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## Supplemental information

# Mitochondrial network adaptations of microglia reveal sex-specific stress response after injury and UCP2 knockout

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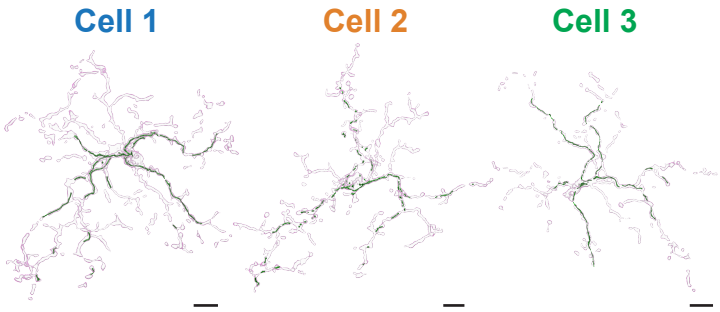
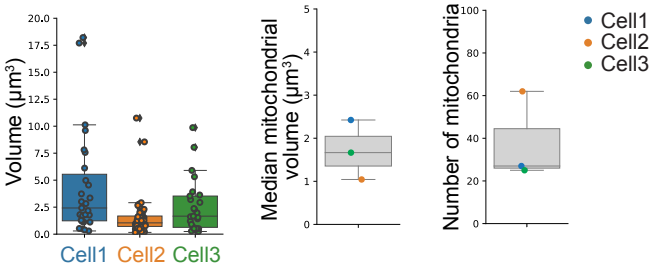
# Figure S1.

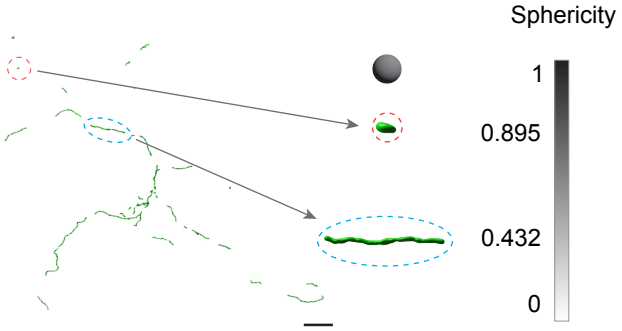
**A** *Example 3D-surface reconstruction*




**B** *Parameter for median mitochondrial volume and number of mitochondria*




**C** *Parameter for mitochondrial sphericity*

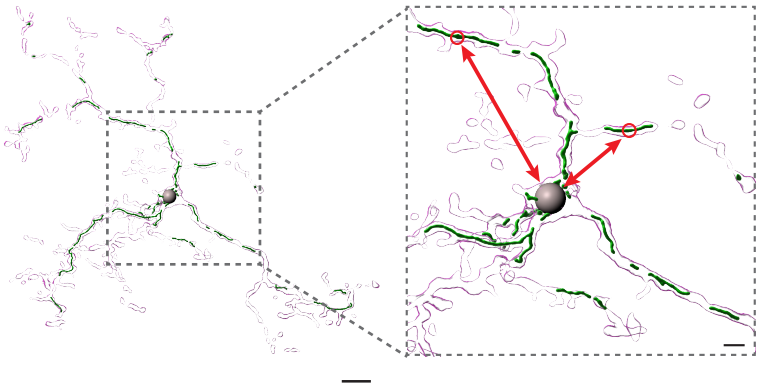



**D** *Parameter for mitochondrial content per microglia*

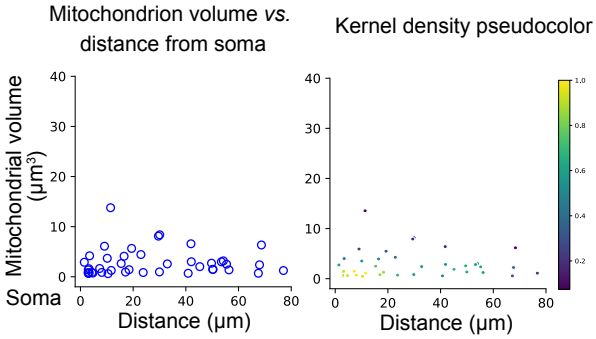
$$\frac{\sum_{i=1}^{n} mv_i}{\text{Microglia volume}} \times 100 = \textbf{\% Mitochondria volume per microglia}$$

