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Induction of specific brain oscillations may restore neural circuits and be used for the treatment of Alzheimer's disease

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Abstract. Brain oscillations underlie the function of our brains, dictating how we both think and react to the world around us. The synchronous activity of neurons generates these rhythms, which allow different parts of the brain to communicate and orchestrate responses to internal and external stimuli. Perturbations of cognitive rhythms and the underlying oscillator neurons that synchronize different parts of the brain contribute to the pathophysiology of diseases including Alzheimer's disease, (AD), Parkinson's disease (PD), epilepsy and other diseases of rhythm that have been studied extensively by Gyorgy Buzsaki. In this review, we discuss how neurologists manipulate brain oscillations with neuromodulation to treat diseases and how this can be leveraged to improve cognition and pathology underlying AD. While multiple modalities of neuromodulation are currently clinically indicated for some disorders, nothing is yet approved for improving memory in AD. Recent investigations into novel methods of neuromodulation show potential for improving cognition in memory disorders. Here, we demonstrate that neuronal stimulation using audiovisual sensory stimulation that generated 40-HZ gamma waves reduced AD-specific pathology and improved performance in behavioural tests in mouse models of AD, making this new mode of neuromodulation a promising new avenue for developing a new therapeutic intervention for the treatment of dementia.

Keywords: Alzheimer's disease, gamma oscillations, neural circuitry, neural oscillations, neuromodulation, translational research.

The offbeat brain

The timing and specific firing patterns of neurons govern all our perceptions and brain functions. Neural oscillations are defined by different frequency bands including δ (delta: 1–4 Hz), θ (theta: 4–7 Hz), α (alpha: 7– 13 Hz), β (beta: 14–30 Hz) and γ (gamma: 30–100 Hz) [1]. Certain frequencies dominate the electroencephalographic (EEG) recording as the brain performs specific functions. For example, slow wave sleep is characterized by the emergence of delta band oscillations and the awake state brings about beta waves.

Abnormalities in neuronal network electrical activity result in neurodegenerative diseases such as PD and AD. PD is a circuitopathy caused by the demise of dopaminergic neurons in the substantia nigra pars compacta. Downstream striatal circuits are thrown into dysfunction, leading to imbalance of direct and indirect pathways through the basal ganglia. The timing of oscillations in the nuclei of the basal ganglia is normally independent, but in PD, beta oscillations become hypersynchronized across the globus pallidus interna, globus pallidus externa, subthalamic nucleus and substantia nigra pars compacta.

Network abnormalities can also be detected in cognitive disorders such as AD including network hypersynchrony and altered oscillatory activity that can precede clinical disease onset [2,3]. In

healthy people, cognitive tasks can increase activation of specific brain regions depending on the task. In these cases, there is concurrent deactivation of the default mode network (DMN), which is a constellation of brain regions that is most active during inwardly oriented mental activities such as introspection, wakeful rest, imagination and recalling [4]. Tasks such as learning can activate the hippocampus while dampening the DMN [5]. However, inadequate deactivation of the DMN during learning is associated with poor memory formation in healthy people and in patients with early-stage AD [6,7]. Hyperactivation of the hippocampus with reduced deactivation of the DMN was found consistently in cognitively normal individuals with cerebral amyloid deposits, in presymptomatic carriers of familial AD mutations and in patients with mild cognitive impairment thought to be due to AD [2,8,9]. Exploring these abnormal, large-scale brain networks has led to possible therapeutic targets and opportunities for earlier intervention for the treatment of AD.

Ways to train the brain

Neuromodulation is a class of therapeutics designed to control local and network-wide neuronal activity by delivering stimulation to targeted sites in the neuroaxis. This stimulation is typically electricity-based; emerging applications have explored alternative modalities including magnetic fields. Methods also include invasive, targeted neurosurgical implantation of electrodes in the case of deep brain stimulation (DBS), optogenetics (used in mice, although there are trials to apply to human diseases that we will not discuss here) and non-invasive methods such as sensory stimulation and transcranial electrical or magnetic stimulation. The underlying theory is that neuromodulation can modify abnormal circuitry so that it returns to a normal physiological state [10]. These methods of neuromodulation work via entrainment, which occurs when an independently oscillating source (i.e. electrical stimuli from an implanted DBS) affects the endogenous, naturally occurring oscillations in the brain so that both systems are synchronized.

Brain stimulation can be categorized into invasive and non-invasive approaches. Invasive approaches for neuromodulation include DBS, spinal cord stimulators used for pain and other nerve stimulators such as vagal or sacral nerve stimulations that target specific nerves. Non-invasive approaches deliver electrical activity to wide areas of the brain; one example is electroconvulsive therapy (ECT), which began in the 1930s for the treatment of schizophrenia. Newer, more refined methods of non-invasive approaches were developed to enable more spatially targeted areas of brain stimulation; these include transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (TES), which includes transcranial direct current stimulation (tDCS) and transcranial alternative current stimulation (tACS). TES methods are still in investigational phases and not yet currently FDA-approved for use in the treatment of human disease.

The differences in risk between invasive and non-invasive neuromodulation are the inherent neurosurgical risks. Fortunately, as neurosurgical techniques and technology have improved over time, the rate of adverse effects has steeply declined. At present, the worst adverse effects after implantation of electrodes for DBS include death in 0.6% and permanent neurological sequelae in 2.8% [11]. Adverse effects due to surgical complications that did not result in permanent neurological sequelae included infection (5.6%), intracranial haemorrhage (3.1%) and post-procedural seizures (1.1%) [11]. Some of these post-surgical complications do require repeat neurosurgery to reposition or remove leads along with additional risks for the patient[12]. In addition to neurosurgical risks, stimulation-related adverse effects of DBS include paraesthesias, facial contractions, dysarthria, dizziness, gait imbalance, hemiballismus and eyelid-opening apraxia, which can sometimes be addressed by manipulating the electrical current or stimulation paradigm [11,13]. DBS adverse effects include (i) cognitive impairment, especially decline in verbal fluency [14–16] and (ii) psychiatric side effects such as apathy or frontal disinhibition and rare suicidality [14]. Despite this adverse effect profile, the benefits clearly exceed the risks in selecting DBS as a treatment option for carefully selected patients with PD: four pivotal randomized, controlled clinical trials (RCTs) done to evaluate the effects of DBS on PD showed that DBS causes improved 'on' time with reduced 'off' states, thereby reducing the need for oral levodopa treatment, which in turn limits dyskinesias [15,17–19].

