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Supplemental information

Microglial identity and inflammatory responses are controlled by the combined effects of neurons and astrocytes

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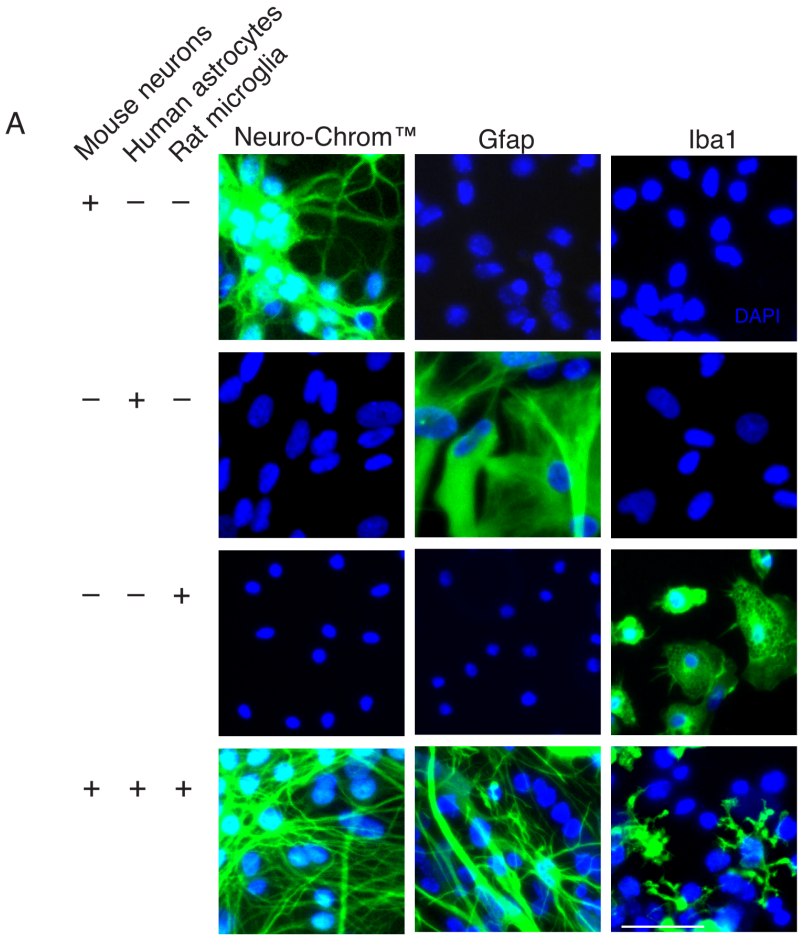
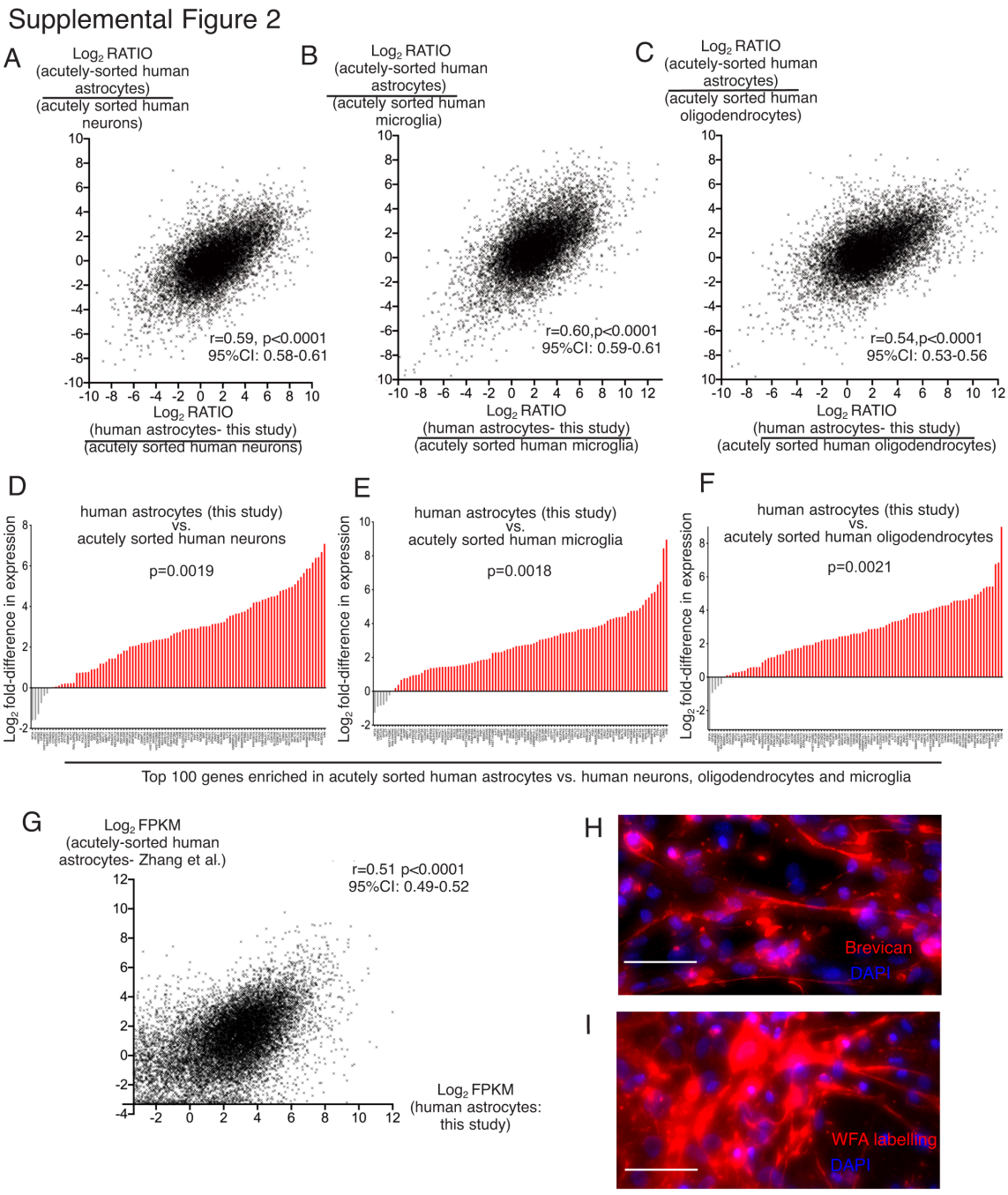
Supplemental Figures

