Supplemental Materials and Methods

Primary cortical cultures

Astrocyte cultures, mixed neuron/astrocyte cultures and astrocyte-free neuronal cultures were prepared as previously described [1-3]. For mixed-species co-cultures, rat neurons were plated on top of a confluent layer of DIV14 mouse astrocytes and both astrocyte mono-cultures and astrocyte-neuron co-cultures were subsequently kept in Neurobasal-A medium containing B27 (both Life Technologies), but devoid of serum. Note that the rat neuronal cells plated do contain a small number of non-neuronal cells (principally astrocytes), but since the focus of the study is the mouse astrocytic transcriptome, this was deemed acceptable. Genotype/age use was E17.5 wild-type, $Nrf2^{-/-}$ and $Keap1^{-/-}$ mice embryos, originally developed by Prof. M. Yamamoto laboratory (University of Tohoku) [4,5]. $Nrf2^{-/-}$ mice have been backcrossed over six generations onto C57BL/6 genetic background [6]. Offspring of $Nrf2^{-/-}$ mice was generated through breeding of $Nrf2^{-/-}$ females and males. Matching C57BL/6 WT animals were used to generate parallel wild-type cultures. Keap1 heterozygote males and females were mated to produce $Keap1^{+/+}$ and $Keap1^{-/-}$ littermates which were used for comparison. The offspring genotypes were verified through multiplex PCR analysis of cerebellum genomic DNA using DNA easy blood and tissue kit (Qiagen UK, Crawley, UK). The primers used are listed in (Table 1) and the PCR conditions were as follows: 5 minutes at 95°C followed by 34 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute, followed by an extension step at 72°C for 10 minutes. The size of the bands produced by this reaction are distinctive based on the genotype of the sample DNA whereby Keap1 WT and KO samples generate bands of 235 and 420 base pairs (bp) respectively.

PCR Primer	Sequence
D123	5'-CGGGATCCCCATGGAAAGGCTTATTGAGTTC-3'
2nd Ex 3'	5'-GAAGTGCATGTAGATATACTCCC-3'
Tv Neo	5'-TCAGAGCAGCCGATTGTGTGTTGTGCCCAGTCA-3'

Application of oxidative stressors and Nrf2 activators

Before treatment, cells were incubated in serum-free trophically deprived transfection medium (TMo) for 2-4h containing 10% minimum essential media (Life Technologies Ltd) and 90% Salt-Glucose-Glycine (SGG) medium, which is comprised of 114mM NaCl, 0.219% NaHCO3, 5.292mM KCl, 1mM MgCl2, 2mM CaCl2, 10mM HEPES, 1mM Glycine, 30mM Glucose, 0.5mM sodium pyruvate, 0.1% Phenol Red. Working stock of tBHQ (Sigma-Aldrich) was prepared at a concentration of 5mM in DMSO in aliquots that were stored at -20°C until needed. At the time of experiment tBHQ was added

at a final concentration of 10μM. Working stock of hydrogen peroxide solution (3%w/w, equivalent to 880mM, Sigma-Aldrich), was prepared at 8.8mM in dH_2O on the same day of the experiment.

Assessment of cell viability

For cell death quantification, mixed astrocyte/neuronal cultures were fixed with 4% paraformaldehyde (PFA) and subjected to 4',6' diamidino-2-phenylindole (DAPI) (Vector Laboratories, California, USA) nuclear staining and cell death was quantified by counting the number of pyknotic nuclei as a percentage of the total. Approximately 1500 cells were scored across several random fields within 3-4 independent experiments.

Transient transfections and plasmids

All transfections were performed using Lipofectamine 2000 (Invitrogen) on cells cultured in 24-well plates. We applied a protocol that preferentially targets the astrocytic population in our cultures [7,8]. Briefly, on DIV2 cells were transferred to TMo 2-4h prior to transfection. For each well, a DNA (μg): Lipofectamine (μl) ratio of (1:3.88) was applied and the cells were incubated with transfection complexes for 3-4h before they were returned to growth medium. Cells were maintained in culture for 6 days (DIV8) before experimentation. For a complete list of all the plasmids used in this study and information on the generation of GBD-Neh5 mutant construct (C191A) see Table S2 and Supplementary Material.

For the GBD-Neh5 and pEF-Nrf2 mutation studies, the reactive cysteine residue 191 (Cys-191) was substituted with alanine. To obtain this, QuikChange II XL site-directed mutagenesis kit (Stratagene) was used along with the mutagenic primer and its reverse complementary sequence containing the mismatches 5'-CATTCCCGAATTA CAG**GCC**CT TAATACCGAAAACAAG-3' (bold indicates nucleotides changed, underlined sequence indicates creation of EcoO109I diagnostic site). As for pGFP-Nrf2 mutant version, the corresponding cysteine residue in the human transcript (Cys-199) was substituted with alanine as well using the same kit and the following mutagenic primer and its reverse complementary sequence containing the mismatches 5'-CGGAGTTACAG**GC**TCT**G**AATATTGAAAATGACAAGC-3' (bold indicates nucleotides changed, underlined sequence indicates creation of AlwNI diagnostic site). All mutants were verified by performing diagnostic cuts with the indicated restriction enzymes and were further confirmed by sequencing (SBS sequencing service, University of Edinburgh). A list of all the plasmids used in this study are shown below:

