Supplementary Information

Microglial Tunneling Nanotubes Restrain Microglial Activation Induced by Methamphetamine

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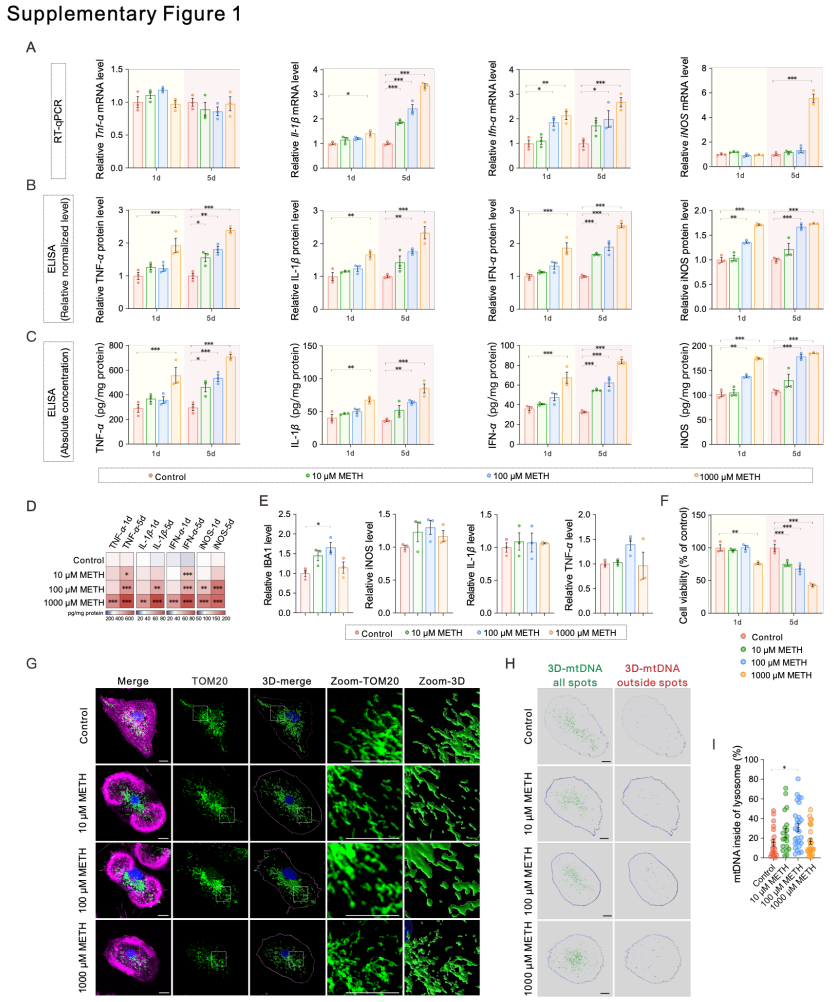
Supplementary Figure Titles and Legends



Supplementary Figure 1. Short-term METH exposure causes an apparent release of mtDNA without noticeably impacting cell viability or overall mitochondrial morphology. (A) Bar graphs showing the relative mRNA levels of *Tnf-α*, *Il-1β*, *Ifn-α*, and *iNOS* in primary microglia treated with 10, 100, or 1000 μM METH for 1 day or 5

days corresponding to Figure 1G. *n* = 3 independent biological experiments; two-way ANOVA followed by Dunnett's multiple comparisons test. (**B**) Bar graphs showing the relative protein levels of TNF-α, IL-1β, IFN-α and iNOS s in primary microglia treated with 10, 100, or 1000 μM METH for 1 day or 5 days corresponding to Figure 1G. ELISA data were normalized to total protein and expressed as fold change over vehicle control, *n* = 3 independent biological experiments; two-way ANOVA followed by Dunnett's multiple comparisons test. (**C**) Absolute concentrations of TNF-α, IL-1β, IFN-α, and iNOS measured by ELISA and normalized to total protein in (B). n = 3 independent biological experiments; two-way ANOVA followed by Dunnett's multiple comparisons test. (**D**) Heatmap representation of the protein-normalized ELISA data shown in (C). n = 3 independent biological experiments; two-way ANOVA followed by Dunnett's multiple comparisons test. (**E**) Bar graph showing the protein levels of IBA1, iNOS, IL-1β, and TNF-α in primary microglia following exposure to 10, 100, or 1000 μM METH for 1 day corresponding to Figure 1H. *n* = 3 independent biological experiments; ordinary one-way ANOVA. (**F**) Quantification of cell viability following 1 day and 5 days of METH treatment. *n* = 3 independent biological samples, two-way ANOVA followed by Dunnett's multiple comparisons test. (**G**) Representative immunofluorescence and 3D-reconstructed images of mitochondrial morphology after the short-term METH treatment. Scale bars, 10 μm. (**H**) 3D-reconstructed images showing the total mtDNA puncta (green) and the cytosolic mtDNA puncta located outside mitochondria (red). Scale bars, 10 μm. **(I)** Scatter plot showing the proportion of mtDNA inside of lysosome. *n* = 19–28 cells from three independent experiments;

