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Gamma Visual Stimulation Induces a Neuroimmune Signaling Profile Distinct from Acute Neuroinflammation

Abbreviated Title: Gamma stimulation induces neuroimmune signaling

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CONFLICTS OF INTERESTS

A.C.S. owns shares in Cognito Therapeutics, which aims to develop gamma stimulation related products. Her conflicts of interest have been disclosed to and are managed by the Georgia Institute of Technology Office of Research Integrity Assurance.

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ABSTRACT

Many neurodegenerative and neurological diseases are rooted in dysfunction of the neuroimmune system, therefore, manipulating this system has strong therapeutic potential. Prior work has shown that exposing mice to flickering lights at 40Hz drives gamma frequency (~40Hz) neural activity and recruits microglia, the primary immune cells of the brain, revealing a novel method to manipulate the neuroimmune system. However, the biochemical signaling mechanisms between 40Hz neural activity and immune recruitment remain unknown. Here, we exposed wildtype male mice to 5-60 minutes of 40Hz or control flicker and assessed cytokine and phospho-protein networks known to play a role in immune function. We found that 40Hz flicker leads to increases in expression of cytokines which promote microglial phagocytic states, such as IL-6 and IL-4, and increased expression of microglial chemokines, such as M-CSF and MIG. Interestingly, cytokine effects differed as a function of stimulation frequency, revealing a range of neuroimmune effects of stimulation. To identify possible mechanisms underlying cytokine expression, we quantified flicker's effect on intracellular signaling pathways known to regulate cytokine levels. We found that 40Hz flicker upregulates phospho-signaling within the NFκB and MAPK pathways. While cytokine expression increased after 1 hour of 40Hz flicker stimulation, protein phosphorylation in the NFκB pathway was upregulated within minutes. Importantly, the cytokine expression profile induced by 40Hz flicker was different from cytokine changes in response to acute neuroinflammation induced by lipopolysaccharides. These results are the first, to our knowledge, to show how visual stimulation rapidly induces critical neuroimmune signaling in healthy animals.

Significance statement: Prior work has shown that exposing mice to lights flickering at 40Hz induces neural spiking activity at 40Hz (within the gamma frequency) and recruits microglia, the primary immune cells of the brain. However, the immediate effect of 40Hz flicker on neuroimmune biochemical signaling was unknown. We found that 40Hz flicker leads to significant increases in the expression of cytokines, key immune signals known to recruit microglia. Furthermore, we found that 40Hz flicker rapidly changes phosphorylation of proteins in the NFκB and MAPK pathways, both known to regulate cytokine expression. Our findings are the first to delineate a specific rapid immune signaling response following 40Hz visual stimulation, highlighting both the unique nature and therapeutic potential of this treatment.

INTRODUCTION

Interactions between the brain and immune system play critical roles in neurological and neuropsychiatric disorders (Calsolaro and Edison, 2016; Wohleb et al., 2016) . The neuroimmune system, including glial cells and immune signaling molecules, offers a unique target to treat disease and improve brain health. However, little is known about how to non-pharmacologically manipulate the brain's immune system. Recent research has shown that exogenously stimulating neural electrical activity at gamma frequency (30-50Hz), through sensory stimulation, promotes microglial activity, a component of the neuroimmune system, and decreases amyloid-beta (Aβ), one of the key proteins that build up in Alzheimer's disease (AD) (Iaccarino et al., 2016; Adaikkan et al., 2019). While this recent research shows that neural electrical activity manipulates a component of the neuroimmune system and has therapeutic implications, a broader understanding of how exogenous stimulation of neural activity affects neuroimmune function is required. Indeed, the immediate effects of exogenous gamma stimulation on biochemical signals that control immune function in the brain are unknown. While the previous literature in this area has focused on Alzheimer's disease pathology, it is important to understand these basic biological interactions between neural activity and immune function in healthy animals outside the context of AD. Furthermore, while 40Hz visual stimulation is hypothesized to recruit the immune system, the effects of different frequencies of visual stimulation on immune signaling remains unknown. Determining how different frequencies of visual stimulation affect the immune system in the brain will enable new strategies to manipulate brain function.

Cytokines, extracellular soluble signaling proteins of the immune system, are a main communication signal between neurons and immune cells (Hanisch, 2002; Prieto and Cotman, 2017). Chemotactic cytokines, or chemokines, are responsible for attracting immune cells to a site of injury and pro- and anti-inflammatory cytokines promote or reduce inflammation, respectively. While molecular signaling between neurons and microglia has been largely attributed to neuronal expression of the cytokine fractalkine (CX3CL1), which attenuates microglial over-activation (Sheridan and Murphy, 2013), many other cytokines may play a role in signaling between neurons and microglia. For example, glial-derived TNF-α is necessary for proper neuronal synaptic scaling and is also a modulator of microglia neuronal phagocytosis (Stellwagen and Malenka, 2006; Goshen et al., 2007; Neniskyte et al., 2014). Besides regulating microglial activation and recruitment, cytokines are also involved in diverse neuronal and synaptic functions. Still, little is known about how neuronal electrical activity affects cytokine expression.

Cytokine expression and its effects on immune function are regulated by intracellular signaling, such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the mitogen activated protein kinase (MAPK) immunomodulatory pathways. These pathways are activated through the subsequent phosphorylation of proteins in a cascade, or phospho-signaling, and they culminate in the activation of transcription factors, which regulate cytokine transcription, expression, and release from the cell. Both the NFκB and MAPK immunomodulatory pathways strongly regulate expression of diverse cytokines involved in immune responses, like microglial activation and recruitment (e.g., M-CSF, MCP-1) (Hommes et al., 2003;

Cloutier et al., 2007; Liu et al., 2017). These pathways also control expression of neurotrophic and synaptotrophic factors and are involved in mechanisms of learning and memory (Sweatt, 2001; Kaltschmidt et al., 2006; Mattson and Meffert, 2006; Chen et al., 2017). Recent studies have shown 40Hz flicker changes protein phosphorylation patterns after weeks of exposure compared to no stimulation, but the immediate effect of flicker on protein phosphorylation in general and the immediate effects of flicker on these two key phospho-protein pathways is still unknown (Adaikkan et al., 2019).

In the present study, we determined how 40Hz visual stimulation affects expression of key cytokines involved in the brain's immune response, synaptic plasticity, and neuronal health. Furthermore, since cytokine expression is regulated by intracellular signaling, we assessed if NFκB and MAPK phospho-signaling changes prior to increases in cytokine expression in mice exposed to 40Hz visual stimulation compared to control visual stimuli. We tested this by exposing mice to varying durations of light emitting diode (LED) light strips flickering at 40Hz, which is known to induce gamma neuronal activity, as well as several control conditions (Gray et al., 1989; Iaccarino et al., 2016; Singer et al., 2018; Adaikkan et al., 2019). Our analysis showed that 1 hour of 40Hz flicker stimulation induces phospho-signaling within the NFκB and MAPK pathways (~15-60 minutes) and protein expression of diverse cytokines. Interestingly, 20Hz flicker, random flicker, and constant light each induced unique cytokine expression profiles, revealing that different frequencies of visual stimulation induce unique immune signaling patterns. We then determined if the cytokine response after 40Hz flicker differed from that due to a model of acute inflammation induced by lipopolysaccharides (LPS) administration. We found that the cytokine profile of acute

inflammation due to LPS administration was distinct from the cytokine response to 40Hz flicker. There results show that 40Hz flicker induces NFκB phospho-signaling followed by MAPK phospho-signaling and increased cytokine expression distinct from inflammation. Importantly, the cytokines assessed here, as well as the NFκB and MAPK pathways, play key roles in many different cellular functions. Furthermore, different frequencies of stimulation induced unique cytokine expression patterns. Thus, our results suggest that stimulation of different patterns of activity may be used to regulate expression of genes that promote neuronal health, synaptic plasticity, and healthy immune activity.

