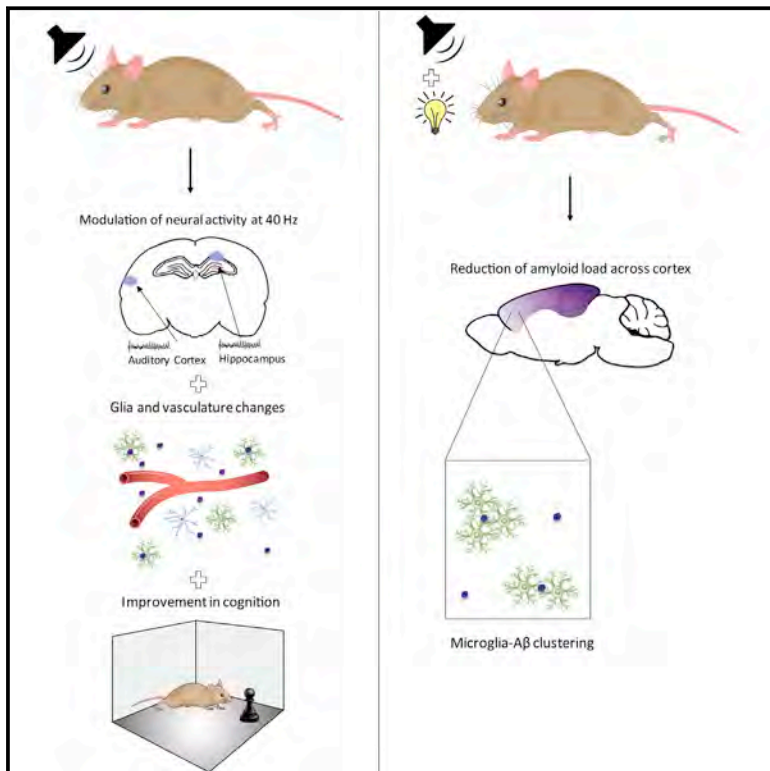


Multi-sensory Gamma Stimulation Ameliorates Alzheimer's-Associated Pathology and Improves Cognition

Graphical Abstract



Authors

Anthony J. Martorell, Abigail L. Paulson, Ho-Jun Suk, ..., Edward S. Boyden, Annabelle C. Singer, Li-Huei Tsai

Correspondence

lhtsai@mit.edu

In Brief

Auditory stimulation combined with light-induced gamma oscillations in the hippocampus CA1 and auditory cortex regions of the brain reduces amyloid levels and improves memory in animal models of Alzheimer's disease.

Highlights

- Auditory gamma entrainment using sensory stimuli (GENUS) boosts hippocampal function
- GENUS affects microglia, astrocytes, and vasculature in auditory cortex and hippocampus
- Auditory plus visual GENUS induces microglia clustering around plaques
- Auditory plus visual GENUS reduces amyloid pathology throughout neocortex



Multi-sensory Gamma Stimulation Ameliorates Alzheimer's-Associated Pathology and Improves Cognition

Anthony J. Martorell,^{1,2,13} Abigail L. Paulson,^{3,13} Ho-Jun Suk,^{4,5,6,12} Fatema Abdurrob,^{1,2,12} Gabrielle T. Drummond,^{1,2} Webster Guan,⁷ Jennie Z. Young,^{1,2} David Nam-Woo Kim,^{1,2} Oleg Kritskiy,^{1,2} Scarlett J. Barker,^{1,2} Vamsi Mangena,⁶ Stephanie M. Prince,³ Emery N. Brown,^{1,2,8,10,11} Kwanghun Chung,^{1,2,7,8,9} Edward S. Boyden,^{2,4,5} Annabelle C. Singer,³ and Li-Huei Tsai^{1,2,9,14,*}

¹Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

²Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

³Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University School of Medicine, Atlanta, GA 30332, USA

⁴McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

⁵MIT Media Lab, Departments of Biological Engineering and Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

⁶Harvard-MIT Health Sciences and Technology, Cambridge, MA 02139, USA

⁷Department of Chemical Engineering, MIT, Cambridge, MA, USA

⁸Institute for Medical Engineering and Science, Massachusetts Institute of Technology (MIT), Cambridge, MA, USA

⁹Broad Institute of Harvard and MIT, Cambridge, MA 02139, USA

¹⁰Department of Anesthesia, Critical Care, and Pain Medicine, Massachusetts General Hospital, Boston, MA, 02114, USA

¹¹Harvard Medical School, Boston, MA 02115, USA

¹²These authors contributed equally

¹³These authors contributed equally

¹⁴Lead Contact

*Correspondence: lhtsai@mit.edu

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SUMMARY

We previously reported that inducing gamma oscillations with a non-invasive light flicker (gamma entrainment using sensory stimulus or GENUS) impacted pathology in the visual cortex of Alzheimer's disease mouse models. Here, we designed auditory tone stimulation that drove gamma frequency neural activity in auditory cortex (AC) and hippocampal CA1. Seven days of auditory GENUS improved spatial and recognition memory and reduced amyloid in AC and hippocampus of 5XFAD mice. Changes in activation responses were evident in microglia, astrocytes, and vasculature. Auditory GENUS also reduced phosphorylated tau in the P301S tauopathy model. Furthermore, combined auditory and visual GENUS, but not either alone, produced microglial-clustering responses, and decreased amyloid in medial prefrontal cortex. Whole brain analysis using SHIELD revealed widespread reduction of amyloid plaques throughout neocortex after multi-sensory GENUS. Thus, GENUS can be achieved through multiple sensory modalities with wide-ranging effects across multiple brain areas to improve cognitive function.

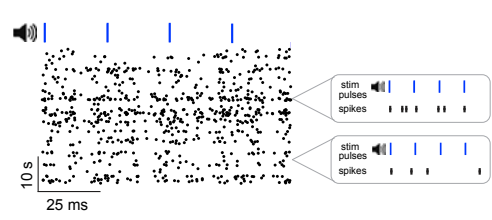
INTRODUCTION

Alzheimer's disease (AD) is the most prevalent form of dementia and is characterized by the progressive loss of cognition, for which there are currently limited treatment options. Pathological hallmarks of the disease include extracellular aggregates of amyloid- β (A β) peptides and tau-associated pathology (Hardy and Higgins, 1992; Selkoe et al., 1996; Yankner and Lu, 2009; Verret et al., 2012). Aberrant neural activity can also exacerbate the progression of AD pathology, which ultimately disrupt neural circuits involved in higher cognitive functions (Palop et al., 2007; Canter et al., 2016). Conversely, neural activity can also be modulated to reduce AD pathology (Cirrito et al., 2005; Bero et al., 2011; Iaccarino et al., 2016), but whether it can improve cognitive function in AD remains to be determined.

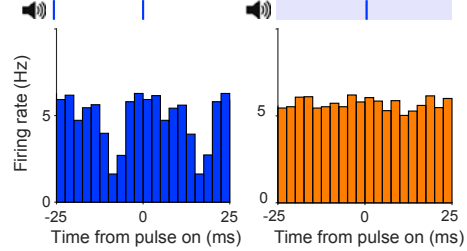
For instance, gamma oscillations (30–90 Hz), associated with numerous higher-order cognitive functions (Fries, 2009), are disrupted in several AD mouse models (Verret et al., 2012; Gillespie et al., 2016; Iaccarino et al., 2016). We and others have shown that gamma oscillations can be evoked with optogenetics (Cardin et al., 2009; Sohal et al., 2009) or non-invasively, which we term gamma entrainment using sensory stimuli (GENUS), via a light programmed to flicker at 40 Hz (Rager and Singer, 1998; Iaccarino et al., 2016). In our previous study (Iaccarino et al., 2016), we used visual GENUS to entrain gamma oscillations in the primary visual cortex (VC) and found a reduction in amyloid load and morphological changes in microglia—such as enlarged soma size and increased co-localization with A β —consistent



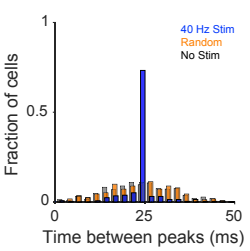
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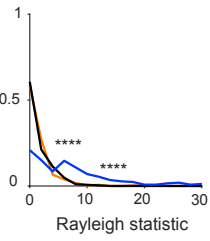
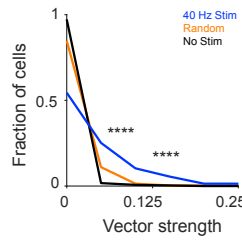
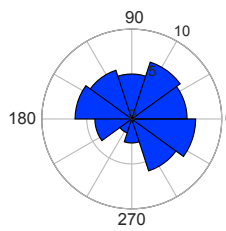
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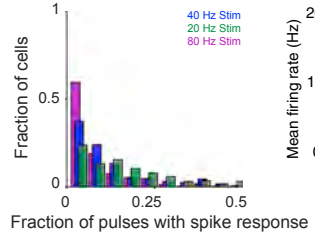
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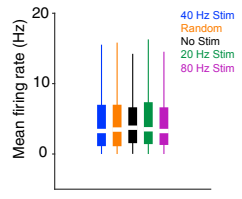
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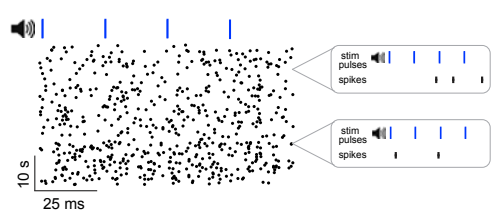
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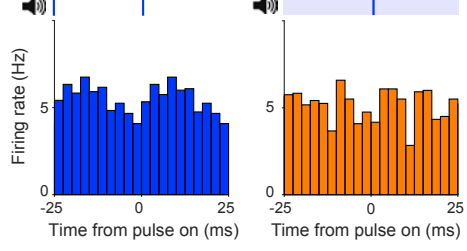
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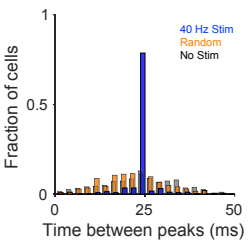
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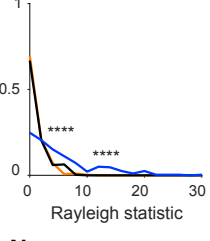
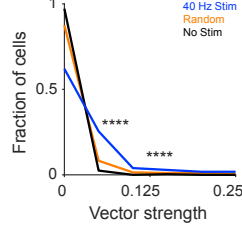
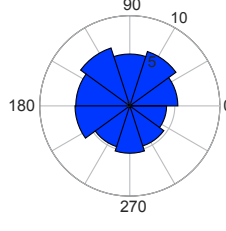
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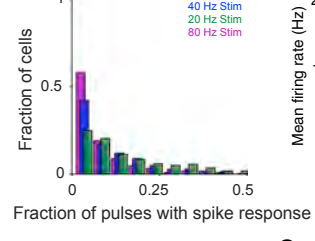
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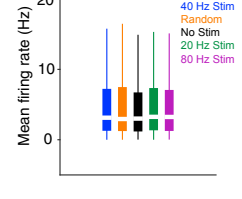
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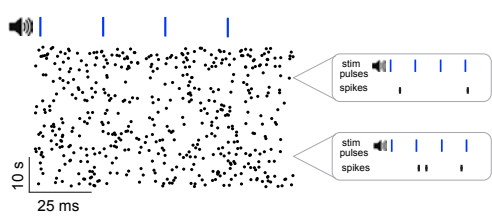
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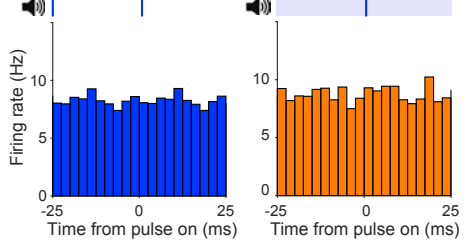
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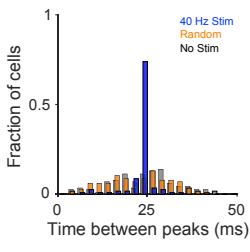
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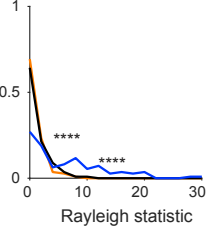
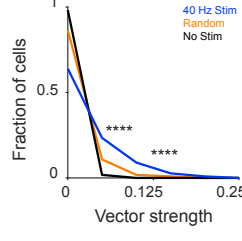
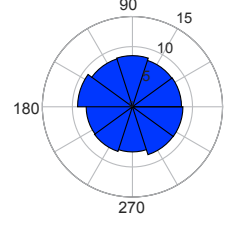
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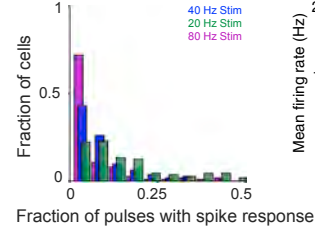
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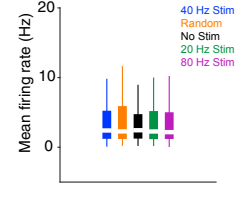
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with a more phagocytic state (Saijo and Glass, 2011; Walker and Lue, 2015). These findings showed that visual GENUUS can affect local network activity and induce a protective molecular and cellular response in VC of 5XFAD mice. However, AD affects multiple brain centers critical for learning and memory and other higher-order brain functions such as the hippocampus (HPC) and medial prefrontal cortex (mPFC) (Seeley et al., 2009; Sperling et al., 2010; Yamamoto et al., 2014; Spellman et al., 2015; Kim et al., 2016). Thus, some outstanding questions in the application of GENUUS include: can GENUUS (1) be induced through other sensory modalities to reduce AD pathology, (2) have impact beyond primary sensory areas, (3) improve cognitive function in AD, and (4) be achieved through multi-sensory stimuli to further impact downstream brain regions?

Here, we tested the application of GENUUS using auditory stimulation with a train of tones flickering at 40 Hz. We show that auditory GENUUS significantly improved performance on several hippocampal-dependent tasks. Concurrently, we observed reduced amyloid load and microglial responses consistent with increased phagocytosis in the auditory cortex (AC) and HPC of 5XFAD mice. Auditory GENUUS also increased the number of reactive astrocytes and elicited a vasculature response in these brain regions. Finally, we found that combined auditory and visual GENUUS uniquely induced a microglia-clustering response in AC, HPC, as well as mPFC, and reduced amyloid load not just in these brain regions but throughout the neocortex. Our observations demonstrate a non-invasive approach to elicit system-wide effects on AD-related pathology and improvements in cognition in an AD mouse model.

RESULTS

40 Hz Auditory Stimulation Modulates Spiking Activity in AC, CA1, and mPFC

We first determined whether auditory tone stimulation could produce GENUUS in AC, area CA1 of HPC, and mPFC. We presented animals with trains of tones repeating at 20 Hz, 40 Hz, 80 Hz, or with trains of randomly spaced tones (1 ms long, 10 kHz tones played every 12.5 ms, 25 ms, 50 ms, or with random inter-tone intervals, henceforward referred to as “auditory stimulation,” STAR Methods). We recorded neural activity in AC, CA1, or mPFC during tone presentation using 32-channel silicon probes in 3- to 8-month-old male wild-type (C57BL6J) mice as they ran or rested on a spherical treadmill. The firing rate of putative single units increased and decreased periodically with each tone thereby entraining to the 40 Hz auditory stimulation (Figures 1A, 1B 1G, 1H, 1M, and 1N, blue). Units were also modulated by random stimulation: when all random pulses were aligned, there was a change in firing rate modulation following the stimuli, indicating that single units responded to the random stimuli pulses. However, the random train of auditory tones did not induce periodic firing modulation because the stimuli themselves were not periodic (Figures 1B, 1H, and 1N, orange). Entrainment to auditory stimulation varied between single units in both phase distribution and amplitude. During auditory stimulation, neurons fired as a function of the stimulus, but did not fire on every cycle and often at a wide range of phases: in response to 40 Hz auditory stimulation most neurons fired every 0–22 pulses in AC, 0–30 pulses in CA1, and 0–34 pulses in mPFC (1st–3rd quartiles

Figure 1. 40 Hz Auditory Stimulation Modulates Spiking Activity in AC, CA1, and mPFC

(A) Left: example putative single unit spiking response to 40 Hz auditory stimulation with many 10 s stimulation blocks shown wrapped every 100 ms. Right: examples of spiking response to four consecutive pulses.

(B) Firing rate modulation of unit shown in (A) during 40 Hz auditory (blue) and random stimulation (orange) in AC. Blue ticks, auditory pulses; light blue bar, randomly distributed pulses.

(C) Intervals between peaks in firing rate in AC for no (gray, labeled no stim), random (orange, labeled random), and 40 Hz auditory stimulation (dark blue, labeled 40 Hz stim) conditions for all single units ($n = 292$ units in 9 recording sessions in 5 mice). Proportion of intervals around inter-stimulus interval: $p = 0$ 40 Hz versus no stim, $p = 0$ 40 Hz versus random; z test for two proportions. For all statistics reported, results are significant after controlling for multiple comparisons using the Bonferroni correction unless otherwise stated.

(D) Example polar plot of firing rate modulation relative to stimulus onset during 40 Hz auditory stimulation (left, stimulus onset at 0), vector strengths of single unit firing rate modulation during 40 Hz auditory, random, and no stimulation (center, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 9 units had 40 Hz stim VS values >0.25 ; 6 units had random stim VS values >0.25), and Rayleigh statistic values of single unit firing rate modulation (right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 40 units had 40 Hz stim RS values >30 ; 2 units had random stim RS values >30).

(E) Fraction of pulses with spiking response from single units in AC for 20 Hz, 40 Hz, and 80 Hz auditory stimulation.

(F) Mean firing rates for stimulation conditions in AC.

(G) Same as (A) for CA1.

(H) Same as (B) for CA1.

(I) Same as (C) for CA1 ($n = 338$ units in 10 recording sessions in 5 mice. $p = 0$ 40 Hz versus no stim, $p = 0$ 40 Hz versus random; z test for two proportions).

(J) Same as (D) for CA1 (center, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 11 units and 2 units had VS values >0.25 during 40 Hz or random, respectively; right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 7 units had 40 Hz stim RS values >30).

(K) Same as (E) for CA1.

(L) Same as (F) for CA1.

(M) Same as (A) for mPFC.

(N) Same as (B) for mPFC.

(O) Same as (C) for mPFC ($n = 115$ units in 7 recording sessions in 4 mice. $p = 0$ 40 Hz versus no stim, $p = 0$ 40 Hz versus random; z test for two proportions).

(P) Same as (D) for mPFC (center, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 2 units had 40 Hz stim RS values >30).

(Q) Same as (E) for mPFC.

(R) Same as (F) for mPFC.

See also Figure S1.

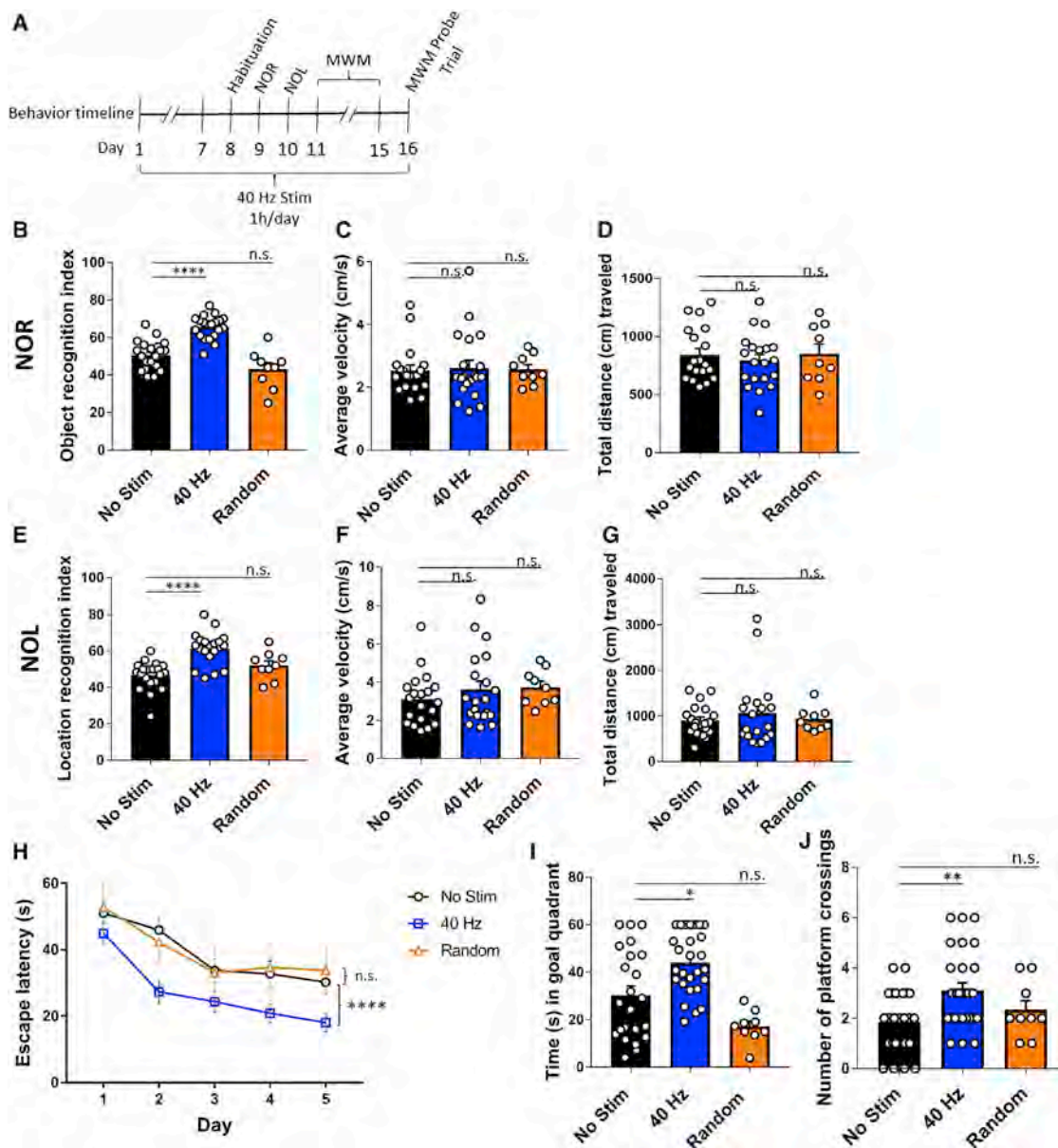


Figure 2. Auditory GENUS Improves Recognition and Spatial Memory Task Performance in 5XFAD Mice

(A) Timeline of behavior experiments.