Plasmid name	Description	Reference
pGL2-SV40-Luc	SV40-driven empty Luciferase reporter control	Promega
pTK-RL	Thymidine kinase promoter driven Renilla luciferase reporter	Promega
Gal4-Luc	Luciferase reporter bearing 4 copies of Gal4-binding site	Promega
GBD-Nrf2	Gal4 DNA binding domain fused to the full length mouse Nrf2	[9]
GBD-Neh5	Gal4 DNA binding domain fused to Neh5 domain (153-227 amino acids)	
GBD-Neh(2-4)	Gal4 DNA binding domain fused to both Neh2 and Neh4 domains (1-156 amino acids)	
GBD	Gal4 DNA binding domain only	
Neh2-Luc	Neh2 domain fused to luciferase gene (Neh2-Luc)	[10]
pEF-Nrf2	mouse full length Nrf2 was cloned into pEF1-α neo expression vector	(Kotkow and Orkin 1995) doi: 10.1128/MCB.15.8.4640.
pGFP-Nrf2	human full length Nrf2 was cloned into pGFP/pOB125 expression vector	(Numazawa et al 2003) https://doi.org/10.1152/ajpcell.00043.2003

Luciferase reporter gene assay

Luciferase assays were carried out on cells transfected with the desired constructs at the following DNA ratio 2:1:4 for luciferase-reporter: Tk-Renilla (normalization control): effector plasmid. For the Gal4-based reporter assay, transfections were carried out using the reporter plasmid Gal4-Luc and pTK-renilla and either of the effector plasmids; GBD-Nrf2, GBD-Neh(2-4), GBD-Neh5 or GBD only. As for the Neh2-Luciferase reporter system, cells were transfected with either Neh2-Luc or Con-Luc along with pTK-renilla in the following proportion 4:1 of reporter gene to pTK-renilla.

RNA extraction and qPCR

Total RNA was isolated using High Pure RNA Isolation Kit (Roche, Welwyn Garden City,UK) including a DNase-treatment step to degrade genomic DNA. cDNA was synthesized from 1-5µg RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). Each qPCR reaction contained 6 ng of cDNA mixed with FS Universal SYBR Green MasterRox (Roche) and was carried out in an Mx3000P qPCR system (Agilent Technologies, Cheshire, UK). In each experiment, technical duplicates were used for every sample including NoRT and No-template controls. The cycling parameters were as follows: 10 minutes of initial denaturation at 95°C; 40 cycles of 30 seconds at 95°C, 40 seconds of annealing at 60°C with detection of fluorescence and 30 seconds of extension at 72°C; followed by one cycle of 1 minute at 95°C, the temperature was ramped from 55°C to 95°C over 30 seconds at 1°C per step with continuous fluorescence detection (for dissociation curve analysis to confirm the amplification of a

single product). Gene of interest expression was normalized to Gapdh levels and subsequently compared to levels in control samples using the 2(-ΔΔCt) efficiency corrected method [11]. Primer sequences used are listed below:

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Gapdh	GGGTGTGAACCACGAGAAT	CCTTCCACAATGCCAAAGTT
Slc7a11/xCT	ATACTCCAGAACACGGGCAG	AGTTCCACCCAGACTCGAAC
Srxn1	GACGTCCTCTGGATCAAAG	GCAGGAATGGTCTCTCTCTG
Hmox1	AGCACAGGGTGACAGAAGAG	GGAGCGGTGTCTGGGATG

RNA-seq and Species-specific sorting of mixed species reads

To generate RNA-seq data, barcoded RNA-seq libraries were prepared by Edinburgh Genomics using the Illumina TruSeq stranded mRNA-seq kit, according to the manufacturer's protocol (Illumina). The libraries were pooled and sequenced to 75 base paired-end on an Illumina NovaSeqTM 6000. For single-species RNA-seq experiments sequencing was performed to a depth of approximately 50 million paired-end reads per sample, whereas for mixed species RNA-seq a greater depth of approximately 100 million (for two species) paired-end reads per sample was done. Species-specific separation of RNA-seq reads was performed using version 1.2 of Sargasso [12] (during which reads were mapped to the mouse, rat and human genomes using version 2.5.3a of STAR [13]). The protocol is described in detail elsewhere [12]. Subsequently, per-gene read counts were summarised using featureCounts version 1.5.2. For read mapping and feature counting, genome sequences and gene annotations were downloaded from Ensembl version 94. Differential expression (DGE) analysis on data sets was performed using DESeq2 [14] (R package version 1.18.1) using a significance threshold set at a Benjamini-Hochberg-adjusted p-value of 0.05. Before arriving at a final DGE dataset for the microglia, we carried out an additional control (as recommended in our published protocol [12]) by performing RNA-seq on a two-species co-culture of mouse neurons and human astrocytes, and determining whether the Sargasso workflow resulted in any human or mouse reads being incorrectly called as rat. We took a conservative approach and discarded any genes for which we estimated >10% of rat reads within the mixed species co-culture could be due to incorrectly called human or mouse reads.

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