Stimulating the brain

Much of what we know about neuromodulation is garnered from the treatment of the cardinal

symptoms of PD with DBS. In 1997, DBS was approved by the FDA first for the treatment of first essential tremor (ET) and then PD. Since then, DBS has been approved for symptomatic treatment of dystonia and refractory epilepsy and obsessive–compulsive disorder. Investigations into the use of DBS for treatment of psychiatric disorders such as depression [20], schizophrenia and other neurodegenerative disorders such as AD are ongoing.

The mechanisms underlying how this brain stimulation translates into symptomatic management are still unknown [21–23]. The timeline of symptomatic improvements with DBS can be categorized into immediate, short term and long term. Benefits are first seen in the abatement of tremor, followed by rigidity, bradykinesia and then axial symptoms (e.g. postural instability); this pattern implies that the effects of neuromodulation act locally initially, and then through changes in neuroplasticity, neuronal stimulation can lead to neuronal reorganization for more widespread benefits [22]. DBS may act locally first in the reticular nucleus–ventrobasal thalamic circuit, which is where the rhythm-generating substrate for Parkinsonian tremor is located [24]. DBS might achieve therapeutic efficacy by disrupting the hypersynchronization between the nuclei of the basal ganglia [25]. Understanding the pivotal role the subthalamic nucleus (STN) plays in this circuit has led to its emergence as one of the surgical targets for DBS in the treatment of PD symptoms [26].

Another class of invasive neuromodulation involves stimulation of the spinal cord or specific nerves. Spinal cord stimulators (SCS) are electrodes with IPGs that are implanted in the dorsal root ganglia (DRG) to modulate dysregulated pain pathways [27]. While effective, the mechanisms of action of how this works are still unknown but may involve increases in gamma-aminobutyric acid (GABA) release in the DRG or other inhibitors of pain [28–30]. As with other techniques of invasive neuromodulation, the risks are mainly surgical. The overall complication rate from SCS ranges from 5.3 to 40% [31,32] and includes hardware-related issues, infection, dural puncture that can lead to CSF leak, and spinal epidural haematoma or spinal cord trauma, which are rare but can cause devastating paralysis [33,34].

As noted above, non-invasive neuromodulation began with electroconvulsive therapy (ECT), which was initially used to treat schizophrenia in 1938 [35] and is now also FDA-approved for the treatment of schizoaffective disorder, depression, bipolar disorder, catatonia and neuroleptic malignant syndrome. ECT involves directly applying electricity to the scalp to induce seizure activity. For this therapy to be tolerated, anaesthesia for sedation is required. This has been shown to be safer and more efficacious than any other treatment used for severe depression, resulting in a prompt improvement of symptoms with several large studies showing 70 - 90% remission rate [36–39]. With an unknown mechanism of action, it is hypothesized that ECT corrects underlying neural circuit dysfunction in these disorders by increasing GABA concentration [40] and may also affect neuronal structure and synaptic plasticity [41]. Compared with invasive neuromodulation techniques, the adverse effects of ECT are less significant. They include headache, nausea, myalgias and aspiration pneumonia [42] and can largely be avoided with proper preparation before sessions or can be treated with adjunctive medications. The risk of death due to ECT is low and is comparable to mortality associated with minor procedures involving general anaesthesia [43]. The major adverse effect of ECT is memory loss, which can be found in 50 – 80% of patients as described in seven observational studies of greater than 1000 patients [44]. However, cognitive impairment is thought to be generally short-lived with return to near baseline after more than 2 weeks after ECT [45]. As with other non-invasive brain stimulation approaches, access to centres that offer ECT is variable and limited, limiting the utility of this therapeutic.

TMS is another non-invasive brain stimulation approach with FDA approval for treating depression. A metal coil is placed against the scalp to generate rapidly alternating magnetic fields that can cause neuronal depolarization in specific, targeted areas of the cortex and can thus be used to modulate neural network activity [46]. This is better tolerated than ECT as it requires neither anaesthesia nor the induction of seizures, but it is not as efficacious as ECT for the treatment of depression [47]. Side effects can include rare tonic–clonic seizure, which can occur in 0.1 – 0.5% of patients, but more commonly, patients complain of headaches, scalp pain, transient hearing issues or vasovagal syncope [48].

Electrical stimulation can also be applied transcranially for neuromodulation, such as with tDCS and tACS. These approaches are still

investigational, but small studies have shown efficacy in improving mood in unipolar major depression and bipolar disorder [49,50]. However, there are reports that treatment with escitalopram was more efficacious than active tDCS for the improvement in depression [51]. tDCS is generally safe and well tolerated with only transient adverse effects such as uncomfortable scalp sensations, tinnitus or nervousness [51]. There are conflicting reports as to whether tDCS causes adverse effects on cognition, but one study showed that memory can improve with active treatment [52]. tACS has been studied extensively to use in psychiatric disorders and to improve memory by correcting neural circuits involved in cognition [53]. This technique is unique in that it can link specific frequency ranges to cognitive processes by generating sinusoidal currents that are bound to a single frequency [54]. It has been used to dissect the influence of specific brain oscillations related to memory. While generally well tolerated, adverse effects are similar to tDCS including skin reactions such as itching, tingling and redness under the electrodes used to deliver current [55].