(B) Recognition index of novel object recognition (NOR) test (n = 20 mice no stim [black], n = 20 mice 40 Hz [blue], n = 9 random frequency [orange], ****p < 0.0001, Kruskal-Wallis [KW] test with Dunn's multiple comparison test).

(C) Average velocity (cm/s) during novel object recognition test (n = 20 mice no stim, n = 20 mice 40 Hz, n = 9 random frequency, KW test with Dunn's multiple comparison test).

(D) Total distance (cm) traveled during novel object recognition test (n = 20 mice no stim, n = 20 mice 40 Hz, n = 9 random frequency, KW test with Dunn's multiple comparison test).

(E) Recognition index of novel object location (NOL) test of 5XFAD auditory GENUS mice (n = 20 mice no stim, n = 20 mice 40 Hz, n = 9 random frequency, ****p < 0.0001, KW test with Dunn's multiple comparison test).

(F) Average velocity (cm/s) during novel object location test (n = 20 mice no stim, n = 20 mice 40 Hz, n = 9 random frequency, KW test with Dunn's multiple comparison test).

(G) Total distance (cm) traveled during novel object location test (n = 20 mice no stim, n = 20 mice 40 Hz, n = 9 random frequency, KW test with Dunn's multiple comparison test).

(H) Escape latencies (s) of 5XFAD mice in the Morris water maze (n = 25 mice no stim, n = 28 mice 40 Hz, n = 9 random frequency, ****p < 0.0001, 2-way ANOVA with Tukey's multiple comparison test).

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reported; **Figures 1B, 1E, 1H, 1K, 1N, and 1Q**), although the interval between peaks in firing rate was around 25 ms (equivalent to 40 Hz) in the majority of single units (**Figures 1C, 1I, and 1O**). In contrast, during baseline periods with no tones and periods with random tones, the interval between peaks had a broad distribution around 25 ms (i.e., the firing rate was not modulated at 40 Hz) (**Figures 1C, 1I, and 1O**). Modulation strength was quantified by considering single unit firing rate as a function of the stimulus phase and calculating its vector strength (VS) (**Figures 1D, 1J, and 1P, left**). Vector strength values range from 0 to 1: 0 represents a uniform distribution of firing not modulated by the stimulus ($VS = 0$), and 1 represents a distribution where a neuron only fired to a specific stimulus phase ($VS = 1$). The distribution of vector strengths of single-unit response to 40 Hz auditory stimulation was significantly higher than no stimulation and random stimulation (**Figures 1D, 1J, and 1P, center**). Random stimulation vector strengths were also significantly higher than no stimulation (because vector strength measures modulation by a stimulus), but it did not induce periodic firing modulation. Similarly, the distribution of Rayleigh statistics for single units during 40 Hz auditory stimulation was significantly higher than that of no stimulation and random stimulation controls (**Figures 1D, 1J and 1P, right**). Differences in vector strength and Rayleigh statistics between stimulation conditions within single units showed that neurons were more strongly modulated by periodic stimuli, and single units were significantly more strongly modulated by lower frequencies of stimulation (**Figures S1G, S1N, and S1U**). The mean firing rate of single neurons was similar between 40 Hz auditory stimulation and no stimulation, random stimulation, 20 Hz, and 80 Hz auditory stimulation controls (**Figures 1F, 1L, 1R, S1D, S1K, and S1R**). Local field potentials in AC displayed elevated power at 40 Hz during 40 Hz auditory stimulation, but the effects varied between recording locations, recording sessions, and response latency to mapping tones (**Figures S1B, S1I, and S1P**). These findings suggest that 40 Hz auditory stimulation induces GENUS robustly in AC, CA1, and mPFC.

Auditory GENUS Improves Memory Performance in 5XFAD Mice

Given that auditory GENUS influences hippocampal neural activity, we next assessed its effects on hippocampus-dependent learning and memory in 6-month-old 5XFAD mice (**Figure 2A**), the age at which behavioral impairments first become evident (**Oakley et al., 2006**). Mice were first habituated to the behavior chamber 24 h prior to running novel object recognition (NOR) and novel object location (NOL) tasks, which test memory for the identity or placement of an object in a specific context as measured by the recognition index (**STAR Methods**) (**Leger et al., 2013**). During habituation, neither auditory GENUS nor random frequency groups differed significantly from no stimulation controls in average velocity, total distance, time spent in the

center, and time spent in the periphery, indicating no differences in general activity or anxiety-like behavior (**Figures S2E–S2H**). Following auditory GENUS, 5XFAD mice exhibited a significantly higher recognition index in NOR ($65.50\% \pm 1.40\%$) and NOL ($61.41\% \pm 2.0\%$) tasks, whereas no stimulation and random frequency controls had a recognition index around chance on both tasks (**Figures 2B and 2E**)—i.e., mice following auditory GENUS spent significantly more time with the novel object in NOR (**Figure S2A**) or object in a novel location in NOL, whereas no stimulation or random frequency controls showed no preference in either tasks (**Figure S2C**). Multiple comparisons (Kruskal-Wallis test with Dunn's multiple comparison test) between all frequency groups in this article are shown in **Table S1**. Auditory GENUS and random frequency groups did not differ significantly in distance traveled nor average velocity during the task periods versus no stimulation controls, indicating that higher recognition index is not due to general differences in activity (**Figures 2C, 2D, 2F, and 2G**). We also observed no significant difference in the time it took to reach the object exploration requirement of 20 s between auditory GENUS and random frequency groups versus no stimulation controls (**Figures S2B and S2D**).

We next applied the Morris water maze (MWM) to test spatial memory for the location of a hidden platform with respect to surrounding contextual cues (**Morris, 1984; Vorhees and Williams, 2006**). All three groups identified the hidden platform over successive training trials (**STAR Methods**), although the escape latency for the auditory GENUS group was consistently and significantly shorter than both no stimulation and random frequency control groups (**Figure 2H**). There was no significant difference in swim velocity between auditory GENUS and random frequency groups versus no stimulation controls (**Figure S2I**). During the probe trial (**STAR Methods**), mice that received auditory GENUS, but not random frequency, spent a significantly longer time exploring the quadrant that previously contained the platform and had a higher number of crossings over the platform location, versus no stimulation controls (**Figures 2I and 2J**). We additionally examined the activity of 5XFAD mice during exposure to 1 h auditory GENUS, random frequency, or no stimulation. We observed no significant differences in average velocity (cm/s), distance traveled (cm), nor periods of quiescence (the amount of time mice spent under 2 cm/s) during the 1 hr of GENUS or random stimulation versus no stimulation controls (**Figures S2J–S2L**). We also performed NOR, NOL, and MWM tests on 12-week-old C57BL/6J mice following 7 days of auditory GENUS, random frequency, or no stimulation. In both NOR and NOL tests, we found no significant difference in recognition index between auditory GENUS and random frequency groups versus no stimulation controls. In MWM, we observed no significant difference in the training curves nor probe tests between auditory GENUS and random frequency versus no stimulation controls (**Figure S8**). Together, these results show that

(I) Time (s) spent swimming in the goal quadrant during the probe trial ($n = 25$ mice no stim, $n = 28$ mice 40 Hz, $n = 9$ random frequency, $*p < 0.05$, KW test with Dunn's multiple comparison test).

(J) Number of platform crossings during probe trial ($n = 25$ mice no stim, $n = 28$ mice 40 Hz, $n = 9$ random frequency, $**p < 0.01$, KW test with Dunn's multiple comparison test). Circles indicate "n," mean \pm SEM in bar graphs unless otherwise noted, n.s., not significant.

See also **Figure S2** and **Table S1**.

auditory GENUS can improve recognition and spatial memory in 6-month-old 5XFAD mice.

Auditory GENUS Reduces Amyloid Load in AC and Hippocampus in 5XFAD Mice

The effects of auditory GENUS on hippocampal function led us to investigate if amyloid pathology could be affected in HPC. We observed that soluble $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were reduced in AC ($A\beta_{1-42}$: 51.84% \pm 4.98%, $A\beta_{1-40}$: 20.65% \pm 3.21%) and HPC ($A\beta_{1-42}$: 46.89% \pm 3.89%, $A\beta_{1-40}$: 34.15% \pm 4.83%) after 7 days of auditory GENUS for 1 h/day, but not in other frequency groups, versus no stimulation controls (Figures 3A and S3A), and insoluble $A\beta_{1-42}$ levels were similarly reduced in AC (36.68% \pm 3.21%) and HPC (43.84% \pm 2.42%) (Figure 3B). Insoluble $A\beta_{1-40}$ was not detectable via ELISA in auditory GENUS nor in no stimulation controls. Amyloid reduction was specific to 40 Hz stimulation, as neither 8 Hz, 80Hz, nor random frequency stimulation significantly changed $A\beta$ levels, versus no stimulation controls. We also examined $A\beta$ levels after 7 days of auditory GENUS in another AD mouse model using 6-month-old APP/PS1 mice, and found significantly reduced soluble $A\beta_{1-42}$ in AC (48.39% \pm 3.50%) and in HPC (35.54% \pm 4.27%), versus no stimulation controls (Figure S3B).

We next examined plaque load in 6-month-old 5XFAD mice after auditory GENUS versus no stimulation controls. Immunostaining with a β -amyloid specific antibody D54D2 (Figures 3C and 3D) revealed significantly reduced plaque numbers (AC: 45.73% \pm 2.818%; CA1: 59.30% \pm 2.083%) (Figure 3E) and size (AC: 54.37% \pm 5.603%; CA1: 40.70% \pm 5.321%) (Figure 3F) after auditory GENUS. Immunostaining with the $A\beta_{1-42}$ -specific antibody 12F4 also showed significant reductions (AC: 45.35% \pm 0.011%; CA1: 43.21% \pm 0.0285%) (Figures 3G–3I). We carried out similar analysis in another AD model, 9-month-old APP/PS1 mice (6-month-old APP/PS1 mice do not have significant plaque load). Consistent with our findings in 5XFAD mice, we saw significantly reduced plaque numbers (AC: 52.65% \pm 7.53%; CA1: 62.90% \pm 15.5%) and size (AC: 67.90% \pm 6.18%; CA1: 64.06% \pm 15.2%) after auditory GENUS. Immunostaining for $A\beta_{1-42}$ (12F4) also showed significant reductions (AC: 38.77% \pm 4.21%; CA1: 47.63% \pm 6.08%) (Figures S3C–S3E). To examine the dynamics of plaque load after auditory GENUS, we analyzed β -amyloid (D54D2) immunostaining in 6-month-old 5XFAD mice one week (with no stimulation) after 7 days of auditory GENUS concluded. We observed slight, non-significant decreases in average plaque number, size, and amyloid intensity, one week after auditory GENUS versus no stimulation controls (Figures S3F–S3H).

Collectively, these results demonstrate that auditory GENUS can reduce amyloid load in areas outside of primary sensory cortices in multiple AD mouse models.

Auditory GENUS Induces a Glia and Blood Vessel Response in 5XFAD Mice

Accumulating evidence suggests that microglia are responsive to changes in neuronal activity, and play a role in AD pathology (Allen and Barres, 2005; Mosher and Wyss-Coray, 2014; Walker and Lue, 2015). We previously observed morphological changes in microglia in 3-month-old 5XFAD after 1 hr of visual GENUS

(Iaccarino et al., 2016). We thus immunostained for the microglia marker Iba1 in AC and CA1 of 6-month-old 5XFAD mice after auditory GENUS and compared to no stimulation controls (Figures 4A and 4B). We observed \sim 60% more microglia in both AC and CA1 after auditory GENUS (Figure 4C). Microglia cell body diameter was increased in both areas (AC: 70.60% \pm 4.78%; CA1: 117.17% \pm 10.4%) (Figure 4D), and additionally, microglia process length decreased (AC: 46.44% \pm 3.2%; CA1: 50.88% \pm 4.8%) and arborization increased (AC: 36.00% \pm 9.5%; CA1: 143.813% \pm 29.9%) after auditory GENUS (Figures 4E and 4F). We co-immunostained for $A\beta_{1-42}$ (12F4) with microglia (Iba1) to evaluate microglia uptake of $A\beta$ and observed an increased percentage of co-labeled microglia in AC (58.75% \pm 1.25%) and CA1 (61.33% \pm 3.71%) after auditory GENUS (Figure 4G). We also examined microglia morphology after auditory GENUS in another AD mouse model, 9-month-old APP/PS1 mice. Consistent with our findings in 5XFAD mice, we observed a significant increase in microglia numbers and cell body diameter, with a significant decrease in average processes length in AC and CA1 after auditory GENUS, compared to no stimulation controls (Figures S4A–S4C). To examine the longitudinal effects of auditory GENUS on microglia morphology, we analyzed Iba1 immunostaining in 6-month-old 5XFAD mice 1 week (of no stimulation) after the 7 days that auditory GENUS concluded. We observed no significant change in microglia number, cell body diameter, nor average processes length in AC 1 week after auditory GENUS, versus no stimulation controls (Figures S4D–S4F). We did, however, see a significant increase in microglia count in CA1 (41.70% \pm 6.75%) in the auditory GENUS group (Figure S4F).

Astrocytes are another primary glial cell in the CNS and are critical for many important biological processes including waste clearance (Chung et al., 2015; Kisler et al., 2017). We first determined that 5XFAD mice have significantly fewer reactive-like astrocytes that immunostain positive for glial fibrillary acidic protein (GFAP) than wild-type (WT) controls (Figures S4G and S4H), consistent with reports of glia atrophy in other AD mouse models (Rodríguez et al., 2009). We then immunostained for GFAP after auditory GENUS and found increased numbers in AC (GFAP: 27.66% \pm 0.954%) and CA1 (18.14% \pm 0.799%), versus no stimulation controls (Figures 4H and 4J). We confirmed the effects of auditory GENUS on reactive-like astrocytes using a second marker, S100 calcium-binding protein B (S100B) (AC: 21.83% \pm 1.07%; CA1: 15.57% \pm 0.869%) (Figures 4I and 4K). Astrocytes are also capable of regulating the brain's vascular network (Kyrtos and Baras, 2015), which can play a role in amyloid clearance from the brain (Kyrtos and Baras, 2015; Storck et al., 2016; Iliff et al., 2012). We first immunostained for tomato lectin, a marker of vascular endothelium, to assess vasodilation (Figures 5A and 5B) and observed increased blood vessel diameter after auditory GENUS (AC: 49.70% \pm 7.80%; CA1: 104.70% \pm 10.96%), versus no stimulation controls (Figure 5C). Amyloid transcytosis through the brain endothelium is mediated by lipoprotein receptor-related protein 1 (LRP1) (Kanekiyo et al., 2013; Storck et al., 2016). We thus co-immunostained for $A\beta$ with LRP1 (Figures 5D and 5E) and observed increased co-localization of the two after auditory GENUS (AC: 53.87% \pm 2.70%; CA1: 57.78% \pm 1.73%) (Figure 5F). Together, these results suggest auditory

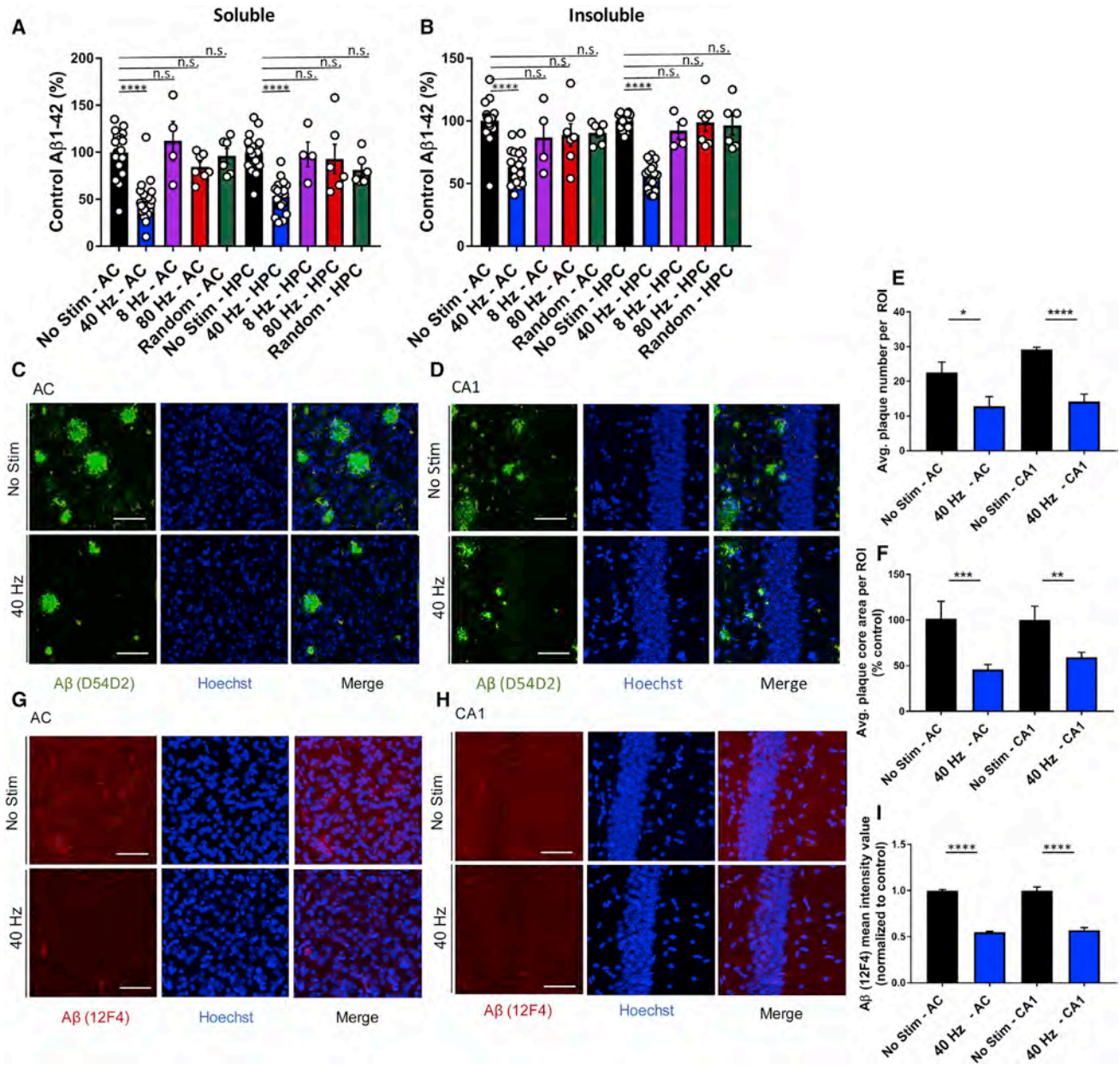


Figure 3. Auditory GENUS Reduces Amyloid Load in AC and HPC in 5XFAD Mice

(A) Relative soluble Aβ₁₋₄₂ levels in AC and HPC in 6-month-old 5XFAD mice following 40 Hz, 8 Hz, 80 Hz, or random auditory stimulation for 1 h/day for 7 days (1 week), normalized to non-stimulation control (n = 19 mice no stim, n = 19 mice 40 Hz, n = 4 mice 8 Hz, n = 7 80 Hz, n = 6 random frequency, ****p < 0.0001, KW test with Dunn's multiple comparison test).

(B) As in (A) for insoluble Aβ₁₋₄₂.

(C) Immunohistochemistry with anti-Aβ (D54D2, green) antibody in 6-month-old AC of 5XFAD mice after auditory GENUS or no stimulation, for 1 week (n = 7 mice/group; scale bar, 50 μm).

(D) As in (C) for CA1.

(E) Average number of Aβ-positive plaques in AC and CA1 (n = 7 mice/group, *p < 0.05, ****p < 0.0001; unpaired Mann-Whitney [MW] test).

