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Supplementary Materials for

Brain rhythms control microglial response and cytokine expression via NF-κB signaling

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary Figures

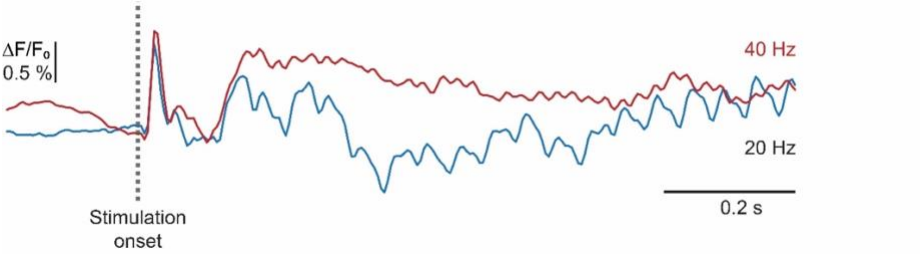

Fig. S1. Mean ΔF/F$_0$ trace from 2×2-pixel (200 µm × 200 µm) at primary visual cortex. Lines indicate the oscillatory response during 40Hz (red) or 20Hz (blue) visual flicker. The vertical line indicates flicker onset. N = 6 mice and 60 trials, 10 trials/mouse.

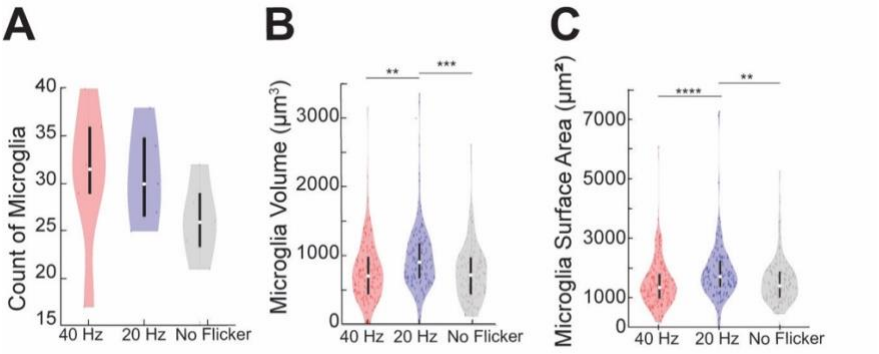

Fig. S2. Visual flicker affects microglia volume and surface area in a frequency specific manner in WT mice. Analysis of IBA-1+ microglia after animals were exposed to 1hr of 40 Hz Flicker (red, n=6 mice, 174 microglia), 20 Hz Flicker (blue, n=5 mice, 140 microglia), or No Flicker (grey, n=5 mice, 132 microglia) stimulation for **(A)**, number of microglia per animal (F=0.9797, p=0.4015) (F(2,13)=0.9797, p=0.4015; post-hoc t-tests: 40Hz vs. 20Hz: p = 0.9999, 40Hz vs No Flicker: p = 0.446, 20Hz vs No Flicker: p = 0.47997; Tukey's HSD corrected for multiple comparisons) **(B)**, Volume of IBA-1+ microglia (including cell body and processes) after 40Hz, 20Hz, or No Flicker stimulation (F(2,443)= 9.9694, p=0.0001; post-hoc t-tests: 40Hz vs. 20Hz: p = 0.0002, 40Hz vs No Flicker: p = 0.9957, 20Hz vs No Flicker: p = 0.0004; Tukey's HSD corrected for multiple comparisons) and **(C)**, surface area of whole IBA-1+ microglia after 40Hz, 20Hz, or No Flicker stimulation (F(2,443)= 9.4815, p=9.39E-5; post-hoc t-tests: 40Hz vs. 20Hz: p = 9.39E-5, 40Hz vs No Flicker: p = 0.7809, 20Hz vs No Flicker: p = 0.0035; Tukey's HSD corrected for multiple comparisons). Box plots inside violin plots indicate median and quartiles, dots indicate individual microglia. F- and p-values were generated from one-way, two-tailed, unpaired ANOVA tests. Differences between groups were found from one-way, two-tailed, t-tests with Bonferroni correction. **p<0.01, ***p<0.001, ****p<0.0001.