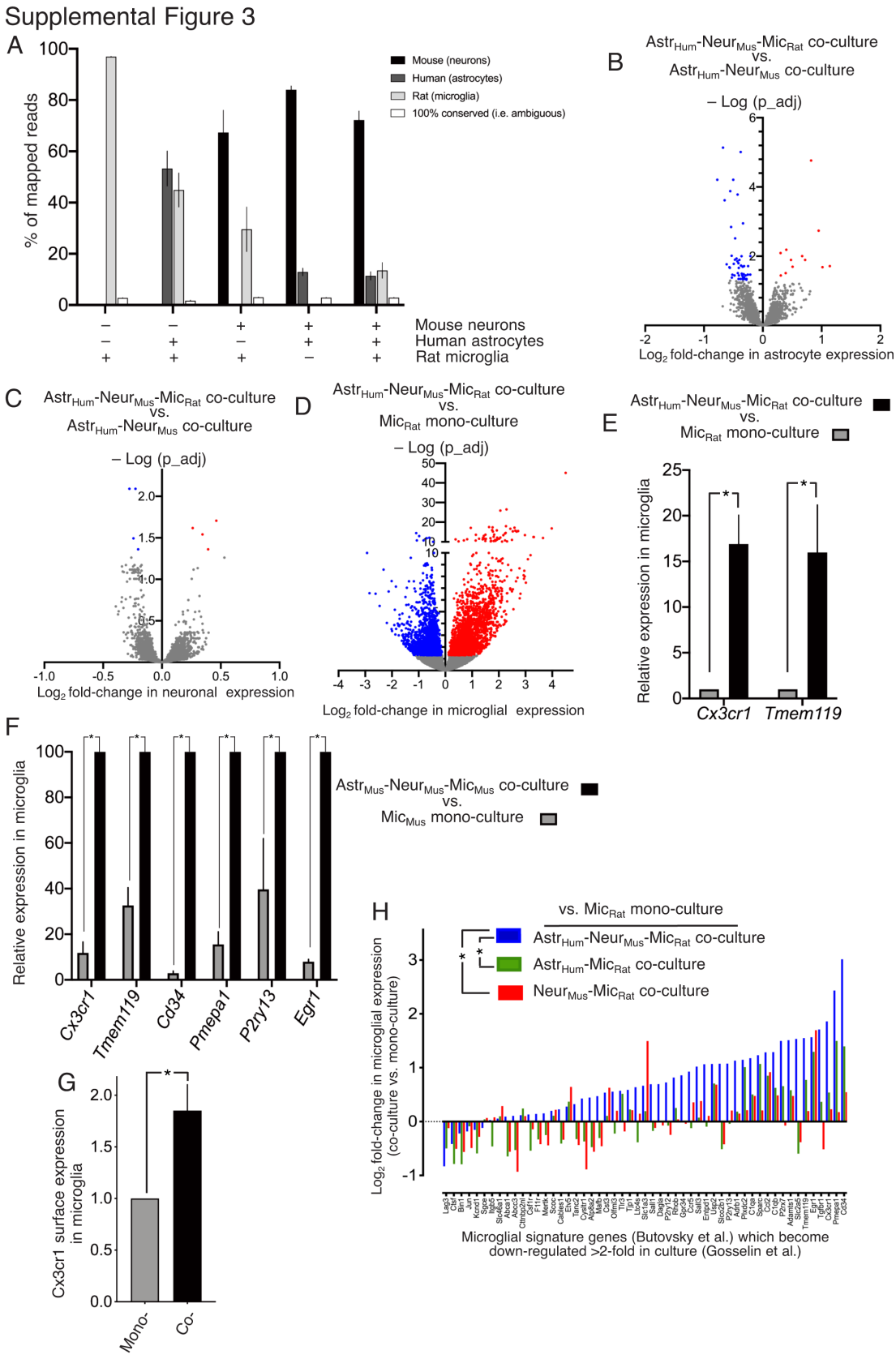
Supplemental Figure 1



Supplemental Figure S1. **Related to Figure 1. A)** Immunofluorescence staining of the indicated cell types, alone or in combination, in mono- or three-way co-culture, as indicated. In all cases DAPI-stained nuclei are blue, and the neuronal (Neuro-Chrom), astrocytic (Gfap) and microglial (Iba1) markers in green. Scale bar: 50 µm.