mv = mitochondrion volume

**E** *Distance measurement from soma center to organelle centroid*

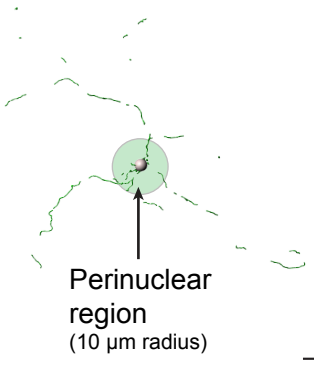


**F** *Distance from soma vs. mitochondrion volume*




Each mitochondrion = marker

**G** *Parameter for mitochondria in the perinuclear region*




$$\textbf{\% Mitochondria perinuclear} = \frac{\sum_{i=1}^{n} mvp_i}{\sum_{i=1}^{n} mv_i} \times 100$$

mvp = mitochondrion volume in perinuclear region
mv = mitochondrion volume

**Figure S1. Analyzed mitochondrial parameters in microglia, Related to Figure 1**

Introduction to the mitochondrial parameters based on three 3D-surface reconstructions of IBA1-immunostained microglia (magenta) from IPL WT (magenta) expressing mito-Dendra2 (green, **A**). Scale bar: 10 µm.

(**B**) Left, boxplot for each cell indicating the volume of every mitochondrial organelle per cell. Each dot, a mitochondrion. Center, median mitochondrial volume and right, number of mitochondria for each cell at left. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond points: outliers outside 1.5 IQRs.

(**C**) Mitochondrial sphericity. Left, example mitochondrial 3D-surface (green) from a WT microglia. Ratio of mitochondrion volume to mitochondrion surface area represents sphericity with two exemplar organelles (orange, sphericity =0.895) and elongated (blue, sphericity = 0.432). Sphericity is represented as a mean sphericity value of all mitochondria per cell. Scale bar: 15 µm.

(**D**) Percentage of the sum of mitochondrial volume in a cell (**Figure S1B, left**) over the microglial volume represents mitochondrial content per cell.

(**E**) Vesicle distance measurement. Example 3D-volume surfaces of an IBA1-immunostained microglia (magenta) with mito-Dendra2 expression (green). Grey sphere: cell soma. Right, zoom-in of region of interest (dashed line). Example red circles: centroid of mitochondrion. Arrows: distance from centroid to soma determined from the centroid of each mitochondrion/vesicle to the center of the soma-sphere (red arrows). Scale bar: 15 µm, zoom-in: 5 µm.

(**F**) Mitochondria or CD68 vesicle localization parameter exemplified using mitochondrial localization. Scatterplot depicts the distance from the soma (µm) *vs.* the mitochondrial volume ($\mu m^3$) for each mitochondrion shown in (**Figure S1E**). To illustrate overlapping mitochondria, scatterplot markers were pseudo-colored with kernel density estimations.

(**G**) Example mitochondrial surface (green) from microglia depicted in (**Figure S1E**). The perinuclear region (shaded green sphere) was determined as the region within a 10 µm radius from the cell soma point. The percentage of mitochondria in the perinuclear region is the sum volume within the perinuclear region over the total mitochondrial volume. Scale bar: 15 µm.

