

Mechanistic investigation of Temporal Interference brain stimulation and its translational application

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Abstract

Temporal Interference (TI) is a recently proposed non-invasive deep brain stimulation technique. It uses two kHz electric fields that differ by a small frequency difference, Δf , producing amplitude-modulated signal where the fields overlap. Early work showed that neurons responded to the Δf oscillation but filtered out the kHz fields, suggesting TI could achieve targeted stimulation away from the electrodes.

The mechanism of action of TI is not completely understood and has so far been primarily investigated at the single-cell level. In this thesis, I extend the mechanistic investigation by characterising TI-evoked network-level neuronal activity *in vivo*. Using widefield calcium imaging, I mapped, for the first time, the direct spatiotemporal effects of TI relative to control, low frequency stimulation. My results highlight important differences between these conditions and point towards the potential contribution of network-level interactions to the effects observed. At the spatial level, responses measured were in agreement with the existing finite element method models. At the temporal level, TI, surprisingly, induced contralateral activation and a secondary harmonic response at $2\Delta f$, suggesting possible recruitment of different cell types. I also found that pure kHz fields can induce transient onset responses, which can be further mitigated, but did not lead to a depolarisation block.

Having established the network-level characteristics of TI, I investigated the therapeutic potential of different frequency protocols to target circuit dysfunction in the App^{NL-G-F} model of Alzheimer's Disease. Two pilot studies performed suggest that TI may be able to affect relevant disease biomarkers. Firstly, stimulation in the delta range transiently reduced cortical excitability. Secondly, theta stimulation increased hippocampal neurogenesis markers and, potentially, microglial presence after eight days of repeated stimulation in freely moving mice.

Overall, these results suggest that TI can effectively drive network-level neuronal dynamics and potentially correct aberrant activity patterns, highlighting its usefulness for translational applications.

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Statement of Publications

A subset of the data presented in **Chapter 3** has been published in Brain Stimulation journal (Luff et al., 2024a).

The data presented in **Chapter 3** is being further analysed and prepared for publication.

Further experiments are being conducted before the data presented in **Chapters 4** and **5** are submitted for publication.

Statement of Originality

Surgical procedure for calcium imaging experiments was developed jointly with Nawal Zabouri. Surgical preparation for and electric field recordings reported in **Chapter 3** were primarily done by Maria Garcia Garrido with my assistance. The technical setup of the equipment for the electric field recordings was done by Xiaoqi Zhu. Pulse-width modulated TI figure (**Figure 3.19**) was created by Charlotte Luff. **Figure 5.1A** and experimental schematics in **Figure 5.13** and **Figure 5.14** were created by Maria Garcia Garrido.

Experimental design and data collection for **Chapter 4** was done jointly with Joseph Airey.

Experimental design, surgeries and data collection reported in **Chapter 5** were done jointly with Maria Garcia Garrido. Behavioural data analysis was performed by Maria Garcia Garrido. Immunohistochemistry was done by Marcelina Wojewska, Maria Otero Jimenez, Maria Garcia Garrido, Sofia Peressotti and Alia Tanbouz. Manual part of the IHC analysis (ROI selection) was done jointly with Marcelina Wojewska, Maria Otero Jimenez, Maria Garcia Garrido and Sofia Peressotti.

All other work contained in this thesis is my own and conforms to the rules and guidelines set out for PhD theses by Imperial College London.

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List of Abbreviations

A β	Amyloid- β
aCSF	Artificial Cerebrospinal Fluid
AD	Alzheimer's Disease
AM	Amplitude Modulation
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
AP	Action Potential
APP	Amyloid Precursor Protein
AUC	Area under the Curve
Base	Baseline
BrdU	5-bromo-2'-deoxyuridine
Ca	Calcium
DAB	3,3'-Diaminobenzidine
DBS	Deep Brain Stimulation
DI	Discrimination Index
DG	Dentate Gyrus
E/I	Excitation/Inhibition
EEG	Electroencephalogram
f	Frequency
f _{carrier}	Carrier Frequency
f _{stim}	Stimulation Frequency
FEM	Finite Element Method
FM	Frequency Mixing

FOI	Frequency of Interest
FOV	Field of View
GABA	Gamma-Aminobutyric Acid
GECI	Genetically Encoded Calcium Indicator
H	Haematoxylin
HH	Hodgkin-Huxley
HIFU	High-Intensity High-Frequency
IHC	Immunohistochemistry
IPI	Interpulse Interval
IPS	Intersectional Pulse Simulation
IT	Intratelencephalic
K	Potassium
KI	Knock-In
LED	Light-Emitting Diode
LF	Low Frequency
LFP	Local Field Potential
LIF	Leaky Integrate-and-Fire
LMM	Linear Mixed-Effect Model
LTD	Long-Term Depression
LTP	Long-Term Potentiation
Na	Sodium
NHS	Normal Horse Serum
NIBS	Non-Invasive Brain Stimulation Technique

NMDA	N-methyl-D-aspartate
OLM	Object Location Memory
PBS	Phosphate Buffered Saline
PD	Parkinson's Disease
PFA	Paraformaldehyde
PSD	Power Spectral Density
PWM	Pulse-Width Modulation
ROI	Region of Interest
rTMS	Repeated Transcranial Magnetic Stimulation
SEM	Standard Error of the Mean
SNR	Signal-to-Noise ratio
STA	Stimulus-Triggered Average
Stim	Stimulation
tACS	Transcranial Alternating Current Stimulation
tDCS	Transcranial Direct Current Stimulation
TES	Transcranial Electric Stimulation
tFUS	Transcranial Focused Ultrasound
Tg	Transgenic
TI	Temporal Interference
TMS	Transcranial Magnetic Stimulation
tRNS	Transcranial Random Noise Stimulation
V1	Primary Visual Cortex
WT	Wild Type

Chapter 1. Introduction

1.1 Brain stimulation methods

1.1.1 Neurons as the fundamental information encoding units

Neurons are the main units of the nervous system, the system that controls much of our bodies functioning. This includes relatively simple functions, such as limb movement or detection of sensory stimuli, but also more complex ones such as cognitive processes. The brain coordinates the synchronised functioning of this highly intricate system and is hence arguably the most complex organ in the human body. As such, it remains a fascinating subject of study. Malfunctioning in the communication between neurons in the brain has been linked to many neurological disorders. For example, in the Parkinson's Disease (PD), the loss of dopaminergic neurons in basal ganglia results in pathological firing patterns (Ashkan et al., 2017). In Alzheimer's Disease (AD), on the other hand, the disease first leads to hyperactivity of hippocampal and cortical neurons, followed by their hypoactivity (Zott et al., 2018).

Balanced neuronal circuit activity is crucial for a healthy brain. At the most fundamental level, neurons communicate by generating action potentials (APs) whenever the combined synaptic input they receive is large enough. During AP generation, the neuronal transmembrane voltage depolarises sufficiently from its resting potential, resulting in a large voltage spike propagating across the axon. It has been shown that both frequency and timing of this spiking activity encodes important information (Huxter, Burgess & O'Keefe, 2003; Ainsworth et al., 2012). Single-cell level firing patterns can be synchronised across many cells, resulting in large-scale oscillatory circuit activity within specific frequency bands, corresponding to different brain states (Buzsáki & Draguhn, 2004), or cognitive functions (Colgin, 2016). Similarly to electrical circuits exhibiting resonance effects, many neuronal types are tuned to, i.e. are more responsive to, specific input frequencies (Hutcheon & Yarom, 2000).

1.1.2 Neurons as electrical circuits

The discovery of AP generation led to first electrical circuit descriptions of functional neuronal activity. One of the earliest and simplest models, leaky integrate-and-fire (LIF), approximated the neuronal membrane as a simple resistor and capacitor circuit

that integrated the incoming signals (Brunel & van Rossum, 2007; Abbott, 1999). Such model was sufficient to mimic the low-pass filtering properties of neurons but did not explain how the action potential was generated. The classic model developed by Hodgkin and Huxley (Hodgkin & Huxley, 1952) proposed the actual mechanism of action behind the spiking activity, by modelling the membrane as a circuit consisting of resistors, corresponding to its two main ion channels, sodium (Na) and potassium (K), and leaky membrane components, and a capacitor (**Figure 1.1**). This prediction was later confirmed to be true, making the Hodgkin-Huxley (HH) model one of the most popular single-cell neuronal models to this day. Combined with cable theory (Rall, 1977), which models the spread of electrical current through axons and dendrites, these models allowed for biophysical modelling of individual cells with complex morphologies in software such as NEURON (Hines & Carnevale, 1997). On the other scale, simple LIF models also became increasingly more sophisticated with time (Brette & Gerstner, 2005; Platkiewicz & Brette, 2011) allowing for representations of more complex neural network operations.

Development of mathematical tools enabled scientists to hypothesise about and investigate the mechanisms behind neuronal biophysics at the single-cell level, such as the frequency filtering and resonance effects (Hutcheon & Yarom, 2000), or the effects of extracellular stimulation for different cell types (Gold et al., 2006; Abera, Peterchev & Grill, 2018), but also at the neural network level, for example by exploring the role of excitation and inhibition in plasticity (Vogels et al., 2011).

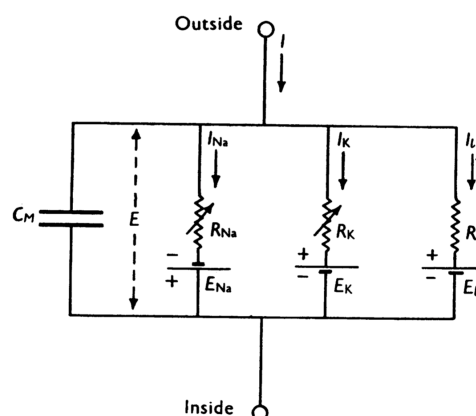


Figure 1.1 | The Hodgkin-Huxley model of the neuron. Hodgkin and Huxley introduced the ion channels into their electrical circuit model of the neuron, leading to much better understanding of the biophysical mechanism of the action potential generation. Figure reproduced from (Hodgkin & Huxley, 1952) with permission of the rights holder.

1.1.3 The principle of neuronal stimulation

Neuronal input can come from various sources. Apart from synaptic transmission, in which a cell receives direct electrical input from other cells, it has been hypothesised that neuronal activity can be influenced by endogenous electric fields via the mechanism of ephaptic coupling (Jefferys, 1995). These extracellular fields can be produced by e.g. a large-scale synchronised circuit activity, although the extent to which such inputs contribute to overall neuronal function remains disputed (Anastassiou & Koch, 2015).