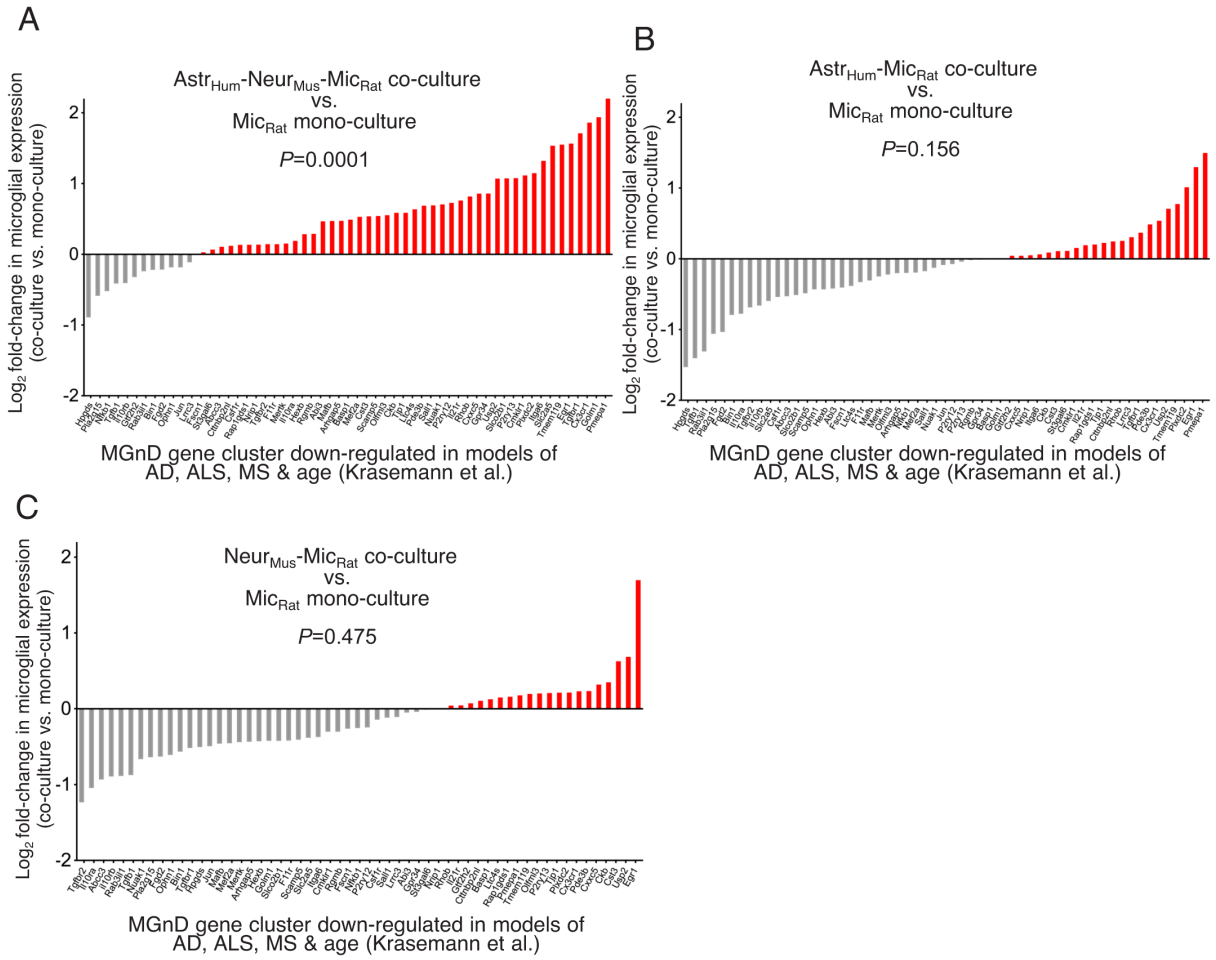
Supplemental Figure S2. **Related to Figure 1. A-C)** For acutely sorted human astrocytes (Zhang et al., 2016); y-axis, and astrocytes used in our study; x-axis, the ratio of gene expression was calculated compared to acutely sorted neurons (A), microglia (B) or oligodendrocytes (C), using data from Zhang et al, and the correlation calculated. A significant positive correlation is observed in all cases showing that genes enriched or de-enriched in astrocytes compared to other brain cell types show similarity between acutely sorted human astrocytes, and the human astrocytes used in this study. **D-F)** Genes expressed >1 FPKM were taken and genes ranked by their enrichment in astrocytes relative to human neurons, microglia and oligodendrocytes (the minimum enrichment of the 3 was taken to ensure stringency). The top 100 genes were taken and the level of expression of the astrocytes used in our study calculated relative to neurons (D), microglia (E) and oligodendrocytes (F). The p-value is the product of a paired t-test between FPKM in the astrocytes used in our study relative to neurons (D), microglia (E) and oligodendrocytes (F). **G)** A comparison of gene expression in acutely sorted human astrocytes (Zhang et al., 2016) vs. that in the astrocytes used in our study. **H,I)** Immunohistochemistry of astrocyte/neuron co-cultures using an antibody against extracellular chondroitin sulfate proteoglycan brevican (H) and labelling of N-acetylgalactosamines beta 1-modified glycoproteins using fluorescently labelled Wisteria floribunda agglutinin (WFA), (I). Scale bar: 50 µm.