# Figure S2.

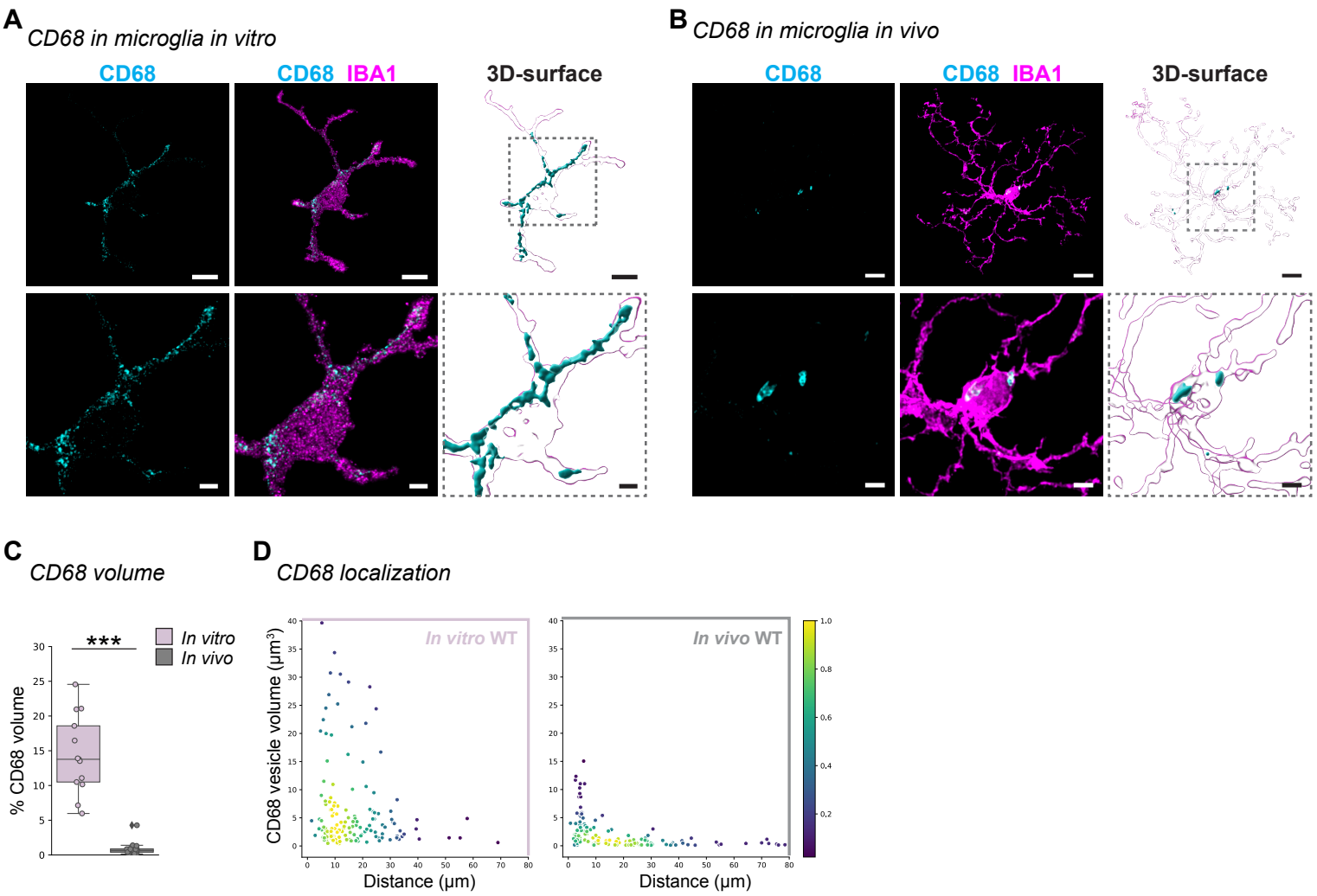

A
CD68 in microglia in vitro
CD68
CD68 IBA1
3D-surface
B
CD68 in microglia in vivo
CD68
CD68 IBA1
3D-surface
C
CD68 volume
***
In vitro
In vivo
% CD68 volume
D
CD68 localization
In vitro WT
In vivo WT
CD68 vesicle volume (µm³)
Distance (µm)
Distance (µm)

**Figure S2. Microglia *in vitro* exhibit increased CD68 expression, Related to Figure 1**

(**A**-**B**) IBA1-immunostained microglia (magenta) from **Figure 1F**-**G** co-labeled with CD68 (cyan) from *in vitro* (**A**) or *in vivo* microglia (**B**). Next, corresponding 3D-surfaces. Below: Zoom-in of region of interest (dashed outline) from image and 3D-surface. Scale bar: 10 µm, zoom-in: 3 µm.

(**C**) Percentage of total CD68 volume per microglia volume. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond points: outliers outside 1.5 IQRs. Wilcoxon rank sum test: $p < 0.0001$.

(**D**) CD68 vesicle localization. Scatterplot depicting CD68 vesicle volume *vs.* distance from the cell soma (0, origin) of the population of vesicles in *in vitro* (left, dusty pink) and *in vivo* (right, grey) conditions. Point density, pseudo-colored.