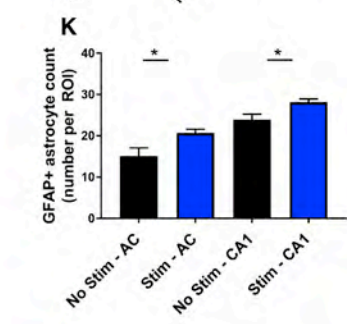
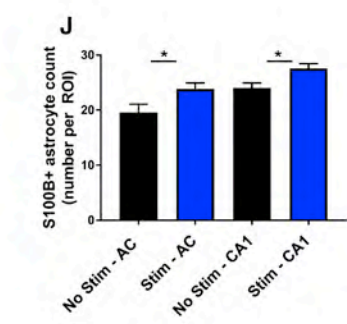
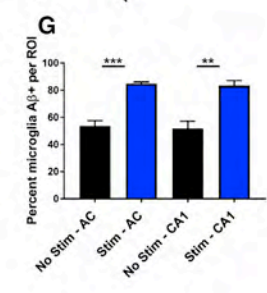
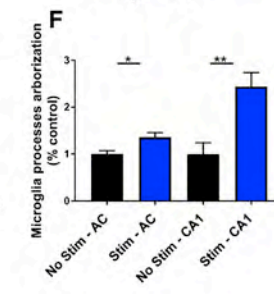
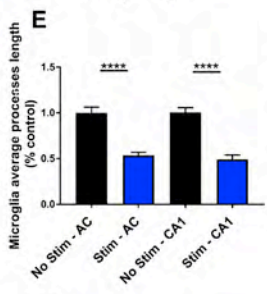
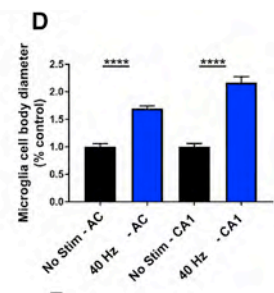
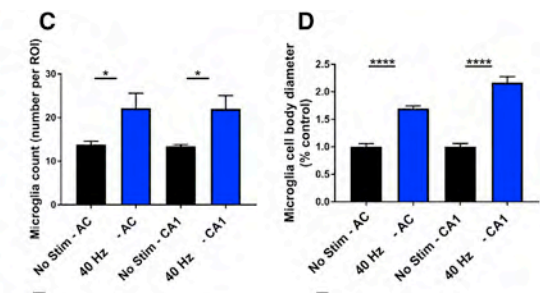
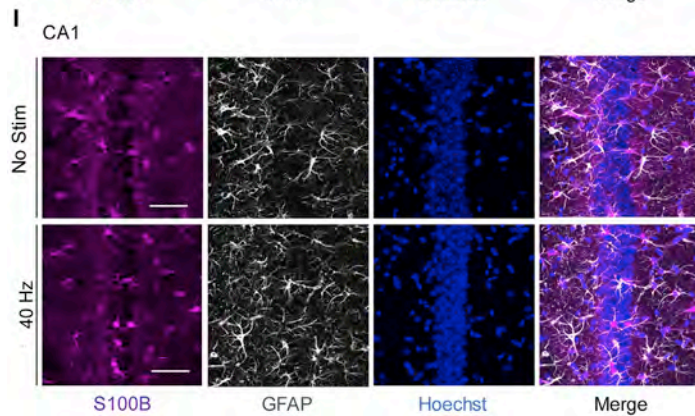
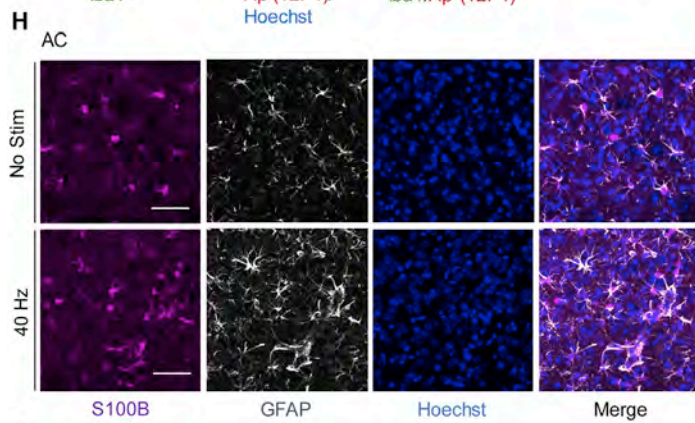
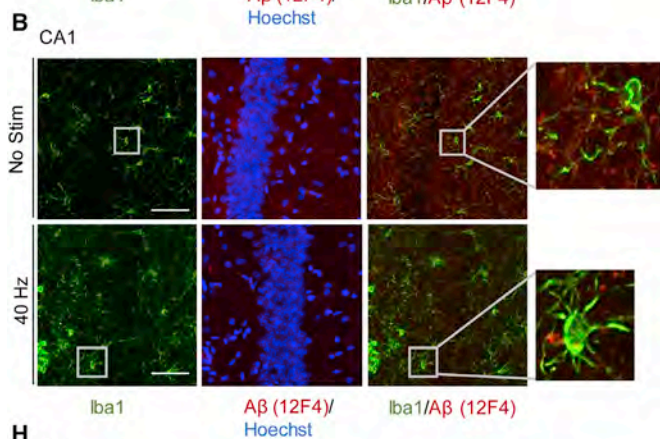
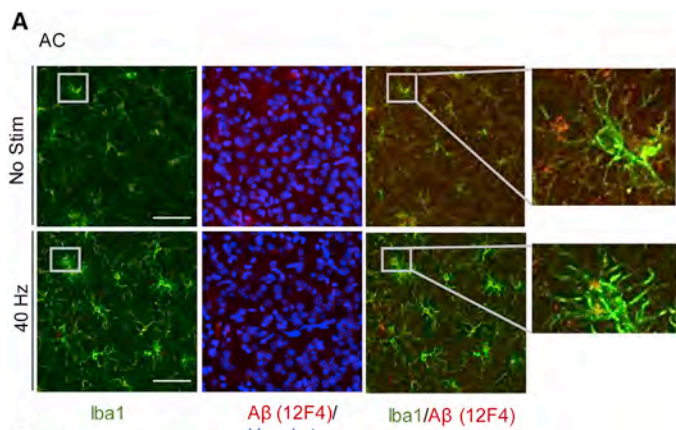
(F) Average area of Aβ-positive plaques in AC and CA1 (n = 7 mice/group, **p < 0.01, ***p < 0.001; unpaired MW test).

(G) Immunohistochemistry with anti-Aβ (12F4, red) antibody in 6-month-old AC of 5XFAD mice after auditory GENUS or no stimulation, for 1 week (scale bar, 50 μm).

(H) As in (G) for CA1.

(I) Aβ (12F4) mean intensity value (12F4 antibody) normalized to non-stimulated controls (n = 7 mice/group, ****p < 0.0001, unpaired MW test). Circles indicate "n," mean ± SEM in bar graphs unless otherwise noted, n.s., not significant.

See also [Figures S3](#) and [S5](#) and [Table S1](#).



(legend on next page)

GENUS may elicit increased clearance of A β through microglia and changes in the vasculature.

Auditory GENUS Reduces Tau Phosphorylation and Seeding in AC and Hippocampus

Tau phosphorylation at specific amino acid residues is a classical AD hallmark, and we previously showed that visual GENUS reduced tau hyperphosphorylation in another AD-associated mouse model, Tau P301S mice (Yoshiyama et al., 2007). We similarly investigated the effects of auditory GENUS by immunostaining (Figures S5A, S5B, S5D, and S5E) and saw reduced tau phosphorylation at threonine-181 (T181) (AC: 36.20% \pm 2.828%; CA1: 38.70% \pm 2.737%) and serine-396 (S396) (AC: 37.90% \pm 3.469%; CA1: 40.80% \pm 4.528%), versus no stimulation controls (Figures S5C and S5F). Tau phosphorylation at S396 was confirmed by western blot (WB) (AC: 33.83% \pm 0.20%; whole HPC: 43.20% \pm 1.50%) (Figures S5G, S5H, S5J, and S5K). WB analysis confirmed reduction in tau T181 phosphorylation in HPC (34.50% \pm 1.61%), although the difference was not significant in AC (AC data not shown) (Figures S5I and S5L). We performed a tau seeding assay to investigate effects of auditory GENUS on proteopathic tau propagation and observed significantly decreased average change in fluorescence resonance energy transfer (FRET) signal (STAR Methods) (Holmes et al., 2014) in AC (72.5% \pm 9.2%) and HPC (77.5% \pm 5.6%) (Figure S5M). Overall, our results show that auditory GENUS can reduce the levels of hyperphosphorylated tau epitopes and seeding in a tauopathy mouse model.

Combined Auditory and Visual GENUS Induces a Clustering Phenotype Response by Microglia

Having shown that GENUS can be applied through both visual (Iaccarino et al., 2016) and auditory stimulation, we next aimed to investigate if a combination of 40 Hz auditory tone stimulation with 40 Hz light flicker (combined GENUS) could entrain neural responses in AC, CA1, and mPFC and possibly have stronger effects than either sensory modality alone. We presented 3- to 8-month-old male WT (C57BL6J) mice with 1 ms-long auditory tones coupled with 12.5 ms-long light pulses (auditory plus visual, or A+V, stimulation) at 40 Hz while recording neural activity in AC, CA1, or mPFC using 32-channel silicon probes as animals ran or rested on a spherical treadmill (STAR Methods). Single unit

firing rate increased and decreased periodically with each tone and light-on period, thus entraining to 40 Hz during combined GENUS (Figures 6A–6C, left). Across AC, CA1, and mPFC, vector strength distributions were significantly higher to 40 Hz A+V, illustrating entrained spiking of single neurons, compared to random and no stimulation periods (Figures 6A–6C, right). We observed elevated power in the LFP at 40 Hz in AC, CA1, and mPFC during 40 Hz A+V stimulation (Figures S6A, S6H, and S6O). Although the increase in LFP power was very small in mPFC, the median distribution of mean firing rate differences during A+V stimulation, compared to no stimulation, differed significantly from zero (Figures S6O and S6R) whereas neither effects were seen in mPFC with auditory GENUS alone (Figures S1P and S1R). Thus, combined tone plus light stimulation at 40 Hz induced GENUS in AC, CA1, and mPFC. Significant entrainment was also observed in all three regions with 20 Hz, 80 Hz, and random frequency A+V stimulation, although the latter did not induce periodic firing modulation (Figures S6B–S6G, S6I–S6N, and S6P–S6U).

We next examined the morphological features of microglia and their interactions with A β in AC, VC, CA1, and mPFC in response to combined (A+V) GENUS (Figures 6D and 6E). Microglia exhibited a significant increase in number and soma area while projection length significantly decreased following combined GENUS versus no stimulation controls (Figures 6F–6H). With auditory or visual GENUS alone, microglia displayed enlarged soma area with reduced projection length and increased number in AC, VC, and CA1, but not in mPFC (Figures S7A–S7E and S7G–S7I). Additionally, we observed a unique encapsulating effect by microglia around amyloid deposits after combined GENUS. We applied IMARIS to quantify the number of microglia within a 25 μ m radius of an amyloid deposit from three-dimensional renderings of AC, VC, CA1, and mPFC images following combined GENUS versus no stimulation controls (Figures 6D and 6E, far right inset; Videos S1 and S2; STAR Methods). We observed a significant increase in the number of microglia per plaque in AC (48.88% \pm 0.651%), VC (31.56% \pm 1.11%), and mPFC (38.64% \pm 0.959%) (Figure 6I), and a non-significant increase in CA1 (33.05% \pm 2.65%). In contrast, auditory or visual GENUS alone had no significant effect on the number of microglia per plaque versus no stimulation controls in any brain region (Figures S7F and S7J). Overall, our findings show that combined

Figure 4. Auditory GENUS Induces Glial Response in AC and CA1 in 5XFAD Mice

(A) Immunohistochemistry with anti-Iba1 (019-19741, green) and anti-A β (12F4, red) antibodies in AC of 5XFAD mice after 7 days of 1 h/day (1 week) no stimulation or auditory GENUS (n = 8 mice/group; scale bar, 50 μ m).

(B) As in (A) for CA1.

(C) Number of Iba1-positive microglia in AC and CA1 (n = 8 mice/group, *p < 0.05; unpaired MW test).

(D) Diameter of Iba1-positive microglia cell bodies in AC and CA1 normalized to non-stimulated controls (n = 8 mice/group, ****p < 0.0001; unpaired MW test).

(E) Average length of Iba1-positive microglia primary processes in AC and CA1 normalized to non-stimulated controls (n = 8 mice/group, ****p < 0.0001; unpaired MW test).

(F) Average processes arborization of Iba1-positive microglia in AC and CA1 normalized to non-stimulated controls (n = 8 mice/group, *p < 0.05, **p < 0.01; unpaired MW test).

(G) Percentage of Iba1-positive microglia cell bodies that are also A β -positive in AC and CA1 (n = 8 mice/group, **p < 0.01, ***p < 0.001; unpaired MW test).

(H) Immunohistochemistry with anti-S100B (ab868, purple) and anti-GFAP (ab4674, gray) antibodies in AC of 5XFAD mice after 1 week no stimulation or auditory GENUS (n = 8 per group; scale bar, 50 μ m).

(I) As in (H) for CA1.

(J) Number of S100B-positive astrocytes in AC and CA1 (n = 8 mice/group, *p < 0.05; unpaired MW test).

(K) As in (J) for GFAP-positive astrocytes. Circles indicate "n," mean \pm SEM in bar graphs unless otherwise noted, n.s., not significant.

See also Figure S4.

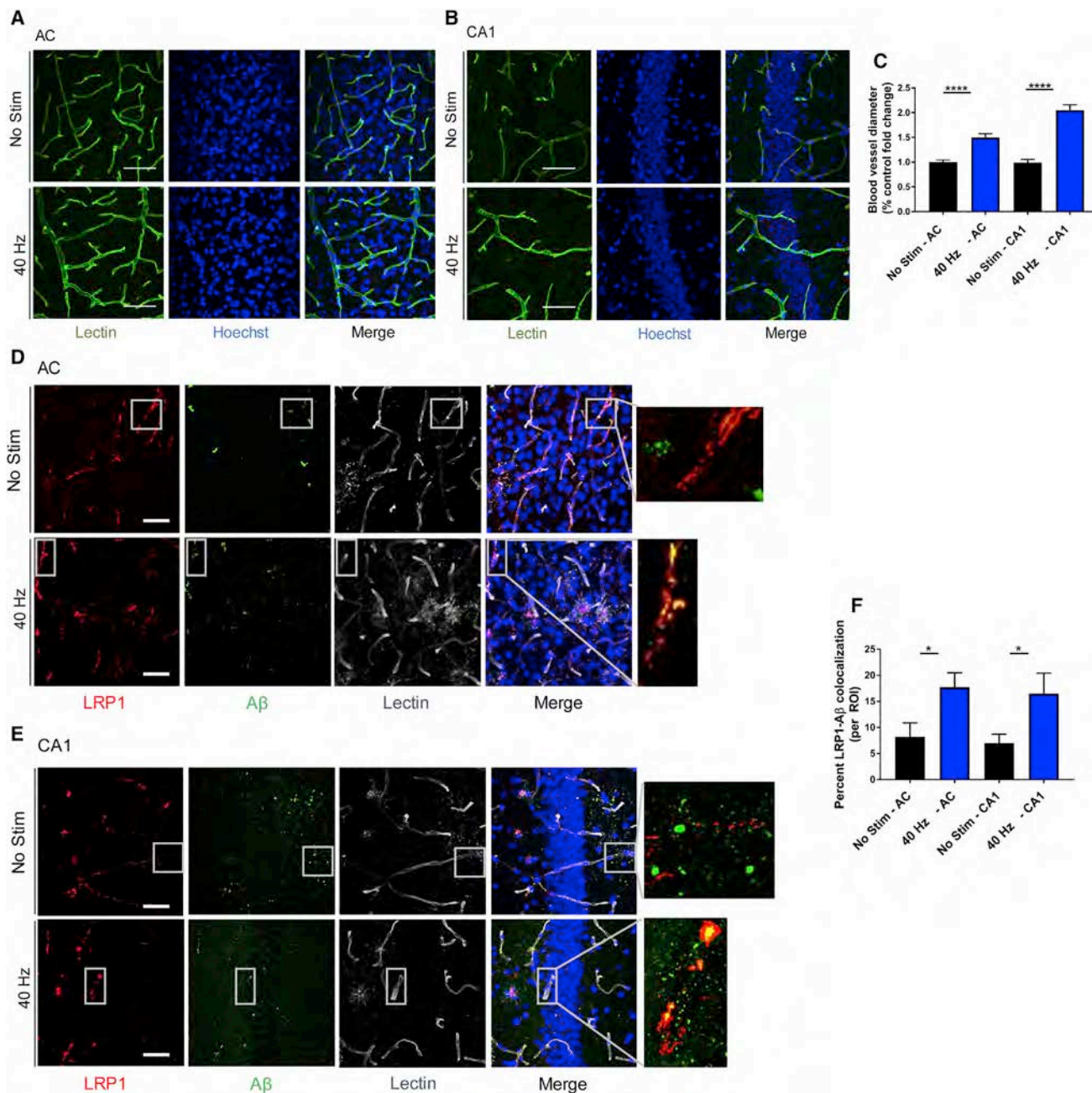


Figure 5. Auditory GENUS Increases Amyloid-Vasculature Associations

(A) Immunohistochemistry with lectin stain (DL-1174, green) in AC of 6-month-old 5XFAD mice after 7 days of 1 h/day (1 week) no stimulation or auditory GENUS (scale bar, 50 μ m).

(B) As in (A) for CA1.

(C) Percent fold change in blood vessel diameter in AC and CA1 of 6-month-old 5XFAD mice after 1 week no stimulation or auditory GENUS, normalized to no stimulation control (n = 7 mice/group, ****p < 0.0001; unpaired MW test).

(D) Immunohistochemistry with anti-LRP1 (28320, red), anti-A β (AB9234, green), and lectin stain (DL-1174, gray) antibodies in AC of 6-month-old 5XFAD mice after 1-week no stimulation or auditory GENUS (n = 8 mice/group, scale bar, 50 μ m).

(E) As in (D) for CA1.

(F) Percentage of A β -LRP1 co-localization in AC and CA1 of 5XFAD mice after 1 week no stimulation or auditory GENUS (n = 8 mice/group, *p < 0.05; unpaired MW test). Circles indicate "n," mean \pm SEM in bar graphs unless otherwise noted, n.s., not significant.

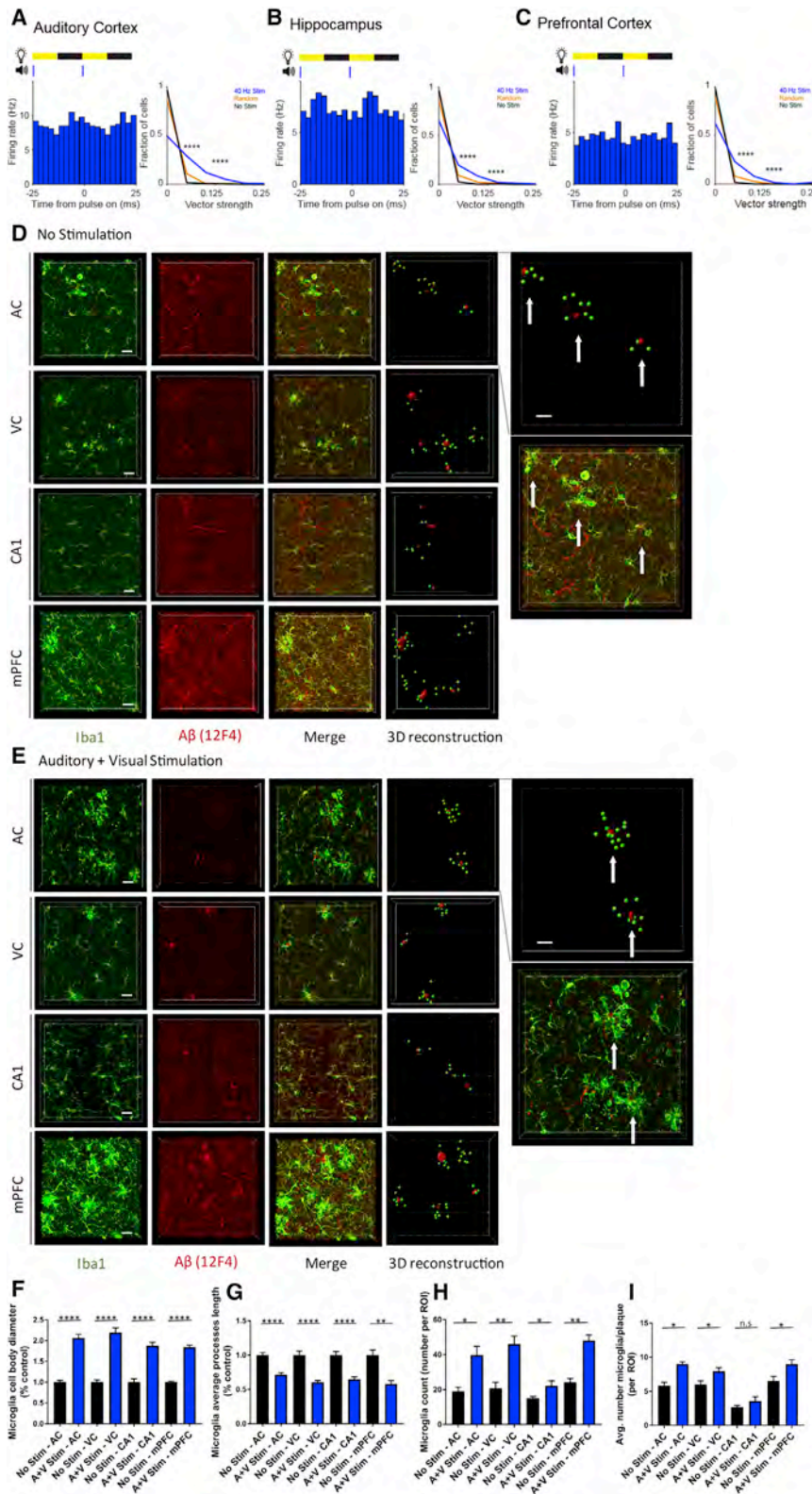


Figure 6. Combined Auditory and Visual GENUS Induces a Clustering Phenotype Response by Microglia

(A) Firing rate modulation of a single unit during 40 Hz audio-visual stimulation (left). Vector strength of responses to 40 Hz A+V stimulation, random A+V stimulation, and no stimulation periods (right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 9 units had 40 Hz stim VS values >0.25 ; 3 units had random stim VS values >0.25). In all statistical tests for (A)–(C), results are significant after controlling for multiple comparisons using the Bonferroni correction unless otherwise stated.

(B) Same as (A) for CA1 (right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 8 units and 3 units had VS values >0.25 for 40 Hz or random stim, respectively).

(C) Same as (A) for mPFC (right, **** $p < 0.00005$ 40 Hz versus no stim, 40 Hz versus random; Kolmogorov-Smirnov test; 5 units had 40 Hz stim VS values >0.25).