Training the brain to improve memory

Memory formation is thought to rely on changes in synaptic connectivity and synchrony between populations of neurons. An emerging body of work now supports that specific brain oscillations affect neural mechanisms involved in the formation, maintenance, consolidation and retrieval of memories [56]. Different memory processes are thought to be related to specific oscillatory signatures [56], and recently, different brain stimulation approaches have been used to entrain the brain to probe the causal relationship between memory and various frequencies of brain oscillations (see Table 1).

Sensory stimulations such as light flicker or audiovisual combinations have been used in small clinical studies to see whether inducing oscillatory entrainment would affect memory [57,58]. Light flicker at specifically 10Hz improved memory encoding as compared to other frequency controls (8.7 and 11.7Hz); however, this experimental paradigm was aimed at evaluating immediate recognition memory and not long-term effects on memory [57]. Clouter et al showed that synchronized auditory and visual stimuli in the form of movies at 4Hz lead to improved memory, which supports the possibility of using sensory stimulation to possibly augment cognition in diseases such as AD [58].

TACS in the gamma frequency range has also been used to improve abstract reasoning, working memory and insight in cognitively normal people [59–61]. In studies performed by Sartanarnecchi et al, TACS is used at 40Hz to target specific brain regions, such as the prefrontal cortex in this case, to improve fluid intelligence, while other frequencies tested including 5Hz and random frequency stimulation (101 – 640Hz) were not effective at improving response speed in cognitively normal subjects [60]. TACS at 40Hz but not at 10Hz over the temporal lobe improved accuracy on a verbal insight task [61]. These studies suggest that externally influenced neuromodulation in the gamma range can be used to improve cognition.

DBS has also been studied to see whether neuromodulation is effective for improving cognition in AD. The fornix is a thick bundle of axons that serves as a major pathway in the Papez circuit important for memory. It constitutes a major inflow and outflow pathway between the hippocampus, hypothalamus and other entorhinal cortex, allowing for the formation of memories. Lesions in the fornix can cause amnesia and learning impairment [62–64]. The fornix was discovered as a potential target for DBS for the treatment of AD when the ventromedial and lateral hypothalamus was stimulated for the treatment of morbid obesity [65]. Hypothalamic lesion surgeries were previously used to treat obesity, and thus, DBS was attempted in this region to control appetite. During the intraoperative trial of DBS, detailed autobiographical memories were provoked with increasing vividness as the voltage was increased >3V. This was replicated when fornix-region DBS was used in a randomized trial involving 42 patients with mild AD [66]. With increasing stimulation, 20 patients (48%) experienced retrieval of distinct autobiographical episodes with acute high-intensity stimulation. These episodes were often described as flashbacks that are consistent with the iconic reports of Penfield and Perot of complex 'experiential phenomena' with cortical electrical stimulation [66–68].

Standardized low-resolution electromagnetic tomography (sLORETA) was used to map areas of focal change in EEG activity in response to hypothalamic DBS showing that unilateral stimulation of the electrode closest to the fornix evoked

Table 1 *Cognitive rhythms currently explored for improving cognition and mood using neuromodulation*

Frequency	Function	Therapeutic uses	References
δ (delta: 1–4 Hz)	Deep sleep, coma, repair	rTMS impacts spatial WM	Lafon et al. 2017 [120] Ribeiro et al 2018 [121]
θ (theta: 4–7 Hz)	Drowsiness	Synced audiovisual entrainment with video and sound at 4hz improves memory TMS delivering theta burst stimulation for treatment-resistant depression	Clouter et al. 2017[58] Lafon et al. 2017 [120] Fitzgerald et al. 2020 [47]
α (alpha: 7– 13 Hz)	Alert, physically and mentally relaxed	Alpha tACS stabilizes visual attention Alpha TMS improves behavioural and functional outcomes in children with autism 10-Hz light flicker improves recognition memory	Clayton et al. 2018 [122] Dickinson et al. 2018 [123] Kang et al. 2019 [124] Williams et al. 2001 [57] Zhang et al. 2019 [125] Dombrowe et al. 2015 [126]
β (beta: 14–30 Hz)	Perception, mental activity, thinking, focusing and sustained attention	Modulates risk-taking, decision-making, frequency-specific	Yaple et al. 2017 [127]
γ (gamma: 30–100 Hz)	Extreme focus, learning, higher cognitive processing	tDCS with cognitive training increases functional connectivity in obese patients Modifies fluid intelligence through a few modalities tACS at 40Hz enhances face and object perception tACS at 40Hz enhances abstract reasoning, fluid intelligence and insight	Forcano et al. 2020 [128] Brem et al. 2018 [129] Lundqvist et al. 2016 [130] Gonzalez-Perez et al. 2019 [92] Santarnecchi et al. 2013 [59] Santarnecchi et al. 2016 [60] Santarnecchi et al. 2019 [61]

increased activity in the ipsilateral hippocampus and parahippocampal gyrus [65]. This supported the hypothesis that neuromodulation of the fornix evokes autobiographical memory by activating medial temporal lobe structures. Furthermore, after 3 weeks of continuous stimulation, the patient with hypothalamic/ fornix DBS showed improved performance on the California Verbal Learning Test and the Spatial Associative Learning Test compared with his preoperative baseline scores [65,69].

Rodent studies of fornix stimulation using DBS show that there is a rapid activation of the hippocampus, as well as an increase in neurotrophic factors (e.g. brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF)) and synaptic markers (e.g. growth-associated protein 43 (GAP-43), synaptophysin) [70]. These results suggest that forniceal DBS improves memory processing by activating the hippocampus and strengthening synaptic connections. Forniceal DBS or stimulation of other nodes of the Papez circuit in rodent models was shown to improve hippocampal memory [71,72].