Neuronal inputs are not limited to endogenous activity arising from local circuit dynamics. As neurons are effectively electrical circuits, electrical input can be applied to them via electrical stimulation methods. Similarly to endogenous inputs, one can provide direct input into each cell **intracellularly**, using methods such as the patch-clamp technique (Neher & Sakmann, 1976) or **extracellularly**, by changing voltage surrounding the neuron in its extracellular medium (Ozen et al., 2010).

Regardless of how the input is applied to the neuron, it results in the change of potential across the cell membrane. This potential difference generates large electric fields which leads to conformational changes in various voltage-sensitive proteins located in the membrane. These include G-protein coupled receptors, membrane pumps, transporters, and, most notably, voltage-gated Na^+ , K^+ and Ca^{2+} (calcium) ion channels (Bezanilla, 2008).

The neuronal response will depend on the strength of the stimulus. As mechanistically explained by the HH model, sufficient current input is required to trigger action potential generation cascade resulting in **suprathreshold** stimulation. If the input is weaker than the critical threshold, the stimulus is **subthreshold** and while it does not trigger immediate response, increasing baseline firing frequency, it may reduce the threshold needed for other incoming stimuli (such as endogenous inputs from nearby cells) and thus bias the cell spiking time (e.g. Krause et al., 2019).

Invasive stimulation methods such as electrical microstimulation induce strong electric fields that drive neuronal firing in a subset of cells around the electrode (Histed, Bonin & Reid, 2009), most likely due to direct activation of closest neighbouring axons and

axon terminals, as suggested by modelling work (McIntyre & Grill, 1999; Histed, Bonin & Reid, 2009; Aberra, Peterchev & Grill, 2018).

Non-invasive electrical stimulation methods tend to be much weaker than the invasive ones. Currents applied to the scalp using well-studied transcranial electrical stimulation (TES) methods can be attenuated by around 75% due to the shunting by skin and skull (Vöröslakos et al., 2018). Neurophysiological effects of TES techniques can thus be varied and have been described to include stochastic resonance, rhythm resonance, temporal biasing of spikes, network entrainment and, if strong enough, imposed stimulus pattern (Liu et al., 2018).

Alongside electrical stimulation, other techniques can be used to affect neuronal activity. These include applying pharmacological compounds, magnetic and ultrasound stimulation, or optogenetics (**Figure 1.2**). These invasive and non-invasive methods have varied spatial and temporal resolutions and thus have their own sets of strengths and limitations (Polanía, Nitsche & Ruff, 2018).

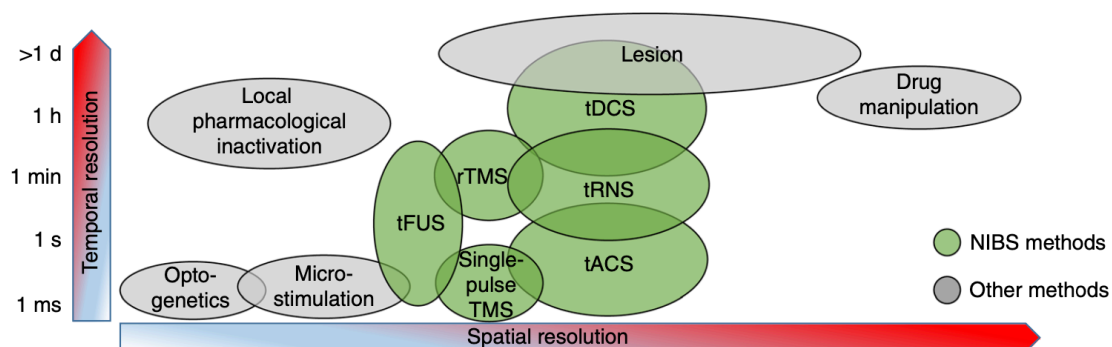


Figure 1.2 | Overview of the invasive and non-invasive brain stimulation (NIBS) methods. Figure reproduced from (Polanía, Nitsche & Ruff, 2018) with permission of the rights holder.

1.1.4 Invasive brain stimulation techniques

The most common invasive stimulation method used in humans is microstimulation via implanted electrodes. Deep Brain Stimulation (DBS) therapy for Parkinson's Disease uses stimulation electrodes implanted into the deep brain structures of the basal ganglia (Ashkan et al., 2017). Less invasive electrocorticographic (ECoG) arrays, placed on top of or below dura, can stimulate nearby neurons and are used for brain mapping purposes; however, their use is limited to superficial cortical cells only (Caldwell et al., 2019).

Optogenetics is a relatively new technique that uses genetic engineering to express photoreceptors into neuronal cells, making them sensitive to light activation (Boyden et al., 2005). This technique allows for unique targeting of specific cell types based on their genetic signature; however, due to safety concerns, the method was only used in animal experiments for a long time. The first human trials using optogenetic stimulation of retinal ganglion cells are starting now (Sahel et al., 2021) and most clinically relevant brain stimulation applications have been restricted to animal studies so far (Iaccarino et al., 2016; Valverde et al., 2020).

These methods, while highly effective, remain limited in their application due to their invasive nature and the associated risks.

1.1.5 Non-invasive brain stimulation techniques

Many non-invasive brain stimulation (NIBS; green in **Figure 1.2**) methods have emerged in the recent years as alternative neuromodulation strategies, with a much better safety profile and ease of use. These methods apply the electric, magnetic and ultrasonic pressure waves to affect neuronal activity through the intact skull.

The most well-established technique, transcranial electric stimulation (tES) uses low-intensity currents (1-2 mA) (Polanía, Nitsche & Ruff, 2018) that are applied to the subject scalp. The tES techniques most famously include transcranial direct (tDCS) and alternating (tACS) current stimulation but also encompass modified methods such as transcranial random noise stimulation (tRNS) (Reed & Cohen Kadosh, 2018), or intersectional pulse stimulation (IPS) (Vöröslakos et al., 2018). Transcranial magnetic stimulation (TMS) uses magnetic fields to generate stronger electric fields, capable of inducing motor-evoked potentials (Hallett, 2007). TMS has been applied in both online context-dependent tasks to affect ongoing neuronal activity (single pulse stimulation), but also in repetitive protocols (rTMS) to induce plasticity changes, such as long-term depression (LTD) or potentiation (LTP) (Polanía, Nitsche & Ruff, 2018). Finally, transcranial focused ultrasound (tfUS) is a recent, highly promising neuromodulation technique that offers improved spatial resolution and the ability to reach deep brain structures (Legon et al., 2014).

1.1.6 Deep brain stimulation

The aim of deep brain stimulation (DBS) is to deliver electrical stimulation to specific brain structures, typically via chronically implanted electrodes. The method is famous for its success in movement disorders, especially Parkinson's Disease. In this condition, the loss of dopaminergic neurons in substantia nigra pars compacta leads to abnormal firing patterns of the basal ganglia output structures. These effects vary depending on the brain circuits, and include both increased (in globus pallidus internus and subthalamic nucleus), and decreased (globus pallidus externus), firing rates (Ashkan et al., 2017)

Electrical stimulation of basal ganglia circuits has been shown to significantly reduce the most notable of the disease symptoms, tremor, resulting in marked improvement in the quality of life of those affected. Despite widespread use, the mechanism of this DBS method remains unclear and is likely multimodal, including short-term modulatory, plasticity and long-term reorganisation effects (Ashkan et al., 2017). One of the hypotheses suggests that the high frequency stimulation (~100 Hz) used in DBS disrupts abnormal neuronal activity present in basal ganglia centres (Chiken & Nambu, 2015). Correcting firing patterns in affected circuits, either by overriding or biasing ongoing activity, could be used to slow down or treat many health conditions. Apart from movement disorders, DBS is also used in pain management and other cognitive disorders, such as treatment-resistant depression or obsessive-compulsive disorder (Ashkan et al., 2017). More preclinical and clinical trials in diseases such as Alzheimer's Disease, Tourette's syndrome or schizophrenia are also ongoing (Lozano et al., 2019). Despite the advances in the DBS field, such as closed-loop stimulation paradigms, improved electrode design or improved battery charging options, the invasiveness of the technique raises ethical considerations (Lozano et al., 2019). Due to the highly invasive, risky nature of the surgery required and associated complications (Doshi, 2011; Oh et al., 2002), but also the high cost associated with the treatment (Lozano et al., 2019), it remains hard to explore the full potential of DBS in wider range of disorders and stimulation targets.

1.1.7 Potential non-invasive DBS strategies and their limitations

A safe, easy-to-use non-invasive DBS method would allow to overcome these obstacles, paving way for its much wider clinical adoption. Additionally, such methods

could also be used to probe neuronal function in healthy individuals. Unfortunately, existing non-invasive approaches have many limitations, and their clinical usefulness remains a subject of critical debates (Polanía, Nitsche & Ruff, 2018).

Traditional electrical and magnetic transcranial techniques remain less focal than direct microstimulation with implanted electrodes and are not able to reach deep structures without strongly stimulating the overlying tissue (Polanía, Nitsche & Ruff, 2018). Improving stimulation strength, for example by using stronger H-coils, capable of achieving deep brain TMS (Zangen et al., 2005), has been suggested to address this. However, such methods would lead to even stronger activation of overlying structures, leading to potential undesirable stimulation effects.

The relatively weak strength of the tES methods is another limitation. To directly affect neuronal spiking, electric fields of at least ~ 1 V/m have been reported necessary (Ozen et al., 2010; Vöröslakos et al., 2018). This corresponds to currents exceeding 4-6 mA in a recent cadaver study, suggesting a lot of previous work on tES may have been reporting indirect effects only (Vöröslakos et al., 2018). Electrical stimulation with such strong currents, however, may lead to unpleasant side effects including metallic taste in the mouth, dizziness, or feeling of burning of the skin (Vöröslakos et al., 2018).

tFUS is a promising technique that may be able to stimulate deeper structures without these constraints, in a relatively focal manner. Due to the previous use of high-intensity high-frequency (HIFU) ultrasound for tissue ablation (Ghanouni et al., 2015), safety of the lower intensity and lower frequency of tFUS (Legon et al., 2014) must be thoroughly demonstrated. FUS has been shown to exert both mechanical and thermal effects on the tissue, depending on the parameters used (Blackmore et al., 2019). The leading hypotheses on the mechanism of action of tFUS include thermal effects, sonoporation, and mechanical pressure exertion (Blackmore et al., 2019), with recent experimental evidence pointing towards activation of mechanosensitive receptors (Yoo et al., 2022). Safety reviews to date raise rare but serious side effects, which include unintended opening of the blood-brain barrier, haemorrhage and cell death (Pasquinelli et al., 2019). Further standardisation of safe stimulation protocols for tFUS is thus of utmost importance.

While the quest for an effective and safe non-invasive DBS method is still unresolved, new technologies are being continuously developed to address this pressing need.

One of the new, promising techniques offering deep brain stimulation non-invasively is Temporal Interference.