*** $p < 0.001$. See **Table S7** for experimental, retina and cell numbers, statistical tests and corresponding data.

# Figure S3.

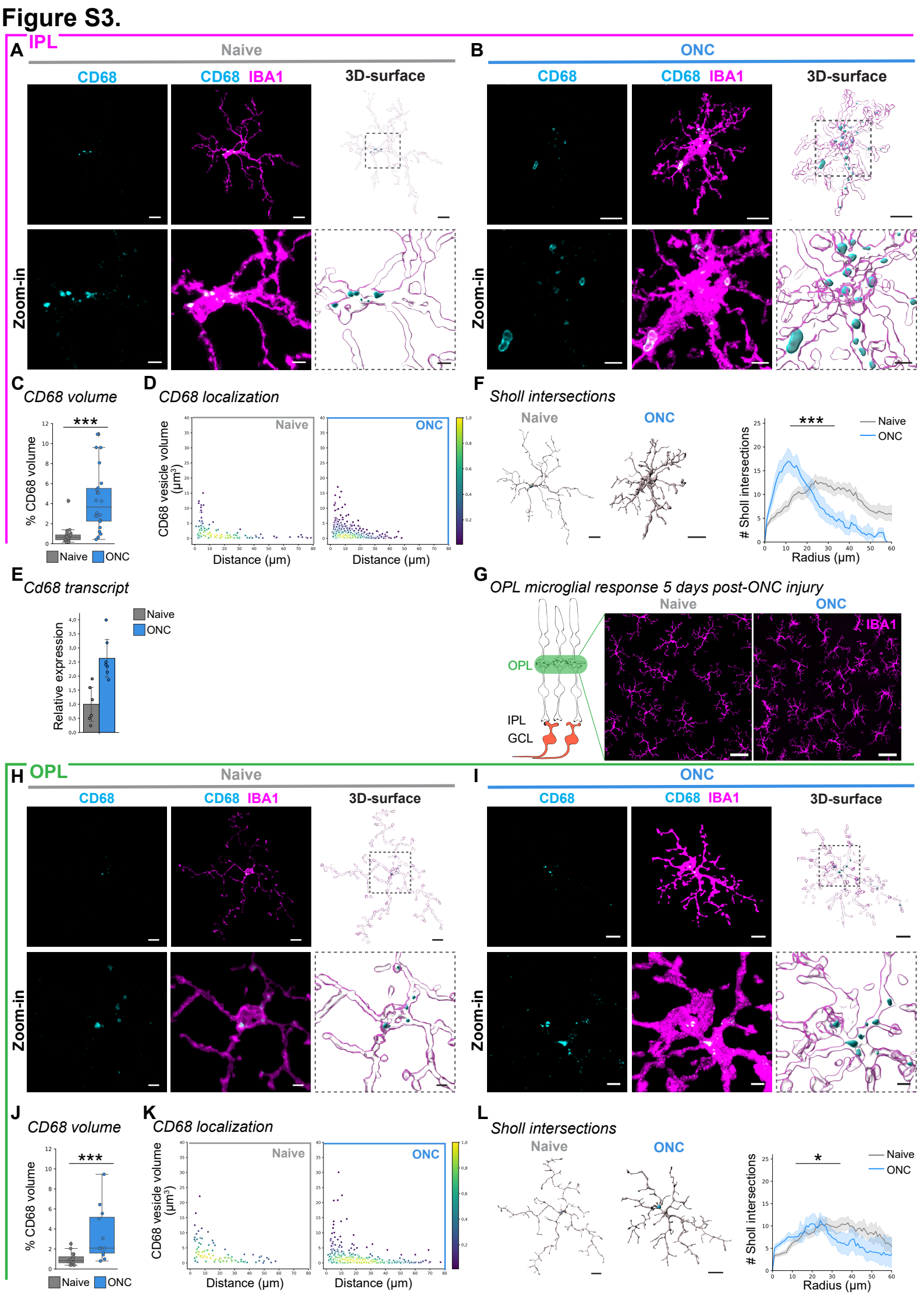

A
IPL
Naive
CD68
CD68 IBA1
3D-surface
Zoom-in
B
ONC
C
CD68 volume
% CD68 volume
***
Naive
ONC
D
CD68 localization
CD68 vesicle volume (µm³)
Distance (µm)
F
Sholl intersections
# Sholl intersections
Radius (µm)
E
Cd68 transcript
Relative expression
G
OPL microglial response 5 days post-ONC injury
OPL
IPL
GCL
IBA1
H
OPL
I
J
K
L
*

**Figure S3. CD68 and microglial morphology changes in ONC-responsive IPL and OPL microglia, Related to Figure 2**

(**A-D, F**) Microglial response in IPL WT microglia.

(**A**-**B**) Representative IBA1-immunostained IPL microglia (magenta) from **Figure 2D**-**E** co-labeled with CD68 (cyan) in naïve WT (**A**, grey) or ONC WT microglia (**B**, blue). Next, corresponding 3D-surfaces. Below: Zoom-in of region of interest (dashed line) from image and 3D-surface. Scale bar: 10 µm, zoom-in: 3 µm.

(**C**) Percentage of total CD68 volume per microglia volume. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond points: outliers outside 1.5 IQRs. Wilcoxon rank sum test: $p < 0.0001$.