With these encouraging results, Phase I and Phase II trials were done in patients with mild-to-

moderate AD to assess the safety and feasibility of using bilateral fornix DBS to treat AD [73,74]. In Phase I, 6 patients with mild-to-moderate AD had DBS to the fornix at 3.0 to 3.5V with the frequency set at 130Hz and pulse width at 90 microseconds. After continuous stimulation for 12 months, neural activity was increased in the memory circuit including the entorhinal and hippocampal areas. The default mode network (DMN), which we noted is important for memory, and for self-knowledge or for thinking of others, is known to be dysregulated in AD. After 12 months of forniceal DBS, there was improved connectivity in the DMN [74]. In addition, there was reversal of impaired glucose utilization in the temporal and parietal lobes that was first seen at one month after continuous forniceal DBS and was persistently maintained after 12 months of usage. With no serious adverse events, 42 patients with mild AD were enrolled in a Phase II multicentre, double-blinded RCT to determine whether forniceal DBS would improve aberrant circuitry caused by AD [73]. While DBS for AD was found to increase cerebral glucose metabolism after 12 months of continuous stimulation, there was no difference in cognitive outcomes between the stimulated and sham groups. Forniceal DBS therefore seems to activate the fornix, thereby driving trans-synaptic activity to modulate the dysfunctional brain networks in AD [73]. A larger Phase III trial is required to determine the efficacy of forniceal DBS as a treatment of AD moving forward.

Traditionally, therapies were targeted against known pathophysiology seen in the course of AD (Table 2). More recent studies focusing on neural circuits and others noted in Table 1 employ different methods of neuromodulation including tDCS, tACS, TMS, sensory stimulation (i.e. light and sound) and DBS to improve memory and serve as proof of concept that neural oscillations can be manipulated and leveraged to improve cognition and pathology underlying AD.

Driving gamma in AD mouse models

Gamma oscillations (30-80hz) in the brain have been observed to play a critical role in a variety of memory functions across mice, rats, non-human primates and humans [75–77]. Additionally, gamma oscillatory activity has been observed to be altered in the hippocampus of rats performing spatial working memory tasks [78], in the visual cortex of cats encoding of visual features of stimuli [79], and in working memory function in humans (Howard, 2003). Given the important role of gamma in memory encoding and recall, it is fitting that we also see dysfunction of this activity in neuropsychiatric disorders. In patients with schizophrenia, it has been observed that decreases in gamma activity correlate with negative symptoms, while increased gamma activity is produced during positive symptoms, such as hallucinations [80]. In Alzheimer's dementia, MEG recordings have shown that patients exhibit significant decreases in gamma band synchronization [81]. A reduction in this synchronous activity has been observed in both patients with AD and mouse models of the disorder [81–83].

Given their relevance to a variety of memory functions, several methods of inducing gamma oscillations directly have been investigated. Experimental and theoretical modelling previously summarized by Bartos, et al. indicates that gamma oscillations, specifically in the hippocampus, rely on local networks of synaptically connected GABAergic interneurons [84]. First, basket cells have been shown to produce action potentials at high frequency during gamma activity *in vivo*, with single spikes phase-locked to the oscillations of the field potential [85]. Secondly, chemically isolated networks of these inhibitory interneurons *in vitro* have been observed to oscillate at gamma frequency in response to glutamate receptor activation [86]. Finally, computational modelling has shown that mutually connected interneurons are able to generate coherent action potential activity in the gamma frequency range given a consistent excitatory input [84]. Furthermore, chemical application of kainate receptor agonists was also found to be able to induce gamma oscillations in the hippocampus through the production of phase-locked action potentials generated from fast-spiking interneurons within the region [87]. Overall, these previous studies indicate that fast-spiking interneurons play a crucial role in the generation and maintenance of hippocampal gamma oscillations.

Using more direct methods, Cardin, et al. used optogenetics to directly drive these fast-spiking parvalbumin interneurons and were able to show that this subset of neurons plays a major role in inducing and synchronizing gamma oscillations within the mouse cortex [84,88,89]. The phase of this induced gamma cycle had a significant impact on the response amplitude to visual stimuli, further supporting the hypothesis that gamma oscillations play a role in gating of sensory stimuli.

Table 2 *Targeted therapies against specific Alzheimer's pathology*

AD Pathology	Mechanism of targeted therapies	Examples
Circuit abnormalities	Gamma frequency abnormalities	Sensory stimulation
		TACS
	Papez circuit	DBS fornix
Amyloid plaques	Amyloid transport	Targeting LRP1
	Secretase enzymes	Bryostatin 1
		GRL-8234
		Semagacestat
	Amyloid aggregation	Tramiprosate
		ELND005
		Colostrinin
	Amyloid clearance	Somatostatin
		Plasminogen inhibitor
	Anti-amyloid vaccinations	Aducanumab
Tau tangles	Blocking aggregation	Methylene blue
	Anti-tau vaccinations	Semorinemab (passive)
		ACI-35 (active)
		AADvac1 (active)
	Stabilizing microtubules	Epothilone D
		Abeotaxane
		Davunetide
	Modulating tau phosphorylation	Lithium
		Tideglusib
		Saracatinib
	Reducing tau oligomerization	Lansoprazole
		Astemizole
		Methylene blue
	Tau clearance	Nilotinib
	Reducing tau expression	BIIB080 (antisense oligonucleotide)
Neurotransmitter imbalance	Acetylcholinesterase inhibitors	Donepezil[a]
		Rivastigmine[a]
		Galantamine[a]
	NMDA receptor antagonists	Memantine[a]
	GABA modulation	Etazolate
		SGS-742

[a]FDA-approved therapies.