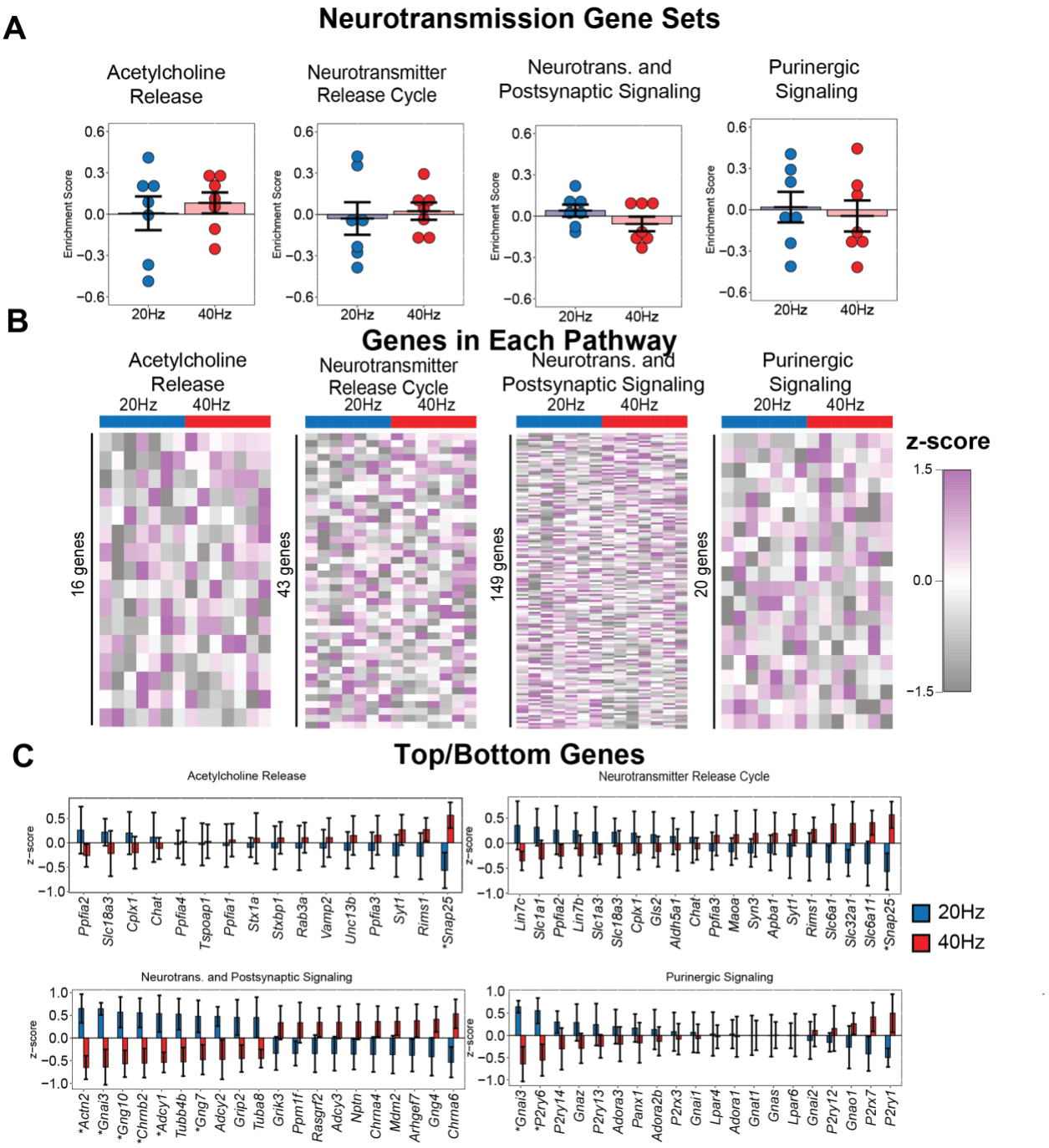

Fig. S3. Flicker audio/visual stimulation for 1 hr at 40 Hz and 20 Hz does not significantly modulate gene sets associated with neurotransmission. (**A**) Gene set variation analysis did not identify significant enrichment immune-related gene sets (mean±SEM, statistical testing via gene set permutation analysis, dots indicate individual animals). (**B**) Heatmap representation of all genes within each pathway (rows are z-scored). (**C**) Few genes within neurotransmission-related pathways are individually significantly different. (DESeq2, *p<0.05 un-adjusted).

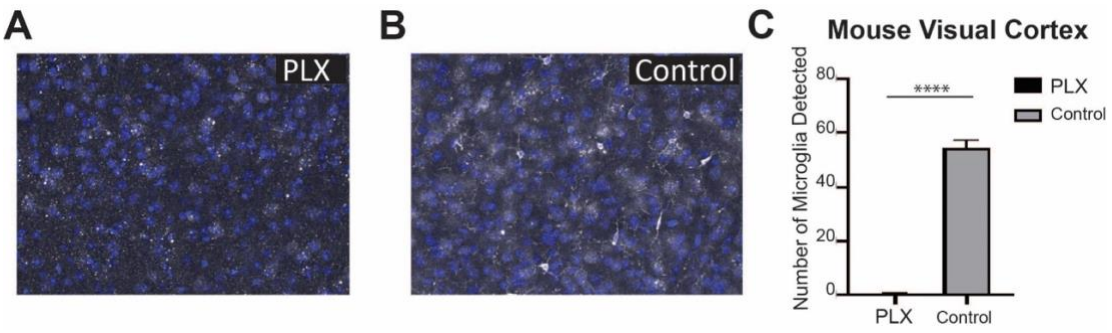

Fig. S4. PLX 3397 successfully depletes microglia while cytokines remain expressed. Microglia were depleted using Pexidartinib (PLX3397, MedChem) incorporated into the Open Standard Diet with 15Kcal% fat (Research Diets, INC) at a dose of 290mg/kg for 3 weeks. A control group was fed a diet of the Open Standard Diet with 15Kcal%. (A) Representative immunohistochemistry for IBA1+ microglia in the mouse visual cortex following 3-weeks of PLX diet compared to (B) mice fed control chow. (C) Number of microglia in visual cortex in mice fed PLX (black) and control (grey) diets ($t(8)=17.35$, $p<0.000001$). Error bars indicate mean±SEM. ****$p<0.0001$.