(D) Immunohistochemistry and 3D reconstruction using IMARIS (STAR Methods) of anti-Iba1 (019-19741, green) and anti-Aβ (12F4, red) antibodies in AC, VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h/day (1 week) of no stimulation ($n = 6$ mice/group, top inset: example of using IMARIS to quantify the number of microglia surrounding a 25 μm radius around amyloid plaques). Plaques as red dots, microglia as green dots, and white arrows point to clusters. Bottom inset: enlarged merged image from AC. Scale bar, 20 μm.

(E) As in (D) for combined GENUS.

(F) Average microglia cell body diameter in AC, VC, CA1, and mPFC of 6-month-old 5XFAD mice after 1 week no stimulation or combined GENUS, normalized to no stimulation control ($n = 6$ mice no stim, $n = 7$ mice combined GENUS, **** $p < 0.0001$; unpaired MW test).

(G) Average microglia process length in AC, VC, CA1, and mPFC of 6-month-old 5XFAD mice after 1 week no stimulation or combined GENUS, normalized to no stimulation control ($n = 6$ mice no stim, $n = 7$ mice combined GENUS, ** $p < 0.01$, **** $p < 0.0001$; unpaired MW test).

(H) Microglia count per region of interest in AC, VC, CA1, and mPFC of 6-month-old 5XFAD mice after 1 week no stimulation or combined GENUS ($n = 6$ mice no stim, $n = 7$ mice combined GENUS, * $p < 0.05$, ** $p < 0.01$, unpaired MW test).

(I) Average number of microglia surrounding 25 μm radius of a plaque in AC, VC, CA1, and mPFC following no stimulation or combined GENUS ($n = 6$ mice/group, * $p < 0.05$; unpaired MW test). Circles indicate “n,” mean \pm SEM in bar graphs unless otherwise noted, n.s., not significant.

See also Figures S6 and S7 and Videos S1 and S2.

GENUS elicits a unique microglia response that extends to mPFC, and is not seen with auditory or visual stimulation alone.

We next addressed the frequency-specificity of microglia response in AC, CA1, and mPFC using auditory GENUS, combined GENUS, 80 Hz A+V, or random frequency A+V stimulation. We observed significant increases in microglia number and cell body diameter with decreased average process length in AC and CA1 following auditory and combined GENUS, but not auditory or A+V stimulation at other frequencies, compared to no stimulation controls. Only combined GENUS elicited a microglia response in mPFC, but not auditory GENUS, nor auditory or A+V stimulation at other frequencies, versus no stimulation controls (Figures S7K–S7M). We further examined the frequency-specificity of astrocyte reactivity in AC and CA1 using auditory GENUS, combined GENUS, 80 Hz A+V, or random frequency A+V stimulation. Auditory GENUS increased the number of reactive-like astrocytes in both areas, as before (Figures 4H–4K). Combined GENUS, but not other frequency A+V groups, elicited a significant increase GFAP-positive astrocytes in AC ($63.7\% \pm 4.3\%$) and non-significant increase in S100B-positive astrocytes ($27.8\% \pm 2.1\%$) (Figures S7N and S7O). We observed a non-significant increase in reactive-like astrocytes in CA1 (GFAP: $17.2\% \pm 2.1\%$; S100B: $21.6\% \pm 2.8\%$) after combined GENUS, but not other frequency A+V groups, versus no stimulation controls (Figure S7O).

Taken together, these results demonstrate that auditory and combined GENUS elicit profound glial responses in primary sensory cortices and HPC, with the latter further impacting mPFC. Moreover, these effects are specific to A+V stimulation at 40 Hz.

Concurrent Auditory and Visual GENUS, but Not Auditory or Visual Alone, Reduces Amyloid Load in the mPFC

Our observation of a microglial response in AC, VC, CA1, and mPFC led us to investigate whether combined GENUS could change amyloid levels in those regions. Immunostaining for A β (D54D2) revealed reduced plaque area (AC: $56.34\% \pm 6.3\%$; VC: $71.50\% \pm 6.5\%$; CA1: $69.73\% \pm 6.4\%$) and number (AC: $50.02\% \pm 3.7\%$; VC: $50.60\% \pm 10.9\%$; CA1: $48.80\% \pm 11.1\%$) following combined GENUS, versus no stimulation controls. Combined GENUS uniquely reduced plaque area ($58.39\% \pm 11.7\%$) and number ($58.82\% \pm 7.67\%$) in mPFC (Figures 7A–7D), whereas neither auditory nor visual GENUS alone affected amyloid in mPFC (Figures S7P–S7U). Multi-comparisons between frequencies and between stimuli modalities in the same dataset showed that combined GENUS had significant effects in the mPFC when compared to auditory or visual GENUS alone, and summed auditory and visual stimulations (Figures S7Y and S7Z; Table S1). Amyloid reduction in mPFC is frequency-specific, as we observed no significant decreases in amyloid plaque area nor number with 80 Hz A+V or random A+V stimulation (Figures 7C and D). We carried out A β -ELISA to support our immunostaining results and found that combined GENUS reduced A β_{1-42} levels in AC, HPC, as well as mPFC (soluble A β_{1-42} : $59.58\% \pm 7.2\%$; insoluble A β_{1-42} : $34.17\% \pm 8.2\%$) (Figures 7E and F) that was not affected by auditory GENUS alone (Figures S7V and S7W). Additionally, A+V stimulation at 8 Hz and random frequency did not impact A β_{1-42} levels in AC, HPC, and mPFC, compared to no stimulation

controls (Figures S7V and S7W). Immunostaining for A β_{1-42} (12F4) showed a unique reduction in amyloid in mPFC after combined GENUS (but not auditory GENUS alone), and no effect of 80 Hz A+V or random frequency A+V stimulation across all three brain regions, compared to their respective no stimulation controls (Figure S7X). In sum, these results demonstrate that combined GENUS uniquely reduces amyloid in mPFC (in contrast to auditory or visual GENUS alone), and this effect is specific to A+V stimulation at 40 Hz.

To gauge how combined GENUS impacts amyloid plaque abundance on a larger scale, we next performed whole brain SHIELD processing in 6-month-old 5XFAD mice followed by immunostaining for β -amyloid (D54D2) (STAR Methods). Analysis of amyloid plaques in neocortex revealed significant reductions in total plaque volume (37%) and number (34%) after 1 week of combined GENUS versus no stimulation controls (Figures 7G–7J; Videos S3 and S4). These results suggest that combined GENUS may be able to effect widespread reductions of amyloid across broad cortical regions.

DISCUSSION

In this study, we applied the GENUS approach with auditory tones repeating at 25-ms intervals (40 Hz) to effect gamma entrainment and reduce AD pathology in brain regions beyond primary sensory cortex. A key result of auditory GENUS was improved hippocampal-dependent recognition and spatial memory tasks in 5XFAD mice. Alongside significant reductions in amyloid load in AC and HPC, we also observed responses in microglia, astrocytes, and vasculature. Additionally, we show that combined auditory and visual GENUS had unique effects from either sensory modality alone, and reduced amyloid in mPFC and broader cortical regions.

Slow auditory click-train stimuli can elicit a synchronized, phase-locked spiking response from AC neurons although each click becomes more modulatory as the frequency increases (Ma et al., 2013). Consistent with these results, we found that AC neurons entrain to tones repeating at 20 Hz, 40 Hz, and 80 Hz, and for faster frequencies neurons fire in response to a smaller fraction and at a wider range of phases relative to individual tones. Although there is extensive evidence that CA1 and mPFC neurons can respond to sensory cues including auditory stimuli (Aronov et al., 2017; Mears et al., 2006; Miller and Freedman, 1995), we show for the first time that 40 Hz auditory, or A+V, stimulation elicits small but significant firing rate entrainment at 40 Hz in these brain regions (Figures 1B, 1H, 1N, and 6A–6C). As in AC, single units in CA1 and mPFC show modulation, meaning the firing rate changed as a function of stimulus phase, although they do not fire in response to every pulse. Weaker spiking modulation may be expected in HPC and mPFC as sensory inputs reach these regions through multiple indirect pathways that likely low-pass filter spiking entrainment.

Studies of rate coding show that neurons can encode click trains using firing rate without synchronizing to the stimulus (Lu et al., 2001; Ma et al., 2013). In each brain region, we found that some neurons fire at a different rate depending on the frequency of the auditory train, although the population as a whole did not fire more or less to the different stimulation frequencies

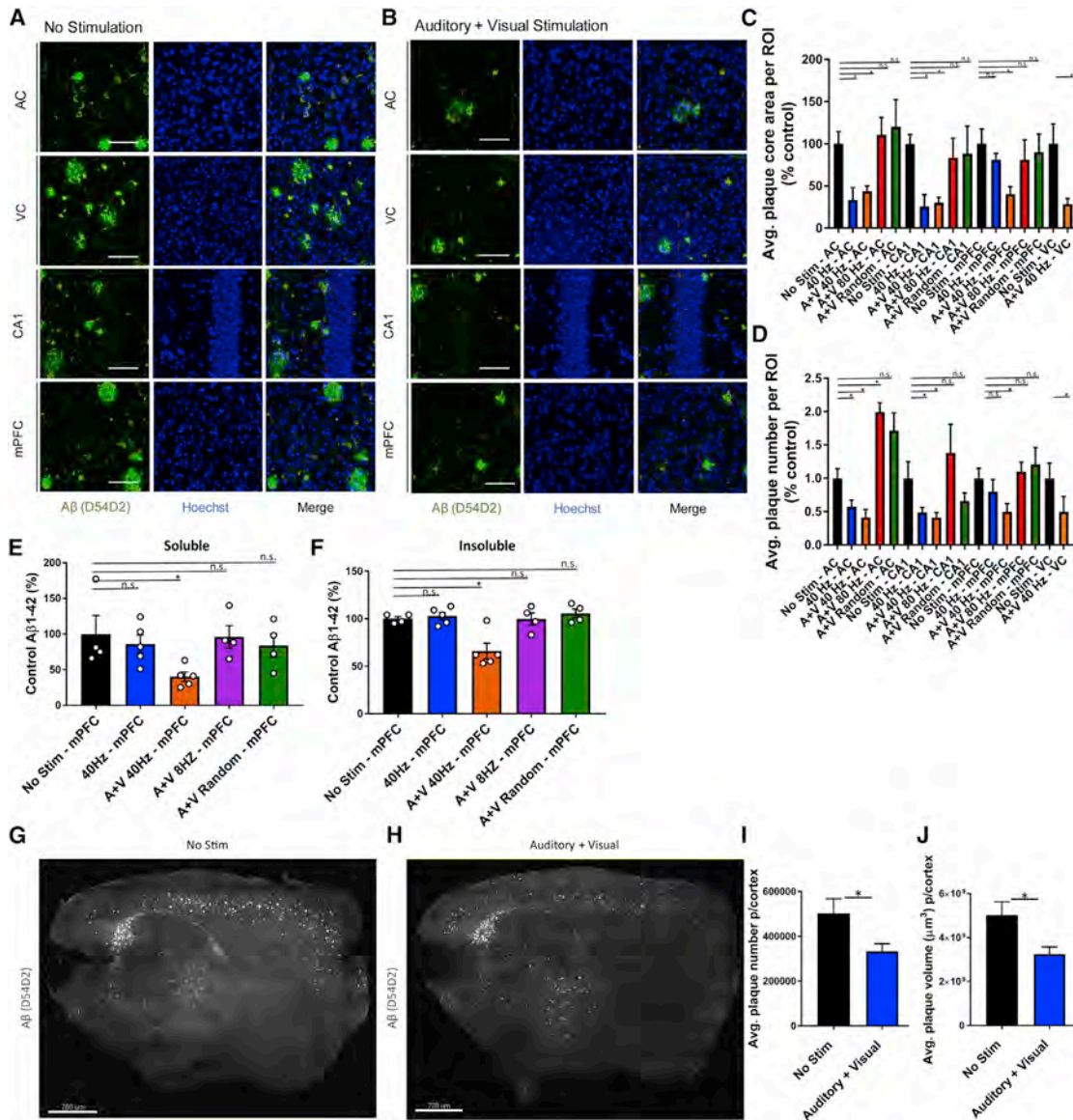


Figure 7. Combined Auditory and Visual GENUS Reduces Amyloid Load in the mPFC and Neocortex

(A) Immunohistochemistry of anti-Aβ plaques (D54D2, green) antibodies in AC, VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h/day (1 week) no stimulation (40× objective; scale bar, 50 μm).

(B) As in (A) for combined GENUS.

(C) Average plaque core area in AC, CA1, mPFC, and VC in 6-month-old 5XFAD mice following 1 week no stimulation, 40 Hz auditory stimulation, combined GENUS, combined 80 Hz, and combined random frequency stimulation, normalized to no stimulation control (n = 12 mice/group, *p < 0.05, Kruskal-Wallis [KW] test with Dunn's multiple comparison test).

(D) Average plaque number in AC, CA1, mPFC, and VC in 6-month-old 5XFAD mice following 1 week no stimulation, 40 Hz auditory stimulation, combined GENUS, combined 80 Hz, and combined random frequency stimulation, normalized to no stimulation control (n = 12 mice/group, *p < 0.05, KW test with Dunn's multiple comparison test).

(E) Relative soluble Aβ₁₋₄₂ levels in mPFC of 6-month-old 5XFAD mice following 1 week 40 Hz auditory stimulation, combined GENUS, combined 8 Hz, or combined random frequency stimulation, normalized to non-stimulation control (n = 4–5 mice per group, *p < 0.05, KW-test with Dunn's multiple comparison test).

(F) As in (E) for insoluble Aβ₁₋₄₂ (*p < 0.05).

(G) Immunohistochemistry of SHIELD-treated whole brain (sagittal plane of 25-μm section of brain) of anti-Aβ plaques (D54D2, white) antibodies of 6-month-old 5XFAD mice after 1 week no stimulation (light-sheet microscope; scale bar, 700 μm).

(H) As in (G) for combined GENUS.

(I) Average cortical plaque number following no stimulation or combined GENUS (n = 6 mice/group, *p < 0.05; unpaired MW test).

(J) Average cortical plaque volume (μm³) following combined GENUS (n = 6 mice/group, *p < 0.05; unpaired MW test). Circles indicate "n," mean ± SEM in bar graphs unless otherwise noted, n.s., not significant.

(Figures 1F, 1L, 1R, S6D, S6K, and S6R). Therefore, we conclude that our observed changes—in microglia, astrocytes, vasculature, and amyloid levels, as well as behavioral performance—in response to 40 Hz auditory stimulation, but not other frequencies, cannot be explained by overall changes in firing rate.

Microglia display an array of responses, ranging from neurotoxic inflammation to phagocytic to neuroprotective, and their dysfunction is known in AD (Heneka et al., 2014; Wolf et al., 2017). Building on our previous study using visual stimuli (Iaccarino et al., 2016), we show here that auditory GENUS induces a microglia morphological response, indicative of increased A β -uptake, in primary sensory cortex as well as HPC (Figures 4A and 4B). Combining auditory and visual GENUS elicited unique effects of microglia clustering around amyloid plaques, and impacted mPFC in addition to AC and HPC (Figures 6D, 6E, and 6I). Our results also indicate a range in microglial response that can be elicited by GENUS (Ransohoff and Cardona, 2010) and is supported by accumulating evidence that microglia are heterogeneous and display a spectrum of activated states (Walker and Lue, 2015; Mathys et al., 2017; Keren-Shaul et al., 2017).

We further investigated effects of GENUS on astrocytes, another primary glial cell type, and found an increase in astrocytes that were positive for markers of reactivity, such as GFAP and S100B (Figures 4H–4K, S7N, and S7O). Astrocytes are known to be intermediaries between neuronal activity and vasculature (Attwell et al., 2010; Filosa and Iddings, 2013). Indeed, we observed an increase in vascular dilation following auditory GENUS (Figures 5A–5C). Significantly, mice were sacrificed and analyzed 24 h post-stimulus, suggesting that vascular changes following GENUS may not be solely due to immediate stimulus response. Vascular clearance, such as through LRP1, may be an additional route for reduced amyloid levels in the brain and, consistent with this hypothesis, we observed increased A β -LRP1 co-localization following auditory GENUS (Figures 5D–5F).

Our results indicating increased A β -uptake by microglia, vascular-dilation response, and potential amyloid transvascular transport all suggest an enhancement in A β clearance by auditory GENUS through multiple mechanisms. An intriguing finding is that combined GENUS reduced amyloid load uniquely in mPFC (Figures 7A–7F) compared to auditory or visual GENUS alone and summed auditory and visual stimulations (Figures S7Y and S7Z; Table S1). Whole brain SHIELD revealed a reduction in amyloid load across the whole cortex following combined GENUS versus no stimulation controls (Figures 7G–7J) and suggests the potential for combined GENUS to ameliorate AD-like pathology spanning a larger network. However, it remains to be seen if the effects of multi-sensory GENUS on A β are synergistic or simply additive through individual sensory modalities.

A major finding from our study is the improvement of hippocampus-dependent cognitive function in spatial and recognition memory tasks in AD model mice following auditory GENUS (Figure 2). We observed no effects on these tasks in WT mice (Figure S8). Together with our previous study (Iaccarino et al., 2016), our overall findings suggest that GENUS elicits effects across multiple cell types—neurons, microglia, astrocytes, and vasculature—and brain regions to drive the attenuation of AD-related pathology in a circuit-wide manner that is consistent with

improved cognitive function. It remains unknown how GENUS relates to endogenous gamma and, for example, whether GENUS affects the capacity for information transmission and sensory processing between neuronal systems (Fries, 2009; Knoblich et al., 2010). Importantly, because our experiments were performed in AD mouse models, future studies are essential to determine whether the benefits of our non-invasive GENUS approach is translatable to humans.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found with this article online at <https://doi.org/10.1016/j.cell.2019.02.014>.

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AUTHOR CONTRIBUTIONS

A.J.M., A.C.S., A.L.P., J.Z.Y., E.N.B., E.S.B., K.C., and L.-H.T. designed experiments. A.C.S. and A.L.P. performed and analyzed electrophysiology data. S.M.P. assisted with electrophysiology experiments. A.J.M. and D.N.-W.K. performed and analyzed ELISA experiments. O.K. performed western blots. A.J.M. performed behavior experiments. D.N.-W.K. and F.A. analyzed behavior experiments. A.J.M., D.N.-W.K., and F.A. performed and analyzed imaging experiments. F.A. performed and analyzed 3D microglia clustering quantification. G.T.D., W.G., and V.M. performed and analyzed whole brain SHIELD experiments. A.J.M., D.N.-W.K., and F.A. performed auditory and combined GENUS experiments. H.-J.S. and A.C.S. engineered auditory and combined GENUS sounds and equipment. A.J.M., A.C.S., A.L.P., J.Z.Y., F.A., and L.-H.T. wrote the manuscript. All authors read and revised the manuscript.

DECLARATION OF INTERESTS

L.-H.T. and E.S.B. are co-Scientific Founders and serve on the scientific advisory board of Cognito Therapeutics, Inc. A.C.S. owns shares of Cognito Therapeutics, Inc.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
β -Amyloid (D54D2) XP [®] Rabbit mAb	Cell Signaling Technology	Cat#: 8243
anti-Iba1	Wako Chemicals	Cat#: 019-19741
anti-gliial fibrillary acidic protein (GFAP)	Abcam	Cat#: ab4674
anti-S100B	Abcam	Cat#: ab868
anti-LRP1	Abcam	Cat#: 28320
DyLight 488 labeled Lycopersicon Esculentum (tomato) lectin	Vector laboratories	Cat#: DL-1174
anti-amyloid oligomer	Millipore Sigma	Cat#: AB9234
anti-phospho-tau (Ser396)	Cell Signaling Technology	Cat#: 9632
anti-phospho-tau (Thr181)	Cell Signaling Technology	Cat#: 12885
Total tau (1:1000)	Santa Cruz Biotechnology	Cat#: sc-5587, H-150
Hoechst 33342	Thermo Fisher Scientific	Cat#: H3570
Purified anti- β -Amyloid, 1-42 (12F4) Mouse	Biolegend	Cat#: 805501
Cy2-AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat#: 715-225-150
Cy3-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	Cat#: 711-165-152
Cy5-AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	Cat#: 715-175-150
Alexa Fluor [®] 594 AffiniPure Donkey Anti-Mouse IgG	Thermo Fisher Scientific	Cat#: A-11032
Alexa Fluor [®] 647 AffiniPure Donkey Anti-Rabbit IgG	Thermo Fisher Scientific	Cat#: A-31573
Alexa Fluor [®] 633 AffiniPure Goat Anti- Chicken IgG	Invitrogen	Cat#: A-21103
IRDye 800CW	LI-COR	N/A
IRDye 680RD	LI-COR	N/A
IRDye 680LT	LI-COR	N/A
Chemicals, Peptides, and Recombinant Proteins		
Paraformaldehyde	Electron Microscopy Sciences	Cat#: 15714S
Triton X-100	Fischer Biotech	Cat#: BP151-100
Normal donkey serum	EMD Millipore Corp	Cat#: S30-100ML
Phosphate buffered saline (PBS)	Sigma Life Sciences	Cat#: P5368-10PAK
Fluoromount G Mounting Medium	Electron Microscopy Sciences	N/A
Sodium azide	Millipore Sigma	Cat#: S2002
Glutaraldehyde	Electron Microscopy Sciences	Cat#: 16110
Sodium dodecylsulfate	Sigma-Aldrich	Cat#: L3771
Lithium hydroxide monohydrate	Sigma-Aldrich	Cat#: 254274-50G
Boric acid	Sigma-Aldrich	Cat#: B7901
Diatrizoic acid	Sigma-Aldrich	Cat#: D9268
Iodixanol	Sigma-Aldrich	Cat#: D1556
N-methyl-d-glucamine	Sigma-Aldrich	Cat#: M2004
Glycine	Sigma Aldrich	Cat#: G7126
Sodium hydroxide	Sigma Aldrich	Cat#: 795429
Bovine serum albumin	Gemini Bio-Products	Cat#: 700-100P
KH ₂ PO ₄	Mallinckrodt Chemicals	Cat#: 7100-12
Na ₂ HPO ₄	Sigma-Aldrich	Cat#: S7907
Acrylamide 40% solution	Biorad	Cat#: 161-0140
Histodenz	Sigma-Aldrich	Cat#: D2158-100G

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Amyloid beta 40 Human ELISA kit	Thermo Fisher Scientific	Cat#: KHB3481
Amyloid beta 42 Human ELISA kit	Thermo Fisher Scientific	Cat#: KHB3442
Experimental Models: Organisms/Strains		
Mouse: 5XFAD: B6SJL-Tg(APPswFLon,PSEN1* ^{M146L} * ^{L286V})6799Vas/Mmjax	Jackson Laboratory	Stock No: 34840-JAX
Mouse: P301S tau-tg: B6;C3-Tg(Prnp-MAPT* ^{P301S})PS19Vle/J	Jackson Laboratory	Stock No: 008169
Mouse: APP/PS1	Gift from Susumu Tonegawa lab, MIT	N/A
Software and Algorithms		
MATLAB R2017b	Mathworks Inc.	Natick, Massachusetts, United States
Imarisx64 8.3.1	Bitplane	Zurich, Switzerland
ImageJ	N/A	https://imagej.nih.gov/ij/download.html
TSEsystem	TSE Systems	tse-systems.com
Zen Black 2.1	Carl Zeiss Microscopy	Jena, Germany
GraphPad Prism	GraphPad	graphpad.com

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead Contact, Li-Huei Tsai (lhtsai@mit.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animal work was approved by the Committee for Animal Care of the Division of Comparative Medicine at the Massachusetts Institute of Technology and by the Institutional Animal Care and Use Committee at Georgia Institute of Technology. Mice were housed in groups no larger than five on a standard 12-h light/12-h dark cycle; all experiments were performed during the light cycle. Electrophysiology experiments were performed at Georgia Institute of Technology, male (1-3 month-old) WT mice (C57BL/6) were obtained from the Jackson laboratory. Mice were housed on a reverse 12 h light/12 h dark cycle and all experiments were performed during the dark cycle. Food and water were provided without restriction. For all experiments, mice from the same litter were divided into different conditions, respectfully. If additional groups were added, respective controls were always repeated concurrently.

