SI Appendix

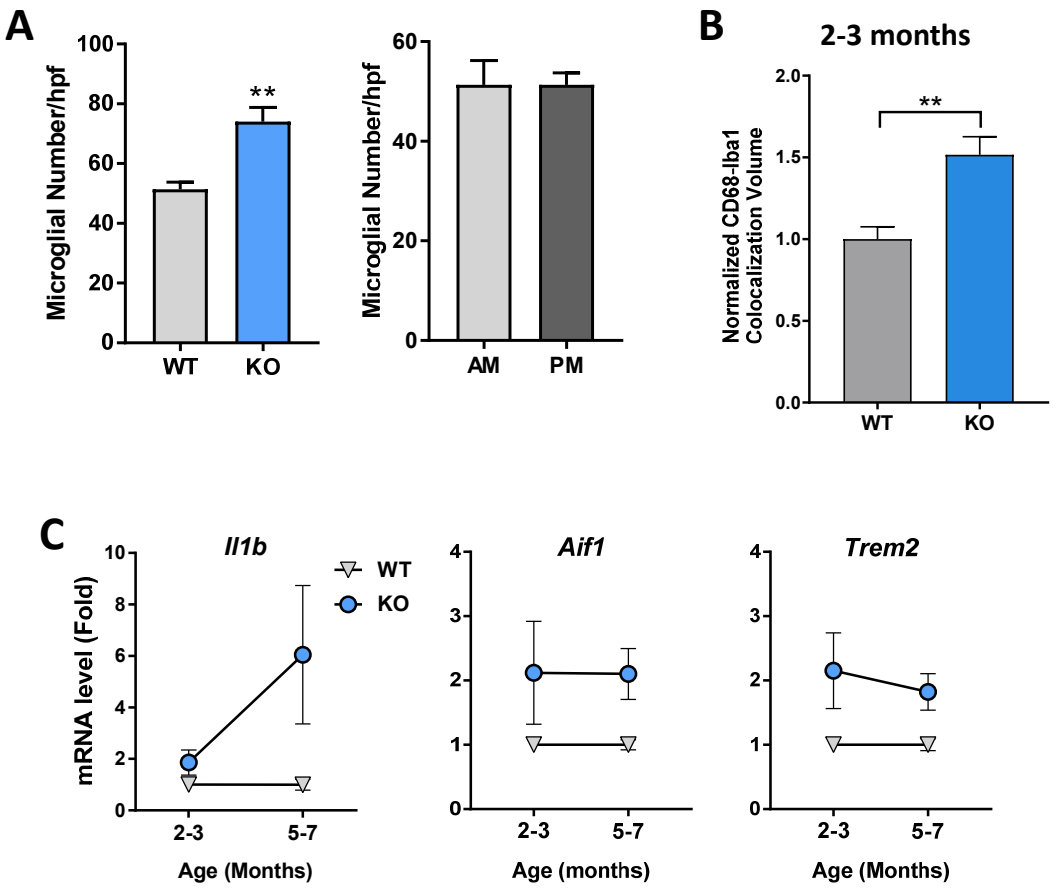

Figure S1: Rev-erbα increases microglial number and induces activation in young mice. A. Left panel: Number of Iba1+ cells per 10x field of the hippocampus from WT or Rev-erbα$^{-/-}$ mice sacrificed at CT10 (AM). N = 6 mice per group. Right panel: Number of Iba1+ cells per 10x field of the hippocampus from WT mice harvested at CT10 (AM) or CT22 (PM). B. Normalized Iba1-CD68 colocalization volumes for 2-3mo WT or Rev-erbα$^{-/-}$ mice. N = 3 mice/genotype, n = 9 40X fields of view per genotype. C. qPCR for proinflammatory marker transcripts stratified by age groups 2-3mo and 5-7 mo in WT or Rev-erbα$^{-/-}$ mice. N = 6 mice per genotype. **