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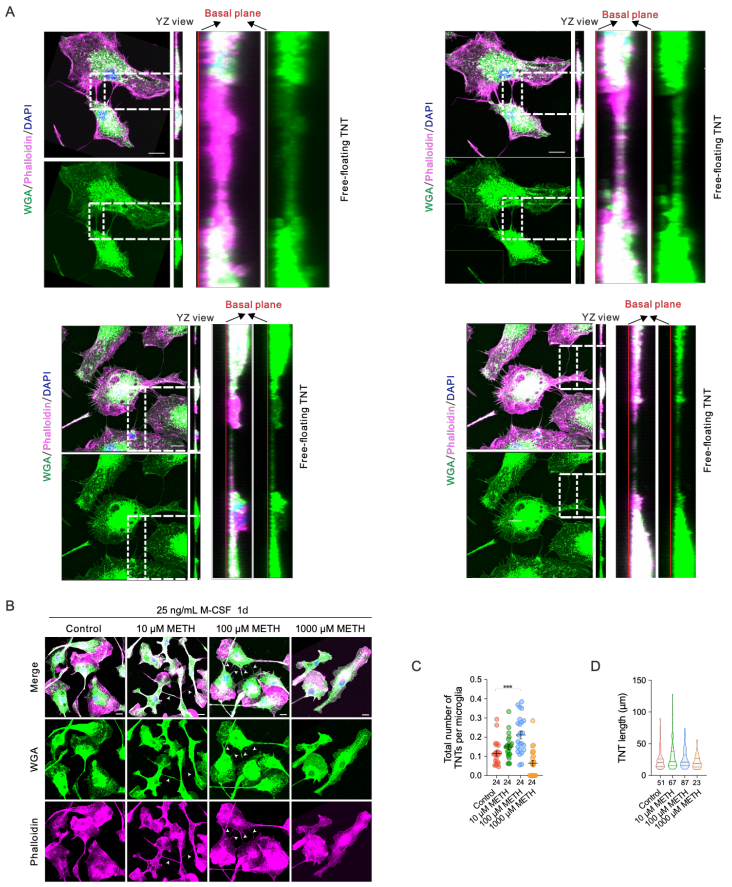
ordinary one-way ANOVA. Data present as mean ± SEM; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

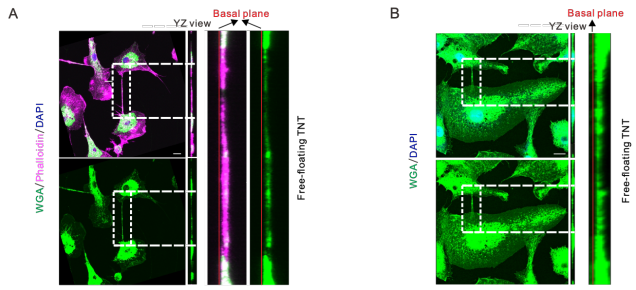
Supplementary Figure 2



Supplementary Figure 2. Orthogonal-view validation of suspended TNTs and M-CSF-supported TNT formation in primary microglia after short-term METH exposure. (A) Y–Z orthogonal-view analysis of the TNT structure shown in Figure 2E. Scale bars, 10 μm. **(B)** Representative immunofluorescence images showing homotypic