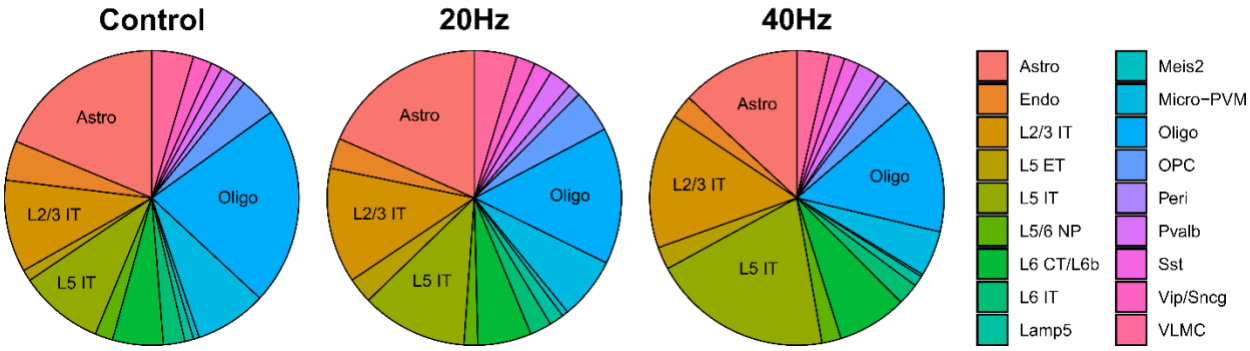

Fig. S5. Cell type proportions are approximately even throughout visual flicker stimulation groups. Proportion of each identified cell type by Azimuth reference-based mapping to mouse primary motor cortex cell atlas.