1.2 Temporal Interference stimulation

1.2.1 Principle of Temporal Interference

Temporal Interference (TI) is a novel non-invasive DBS technique that uses kHz electric fields (Grossman et al., 2017). In TI, two high frequency electric fields, with frequencies $f_1 \geq 1\text{kHz}$ and $f_2 = f_1 + \Delta f$, where frequency difference Δf is in the neurophysiological range, are simultaneously applied to the subject scalp (**Figure 1.3A**). In the area of the overlap between the two fields, the resulting signal is amplitude-modulated (AM) at Δf (**Figure 1.3Bii**), while the cells underneath the electrodes are exposed to individual kHz fields (**Figure 1.3Bi**). The original study proposed that neurons would only spike when exposed to AM fields but would not respond to pure kHz stimuli. This was based on the idea that neurons filter out high frequency inputs and effectively act as low-pass filters (Deans, Powell & Jefferys, 2007), a phenomenon stemming from the resistive and capacitive components of their cell membrane (Hutcheon & Yarom, 2000). Thus, the area of stimulation can be located away from the electrodes by appropriate positioning of the electrodes (**Figure 1.3C**) and further steered by varying the current ratio between the two fields applied. Electrophysiological experiments showed that neurons were unable to follow the original kHz frequencies of 2 kHz (**Figure 1.3F**) but responded reliably at the overlap of the two fields at $\Delta f = 10\text{ Hz}$ (**Figure 1.3D**), similarly to when they were exposed to 10 Hz directly (**Figure 1.3E**). Pure kHz frequencies induced a response at the onset of the stimulation (**Figure 1.3F**), which was further reduced when the current ramp-up period was extended from 0.25 to 0.5 s.

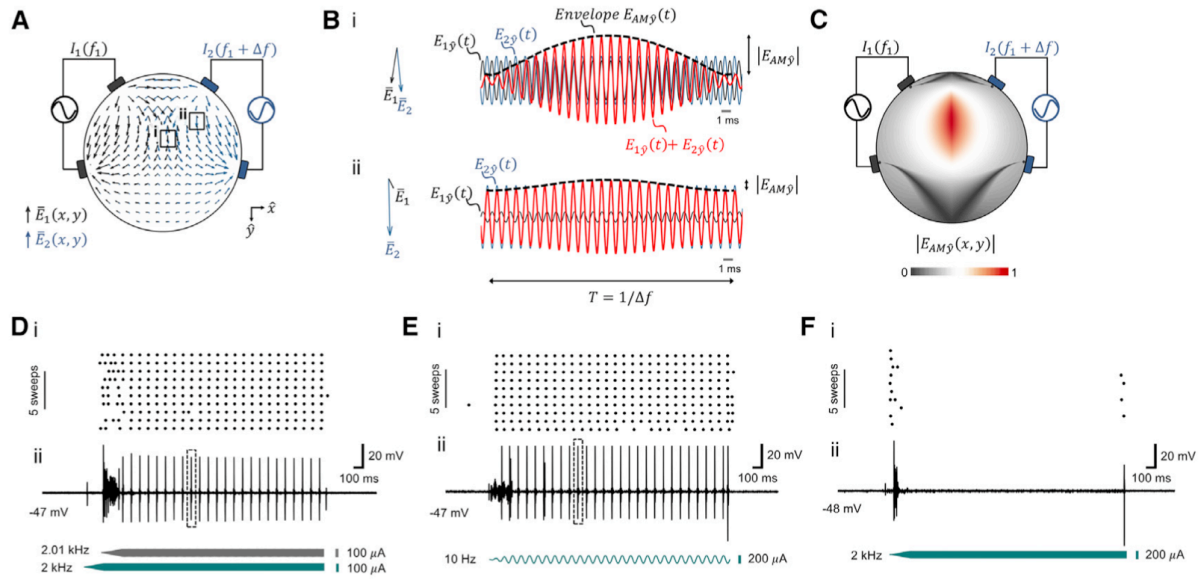


Figure 1.3 | Principle of Temporal Interference brain stimulation. **A.** Electric field (E_1 , E_2) distributions produced by two applied alternating currents (I_1 , I_2) of frequencies f_1 and $f_2 = f_1 + \Delta f$. **B. i.** In the area of the overlap of the two fields, the interference signal is amplitude modulated (AM) with envelope frequency equal to Δf . **ii.** Closer to the second electrode pair, the interference is dominated by the second current, I_2 . **C.** Map of the normalised maximum AM distribution, in the y-axis, shows the expected location of the activation at Δf . **D-F.** Neuronal responses to TI (**D**), low frequency (**E**) and kHz (**F**) stimulation *in vivo*, shown as spike count over 5 repeats (**i**) and membrane voltage (**ii**) during stimulation. Membrane voltage TI stimulation at 2000 and 2010 Hz induces spiking activity at the frequency difference of 10 Hz (**D**), similarly to the direct application of 10 Hz (**E**). kHz stimulation alone induces no activation, except for the transient onset effects. Figure reproduced from (Grossman et al., 2017) under Creative Commons CC-BY 4.0 license.

1.2.2 kHz stimulation

Despite their general low-pass filtering properties, nerve cells can be affected by strong enough kHz stimulation. Similarly to electrical low-pass filters, neurons do not filter out all frequencies but simply attenuate frequencies larger than the cut-off frequency (Deans, Powell & Jefferys, 2007). Several electrical stimulation techniques using kHz AC fields, including interferential currents applied in peripheral nerve stimulation, have been used in the context of nerve and muscle stimulation since the 1950s. A few theories, such as signal summation, have been suggested to explain their mechanism of action, with no conclusive evidence established (Ward, 2009). Pure kHz stimulation was also found capable of inducing reversible conduction block of peripheral nerves at large current amplitudes (Kilgore & Bhadra, 2014; Peña, Pelot & Grill, 2021).

Up-to-date evidence suggests that at amplitudes used for TI stimulation, kHz frequencies can induce transient onset effects, but no sustained steady-state responses or suspected depolarisation blocks were observed (Grossman et al., 2017).

1.2.3 Mechanism of action of TI

The mechanism of action of TI remains unclear, but the neuronal activity at Δf could indicate a non-linear response to the interfering fields. Given no major response to kHz fields applied alone (**Figure 1.3F**), a purely linear system would be expected to filter both f_1 and f_2 frequencies in an equal fashion, producing no response. (Grossman et al., 2017) suggested that the area of stimulation at Δf corresponds to the area of maximum amplitude modulation, with cells capable of demodulating the envelope of the superimposed fields. In theory, detection of Δf could be achieved by different non-linear processes: 1) reaching a critical amplitude of signal for a spiking threshold (summation), 2) signal rectification, followed by filter smoothing (amplitude demodulation), or 3) frequency mixing (FM), where neurons compute multiplication products of the two fields, Δf and $\Sigma f = f_1 + f_2$ (Ahrens et al., 2002; Haufler & Paré, 2019).

Recent single-cell experimental evidence suggests that neurons can perform frequency mixing of inputs spanning a wide frequency range (~10-5000 Hz), suggesting this process may play a role in the neuronal response to TI stimulation. Voltage-gated sodium channels were found to be required for mixing (Luff et al., 2024b). Other modelling work further supports the potential role of the ion channels (Cao et al., 2020), and specifically nonlinear rectification by the Na^+ channels in TI (Mirzakhilili et al., 2020).

Detection of Δf is directly linked to other mechanistic questions about TI. Which compartment of the neuron is the most sensitive, i.e. polarises the most, when exposed to interfering fields? (Mirzakhilili et al., 2020) modelling work suggests that, similarly to other tES modalities, axons may also be the most important compartment. The study also shows that when ion channels and myelination are included in the axon model, Δf is detected in the membrane potential. The potential need for myelination aligns with the previously reported lack of phosphene induction, commonly associated with tACS, when using kHz TI stimulation (Thiele et al., 2021). Other modelling work also indicates sensitivity to TI is increased in components with faster time constants,

such as axons, and postulates that inclusion of inhibitory GABA_B cells in the network model is sufficient to explain neuromodulatory effects of TI on hippocampal oscillations *in vitro* (Esmaeilpour et al., 2021). Apart from axons, dendritic trees are another compartment known for their nonlinear processing of synaptic inputs (Silver, 2010). For example, dendritic spines can have variable, i.e. nonlinear, neck resistance arising from nanoscale electrodiffusive effects which may in theory be able demodulate the AM signals (Cartailler et al., 2018). Other non-linear dendritic processes may also be involved in the FM of low frequency synaptic signals, as indicated by attenuation of FM products when blocking synaptic inputs (Luff et al., 2024b).

These modelling studies highlight that biophysical properties affecting sensitivity to TI may differ for different compartments within a cell but also for different cell types. Further experimental work is needed to confirm these hypotheses into the mechanism of TI detection, its origin within a single cell, and cell type specificity.

More experimental evidence is required to directly verify these hypotheses, especially at the network-level. The underlying mechanism behind TI stimulation could affect the spatial distribution of the response; it is therefore essential to uncover it to reliably target the desired brain regions. In particular, spatial profile of TI stimulation (and other frequency mixed signals) has not been tested directly *in vivo* and only modelled based on the maximum AM response assumption. If neurons act as non-linear mixers, it is possible that the stimulation area induced by such signals will not correspond simply to the summation, with maximum amplitude modulation demodulated, but to the product of the two fields, or even a combination of both sum and product. This may affect the capability to steer the stimulation site of TI and is essential to validate the method before further improvements can be implemented.

1.2.4 Applications of TI

While mechanistic investigation is still ongoing, TI has already been tested in multiple applications. This includes experimental animal work, such as replacing implanted probes to evoke seizure-like events (Missey et al., 2021), suppress hippocampal epileptogenic biomarkers (Acerbo et al., 2022), or restore breathing after spinal cord injury (Sunshine et al., 2021). First experiments in humans used TI to modulate hippocampus during memory tasks in healthy adults (Violante et al., 2023). This study also demonstrated that TI is a safe stimulation technique, with only minor side effects,

such as itchiness, reported. Despite higher current intensities used (2mA), TI resulted in less sensation and was overall well tolerated. Other human work using TI included striatal stimulation to either disrupt (Vassiliadis et al., 2022) or enhance (Wessel et al., 2023) motor skill learning in younger and older participants, respectively.

TI is a promising neuromodulation strategy under active investigation in multiple clinical studies worldwide (e.g. NIH IDs NCT03747601, NCT04761471, NCT05805215). As a non-invasive DBS method, it can target arbitrary structures and thus holds a great clinical potential, especially for neurodegenerative diseases such as Alzheimer's Disease, where new treatment options are much needed.