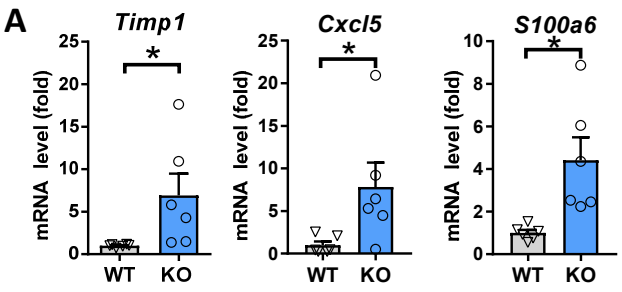

Figure S2: A. qPCR validation of selected transcripts from the microarray data in a separate cohort of mice (n=5-6/group). *p<0.05 by Mann-Whitney test.

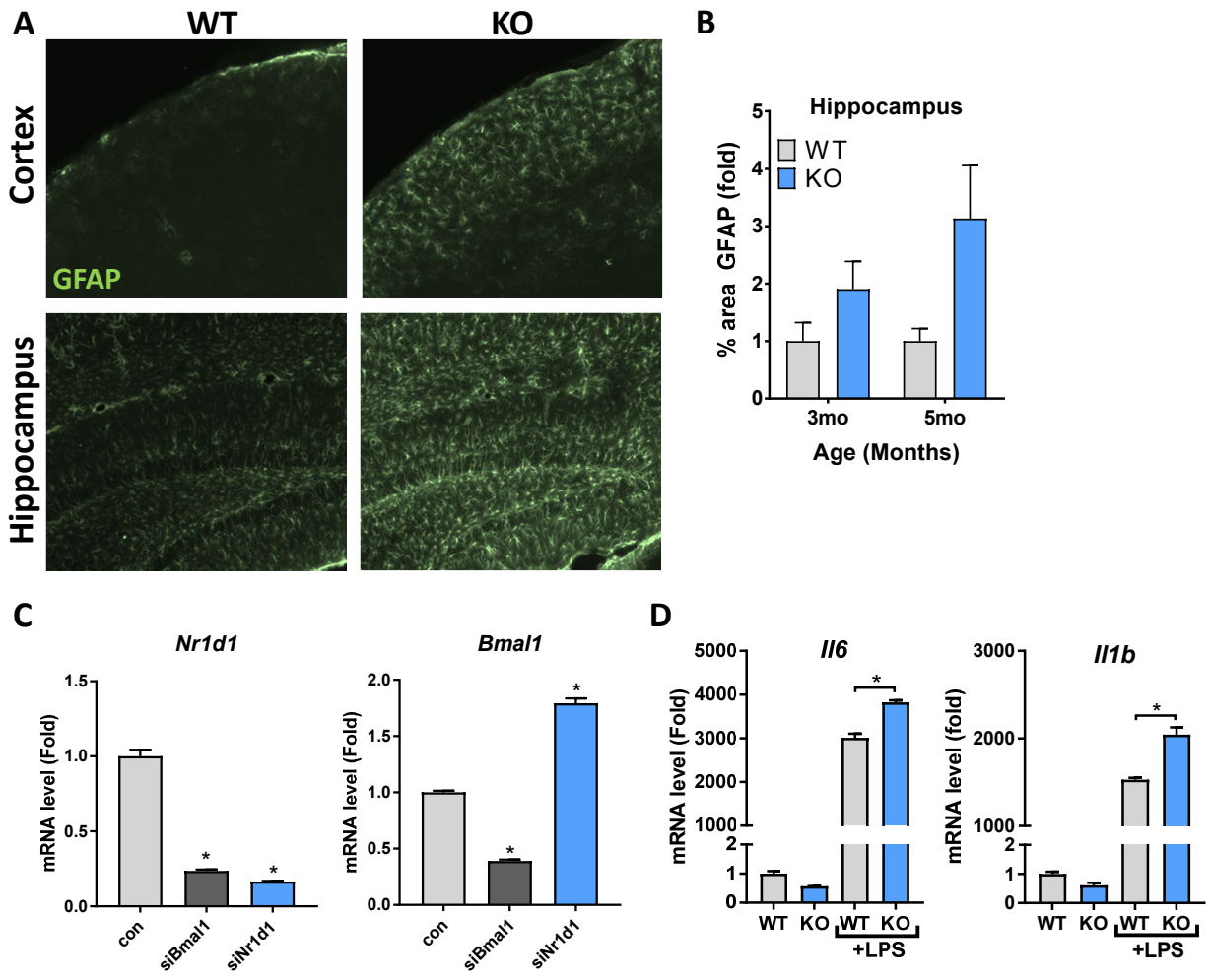

Figure S3. Rev-Erbα deletion induces astrocyte activation in vivo and alters response to LPS. A. GFAP staining in cortex (top panels) and hippocampus (bottom panels) of 3mo WT and Rev-erbα$^{-/-}$ mice shows early involvement of hippocampus and outer cortical layers. B. Quantification of percent area GFAP immunoreactivity in the hippocampus of 3mo and 5mo WT and Rev-erbα$^{-/-}$ mice. C. qPCR for *Nr1d1* and *Bmal1* mRNA expression in primary WT astrocytes treated with control siRNA as well as siRNA targeting *Bmal1* and *Nr1d1*. D. mRNA expression of *Il6* and *Ilb* in WT and Rev-erbα$^{-/-}$ astrocytes at baseline and treated with LPS. *p<0.05 by 2-tailed T-test.