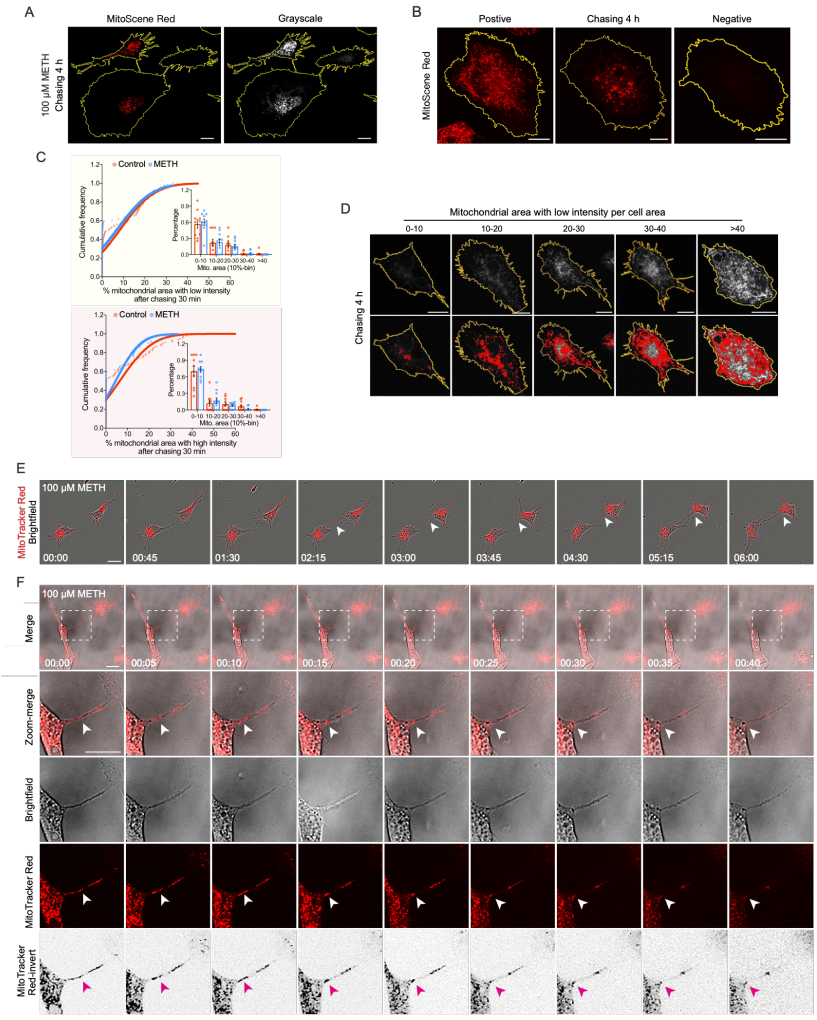
TNTs among primary microglia treated with 25 ng/mL M-CSF in the presence or absence of 10, 100, or 1000 μM METH for 1 day. WGA and phalloidin were used to label the cell membrane and F-actin, respectively. White arrowheads indicate TNT. Scale bars, 10 μm. **(C)** Scatter plot showing the proportion of TNT-connected cells relative to total microglia within regions of interest (ROIs). The numbers below the x-axis indicate the number of ROIs analyzed in each group. n = 24 ROIs from three independent experiments, ordinary one-way ANOVA. **(D)** Quantitative analyses of TNT length demonstrate comparable morphological characteristics. The numbers below the x-axis indicate the number of individual TNTs measured in each group. *n* = 23–87 TNTs from three independent experiments, ordinary one-way ANOVA. Data present as mean ± SEM; ***$P < 0.001$.

Supplementary Figure 3



Supplementary Figure 3. Y–Z orthogonal-view validation of TNTs shown in Figure 3A and Figure 3B. (A) Representative Y–Z orthogonal view of the TNT structure shown in Figure 3A. Scale bars, 10 μm. **(B)** Representative Y–Z orthogonal view of the TNT structure shown in Figure 3B. Scale bars, 10 μm.