(**D**) CD68 vesicle localization. Scatterplot depicting CD68 vesicle volume *vs.* distance from the cell soma (0, origin) of the population of vesicles in naïve WT (left) and ONC WT (right) conditions. Point density, pseudo-colored.

(**E**) Bar plot depicting relative *Cd68* transcript expression from FACSed mito-Dendra2$^{+}$ microglia in naïve WT or ONC WT retinas. Welch's t-test: $p = 0.0137$.

(**F**) 3D-filament tracings of microglia from naïve WT (left) or ONC WT (right). Scale bar: 10 µm. Line plot for mean number of Sholl intersections per radial distant from the soma (µm) with 95% confidence interval band. Linear mixed effects model: $p < 0.0001$.

(**G**) Left, retinal side-view schematic. OPL, outer plexiform layer. IPL, inner plexiform layer. GCL, ganglion cell layer. Overview confocal image of immunostained OPL microglia (IBA1, magenta) in naïve WT (left) and 5 days after ONC WT (right) condition. Scale bar: 30 µm.

(**H**-**L**) Microglial response in OPL WT microglia.

(**H**-**I**) CD68 (cyan) immunostaining in the IBA1-immunostained OPL microglia (magenta) from **Figure 2I**-**J** for naïve WT microglia (**H**) or ONC WT microglia (**I**). Next, corresponding 3D-surfaces. Below: Zoom-in of region of interest (dashed outline) from image and 3D-surface. Scale bar: 10 µm, zoom-in: 3 µm.

(**J**) Percentage of total CD68 volume per microglia volume. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond points: outliers outside 1.5 IQRs. Wilcoxon rank sum test: $p = 0.0007$.

(**K**) CD68 vesicle localization. Scatterplot depicting CD68 vesicle volume *vs.* distance from the cell soma (0, origin) of the population of vesicles in naïve (left, grey) and ONC (right, blue) conditions. Point density, pseudo-colored.

(**L**) 3D-filament tracings of microglia from naïve WT (left) or ONC WT (right). Scale bar: 10 µm. Line plot for mean number of Sholl intersections per radial distant from the soma (µm) with 95% confidence interval band. Linear mixed effects model: $p = 0.0302$.

* $p < 0.05$, *** $p < 0.001$. See **Table S8** for retina and cell numbers, statistical tests and corresponding data.