MATERIALS AND METHODS

Animals

All animal work was approved by The Georgia Tech Institutional Animal Care and Use Committee. Adult (2-3 month old) male C57BL/6J mice were purchased from The Jackson Laboratory. Mice were pair housed upon arrival and allowed to acclimate to the environment for at least 5 days before being handled.

For all animals, food and water were provided *ad libitum*. For three days before the experiment, mice were single-housed and briefly handled (about 1 minute per mouse). All experiments were performed during the light cycle and began between 8 and 9 AM. For experiments with flicker durations of less than 1 hour, animals undergoing the same stimulation condition were interspersed with animals undergoing different conditions, to ensure circadian rhythms did not impact results. For experiments

with duration of flicker exposure at 1 hour, we conducted multiple experiments, at different times of day, with varying order of stimulus presentation, to ensure results remained consistent (data not shown).

Visual Stimulation Exposure

To habituate and reduce visual stimulation, mice were placed in a dark room in the laboratory, for at least one hour, before beginning each experiment. To commence the experiment, mice were transferred from their home cage to a similar cage without bedding, termed a flicker cage. The flicker cage was covered in dark material on all but one side, which was clear and faced a strip of LED lights. Animals remained in the dark room, where they were exposed to either LED lights flashing at 40Hz frequency (12.5ms light on, 12.5ms light off), 20Hz frequency (25ms light on, 25ms light off), a random frequency which averaged at 40Hz (12.5ms light on, variable duration light off), or constant light on stimulation, utilizing a dimmer to ensure the same total lux (about 150 lx) as 40Hz light flicker (**Video 1**)(Singer et al., 2018). These conditions allowed us to control for light stimulation (light), light stimulation flashes (random), and frequency specific stimulation (20Hz). Animals were exposed to visual stimulation for 5 minutes, 15 minutes, or 1 hour.

Immediately after stimulation exposure, mice were anesthetized with isoflurane, and within 3 minutes mice were decapitated and brains were removed. The left hemisphere's visual cortex was micro-dissected, placed in an Eppendorf tube, and flash frozen using liquid nitrogen.

Cytokine analysis was conducted using six animals per group. Phospho-protein experiments were conducted and combined across several cohorts of animals, utilizing a total of 72 animals. More animals were utilized for experiments examining 5 minutes of stimulation due to concern that initial signals at this time point may be transient and hard to detect.

Lipopolysaccharides (LPS) Stimulation

Animals were intraperitoneal (i.p.) injected with 5mg/kg LPS (n=6), diluted in saline (Sigma lot #039M-4004V) or saline (veh) (n=5). 3 hours after injection, mice were anesthetized with isoflurane, and within 3 minutes mice were decapitated and brains were removed. The left hemisphere's visual cortex was micro-dissected, placed in an Eppendorf tube, and flash frozen using liquid nitrogen. Cytokine analysis was conducted as described below.

Cytokine and phospho-protein assays

For signaling and cytokine analysis, the visual cortex was thawed on ice and lysed using Bio-Plex lysis buffer (Bio-Rad). After lysing, samples were centrifuged at 4C for 10 minutes at 13,000 RPM. Protein concentrations in each sample were determined using a Pierce BCA Protein Assay (Thermo Fisher). Total protein concentrations were normalized in each sample using Bio-Plex lysis buffer (Bio-Rad). For MAPK and NFκB pathway analysis, 1.5 µg of total protein was loaded, and 6µg was loaded for cytokine analysis. Preliminary experiments determined these protein concentrations fell within the linear range of bead fluorescent intensity versus protein concentration for detectable

169 analysis. Multiplexed phospho-protein analysis was conducted by adapting the
170 protocols provided for the Milliplex MAP MAPK/SAPK Signaling Magnetic Bead 10-Plex
171 (p-ATF2, p-Erk, p-HSP27*, p-JNK, p-c-Jun, p-MEK1, p-MSK1, p-p38*, p-p53*) kit and
172 the Milliplex MAP NFκB Signaling Magnetic Bead 6-Plex Kit(c-Myc*, p-FADD, p-IκBα*,
173 p-IKKα/β, p-NFκB, TNFR1). Cytokine analysis was conducted by adapting protocols
174 provided for the Milliplex Mouse MAP Mouse Cytokine/Chemokine Magnetic Bead
175 Panel 32-Plex Kit (Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5,
176 IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-
177 1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF). All kits were
178 read on a MAGPX system (Luminex, Austin, TX). Asterisks denote analytes that did not
179 fall within the linear range and were therefore excluded from our analysis.
180
181 **Partial least squares discriminant analysis**
182 Data was z-scored before analysis. A partial least squares discriminant analysis
183 (PLSDA) was performed in MATLAB (Mathworks) using the algorithm by Cleiton Nunes
184 (Mathworks File Exchange) (Eriksson, L.; Byrne, T.; Johansson, E.; Trygg, J.; Vikström,
185 2006). To identify latent variables (LVs) that best separated conditions, an orthogonal
186 rotation in the plane of the first two latent variables (LV1-LV2 plane) was performed.
187 Error bars for LV1 figures represent the mean and standard deviations after iteratively
188 excluding single samples (a leave-one-out cross validation), one at a time, and
189 recalculating the PLSDA 1000 times.
190 To test different flicker conditions and durations, phospho-protein experiments
191 were performed over several cohorts and samples were normalized by removing batch

effects. We performed a batch effects analysis (limma package in R v 3.5.2) to remove any between-experiment-variability before conducting the PLSDA. Outliers were removed by performing a principle component analysis on the data and iteratively removed data points which fell outside of a 99.5% confidence ellipse (mahalanobisQC in R v 3.5.2).

Animal behavior assays

To analyze animal behavior, mice were placed in a polycarbonate cage (210 x375 x 480mm), covered on all sides but one with 100% polyester black material (similar to cage in visual flicker experimental protocol cage described above, but larger and without cage top). In order to record the mice in the dark, an infrared (IR) light was placed above the cage. To record mice, without the flicker stimulation affecting behavior tracking, a Basler Ace monochrome IR-sensitive camera with Gigabit Ethernet interface with attached IR-only filter was used for recording. Ethernet connection from the camera to the computer allowed for Ethovision to record and analyze the experiment in real time.

Three days before recording, mice were handled as described in flicker experiment section above. On the third handling day, each mouse was placed in the behavior cage to habituate for five minutes. On the fourth day, each mouse was recorded individually for an hour and five minutes while receiving one of four flicker treatments (40Hz, 20Hz, random, light). Behavior assays were conducted using a total of six animals, with a within-subjects design. Each mouse received each treatment

separated by 1 day in a randomized order. Experimenter was blinded to treatment type during experiment and analysis.

Ethovision XT version 14.0 was used to track and analyze behavior. First, the arena was defined, and a zone group defined which split the bottom of the cage in to two halves, front and back. A second zone group defined as center consisted of half of the total area in the center of the cage. Detection settings were made using Ethovision's automatic detection function and were adjusted after preliminary analysis. Automated video analysis recorded the animals' center point and all recordings were checked and manually corrected through software interpolation for any time points in which the mouse could not be automatically detected. Activity level was designated as active or inactive with inactive indicating freezing. Inactivity was determined to be less than 0.01% of the total arena having activity and lasting for a duration longer than half a second. Data for each variable was exported and analyzed using GraphPad. A one-way analysis of variance (ANOVA) was conducted to assess differences between groups for each variable of interest.