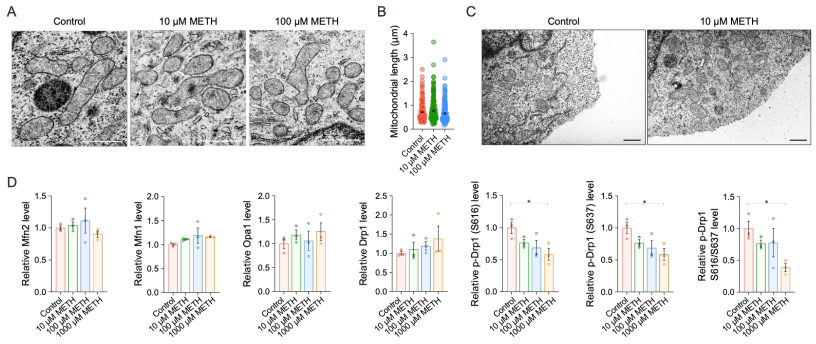
Supplementary Figure 4



Supplementary Figure 4. Homotypic TNT-mediated mitochondrial transfer is essential for maintaining microglial homeostasis in response to METH exposure. (**A**) Representative fluorescence and corresponding grayscale images of donor and acceptor microglia after 4 h of co-culture in the 100 μM METH-treated group. MitoScene Red-labeled mitochondria are shown in red, and cell boundaries are outlined

in yellow. Scale bar, 10 μm. (**B**) Representative fluorescence images corresponding to the grayscale images shown in Figure 3E, including the positive, 4 h chasing, and negative groups. Scale bar, 10 μm. (**C**) Quantification of the cumulative frequency of microglia containing low- and high-intensity mitochondria at 30 min. The proportion of microglia containing mitochondria was binned in 10% intervals (insets). $n = 10$ fields per sample from three independent experiments; two-way repeated measures ANOVA. (**D**) Representative immunofluorescence images of mitochondrial area with low intensity per cell area at every 10% intervals at 4 h. Scale bars, 10 μm. (**E**) Time-lapse live-cell imaging showing mitochondrial movement within TNTs in primary microglia treated with 100 μM METH. White arrowheads indicate MitoTracker Red labeled mitochondria moving along TNTs. Scale bars, 20 μm. (**F**) Time-lapse live-cell imaging corresponding to Figure 3I showing MitoTracker-labeled mitochondria trafficking along TNTs in primary microglia following treatment with 100 μM METH. Scale bars, 20 μm.