# Figure S4.

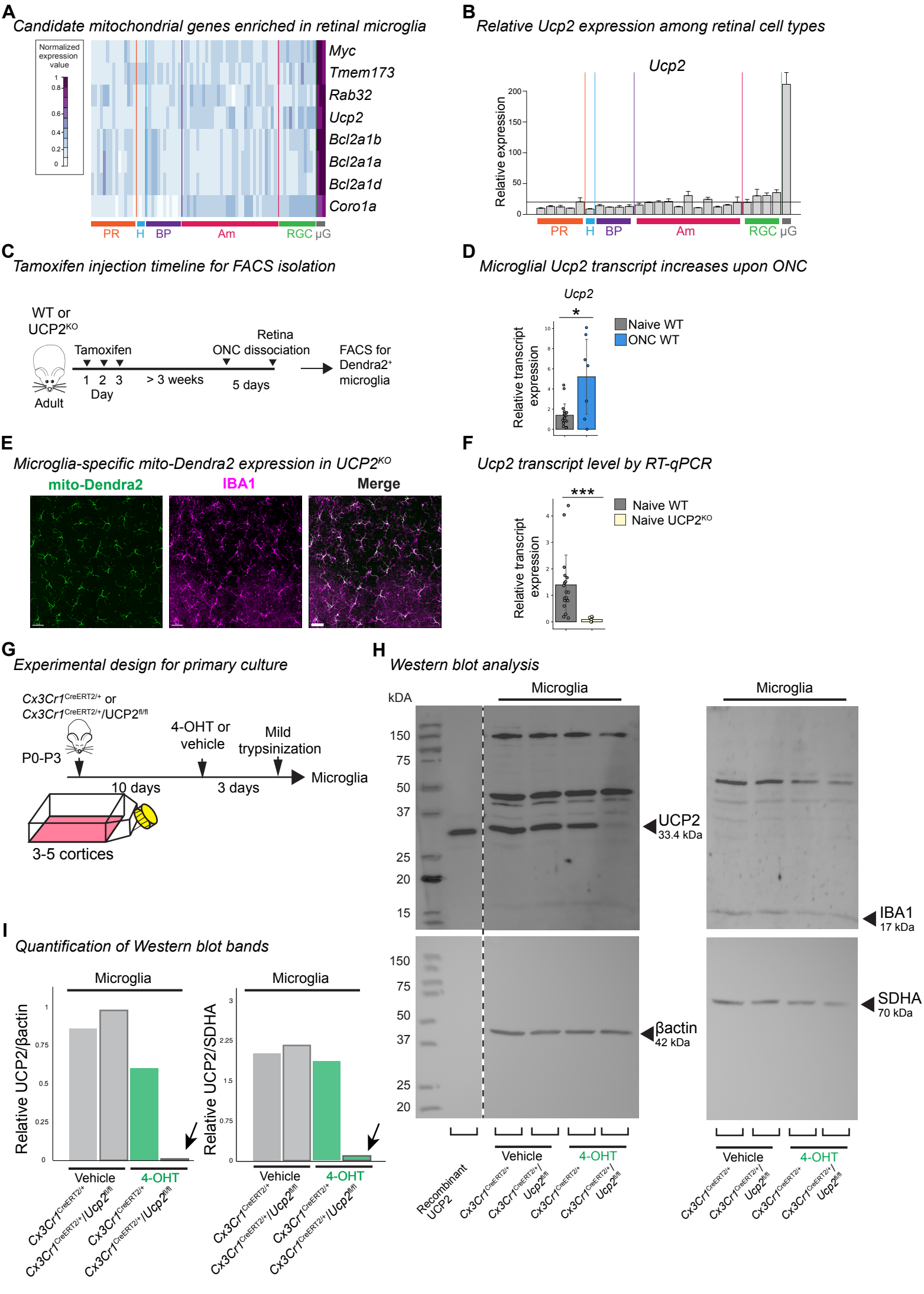

A
Candidate mitochondrial genes enriched in retinal microglia
Normalized expression value
Myc
Tmem173
Rab32
Ucp2
Bcl2a1b
Bcl2a1a
Bcl2a1d
Coro1a
PR H BP Am RGC µG
B
Relative Ucp2 expression among retinal cell types
Ucp2
Relative expression
PR H BP Am RGC µG
C
Tamoxifen injection timeline for FACS isolation
WT or UCP2KO
Tamoxifen
1 2 3 Day
> 3 weeks
Retina ONC dissociation
5 days
FACS for Dendra2+ microglia
Adult
D
Microglial Ucp2 transcript increases upon ONC
Ucp2
Relative transcript expression
Naive WT
ONC WT
E
Microglia-specific mito-Dendra2 expression in UCP2KO
mito-Dendra2
IBA1
Merge
F
Ucp2 transcript level by RT-qPCR
Relative transcript expression
Naive WT
Naive UCP2KO
G
Experimental design for primary culture
Cx3Cr1CreERT2/+ or Cx3Cr1CreERT2/+/UCP2fl/fl
P0-P3
4-OHT or vehicle
Mild trypsinization
10 days
3 days
Microglia
3-5 cortices
H
Western blot analysis
kDA
Microglia
UCP2 33.4 kDa
IBA1 17 kDa
βactin 42 kDa
SDHA 70 kDa
Recombinant UCP2
Vehicle
4-OHT
I
Quantification of Western blot bands
Relative UCP2/βactin
Relative UCP2/SDHA
Vehicle
4-OHT

**Figure S4. Selective expression and knockout of UCP2 in retinal microglia, Related to Figure 3**

(**A**-**B**) Relative fold change expression level of mitochondrial-associated genes (**A**) and *Ucp2* (**B**) among retinal cell types. Plotted from available data in *Siegert et. al*. (1). Purple and blue: high and low normalized expression value, respectively. PR: photoreceptors, H: horizontal cells, BP: bipolar cells, Am: amacrine cells, RGC: retinal ganglion cells, µG: microglia.

(**C**) Timeline for tamoxifen administration to induce mito-Dendra2 expression or in combination $UCP2^{KO}$ in microglia used for FACS (fluorescence activated cell sorting).

(**D**) Boxplot of relative *Ucp2* transcript expression from $Dendra2^{+}$-FACSed retinal microglia in naïve WT or 5 days ONC WT. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond: outliers outside of 1.5 IQRs. Naïve: grey. ONC: blue. Wilcoxon rank sum test: $p = 0.0474$.

(**E**) Microglial specificity of mito-Dendra2 labeling in $UCP2^{KO}$ mouse model. Overview image of immunostained microglia (IBA1, magenta) in the inner plexiform layer (IPL) expressing the mitochondrial label (Dendra2, green) in retinal wholemounts. Scale bar: 50 µm.