Experimental design and statistical analysis

Animals were randomly assigned to flicker exposure groups and experimenters were blind to flicker exposure conditions during analysis for all experiments. Sample sizes were determined on preliminary data with a 0.80 power and alpha of 0.05, and samples sizes were adjusted based on high variability. Control groups (20 Hz, random, and light) were based on prior experiments and preliminary data.

For multi-plex analysis, either a one-way ANOVA (more than two groups) or two-tailed unpaired t-test (two groups) was used to determine if there was a significant LV1 separation between groups. The top correlated cytokines and/or phospho-proteins on the LV1 were isolated and an ANOVA or two-tailed unpaired t-test was performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA) to determine statistical significance between groups. These tests were followed by a post-hoc Dunnett's multiple comparisons test to determine differences between specific groups or a Tuckey's multiple comparisons test to compare differences in phosphorylation levels across time. Levels of significance were set to *$p<0.05$, ** $p<0.01$, ****$p<0.001$.

To further confirm significant differences between groups using PLSDA, a permutation analysis was conducted, which randomly assigned animals into experimental groups and ran the PLSDA based on these shuffled values 1000 times (Golland et al., 2010). For each test, true group assignment showed $p_{permute}<0.05$ compared to the randomly permuted distribution, further confirming the validity of our data.

For analyzing changes in animal behavior, a one-way repeated measures analysis of variance (RM-ANOVA) was to compare results between different stimulation types.

For comparing between LPS-treated and 40Hz-stimulated animals, each group was normalized to control. Specifically each cytokine level in each animal was divided by the mean cytokine level in the related control group (vehicle for LPS and random for 40Hz). Multiple t-tests were then conducted to compare each stimulation to its control; multiple comparisons were corrected using the Holm-Sidak method.

Code accessibility

Data was analyzed using custom code that is available upon request.

RESULTS

40Hz flicker induces increased cytokine profile

Given previous findings showing that exposing mice to 40Hz light flicker leads to morphological changes in microglia, we speculated that 40Hz flicker affects neuroimmune signaling. Specifically, since cytokines are key regulators of immune activity in the brain, we hypothesized that 40Hz light flicker promotes cytokine expression in the visual cortex. To test this, we exposed animals to 1 hour of 40Hz flickering light, light flickering at a random interval averaging at 40Hz (random), 20Hz flickering light (20Hz), and constant light (light)(**Fig. 1A, Video1**). After visual stimulation, mice were euthanized, and the visual cortex was rapidly (<3min) micro-dissected and flash frozen. Using a Luminex multi-plexed immunoassay we quantified expression levels of thirty-two cytokine proteins in the visual cortex (**Fig. 1B**). To account for the multi-dimensional nature of the data, we used a PLSDA to identify profiles of cytokines that distinguished the effects of 40Hz stimulation from the control groups (**Fig. 1C**) (Eriksson, L.; Byrne, T.; Johansson, E.; Trygg, J.; Vikström, 2006).

We observed overall higher cytokine expression levels across animals exposed to 40Hz flicker compared to other groups. PLSDA identified a latent variable 1 (LV1), consisting of a weighted linear combination of cytokines, as being most upregulated in

40Hz (higher LV1 scores) or from all control conditions ((negative) (lower LV1 scores), **Fig. 1D**). LV1 best separated 40Hz from all control groups (**Fig. 1C, Fig.1D).** LV1 scores differed significantly between groups confirming the groups were statistically separate, specifically, 40Hz flicker versus light and 20Hz stimulation were best separated ($F(3, 20) = 5.855$, $p=0.0049$, one-way ANOVA) (**Fig. 1E**). LV2 significantly separated control groups from each other ($F(3,20)=21.77$, $p<0.0001$, **Fig.1C, Fig. 2B**).

The top four identified by the PLSDA to contribute to differences between groups were M-CSF, IL-6, MIG, and IL-4 (**Fig. 1F**). Of these top upregulated cytokines, IL-6 and MIG were significantly different between groups when each one was analyzed individually (IL-6: $F(3,20)=3.775$, $p=0.0269$; MIG: $F(3,20)=4.532$, $p=0.0140$, one-way ANOVA) (**Fig. 1F**). We found IL-6 had significantly higher expression in 40Hz than 20Hz flicker and light; and MIG had significantly higher expression in 40Hz than 20Hz flicker (IL-6: 40Hz versus 20Hz: mean difference =1.917, adjusted $p=0.0452$; IL-6: 40Hz versus light: mean difference= 2.33, adjusted $p=0.0135$; MIG: 40Hz versus 20Hz: mean difference =2.083, adjusted $p=0.0123$, Dunnett's test). M-CSF expression was higher in 40Hz flicker than light exposed animals (mean difference = 1.750, adjusted $p=0.0360$. Dunnett's test). We found no significant differences in IL-4 between groups although expression levels trended towards being higher in 40Hz flicker animals ($F(3,20)=2.051$,$p=0.1391$, one-way ANOVA). Based on observed differences between groups in a heatmap of these cytokines (**Fig.1B**), we ran additional analysis on a subset of cytokines and found significant differences in IL-12p70, IL-1β, IFN-γ, GM-CSF, Il-7 between 40Hz flicker and control conditions (see **Fig. 1G**). These findings show that 1

hour of 40Hz flicker drives a significant neuroimmune signaling response, and this response is distinct from other flicker frequencies or exposure to constant light.

Interestingly, while animals exposed to 40Hz flicker showed a higher cytokine expression profile, we also observed distinct cytokine expression profiles for random, 20Hz, and light stimulation conditions (**Fig.1B, Fig. 2C**). Indeed, our analysis revealed a second latent variable (LV2), which best separated these three control groups (**Fig. 1C, Fig.2A**). Quantification of these effects showed constant light leads to an upregulation of a subset of cytokines, unique from those upregulated by 40Hz flicker. For example, when analyzed individually, IL-2, VEGF, IL-9, IL-1α, and IL-13 all showed significant differences in expression levels between groups, with higher upregulation after light exposure than at least one other group (IL-2: $F(3,20)=9.366, p=0.0005$; VGEF: $F(3,20)=6.790$, $p=0.0024$; IL-9: $F(3,20)=4.076, p=0.0207$; IL-1α:$F(3,20)=4.399$, $p=0.0157$; IL-13: $F(3,20)=3.802$, $p=0.0263$; one-way ANOVA, Dunnett's multiple comparisons method was used to control for 3 comparisons, **Fig. 2E-2I)** In contrast, random stimulation uniquely upregulated IL-10 across all samples, when compared to other stimulation types ($F(3, 20) =27.29$, $p<.0001$, **Fig. 2D)** These results highlight that while 40Hz flicker modulated cytokine activity, 20Hz, random, and light exposure also led to distinct cytokine activity.

40Hz flicker induces NFκB and MAPK signaling

Cytokine expression is regulated by several canonical intracellular phospho-signaling pathways, including the NFκB and MAPK pathways. Thus, to gain better insight into intracellular signaling changes that underlie changes in cytokine expression

after visual stimulation, we examined how 40Hz flicker impacted these two phospho-protein pathways. Because phospho-signaling is usually transient, we examined this signaling after 5, 15, and 60 minutes of 40Hz and random flicker (Janes et al., 2005). We focused on random flicker as our control group for this comparison specifically because it controls for the average number of times the lights turn on and off, the percent of time the light is on, and the frequency of light presentation without inducing periodic neural activity (Iaccarino et al., 2016; Singer et al., 2018; Adaikkan et al., 2019). As with cytokine experiments, immediately following flicker exposure, mice were euthanized, and visual cortex was rapidly micro-dissected. We used Luminex assays and PLSDA to quantify differences in the NFκB and MAPK phospho-protein pathways in 40Hz versus random flicker exposure animals after different durations of flicker (**Fig. 3, 4**).