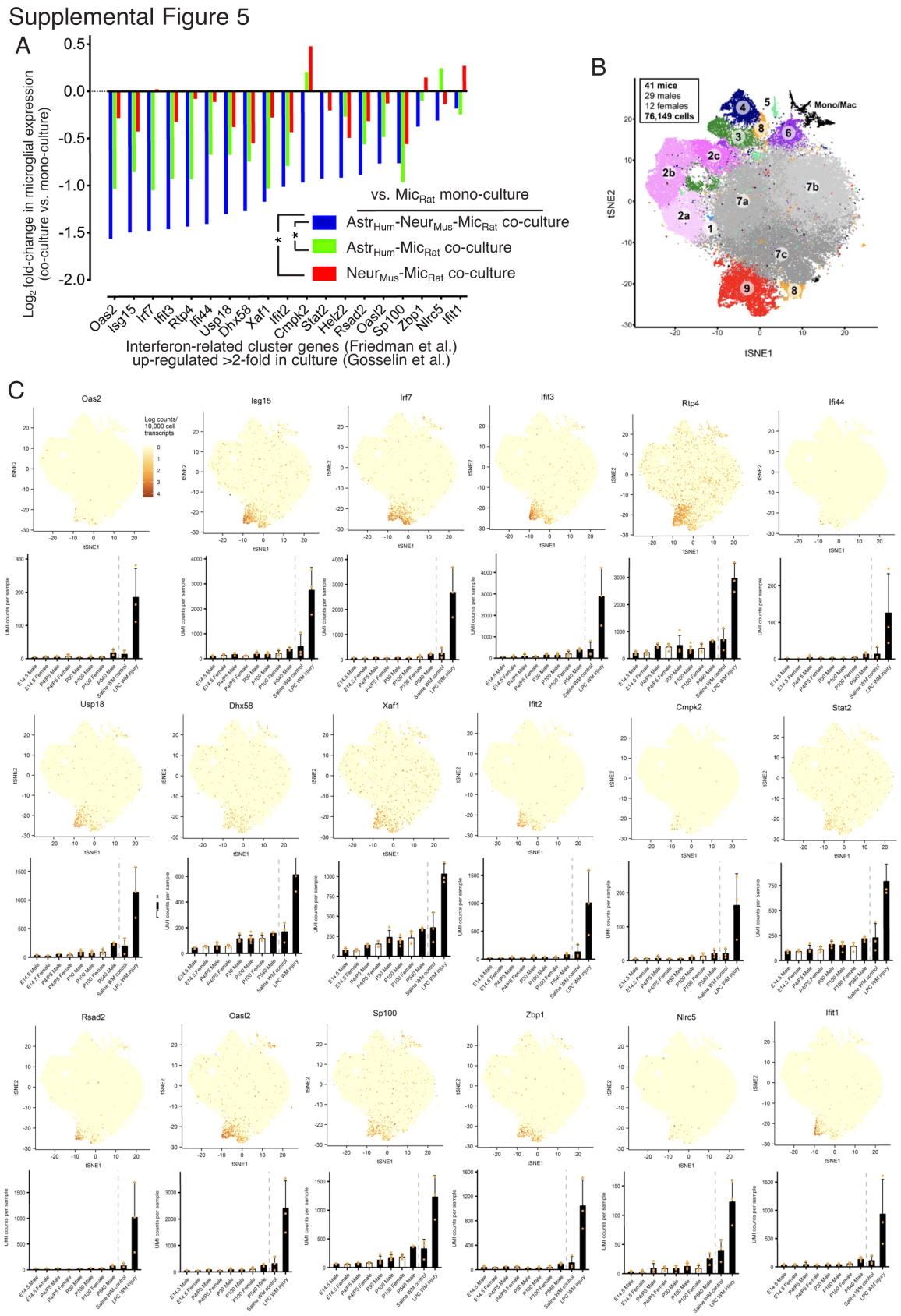
Supplemental Figure S3. Related to Figure 1. A) For the indicated co-cultures and mono-cultures on which RNA-seq was performed, the % of reads mapped to each species is shown, as well as the percentage of reads discarded due to 100% conservation of the paired-end read to two or more species. **B,C)** A volcano plot showing the effect of the presence of microglia on astrocytes (B) and neurons (C) within an astrocyte/neuron co-culture. RNA-seq was performed on the indicated co-cultures and astrocyte (human) and neuron (mouse) reads sorted by species prior to differential gene expression analysis. D) A volcano plot showing the effect of astrocyte/neuron co-culture on the microglial transcriptome, compared to microglial mono-cultures RNA-seq was performed on the indicated cultures and microglial reads sorted by