(**F**) Relative *Ucp2* transcript expression from tamoxifen-induced naïve WT or naïve $UCP2^{KO}$ FACSed-microglia from the retina. Wilcoxon rank sum test: $p < 0.0001$.

(**G**) Experimental timeline for primary murine mixed glial cultures to obtain microglia and astrocyte-enriched cultures for protein analysis. P0-P3: postnatal day 0 to 3. 4-OHT: 4-hydroxytamoxifen. Next: IBA1-immunostained (magenta) mixed glial culture at day 10 prior to trypsinization. Scale bar: 50 µm.

(**H**) Western blot analysis of UCP2 protein expression (left, top), β-actin loading control (left, bottom), IBA1 microglial marker (right, top), or SDHA (Succinate dehydrogenase complex flavoprotein subunit A) mitochondrial loading control (right, bottom) in primary microglia of either $Cx3cr1^{CreERT2/+}$ or $Cx3cr1^{CreERT2/+}$/$UCP2^{fl/fl}$ with vehicle or 4-OHT (n = 1). kDa, kilodaltons.

(**I**) Quantification of UCP2 band intensity relative to β-actin loading control (left) or relative to SDHA (right) mitochondrial loading control.

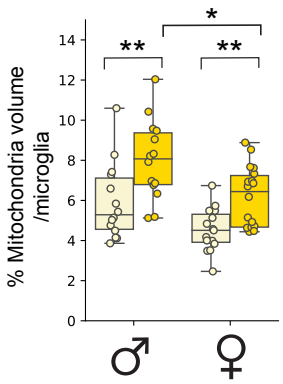
* $p < 0.05$, *** $p < 0.001$. See **Table S9** for retina and cell numbers, statistical tests and corresponding data.

# Figure S5

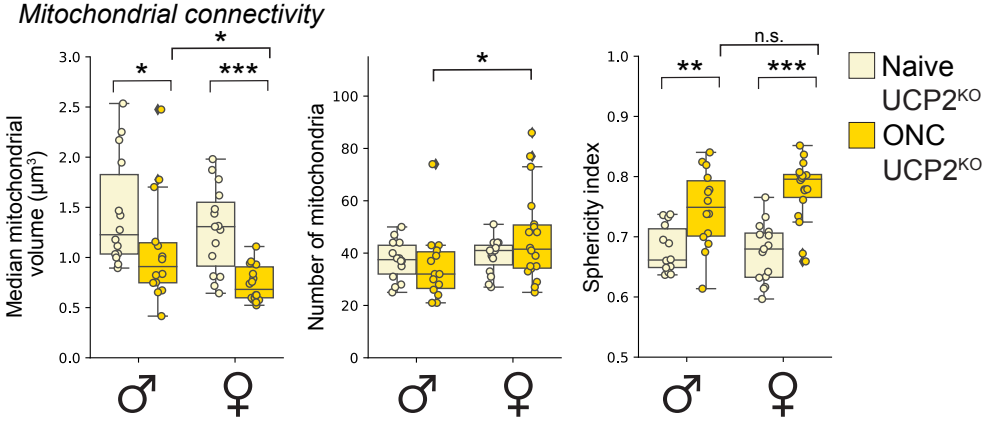
**A** *Principal component loadings*

| | PC 1 | PC 2 |
|---|---|---|
| **% Mitochondria Perinuclear** | 0.4915 | 0.0797 |
| **Median mitochondrial volume** | -0.3809 | 0.5161 |
| **Number of mitochondria** | 0.2067 | -0.5712 |
| **% Mitochondria volume/microglia** | 0.3184 | 0.6000 |
| **Sholl index** | 0.5110 | 0.1991 |
| **CD68 volume** | 0.4563 | -0.0379 |