We found phospho-signaling in the NFκB pathway was significantly upregulated after 15 minutes, but not 5 or 60 minutes, of 40Hz versus random flicker. Experiments were performed across several cohorts and animals were removed based on an outlier analysis; data was z-scored according to batch-corrected data (see **Methods**). Our analysis revealed a trend of increased phosphorylation after 5 minutes of flicker, driven specifically by increases in TNFR1 and pIKKα/β, but 40Hz and random groups were not significantly different ($t(21) = 2.056$, $p=0.0525$, unpaired t-test, **Fig.3B,3E**). After 15 minutes of flicker exposure, NFκB phosphorylation significantly differed between groups as indicated by significant separation of NFκB LV1 scores ($t(16)=4.456$, $p=0.0004$,unpaired t-test, **Fig. 3C,3F**). At 60 minutes, there was no significant difference between groups but phosphorylation of NFκB proteins appeared lower in 40Hz than

random groups (t (8) =1.415, p=0.1948, unpaired t-test, **Fig.3D,3G**). These results show that 40Hz flicker transiently upregulates the NFκB pathway after approximately 5 to 15 minutes and this difference in upregulation is no longer detectable after 60 minutes of flicker.

We found that MAPK phosphorylation patterns were similar to those of NFκB but with different kinetics. MAPK phospho-signaling was significantly different between 40Hz and random groups after 60 minutes of flicker but not after 5 or 15 minutes. Phosphorylation of MAPK proteins following 5 minutes of 40Hz flicker did not significantly differ from random stimulation as indicated by no significant LV1 separation (t (32) =1.870, p=0.0707, unpaired t-test) (**Fig. 4B,4E**). While phosphorylation of MAPK proteins also did not significantly differ following 15 minutes of 40Hz or random flicker (t(8.582)=1.99, p=0.2624, welch corrected unpaired t-test), the heatmap of phospho-protein levels showed a trend for more phosphorylated MAPK proteins in 40Hz than random group (**Fig. 4C,4F**). MAPK phospho-signaling did significantly differ between 40Hz and random stimulation following 60 minutes of flicker exposure, as revealed by significantly separated LV1 scores (t(10)=3.709,p=0.004, unpaired t-test, **Fig. 4D,4G**). This effect was mostly driven by differences in phosphorylated MSK1, and MEK1 (**Fig. 4J**). These results show that 40Hz flicker upregulates the MAPK pathway after approximately 60 minutes.

Correlations between phosphorylation of proteins increases after minutes of 40Hz flicker

Because phospho-signaling is transient, another way of assessing activity in these pathways is to measure coordinated phosphorylation among proteins. We thus hypothesized that activation of these pathways would lead to coordinated phospho-protein levels at time points prior to our observed increases in cytokine expression. Therefore, we examined how coordination both within and between NFκB and MAPK networks changed over the course of 40Hz flicker exposure. In order to do so, we utilized variability across animals and investigated protein co-variation across animals by calculating the correlation coefficients of each protein pair from NFκB and MAPK pathways after 5, 15 and 60 minutes of 40Hz flicker stimulation (Eisen et al., 1998; Kanonidis et al., 2016) (**Fig. 5**). In other words, this analysis revealed protein phosphorylation levels that significantly increased or decreased together across animals (**Fig. 5,** $q < 0.1$, false discover rate correction for 135 comparisons, Pearson correlation test).

Over the time points assessed, we found the number of significant correlations between proteins within each pathway was highest after 15 minutes of 40Hz flicker. After 5 minutes of 40Hz flicker, five proteins were significantly correlated within the NFκB pathway and four within the MAPK pathway (**Fig. 5A**). After 15 minutes of 40Hz flicker, more protein correlations were found: 6 significant correlations in the NFκB pathway and 10 significant correlations in the MAPK pathway (**Fig. 5B**). Interestingly, the proteins that were significantly correlated after 15 but not 5 minutes of flicker were farther downstream in the MAPK pathway: pERK1/2, pMSK1, pATF-2, and pSTAT1

(**Fig. 4A**). After 60 minutes of 40Hz flicker, only 3 significant correlations were found in both pathways (**Fig. 5C**). Of note, all significant correlations were within and not across NFκB and MAPK pathways in all time points assessed.

Animal behavior is similar across flicker conditions

Given our findings indicating cytokine and phospho-protein upregulation induced by 40Hz flicker, we sought to determine if behavioral differences during flicker could explain these results. We assessed both overall activity levels, measured as percent time active and total distance traveled, as well as exploratory versus anxiety-like behavior, assess by the percent of time animals spent in the center of the environment versus along the walls (perimeter). More anxious animals stay close to the walls of an enclosure. We recorded mice during 1 hour of either 40Hz flicker, random flicker, 20Hz flicker, or light and tracked mouse movement (**Fig. 6A**). Our results revealed no significant differences between different flicker conditions in the amount of time spent in the center versus the perimeter of the enclosure, time spent active instead of freezing, time spent in the front (near the light source) versus the back half of the enclosure, and total distance traveled (Center versus perimeter: **Fig. 6B**, $F(3,15)=0.8754$, $p=0.4757$, RM-ANOVA; Activity: (**Fig. 6C**, $F(3,15)=0.9304$, $p=0.4502$, RM-ANOVA; Front versus Back: **Fig.6D**, $F(3,15)=0.1727p=0.9132$, RM-ANOVA; Distance: **Fig.6E**, $F(3,14)=0.3392$, $p=0.7973$, RM-ANOVA). These results show different visual stimulation conditions including different frequencies of flicker, periodic or aperiodic flicker, or constant or flickering light stimuli do not differentially affect animal behavior or induce an

anxiety-like phenotype. Thus, the molecular changes we found in response to different visual stimulation conditions are not due to changes in animal behavior.

40Hz flicker induces neuroimmune profile distinct from acute inflammation

Next, we wondered how the cytokine profile in response to 40Hz flicker differed from acute inflammation. Thus we assessed cytokine expression in the visual cortex of animals intraperitoneally injected with lipopolysaccharides (LPS), a traditional, acute model of inflammation (Gabellec et al., 1995; Qin et al., 2007; Meneses et al., 2018). As in our flicker experiments, we characterized cytokine expression in visual cortex of mice injected with LPS versus vehicle injection and separated these differences using a PLSDA (**Fig. 7**). The latent variable that best separated LPS- and vehicle-injected animals (LV1, *orange*) was most heavily weighted by expression of MIG, IP-10, IL-3, and IL-17 (**Fig.7A**). The profile of cytokines weights that separated LPS from vehicle injected animals (LV1, *orange*) differed from the profile that separated 40Hz versus random stimulation exposed animals (LV1, *blue,* **Fig. 7A**). These results show that 40Hz flicker and LPS stimulation lead to distinct immune profiles.