1.3 Neuromodulation for Alzheimer's Disease

1.3.1 AD pathology

Alzheimer's Disease (AD) is a progressive neurodegenerative disease, affecting estimated 10-30% of people over 65 years. AD is a form of dementia characterised by cognitive deficits and memory impairment. The disease progression is associated with accumulation of amyloid- β ($A\beta$) protein and neurofibrillary tangles, aggregates of phosphorylated tau protein (Masters et al., 2015). The accumulation of $A\beta$ is thought to be the initial point of the disease, which begins 15-20 years before the clinical symptoms occur, and starts in hippocampus and entorhinal cortex (Panza et al., 2019). $A\beta$ peptides are generated during the metabolism of amyloid precursor protein (APP) and exist in multiple forms, including the most common $A\beta_{1-40}$ and the less soluble $A\beta_{1-42}$. These monomers often aggregate and form oligomers, protofibrils, fibrils, and, finally, $A\beta$ plaques (Panza et al., 2019).

Prevalence of $A\beta$ in the early stages of the disease led to the amyloid cascade hypothesis which postulated that the $A\beta$ accumulation is the disease trigger that causes further disease pathology, including tau deposition, neuronal cell loss and dementia (Hardy & Higgins, 1992). As the number of $A\beta$ deposits in the brain was found not to correlate with cognitive symptoms, the theory has since been revised to emphasise the role of $A\beta$ oligomers specifically (Hardy & Selkoe, 2002), considered to be the most neurotoxic species (Panza et al., 2019).

So far, clinical trials targeting different A β forms have been mostly unsuccessful, with only 2% of successful phase II and III trials since 2003 (Kim et al., 2022). This could indicate that the trialled treatments have been applied too late in the disease progression (although some early-stage trials also failed), the drugs identified in the preclinical studies of familial AD models were unsuitable for sporadic AD, the most common form in humans (>99% of cases) (Masters et al., 2015), or that the accumulation of A β is the symptom rather than the cause of AD (Panza et al., 2019). Investigation of new, more varied treatment approaches is, therefore, desperately needed.

1.3.2 Neuronal dysfunction in AD

While AD ultimately results in neuronal cell death, increasing evidence suggests that the early stages of the disease are characterised by hyperactivity. It may be the hyperactivity that ultimately leads to hypoactivity (Busche & Konnerth, 2015), which may potentially be further mediated by the presence of tau in the later stages of the disease (Busche & Hyman, 2020). Hyperactivity seen in amyloidosis may be linked to increased incidence of epileptic events (Busche & Konnerth, 2015), also seen in human AD patients (Vossel et al., 2016; Cope, Murai & Sukoff Rizzo, 2022).

Early work on the effects of amyloid plaques on neuronal activity has shown that, in the transgenic (Tg) APP23xPS45 mouse model of amyloidosis, there were almost as many hyperactive (~20%) as hypoactive cortical neurons (~30%) present. These hyperactive cells were located preferentially next to the plaques while the hypoactive cells were distributed uniformly (Busche et al., 2008). Since then, it has been shown that hyperactivity in hippocampus precedes both the formation of plaques and the presence of hypoactive cells, suggesting soluble A β may be responsible for the effect (Busche et al., 2012). In humans, hippocampal hyperactivity has also been demonstrated early in the disease (Zott et al., 2018). Current research suggests that the relationship between hyperactivity and A β is bidirectional, with higher neuronal activity causing increased levels of A β (Yamamoto et al., 2015) and, in turn, A β presence leading to increased neuronal activity via suppressed glutamate reuptake (Zott et al., 2019).

The excitation/inhibition (E/I) balance is disturbed in the presence of A β but correcting it in the early stages of the disease may prevent further circuit dysfunction and disease progression (Busche & Konnerth, 2016; Haberman, Branch & Gallagher, 2017). For example, benzodiazepines, GABA receptor agonists, have been shown to restore the reduced long-range coherence and improve behavioural performance in Tg models of amyloidosis (Busche et al., 2015). In recent years, alongside pharmacological interventions that increase inhibition, novel neuromodulation strategies to restore the disturbed E/I balance have started to emerge.

1.3.3 Brain stimulation for neuronal dysfunction

There are three major types of oscillatory rhythms present in the hippocampus: theta (~4-12 Hz), gamma (~25-100 Hz), and sharp wave-ripples (Colgin, 2016). Theta and gamma have been shown to be tightly linked to each other, with established coupling between the phase of theta and the power of gamma oscillation (Canolty et al., 2006; Sirota et al., 2008). They both have also been associated with ongoing cognitive processes, with theta being involved in learning new information (Colgin, 2016) and gamma in memory encoding (Palop & Mucke, 2016). Fast-spiking parvalbumin-positive (PV+) interneurons have been shown to generate hippocampal gamma oscillations (Palop & Mucke, 2016; Hijazi, Smit & van Kesteren, 2023), with their impairment playing a critical role in AD circuit dysfunction and the associated cognitive decline (Hijazi, Smit & van Kesteren, 2023).

For example, hippocampal gamma power has been shown to be reduced in the Tg 5XFAD model (Iaccarino et al., 2016). Optogenetic gamma stimulation of the PV cells was able to rescue this deficit and led to numerous improvements in the AD pathology markers. This included reduction in amyloid load, increased microglial activation, and higher colocalization of microglia with A β plaques following a stimulation period of just 7 days. Similar effects were achieved with purely somatosensory gamma stimulation approaches of visual (Iaccarino et al., 2016; Adaikkan et al., 2019), auditory and combined visual and auditory stimuli (Martorell et al., 2019), with associated improvements in memory tasks (Adaikkan et al., 2019; Martorell et al., 2019).

Such somatosensory stimulation approaches show potential to non-invasively affect the AD pathology by targeting and correcting functional dysfunction caused by the disease. While they have been strong enough to affect neuronal dysfunction in

hippocampus, their application is likely limited to regions directly connected to somatosensory areas. More focused neuromodulatory intervention that can be applied to any brain region would be more versatile and could extend the use of therapy to other disorders. Temporal Interference is a non-invasive stimulation method capable of reaching both superficial and deep brain structures and is, therefore, ideally suited for exploring such applications.

1.4 Thesis overview

In this thesis, I investigate, for the first time, direct large-scale cortical responses to Temporal Interference *in vivo*. **Chapter 3** focuses on characterisation of TI parameter space and exploring its mechanism of action at the network level, defined here as bulk neuronal activity, using widefield calcium imaging. Using the same experimental setup, I then develop and apply a short-term, low-frequency stimulation paradigm in the App^{NL-G-F} Alzheimer's Disease model, with the aim to reduce the cortical hyperexcitability (**Chapter 4**). Finally, I apply multiple longitudinal stimulation paradigms in the same AD model, including the one developed in **Chapter 4**, directly to the hippocampus, the area earliest affected by AD, and assess their effects on behaviour and AD biomarkers (**Chapter 5**).

Chapter 2. Materials and methods

2.1 Overview

Parts of the Materials and Methods reported here have been adapted from the manuscript published at Brain Stimulation (Luff et al., 2024a).

2.1.1 Experiment types

The work presented in this thesis used two main experimental approaches to measure the effects of TI stimulation: simultaneous cortical stimulation and widefield calcium imaging *in vivo* (**Chapters 3 and 4**), and hippocampal stimulation *in vivo* combined with behavioural assessment and immunostaining of the fixed tissue (**Chapter 5**).

The methods chapter is split into shared methods that apply to all chapters (sections **2.2-2.6**), common cortical stimulation section that applies to **Chapter 3** and **4** (section **2.7**), subsections relevant for **Chapter 3** (sections **2.8-2.10**), **Chapter 4** (section **2.11**) and, finally, hippocampal stimulation section that applies to **Chapter 5** (section **2.12**).

2.1.2 Recording neuronal activity during electrical stimulation

Electrophysiological recording techniques, such as the traditional single-cell voltage patch-clamp (Neher & Sakmann, 1976) or modern high-density probes (Jun et al., 2017), have superb temporal resolution. These methods can reliably record neuronal activity at the level of individual spikes with millisecond precision, and are thus often used to validate the effects of novel neurotechnologies (Boyden et al., 2005; Chen et al., 2012). The initial work on Temporal Interference stimulation used patch-clamp recordings to verify the effects of TI stimulation on individual neurons (Grossman et al., 2017) and to test the role of synaptic currents and sodium channels in neuronal mixing (Luff et al., 2024b).

While tracking neuronal responses with excellent temporal detail, electrophysiological techniques are difficult to implement during concurrent electrical stimulation due to large electrical artefacts present (von Conta et al., 2022; Luff et al., 2024b). With its distinctive shape and temporal profile, action potential generated by electrical stimuli can be detected. Recording low frequency signals, such as subthreshold single-cell activity and network-level local field potential (LFP) or electroencephalography (EEG),

however, remains particularly challenging, although new approaches to filtering out the artefacts start to appear (Helfrich et al., 2014; Zhou et al., 2019; Haslacher et al., 2021).

While kHz TI stimulation does not contain low frequency components, the nonlinearities in the recording equipment may also lead to artificial generation of a frequency component at Δf in the measured signal (Luff et al., 2024b). Because of this, any electrophysiological recording setup used to monitor the effects of TI stimulation needs to be thoroughly tested to ensure the recordings are artefact-free.

New approaches to recording neuronal activity, especially at the meso- and macroscopic scales, commonly use functional imaging to simultaneously map the activity of many cells in multiple brain regions in bulk (Ma et al., 2016; Cardin, Crair & Higley, 2020; Pinto et al., 2019) or with a single cell resolution (Sofroniew et al., 2016; Fişek et al., 2023). These approaches usually rely on the expression of genetically encoded calcium indicators (GECI) in a subset of neurons of desired genetic signature. Calcium influx into these cells leads to conformational changes of fluorescent proteins present (green fluorescent protein for GCaMP) and the corresponding neuronal fluorescence (Lin & Schnitzer, 2016). Apart from the ability to cover wide areas, these methods also allow for concurrent electrical stimulation without the electrical artefact considerations present in all electrophysiological recordings (Histed, Bonin & Reid, 2009; Ghanbari et al., 2019; Michelson et al., 2019).

2.2 Animal experiments

All experiments were conducted according to the United Kingdom Animals (Scientific Procedures) Act 1986. Mice were single-housed in standard cages in the Imperial College London animal facility, with ad libitum food and water in a controlled light-dark cycle environment, with standard monitoring by veterinary staff.

2.3 Mouse models

Two main mouse models were used in the experiments reported in this thesis, both of which were on the C57BL/J6 background.