Additionally, non-invasive technologies have been shown to be capable of inducing oscillations within the gamma range. Iaccorino, et al. used LFP recordings to show increased 40-Hz power during visual presentation of a 40-Hz flicker [90,91]. Transcranial alternating current stimulation administered to human subjects at 40 Hz was also found to have a positive impact on gamma oscillation-related memory tasks, such as face and object perception [92]. Leveraging non-invasive methods to induce gamma oscillations to improve memory is currently an active focus of research in our laboratory.

GENUS in AD mouse models

Our group previously demonstrated that gamma frequency entrainment (i.e. enhancement of gamma oscillations in response to periodic stimulation) reduces amyloid load and modifies microglia in AD mouse models, effectively reducing AD pathology [91]. In 5xFAD mice, a well-established AD mouse model, 40-Hz optogenetic stimulation of fast-spiking, parvalbumin-positive (FS-PV) interneurons in hippocampal subregion CA1 led to a sharp increase in the 40-Hz power in the LFP recordings [91]. After one hour of the stimulation, amyloid-β (Aβ) levels in CA1 were significantly reduced [91]. Interestingly, optogenetically driving other cell types at 40 Hz or driving FS-PV interneurons at frequencies other than 40 Hz did not reduce amyloid levels, suggesting that the effect of gamma stimulation on amyloid load is cell-type- and frequency-specific. Significant decreases in the levels of cleavage intermediates of amyloid precursor protein (APP) and increase in the number of phagocytic microglia and microglia-Aβ co-localization (suggesting more Aβ uptake by microglia) indicate that the reduction in amyloid levels was likely due to lower Aβ production coupled with improved Aβ clearance (Fig. 1).

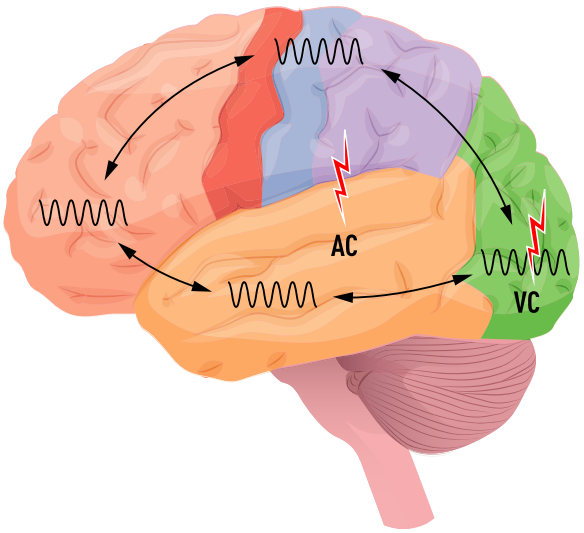

Fig. 1 *Visual and auditory stimulation induces gamma entrainment at 40Hz in the auditory and visual cortex leading to enhanced gamma frequency synchronization in the visual cortex, somatosensory cortex, temporal lobe and prefrontal cortex. AC: auditory cortex. VC: visual cortex.*

Based on these findings, we explored a non-invasive sensory approach, which we call gamma entrainment using sensory stimuli (GENUS), to test the hypothesis that visual stimulation with LED lights flickering at 40 Hz can also lead to gamma frequency entrainment and a reduction of amyloid levels in the primary visual cortex (VC) of AD mouse models. Similar to the optogenetic stimulation, the 40-Hz visual stimulation in 5xFAD mice increased the 40-Hz power in the LFP recordings from VC, and an hour-long stimulation resulted in significantly reduced Aβ levels, more phagocytic microglia and increased microglia-Aβ co-localization in VC compared with the exposure to a dark condition for the same duration. The reduction in Aβ levels in 5xFAD mice was specific to the 40-Hz flicker frequency: Constant light and 20-Hz, 80-Hz and random frequency flicker conditions did not show a significant change from the dark condition. 40-Hz light was also effective at decreasing the Aβ levels in another AD mouse model, APP/PS1, suggesting that the effect of 40-Hz visual stimulation on the amyloid load is not restricted to a specific transgenic expression. In addition, daily stimulation with the 40-Hz light for seven days (one hour/day) decreased the number and size of amyloid plaques in VC of 5XFAD mice. Tau phosphorylation, another major AD-related pathology, was also significantly reduced in VC of TauP301S tauopathy mice following seven days, one hour/day exposure to the 40-Hz flickering LED lights [93]. These results suggest that visual GENUS can potentially provide an effective method for ameliorating AD pathology.

Our group recently demonstrated that repeating auditory tones at 40 Hz is another effective sensory-based method of inducing gamma entrainment and affecting AD pathology [94]. In the auditory cortex (AC) and hippocampal area CA1, we found strong 40-Hz modulation of spiking activity in response to a train of 10-kHz tones repeated at 40 Hz. When this auditory stimulation was used for seven days (one hour/day) on 5xFAD mice, their performance on hippocampal-dependent memory tasks was significantly improved compared with the non-stimulated mice. This week-long stimulation also reduced amyloid load (in 5XFAD mice) and tau phosphorylation (in TauP301S mice) in both AC and CA1. Similar to the 40-Hz visual stimulation, microglia seem to be playing a role in affecting AD pathology following the 40-Hz auditory stimulation, as demonstrated by a significantly higher number of phagocytic

microglia and more microglia-Aβ co-localization in both AC and CA1 of 5xFAD mice compared with the no-stimulation condition. The effect of 40-Hz auditory stimulation also extended to astrocytes and vasculature, with the week-long, one hour/day stimulation significantly increasing the number of reactive-like astrocytes, blood vessel diameter and co-localization of lipoprotein receptor-related protein 1 (LRP1) and Aβ in both AC and CA1 of 5xFAD mice. Since astrocytes are implicated in waste clearance [95,96], and the vasculature in the brain may contribute to amyloid clearing via LRP1-mediated amyloid transcytosis [97,98], the effect of the 40-Hz auditory stimulation on astrocytes and vasculature may be increasing Aβ clearance and consequently contributing to reduced amyloid load. Furthermore, concurrent visual and auditory stimulation at 40 Hz extended the gamma frequency entrainment and amyloid reduction to the prefrontal cortex that was not affected by either visual or auditory stimulation alone. The combination of different stimulation modalities thus provides a way of propagating the beneficial effects of gamma frequency entrainment beyond the primary sensory areas of the brain and can potentially influence AD pathology at a larger scale (Fig. 2).