We further analyzed the differences between LPS and 40Hz flicker by comparing individual cytokines following LPS or 40Hz flicker relative to their controls. These comparisons revealed that most cytokines differed between LPS and 40Hz flicker, both in terms of whether or not each cytokine was modulated by the stimulus and in terms of the magnitude of the response relative to control (**Fig. 7B,C,D**). For example, GM-CSF and IL-2 significantly differed between animals exposed to 40Hz flicker versus random stimulation but did not differ between animals injected with LPS or vehicle (*40Hz versus*

random: GM-CSF: t(10)=3.114, p=0.011; IL-2: t(10)=3.613, p=0.0047; *LPS versus vehicle*: GM-CSF: t(9)=1.646, p=0.134; IL-2: t(9)=0.167, p=0.871) (**Fig. 7B**). In contrast, G-CSF, KC, MIG, RANTES, IP-10, and IL-6 significantly differed between LPS and vehicle but not between 40Hz and random (*LPS versus vehicle*: G-CSF: t(9)=4.069, p=0.003; KC: t(9)=3.883, p=0.004; MIG: t(9)=12.16, p<0.000001; RANTES: t(9)=3.502,p=0.007; IP-10: t(9)= 14.86, p<0.000001: IL-6: t(9)= 3.107, p=0.013; *40Hz versus random*: G-CSF: t(10)=1.661, p=0.128; KC: t(10)=0.450, p=0.662, MIG: t(10)=0.2928,p=0.776; RANTES: t(10)=1.335,p=0.212, IP-10: t(10)=0.3421, p=0.739; IL-6: t(10)=1.437, p=0.1813) (Fig. 7C). Lastly, there was a subset of cytokines, IL-12p70, Eotaxin, TNF-α, and MCP1, that significantly differed in both LPS versus vehicle and 40Hz versus random (*LPS versus vehicle*:IL-12p70: t(9)=2.359,p=0.043;Eotaxin: t(9)=2.710,p=0.024 ;TNF-α:t(9)=3.561,p=0.007;MCP1: t(9)= 2.884,p=0.018; *40Hz versus random*: IL-12p70:t(10)=3.729,p=0.004;Eotaxin:t(10)= 2.318,p=0.043;TNF-α: t(10)= 3.512,p=0.006; MCP1: t(10)= 2.785,p=0.019) (Fig. 7D). For those cytokines that significantly increased expression in response to both LPS and 40Hz stimulations relative to controls, the scale of the difference between the two comparisons was usually much larger when comparing LPS versus vehicle. For example, the difference in expression of MCP1 was over 10 fold larger between LPS and vehicle than between 40Hz and random, though both differences were significant (**Fig. 7D**). Overall these results show that acute inflammation via LPS and 40Hz flicker produce different immune responses.

DISCUSSION

In this study, we utilized multiplexed immunoassays to profile expression of cytokines and phospho-signaling within key pathways that regulate the brain's immune response to different forms of visual stimulation. We found that 40Hz light flicker rapidly and transiently stimulated phospho-signaling within the NFκB pathway in mouse visual cortex. NFκB phospho-signaling was followed by increased phospho-signaling within the MAPK pathway and increased expression of a diverse profile of cytokines involved in microglial recruitment (e.g., M-CSF), neurotrophic properties (e.g., IL-6), and synaptic plasticity (e.g., IL-1). Furthermore, we found distinct cytokine responses to different visual stimuli, including 20 Hz flicker, random flicker, and constant light. These results show that specific frequencies and patterns of visual stimulation lead to different cytokine responses. Because these assays were performed in wild-type animals, the results reveal that 40Hz flicker stimulation modulates immune function independent of disease pathology. Thus, these findings suggest this approach could be applied to many diseases that affect brain immune function. Importantly, we found that 40Hz flicker induced a cytokine signature that differs from an LPS model of inflammation. Together, our data show that visual flicker rapidly activates canonical intracellular signaling pathways and induces a unique cytokine expression profile. Due to the multifunctional nature of these pathways and the broad range of cytokines expressed, the effects of visual flicker likely extend beyond neural immune activity to changes in neuronal health, synaptic plasticity, and other functions.

40Hz flicker-induces a unique cytokine profile in the visual cortex

Because nothing is known about the effect of gamma oscillatory activity on cytokine expression levels, we used a multi-plex approach to determine the global impact of this activity on the brain's main immune signals. Our results establish that 40Hz flicker upregulated a combination of cytokines in the visual cortex resulting in a neuroimmune profile distinct both from other types of flicker stimulation and from acute inflammation induced by LPS. Interestingly, LV1 better separated 40Hz stimulation from

20Hz and light than from random stimulation. This point is reinforced by our finding that the top LV1-contributing cytokines did not differ significantly between 40Hz and random stimulation. However other cytokines, such as IFN-γ, IL-12p70, and GM-CSF, showed significant differences between 40Hz and random stimulation (**Fig.1G**). Thus, random stimulation leads to an immune profile with an overlap in expression of some cytokines to that of 40Hz but distinct expression levels of other cytokines (**Fig. 2**). While random stimulation does not induce gamma oscillations, randomized pulses of light slightly alter neural activity in visual cortex over a much broader ranges of frequencies than gamma frequency light flicker (Iaccarino*, Singer* et al., 2016). Therefore, random flicker has some similar and some different effects on cytokines as 40Hz flicker but taken together this still produces a distinct immune profile.

Random flicker, 20Hz flicker, and light stimulation each induce expression of unique subsets of cytokines (**Fig. 2**). When comparing cytokine expression, LV1 best separated 40Hz stimulation from the other controls. In contrast, LV2 best separated all of the control groups from each other. Interestingly, while LV1 separated animals stimulated with 20Hz and light stimulation from those stimulated with 40Hz visual flicker, these groups were not as well separated by LV2. In fact, LV2 best separated animals stimulated with random visual flicker from other groups. When examining individual cytokines, plotted in Figure 2, there is a significant difference in expression of IL-10 and RANTES between 40Hz and 20Hz flicker, and significant difference in expression of IL-10, IL-15, and RANTES between 40Hz flicker and light. Together, these results demonstrate that 40Hz stimulation leads to increased expression of the many cytokines

in the visual cortex compared to other conditions, but each of our control stimulations produces its own unique profile of cytokine expression.

40Hz flicker-induced cytokines have neuroprotective functions

We found that 40Hz flicker is upregulating a unique combination of cytokines, and we hypothesize that no one cytokine in particular is responsible for neuroprotective function, but rather the combinatory profile is necessary. This effect would coincide with what is known about gamma oscillations, that no one neuron is responsible for gamma oscillations, but rather the combination of neuronal activity.

By focusing on the functions of the top five cytokines distinguishing 40Hz flicker from all other groups M-CSF, IL-6, MIG, IL-4, and KC (also known as CXCL1), it becomes apparent that each cytokine's unique immune function may contribute to an overall neuroprotective effect (**Fig.1**)., M-CSF (encoded by *Csf1*) transforms microglia morphologically and has been reported to enhance phagocytosis of amyloid beta (Imai and Kohsaka, 2002; Mitrasinovic and Murphy, 2003). Furthermore, the primary receptor for M-CSF, Csf1r, is primarily expressed by microglia in the brain (Elmore et al., 2014). Thus, M-CSF may be responsible for previously observed morphological transformation of microglia in 5XFAD mice (after 1 hour of 40Hz light flicker stimulation followed by 1 hour of no stimulation) because those microglial changes are associated with engulfment (Iaccarino et al., 2016).

Interestingly, IL-6, the second top cytokine that increased following 1 hour of 40Hz flicker, is a pleiotropic cytokine with both protective and pathogenic effects. The potential pathogenic effects of IL-6 include stimulating amyloid precursor protein

expression (Ringheim et al., 1998). In contrast, the neuroprotective effects of IL-6 include increasing synaptic density and inhibiting macrophage expression of TNF-α, a cytokine known to have pro-inflammatory and neurotoxic properties (Decourt et al., 2017). In our own work, increased expression of IL-6 was correlated with a resiliency to Alzheimer's pathology; defined as human subjects with no neuronal loss or cognitive impairment even in the presence of plaques and tangles associated with Alzheimer's (Barroeta-Espar et al., 2019). IL-6 is also important for neural progenitor cell health as IL-6 knockout significantly reduced neural progenitor cell density in multiple brain regions (Bowen et al., 2011). Furthermore, IL-6 levels correlate with improved learning and memory performance in both animals and humans (Baier et al., 2009; Donegan et al., 2014; Wang et al., 2017).