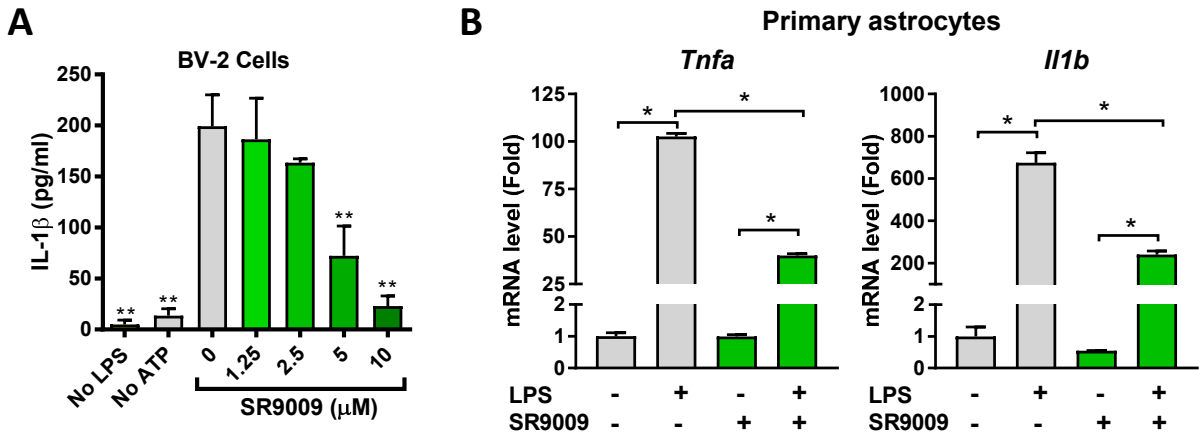

Figure S4. Rev-erb agonist mitigates proinflammatory mediator release in cell lines, primary astrocytes and in vivo. A. ELISA measuring IL-1β levels in the media from BV-2 cells treated with LPS and increasing concentrations of SR9009 to find an optimal dose for primary cell treatment as well as ATP. B. qPCR for *Tnfa* and *Il1b* mRNA expression in WT primary astrocytes treated with LPS and/or SR9009. mRNA expression of hippocampus from WT mice pretreated with SR9009 or vehicle, and then stimulated with i.p. LPS for 6 hours. **p<0.01 vs. No SR9009+LPS condition by 1-way ANOVA with Sidak's multiple comparison test. *p<0.05 by 2-way ANOVA with Holm-Sidak selected multiple comparisons test.