METHOD DETAILS

Surgical procedures

Adult (2-3 month-old) mice were anesthetized with isoflurane and fixed in a stereotaxic frame. Ophthalmic ointment (Puralube Vet Ointment, Dechra) was applied to the eyes, and the scalp was shaved and sterilized with povidone-iodine (Dynarex) and 70% ethanol. A custom stainless steel headplate was fixed using dental cement (C&B Metabond, Parkell) and the target craniotomy site for LFP recordings was marked on the skull (in mm, from bregma: -2.0 anterior/posterior, ± 1.8 medial/lateral for targeting CA1, -2.0 to -3.0 anterior/posterior, ± 1.8 medial/lateral for targeting auditory cortex, and $+1.3$ to $+1.4$ anterior/posterior, ± 1.0 medial/lateral for targeting prefrontal cortex). A craniotomy was later performed in 3-8 month-old mice. The day before or day of the first recording session, craniotomies (200-500 μm diameter) were made by thinning the skull with a dental drill and then making a hole with a 27-gauge needle. When not recording, the craniotomy was sealed with a sterile silicon elastomer (Kwik-Sil WPI).

Electrophysiology recordings

During recordings, head-fixed animals ran on an air-floating 8-inch spherical treadmill. All animals had previously learned to maneuver on the treadmill until they were comfortable while occasionally receiving sweetened condensed milk (1:2 water dilution). Animals were on the ball for a maximum of 5 hs and had multiple periods of running and rest during this time. Single shank 32-channel probes (NeuroNexus) were advanced to the target location. Recording sites spanned 250 μm . For auditory cortex recordings, the probe was advanced at a 45° angle from vertical parallel to the coronal plane to a depth of 3-4.15mm. A series of 50 ms tones of 5, 10, 15, and 20 kHz were presented to detect auditory response in the mean LFP. For CA1 recordings, the probe was advanced vertically through

the craniotomy to a depth of 1.14–2.05mm until hippocampal pyramidal layer electrophysiology characteristics were observed (large theta waves and sharp wave ripples, 150+ μ V spikes on multiple channels). For prefrontal cortex recordings, the probe was advanced at a 20° angle from vertical, at a 49° angle from the coronal plane to a depth of 1.48–2.15mm. If data were collected at multiple depths during the same recording session; new depths were mapped in order to ensure the location of the recording sites remained in the target location ($n = 9$ recording depths from 9 sessions in 5 mice for AC and 12 recording depths from 10 sessions in 5 mice for CA1, $n = 7$ recording depths from 7 sessions in 4 mice for mPFC). Data were acquired with a sampling rate of 20 kHz using an Intan RHD2000 Evaluation System using a ground pellet as reference.

Auditory and visual stimuli for electrophysiology recordings

Animals were presented with 10 s stimulation blocks interleaved with 10 s baseline periods. Stimulation blocks rotated between auditory-only or auditory and visual stimulation at 20 Hz, 40 Hz, 80 Hz, or with random stimulation (pulses were delivered with randomized inter-pulse intervals determined from a uniform distribution with an average interval of 25 ms). Stimuli blocks were interleaved to ensure the results observed were not due to changes over time in the neuronal response. 10 s long stimulus blocks were used to reduce the influence of onset effects, and to examine neural responses to prolonged rhythmic stimulation. All auditory pulses were 1 ms-long 10 kHz tones. All visual pulses were 50% duty cycle of the stimulation frequency (25 ms, 12.5 ms, or 6.25 ms in length). For combined stimulation, auditory and visual pulses were aligned to the onset of each pulse.

Prefrontal cortex histology

During the final mPFC recording in each animal, the probe was coated with Dil (Thermo Fisher) and inserted to target depth. Mice were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) under anesthesia (isoflurane), and the brains were post-fixed overnight in 4% paraformaldehyde in 1xPBS. Brains were sectioned 100 μ m thick with a Leica VT1000S vibratome (Leica). Sections were stained with 0.2% 1mMol DAPI (PanReac AppliChem) in 1xPBS and mounted onto microscopy slides with Vectashield mounting medium (VWR). Images were acquired on a Zeiss Axio Observer Z1 inverted epifluorescent microscope with the accompanying Zen Blue 2 software.

Spike sorting and single unit stability

Spike detection and sorting was carried out using MountainSort automated spike sorting followed by manual curation based on visual inspection of waveforms and cross-correlograms (Chung et al., 2017). Prior to manual curation, quality thresholds were applied to only include units with peak SNR greater than or equal to 1, less than 10% overlap with noise, and greater than 95% isolation against other units which resulted in well-isolated single units. To account for periods of instability in the recordings during which single units were lost, stability criteria were applied such that only stable periods (no sudden loss of a single unit's firing rate) would be considered in analysis. Firing rate (FR) for each unit was computed over the course of the recording session. Firing rate was clustered into two distributions, low FR and high FR, using k-means clustering. For units with FR that dropped below 10% of the high FR mean, further analyses identified a stable recording period defined as the longest length of time that the FR was 2 standard deviations above the low FR mean.

LFP

LFP was obtained by downsampling raw traces to 2kHz and bandpass filtering between 1–300Hz.

Power spectrum

Power spectral density analysis was performed using multitaper methods from the Chronux toolbox (time-bandwidth product = 3, number of tapers = 5). LFP traces were divided into 10 s trials of each stimulation condition. The average power spectral density was computed for each animal (within the same recording day and recording depth) over these trials, referencing to a ground pellet in saline above the skull. Power spectral density analysis was initially computed for all recording sites in AC, CA1, and mPFC. From each recording depth, the traces with the largest 40 Hz peak in response to 40 Hz flicker stimuli were included in the analysis. The per-depth traces displayed in the presented data had the largest 40 Hz peak in response to auditory flicker stimuli.

Firing during flicker stimulation

The single unit peri-stimulus time histograms (PSTH) for each stimulus frequency encompassed two stimulus cycles (where one cycle = $1/\text{stimulus frequency}$ sec), with 10 bins per cycle, to show spiking across trains of stimuli. Displaying spiking modulation over multiple cycles is typical for displaying modulation by oscillations (Csicsvari et al., 1999). PSTHs were computed for all single units by binning spikes for 1 stimulus cycles before and after the start of each light-on or audio-on pulse. No stimulation (baseline) histograms were calculated using randomly distributed pulse times, as in the random stimulation condition. Firing rate was computed in each bin by dividing the number of spikes per bin by the total time in that bin (the total number of pulses times the bin size). To quantify firing rate periodicity in relation to the stimulus frequency, the time interval between firing rate peaks was calculated for all single unit histograms. The peaks of each PSTH was the maximum firing rate within one stimulus interval. To quantify firing rate modulation by the stimulus and compute circular statistics, peri-stimulus spike times were converted into radians: $((\text{peri} - \text{stimulus spike time}) * 2\pi * (\text{stimulus frequency}))$ and vector strengths and Rayleigh statistics were computed. Vector strength was computed using methods from the CircStat toolbox; the Rayleigh statistic was computed using the equation $RS = 2nVS^2$, where n is total spike count, and VS is vector strength (Berens, 2009;

Ma et al., 2013). Differences in vector strength and Rayleigh statistic values were computed by taking the differences in these values between stimulus conditions for each unit. Heatmaps showing the firing rate response to flicker for all recorded single units were computed over four consecutive stimulus cycles. In order to show the response of all neurons, we show four consecutive stimulus cycles of each stimulation period. To do this, we aligned the 10 s presentation periods of each stimulus condition, and then excluded the first 100ms of each presentation period to prevent onset effects from obscuring entrainment. Then, we averaged spiking response over the next four stimulus cycles (200 ms for 20 Hz, 100 ms for 40 Hz, and 50 ms for 80 Hz) to obtain the firing rate response to flicker. Firing rate for each single unit was computed in 1 ms bins, smoothed with a Gaussian windows proportional to each stimulus frequency ($N = 1 / \text{Stimulus frequency sec}$, $\alpha = 3$), and z-scored. Neurons were aligned by their average stimulus phase preference in the analyzed four cycles.

Mean Firing Rate

Mean firing rate was computed for each single unit for each stimulus condition. Only stable periods for each unit contributed to the mean FR calculation (see Spike sorting and single unit stability, above). Difference in mean firing rate between stimulus conditions was computed within each unit by taking the difference in mean FR in each condition for that unit.

40 Hz visual flicker stimulation protocol

For biochemical and Immunohistochemical analysis, 5XFAD mice were placed in a dark chamber illuminated by a light-emitting diode (LED) bulb and were exposed to one of four stimulation conditions: dark, 8 Hz, 40 Hz (12.5 ms light on, 12.5 ms light off, 60 W), or random (light pulses were delivered with a random interval determined by a uniform distribution with a mean of 25 ms) stimulation for 1-h for seven days.

40 Hz auditory tone train stimulation protocol

For biochemical, Immunohistochemical, or behavioral analysis, 5XFAD, APP/PS1, or P301S mice were placed in a dimly lit chamber in a quiet room insulated with sound-proof foam (McMaster-Carr, 5692T49). Speakers (AYL, AC-48073) were placed out-of-reach from the mouse above the chambers. Mice were exposed to one of five stimulation conditions: no tones, tones at 8 Hz, tones at 40 Hz, tones at 80 Hz, or tone delivered at random (auditory tones were delivered with a random interval determined by a uniform distribution with a mean of 25ms) stimulation. Tones for the stimulation conditions consisted of a 10 kHz tone that was 1 ms in duration and delivered at 60 dB. For electrophysiology recordings, after probe placement, the lights in the room were turned off and the animals were presented with alternating 10 s periods of audio-only and visual-audio stimulation interleaved with 10 s periods of no light or tones. For audio-only stimulation, a 10 kHz tone was played at 40 Hz with a 4% duty cycle. For visual-audio stimulation, the audio stimulation was accompanied with surrounding light flickered at 40 Hz for 10 s periods with a 50% duty cycle. Stimuli were presented in this manner for 20 min sessions, with 1-10 min pauses in between sessions to check on the animals' behavior.

Concurrent 40 Hz auditory and visual stimulation protocol

For biochemical, Immunohistochemical, or behavioral analysis, 5XFAD mice were placed in a dark chamber illuminated by an LED bulb and exposed to an auditory tone train, simultaneously. Mice were exposed to one of four stimulations: dark/quiet, 40 Hz light flicker, 40 Hz auditory tone train, concurrent 40 Hz light flicker and auditory tone, or random light flicker/tone stimulations.

Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) under anesthesia (2:1 of ketamine/xylazine), and the brains were post-fixed overnight in 4% paraformaldehyde in PBS. Brains were sectioned 40 μm thick with a Leica VT1000S vibratome (Leica). Sections were permeabilized and blocked in PBS with 0.3% Triton X-100 and 10% donkey serum at room temperature for 2-hs. Sections were incubated overnight at 4°C in primary antibody containing PBS with 0.3% Triton X-100 and 10% donkey serum. Primary antibodies were: anti- β -amyloid (Cell Signaling Technology; D54D2), anti-Iba1 (Wako Chemicals; 019-19741), anti-glia fibrillary acidic protein (GFAP)(Abcam; ab4674), anti-S100B (Abcam; ab868), anti-LRP1 (Abcam; 28320), Dy-Light 488 labeled Lycopersicon Esculentum (tomato) lectin (Vector laboratories; DL-1174), anti-amyloid oligomer (Millipore Sigma; AB9234), anti-phospho-tau (Ser396) (Cell Signaling Technology; 9632), anti-phospho-tau (Thr181) (Cell Signaling Technology, 12885), Hoechst 33342 (Thermo Fisher Scientific; H3570). The anti-A β antibody 12F4 was used because it does not react with APP, allowing us to determine whether our labeling was specific to A β , as well as allowing for co-labeling with Iba1. Anti-amyloid oligomer antibody AB9234 was used for co-labeling with LRP1. The following day, brain sections were incubated with fluorescently conjugated secondary antibodies (Jackson ImmunoResearch) for 2 hs at room temperature, and nuclei were stained with Hoechst 33342 (Invitrogen). Images were acquired using a confocal microscope (LSM 710; Zeiss) with a 40 \times objective at identical settings for all conditions. Images were quantified using ImageJ 1.42q by an experimenter blind to treatment groups. For each experimental condition, two coronal sections from each animal were used for quantification. Scale bars are 50 μm unless otherwise noted in figure legends. ImageJ was used to measure the diameter of Iba1+ cell bodies and to trace the processes for length measurement. In addition, the Coloc2 plugin was used to measure co-localization of Iba1 and A β . Microglia processes arborization was quantified using Imarisx64 8.1.2 (Bitplane, Zurich, Switzerland). The 'analyze particles' function in ImageJ was used for counting plaque number and area, deposits of at least 10 μm were included and a set threshold was used for both control and experimental groups.

Vasculature- A β colocalization analysis

ImarisColoc module was used to quantify colocalization of signal between two separate source channels (i.e., Lectin and AB, Lectin and LRP1) in 3D. These source channels were thresholded to mask any intensity coming from noise or background signal. Imaris-Coloc then generates a new channel containing only voxels that colocalize within the thresholds set for the source channels, and presents the associated statistical analyses.

Microglia-A β clustering analysis

IMARIS was used to analyze the microglial clustering pattern around amyloid plaques in 40 μ M slices. The surfaces module was utilized to detect and 3D render plaques (red) based on 12F4 signal. Iba1-positive microglia were then counted using the spots module, placing a sphere at the soma of each cell (green). Finally, the Spots Close To Surface XTension was run to find the subset of spots that are closer to the surface objects than the defined 25 μ M threshold, and exclusion of spots that fall outside this range. The algorithm measures the distance from the center of the spot to the nearest point of the surface object in 3D space, allowing for the quantification of microglial aggregation near plaques.

CLARITY immunostaining in brain slices

Mice were perfused with ice-cold PBS (1X) followed by ice-cold 4% PFA, 1% glutaraldehyde in 1xPBS. Brains were dissected out and post-fixed in 4% PFA/1% glutaraldehyde solution for 72 hs at 4°C. Fixation was terminated by incubating brains in inactivation solution (4% acrylamide, 1 M glycine, 0.1% Triton X-100 in 1X PBS) for 48 hs at RT. After washing in 1xPBS, brains were sliced into 100 μ M coronal sections on a vibratome (Leica VT100S) in 1xPBS. Sections containing regions of interest (i.e., auditory cortex and hippocampus) were selected, with reference to the Allen Mouse Brain Atlas, and incubated in clearing buffer (pH 8.5-9.0, 200mM sodium dodecylsulfate, 20mM lithium hydroxide monohydrate, 4mM boric acid in ddH₂O) for 2-4 hs, shaking at 55°C. Cleared sections were washed 3 x15mins in 1xPBST (0.1% Triton X-100/1XPBS) and put into blocking solution (2% bovine serum albumin/1xPBST) overnight at RT. Subsequently, three 1h washes in 1x PBST were performed, shaking at RT. Sections were incubated in weak binding buffer (pH 8.5-9.0, 37.75 mM Na₂HPO₄, 3.53 mM KH₂PO₄, 0.02% sodium azide in PBST) for 1 h at RT, then transferred to primary antibody, diluted to 1:100 in 1x weak binding buffer for 12 hs at 37°C. Reversal buffer (pH 7.4, 37.75 mM Na₂HPO₄, 3.53 mM KH₂PO₄ in 0.02% sodium azide in PBST) is then added in even aliquots over 6 hs, to equal the volume of primary antibody solution plus the volume of the tissue. Another set of 3x1 h washes in 1xPBST was conducted before sections were incubated for 12 hs at RT, in a mixture of Hoechst 33258 (1:250) (Sigma-Aldrich, 94403) and secondary antibody (1:100) in 1xPBS. Sections were then washed overnight in 1xPBS and incubated in RIMS (Refractive Index Matching Solution: 75 g Histodenz, 20mL 0.1M phosphate buffer, 60mL ddH₂O) for 1 h at RT prior to mounting. Brain sections were mounted onto microscopy slides with coverslips (VWR VistaVision, VWR International, LLC, Radnor, PA) in RIMS.

Images were acquired on a Zeiss LSM 880 microscope with the accompanying Zen Black 2.1 software (Carl Zeiss Microscopy, Jena, Germany). Z stack images were taken with a step size of 0.4-0.5 μ m, pixel dwell 4.1 ms, averaging of 2, resolution 1024x1024 suitable for 3D reconstruction. Imarisx64 8.3.1 (Bitplane, Zurich, Switzerland) was used for 3-D rendering and analysis.

Whole mouse brain processing and clearing

5XFAD mouse brains were processed according to the SHIELD protocol (Park et al., 2018). Briefly, 5XFAD mice were transcardially perfused with ice-cold PBS followed by 20 mLs of SHIELD-OFF solution containing 4% PFA. Brains were dissected and post-fixed in the same solution for 24 hs at 4°C. Brains were then incubated overnight in SHIELD-OFF solution without PFA at 4°C. Brains were then incubated in the SHIELD-ON solution for 24 hs at 37°C. Following fixation, brains were incubated in an aqueous clearing solution containing 200mM sodium dodecyl sulfate (SDS), 20mM lithium hydroxide monohydrate, 40mM boric acid, pH 8.5-9.0. Brains were then cleared using SmartClear Pro (LifeCanvas Technologies, Cambridge, MA) based on stochastic electrotransport (Kim et al., 2015) for several days, until transparent.

Immunostaining of cleared whole hemispheres

Cleared hemispheres were stained with 15 μ l of beta-amyloid antibody conjugated with Alexa Fluor-488 (CST, #51374) over 2 days, using eFLASH, an unpublished technique that integrates stochastic electrotransport method (Kim et al., 2015) and SWITCH method (Murray et al., 2015).

Light-sheet microscopy

Immunostained samples were incubated with hPROTOS (3g diatrizoic acid, 5g N-methyl-d-gludamine, 125 g iohexol in 105ml DI-water) for optical clearing and then mounted to acrylic holder using 2% low-temperature melting agarose in hPROTOS. Custom-made light-sheet microscope equipped with 10 \times CLARITY-optimized objective was used to image whole hemispheres using the 488 channel for beta-amyloid visualization and the 647 channel for autofluorescence.

Cleared whole brain image processing, plaque detection, and atlas alignment

Acquired image data were illumination corrected using CIDRE, an open-source software package implemented in MATLAB, and the resulting processed images were stitched together using Terastitcher. Imaris (Bitplane, <http://www.bitplane.com/Imaris/Imaris>) was

used for 3D visualizations, and ImageJ (NIH, <https://imagej.nih.gov/ij/>) was used to create representative slice-by-slice 2D visualizations. Automated plaque detection was performed using a combination of the open-source ClearMap software, a custom cell classification neural network model, and Elastix. Candidate plaques were located as “spots” with ClearMap’s spot detection module. First, background subtraction was done slice-by-slice by using a gray-scale morphological top-hat transformation with a disk structure element with major and minor diameter pixel sizes of (21,21). Next, local maxima of the data are detected by applying a 3D maxima filter with disk structure element of size (7,7,4), and these local maxima are filtered with an intensity threshold of 100. The pixel volumes corresponding to each spot center location are also computed using a 3D watershed transform with spot centers as seed points. All candidate plaques with volume less than a sphere with 10-micron diameter were then filtered out. True plaques were identified from the candidate plaques using a convolutional neural network (CNN) model as a categorical plaque / non-plaque classifier implemented in Keras (<https://keras.io/>) with a Theano backend (<https://github.com/Theano/Theano>). The CNN input is a 32-by-32 pixel bounding box centered at a candidate plaque center, and the output is a two element one-hot vector representing the plaque and non-plaque categories. The architecture consists of 12 total convolutional layers, each with a rectified linear unit (ReLU) activation and followed by batch normalization: 3 with 64 2x2 kernels, 3 with 128 2x2 kernels, followed by 3 with 192 2x2 kernels, 1 with 256 2x2 kernels, 1 with 256 1x1 kernels, and 1 with 2 1x1 kernels. 2x2 subsampling is done after the third, sixth, and ninth convolutional layer, and Dropout with a rate of 0.5 is applied after the last nine convolutional/batch normalization layers for regularization. After the final convolutional layer, global average pooling followed by softmax activation is applied to generate the final categorical vector. During training, a categorical cross entropy loss was used with the Adam optimizer with default parameters. The CNN was trained for 400 epochs with batch size of 64 on ~10,000 manual plaque annotations augmented with random rotations, shears, and reflections using the Keras Image Data Generator. The resulting model was then used to classify plaques from detected spots for all samples. To perform atlas alignment, autofluorescence channel images were first downsampled to the atlas resolution, and then Elastix was used to calculate affine and B-spline transformation parameters to do 3D image registration, with the resampled autofluorescence image as the fixed image and the atlas as moving image. The resulting alignment parameters were applied on the plaque locations (output from the CNN model) to transform the plaques into the atlas space, after which a CSV file with plaque count and volume information for each brain region (segmentation according to the Allen Brain Atlas) is generated.

