent experiments, fold change normalized per experiment to siScramble controls. **=p<0.005 by t-test.

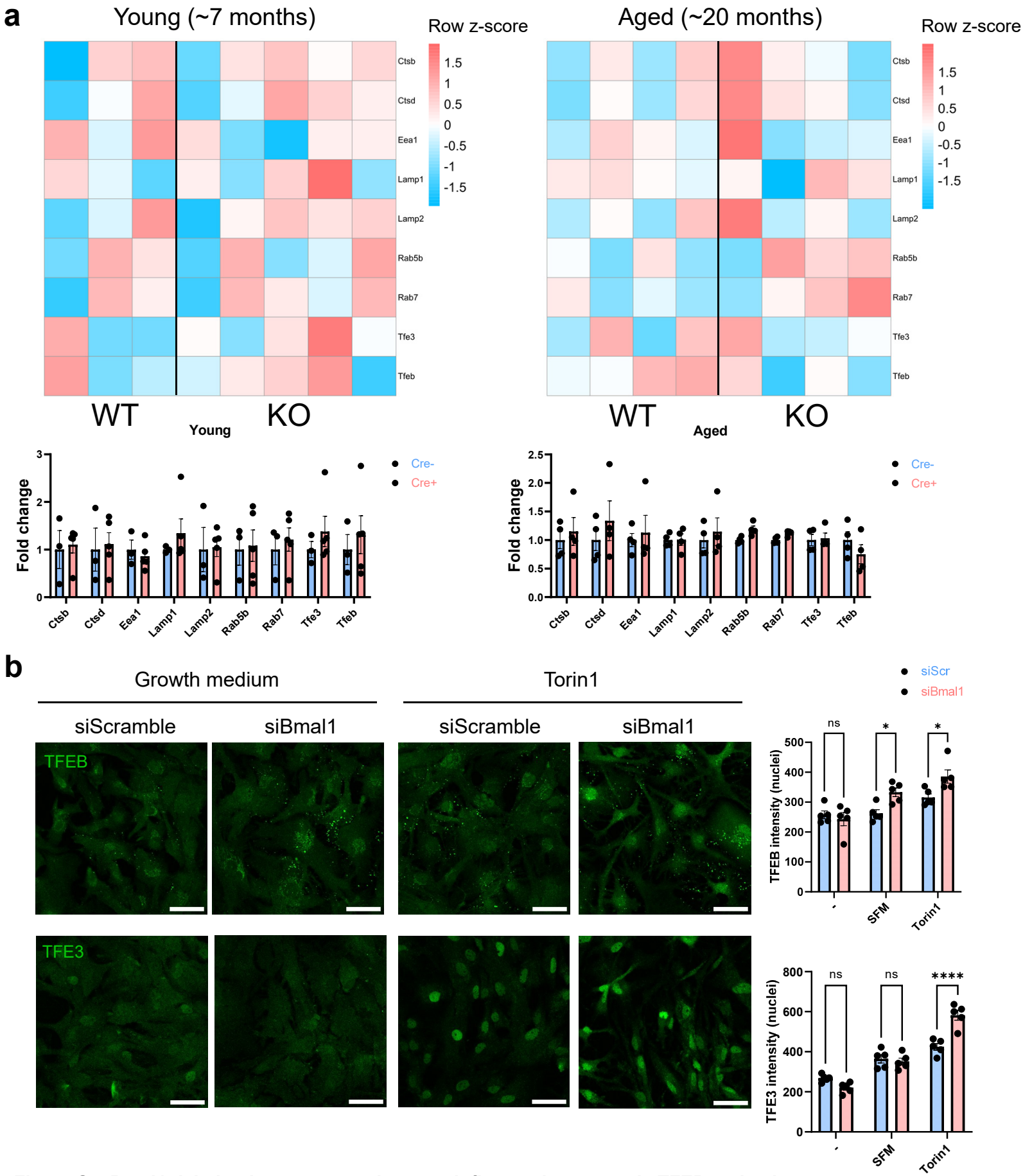

Figure S7. Bmal1 deletion in astrocytes does not influence homeostatic TFEB activation

A) RNAseq analysis comparing isolated astrocytes as shown in Figure 1. Top: Heatmap of genes listed represent TFEB, TFE3, or their target genes including lysosomal proteases, lysosomal membrane proteins, and GTPases. Bottom: RNAseq individual plotted expression of TFEB-related genes, normalized to Cre- controls. B) Confocal imaging and quantification of TFEB and TFE3 in the nuclei of transfected astrocytes treated with normal growth medium or Torin1 in SFM for 3 hours. Scale bar =50μm. N=5 wells per condition, representative of 3 independent experiments. * =p<0.05, **** =p<0.0001.

SI: Methods

Microglial Cultures:

For microglia, the same methods as were used to generate mixed glia cultures from P2-3 pups as described in Methods, but with the addition of 5ng/mL GM-CSF (Biolegend) to the growth medium which was maintained in all subsequent media changes until siRNA transfection (see below). After reaching 100% confluence (~10 days), cells were shaken in a heated shaker at 225RPM for 2 hours to lift adherent microglia. Medium containing detached microglia was then collected and immediately plated onto PDL-coated plates for experiments.

Cell synchronization

Astrocytes for rhythmic experiments were plated for synchronization on separate plates for each time point. Astrocytes were synchronized via medium change with normal growth medium and returned back to a 37°C incubator. After media changes, care was taken not to shake or disturb cells until the end of the experiment. Cells were synchronized at 5 time points with 6-hour intervals for a total of 1.25 circadian cycles. Lysotracker Green was applied as described below for flow cytometry analysis. RNA was collected at the same time as flow cytometry experiments to compare transcriptional rhythms to Lysotracker data within the same batch of cells. qPCR data were imported into the Nitecap circadian analysis web application[59] for rhythm analysis.