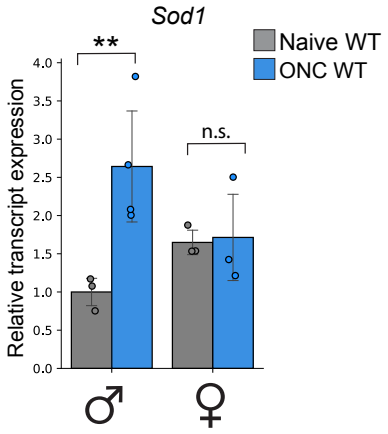
**B** *Mitochondrial content*




**C** *Mitochondrial connectivity*




**D** *Cellular ROS in WT male and female*




**E** *Mitochondrial ROS in WT ONC male and female*

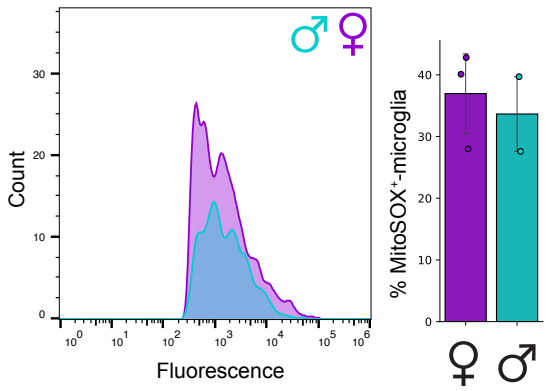


**Figure S5. Sex-separated UCP2$^{KO}$ mitochondrial parameters, Related to Figure 5**

(**A**) Table of principal component (PC) loadings from **Figure 5**. Green highlight: Highest loading for PC2.

(**B**-**C**) Mitochondrial parameters. Boxplot minimum and maximum: InterQuartile Range (IQR) around median (center line). Whiskers: 1.5 IQRs. Black diamond: outliers outside 1.5 IQRs. Each overlaid point represents data point of a single microglia. Naïve UCP2$^{KO}$: tan. ONC UCP2$^{KO}$: gold.

(**B**) Percentage of mitochondrial volume per microglial volume. Kruskal-Wallis test: $p < 0.0001$. Selected Conover's post-hoc comparisons with Holm p-adjustment: Naïve ♂UCP2$^{KO}$ *vs.* ONC ♂UCP2$^{KO}$, $p = 0.0076$; Naïve ♀UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p = 0.0073$; ONC ♂UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p = 0.0266$.

(**C**) Mitochondrial network connectivity determined by median mitochondrial volume (left, Kruskal-Wallis test: $p < 0.0001$. Selected Conover's post-hoc comparisons with Holm p-adjustment: Naïve ♂UCP2$^{KO}$ *vs.* ONC ♂UCP2$^{KO}$, $p = 0.0348$; Naïve ♀UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p < 0.0001$; ONC ♂UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p = 0.0303$), number of organelles (center, Wilcoxon rank sum test: $p = 0.0383$) and mean sphericity (right, Kruskal-Wallis test: $p < 0.0001$. Selected Conover's post-hoc comparisons with Holm p-adjustment: Naïve ♂UCP2$^{KO}$ *vs.* ONC ♂UCP2$^{KO}$, $p = 0.0029$; Naïve ♀UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p < 0.0001$; ONC ♂UCP2$^{KO}$ *vs.* ONC ♀UCP2$^{KO}$, $p = 0.2323$).

(**D**) Bar plot depicting relative *Sod1* transcript expression from FACSed mito-Dendra2$^{+}$ microglia in naïve WT and ONC WT male and female microglia. Transcript expression relative to male naïve WT. Kruskal-Wallis test: $p = 0.0269$. Selected Conover's post-hoc comparisons with Holm p-adjustment: ♂WT Naïve *vs.* ♂WT ONC: $p = 0.0026$, ♀WT Naïve *vs.* ♀WT ONC: $p = 0.8554$.

(**E**) Frequency plot of MitoSOX fluorescence from FACSed mito-Dendra2$^{+}$ retinal microglia for WT ONC male (turquoise) and female (purple). Corresponding bar plot of percentage of microglia that are MitoSOX$^{+}$. ** $p < 0.01$, *** $p < 0.001$, $^{n.s.}$ $p > 0.05$: not significant. See **Table S10** for retina and cell numbers, statistical tests, other post-hoc comparisons and corresponding data.

**Table S11**. Primer sequences, Related to STAR Methods.

| Gene | Primer | Sequence | Product length |
|---|---|---|---|
| **GAPDH** | FW | ACAGCAACTCCCACTCTTC | 131 bp |
| | REV | CATTGTCATACCAGGAAATGAGC | |
| **IBA1** | FW | ATGAGGATCTGCCGTCCAAAC | 114 bp |
| | REV | CAAGTTTCTCCAGCATTCGCTTC | |
| **UCP2** | FW | AAGTGTTTCGTCTCCCAGCC | 188 bp |
| | REV | GGGACCTTCAATCGGCAAGA | |
| **CD68** | FW | CCTCTGTTCCTTGGGCTATAAG | 105 bp |
| | REV | ATTGAGGAAGGAACTGGTGTAG | |
| **SOD1** | FW | CACTTCGAGCAGAAGGCAAGC | 186 bp |
| | REV | ATGCCTCTCTTCATCCGCCG | |