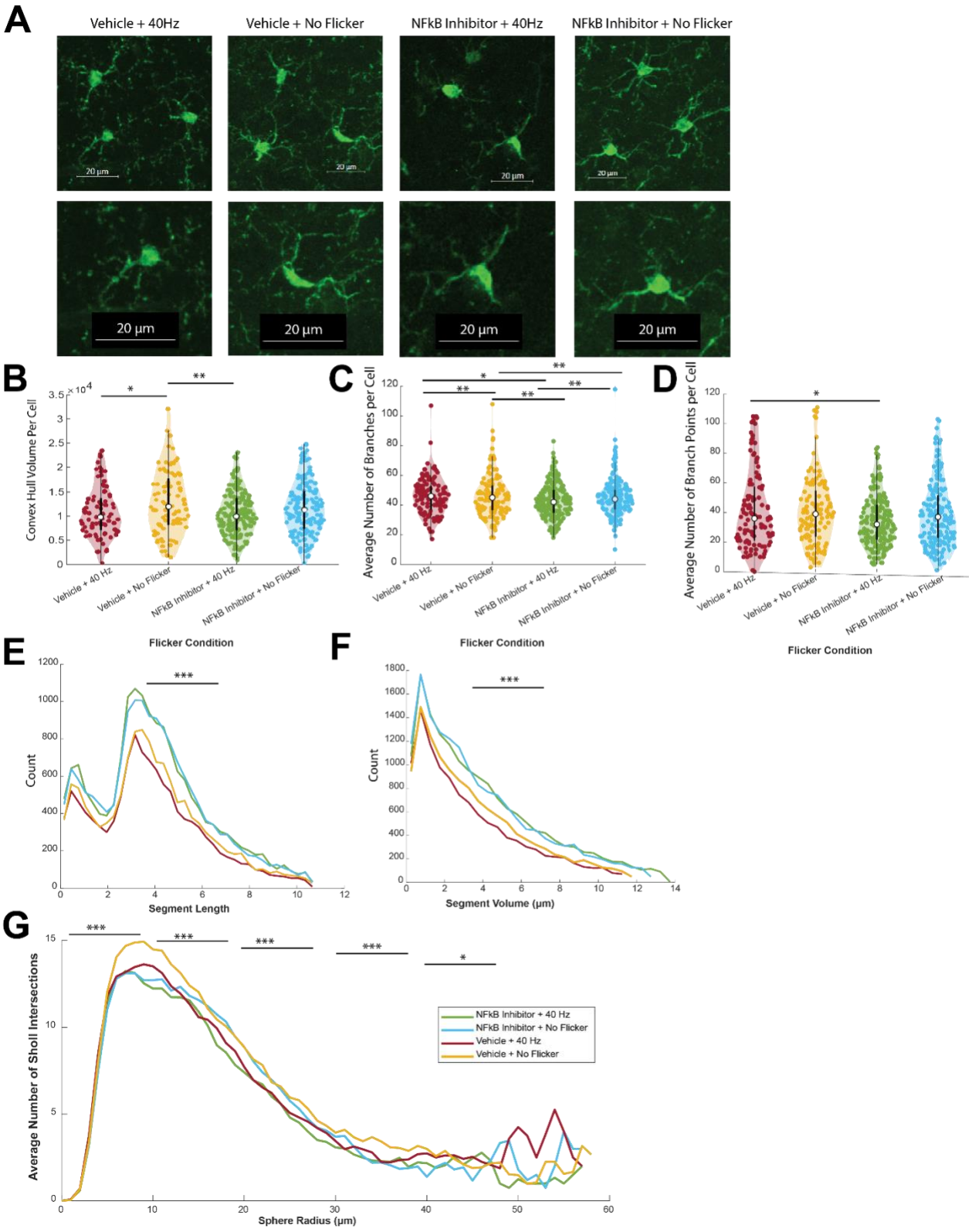

Fig. S6. 40Hz flicker-induced microglia changes are mediated by NFκB phospho-signaling pathways. (A) Example images of IBA-1+ microglia (green) from each experimental condition

show clusters of representative microglia (top row, taken at 20x) and single representative microglia (bottom row, taken at 40x). Scale bar indicates 20 μm. **(B)** There were significant differences for convex hull volume ($F(3,504) = 4.84$, $p = 0.003$), and post-hoc t-tests: Vehicle + 40Hz vs Vehicle + No Flicker $p = 0.034$, Vehicle + No Flicker vs NFκB Inhibitor + 40Hz $p = 0.0017$; Tukey's HSD corrected for multiple comparisons). **(C)** Number of branches ($F(3,27125) = 16.24$, $p < 0.001$), and post-hoc tests: Vehicle + 40Hz vs Vehicle + No Flicker $p = 0.002$, Vehicle + 40Hz vs NFκB Inhibitor + 40Hz $p = 0.015$, Vehicle + No Flicker vs NFκB Inhibitor + 40Hz $p < 0.002$, Vehicle + No Flicker vs NFκB Inhibitor + No Flicker $p = 0.0019$, NFκB Inhibitor + 40Hz vs NFκB Inhibitor + No Flicker $p = 0.0015$. **(D)** Number of branch points ($F(3,587)=4.07$, $p = 0.007$), and post-hoc t-tests: Vehicle + 40Hz vs NFκB Inhibitor + 40Hz $p = 0.01$. **(E)** Segment length ($F(3,56772)=19.46$, $p < 0.001$, and post-hoc tests: Vehicle + 40Hz vs Vehicle + No Flicker $p = 0.01$, Vehicle + 40Hz vs NFκB Inhibitor + 40Hz $p < 0.0003$, Vehicle + 40Hz vs NFκB Inhibitor + 40Hz + No Flicker, $p < 0.0003$, Vehicle + No Flicker vs NFκB Inhibitor + 40Hz $p = 0.0023$, Vehicle + No Flicker vs NFκB Inhibitor + No Flicker $p = 0.0024$. **(F)** Segment volume ($F(3,55815)=141.99$, $p < 0.0001$, and post-hoc t-tests were significant across all groups $p < 0.001$. **(G)** Sholl analysis of IBA-1+ microglia shows significant differences in the number of branches between groups (linear mixed model with repeated measures) and at different radii extending from the soma ($F(59,21534)=293.6$, $p < 0.001$) and groups ($F(3,21534) = 7.22$, $p < 0.001$). There were significant differences for post-hoc t-tests between groups NFκB Inhibitor + 40Hz vs Vehicle + 40Hz $p=0.014$, NFκB Inhibitor + 40Hz vs Vehicle + No Flicker $p < 0.001$. These differences between groups occurred at specific radii: <u>at bin 0-10</u> significant differences between NFκB Inhibitor + 40Hz vs Vehicle + No Flicker groups $p<0.001$; <u>at bin 11-20</u> NFκB Inhibitor + 40Hz vs NFκB Inhibitor + No Flicker $p<0.001$, NFκB Inhibitor + 40Hz vs Vehicle + No Flicker $p = <0.001$, Vehicle + 40Hz vs Vehicle + No Flicker $p < 0.001$; <u>at bin 21-30</u> NFκB Inhibitor + 40Hz vs NFκB Inhibitor + No Flicker $p < 0.001$, NFκB Inhibitor + 40Hz vs Vehicle + No Flicker $p < 0.001$, NFκB Inhibitor + No Flicker vs Vehicle + 40Hz $p = 0.043$, Vehicle + 40Hz vs Vehicle + No Flicker $p < 0.001$; <u>at bin 31-40</u> NFκB Inhibitor + 40Hz vs Vehicle + 40Hz $p = 0.033$, NFκB Inhibitor + 40Hz vs Vehicle + No Flicker $p < 0.001$, NFκB Inhibitor + No Flicker vs Vehicle + No Flicker $p < 0.001$, Vehicle + 40Hz vs Vehicle + No Flicker $p = 0.009$; <u>at bin 41-50</u> and NFκB Inhibitor + 40Hz vs NFκB Inhibitor + No Flicker $p = 0.032$, NFκB Inhibitor + 40Hz vs Vehicle + 40Hz $p = 0.036$. * $p< .05$, ** $p<0.01$, *** $p<.001$.