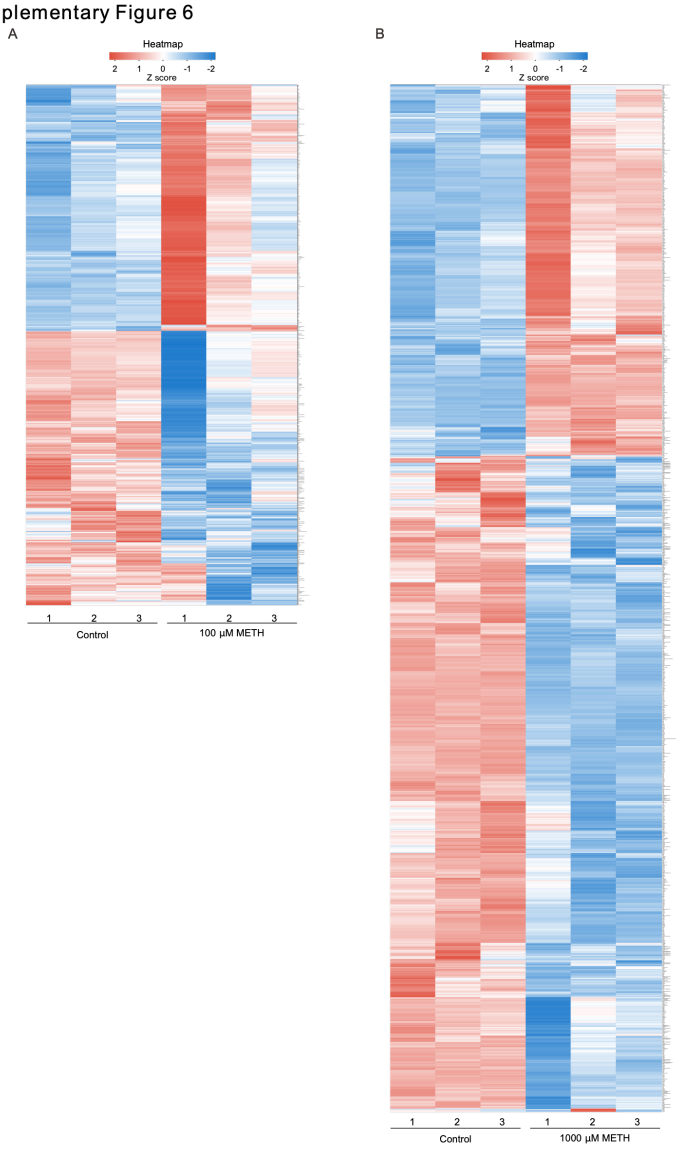
Supplementary Figure 5



Supplementary Figure 5. Short-term moderate METH exposure has minimal

effects on mitochondrial ultrastructure and dynamics-related proteins. (A) Representative electron micrographs showing mitochondrial ultrastructure in primary microglia following METH treatment. Scale bars, 1 μm. **(B)** Quantification of mitochondrial length measured from electron micrographs. n = 190–276 mitochondria from three independent biological samples; ordinary one-way ANOVA. **(C)** Representative electron micrographs showing the ultrastructure of the cell edge in primary microglia under control and 10 μM METH-treated conditions. Scale bars, 1 μm. **(D)** Quantitative analysis of mitochondrial dynamics-related proteins corresponding to Figure 3L, including Mfn2, Mfn1, Opa1, Drp1, p-Drp1 (S616), p-Drp1 (S637), and the p-Drp1 S616/S637 ratio, in primary microglia following METH treatment. n = 3 independent biological experiments; ordinary one-way ANOVA. Data present as mean ± SEM. *P < 0.05.

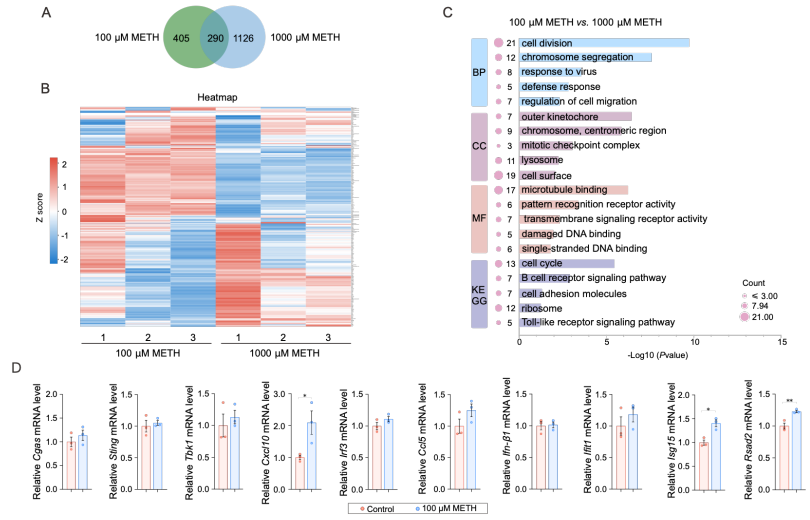
Supplementary Figure 6



Supplementary Figure 6. Expression profile of DEGs following different METH treatments. (**A-B**) Heatmap of gene expression for all DEGs in the 100 μM METH-treated group (A) and the 1000 μM METH-treated group (B). Each column represents

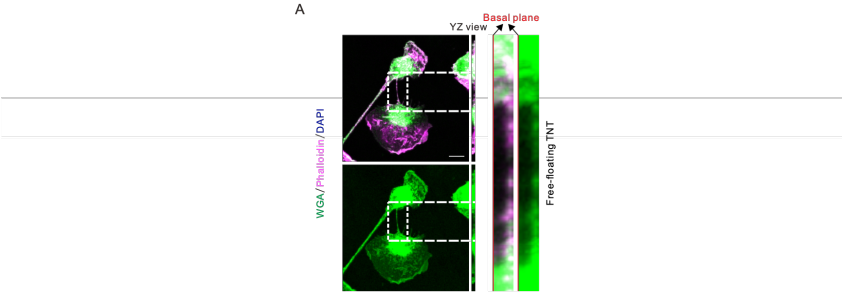
an experimental treatment, and each row represents a DEG.