Western blot

Hippocampus and auditory cortex from 6-month-old tau P301S mice were dissected and homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitors. Lysates were incubated on ice for 15 min and spun at 12,000 RPM for 15 minutes. Then, supernatants were transferred to fresh tubes and analyzed for protein concentration (Bio-Rad Protein Assay). Equal amounts of protein (20 ug/lane) was resolved on a SDS-polyacrylamide gel and blotted onto a PVDF membrane. This membrane was incubated in blocking buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% (v/v) Tween 20 (TBS-T) plus 5% dry milk (m/v) for 1 h at room temperature followed by incubation overnight at 4°C in primary antibodies and then secondary antibodies at room temperature for 1 h. Primary antibodies were anti-phospho-tau (Ser396) and anti-phospho-tau (Thr181). Secondary antibodies were LI-COR IRDye secondary antibodies. Signal intensities were quantified using ImageJ 1.46a and normalized to values of total tau Tau5 (Thermo Fisher Scientific; AHB0042).

Tau seeding activity assay

2-month old tau P301S brain sections were homogenized in 1 × TBS supplemented with protease inhibitors (Roche complete mini tablets) using a probe sonicator (30% power; 15 pulses). After sonication, the lysates were centrifuged at 14,000 × g for 15 min to eliminate large, insoluble material. The supernatant was stored at –80°C and used for all future experiments. Protein concentration was determined using a Bio-Rad Protein Assay Dye. Fluorescence resonance energy transfer (FRET) biosensor cell lines described previously (Holmes et al., 2014) were provided by Marc I. Diamond. Cells were grown in DMEM (Invitrogen) augmented with 10% FBS and 1 × penicillin/streptomycin and maintained at 37°C and 5% CO₂ in a humidified incubator. For the assay, cells were plated in a 96-well plate at a density of 40,000 cells/well. Sixteen hs later, at 50% confluence, brain homogenate samples were transduced into cells using 1.2 μL Lipofectamine/well. After a 24 h incubation at 37°C, cells were harvested with 0.25% trypsin, fixed in 2% PFA (Electron Microscopy Services) for 15 min, and then resuspended in PBS. An LSR II HST-2 flow cytometer was used to measure the FRET signal within each cell. FRET quantification was accomplished using FlowJo version 10 software (TreeStar). Integrated FRET density was derived by multiplying the percentage of FRET-positive cells in each sample by the median FRET intensity of those cells.

ELISA

Primary auditory cortices, medial prefrontal cortices, and hippocampi were isolated from 6-month-old 5XFAD males and subjected to Aβ measurement using Aβ₄₂ or Aβ₄₀ ELISA kits (Invitrogen) according to the manufacturer’s instructions. Insoluble Aβ was treated with 5M guanidine/50 mM Tris HCL (pH 8.0) buffer before ELISA measurement.

Behavioral Experiments

Novel Object Recognition

The novel object recognition (NOR) task consisted of a habituation phase followed by training and testing performed the following day, as previously described (Leger et al., 2013). 24 hs before training, mice were habituated to an open testing arena

(40 cm L x 40 cm W x 35 cm H) for 5 min, during which total distance (cm), time in the center (s), and velocity (cm/s) were calculated (TSE Systems). During training, mice were placed into the same box with two identical objects placed in opposite corners. Mice were allowed a total of 20 s of object interaction time (within a maximum time frame of 10 minutes), and then immediately removed from the arena. Object memory was tested 1 hr later using the same procedure during training, except one object was replaced with a novel one in its place. Object exploration was recorded when the snout contacted either object and was calculated by a recognition index, $RI = T_{novel}/(T_{novel}+T_{familiar})$, where T_{novel} and $T_{familiar}$ indicate the time spent with the novel and familiar object, respectively.

Novel Object Location

The novel location recognition (NOL) task was performed using the same procedure as the object recognition task, except two identical objects were used for both training and testing, and one object was displaced to a novel location during testing.

Morris Water Maze Test

Spatial reference memory testing was performed in a circular tank (diameter, 1.2 m) filled with white opaque water at approximately 22°C. Reference cues consisting of different colors and shapes were placed along the walls surrounding the tank. Within the tank was a fixed platform (diameter, 10cm) located in a target quadrant. During testing, the platform was submerged and the mice were placed into the tank at one of seven points randomly facing the wall of the tank. Mice were provided 60 s to search for the platform, which if not found, were gently guided to it. Animals were kept on the platform for 15 s. Two trials a day were conducted with a 1 h intertrial interval. Between the trials, mice were gently patted dry and warmed on a heating pad. Mouse behavior was video-recorded using TSE Systems. The escape latency, or the time it took for the mouse to reach the platform, was scored for each trial and averaged per testing day. On day 6, the platform was removed and a memory test (probe test) was performed. The time spent in each of the 4 quadrants and the number of crossing of the area where the platform used to be was recorded. Swimming velocity was recorded automatically.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Statistical significance between two groups for non-electrophysiology experiments was calculated by two-tailed unpaired Mann-Whitney test. Significance between three groups or more was calculated by Kruskal-Wallis test with Dunn's multiple comparisons test. Prism 7 software was used to calculate the values. For electrophysiology data, differences between two stimulus conditions were assessed using non-parametric, two-sample Kolmogorov-Smirnov goodness of fit tests to compare circular statistic value distributions or using z-Test for two proportions to compare proportions. The significance of difference distributions was assessed using two-sided Wilcoxon signed rank test for zero median (e.g., differences in firing rates between stimulus conditions). Multiple comparisons were controlled for using the Bonferroni correction. Significance values are $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) unless otherwise specified. Specific statistical parameters are detailed in the figure legends.

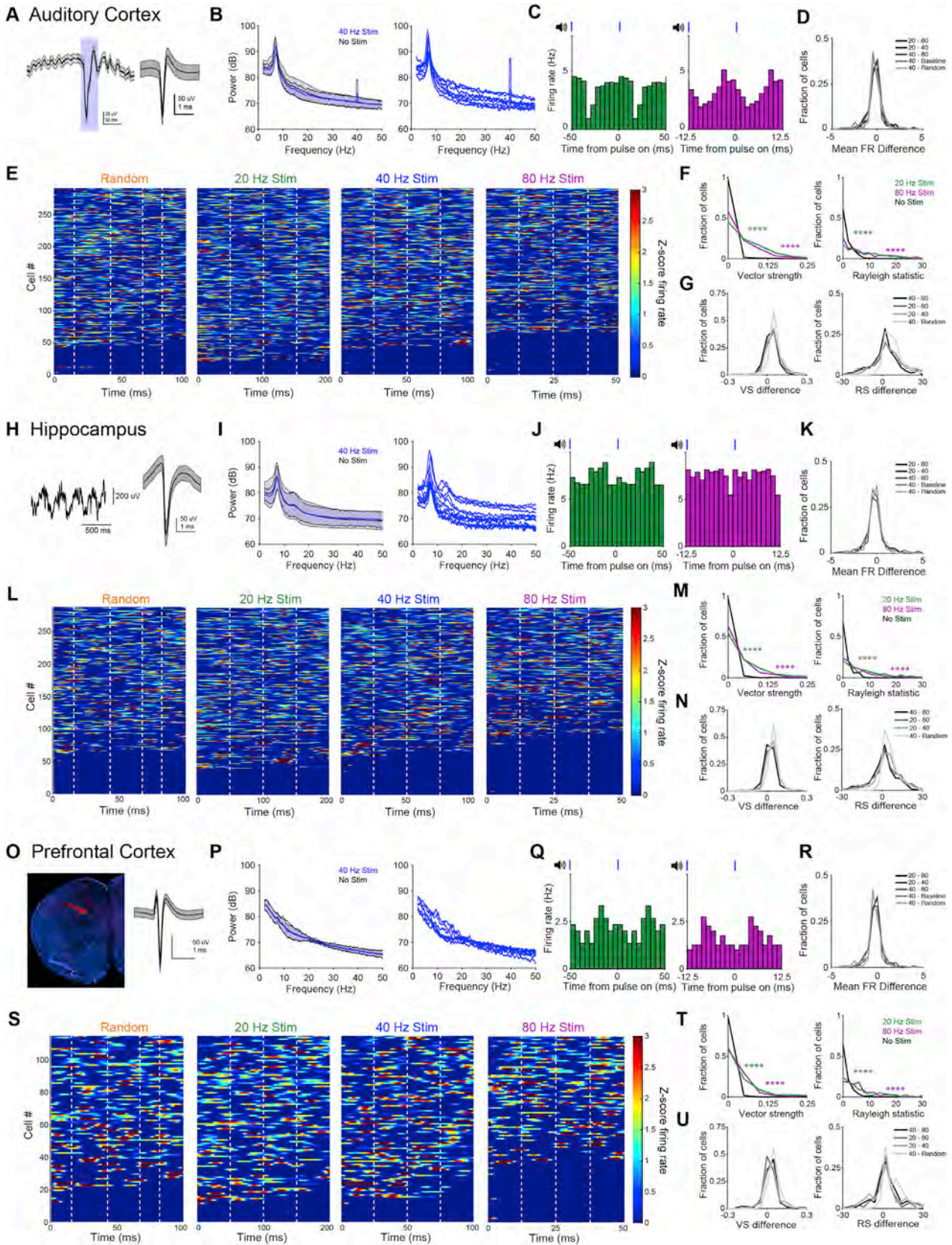


Figure S1. 20 Hz and 80 Hz Auditory Stimulation Modulates Activity in AC, CA1, and mPFC, Related to Figure 1

- (A) Mean LFP response to auditory mapping tones used to detect auditory cortex (*left*). The blue region indicates when the 50 ms mapping tone played. Example of a clustered putative single unit (*right*).
- (B) Power spectral density (PSD) response to 40 Hz auditory flicker stimuli and no stimulation periods, with mean and standard deviation across recording days (*left*), power spectrum LFP response to auditory flicker of all recording days in AC (recording site with largest 40 Hz peak during 40 Hz auditory flicker per recording depth is shown, see Methods) (*right*).
- (C) Firing rate modulation of a putative single unit in response to 20 Hz audio flicker stimulation (*left*, green) and 80 Hz auditory flicker (*right*, purple)
- (D) Mean firing rate difference between multiple stimulation conditions of single units in AC centers around 0 Hz ($p > 0.01$ 20 Hz – 40 Hz, n.s. after controlling for five comparisons; **** $p < 0.00002$ 40 Hz – no stimulation; all others n.s.; Wilcoxon signed rank test for zero median. In all statistical tests, significance remains after controlling for multiple comparisons using the Bonferroni correction, unless otherwise stated).
- (E) Firing rate response of all isolated single units in AC to Random, 20 Hz, 40 Hz, and 80 Hz auditory stimulation. Z-scored response to four consecutive stimulus cycles is shown. Units are ordered by their average stimulus phase preference in the analyzed four cycles. White dashed lines indicate auditory pulse timing.
- (F) Vector strength distribution of 20 Hz and 80 Hz auditory stimulation versus no stimulation condition (*left*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 11 units had 20 Hz stim VS values greater than 0.25; 6 units had 80 Hz stim VS values greater than 0.25) and Rayleigh statistic distribution of 20 Hz and 80 Hz auditory stimulation versus no stimulation (*right*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 74 units had 20 Hz stim RS values greater than 30; 41 units had 80 Hz stim RS values greater than 30).
- (G) Distribution of within cell differences in vector strength values between all frequencies of auditory stimulation (*left*, **** $p < 0.000025$ 20 Hz – 80 Hz, 20 Hz – 40 Hz, 40 Hz – Random; 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median). Within cell differences in Rayleigh statistic values between all frequencies of auditory stimulation (*right*, **** $p < 0.000025$ 20 Hz – 80 Hz, 20 Hz – 40 Hz, 40 Hz – Random; 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median).
- (H) Example of theta rhythm, a hallmark of hippocampus, used to detect CA1.
- (I) Same as (B) for CA1.
- (J) Same as (C) for CA1.
- (K) Same as (D) for CA1 ($p > 0.01$ 40 Hz – no stimulation, n.s. after controlling for five comparisons; all others n.s.; Wilcoxon signed rank test for zero median).
- (L) Same as (E) for CA1.
- (M) Same as (F) for CA1 (*left*, **** $p < 0.00005$, 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 12 units had 20 Hz stim VS values greater than 0.25; 10 units had 80 Hz stim VS values greater than 0.25; *right*, **** $p < 0.00005$, 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 4 units had 20 Hz stim RS values greater than 30; 5 units had 80 Hz stim RS values greater than 30).
- (N) Same as (G) for CA1 (*left*, ** $p < 0.0025$ 20 Hz – 80 Hz; *** $p < 0.00025$ 20 Hz – 40 Hz; **** $p < 0.000025$ 40 Hz – random, 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median; *right*, $p > 0.0125$ 20 Hz – 80 Hz, n.s. after controlling for four comparisons; ** $p < 0.0025$ 20 Hz – 40 Hz, **** $p < 0.000025$ 40 Hz – Random, 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median).
- (O) Histology image showing probe trace and recording location in mPFC. Red arrow indicates recording location.
- (P) Same as (B) for mPFC.
- (Q) Same as (C) for mPFC.
- (R) Same as (D) for mPFC (*right*, n.s.; Wilcoxon signed rank test for zero median).
- (S) Same as (E) for mPFC.
- (T) Same as (F) for mPFC (*left*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 4 units had 20 Hz stim VS values greater than 0.25; *right*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 5 units had 20 Hz stim RS values greater than 30; 3 units had 80 Hz stim RS values greater than 30).
- (U) Same as (G) for mPFC (*left*, **** $p < 0.000025$ 40 Hz – random, all others n.s.; Wilcoxon signed rank test for zero median; *right*, **** $p < 0.000025$ 40 Hz – Random, all others n.s.; Wilcoxon signed rank test for zero median).

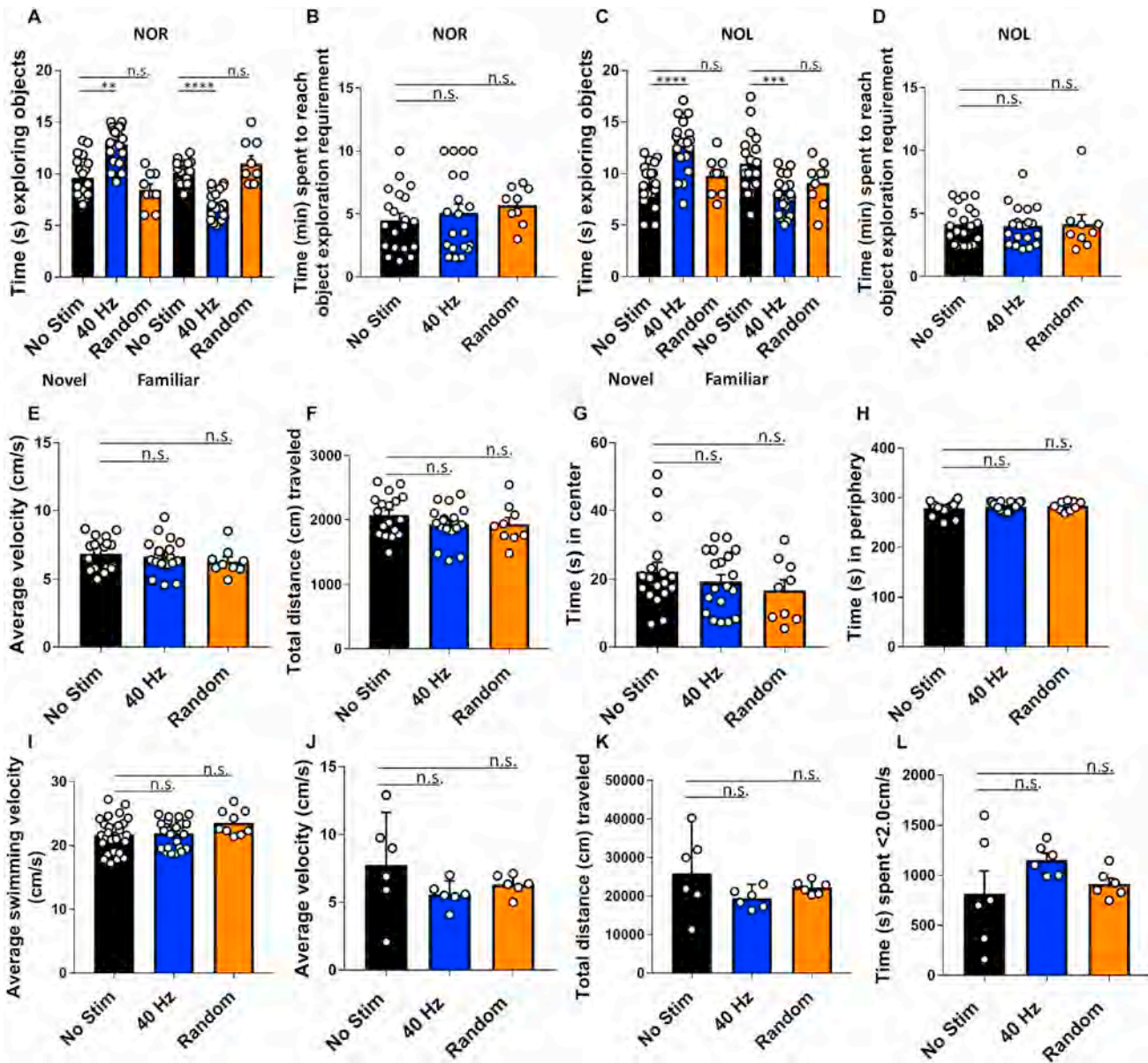


Figure S2. Auditory GENUS Does Not Affect Mouse Behavior, Related to Figure 2

(A) Time (seconds) spent exploring familiar and novel objects during NOR test of 5XFAD non-stimulated, 40 Hz, and random frequency stimulated mice ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, $**p < 0.01$, $****p < 0.0001$, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(B) Time (min) mice required during NOR test to reach the total object exploration requirement of 20 s ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(C) Time (seconds) spent exploring object in familiar and novel location during NOL test of 5XFAD non-stimulated, 40 Hz, and random frequency stimulated mice ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, $***p < 0.001$, $****p < 0.0001$, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(D) Time (min) mice required during NOL test to reach the total object exploration requirement of 20 s ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(E) Average velocity (cm/s) during habituation ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(F) Total distance (cm) traveled during habituation ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(G) Time (seconds) spent in the center of the behavior chamber during habituation ($n = 20$ mice in no stim group, $n = 20$ mice in 40 Hz group, $n = 9$ in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

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(H) Time (seconds) spent in the periphery of the behavior chamber during habituation (n = 20 mice in no stim group, n = 20 mice in 40 Hz group, n = 9 in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(I) Average swimming velocity (cm/s) during Morris water maze (n = 25 mice in no stim group, n = 28 mice in 40 Hz group, n = 9 in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(J) Average velocity (cm/s) during 1-h no stimulation, auditory GENUS, or random frequency stimulation (n = 6 mice in no stim group, n = 6 mice in 40 Hz group, n = 6 in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(K) Total distance (cm) traveled during 1-h no stimulation, auditory GENUS, or random frequency stimulation (n = 6 mice in no stim group, n = 6 mice in 40 Hz group, n = 6 in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(L) Time (seconds) spend under 2 cm/s during 1-h no stimulation, auditory GENUS, or random frequency stimulation (n = 6 mice in no stim group, n = 6 mice in 40 Hz group, n = 6 in random frequency group, mean SEM in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