To allow for widefield calcium imaging experiments, transgenic *Thy1*-GCaMP6s mice (Dana et al., 2014) were chosen. In this mouse model, the calcium indicator,

GCaMP6s, is expressed in a subset of excitatory neurons throughout different brain regions, with strong expression in cortical layers 5, 6, and usually a bit weaker in layer 2/3, and no expression in layer 4 neurons. GCaMP6s is an established calcium indicator with a good signal-to-noise ratio (SNR) but relatively slow response dynamics, with a rise time of ~0.5 s and a decay time of ~1s (Chen et al., 2013).

As a proxy for Alzheimer's Disease, a knock-in (KI) App^{NL-G-F} (Saito et al., 2014) model was used. App^{NL-G-F} mice, harbouring Swedish, Beyreuther/Iberian and Arctic mutations in the APP gene, are the next generation amyloidosis model that elevate amyloid- β peptide presence. Unlike previous transgenic models, they do not overexpress APP and thus do not suffer from artificial phenotypes associated with it. For both experiments in **Chapters 4** and **5**, 5-6-month-old App^{NL-G-F} mice have been used to capture the early stage of the disease. At this age, mice have modest amounts of A β deposits in both cortex and hippocampus that start to rise rapidly with further aging (Saito et al., 2014). It is also around this age when first, subtle behavioural effects of the pathology can be observed (Saito et al., 2014; Whyte et al., 2018; Latif-Hernandez et al., 2019; Saifullah et al., 2020).

2.4 Electrical stimulation

2.4.1 Hardware and software

As opposed to the push-pull electrical approach implemented in the original TI device (Grossman et al., 2017), a novel stimulation setup was developed for the experiments reported in this thesis. This setup enabled the new pulsed TI stimulation paradigm (section **2.4.6**), first reported in my MRes thesis 'Pulsed temporal interference stimulation via frequency modulation' (Imperial College London), and was subsequently also used to generate pulse-width-modulated waveforms (PWM-TI, section **2.4.5**).

The new stimulation setup consists of commercially available devices controlled by a custom-written control software (**Figure 2.1**), making it easily accessible and applicable to many experimental settings. A custom-written MATLAB (MathWorks) GUI was used to generate analogue voltage waveforms output by a multifunction DAQ device (USB-6216 BNC, NI) to drive two current sources (LCI117, Soterix). All waveforms were created in MATLAB with a sampling rate of 250kHz. The laptop

operating GUI (ThinkPad X280, Lenovo) was unconnected from the mains to avoid contamination of the stimulation with electrical noise. When applying frequencies > 200 Hz, auditory transformers (1440-LN-B, Hammond Manufacturing) were used to remove noise and DC shifts, and to ensure additional channel isolation. Passive band pass filters (~0.0002 - 10kHz range) were used for frequencies < 200 Hz. When low and kHz frequencies were applied simultaneously, no filters or transformers were used. Additionally, in the imaging experiments (**Chapters 3 and 4**), an impedance adapter containing a 1 k Ω resistor was added at each current output to allow for constant monitoring of applied current waveforms and impedance across each electrode pair.

With electric field generated proportional to the current density applied, it is important that the stimulation hardware is current controlled. Channel isolation of the modified system was tested on a resistor bridge, similarly to previous testing reported in (Grossman et al., 2017), to ensure channel isolation.

Current density in **Chapter 3** and **4** was estimated by dividing the applied current amplitude by the cross-sectional area of the stimulation electrodes. Reported estimated current density is applied through each individual electrode.

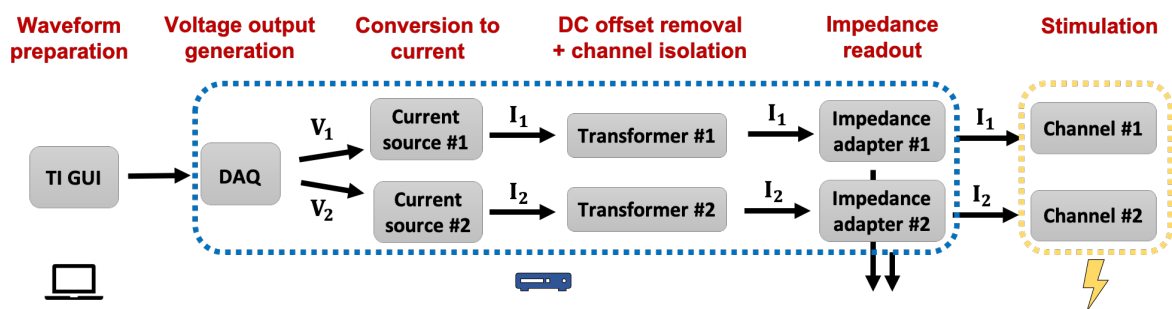


Figure 2.1 | Schematic of the electronic hardware used to deliver TI and control stimulation.

2.4.2 Impedance testing

Impedance across each electrode pair was tested in all mice prior to the experiment start. This was done to ensure the electrical hardware could output sufficient voltage to deliver required current. Standard impedance at 2000 Hz was in the 5-20 k Ω range. Mice with impedance above 40 k Ω at 2000 Hz were excluded from experiments (**Chapter 3** and **4**) or placed into the sham control group (**Chapter 5**).

2.4.3 Control stimulation

Low frequency and pure kHz stimulation was applied as two biphasic sinusoidal waveforms with frequencies $f_1 = f_2$ and phases $\Phi_1 = \Phi_2 = -90^\circ$ unless specified otherwise.

2.4.4 Temporal Interference stimulation

TI stimulation was created by applying two biphasic sinusoidal current waveforms, $I_1 = A_1 \cos(2\pi f_{1t} + \Phi_1)$ and $I_2 = A_2 \cos(2\pi f_{2t} + \Phi_2)$, via two electrode pairs, with frequencies $f_1 = f_{\text{carrier}}$ and $f_2 = f_1 + \Delta f$, e.g. $f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz and $\Delta f = 0.5$ Hz, as in (Grossman 2017), and phases $\Phi_1 = 0^\circ$, $\Phi_2 = 180^\circ$, unless specified otherwise.

Apart from conventional TI stimulation, two other modalities of TI were used in the experiments: PWM-TI and pulsed TI.

2.4.5 Pulse-width modulated TI stimulation (related to Chapter 3)

Pulse-width modulated TI (PWM-TI) is an alternative way to apply temporally interfering electric fields using square rather than sinusoidal waveforms. The resulting combined sum of the two waveforms is pulse-width, rather than amplitude, modulated at the difference frequency. Previous studies (Missey et al., 2021; Acerbo et al., 2022) used this alternative TI method without validating the neuronal responses evoked by it.

Here, PWM-TI stimulation was applied as two constant amplitude biphasic square waveforms with duty cycles of 50% and frequencies $f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz. The resulting waveform had a constant amplitude with a pulse-width that changed periodically at the difference frequency, $\Delta f = 0.5$ Hz (**Figure 3.19**).

2.4.6 Pulsed TI stimulation (Chapters 4-5)

Conventional TI stimulation applies continuous stimulation at a specified frequency difference, with pulse widths of fixed length corresponding to the inverse of Δf . This approach lacks the flexibility needed to generate more complex protocols that may be able to improve the efficacy of the stimulation (Grill, 2018), such as the plasticity protocols used to induce long-term potentiation or long-term depression (Albeni et

al., 2007). Such pulsed protocols could be achieved by simply continuously switching the stimulation on and off. However, this would lead to undesirable stimulation in the vicinity of the electrodes, as TI has been shown to induce extraneous effects at the onset of kHz stimulation (Grossman et al., 2017).

Pulsed TI (pTI) approach uses frequency modulation to achieve creation of amplitude-modulated pulses of desired width at specific points in time (**Figure 4.1B**), without the need to change the current density applied. In this approach, two out-of-phase ($\Phi_1 = 0^\circ$, $\Phi_2 = 180^\circ$) biphasic sinusoidal waveforms are applied via two electrode pairs, with frequencies f_1 and f_2 . Frequency f_1 is fixed while frequency f_2 is modulated throughout the stimulation duration, such that $f_2 = f_1 + \frac{1}{\text{pulse width}}$ during pulse period, generating the desired pulse, and $f_2 = f_1$ during the interpulse interval (IPI), leading to destructive interference of the resulting signal.

The 1 Hz TI stimulation protocol, delivered in **Chapter 4** and **5**, was generated by setting frequencies $f_1 = f_{\text{carrier}} = 2000$ Hz, and $f_2 = f_1 = 2000$ Hz during IPI and $f_2 = f_1 + \frac{1}{\text{pulse width}} = 2005$ Hz during pulse period to generate pulses of 200 ms width.

2.5 Data analysis

All data was analysed in MATLAB (2021a, MathWorks) except for the linear mixed-effect model (LMM) fitting. Phase statistics were done using circular statistics toolbox in MATLAB (Berens, 2009). LMM analysis was done in R using lme4 and emmeans packages.

2.6 Statistical analysis

In most statistical analyses, normality of the data was tested using Lilliefors test. Subsequently, either a parametric (paired or unpaired t-test) or a non-parametric test (Wilcoxon signed-rank or Wilcoxon rank-sum test) was used. Tests were two-sided unless stated otherwise. When performing multiple comparisons, parametric tests were used only if all data was approximately normal. To compare for multiple comparisons, Bonferroni-Holm correction was applied. P values and corresponding significance reported correspond to the original p values that passed multiple

comparisons corrections. P values below the smallest significance level of $p < 0.05$ that did not pass multiple comparisons corrections are indicated on the figures.

2.7 Cortical TI stimulation and widefield calcium imaging (Chapters 3-4)

2.7.1 Surgery

Animals were anaesthetised with 2-3% (vol/vol) isoflurane in oxygen and received a dose of carprofen (5 mg/kg), buprenorphine (0.1 mg/kg) and dexamethasone (0.38 mg) subcutaneously at the start of the surgery. Ophthalmic ointment (Vaseline) was applied to the eyes and the hair was removed using a shaving cream (Veet). The animal was head-fixed in a stereotaxic frame. The scalp was removed, and the skull was cleaned and scraped thoroughly to remove all tissue, including wiping it carefully with acetone. The skull was covered with a thin layer of cyanoacrylate glue (Superglue Brush on, Loctite) and transparent nail polish (Rimmel) to clear the skull, as previously tested by (Steinzeig, Molotkov & Castrén, 2017). A custom-made headbar was attached to the skull with clear dental cement (Paladur) mixed with charcoal. Two holes (0.5 mm diameter) were drilled through the skull down to dura in the V1 visual area (coordinates specified in sections **2.8.1** and **2.11.1**). Two custom-made electrodes (platinum-iridium, 0.25 mm diameter, Alfa Aesar), attached to a socket and smoothed at the ends (by placing wire endings under oxy-acetylene flame for a few seconds to create a ball of ~0.3-0.4 mm diameter at each end), were placed in the holes, glued to the skull using a small amount of glue (Superglue, Loctite) and fixed to the headbar with cement. The skull area and electrodes were covered with another thick layer of nail polish. Mice received carprofen in water for two days post-surgery and were left to recover for 6-7 days before experiments.