species (rat). **E)** Confirmation of the induction of the indicated genes by co-culture by qPCR, using species (rat)-specific primers. **F)** Mouse microglia were cultured in the presence or absence of mouse neurons and astrocytes for 72h, after which they were sorted by MACS and the indicated genes analysed by qPCR *p<0.0001 in all cases (2-way ANOVA plus Sidak's post-hoc test, n=3). **G)** Cx3cr1 surface expression measured by flow cytometry in Cd11b-positive microglia from mono- vs co-culture. *p=0.007, two-tailed t-test on the mean normalized Cx3cr1 expression in n=6 biological replicates. **H)** The data in Figs 2B-D are interleaved for comparison and a repeated measures 1-way ANOVA performed with Tukey's post-hoc test performed on the Log_2-fold change of the indicated genes by astrocyte/neuron co-culture, astrocyte co-culture and neuron co-culture, relative to microglial mono-culture. *p<0.0001 both when comparing the effect of astrocyte/neuron co-culture with astrocyte-only co-culture, and with neuron-only co-culture. There was no significant difference between the effect of astrocyte-only co-culture, and neuron-only co-culture (p=0.78).

Supplemental Figure 4



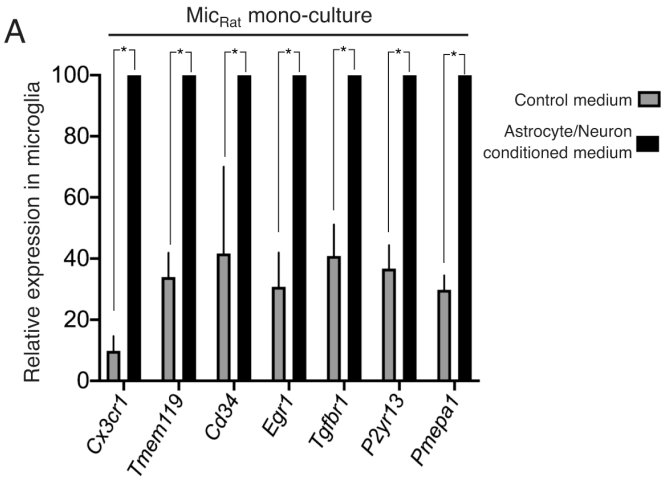
Supplemental Figure S4. Related to Figure 1. A-C) Analysis was performed exactly as in Fig. 2B-D except that the gene set interrogated was the set of genes repressed in the microglial neurodegenerative phenotype (MGnD) (Krasemann et al., 2017). P values are calculated using a 2-way ANOVA.