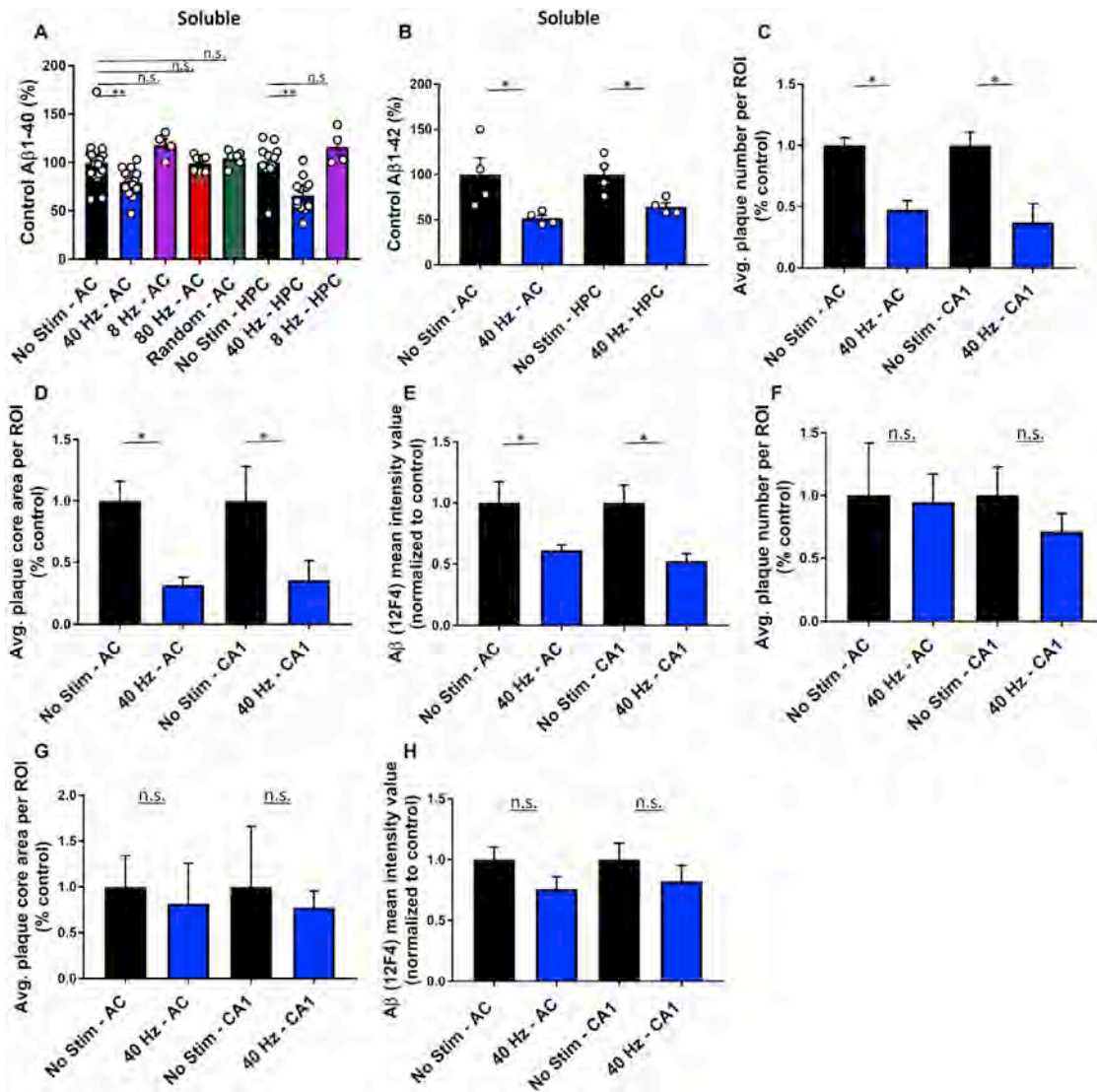


Figure S3. Auditory GENUS Ameliorates Plaque Load in APP/PS1 Mice, Related to Figure 3

(A) Relative soluble $A\beta_{1-40}$ levels in auditory cortex (AC) and hippocampus (HPC) in 6-month-old 5XFAD mice following 40 Hz, 8 Hz, 80 Hz, or random frequency auditory stimulation for 1 h per day for 7 days, normalized to non-stimulation control (note: ELISA for 80 Hz and random frequency HPC samples were unsuccessful and were unable to be reported, $n = 19$ mice in no stim group, $n = 19$ mice in 40 Hz group, $n = 4$ mice in 8 Hz group, $n = 7$ in 80 Hz group, $n = 6$ in random frequency group, mean SEM in bar graphs, ** $p < 0.01$, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(B) Relative soluble $A\beta_{1-42}$ levels in auditory cortex (AC) and hippocampus (HPC) in 6-month-old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 4$ mice in no stim group, $n = 4$ mice in 40 Hz group, mean SEM in bar graphs, * $p < 0.05$, unpaired Mann-Whitney test).

(C) Average plaque number in AC and CA1 ('region of interest', ROI) in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 5$ mice in no stim group, $n = 5$ mice in 40 Hz group, mean SEM in bar graphs, * $p < 0.05$, unpaired Mann-Whitney test).

(D) Average plaque core area in AC and CA1 in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 5$ mice in no stim group, $n = 5$ mice in 40 Hz group, mean SEM in bar graphs, * $p < 0.05$, unpaired Mann-Whitney test).

(E) $A\beta$ (12F4) mean intensity value (12F4 antibody) in AC and CA1 in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 5$ mice in no stim group, $n = 5$ mice in 40 Hz group, mean SEM in bar graphs, * $p < 0.05$, unpaired Mann-Whitney test).

(F) Average plaque number in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 6$ mice in no stim group, $n = 6$ mice in 40 Hz group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).

(G) Average plaque core area in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 6$ mice in no stim group, $n = 6$ mice in 40 Hz group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).

(H) $A\beta$ (12F4) mean intensity value (12F4 antibody) in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control ($n = 6$ mice in no stim group, $n = 6$ mice in 40 Hz group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).

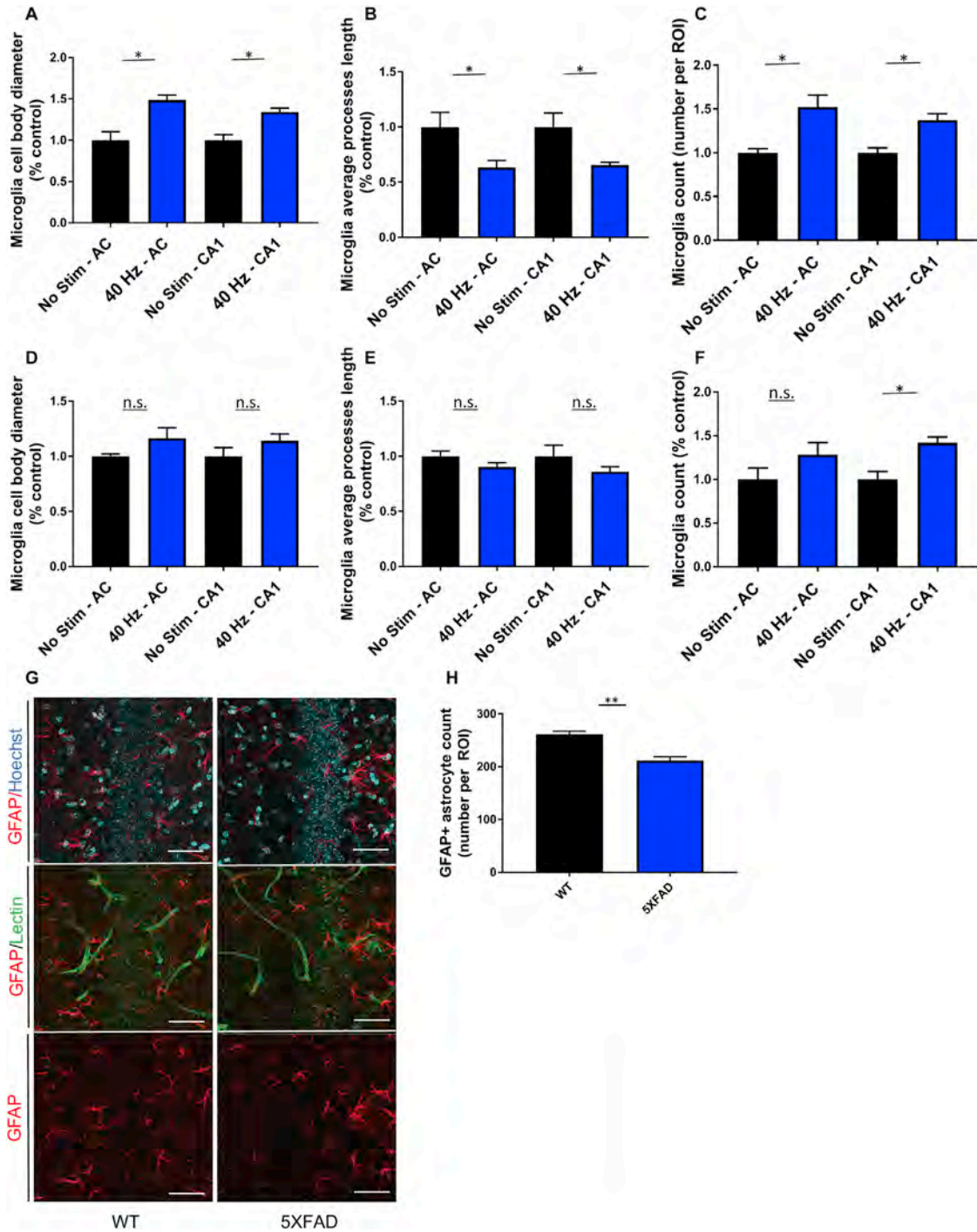


Figure S4. Auditory GENUS Induces Microglia Response in APP/PS1 Mice, Related to Figure 4

(A) Diameter of Iba1-positive microglia cell bodies in AC and CA1 in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 5 mice per group, mean SEM in bar graphs, *p < 0.05, unpaired Mann-Whitney test).

(B) Average length of Iba1-positive microglia primary processes in AC and CA1 in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 5 mice per group, mean SEM in bar graphs, *p < 0.05, unpaired Mann-Whitney test).

(C) Number of Iba1-positive microglia in AC and CA1 in 9-month old APP/PS1 mice following auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 5 mice per group, mean SEM in bar graphs, *p < 0.05, unpaired Mann-Whitney test).

(D) Diameter of Iba1-positive microglia cell bodies in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).

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(E) Average length of Iba1-positive microglia primary processes in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).

(F) Number of Iba1-positive microglia in AC and CA1 in 6-month old 5XFAD mice following 7 days no stimulation post auditory GENUS for 1 h per day for 7 days, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, *p < 0.05, n.s. = not significant, unpaired Mann-Whitney test).

(G) Immunohistochemistry of CLARITY treated brain sections with anti-GFAP (ab4674, red) and lectin stain (DL-1174, green) antibodies in CA1 of 6-month-old WT and 5XFAD mice (n = 3 mice per group, scale bar, 50 μ m).

H(H). Number of GFAP positive cells (per image of interest, using IMARIS) in CA1 of 6-month-old WT and 5XFAD mice (n = 4 mice per group, mean SEM in bar graphs, **p < 0.01, unpaired Mann-Whitney test).

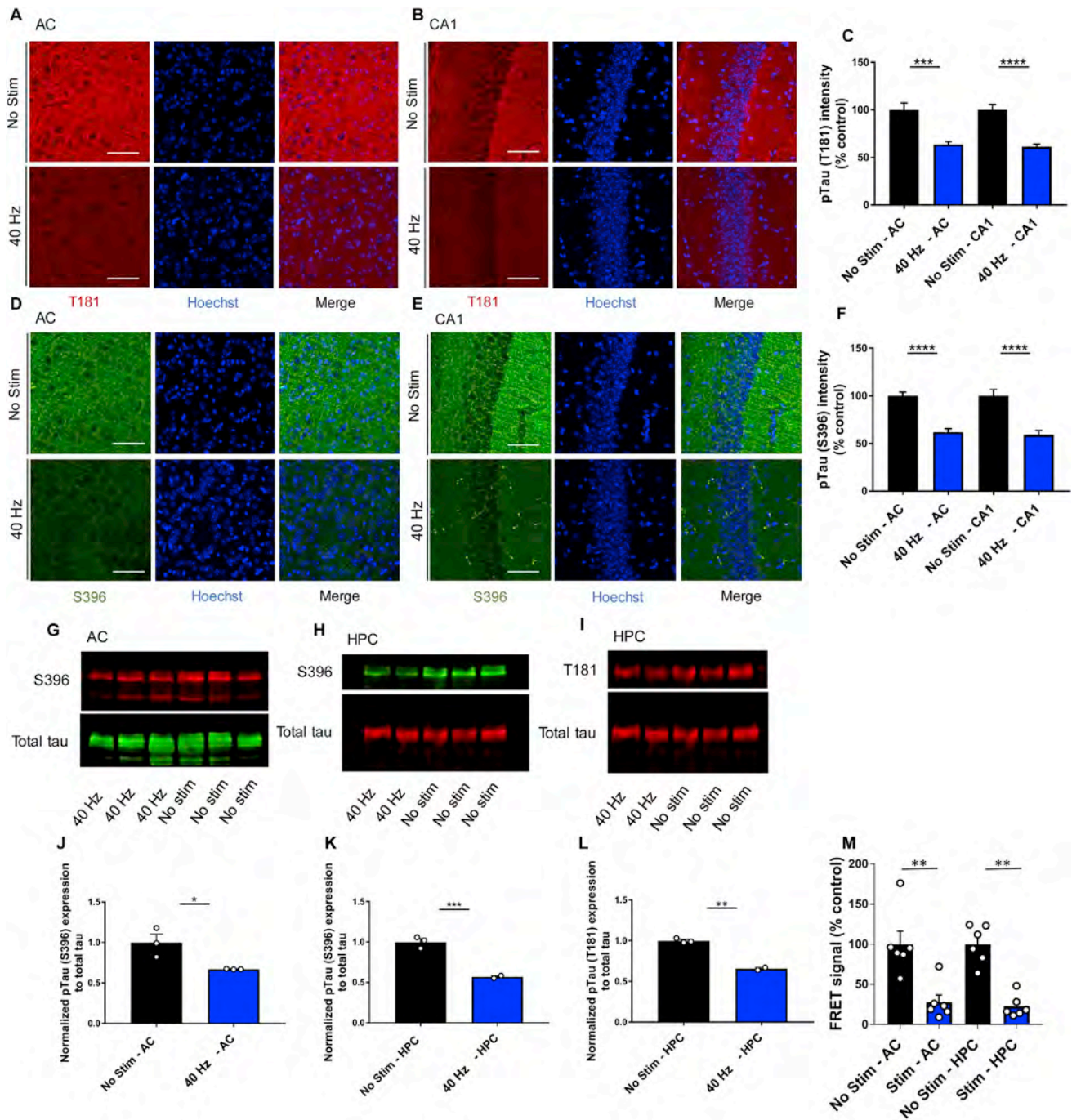


Figure S5. Auditory GENUS Reduces Phosphorylated Tau and Seeding in P301S Mice, Related to Figure 3

(A) Immunohistochemistry with anti-pTau (T181, red) antibodies in AC of 6-month-old P301S mice after 7 days of 1 h per day no stimulation or auditory GENUS (image taken with 40x objective, scale bar, 50 μ m).

(B) As in (A) for CA1.

(C) Relative pTau (T181) intensity levels in AC and CA1 of P301S mice after 7 days of 1 h per day no stimulation or auditory GENUS normalized to non-stimulation control (n = 10 mice per group, ***p < 0.001, ****p < 0.0001; unpaired Mann-Whitney test).

(D) Immunohistochemistry with anti-pTau (S396, green) antibodies in AC of 6-month-old P301S mice after 7 days of 1 h per day no stimulation or auditory GENUS (scale bar, 50 μ m).

(E) As in (D) for CA1.

(F) Relative pTau (S396) intensity levels in P301S mice in AC and CA1 after 7 days of 1 h per day no stimulation or auditory GENUS normalized to non-stimulation control (n = 10 mice per group, ****p < 0.0001; unpaired Mann-Whitney test).

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(G) Representative western blot showing levels of pTau (S396) and total tau in AC of 6-month-old P301S mice after 7 days of 1 h per day no stimulation or auditory GENUSt.

(H) As in (G) for hippocampus.

(I) Representative western blot showing levels of pTau (T181) and total tau in hippocampus of 6-month-old P301S mice after 7 days of 1 h per day no stimulation or auditory GENUSt.

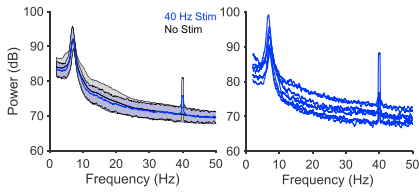
(J) Relative immunoreactivity of pTau (S396) normalized to total tau in AC of P301S mice (from western blot in (G) after 7 days of 1 h per day no stimulation or auditory GENUSt (n = 3 mice per group, mean SEM in bar graphs, *p < 0.05; unpaired Mann-Whitney test).

(K) Relative immunoreactivity of pTau (S396) normalized to total tau in HPC of P301S mice (from western blot in (H) after 7 days of 1 h per day no stimulation or auditory GENUSt (n = 2 mice in 40 Hz group and n = 3 in non-stimulation group, mean SEM in bar graphs, ***p < 0.001; unpaired Mann-Whitney test).

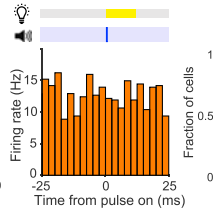
(L) Relative immunoreactivity of pTau (T181) normalized to total tau in hippocampus of P301S mice (from western blot in (I) after 7 days of 1 h per day no stimulation or auditory GENUSt (n = 2 mice in 40 Hz group and n = 3 in non-stimulation group, mean SEM in bar graphs, **p < 0.01; unpaired Mann-Whitney test).

(M) Average change in FRET signal from 2-month-old P301S tau mice following auditory GENUSt for 1 h per day for 7 days, normalized to non-stimulation control (n = 6 in no stim group, n = 6 in 40 Hz group, mean SEM in bar graphs, **p < 0.01, unpaired Mann-Whitney Test).

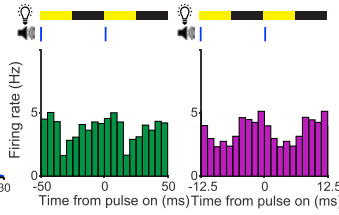
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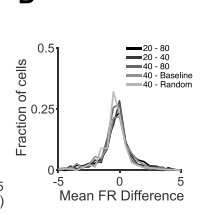
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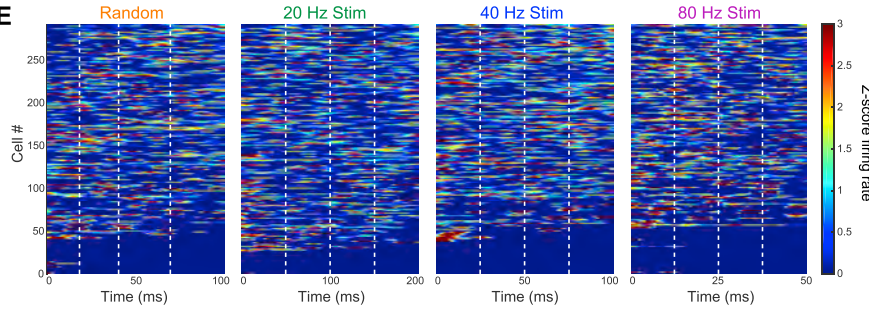
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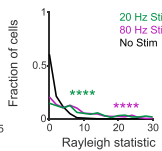
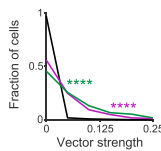
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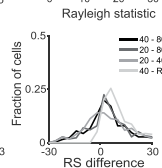
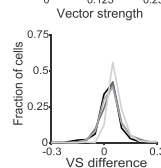
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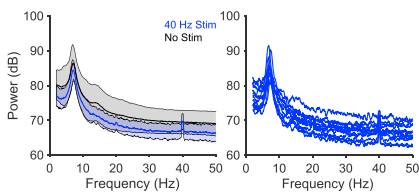
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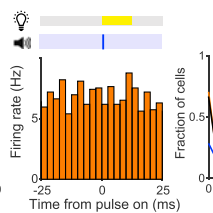
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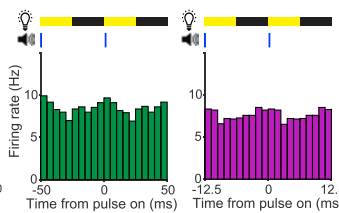
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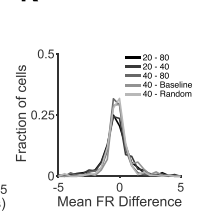
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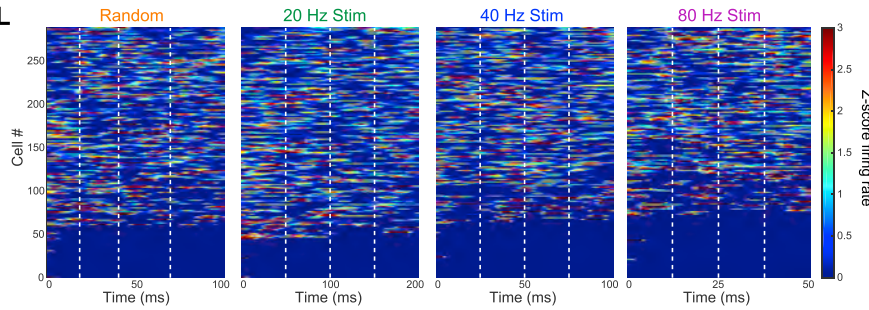
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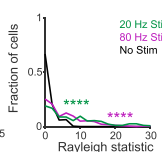
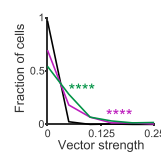
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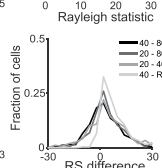
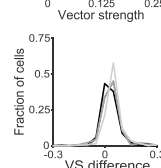
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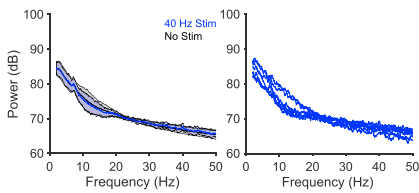
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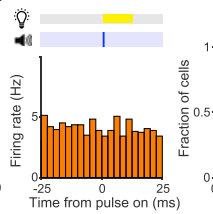
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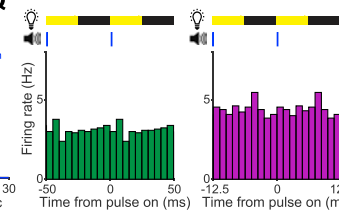
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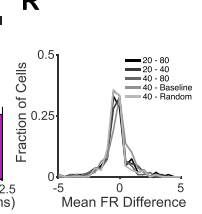
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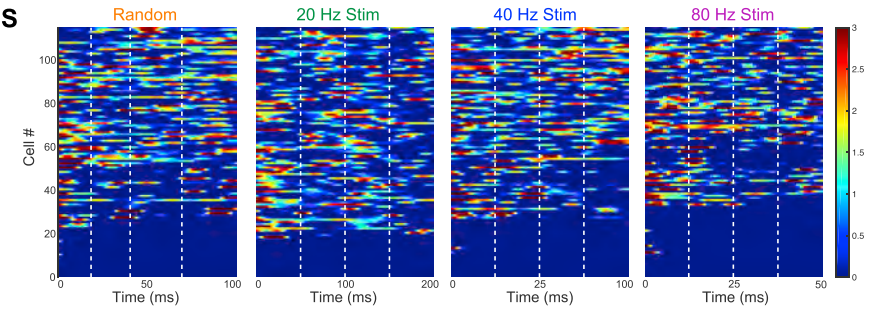
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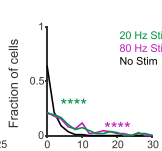
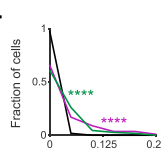
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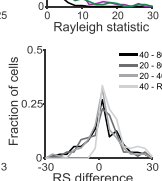
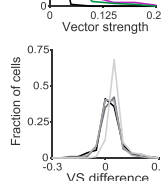