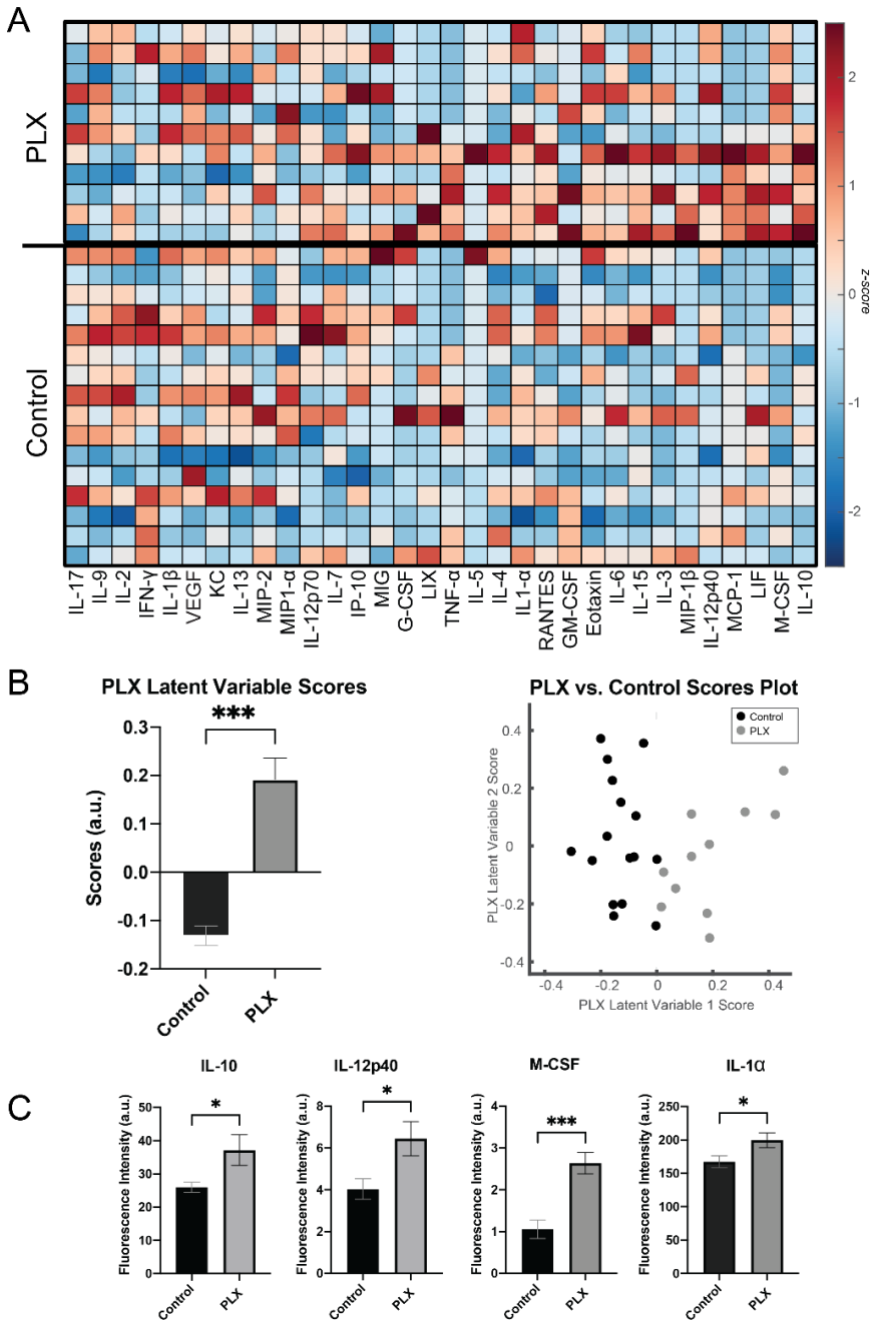

Fig. S7. Cytokines Persist in the Absence of Microglia.

(A) Cytokine expression in visual cortices of mice fed PLX3397 or control diet for three weeks. Each row represents one animal. Cytokines (columns) are arranged in the order of their weights on the LV1. Color indicates z-scored expression levels for each cytokine. **(B)** (**right**) PLSDA identified LV1, the axis that separated animals fed PLX3397 and a control diet, (**left**) LV1 scores were significantly different between the two groups of animals (mean ± SEM; t(25)=7.297, p<0.0001, unpaired, two-tailed t-test). **(C)**, Cytokines with significant differences in expression between both groups, IL-10: t(25)=2.647, p=0.0139; , IL-12p40: t(25)=2.665, p=0.0133; M-CSF: t(25)=4.608, p=0.0001; IL-1α: t(25)=2.309, p=0.0295.SEM, standard error of the mean. * p< .05, ***p≤.001