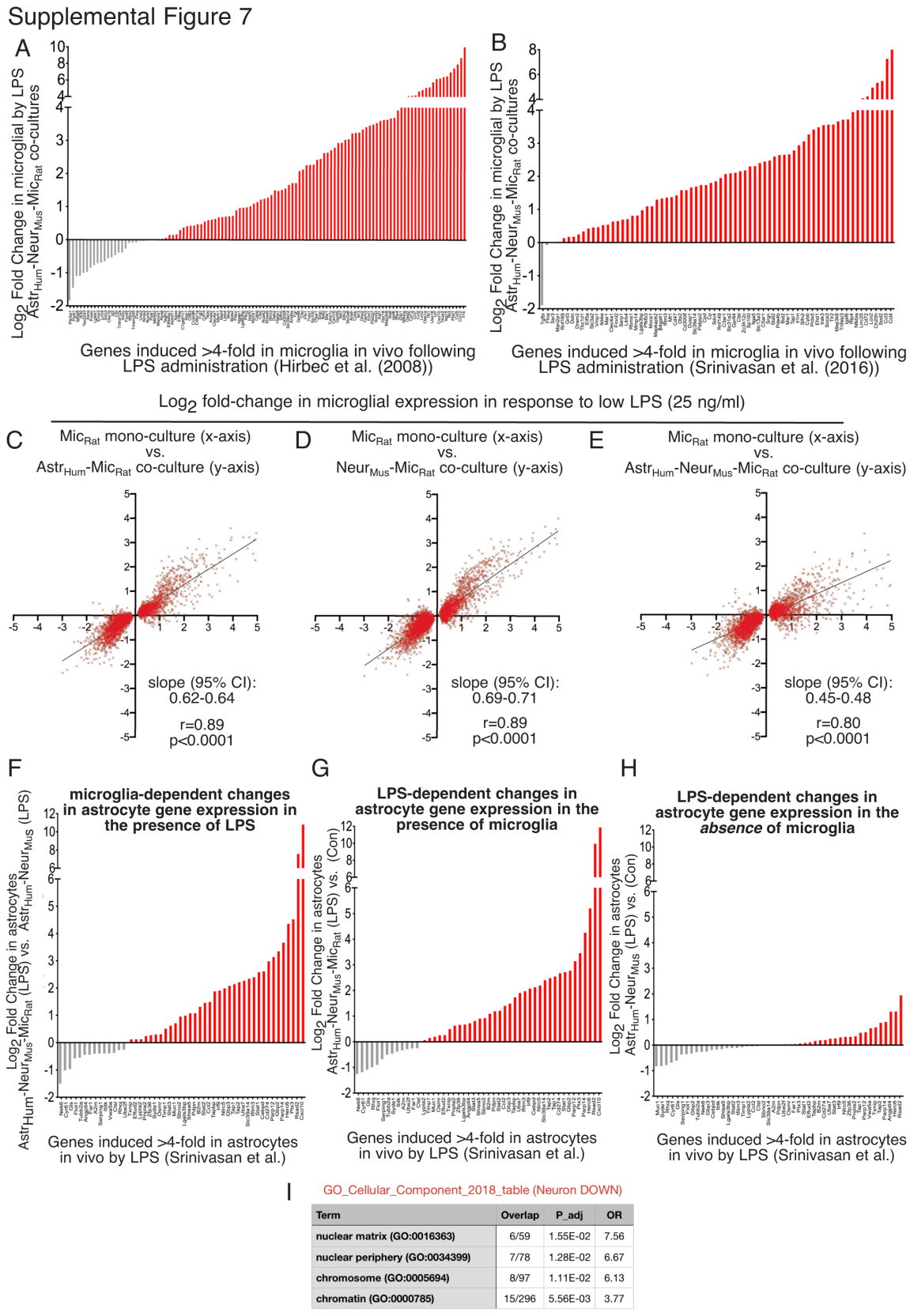
Supplemental Figure S5. Related to Figure 2. A) The data in Figs 3A-C are interleaved for comparison and a repeated measures 1-way ANOVA performed with Tukey's post-hoc test performed on the Log_2-fold change of the indicated genes by astrocyte/neuron co-culture, astrocyte co-culture and neuron co-culture, relative to microglial mono-culture. *$p<0.0001$ both when comparing the effect of astrocyte/neuron co-culture with astrocyte-only co-culture, and with neuron-only co-culture. There was also a significant difference between the effect of astrocyte-only co-culture, and neuron-only co-culture (*$p=0.001$). **B,C)** All interferon-related genes analysed in Fig. 3a-c (with a threshold of 100 UMI counts per sample in at least one sample) were mapped onto a published data set of single cell RNA-seq of microglia