Figure S6. 40 Hz Combined Auditory and Visual Stimulation Modulates Spiking Activity in AC, CA1, and mPFC, Related to Figure 6

- (A) Power spectral density (PSD) response to 40 Hz audio-visual flicker stimuli and no stimulation periods, with mean and standard deviation across recording days (*left*), power spectrum LFP response to audio-visual flicker stimulation of all recording days in AC (recording site with largest 40 Hz peak during 40 Hz audio-visual flicker per recording depth is shown, *STAR Methods*) (*right*).
- (B) Firing rate modulation of putative single unit shown in Figure 6A to audio-visual random stimulation, Rayleigh statistic distribution of single unit response to 40 Hz audio-visual stimulation (*right*, **** $p < 0.00005$ 40 Hz versus No Stim, 40 Hz versus Random; Kolmogorov-Smirnov test; 40 units had 45 Hz stim RS values greater than 30; 5 units had random stim RS values greater than 30. In all statistical tests, significance remains after controlling for multiple comparisons using the Bonferroni correction, unless otherwise stated).
- (C) Firing rate modulation of a putative single unit in response to 20 Hz audio-visual flicker stimulation (*left*, green) and 80 Hz audio-visual flicker stimulation (*right*, purple).
- (D) Mean firing rate difference of single units between multiple stimulation conditions in AC centers around 0 Hz ($p > 0.01$ 40 Hz – no stimulation, n.s. after controlling for five comparisons; all others n.s.; Wilcoxon signed rank test for zero median).
- (E) Firing rate response of each single unit isolated in AC to Random, 20 Hz, 40 Hz, and 80 Hz audio-visual stimulation. Z-scored response to four consecutive stimulus cycles is shown. Units are ordered by their average stimulus phase preference in the analyzed four cycles. White dashed lines indicate auditory pulse timing.
- (F) Vector strength distribution of 20 Hz and 80 Hz audio-visual stimulation versus no stimulation condition (*left*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 12 units had 20 Hz stim VS values greater than 0.25; 10 units had 80 Hz stim VS values greater than 0.25), and Rayleigh statistic distribution of 20 Hz and 80 Hz audio-visual stimulation versus no stimulation (*right*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 86 units had 20 Hz stim RS values greater than 30; 35 units had 80 Hz stim RS values greater than 30).
- (G) Distribution of within cell differences in vector strength values between all frequencies of auditory stimulation (*left*, *** $p < 0.00025$ 20 Hz – 40 Hz; **** $p < 0.000025$ 20 Hz - 80 Hz, 40 Hz – Random; 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median). Within cell differences in Rayleigh statistic values between all frequencies of auditory stimulation (*right*, *** $p < 0.00025$ 20 Hz - 80 Hz; **** $p < 0.000025$ 20 Hz – 40 Hz, 40 Hz – Random; 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median).
- (H) Same as (A) for CA1.
- (I) Same as (B) for CA1 (*right*, **** $p < 0.00005$ 40 Hz versus No Stim, 40 Hz versus Random; Kolmogorov-Smirnov test; 8 units had 40 Hz stim RS values greater than 30).
- (J) Same as (C) for CA1.
- (K) Same as (D) for CA1 ($p > 0.01$ 40 Hz – no stimulation, n.s. after controlling for five comparisons; all others n.s.; Wilcoxon signed rank test for zero median).
- (L) Same as (E) for CA1.
- (M) Same as (F) for CA1 (*left*, **** $p < 0.0005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 16 units had 20 Hz stim VS values greater than 0.25; 7 units had 80 Hz stim VS values greater than 0.25; *right*, **** $p < 0.0005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 21 units had 20 Hz stim RS values greater than 30; 3 units had 80 Hz stim RS values greater than 30).
- (N) Same as (G) for CA1 (*left*, *** $p < 0.00025$ 20 Hz – 40 Hz; **** $p < 0.00025$ 20 Hz – 80 Hz, 40 Hz – random; 40 Hz – 80 Hz n.s.; Wilcoxon signed rank test for zero median; *right*, ** $p < 0.0025$ 20 Hz – 40 Hz; **** $p < 0.000025$ 40 Hz – Random; all others n.s.; Wilcoxon signed rank test for zero median).
- (O) Same as (A) for mPFC.
- (P) Same as (B) for mPFC (*right*, **** $p < 0.00005$ 40 Hz versus No Stim, 40 Hz versus Random; Kolmogorov-Smirnov test; 1 unit had a 40 Hz stim RS value greater than 30).
- (Q) Same as (C) for mPFC.
- (R) Same as (D) for mPFC ($p > 0.01$ 40 Hz – no stimulation, n.s. after controlling for five comparisons; all others n.s.; Wilcoxon signed rank test for zero median).
- (S) Same as (E) for mPFC.
- (T) Same as (F) for mPFC (*left*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 4 units had 20 Hz stim VS values greater than 0.25; 1 unit had a 80 Hz stim VS value greater than 0.25; *right*, **** $p < 0.00005$ 20 Hz versus No Stim, 80 Hz versus No Stim; Kolmogorov-Smirnov test; 5 units had 20 Hz stim RS values greater than 30; 1 unit had a 80 Hz stim RS value greater than 30).
- (U) Same as (G) for mPFC (*left*, **** $p < 0.000025$ 40 Hz – random; all others n.s.; Wilcoxon signed rank test for zero median; *right*, **** $p < 0.000025$ 40 Hz – Random; all others n.s.; Wilcoxon signed rank test for zero median).

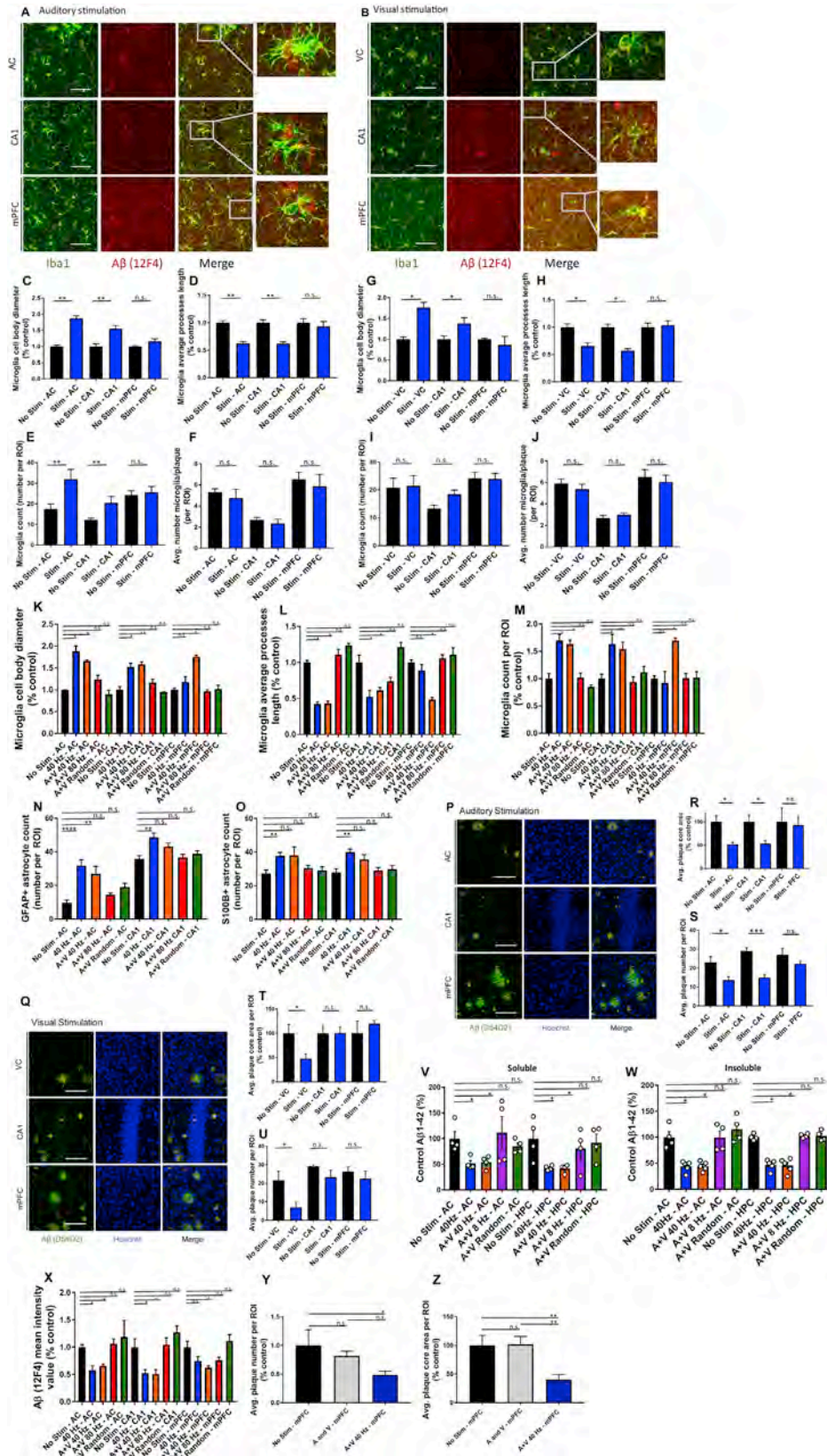


Figure S7. 1 Week of Auditory or Visual GENUS Only Does Not Affect mPFC Pathology, Related to Figures 6 and 7

- (A) Immunohistochemistry of anti-Iba1 (019-19741, green) and anti-A β (12F4, red) antibodies in AC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of auditory GENUS (inset magnification, 100x; scale bar, 50 μ m).
- (B) Immunohistochemistry of anti-Iba1 (019-19741, green) and anti-A β (12F4, red) antibodies in VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of visual GENUS (inset magnification, 100x; scale bar, 50 μ m).
- (C) Average microglia cell body diameter in AC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of auditory GENUS, normalized to no stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01, unpaired Mann-Whitney test).
- (D) Average microglia process length in AC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of auditory GENUS, normalized to no stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01, unpaired Mann-Whitney test).
- (E) Microglia count per region of interest in AC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of auditory GENUS (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01, unpaired Mann-Whitney test).
- (F) Average number of microglia surrounding 25 μ m radius of a plaque in AC, CA1, and mPFC following no stimulation or auditory GENUS (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).
- (G) Average microglia cell body diameter in VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of visual GENUS, normalized to no stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05; unpaired Mann-Whitney test).
- (H) Average microglia process length in VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of visual GENUS, normalized to no stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05; unpaired Mann-Whitney test).
- (I) Microglia count per region of interest in VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of visual GENUS (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).
- (J) Average number of microglia surrounding 25 μ m radius of a plaque in VC, CA1, and mPFC following no stimulation or visual GENUS (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, unpaired Mann-Whitney test).
- (K) Average microglia cell body diameter in AC, CA1, and mPFC of 6-month-old 5XFAD mice following 7 days of 1 h per day 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 80 Hz, or combined (A+V) random frequency stimulation, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, **p < 0.01; unpaired Mann-Whitney test).
- (L) Average microglia process length in AC, CA1, and mPFC of 6-month-old 5XFAD mice following 7 days of 1 h per day 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 80 Hz, or combined (A+V) random frequency stimulation, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, unpaired Mann-Whitney test).
- (M) Microglia count per region of interest in AC, CA1, and mPFC of 6-month-old 5XFAD mice following 7 days of 1 h per day 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 80 Hz, or combined (A+V) random frequency stimulation, normalized to non-stimulation control (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, unpaired Mann-Whitney test).
- (N) Number of GFAP-positive astrocytes in AC and CA1 (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01, ****p < 0.0001; Kruskal-Wallis test with Dunn's multiple comparison test).
- (O) Number of S100B-positive astrocytes in AC and CA1 (n = 6 mice per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01; Kruskal-Wallis test with Dunn's multiple comparison test).
- (P) Immunohistochemistry of anti-A β plaques (D54D2, green) antibodies in AC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of auditory GENUS (n = 6 mice per group, scale bar, 50 μ m).
- (Q) Immunohistochemistry of anti-A β plaques (D54D2, green) antibodies in VC, CA1, and mPFC of 6-month-old 5XFAD mice after 7 days of 1 h per day of visual GENUS (n = 6 mice per group, scale bar, 50 μ m).
- (R) Average plaque core area per region of interest, normalized to no stimulation control (n = 6 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, unpaired Mann-Whitney test).
- (S) Average number of plaques in AC, CA1, and mPFC following auditory GENUS normalized to no stimulation control (n = 6 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, ***p < 0.001, unpaired Mann-Whitney test).
- (T) Average plaque core area per region of interest, normalized to no stimulation control (n = 6 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, unpaired Mann-Whitney test).
- (U) Average number of plaques in VC, CA1, and mPFC following visual GENUS normalized to no stimulation control (n = 6 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, unpaired Mann-Whitney test).
- (V) Relative soluble A β ₁₋₄₂ levels in AC and HPC in 6-month-old 5XFAD mice after 7 days of 1 h per day no stimulation, 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 8 Hz, and combined (A+V) random frequency stimulation, normalized to no stimulation control (n = 4-5 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, Kruskal-Wallis test with Dunn's multiple comparison test).
- (W) Relative insoluble A β ₁₋₄₂ levels in AC and HPC in 6-month-old 5XFAD mice after 7 days of 1 h per day no stimulation, 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 8 Hz, and combined (A+V) random frequency stimulation, normalized to no stimulation control (n = 4-5 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, Kruskal-Wallis test with Dunn's multiple comparison test).
- (X) A β (12F4) mean intensity value (12F4 antibody) in AC, CA1, and mPFC in 6-month old 5XFAD mice following 7 days of 1 h per day no stimulation, 40 Hz auditory stimulation, combined (A+V) GENUS, combined (A+V) 80 Hz, and combined (A+V) random frequency stimulation, normalized to no stimulation control (n = 4-5 mice per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, Kruskal-Wallis test with Dunn's multiple comparison test).
- (Y) Average number of plaques in mPFC following combined GENUS, summed auditory and visual alone GENUS, normalized to non-stimulation control (n = 6-8 per group, mean SEM in bar graphs, n.s. = not significant, *p < 0.05, one-way ANOVA with Tukey's multiple comparison test).
- (Z) Average area of plaques in mPFC following combined GENUS, summed auditory and visual alone GENUS, normalized to non-stimulation control (n = 6-8 per group, mean SEM in bar graphs, n.s. = not significant, **p < 0.01, one-way ANOVA with Tukey's multiple comparison test).

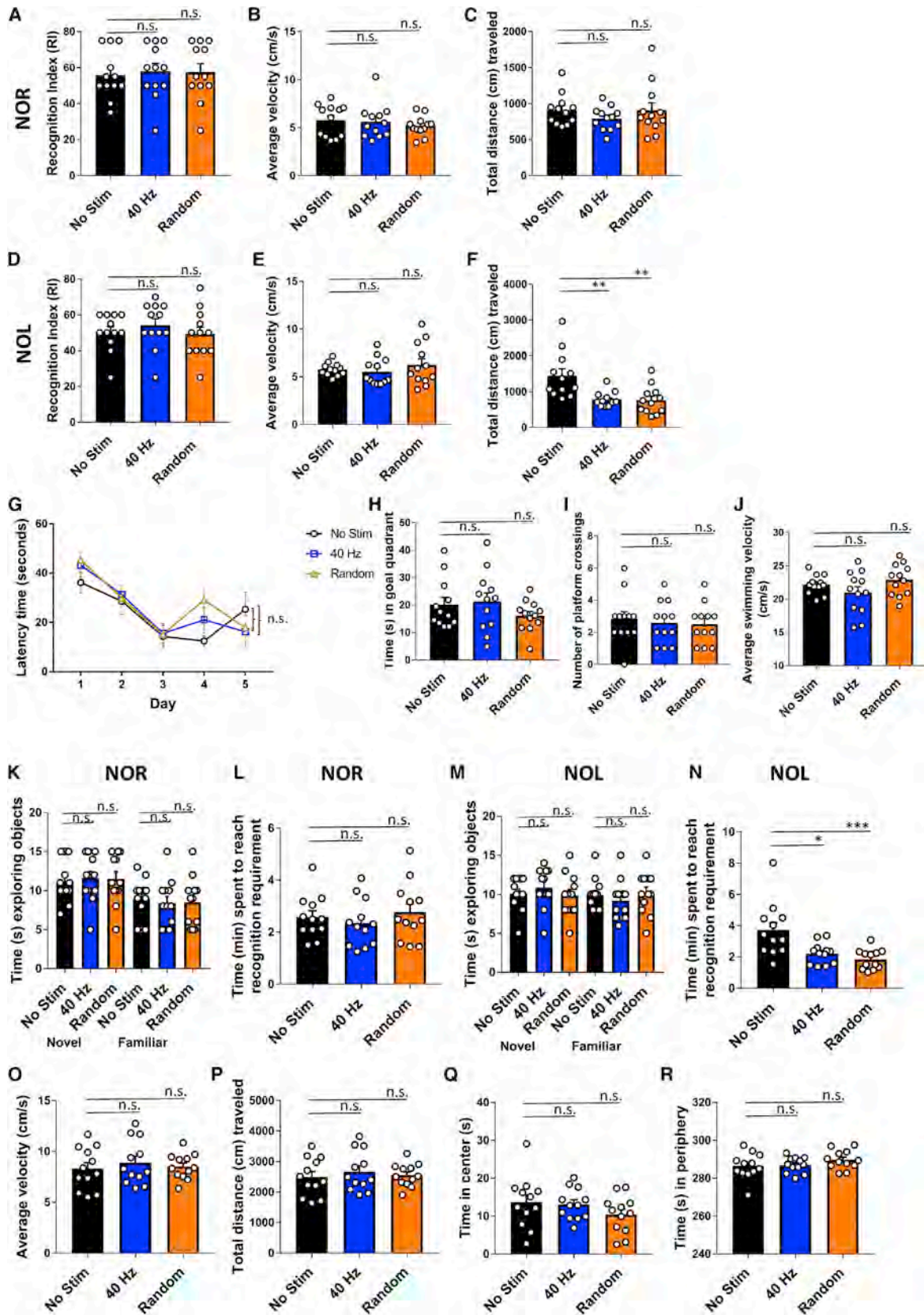


Figure S8. Auditory GENUS Has No Effect on Recognition and Spatial Memory Tasks in WT Mice, Related to Figure 2

(A) Recognition index from novel object recognition (NOR) test in WT mice with auditory GENUS (n = 12 mice in no stimulation group [black], n = 12 mice in 40 Hz group [blue], n = 12 in random frequency group [orange], circles indicate 'n', mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(B) Average velocity (cm/s) during NOR test (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(C) Total distance (cm) traveled during NOR test (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(D) Recognition index of novel object location (NOL) test of WT mice with auditory GENUS (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(E) Average velocity (cm/s) during NOL test (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(F) Total distance (cm) traveled during NOL test (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, **p < 0.01, Kruskal-Wallis test with Dunn's multiple comparison test).

(G) Escape latencies (s) during training in the Morris Water Maze (MWM) of WT with auditory GENUS (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, 2-way ANOVA with Tukey's multiple comparison test).

(H) Time (s) spent swimming in the goal quadrant during the MWM probe trial (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, Kruskal-Wallis test with Dunn's multiple comparison test).

(I) Number of platform crossings during the MWM probe trial (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, Kruskal-Wallis test with Dunn's multiple comparison test).

(J) Average swimming velocity (cm/s) during the MWM probe trial (n = 12 mice in no stimulation group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(K) Time (seconds) spent exploring familiar and novel objects during NOR test of WT non-stimulated, 40 Hz, and random frequency stimulated mice (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(L) Time (min) mice required during NOR test to reach the total object exploration requirement of 20s (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(M) Time (seconds) spent exploring object in familiar and novel location during NOL test of WT non-stimulated, 40 Hz, and random frequency stimulated mice (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(N) Time (min) mice required during NOL test to reach the total object exploration requirement of 20s (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, *p < 0.05, ***p < 0.001, Kruskal-Wallis test with Dunn's multiple comparison test).

(O) Average velocity (cm/s) during habituation (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(P) Total distance (cm) traveled during habituation (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(Q) Time (seconds) spent in the center of the behavior chamber during habituation (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).

(R) Time (seconds) spent in the periphery of the behavior chamber during habituation (n = 12 mice in no stim group, n = 12 mice in 40 Hz group, n = 12 in random frequency group, mean s.e.m. in bar graphs, n.s. = not significant, Kruskal-Wallis test with Dunn's multiple comparison test).