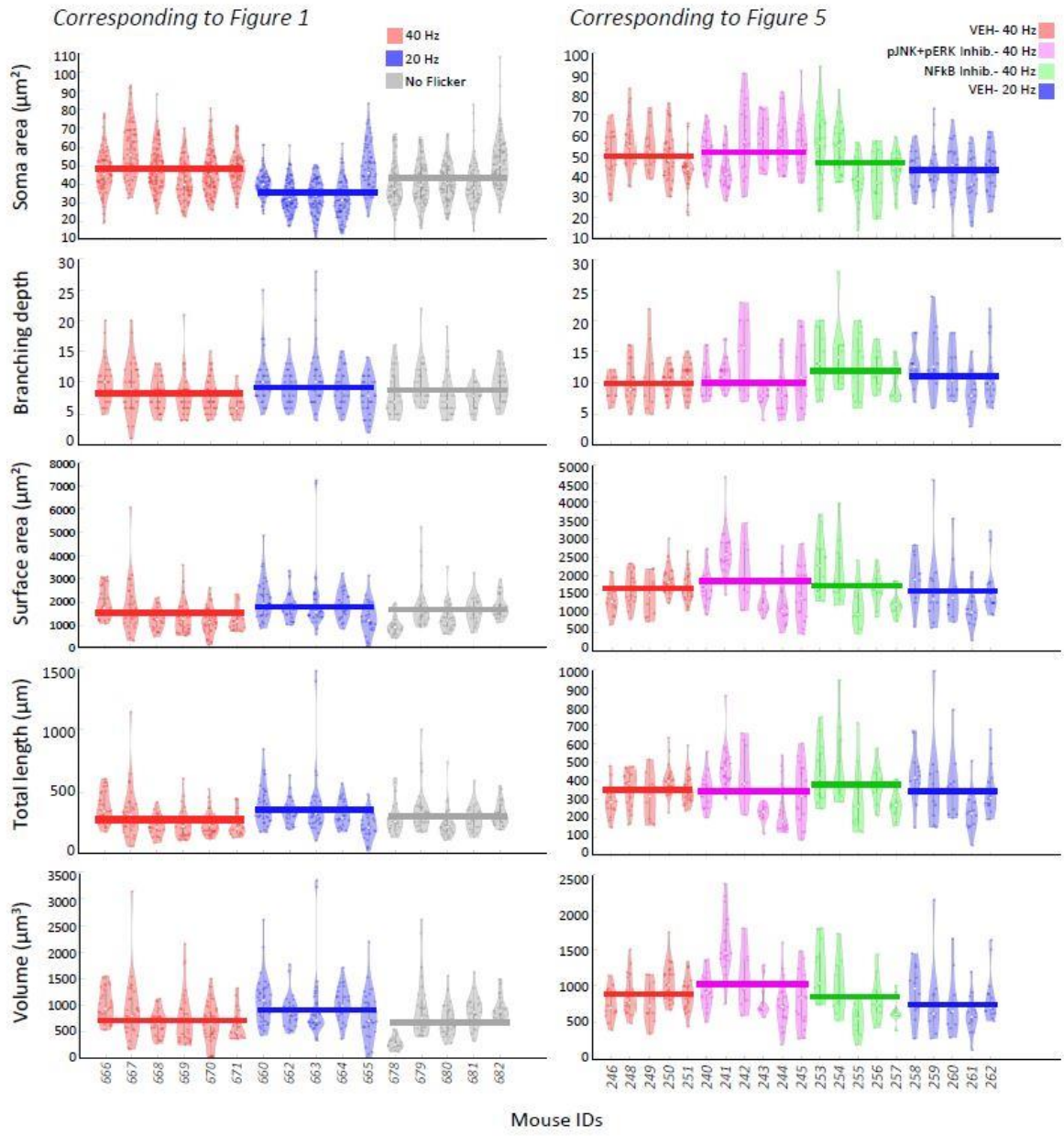

Fig. S8. Microglia morphology measures per animal. Left) As in Fig 1 & Fig S2, Analysis of IBA-1+ microglia after animals were exposed to 1 hour of 40 Hz Flicker (red, n=6 mice, 174 microglia), 20 Hz Flicker (blue, n=5 mice, 140 microglia), or No Flicker (grey, n=5 mice, 132 microglia) stimulation for soma area (µm^2), branching depth, whole-cell surface area (µm^2), total process length (µm), and total microglia volume (µm^2). **Right) As in Fig 5 & Fig S5** Analysis of IBA-1+ microglia measures as in the left column, after animals were exposed to one hour of 40 Hz Flicker and Vehicle Injection (orange, n=5 mice, 128 microglia), 40 Hz Flicker and pJNK+pERK Inhibitor injection (pink, n=6 mice, 129 microglia), 40 Hz Flicker and NFkB Inhibitor injection (green, n=5 mice, 116 microglia), and 20 Hz Flicker and Vehicle injection (blue, n=5 mice, 106 microglia). X-axes represent individual mice IDs. Dark colored line indicated mean per group. **Statistical analyses were performed per group and are shown in the Fig 1 & Fig 5 captions for this data.**