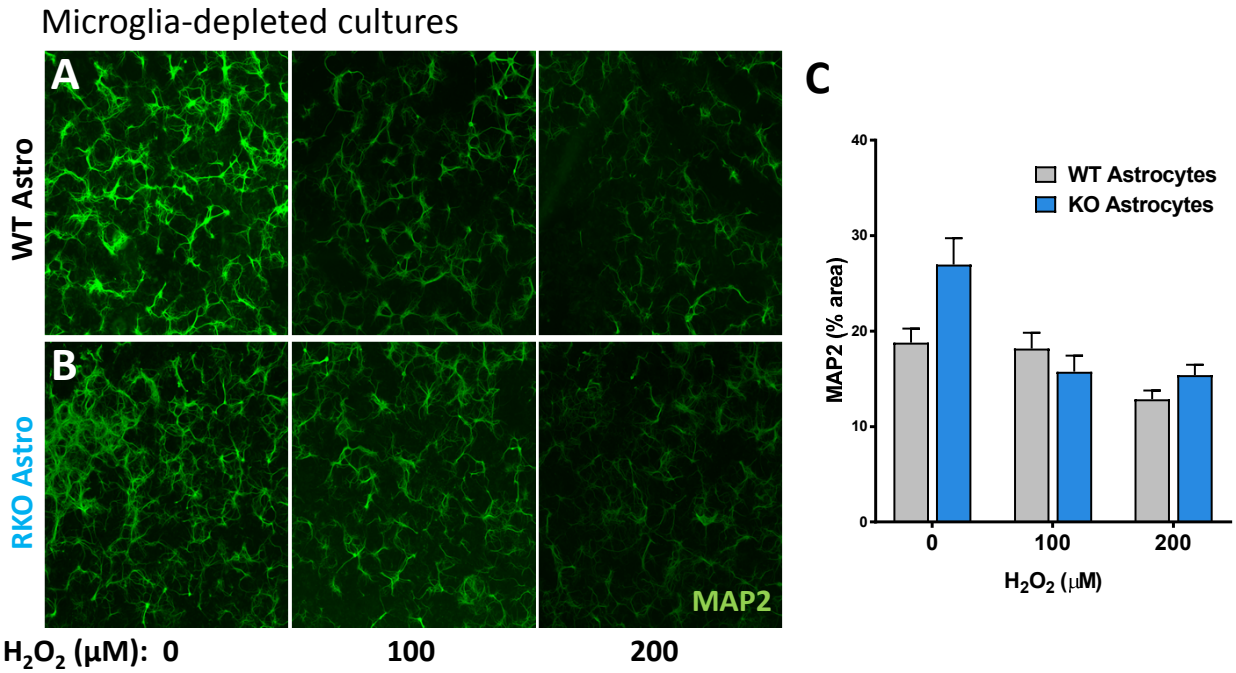

Figure S5. Astrocyte-enriched cultures do not exacerbate H_2O_2-induced neuronal injury. A. MAP2 staining of WT neurons grown on WT primary astrocytes and treated with increasing concentrations of H_2O_2. B. MAP2 staining of WT neurons grown on RKO primary astrocytes and treated with increasing concentrations of H_2O_2. C. The associated quantifications of percent area MAP2 staining in A and B. There were no significant differences between astrocyte genotype, as assessed by multiple T-test with Holm-Sidak correction.

Supplemental Video S1. Videos of 3D surface reconstruction of WT hippocampal microglia with the Iba1 surfaces shown in red and CD68 surfaces shown in yellow.

Supplemental Video S2. Videos of 3D surface reconstruction of RKO hippocampal microglia with the Iba1 surfaces shown in red and CD68 surfaces shown in yellow.