The third flicker-upregulated cytokine, monokine induced by gamma interferon (MIG), is a chemokine involved in microglial recruitment via its receptor, CXCR3 (Rappert, 2004). In brain endothelial cells *in vitro*, MIG is released in response to the presence of other cytokines (Ghersa et al., 2002). It is specifically responsible for recruiting T-cells to the brain and responds to interferon gamma (IFN-γ), suggesting MIG may be released to combat infection. Indeed, injecting mice with an anti-MIG treatment leads to increased viral infection and pathology (Ure et al., 2005).

Finally, the fourth and fifth top cytokines, IL-4 and KC (CXCL1) signal to microglia (Johnson et al., 2011; De Filippo et al., 2013; Fenn et al., 2014). IL-4 has been shown to reprogram microglia to stimulate neurite outgrowth after spinal cord injury (Fenn et al., 2014). In addition, AAV mediated over-expression of IL-4 in APP mice reduced Aβ plaque load (Latta et al., 2015). KC (CXCL1) is a chemokine involved in recruiting

556 neutrophils, a type of white blood cell, signaling to microglia (Johnson et al., 2011; De
557 Filippo et al., 2013). Finally, KC signals to the receptor CXCR2, which is expressed on
558 microglia and has been reported to mediate microglial recruitment (Lee et al., 2012). In
559 total, our data show that 40Hz light flicker stimulates expression of diverse cytokines
560 with both immunomodulatory and neuro-protective properties.

561 It is important to note that our results were based on experiments done with male
562 mice. We chose to focus on males because we wanted to assess how 40Hz flicker,
563 which induces gamma oscillations, impacts neuroimmune signaling, building on
564 previous papers that have only studied this effect in male mice (Iaccarino et al., 2016;
565 Adaikkan et al., 2019; Martorell et al., 2019). However, we acknowledge that this is a
566 limitation of this study because there are effects of sex on immune response and
567 ovarian hormones have been shown to impact cytokine expression (Arakawa et al.,
568 2014; Cai et al., 2016). Future studies should further explore how this effect may differ
569 between the sexes.

570

571 **40Hz flicker cytokine response differs from acute inflammation**

572 We found that 40Hz flicker upregulates a profile of cytokines in a short period of
573 time, similar to an acute pro-inflammatory stimulus. However, previous work has shown
574 that 40Hz flicker has a neuro-protective effect. Therefore, we wanted to compare how
575 40Hz flicker induced cytokine expression differs from a known pro-inflammatory
576 stimulus operating under a similar acute time period.

577 Our analysis showed that 40Hz flicker stimulates expression of diverse cytokines
578 that differs from a model of acute inflammation. This difference was established by

579 comparing the LV1 cytokine profile of gamma sensory stimulation and acute LPS
580 inflammation. While there may be an overlap in some cytokines that were elevated, the
581 overall profiles are distinct between these two stimulations due to the differences in
582 magnitude of responses and the overall cytokines included. Two cytokines, GM-CSF,
583 and Il-2, significantly differed between 40Hz flicker and random stimulation but did not
584 differ between LPS and vehicle administration. The specific response of these two
585 cytokines to 40Hz flicker is especially interesting because GM-CSF may be both
586 neuroprotective and proinflammatory and low-dose IL-2 rescues cognitive and synaptic
587 plasticity deficits in a mouse model of AD (Bhattacharya et al., 2015; Alves et al., 2017;
588 Kiyota et al., 2018). Furthermore, the combination of GM-CSF and IL-2 has been
589 suggested to have synergistic effects. The combination of these cytokines has been
590 used to prevent immune dysregulation in multiple models of disease (Baiocchi et al.,
591 2001; Stagg et al., 2004; Elias et al., 2005). Thus the combination of these two
592 cytokines following 40Hz flicker may be neuroprotective.

593 Some cytokines significantly differed between both 40Hz versus random flicker
594 and LPS versus vehicle administration. An interesting observation from our data is that
595 while there was overlap in some of these cytokines, there was a noticeable difference in
596 the scale of separation between LPS and vehicle versus 40Hz and random stimulation.
597 While both were significantly upregulated by stimulation, LPS had a much larger effect
598 when compared to control condition. The magnitude of the cytokine response has been
599 suggested to alter a cytokine's effects (Saadoun et al., 2011; Shachar and Karin, 2013).
600 Thus, this low-dose increase caused by 40Hz flicker may be more responsible for the
601 neuroprotective effect of 40Hz flicker.

Lastly, IL-12p70, the bioactive form of the p35 and p40 combined IL-12 subunits, was up-regulated in both LPS and 40Hz versus controls. In contrast, IP-10, which is downstream of IL-12, was significantly different between LPS and control but not 40Hz and random. IP-10 is induced by IFN- γ, which is a pro-inflammatory cytokine downstream of IL-12 (Sgadari et al., 1996). This finding suggests that while both LPS and 40Hz flicker may upregulate some similar cytokines, they may be leading to different downstream mechanisms which explain why LPS is known to be neuro-toxic, while 40Hz flicker is known to be neuroprotective (Iaccarino et al., 2016; Martorell et al., 2019).

40Hz flicker induces neuroimmune intracellular signal transduction pathways

Our data show that 40Hz flicker stimulates NFκB and MAPK pathway signaling, which increased following 15 minutes or 60 minutes of 40Hz flicker, respectively, followed by expression of cytokines. These findings are consistent with the known transient kinetics of phospho-signaling and downstream gene and protein expression (Janes et al., 2005; Gierut et al., 2015). While the effect of 5 minutes of 40Hz flicker on the NFκB pathway were not significant, many of the animals did separate on LV1 and this was mostly driven by increases in TNFR1 and FADD phosphorylation. Interestingly, these two proteins are upstream in the NFκB pathway and therefore we would expect these to become phosphorylated earlier than other proteins in the pathway. After 15 minutes of 40Hz flicker, all proteins in the NFκB pathway had elevated phosphorylation levels. Thus, we conclude that phosphorylation of the NFκB pathway may begin after around 5 minutes of 40Hz flicker in some animals, but the full effects may not be

625 detectable until after 15 minutes of 40Hz flicker. A similar trend is observed for the
626 MAPK pathway, but it seems to be later, where 15 minutes of 40Hz flicker begins to
627 upregulate the pathway and 60 minutes shows a distinct separation between 40Hz
628 flicker and random groups. Later onset of MAPK pathway activation may be related to
629 increased expression of cytokines that act through the MAPK pathway.

630 For phospho-protein analysis, we specifically compared 40Hz flicker and random
631 flicker exposure. We chose random flicker as our control because this stimulation type
632 controls for the largest number of stimulation variables. However, we anticipate that if
633 we compared 40Hz to 20Hz stimulation instead, we would see a bigger difference
634 between groups, since 20Hz had a more reduced cytokine expression profile in Figure 1.
635 In contrast, we anticipate that light stimulation may also upregulate signaling pathways.
636 We expect that because light stimulation uniquely upregulates some cytokines, distinct
637 from 40Hz flicker, that light may also upregulate some of the signaling pathways that
638 are known to regulate cytokines.

639 Interestingly, our data show that the NFκB pathway is downregulated below
640 random flicker after 1hour of 40Hz flicker stimulation, suggesting the effects of 40Hz
641 flicker on these pathways are transient. Both NFκB and MAPK pathways are highly
642 regulated via feedback mechanisms (Hutti et al., 2007; Lake et al., 2016). Given that our
643 observed changes are occurring over the course of an hour, negative feedback
644 mechanisms within the pathways are likely responsible for the downregulation of these
645 pathways.