Supplementary Figure 7



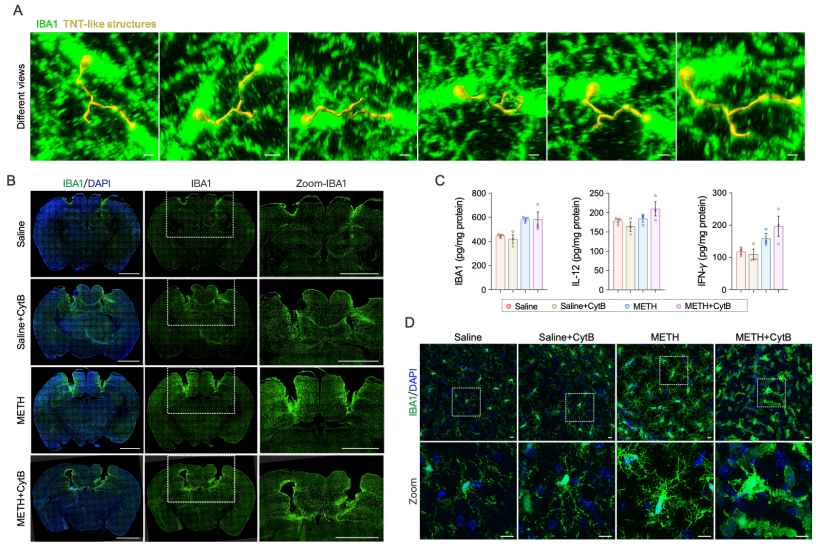
Supplementary Figure 7. Microglial transcriptional responses to METH were dose-dependent, with overlapping DEGs reflecting shared stress-response mechanisms. (**A**) Venn diagram illustrating the overlap of DEGs identified in microglia following 100 μM and 1000 μM METH treatment for 1 day. (**B**) Heatmap of gene expression for overlapped DEGs identified in both the 100 μM and 1000 μM METH-treated groups. (**C**) GO and KEGG pathway enrichment analyses of the overlapped DEGs in both the 100 μM and 1000 μM METH-treated groups. (**D**) RT-qPCR analysis of cGAS–STING pathway gene expression in microglia following 100 μM METH treatment for 1 day. n = 3 independent biological experiments, unpaired two-tailed Student's t-test. Data present as mean ± SEM; *$P < 0.05$, and **$P < 0.01$.

Supplementary Figure 8



Supplementary Figure 8. Y–Z orthogonal-view validation of TNTs shown in Figure 5A. (A) Representative Y–Z orthogonal view of the TNT structure shown in Figure 5A. Scale bars, 10 μm.

Supplementary Figure 9



Supplementary Figure 9. METH injection promotes the TNT formation *in vivo* and intrahippocampal CytB injection induces only mild localized inflammation.

(**A**) Representative 3D-reconstructed images corresponding to Figure 6C and viewed from different angles. TNT-like structure is highlighted in yellow. Scale bars, 2 μm. **(B)** Representative immunofluorescence images showing IBA1 expression in each treatment group. Scale bars, 2 mm. **(C)** ELISA quantification of IBA1, IL-12 and IFN-γ levels in hippocampal tissue from the indicated treatment groups. The measured concentrations were normalized to the corresponding total protein content and expressed as pg/mg protein. n = 3 independent biological experiments; ordinary one-way ANOVA. (**D**) Representative immunofluorescence images of microglial morphology. Scale bars, 20 μm.

Video 1. Time-lapse live-cell imaging corresponding to Figure 3I showing mitochondrial movement along TNTs in primary microglia following 100 μM METH treatment. Mitochondrial movement along TNTs was monitored by confocal microscopy, and images were acquired at 5-min intervals.

Video 2. Time-lapse live-cell imaging corresponding to Supplementary Figure 4F showing mitochondrial movement along TNTs in primary microglia following 100 μM METH treatment. Mitochondrial movement along TNTs was monitored using the Incucyte® CX3 live-cell imaging system, and images were acquired at 45-min intervals.