SI Dataset. List of transcripts which were upregultaed by at least 2 fold with a uncorrected p Value < 0.05 in 5mo Rev-Erbα$^{-/-}$ KO and wt littermate mice (n=3/genotype). Tissue was collected at ZT 6-8. Trancriptomic data is from Agilent Mouse Array 2 microarray analysis. Full array data is available on the EMBL-EBI ArrayExpress database, accession number E-MTAB-7590.

SI: Methods

Mice: Both Rev-erb$\alpha^{+/-}$ on a C57Bl/6 background mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at mouse facilities in BJCIH at Washington University. Heterozygous mice were bred together to generate Rev-erb$\alpha^{+/+}$ (wt) and Rev-erb$\alpha^{-/-}$ (RKO) littermates which were used for experiments. Mice were housed on a 12/12 light/dark cycle unless otherwise noted, and were fed ad libitum. All procedures performed on the mice were approved by the Washington University IACUC.

Antibodies: Primary antibodies used for Western Blotting (WB) and Immunohistochemistry (IHC) are listed with target (IHC or WB, host, company, product number and dilution): GFAP (IHC, rabbit, Dako/Agilent Z0334, 1:5000), GFAP (IHC (for co-staining), mouse, Novus Biologics, Littleton, CO, NBP1-05197, 1:1000), MAP2 (IHC, mouse, EMD/Millipore, AB5622, 1:500), p42/44 ERK (WB, rabbit, Cell signaling Technologies, Danvers, MA, 9102, 1:1000), CD68 (IHC, Rat, BioRad, MCA1957, 1:50) , p65(IHC, mouse, Cell Signaling Technologies, 6956, 1:1000), IκBα (WB, Mouse, Cell Signaling Technologies, 9936, 1:1000) , p-p65 (WB, Rabbit, Cell Signaling Technologies, 9936, 1:1000). The Rev-Erbα antibody used for chromatin immunoprecipitation was developed and validated in-house by the Burris lab[3].

Immunohistochemistry: Mice were anesthetized with intraperitoneal (i.p.) injection of pentobarbital (150mg/kg), followed by pump perfusion for 3 mins with ice cold Dulbecco's modified Phosphate Buffered Saline (DPBS) containing 3g/l heparin sulfate. One hemisphere was drop fixed in 4% paraformaldehyde (PFA) for 24 hours at 4˚C, then cryoprotected with 30% sucrose in PBS also at 4˚C for 48 hours. 50μm serial coronal sections were cut on a freezing sliding microtome and stored in cryoprotectant (30% ethylene glycol, 15% sucrose, 15% phosphate buffer in ddH_2O). Sections were washed 3 times in Tris buffered saline (TBS), blocked for 30 mins in TBS containing 3% goat serum and 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO) then incubated in TBS containing 1% goat serum and 0.25% Triton X-100 with primary antibody overnight at 4˚C. Sections were then washed 3 times and incubated for 1 hour at room temperature with 1:1000 fluorescent secondary antibody and mounted on slides. Epifluorescent images were obtained with the MetaMorph software on the Nikon Eclipse 80i fluorescent microscope. Confocal images were taken on the Nikon Elements software with the Nikon A1Rsi scanning confocal microscope. Z-stacks were taken at a step size of 0.1-0.3µm from dark to dark through the tissue.

Bitplane Imaris Analysis: Bitplane 9 image visualization and analysis software was used at the Washington University Center for Cellular Imaging. For 3-D reconstructions, Z-stacks were saved in the .nd2 file format and loaded into the Arena of the Bitplane software. 3-D surfaces had a surface detail ranging from 0.1-0.3μm. For the Iba1-CD68 colocalization analyses, a colocalization channel was built between the Iba and CD68 channels and the surface volume was built and calculated from this channel. For the morphology visualizations, a surface-surface colocalization volume was built between Iba1 and Iba/CD68 colocalized volume. To quantify the Iba1 volumes, 60X z-stacks were taken with 0.5μm step sizes in the mossy fiber region of the CA3. The .nd2 files were imported into Bitplane 9 and surfaces were built from the immunofluorescence z-stacks based on the Iba1 staining channel. The total volume of the surface for each image was exported into excel for further quantification.