2.7.2 Electrical stimulation

During experiments, animals were connected to the stimulators via implanted sockets and return electrodes placed on shaved animal cheeks covered with electrode gel (SignaGel, Parker Laboratories, Fairfield, NJ, USA). The return electrodes consisted of ~3x3 mm conductive copper tape electrodes (RS Components, London, UK) connected to 26 AWG Teflon-coated silver wires (WPI, Hitchin, UK).

2.7.3 Widefield calcium imaging

Widefield calcium imaging was done using a custom tandem-lens epifluorescence macroscope, with two lenses placed face-to-face (85mm primary lens, 50mm secondary lens), as previously described in (Doostdar et al., 2021). Excitation light was delivered with 405 and 470 nm LEDs (405nm: M405L3, Thorlabs, with excitation filter DC/ ET405/10x, Chroma; 470 nm: M470L3, Thorlabs, with excitation filter DC/ET470/40x, Chroma) and combined with a beam-splitter (DC/ T425lpxr, Chroma) through a dichroic mirror (DC/ET525/50 m, Chroma). Alternating 405 and 470 nm images were acquired at 50 Hz with a CMOS camera (GS3-U3-23S6, FLIR), for a 25 Hz imaging frequency per channel, at a full resolution of 1152 x 1200 pixels. Camera exposure per frame was limited to 10 ms and offset by 5 ms relative to the LED onset to limit crosstalk between the two channels. Camera, LEDs and stimulation were controlled by and synchronised with TTL pulses programmed in WaveSurfer software (HMMI Janelia).

2.7.4 Image preprocessing

All data was downsampled with a box-shaped kernel by a factor of 3 for a 384x400 pixels FOV. For each animal, images were registered between days to the animal reference frame by applying linear rigid transformation (using NoRMCorre package (Pnevmatikakis & Giovannucci, 2017); with the original efficient algorithm of (Guizar-Sicairos, Thurman & Fienup, 2008)). The images were rotated to ensure midline is placed symmetrically in the image, with angle per animal calculated by manually selecting two points on the midline of the reference image. All images were further registered to the scaled 2D top cortical projection of the Allen Brain Atlas reference space using manually selected reference points (base of olfactory bulb, lambda). Each combined recording was deinterleaved to split the data into the 405 and 470 (nm) channels. The two channels record fluorescence pertaining to hemodynamic (405) and calcium (470) activity. Hemodynamic fluorescence is recorded so it can be subtracted from the calcium fluorescence to obtain artefact-free neuronal recordings (Ma et al., 2016), as previously implemented in (Allen et al., 2017). The 405 and 470 channels were individually normalised at the pixel level to the median fluorescence of the first 20 s of each recording (**Chapter 3**) or of the full recording (**Chapter 4**) for dF/F traces. The 405nm channel was temporally smoothed using moving average window

of 400 ms and then subtracted from the 470 nm channel. The resulting data was temporally smoothed with a moving average of 200 ms at a single pixel level and detrended using a robust 3rd-order polynomial fitting method (de Cheveigné & Arzounian, 2018) (10/2019 version), which excluded events of >10% dF/F amplitude from the fit.

2.7.5 Main ROI selection

The location of the stimulating electrodes was manually selected for each mouse. Based on that, the main region of interest (ROI), ~0.5 x 0.5 mm large (18 x 18 pixels), was computed for each mouse. The main ROI (**Figure 3.1B**) was chosen to be located between the two cortical electrodes, in the expected area of maximum amplitude modulation (**Figure 3.12B**).

2.7.6 Data analysis

Example traces show mean dF/F activity from the main ROI for all animals. Power spectral density (PSD) values between original frequency bins were interpolated and the plots were smoothed over a span of 5 samples using linear least squares with a 1st degree polynomial model method.

Stimulus-triggered average (STA) plots were created by aligning average responses to the onset of individual stimulation pulses for all mice. In **Chapter 3**, the STA responses were normalised by subtracting mean of the whole response. In **Chapter 4**, to calculate the amplitude of the responses, the STA responses were normalised by subtracting the mean dF/F amplitude of the 0.2 s preceding the stimulus onset.

2.7.7 Spatial analysis

For spatial analysis of the data, all images were aligned to the common Allen Brain Atlas reference space. Average maps are shown, with the average position of stimulating electrodes for all mice indicated. Individual electrode locations per mouse were removed from the frames by setting their values to zero. One-sided cluster permutation analysis of dependent samples (Maris & Oostenveld, 2007) was performed to compare the tested values, evoked during the full 20 s of stimulation, were significantly higher than those present during the 20 s of baseline activity immediately preceding it, at a significance level of $p < 0.05$. Number of permutations

was limited to 2^n , where n indicates the number of animals. Clusters smaller than 100 pixels in size were excluded. Binary cluster masks were further smoothed with a 5 x 5 box kernel before final cluster boundaries were extracted.

2.8 Characterisation of TI stimulation with widefield calcium imaging (Chapter 3)

2.8.1 Animals

2-4 months old *Thy1*-GCaMP6s mice were used in two sets of imaging experiments. Cohort 1 consisted of 10 mice (6 male, 4 female). Cohort 2 consisted of 10 mice (5m, 5f).

Mice were implanted with two stimulating electrodes placed in the skull above the right primary visual cortex (V1), with the separation between the electrodes set to 2 mm for Cohort 1 (AP -3.5, ML +1.25 and AP -3.5, ML +3.25 mm; Configuration 1) and to 4 mm for Cohort 2 (AP -3.5, ML +0.25 and AP -3.5, ML +4.25 mm; Configuration 2). Most reported results were obtained from animals in Cohort 1 (Configuration 1), unless specified otherwise.

2.8.2 Experimental protocol

Animals were imaged under light anaesthesia (0.5% vol/vol isoflurane in oxygen) on multiple experimental days. Prior to the recordings, mice were allowed to habituate to the setup for a minimum of 5 minutes. Each experimental session consisted of 15-30 conditions, each repeated three times for Cohort 1 and just once for Cohort 2. Each individual recordings lasted 62 s and consisted of 20s of baseline, 22 s of stimulation (1 s ramp up and 1 s ramp down, 20 s full stimulation amplitude), and 20 s of post stimulation activity. Order of conditions within a day and, if possible, sessions between days was randomised.

2.8.3 ROI analysis

Data preprocessing was done as described in section 2.7.4. For Cohort 1, dF/F data was averaged across 3 repeat recordings.

To calculate the dF/F response (%) at a specific frequency of interest (FOI), 20s-long baseline and stimulation traces were bandpass-filtered with a window around FOI

(window size = 0.05 Hz, e.g. for 0.5 Hz, window = 0.475 - 0.525 Hz). dF/F amplitude for each filtered trace was then computed as $\sqrt{2} \times \text{rms}(\text{trace})$. To obtain the relative dF/F (%) measure, baseline dF/F amplitude was subtracted from stimulation dF/F to quantify the relative increase in response at FOI. Current threshold per condition was determined as the minimum amount of current density needed to induce a significant relative dF/F at a specified frequency. Onset response was quantified as the mean or maximum (**Figure 3.22** only) dF/F amplitude recorded during the first 1 s of the full stimulation (following the ramp up period, i.e. 21-22 s). Mean response was calculated as the average dF/F value of the middle 15 s of the stimulation period.

Custom ROIs, close to electrodes 1 and 2, were 0.5 x 0.5 mm (18 x 18 pixels) large, with location adjusted based on a fixed distance from a given electrode, custom for each mouse. The horizontal ROI used for spatial spread quantification was 1 mm (36 pixels) high, placed at the level of the mean electrode location for all mice, and common for all mice. Mean spread values in the average electrode areas (0.35 mm wide each) were excluded when calculating Person correlation coefficient.

Significant stimulation effects were established by comparing them to their corresponding baseline or to spontaneous levels (for onset and mean dF/F). One-tailed tests (paired-sample t-test or Wilcoxon signed-rank test) were used to establish this significance (dF/F response, relative dF/F response, onset dF/F, mean dF/F, phase resultant).

Phase difference between the evoked calcium response and the stimulation was calculated on the traces and the stimulation waveform that were bandpass filtered around FOI (window size = 0.1 Hz) and then Hilbert transformed. The phase difference (angle) between them was then calculated as difference in instantaneous median phase of the two signals. Phase resultant was calculated as the distance correlation between these two instantaneous phases. The same approach was used to compare the phase at Δf between PWM-TI and TI responses.

Linear mixed-effect models (lmer function) were fitted to analyse TI versus PWM-TI responses, with current amplitude and condition type as fixed effects and mouse ID as a random effect. Data used was approximately normally distributed as tested with Lilliefors test. Condition and current were treated as categorical variables, with reference coding scheme (lowest current density and standard TI as references).

2.9 Finite Element Method models (Chapter 3)

A recent anatomical 3D mouse model (IT'IS Foundation) was used to perform the finite element methods (FEM) modelling simulations in the Sim4life platform (Zurich Med Tech), with assigned low frequency tissue conductance properties (Low Frequency Conductivity Database; IT'IS Foundation). Electrodes were modelled as cylinders of 0.3 mm diameter and 2 mm height that were positioned in the approximate right V1 cortical region, normal to and 1.25 and 3.25 mm medial to the virtually created midline. The electrodes were inserted through the skull, close to the cortical surface. Insulating tubes were positioned around them to mimic the experimental removal of the tissue, with the inside and outside diameters of 0.3 and 0.5 mm respectively. Return electrodes were modelled as cylinders of 3 mm diameter and 1 mm height, positioned on the mouse cheeks. Voltage between each electrode pair was scaled for the equivalent current amplitude of 1 mA per channel for all simulations. Reported electric field distributions are normalised to maximum value to show relative location of the stimulation area rather than the absolute expected electric field magnitude. Electromagnetic field simulations were solved using the quasi-electrostatic solver and the maximum amplitude modulation along any direction was calculated using custom Python scripts which follow equations described in the methods of (Grossman et al., 2017).