Our group also investigated whether visual GENUS can directly impact features of neurodegeneration, such as neuronal and synaptic loss and inflammatory response [93]. We first found that, in wild-type C57BL/6J mice, 40-Hz visual stimulation delivered using LED lights increases 40-Hz oscillations in VC, somatosensory cortex (SS1), CA1, and prefrontal cortex (PFC), both in mice naive to the stimulation and those that had been stimulated daily for 42 days (one hour/day), suggesting that visual GENUS persists even after chronic exposure to the stimulation. In Tau P301S (PS19) mice, which have reduced neurons and synapses when they reach 8 months of age [99], we found that 1 hour/day of visual GENUS for 22 days preserved the number of neurons in VC and CA1 when the stimulation was started at 7.5 months of age. We used visual GENUS in another mouse model of neurodegeneration called CK-p25 mice that experience significant neuronal and synaptic loss, as well as cognitive impairment after 6 weeks of p25 induction [100,101]. When we stimulated these mice 1 hour/day with visual GENUS during the 6-week p25 induction period, we found that neurons in VC, SS1, CA1 and cingulate cortex (CC) could be preserved, with significantly less cortical shrinkage and ventricle expansion compared with non-stimulated mice. In addition to the neuroprotective effect, chronic visual GENUS had a positive impact on synapses and neuronal integrity in both CK-p25 and P301s mice, which showed higher levels of synaptic density and lower levels of DNA damage compared with non-stimulated controls after 6 weeks and 22 days of visual GENUS, respectively.

Six weeks of visual GENUS also improved hippocampus-dependent spatial learning and memory in CK-p25 mice, which exhibited significantly lower latencies in finding the platform over training days for the Morris water maze (MWM) task, as well as higher number of platform crossings and time spent in the target quadrant during the task, compared with non-stimulus controls. Visual GENUS delivered for 22 days had the same effect on P301S mice, although the results were not as significant. Interestingly, aged wild-type mice (C57BL/6J mice at 17 months of age) also showed an improvement in spatial learning after five weeks of visual GENUS, which suggests that behavioural performance related to spatial learning and memory can be positively changed by chronic visual GENUS.

Our investigation into the effect of chronic visual GENUS on microglia in CK-p25 mice revealed that the number of microglia is reduced in VC, SS1, CA1 and CC and that these cells show morphological changes related to less inflammatory state compared with no stim controls after 6 weeks of visual GENUS. Similar changes in microglial morphology were observed in P301S mice after 22 days of visual GENUS, suggesting that chronic visual GENUS reduces inflammatory response by microglia. It is worth noting that the morphological changes that were induced after visual GENUS in CK-p25 and P301S mice are seemingly in the opposite direction from those observed in 5XFAD mice (as described above, visual and auditory GENUS in 5XFAD mice led to more phagocytic microglia). This difference suggests that the impact of GENUS on microglia may be disease-specific and favour homeostasis rather than unidirectional influence in every disease and condition (also see Adaikkan and Tsai, 2019, for potential explanation for the difference).

40-Hz stimulation in mouse models also exerts secondary benefits to other systems, including neuroimmune signalling and circadian function, and appears to be even more beneficial when