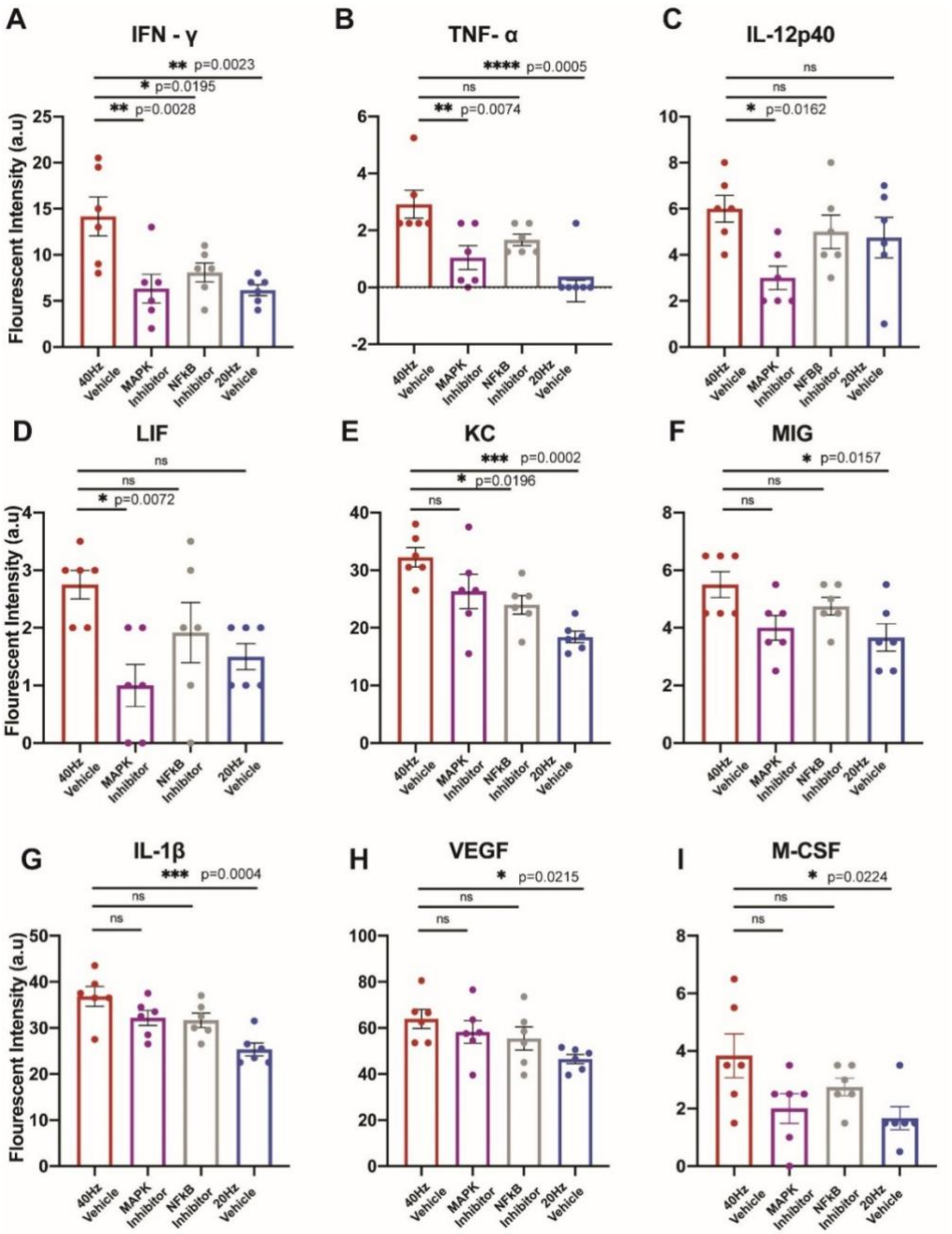

Fig. S9. Inhibition of phosphoprotein pathways impact on key inflammatory cytokines. (A) IFN-γ expression across groups, ($F(3,20) = 6.787$, $p = 0.0024$, one-way ANOVA). The p-values from a Dunnett's multiple-comparison test are listed. **(B)**, As in A for TNF-α, ($F(3,20) = 7.774$, $p = 0.0012$, one-way ANOVA). **(C)**, As in A for IL-12p40, ($F(3,20) = 3.257$, $p = 0.0431$, one-way ANOVA). **(D)**, As in A for LIF, ($F(3,20) = 4.225$, $p = 0.0182$, one-way ANOVA). **(E)**, As in A for KC, ($F(3,20) = 8.57$, $p = 0.0007$, one-way ANOVA). **(F)**, As in A for MIG, ($F(3,20) = 3.782$, $p = 0.0268$, one-way ANOVA). **(G)**, As in A for IL-1β, ($F(3,20) = 7.633$, $p = 0.0014$, one-way ANOVA). **(H)**, As in A for VEGF, ($F(3,20) = 3.007$, $p = 0.0545$, one-way ANOVA). **(I)**, As in A for M-CSF, ($F(3,20) = 3.352$, $p = 0.0395$, one-way ANOVA). Error bars indicate mean ± SEM, dots indicate individual animals.