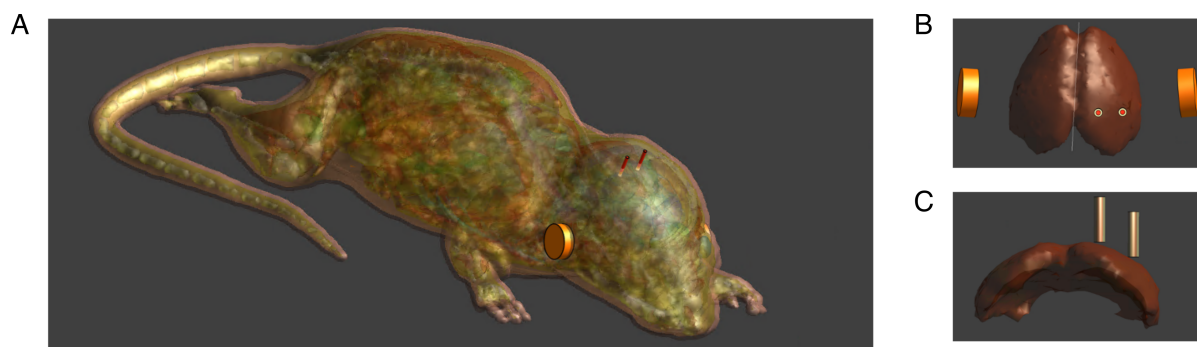


Figure 2.2 | Virtual mouse model used to perform FEM simulations of the spread of the electric field. **A.** Full mouse model with two stimulating electrodes placed in the skull and two return electrodes positioned on the animal cheeks. **B.** Horizontal view of the brain and the corresponding electrode locations. White line indicated the position of the virtual midline. **C.** As in B but sagittal view.

2.10 Electric field measurements (Chapter 3)

Electric field measurements were done in a subset of animals from Cohort 2 (n = 6), with Configuration 2 electrode arrangement, at the end of the experiments, weeks after the imaging sessions, when animals were ~7 months old.

2.10.1 Surgery

Prior to recordings, mice underwent a surgery to create a craniotomy (1 x 0.5 mm) between the two stimulating electrodes (AP -3.25 to -3.75, ML -1.75 to 2.75 mm). Animals were anaesthetised with 2-3% (vol/vol) isoflurane in oxygen and received a dose of carprofen (5 mg/kg) subcutaneously. The glue was scraped off the marked craniotomy location and a drop of bupivacaine hydrochloride (Marcain 0.25%, 2.5 mg/ml solution) was applied to the area for 2 minutes for local analgesia. A thick layer of clear dental cement (SuperBond) was applied to the skull to allow for creation of a well containing aCSF and reference electrode during recordings. Finally, the skull containing the marked craniotomy location was exposed and the craniotomy, including a durotomy, was performed. The craniotomy was then sealed using a silicone sealant (Kwik-CAST, WPI). Mice were allowed to fully recover (at least 2 hours) before subsequent electric field recordings.

2.10.2 Recordings

Similarly to calcium imaging experiments, electric field recordings were done under light anaesthesia (0.5% vol/vol isoflurane in oxygen). Impedance of the electrodes was tested before the experiment start to ensure it remains low enough to perform the recordings (< 30kOhm at 2000 Hz). The craniotomy was exposed and kept hydrated by periodic application of aCSF. The electric recordings were done via a glass pipette inserted into the middle of craniotomy. Pipettes were pulled from glass capillaries (1B120F-4, WPI) using a micropipette puller (P-1000, Sutter Instrument), filled with aCSF and 50µg of a fluorescent dye (FM 1-43FX, Thermo Fisher Scientific Inc.). The reference electrode (EP2 Ag-AgCl Half-Cells, WPI) was placed inside the aCSF well (located around AP +1, ML 0 mm). The recording setup consisted of the pre-amplifier (CV-7B Current clamp and voltage clamp headstage, Molecular Devices Ltd), the amplifier (MultiClamp-700B, Molecular Devices) and digitizer (Digidata 1550B,

Molecular Devices Ltd). Data was acquired with pClamp software (Molecular Devices Ltd) at a sampling rate of 250 kHz.

2.10.3 Experimental protocol

Electric potential was recorded via the glass pipette at six depths, corresponding to different cortical layers at AP -3.5, ML 2.25 mm: 70 (L1), 270 (L2/3), 470 (L4), 670 (L5), 870 (L6a) and 1120 μ m (white matter tract). 3D recordings were done by sampling of two additional points, one lateral (AP -3.5, ML 2.45, DV -0.47 mm) and one anterior (AP -3.2, ML 2.25, DV -0.47 mm).

Recordings were done for three stimulation types: control stimulation (LF; $f_1 = f_2 = 0.5$ Hz, $a_1 = a_2 = 0.5$ mA/mm²), TI ($f_1 = 2000$, $f_2 = 2000.5$ Hz, $a_1 = a_2 = 3$ mA/mm²) and AM (combined TI waveform through the cranial electrode pair; $f_1 = 2000$, $f_2 = 2000.5$ Hz, $a = 3$ mA/mm²). Each condition was done at all depths, with order of conditions randomised across mice. Stimulation was applied for 10 s, with 0.5 s ramp up and 0.5 s ramp down periods.

2.10.4 Analysis

Recorded waveforms were filtered first with a 4-th order low-pass Butterworth filter of the cut-off frequency of 10 kHz, followed by the 1-st order high-pass Butterworth filter with the cut-off of 100 Hz. Electric potential at each location was calculated as the difference between maximum and minimum points in the filtered signals. Electric field between two points was then computed as the absolute difference in the potential between the points divided by the distance between them. AM electric field was calculated in the same way, except that the amplitude of envelope modulation, measured as maximum value of the Hilbert envelope of the signal, was used instead of electric potential. Average 3D electric field was calculated as $\sqrt{E_X^2 + E_Y^2 + E_Z^2}$, where E represents electric field magnitude in X, Y and Z directions.

2.11 Testing slow TI paradigm with widefield calcium imaging in an AD model (Chapter 4)

2.11.1 Animals

Two main experimental groups were tested in this experiment: APP and WT (wild type), corresponding to the AD model and healthy control groups. The APP group consisted of eight ~5-month-old homozygous *App*^{NL-G-F} mice crossed with *Thy1-GCaMP6s* (3m, 5f). The WT group consisted of eight aged-matched *Thy1-GCaMP6s* mice (3m, 5f). Four of the APP mice and four of the WT mice received sham treatment (sham APP and sham WT groups) before undergoing active stimulation.

All mice were implanted with two stimulating electrodes placed in the skull above the primary visual cortex (V1), with a separation between the electrodes set to 2 mm (AP -3.5, ML +1.25 and AP -3.5, ML +3.25 mm).

2.11.2 Experimental protocol

Animals were imaged under light anaesthesia (0.5% vol/vol isoflurane in oxygen) on three consecutive days. Active experimental group received 15 minutes of TI stimulation on each day (section 2.11.3). Sham mice underwent the same surgical and imaging procedure but did not receive TI stimulation. Following the sham experiment, sham mice underwent additional three days of active stimulation paradigm for a total of six experimental days.

A series of tests to assess the stimulation effects was performed before and after each stimulation block (**Figure 4.1C**): 1) a 2-minute-long spontaneous recording, 2) electrically evoked response (EER) test at 0.1 Hz, 3) visually evoked response (VER) test at 0.1 Hz, and 4) VER test at 0.5 Hz, applied only before the first and after the last stimulation block. The EER test consisted of 12 TI pulses of 200 ms length applied every 10 s (current density $a_1 = a_2 = 2.5 \text{ mA/mm}^2$ or 5 mA/mm^2 for 2 WT & 2 APP sham mice only). Similarly, the VER test at 0.1 Hz consisted of 12 visual pulses of 100 ms length applied every 10s. The VER test at 0.5 Hz consisted of 10 visual pulses of 250 ms length applied every 2 s.

2.11.3 Electrical stimulation

Each day, mice underwent a total of 15 minutes of 1 Hz TI stimulation, applied in three 5-minute-long blocks. The stimulation was applied as a pulsed TI protocol (**Figure 4.1B**), at the carrier frequency of 2000 Hz, with pulse frequency of 1 Hz and pulse width of 200 ms, as described in detail in section **2.4.6**. Each stimulation block composed of five periods of 1 minute of stimulation, each with a ramp up period of 1 s and ramp down period of 1 s, at $a_1 = a_2 = 2.5 \text{ mA/mm}^2$ current density.

2.11.4 Analysis

Data preprocessing was done as described in section **2.7.4**. To simplify the analysis, baseline data for each test (before 1 Hz stimulation; Base) was compared with the test data after the full 15-minute stimulation protocol (Post Stim) on each experimental day.

For spontaneous activity, the full recording was split into 12 epochs. Area under the curve (AUC), measured by trapezoid numerical integration (trapz function) of the absolute dF/F trace, was calculated for each epoch and an average was then computed for each mouse. The magnitude of the response to the stimulus was calculated as the average AUC to all stimuli within the response window for each mouse, with the response window of 0.6 s (EER) or 1.5 s (VERs) starting at the stimulus onset.

AUC amplitude for all tests was computed on each day and normalised for each mouse to the baseline of Day 1, for a percentage change in AUC1. This was done to enable comparison between baseline and stimulation within each day ('Between' comparison'), as well as comparison to baseline of Day 1 for baseline and stimulation amplitudes of subsequent days (Day 2 and 3; 'Base v 0' and 'Stim v 0').

2.12 Hippocampal TI stimulation and immunostaining in an AD model (Chapter 5)

2.12.1 Animals

Male and female 5–7-month-old homozygous $\text{App}^{\text{NL-G-F}}$ mice ($n = 27$; 8m, 19f) used in this study were assigned to four experimental groups: sham (control, $n = 7$), delta (1 Hz stimulation, $n = 7$), theta (8 Hz, $n = 6$) and gamma (40 Hz, $n = 7$). Experiments

were done in five cohorts of mice, each containing 4-7 mice that were stimulated and tested within the same two-week window. Mice underwent electrode implantation procedure, with electrode locations chosen to target right hippocampus based on previous studies (Missey et al., 2021; Acerbo et al., 2022).

Due to tissue processing issues in one of the cohorts resulting in big holes in the brain tissue, three mice (one in the delta group, two in the gamma group) were excluded from the immunohistochemistry (IHC) analysis. The tissue in the remaining three mice from this cohort (two in the delta, one in the gamma group) looked unaffected and was kept in the analysis.

2.12.2 Surgery

Mice were anaesthetised with 2-3% (vol/vol) isoflurane in oxygen and received a dose of carprofen (5 mg/kg), buprenorphine (0.1 mg/kg) and dexamethasone (0.38 mg) subcutaneously at the start of the surgery. Ophthalmic ointment (Vaseline) was applied to the eyes and the hair was removed using a shaving cream (Veet). The animal was head-fixed in a stereotaxic frame. The scalp was removed, and the skull was cleaned and scraped thoroughly to remove all tissue. Red activator (Super Bond), containing phosphoric acid, was applied to the exposed skull for 30 s and then carefully washed away with saline to ensure the cement can adhere well to the skull. The electrode coordinates (AP -2, ML -0.7, +0.7, +3.1 and +4.5 mm) were marked using stereotactic measurements, after which the skull area was covered with a layer of clear dental cement (Super Bond). When the cement was completely dry, four 0.7mm holes in the marked locations were drilled through the cement and the skull down to dura. Four stainless steel screws (TX000-1.5FH, 0.86 mm diameter, Component Supply), attached in pairs to sockets pointing upwards, were screwed into the holes. Impedance of the screws was monitored during this procedure and the screws reached the readout of 0.5-1 M Ω DC impedance between all pairs. After the electrode implantation, the skull and the screws were covered with another few layers of dental cement to ensure the electrodes with their sockets remain securely fixed to the skull. Mice received carprofen in water for two days post-surgery and were left to recover for 6-8 days before experiments.