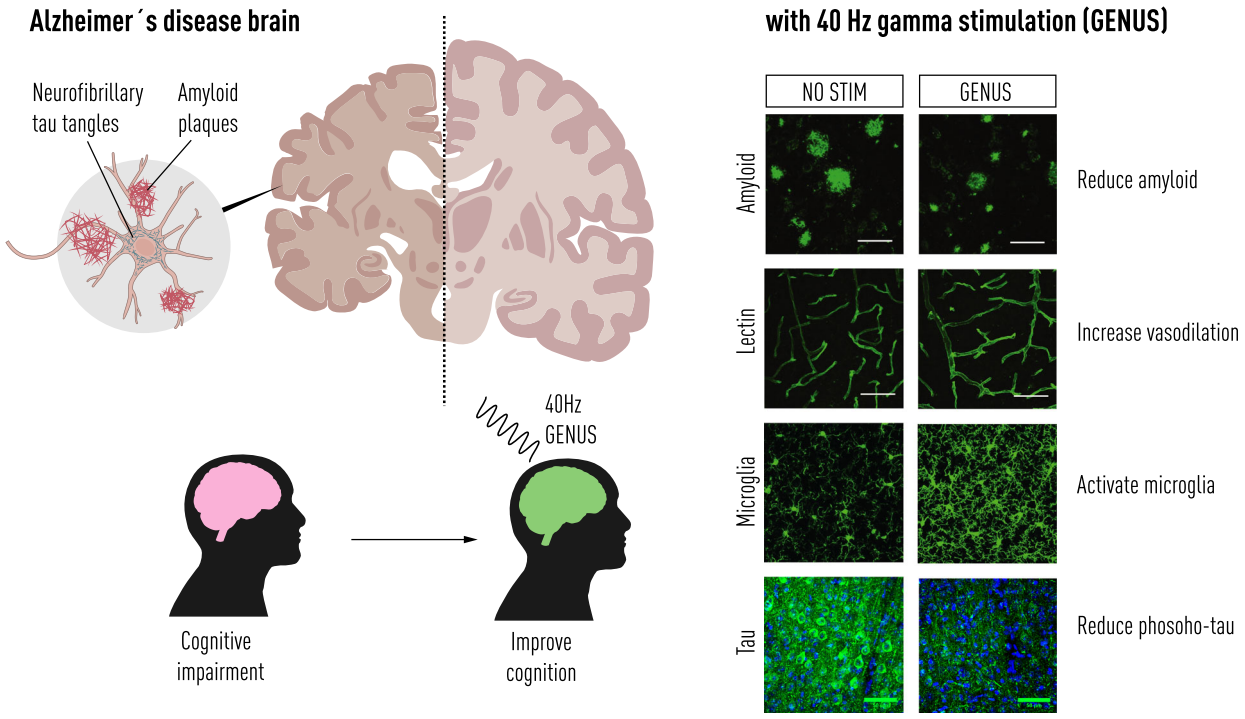

Fig. 2 *Effects of 40-Hz light and sound GENUS on the AD brain. Our studies in mouse models of AD show that chronic exposure to 40-Hz light and sound GENUS reduces amyloid and phosphorylated tau in the brain. We hypothesize that improved synchronization of gamma oscillations in the brain triggers clearance of these pathological markers of AD, which can occur via pathways involving activated microglia or increased blood flow through the brain. Improved gamma oscillations also lead to improved cognition.*

applied simultaneously with exercise [102–104]. One month of daily hour-long 40-Hz light flicker rescues central clock gene expression deficits present in APP/PS1 mice, including CLOCK, BMAL1 and PER2, suggesting that gamma stimulation could be having a direct impact on the suprachiasmatic nucleus and underlying circadian rhythms [102]. In 3xTg-AD mice, 40-Hz visual stimulation for three months combined with an exercise regimen (use of a treadmill) produces a synergistic effect by which improvements in working memory and Aβ and tau burden in the hippocampus are more robust than the effects found in 40-Hz or exercise conditions alone [103]. Additionally, 40-Hz visual stimulation in wild-type mice was found to modulate both NF-KB and MapK signalling pathways, both of which have been shown to play a role in Alzheimer's pathology and cognitive dysfunction [104]. Optimizing the 40-Hz protocol and examining its effects on secondary systems have great potential to grow GENUS as a treatment of AD.

As demonstrated in our mouse studies, visual and auditory GENUS entrains neurons not only in sensory cortices but also in deeper brain regions such as the hippocampus [93,94]. Fast-spiking interneurons are critical for the maintenance and generation of hippocampal gamma oscillations [84,87,88]. Theoretically, fast-spiking interneurons can conduct and coordinate oscillatory activity from sensory areas to other anatomically connected areas such as the hippocampus. In humans, gamma oscillations can conduct through the inferior longitudinal fasciculus which directly connects the occipital and anterior temporal lobes (Fig. 3a) [105]. An additional possibility is that visual gamma stimulation can be carried through the ventral visual pathway from the occipital lobe into inferior and lateral regions of the temporal lobe, where the visual cortex and the hippocampus are located, respectively [106]. Auditory stimulation is perceived in the Heschl's gyrus containing the primary auditory cortex in the human brain and is located directly in the temporal lobe. Visual

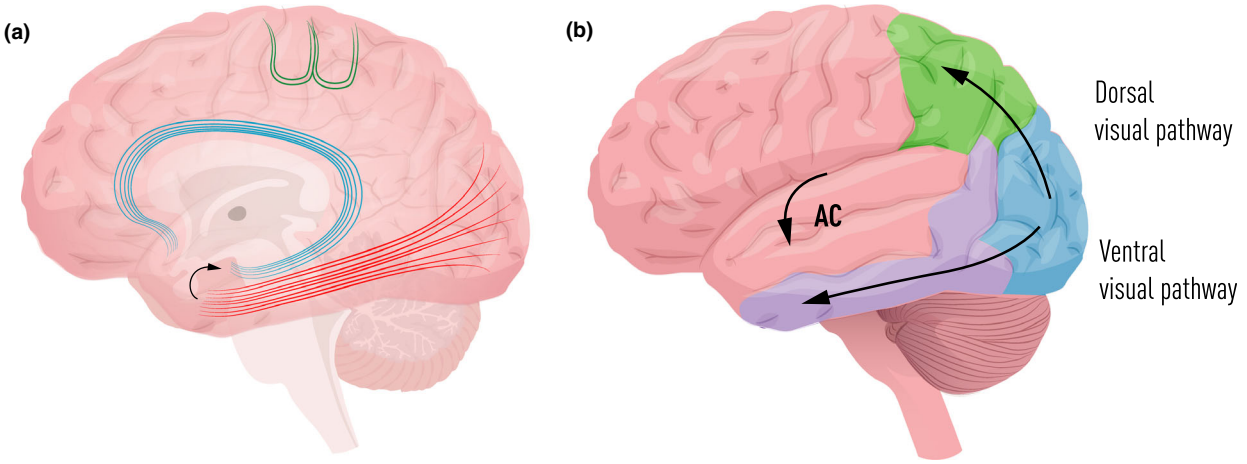

Fig. 3 *Theoretical mechanism of how visual and auditory gamma stimulation entrains sensory cortices and affects the hippocampus. (a) Conductance of visual stimulation from the occipital lobe to the temporal lobe through the inferior longitudinal fasciculus. (b) Conductance of auditory gamma stimulation through the auditory cortex to the hippocampus and visual gamma stimulation from the visual cortex to the temporal lobe through the ventral visual pathway (AC, auditory cortex).*

and auditory stimulation likely conducts LFP in the temporal lobe to the hippocampus through volume conductance (Fig. 3b).