Skeletonize Analysis in ImageJ: 40X z-stacks were imported into ImageJ as a hyperstack. Then we selected the Iba1 staining channel was selected and the skeletonize plugin from the plugins menu applied after drawing ROI's for the microglia. After making the skeleton, we used analyze

particles function to count the number of branches/junctions. 3-4 discrete microglia were analyzed/section, with equivalent numbers of microglia analyzed from each of N=3 mice/genotype.

IL-1β ELISA: We used the ELISA MAX™ Deluxe Set (Biolegend Cat #432606, Lot #B255417) to quantify the IL-1β levels from media collected from BV-2 cells treated with 50ng/mL LPS and increasing concentrations (0,1.25,2.5,5,10μM) of SR9009 to optimize a dose for primary experiment treatment. Following this we collected media from primary microglia. The primary microglia were stimulated with 50ng/mL LPS and/or 10μM SR9009 for 8hrs. Cells were treated with 100mM ATP to allow for inflammasome maturation as well as to enhance IL-1β release and 1mL of media was collected. We first optimized the dilutions by running a plate of increasing concentrations of media from 1:1000 to 1:10 against the provided IL-1β standards. After optimizing the concentrations, we ran 4 replicates for each condition to quantify IL-1β levels.

Immunoreactivity Quantification: GFAP staining was quantified using ImageJ software (NIH). GFAP 8-bit images had ROI's draw by hand to encompass the regions of GFAP immunoreactivity. A threshold value was set and maintained for all sections. GFAP percent area coverage was then calculated for each image. MAP2 images were taken at 10X or 4X for percent area quantifications. 3-5 images were taken per well and thresholded as above.

qPCR: Flash-frozen brain tissue was homogenized with a mechanical handheld homogenizer for 20 seconds in RNA kit lysis buffer (PureLink™ RNA Mini Kit, Life Technologies, Carlsbad, CA) plus 1% β-mercaptoethanol. RNA was then purified using the kit protocol. Cells well collected and lysed in Trizol (Life Technologies). The aqueous layer was collected following chloroform extraction (added at 1:5 then spun at 13000xg for 15 minutes) with RNA isolation protocol. RNA concentrations were then measured using the Nanodrop spectrophotometer and cDNA was made using a high capacity RNA-cDNA reverse transcription kit (Applied Biosystems/LifeTechnologies) with 1μg RNA used per 20μL reaction. Real-time quantitative PCR was performed with ABI Taqman primers and ABI PCR Master Mix buffer on the ABI StepOnePlus 12k Real-Time PCR thermocyclers. β-actin (*Actb*) mRNA levels were used for normalization during analysis.

Rev-erbα ChIP Analyses: Initial search for putative Rev-erbα binding sites were done using the IGV and publicly available ChIP-Seq data sets for the liver (https://chip-atlas.org/view?id=SRX3205285), brown adipocytes (https://chip-atlas.org/view?id=SRX163025) and Ventral Tegmental area [1]. Chromatin immunoprecipitation was performed according to previously published work. [2] Briefly, we used the SimpleChIP® Enzymatic Chromatin IP Kit (Cell Signaling Technology) on isolated WT primary microglia grown to confluency in T75 flasks. Following revers cross-linking, the samples were purified using the QIAquick PCR Purification Kit (Qiagen). Quantitative PCR (qPCR) was performed using the SYBR qPCR master mix (Life Technologies). Chromatin binding was calculated as a percentage of immunoprecipitated DNA relative the input amount. Data were expressed as mean ± standard deviation of duplicate samples from representative assays. The Rev-erbα antibody used was developed in-house by the Burris lab and has been described and validated previously[3]. Good binding was determined as compared to Gapdh binding. The primers had the following sequences
Gapdh Forward: AGTGCCAGCCTCGTCCCGTAGACAAAATG