646 Our hypothesized time-course of pathway activity is further supported when
647 examining correlations between phospho-proteins. After 5 minutes of 40Hz flicker, a

pattern emerged within each pathway. Specifically, in the NFκB pathway, both pFADD and TNFR1 were highly correlated with phosphorylation of each other protein, aligning with increased expression patterns of these two phospho-proteins at the same time course. TNFR1 is upstream in the NFκB signaling pathway, suggesting 5 minutes of flicker begins to modulate the top of the pathway. 15 minutes of 40Hz flicker revealed more correlations among proteins, and these correlations included more proteins downstream in the pathway, suggesting the pathway is progressively activated with longer flicker exposure. However, after 60 minutes of 40Hz flicker, many correlations were no longer present. In fact, in the MAPK pathway, only pATF-2 and pJnk were correlated, downstream in the pathway. These correlation results help support our activation data. However, we note that the small sample size (6 animals), might account for the low significance associated with 60 minutes of stimulation at 40Hz. Further, while the analysis was designed to reveal correlations between any phospho-proteins, all significant correlations remained within each pathway, supporting the idea that each pathway is operating with independent dynamics.

Both the NFκB and MAPK pathways play key roles in immune function, synaptic plasticity, and learning and memory. NFκB and MAPK pathways regulate cytokine levels by activating transcription factors involved in cytokine expression (Hommes et al., 2003; Cloutier et al., 2007; Liu et al., 2017). Increased phosphorylation in the MAPK pathway occurs after spatial memory and is necessary for long-term memory formation (Blum et al., 1999). The NFκB pathway is more traditionally known for its role in inflammation, but is also necessary for long-term potentiation and long-term depression and is increased following synaptic stimulation (C. and P., 1999; Meffert et al., 2003).

671 40Hz light flicker likely stimulates intracellular phospho-signaling and expression
672 of diverse genes by driving neural activity. Several studies have shown that flickering
673 light at a particular frequency drives that frequency neural activity in primary visual
674 areas and 40Hz flicker induces neural activity around 40Hz (Gray et al., 1989; Iaccarino
675 et al., 2016; Singer et al., 2018). Neuronal activity can lead to calcium influx which in
676 turn, stimulates multiple molecular signaling events, including activation of multiple
677 isoforms of protein kinase C (PKC), which can activate the NFκB and MAPK pathways.
678 In fact, inhibition of calcium activity blocks NFκB activation (Meffert et al., 2003). These
679 pathways provide a possible link between gamma frequency activity induced by 40Hz
680 flicker, phospho-signaling, and downstream gene expression.

681

682 **Behavior is similar across different visual stimulation conditions**

683 Cytokine expression can be modified by an animal's behavioral state, for
684 example when an animal is under stress (Minami et al., 1991; Ishikawa et al., 2001).
685 While it is known that chronic 40Hz flicker does not affect anxiolytic behaviors over time,
686 we wanted to ensure 40Hz flicker stimulation did not differentially affect animal behavior
687 in real time over the timescales of our analyses when compared to other stimulation
688 types (Martorell et al., 2019a). We measured total time spent in the center versus
689 surround areas of the enclosure and time spent active versus freezing. We concluded
690 that flicker stimulation did not invoke anxiety-like behavior or changes in overall activity
691 levels. To assess if one stimulation type was more aversive than others, we analyzed
692 time spent in the front of the cage where the lights were positioned versus the back and
693 found no differences across stimulation conditions. Interestingly, animals across all

694 conditions spent somewhat more time (~65%) in the back of the cage, which is not
695 surprising considering that mice tend to stay in dark areas if given the choice (Crawley
696 and Goodwin, 1980). Last, we measured the distance animals traveled within each
697 stimulation type and found no differences across flicker conditions. In short, our analysis
698 revealed no differences in any of our measured animal behaviors between stimulation
699 conditions. Therefore, our observations show that induced phospho-signaling and
700 cytokine expression after 40Hz light flicker cannot be explained by changes in animal
701 behavior.

702 In total, our results show that 40Hz flicker drives rapid signaling within the NFκB
703 and is followed by activation of MAPK pathway and an upregulation of cytokines. The
704 diverse functions regulated by these pathways together with the diversity of cytokine
705 and growth factors expressed in response to 40Hz stimulation suggests that minutes of
706 flicker stimulation may induce changes in various cell and tissue functions that include
707 immune activity and neuronal and synaptic health. Furthermore, different forms of visual
708 stimulation induced unique cytokine profiles. Thus, flicker stimulation may be used to
709 rapidly and non-invasively manipulate signaling and expression of genes that go beyond
710 neural immune activity. Importantly, all our signaling analyses were conducted in wild-
711 type animals, helping establish the effects of 40Hz flicker stimulation independent of
712 disease pathology. Our work provides a foundation for flicker's therapeutic potential to
713 other disorders involving the neuroimmune system.

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FIGURE LEGENDS

Figure 1: One hour of 40Hz flicker increased cytokine expression in visual cortex

A) Experimental configuration for presenting visual stimulation.

B) Cytokine expression in visual cortices of mice exposed to 1 hour of visual stimulation. Each row represents one animal (n=6). Cytokines (columns) are arranged in order of their weights on the first latent variable (LV1) in (D). Color indicates z-scored expression levels for each cytokine. Top four cytokines from LV1 are boxed in red.

C) Partial least squares discriminant analysis (PLSDA) identified LV1, the axis which separated 40Hz flicker exposed animals (red) to the right, 20Hz flicker (blue) and constant light (green) exposed animals to the left and random (black) flicker exposed animals toward the middle (dots indicate individual animals for all graphs in this figure). LV2 separated 20Hz, random, and light conditions.

D) The weighted profile of cytokines that make up LV1 based on which cytokines best correlated with separation of 40Hz (positive) versus flicker control groups (negative). (mean ± SD from a leave one out cross validation).

E) LV1 scores were significantly different for 40Hz group compared to controls (mean± SEM; $F(3, 20) = 5.855$, $p=0.0049$, one-way ANOVA). Each dot represents one animal. Results were confirmed with shuffling analysis (see Methods).

F) There were significant differences in expression of most of the top four analytes, M-CSF, IL-G, MIG, and IL-4 when assessed individually. P-values for comparisons between groups are listed in the figure. For one-way ANOVA comparisons across all groups: M-CSF: $F(3,20)=2.970$, $p=0.0564$, IL-6: $F(3,20)=3.775$, $p=0.0269$, MIG: $F(3,20)=4.532$, $p=0.0140$, and IL-4: $F(3,20)=2.051$, $p=0.1391$.

G) There were significant differences in expression of IFN-γ, IL-12p70, GM-CSF, IL-7, and IL-1β as well. P-values for comparisons between groups are listed in the figure. For one-way ANOVA comparisons across all groups: IFN-γ: $F(3,20)=6.32$, $p=0.0034$), IL-12p70:$F(3,20)=6.428$, $p=0.0032$), GM-CSF: $F(3,20)=4.13$, $p=0.0197$), IL-7: $F(3,20)=9.541$, $p=0.0004$), and IL-1β: $F(3,20)=3.404$, $p=0.0377$).

Figure 2: Flicker control conditions each lead to unique cytokine expression

A) The weighted profile of cytokines that make up LV2 based on which cytokines best correlated with separation of random (positive) versus 20Hz versus light flicker control groups (negative). (Mean ± SD from a leave one out cross validation).

B) LV2 scores were significantly different for all 4 groups (mean± SEM; $F(3, 20) =21.77$, $p<0.0001$, one-way ANOVA). Each dot represents one animal. P-values from Tukey's multiple comparison test are listed.

C) Diagram of cytokine expression, with each cytokine name arranged based on expression per group.

D) One-way ANOVA displays significant difference in IL-10 expression across groups (mean± SEM; $F(3, 20) =27.29$, $p<0.0001$, one-way ANOVA). P-values from Tukey's multiple comparison test are listed.

E) As in **D** for IL-15 expression across groups (mean± SEM; $F(3, 20) =11.33$, $p=0.0001$, one-way ANOVA).

F) As in **D** for RANTES expression across groups (mean± SEM; $F(3, 20) =11.48$, $p=0.0001$, one-way ANOVA).