Inducing gamma entrainment in AD patients

While an expansive body of literature points towards the potential of modulating brain rhythms (including theta and gamma to improve memory), the translational efficacy in AD has yet to be elucidated. Presentation of 40-Hz stimuli in animal models of AD shows promising molecular and behavioural improvements, but the extent to which those findings can be replicated in humans is unknown. Early studies primarily served to confirm that humans can entrain visual or auditory stimuli and that the underlying connections can be improved by the presentation of these rhythmic stimulations. Among various gamma frequencies, the presentation of a 40-Hz auditory stimuli created the highest response, as measured by PSD and optimal regional cerebral blood flow [107]. Most striking, however, is the finding that the entrainment due to 40-Hz stimuli is not limited to the auditory cortex but spreads elsewhere unlike the other gamma frequencies tested. In addition, 40-Hz oscillations may be integral in supporting cortical arousal and sensory processing [108]. The precise dynamics and scope of stimulation are of paramount importance. In order to have a desired outcome, multiple regions must respond. In the left temporal and parietal cortex, which is implicated in visual working memory, anti-phase gamma tACS, between T5 and CP1, leads to the most drastic improvements from baseline relative to other relative phase differences [109]. Gamma oscillations across multiple brain regions must be explored with respect to the particular interaction among those regions and the optimal phase differences that yield improved cognitive outcomes. In a first-in-human study, presentation of visual stimuli at 40 Hz led to marked entrainment in disparate brain regions [110]. This work shows that 40-Hz entrainment can be feasibly translated to humans. Applying 40-Hz protocols in a presymptomatic population will best demonstrate the safety, feasibility and efficacy of such an intervention.

Gamma entrainment in other neurological disorders

Inducing gamma oscillations using non-invasive techniques was used to mitigate cognitive dysfunction due to cerebral ischaemia [111]. In this study, temporary occlusion of bilateral common carotid arteries (i.e. 2 vessel occlusion or 2VO) was used to induce selective ischaemic damage to the hippocampal CA1 pyramidal neurons in mice. Using light flickering at low gamma frequency between 30 and 50 Hz, hippocampal CA1 low gamma

oscillations were restored. Neurons in this region were also protected from ischaemic damage by enhancing RGS12-regulated N-type CaV2.2 voltage-gated calcium channel-dependent synaptic plasticity [111]. Light flicker at 40Hz also mitigated cognitive deficits caused by the 2VO model. While it was postulated that improved synaptic plasticity underlies the efficacy of 40-Hz entrainment in improving cognitive function in this mouse model of cerebral ischaemia, it is also possible that 40 Hz induced improved vasodilation to also play a positive role. Our laboratory found that 40-Hz entrainment robustly enhanced vasodilation in the sensory cortex and the hippocampus after seven days of daily GENUS in AD mouse models [94], which could presumably reduce ischaemia in the 2VO mouse model. Further studies are necessary to evaluate the utility of GENUS in preventing ischaemic damage in the brain.

Gamma as a biomarker for AD

The power of predicting cognitive decline using signatures of brain rhythms as a potential precursor to this decline is of growing interest. In the past, it has been posited that electrophysiology alone cannot diagnose AD, while EEG is a cost- and time-effective measure to screen individuals with distinct signatures of conversion to AD [112]. Previous work has demonstrated the ability to extract relevant biomarker information from electrophysiology, across different bands and stimulation protocols [113–115]. Stimulating the left superior frontal cortex with TMS (0.5 - 0.6 Hz) in young, older and AD patients to assess cortical excitability shows reduced TMS-evoked potential (TEP) specifically in the AD population [116]. In a study of amyloid-positive and amyloid-negative patients as assessed by PET imaging, the EEG alpha frequency reactivity between eyes-closed and eyes-open conditions was reduced in AB+, individuals without dementia, after correction for other influencing factors [117]. Increasing the capacity of clinicians to track more nuanced functions of a patient's cognition using brain rhythms may provide greater insight into the onset of a disease-related decline and dictate proper and informative interventions. Many believe the classical pathologies of AD present as early as a decade before any cognitive deficits present, suggesting this latency is an advantageous period to intervene [118].

Within subjects qualifying as AD preclinical stage 1 as defined by Sperling et al (2011) who also have an amyloid burden over threshold, spectral entropy and PSD in the gamma band are reduced in an inverted 'U' shape manner [119]. Among individuals defined as AD preclinical stage 2, especially at the higher end of amyloid load, multiple EEG metrics are impacted (e.g. reductions in PSD beta and gamma). EEG metrics that were originally trending towards statistical significance among the whole preclinical cohort show stronger inverted U-like shapes in this second stage, suggesting some form of compensatory mechanism that is exacerbated as an individual comes closer to formal AD diagnosis. Tracking of brain rhythms may encourage patients to be enrolled in targeted interventions earlier as well.

Conclusions: The future of GENUS

In an emerging body of work, our laboratory demonstrated that GENUS light and sound at 40 Hz can activate the brain and reverse characteristics of AD pathology in mouse models of the disease and improve performance in memory and behavioural testing. As we work towards delineating the underlying mechanisms that allow for the clearance of amyloid and phospho-tau from the brain during and after GENUS intervention, we have preliminarily shown that GENUS may improve pathology through multiple clearance mechanisms, including activation of microglia and blood vessel dilation for improved elimination. Moreover, GENUS improves performance on recognition and spatial learning and memory tasks in mouse models of AD. We have recently developed prototype devices that safely and non-invasively entrain the human brain at 40 Hz using both patterned light and sound, driven to deliver precise, engineered and synchronized light and sound to entrain the brain at 40 Hz. GENUS prototype devices can be used in the home setting making them highly scalable and accessible. Multiple clinical trials are ongoing to examine early effects of GENUS on patients with mild AD. With these GENUS devices, our laboratory aims to alter the devastating course of AD by manipulating neural circuits to facilitate treatment at a systemic level.

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Conflict of interest

LHT is a scientific co-founder, SAB member and Board of Director of Cognito Therapeutics.

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