Gapdh Reverse: AAGTGGGCCCCGGCCTTCTCCAT

Arntl Forward: ATTGGCTAACGGGAAGAGGC

Arntl Reverse: TACTTTCCGACCAATCCGCT

Traf2 Forward: CGCCGCTCTGACTAATCACC
Traf2 Reverse: AAATAGGCCGCCTTGACTCT
Nfkbib Forward: GGAAACTCAGCGAGCGAATG
Nfkbib Reverse: TAAATAGCTACACCCCGCCC
Nfkb2 Forward: CTTGTGAACCCGCCACTTAC
Nfkb2 Reverse: ATCTAGCGGCCTTTCTGGAG
S100a4 Forward: GTGTCCACCCCATCCAAGTC
S100a4 Reverse: ATGTCATAGCACCCTCGACC

Western Blotting: Tissue/cell samples were homogenized by sonication in radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technologies, Danvers MA). RIPA buffer contained complete protease inhibitors (Roche Life Sciences USA, Indianapolis, IN). The samples were ran on nuPAGE Novex gels with SDS running buffer and transferred on the iBlot2 transfer device (Life Technologies). Bands were visualized with Lumigen TMA-6 chemiluminescence reagents (Lumigen, Southfield, MI) on a Syngene GBOX Imaging System. Band density was calculated using ImageJ software and normalized to ERK as the loading control.

Microarray: 5-6 mo Rev-erb$\alpha^{-/-}$ and WT littermates were placed in constant darkness for 24 hours and then harvested with i.p. injection of pentobarbital in the dark, following pump perfusion. RNA was isolated from flash frozen hippocampus samples as above and submitted to the Genome Technology Access Center at Washington University for quality control, MessageAmp RNA library preparation and Agilent 4x44k mouse microarray. Raw data was normalized and analyzed using Partek Genomics suite v6.6. For Gene Ontology (GO) term analysis, a list of all genes which were upregulated at least 2-fold in KO with an uncorrected P value <0.05 by 2-tailed T-test were uploaded to DAVID v6.8 (https://david.ncifcrf.gov/). Functional annotation analysis was performed for GO terms related to biological processes.

Microarray data access: The microarray data was deposited and is freely accessible in EMBL-EBI ArrayExpress database, accession number E-MTAB-7590.

Primary Cell Cultures: WT Neuronal and astrocyte cultures were obtained from CD1 mice from Charles River Laboratories (Wilmington, MA). Neurons were isolated from E16-E18 pups and astrocytes were isolated from P0-2 pups. For experiments involving Rev-erb$\alpha^{-/-}$ astrocytes or microglia, the cultures were made from mice on a C57BL/6J background. Cortices plus hippocampus were dissected and stripped of meninges in ice-cold DMEM (Life Technologies) and then incubated in 0.05% Trypsin-EDTA at 37˚C for 15 mins (Neurons) or 20 mins (Astrocytes). Tissue was gently triturated in 37˚C DMEM plus 10% FBS (Gibco). For astrocyte cultures cells were then plated in T75 flasks coated for 2 hours at 37˚C with 50µg/mL poly-D-Lysine (PDL – MP Biosciences, Santa Ana, CA) then rinsed with ddH_2O. Astrocytes were then grown to confluence in DMEM, 10% FBS, and 1% Penicillin/Streptomycin (P/S) with or without 5 ng/mL GM-CSF. For primary microglia isolation from GM-CSF containing media, flasks were shaken at 225rpm at 37˚C for 2 hours and replated on plates coated with PDL. For neuronal cultures, triturated cells were transferred to a second tube to remove debris, then diluted in Neurobasal (Life Technologies) plus B27 (Life Technologies) prior to plating on a bed of astrocytes or a PDL-coated plate.
For co-culture experiments, astrocytes were grown to confluence following replating in 24-well plates and neurons were added immediately after primary dissection at a concentration of