across the lifespan as well as following LPC-induced white matter injury (Hammond et al., 2019), analysed on their website http://www.microgliasinglecell.com. The top right scatter plot (B) shows the different clusters of microglia identified (Hammond et al., 2019). For each gene the upper scatters show the expression level of the indicated genes superimposed onto the clusters, with particular enrichment in Cluster 9. The lower graphs show, for each gene, the mean expression level on the different microglial populations that contribute to the single cell data. Note the very high expression in the LPC white matter (WM) injury for all interferon-related genes analysed.

Supplemental Figure 6



Supplemental Figure S6. Related to Figure 4. A) Mono-cultures of rat microglia treated with astrocyte/neuron co-culture conditioned medium or unconditioned medium, RNA extracted, and the indicated genes analysed by qPCR, normalized to Rpl13a. *p<0.0001, <0.0001, 0.0002, <0.0001, 0.0001, <0.0001, <0.0001, 2-way ANOVA plus Sidak's post-hoc (n=5-7).



Term	Overlap	P_adj	OR
nuclear matrix (GO:0016363)	6/59	1.55E-02	7.56
nuclear periphery (GO:0034399)	7/78	1.28E-02	6.67
chromosome (GO:0005694)	8/97	1.11E-02	6.13
chromatin (GO:0000785)	15/296	5.56E-03	3.77

Supplemental Figure S7. Related to Figure 5. And Figure 6 A,B) For genes reported to be induced >4-fold in vivo by LPS injection in microglia in two separate studies (Hirbec et al., 2018; Srinivasan et al., 2016), the Log_2 fold-change in microglial gene expression induced by 500 ng/ml LPS in microglia co-cultured with (human) astrocytes and (mouse) neurons is shown. For both gene sets, there is a significant induction by LPS in the microglia: p=1.81E-05 (A), 1.8E-23 (B), 2-way ANOVA (effect of LPS vs. control). **C-E)** Log_2 fold change of genes induced by low-LPS in microglial monoculture plotted against the corresponding Log_2 fold-change in microglia in the astrocyte/microglial co-culture (C), neuron/microglia co-culture (D) and the astrocyte/neuron/microglia co-culture for comparison (E). Linear regression analysis revealed the slopes indicated, and the extent of their deviation from 1 gives a measure of the effect of co-culture

in repressing the microglial response to low-dose LPS. **F-H)** For genes reported to be induced >4-fold in vivo by LPS injection in astrocytes (Srinivasan et al., 2016), the Log_2 fold-change in astrocyte gene expression in neuron/astrocyte co-cultures in vitro in the presence of LPS (500 ng/ml) ± microglia (F), in the presence of microglia ± LPS (500 ng/ml) (G), and in the absence of microglia ± LPS (500 ng/ml) (H) is shown. Statistical test: 2-way ANOVA measuring main effect of the comparison stated on the y-axis. P=5.8E-26 (F), 4.2E-28 (G). **H)** The only 4 GO cellular Component terms enriched in genes repressed in neurons by activated microglia.