Tamoxifen treatment

CAG-Cre^{ERT2+}, *Bmal1$^{fl/fl}$* and CAG-Cre^{ERT2-} *Bmal1$^{fl/fl}$* pups were used to generate astrocytes as described above. 24 hours after replating, astrocytes were treated with 1.5μM 4-hydroxy tamoxifen diluted in growth medium. Tamoxifen was kept on cells 3-4 days until 24 hours before an experiment, at which point cells were switched back to normal growth medium to avoid any effects of tamoxifen on assays of interest.

RNA sequencing

Sample RNA integrity was determined using a Tapestation and library preparation was performed with 10ng of total RNA for samples with a Bioanalyzer RIN score greater than 8.0. ds-cDNA was prepared using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Takara-Clontech) per manufacturer's protocol. cDNA was then fragmented using a Covaris E220 sonicator using peak incident power 18, duty factor 20%, cycles per burst 50 for 120 seconds. The cDNA was blunt ended, had an A base added to the 3' ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12-15 cycles using primers incorporating unique dual index tags. The fragments for each sample were then pooled in an equimolar ratio and sequenced on an Illumina NovaSeq-6000 using 150 base pair paired end reads. Basecalls and demultiplexing was performed with Illumina's RTA 1.9 software and the reads were aligned to the Mus musculus Ensembl release 76 GRCm38 primary assembly with STAR version 2.5.1a. Gene counts were quantitated with Subread:featureCount version 1.4.6-p5. All gene counts were then imported into the R/Bioconductor package EdgeR and TMM normalization size factors were calculated to adjust the samples for differences in library size. The adjusted TMM size factors and the matrix of counts were then imported into the R/Bioconductor package Limma. Weighted likelihoods were then calculated for all samples and the count matrix was transformed to moderated log 2 counts-per-million with Limma's voomWithQualityWeights.

Western blotting

Cells were cultured in 6-well plates and transfected as described above. On the day of collection, protease and phosphatase inhibitor tablets (Pierce) were dissolved in cold RIPA buffer (Cell Signaling). Cells were washed with dPBS and then incubated in RIPA buffer with inhibitors for 10 minutes on ice. Cell samples were scraped from wells, collected into microfuge tubes, and sonicated before storage at -80°C. A BCA total protein assay (Pierce) was performed according to instructions in order to measure total protein content. Protein samples were diluted in 4x LDS buffer and boiled for 10 minutes before gel loading. 16μg of protein was loaded into each well of a 4-12% Bis-Tris gel (Invitrogen) and run at 120V until the loading dye front reached the bottom of the gel. Protein was transferred onto a PVDF membrane (Millipore) at 30V for 90 minutes. Membranes were blocked with 3% BSA in TBS with 0.1% Triton X-100 (TBST) for 1 hour at room temperature, and then incubated on primary antibodies diluted in blocking solution overnight at 4°C (LC3, 1:1000, Novus Biologicals; p62, 1:2000, Abcam; β-tubulin, 1:2000; Invitrogen). Membranes were then washed with 0.1% TBST and incubated in secondary antibodies (anti-mouse or anti-rabbit HRP, 1:5000, Cell Signaling Technologies) diluted in blocking solution for 1 hour at room temperature. After final washing in TBST and TBS, membranes were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) and imaged on a ChemiDoc MP Imaging System (Bio-Rad). Images were imported into Image Lab software (Bio-Rad, version 6.1.0) for band density quantification. LC3 and p62 band densities were normalized using Tubulin as a housekeeping gene. For LC3I and LC3II bands, rectangular ROIs of equal size were placed manually around bands for all samples and the density was recorded (Fig. S5).

Electron Microscopy

Bmal1 aKO mice were tamoxifen-treated and housed as described above before harvesting at 7 months-of-age. On the day of perfusion, normal Ringer's solution and fixative (2.5 % glutaraldehyde, 2% paraformaldehyde, 0.15M cacodylate buffer and 2mM $CaCl_2$) were prepared and warmed to 37°C. Mice were perfused with warm Ringer's solution for 2 minutes followed by 5 minutes of perfusion with fixative. Brains were carefully removed and stored in cold fixative overnight. Brains were then delivered to the WUCCI and prepared using their standard methods. Post fixation, brain samples were cut into 100 µm thick sections with a vibratome (Leica VT1200S, Vienna, Austria). Sections containing region of interest (hippocampus) were then rinsed in 0.15 M cacodylate buffer containing 2 mM calcium chloride 3 times for 10 minutes each followed by a secondary fixation in 1% osmium tetroxide/1.5% potassium ferrocyanide in 0.15 M cacodylate buffer containing 2 mM calcium chloride for 1 hour in the dark. The samples were then rinsed 3 times in ultrapure water for 10 minutes each and en bloc stained with 2% aqueous uranyl acetate overnight at 4 °C in the dark. After another 4 washes in ultrapure water, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, 90%, 100% x3) for 10 minutes each step. Once dehydrated, samples were infiltrated with LX112 resin (Electron Microscopy Sciences) and flat embedded and polymerized at 60 °C for 48 hours. Post curing, region of interest was excised and mounted on a blank epoxy stub and 70 nm sections were then cut, post-stained with 2% aqueous uranyl acetate and Sato's lead and imaged on a TEM (Jeol JEM-1400 Plus) at 120 kV. Hippocampal astrocytes were identified as containing a round or oval nucleus, electrolucent cytoplasm, and intermediate filament bundles[36,37], which was more pronounced in Bmal1 knockout astrocytes as they express more GFAP. 3-5 astrocytes were imaged and quantified per mouse brain. An observer blinded to genotype counted electrolucent vesicles, electron-dense vesicles, and multilamellar structures in each cell.