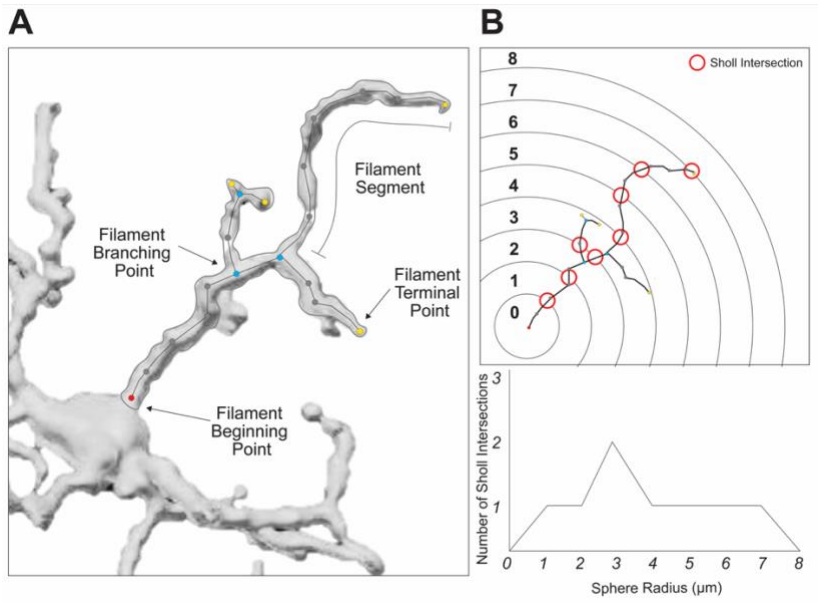

Fig. S10. Microglia reconstruction protocol (**A**) Diagram showing key features of filament segments superimposed on reconstructed microglia, such as filament beginning, terminal, and branching points. (**B**) Diagram demonstrating Sholl radii using a simplified microglia (*top*) and accompanying graph plotting number of Sholl intersections at each radius (*bottom*).

Supplementary Tables

Main Effects	**ANOVA**	**Tukey's HSD Post Hoc Comparisons**
Number of Branches	F (2,15271) = 17.65, $p < 0.001$)	
Radii	(F(1,15271) = 3034.051, $p < 0.001$)	
Frequency	(F(2,4897) = 4.84, p = 0.008)	with significant differences across all 3 frequencies at $p<0.001$ (20Hz t=65.47, 40Hz t=68.6, No Flicker t=59.23)
Radii bins		
11-20	(F(2,4423) = 38.17, $p <0.001$)	with significant difference at all 3 frequencies 20Hz t(4423) = 71.065, $p<0.001$, 40Hz t(4423) = 66.26, $p<0.001$), No Flicker t(4423)=60.914, $p<0.001$
21-30	(F(2,3809) = 39.638, $p <0.001$),	with significant effects across all 3 frequencies 20Hz t(3809) = 5.31, $p<0.001$, 40Hz t(3809)=-3.13, p=0.002, and No Flicker t(3809)=-3.1, p=0.002

Table S1. Panel E Linear Mixed Model sholl analysis of microglia after flicker. Shows significant differences between groups using a linear mixed model (LMM) with repeated measures and fixed effects for flicker frequency and radius. Radius data was separated into bins for frequency-specific effects using post hoc Tukeys HSD corrections.

	Primary	**Secondary**
Set 1	pNFκB rabbit (ab86299) (1:500) NeuN mouse (ab104224) (1:500)	Goat anti-rabbit 555 (A32732) (1:2000) Goat anti-mouse 488 (A11001 (1:2000)
Set 2	pNFκB rabbit (ab86299) (1:500) GFAP mouse (MOB064-05) (1:500)	Goat anti-rabbit 555 (A32732) (1:2000) Goat anti-mouse 488 (A11001 (1:2000)
Set 3	pNFκB rabbit (ab86299) (1:500) IBA1 goat (ab5076) (1:100)	Donkey anti-rabbit 488 (A21206) (1:2000) Donkey anti-goat 555 (A21432) (1:2000)
Set 4	IBA1 rabbit (Wako 019-19741)(1:1000)	Donkey anti-rabbit 488 (ab181346) (1:5000)
ALL	DAPI (PanReac AppliChem A1001) (2μMol)	

Table S2. Antibodies and dilutions used for histology. Primary and secondary antibodies used to visualize pNFκB and NeuN (Set 1), pNFκB and GFAP (Set 2), and pNFκB and IBA1 (Set 3). Due to difficulties with the Abcam IBA1 antibody, additional microglia data with NFκB inhibitor were stained with IBA1 rabbit (Set 4).

Supplementary Movies

Movie S1. (separate file)

Mouse exposure to 40Hz visual flicker.

Supplementary Data

Data S1. (separate file)

Statistical analysis (DESeq2) of individual genes within the pathways highlighted in Figures 2 and S3: Cytokine Signaling, Complement, Cellular Stress, MAPK Pathway, Acetylcholine Release, Neurotransmitter Release Cycle, Neurotransmitter and Postsynaptic Signaling, Purinergic Signaling.

Data S2. (separate file)

Individual DEGs comparing No Flicker vs 40Hz or 20Hz vs 40Hz flicker for each cell type (Wald test FDR-adjusted p-value less than 0.05; log2FC= log2(No Flicker/40) or log2 (20/40)).

Data S3. (separate file)

Cytokine expression levels measured via Luminex for data shown in Figures 3 and 7.