150,000 cells/mL in Neurobasal plus B27 supplement and glutamine (Life Technologies). After 48 hours, 50% of the media was changed to Neurobasal plus anti-oxidant free B-27 (AOF B-27, Life Technologies) and glutamine. Following that, 50% media changes were done using Neurobasal plus AOF B-27 and glutamine every 3 days until neuronal DIV 11 at which the experimental wells received H_2O_2 and the controls received vehicle for 24 hours. All wells were then fixed with 4% PFA and stained.
In general, all cell culture experiments were performed in duplicate and repeated at least 3 times, using primary cells prepared from at least separate dissections.

siRNA transfections: Astrocytes/mixed glial cultures were transfected with siRNA using lipofectamine RNAiMAX (Life Technologies) in OptiMEM (Life Technologies) according to the manufacturer's instructions. After 48 hours media was changed to DMEM plus 10% FBS or Neurobasal plus B-27 for conditioning. siRNAs targeting mouse *Nr1d1* or non-targeting (scrambled and designed to target no known mouse gene) were obtained from Dharmacon (Lafayette, CO). An siRNA to RNAiMAX ratio of 1:1.25 was used and 40 pmol of siRNA (2μL of 20μM stock) was added to each well of a 12 well plate. Media was changed/collected every 48 hours.

Optical Intrinsic Signal Imaging and Functional Connectivity Analysis: Following our previously published protocols[4], mice were anesthetized with ketamine-xylazine (86.9 mg/kg ketamine, 13.4 mg/kg xylazine) and allowed 15 min for anesthetic transition. Mice were placed on a heating pad maintained at 37°C with and the head secured in a stereotactic frame. The head was shaved and cleaned, a midline incision was made to reflect the scalp, and the intact skull was kept moist with mineral oil.
For imaging, sequential illumination was provided at four wavelengths by light emitting diodes (LEDs) placed approximately 10 cm above the head. Diffuse reflected light was detected by a cooled, frame-transfer EMCCD camera (iXon 897, Andor Technologies) at a full frame rate of 30 Hz over the majority of the convexity of the cerebral cortex (approximately 1 cm2). Forty-five minutes of data were collected in each mouse.
Data from all mice were subject to an initial quality check prior to spectroscopic analysis[5,6]. Raw light levels in which temporal variation in reflected light level intensity exceeded 1% for any wavelength were excluded from further analysis. This preliminary analysis resulted in 10–45 minutes of data per mouse. For each pixel the differential light intensity at each wavelength was converted to differential absorption. The multi-wavelength absorption coefficient data were then converted to hemoglobin concentration changes for each pixel and time point. All analyses were performed on oxygenated hemoglobin. Data were filtered over the infraslow band (0.009-0.08 Hz) following previous human fMRI algorithms[7], resampled from 30 Hz to 1 Hz, spatially normalized to the Paxinos atlas, and only shared brain areas across all mice were further analyzed.
For functional connectivity analysis, global signal regression was performed for each mouse prior to any functional connectivity analysis. Zero-lag correlation was computed for all pixel pairs within the shared brain region to create a whole-cortex correlation matrix for each mouse. Regions of interest were generated by decomposing the average whole-cortex correlation matrix calculated for all mice (combined WT and KO groups) using principal component analysis (PCA) as we have done previously[8]. This procedure generated a set of topographical maps corresponding to brain regions common to all mice imaged. ROIs were defined by thresholding the first 2 PCs at the 95% percentile for positive and negative values, resulting in 8 total ROIs. Functional connectivity patterns for each ROI were generated by correlating the average time courses within each region with time courses of all other brain pixels. To assess the topography of pairwise correlation changes between WT and KO groups, we computed the difference

correlation matrices by subtracting the group-averaged, whole cortex KO matrix from that of the WT group, and applied spatial principal components analysis to the difference matrix.

Statistical Analyses: Statistical analyses were performed using GraphPad Prism v7.02. When multiple t-tests were performed, we applied Holm-Sidak correction.

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