G) As in **D** for IL-2 expression across groups (mean± SEM; F(3, 20) =9.366, p=0.0005, one-way ANOVA).

H) As in **D** for VEGF expression across groups (mean± SEM; F(3, 20) =6.790, p=0.0024, one-way ANOVA).

I) As in **D** for IL-9 expression across groups (mean± SEM; F(3, 20) =4.076, p=0.0207, one-way ANOVA).

Figure 3: NFκB phospho-protein signaling in visual cortex increased in response to 15 minutes of 40Hz flicker

A) Simplified diagram of NFκB signaling including phospho-proteins quantified in the present study (blue ovals).

B) NFκB phospho-protein expression in visual cortex in mice exposed to 5 minutes of 40Hz or random flicker (z-scored). Each row represents one animal with groups separated by thicker black horizontal lines. 23 total animals were analyzed, n=17 in 40Hz group 17, n=6 in random group; outliers removed through a principle component analysis on the data and iterative removal of data points outside a 99.5% confidence ellipse (mahalanobisQC in R v 3.5.2).

C) As in (B) for mice exposed to 15 minutes of 40Hz or random flicker. 40Hz n=12, random n=6.

D) As in (B) for mice exposed to 60 minutes of 40Hz or random flicker. 40Hz n=6, random n=6.

E) *Top:* PLSDA on phospho-signaling data for mice exposed to 5 minutes of flicker, separating 40Hz (red) exposed animal to the right and random (black) to the left,

along LV1. *Bottom:* Plot of LV1 scores between groups show a trend toward higher LV1 scores in 40Hz exposed animals (difference between means ± SEM= -0.1943±0.09455; t(21)=2.065,p=0.0525, two-tailed t-test). Dots indicate individual animals for all graphs in this figure.

F) *Top:* As in (E) for mice exposed to 15 minutes of 40Hz or random flicker. *Bottom:* Plot of LV1 scores between groups reveal significantly higher LV1 scores in 40Hz exposed animals (difference between means ± SEM = -0.3721±0.08351; t(16)=4.456,p=0.0004, two-tailed t-test). Results were confirmed with permutation analysis (see Methods).

G) *Top:* As in (E) for mice exposed to 60 minutes of 40Hz or random flicker. *Bottom*: Plot of LV1 scores between groups show no significant difference (difference between means ± SEM = -0.2829±0.2; t(8)=1.415,p=0.1948, two-tailed t-test).

H) The weighted profile of NFκB phospho-proteins that make up LV1 based on which phospho-proteins best correlated with 40Hz (positive) or random (negative) after 5 minutes of flicker exposure (mean ± SD from a leave one out cross validation). pIKKα/β and TNFR1 most strongly contributed to separation between the groups

I) As in (H) for 15 minutes of flicker. pFADD expression in 40Hz flicker group and pNFκB in random group most strongly contributed to separation between groups.

J) As in (H) for 60 minutes of flicker.

Figure 4: MAPK phospho-protein signaling in visual cortex increased in response to 60 minutes of 40Hz flicker

A) Simplified diagram of MAPK signaling diagram including phospho-proteins quantified in the present study (blue ovals).

B) MAPK phospho-protein expression in visual cortex in mice exposed to 5 minutes of 40Hz or random flicker (z-scored). Each row represents one animal with groups separated by thicker black horizontal lines. 34 total animals were analyzed, n=24 in 40Hz group, n=10 in random group; outliers removed through a principle component analysis on the data and iterative removal of data points outside a 99.5% confidence ellipse (mahalanobisQC in R v 3.5.2).

C) As in (B) for mice exposed to 15 minutes of 40Hz or random flicker. 40Hz n=8, random n=6.

D) As in (B) for mice exposed to 60 minutes of 40Hz or random flicker.

E) *Top:* PLSDA on phospho-signaling data for mice exposed to 5 minutes of flicker did not separate 40Hz (red) samples and random (black) along LV1. *Bottom:* Plot of LV1 scores per group show no significant differences between groups (difference between means ± SEM= -0.1181±0.06318; (t(32)=1.870,p=0.0707, unpaired two-tailed t-test). Dots indicate individual animals for all graphs in this figure.

F) *Top:* As in (E) for mice exposed to 15 minutes of 40Hz or random flicker. *Bottom:* Plot of LV1 scores per group reveal a non-significant increase in 40Hz LV1 expression in 40Hz exposed animals. (-0.1572±0.1311; t (8.582) =1.199, p=0.2624, unpaired t test with Welch's correction).

G) *Top:* As in (E) for mice exposed to 15 minutes of 40Hz or random flicker. *Bottom*: Plot of LV1 scores per group show significantly higher LV1 scores in 40Hz exposed animals (-0.4393±0.1185; t(10)=3.709,p=0.0040, two-tailed t-test).

H) The weighted profile of MAPK phospho-proteins that make up LV1 based on which best correlated with 40Hz (positive) or random (negative) flicker after 5 minutes of flicker exposure (mean ± SD from a leave one out cross validation).

I) As in (H) for 15 minutes of flicker.

J) As in (H) for 60 minutes of flicker. pMSK and pMEK expression in 40Hz flicker group most strongly contributed to separation between groups.

Figure 5: Phosphoprotein network correlations are highest after 15 minutes of 40Hz flicker exposure

A) Significant correlations following 5 minutes of 40Hz flicker in NFκB (orange) and MAPK (green) phosphoprotein levels across animals displayed as links, with color indicating the strength of positive (red) or negative correlations (blue). No negative correlations were found in these interactions. $q < 0.1$, false discover rate correction for 135 comparisons, Pearson correlation test. *Right*: Example correlated phospho-proteins.

B) As in (A) for 15 minutes of 40Hz flicker.

C) As in (A) for 60 minutes of 40Hz flicker.

Figure 6: 40Hz flicker does not alter animal behavior during exposure

A) Example heatmap representing the amount of time spent in locations within the flicker enclosure. Delineations indicating the center, front, and back of the cage were used for behavior analysis.

B) Total percentage of time animals in each group spent in the center of the cage (F (3, 15) =0.8754, p=0.4757, RM-ANOVA). Error bars indicate mean ± SEM. A total of 5 animals were used, represented by dots indicating individual animals for all bar graphs in this Figure (Red=40Hz, Black=random, Blue=20Hz, Green=light).

C) Total percentage of time animals in each group spent active (versus freezing) (F (3, 15) =0.9306, p=0.4502, RM-ANOVA).

D) Total percentage of time animals in each group spend in the back half of the cage (versus front) (F (3, 15) =0.1727, p=0.9132, RM-ANOVA).

E) Total distance animals in each group traveled during stimulation (F (3, 15) =0.3392, p=0.7973, RM-ANOVA).

Figure 7: 40Hz flicker differs from LPS-induced acute inflammation.

A) The weighted profile of cytokines that make up LV1 following 40Hz flicker (blue) or LPS (orange) (mean± SD from a leave one out cross validation).

B) GM-CSF and IL-2 expression levels significantly differed in 40Hz versus random ("Flicker") but not LPS versus vehicle ("Acute Inflammation"). P-values for t-test comparisons between groups are listed in figure.

C) IL-12p70, Eotaxin, TNF-α, and MCP1 expression levels significantly differed for both 40Hz versus random and LPS versus vehicle. P-values for t-test comparisons between groups are listed in figure.

D) G-CSF, KC, RANTES, IP-10, IL-4, and MIC expression levels significantly differed for LPS versus vehicle but not 40Hz versus random. P-values for t-test comparisons between groups are listed in figure.

Video 1:

Example video of mouse undergoing flicker stimulation. Note, this video shows 20Hz flicker because 40Hz flicker is too fast to capture with standard movie frame rates.