2.12.3 Habituation

Mice were habituated to the experimenters by a minimum of three short handling sessions (~1-2 minutes) before the stimulation start.

2.12.4 Experimental protocol

Experiments were done in the same location every day, with lights dimmed in the room dimmed. Mice were stimulated on eight stimulation days (S1-S8) for one hour per day while freely moving in a cage-like environment over the period of two weeks (**Figure 5.1A**). In the second week, following the morning stimulation session, mice underwent behavioural testing in the afternoon (a day prior to S5 and S5-S7). Following the last stimulation session (S8), mice were perfused to collect the brain tissue.

2.12.5 Electrical stimulation

On each experimental day, mice were connected to the stimulating equipment. Four stimulation protocols were applied: sham (no stimulation), delta stimulation (1 Hz TI with 200 ms pulse width), theta stimulation (8 Hz), and gamma stimulation (40 Hz). Stimulation was done for one per day in the 10 s on, 10 s off protocol. Each 10 s of stimulation was ramped up for 0.5 s and ramped down for 0.5 s. Current amplitude used for all experiments was in the range of 0.3-0.375 mA per channel.

2.12.6 Brain tissue collection

60 minutes after the stimulation session on the last experimental day, mice were anaesthetised with ketamine (0.1 mg/g) and xylazine (0.01 mg/g) and transcardially perfused with 20 ml of PBS (phosphate buffered saline). Following perfusion, brains were immediately placed in 4% PFA (paraformaldehyde) for a further day of fixation (18 – 24 h).

2.12.7 Immunohistochemistry

Following fixation, the brains were cut in half with a standardised matrix, washed with PBS and embedded in paraffin. They were then cut with a microtome into 7 µm coronal slices and let to air dry for at least 24 h. These were stained for nine different biomarkers as summarised with all custom settings per antibody used in **Table 2.1**. One mouse slice per biomarker was used.

First, the slices were rehydrated by two 5-minute washes in xylene (534056-4L, Honeywell), two washes in 100% ethanol (20821.365, VWR), one wash in 90% ethanol and one in 70% ethanol. Subsequently, they were washed for 5 minutes in deionised water and incubated for 30 minutes in the dark in 0.3% hydrogen peroxide (23622.298, VWR) to prevent endogenous peroxidase activity. Antigen retrieval was done by incubating slices in citrate buffer (pH of 6, ab93678, Abcam) in an electric steamer or in 100% formic acid at room temperature (147932500, Thermofisher). After a 5-minute PBS (003002, Invitrogen) wash, protein blocking was done with 10% normal horse serum (NHS) (S-2000-20, Vector Laboratories) in PBS for 60 minutes. After two PBS washes, slides were incubated with the primary antibody diluted in 10% NHS overnight at 4°C. Next day, after three PBS washes, they were incubated with secondary antibodies (MP-7401-50, Vector Laboratories) for 30 minutes at room temperature. Following three PBS washes, the slices were incubated with 3,3'-Diaminobenzidine (DAB; SK-4105, Vector Laboratories). They were then washed in deionised water for 5 minutes and incubated in haematoxylin (H; MHS32-1L, Mayer's) for 2-3 minutes. The slices were washed in tap water and dehydrated (5-minute washes in 70% ethanol, 90% ethanol, 100% ethanol and three xylene washes). Finally, the slides were mounted using DPX mounting medium (D/5330/05, Fisher Scientific). The imaging of full slices was done at 20x magnification with a brightfield scanner (Aperio AT2, Leica).

Table 2.1 | Immunohistochemistry (IHC) antibodies and custom processing steps.

Biomarker	Antibody	Pre-treatment	Pre-treatment time (min)	Dilution factor	DAB time (min)
mOC23	ab251427, Abcam	100% formic acid	10	1:1000	0.5
12F4	SIG-39142, Biolegend	100% formic acid	10	1:1000	1
CT695	51-2700, Thermofisher	Heat-mediated citrate buffer	30	1:1000	1
c-Fos	E-8, Santacruz	Heat-mediated citrate buffer	30	1:500	1
Iba1	019-19741, Wako, Fujifilm	Heat-mediated citrate buffer	30	1:1000	1
GFAP	Z0334, Dako Agilent	-	-	1:1000	1
Ki67	ab16667, Abcam	Heat-mediated citrate buffer	20	1:500	4.5
DCX	ab18723, Abcam	Heat-mediated citrate buffer	20	1:4000	3
SOX2	ab97959, Abcam	Heat-mediated citrate buffer	20	1:4000	1.5

2.12.8 IHC analysis

Data analysis of brightfield images consisted of multiple steps (**Figure 5.1C**). All analysis, apart from the colour deconvolution step, was done in MATLAB (2021a, MathWorks).

First, the image was split into separate H and DAB channels using built-in 'Colour deconvolution' function in Fiji software (Schindelin et al., 2012). Next, eight ROIs were selected per image, containing right and left dentate gyrus (DG), CA1, CA3 and cortex regions (**Figure 5.1B**). The ROI selection was done in a semi-automated fashion, with manual selection for one image type (typically mOC23) followed by supervised automatic ROI selection for subsequent images, with manual selection enforced as needed. The selected ROI images were then screened for presence of large artefacts (e.g. tissue folds) which were then manually removed by drawing an exclusion area per image. Slice mask, containing only the brain tissue, was then generated for each image. Each image had its brightness levels corrected by adjusting its histogram to that of a reference image (imhistmatch function) preselected for each antibody or by subtracting the mean value of the reference image (c-Fos and Ki67 antibodies only). The images were then denoised with a 3 x 3 box kernel. The antibody detection steps created a binary mask by comparing the DAB channel intensity values with a predefined threshold. For the CT695 antibody, a separate mask with all neuronal cell bodies was created from the H channel and the overlap between the two masks was taken to ensure only intracellular APP is measured. Watershed procedure, with the connectivity of 26 pixels, was done for c-Fos, Ki67 and SOX2 images to spatially separate small cell bodies that are overlapping. Finally, all particles in the mask were inspected and rejected if they were too small (minimum size of 1 μm for CT695; 3 μm for SOX2, DCX, Ki67; 4 μm for c-Fos, GFAP, Iba1; and 6 μm for mOC23, 12F4) or too large ($> 15 \mu\text{m}$ for CT695; 30 μm for c-Fos, Ki67, DCX, Sox2; 50 μm for Iba1, GFAP; and 100 μm for mOC23, 12F4). Approximate cell body radius was also calculated for all particles accepted in this step. The final area covered by the antibody was calculated as the % of the brain tissue present in the image, i.e. excluding the non-tissue area as defined by the slice mask. ROIs with less than 20% of the image covered by brain tissue were excluded from the analysis.

2.12.9 Behavioural testing

All behavioural tasks were done in the same location, in a quiet environment with lights dimmed. Before each behavioural paradigm, mice were left in the experimental room for 30 minutes to acclimatise. Each cohort was tested by one of the two experimenters, the same one who also habituated the mice prior to the experiment start. The

experiments deployed tested two aspects of spatial memory, the long-term (OLM task) and the working (Y-maze) memory.

Object Location Memory (OLM) task

Open field arena setup consisted of a 45 x 45 x 45 cm white acrylic box, with visual cues positioned on one of the inner walls (**Figure 5.13A**), and a webcam camera (C920 HD Pro, Logitech) placed above the setup. The inside of the arena was equally illuminated with light to ensure the mice explore the whole area.

The OLM task consisted of three experimental days. The habituation phase took place a day before the S5 day, on the day with no stimulation. On the day, mice were placed in the open field arena and left to explore it freely for 10 minutes while being recorded. On the training day (S5 day), 24 hour later, two identical objects A and B (falcon tubes filled with water) were placed in the arena, 1.5 cm away from the walls in two neighbouring corners. Two configurations of objects were used (either the two top corners, adjacent to the visual cue, or the two bottom corners), with each frequency group split in half and tested in both configurations. Mice were then recorded while exploring the arena with new objects for 10 minutes. At this stage, it is expected that mice would explore both objects equally. On the test day (S6), 24 hour later, object A was moved to the opposite side of the arena. Mice were again recorded for 10 minutes to test whether they would preferentially explore the moved object, as expected if they remember the spatial configuration from the prior day.

Y-maze task

The experimental setup consisted of a 35 x 5 x 10 cm (length x width x height) white acrylic Y-maze with three arms (A, B, C) (**Figure 5.14Ai**), two spatial cues placed on the walls surrounding the maze, and the video camera placed above the setup. The experiment, performed on day S7, involved placing the mouse in one of the arms (with each frequency group split into three subgroups, each starting in a different arm), immediately closing the Y-maze with transparent covers to prevent mice from escaping the arena, and recording their spontaneous behaviour over the 5-minute period.

2.12.10 Behavioural analysis

The mouse behaviour was extracted from the videos using pose estimation deep learning models implemented in the DeepLabCut software, version 2.2.1 in Python (Mathis et al., 2018; Nath et al., 2019). A model was first trained on the reference recordings and manually curated to ensure reliable tracking is achieved for both behavioural tasks. The experimental data was then extracted by the trained DL model and used for further behavioural analysis in MATLAB.

Object Location Memory (OLM) task

First, to ensure that mice sufficiently engaged with the task, the exploration time of the two objects was computed for all experimental groups during the training day. This was calculated as the total time the mouse nose was within 1 cm of either of the objects. The minimum time needed was set to 20 s and all mice met that threshold. Then, to measure their performance on the next two days, the discrimination index was used to assess whether mice preferentially explored one object (A), defined as Discrimination Index (DI): $DI = \frac{\text{object A time}}{\text{object A time} + \text{object B time}}$. A DI of 0.5, equal to chance level, indicates mice have no object preference (expected on the training day). A DI of ~0.6 is expected on the test day in WT mice, indicating mice retain the spatial memory of previous configuration and recognise that one of the objects moved (Leger et al., 2013).

Y-maze task

The Y-maze task measures whether mice tested alternate the three maze arms, indicating intact working memory. This was calculated using three metrics.

Spontaneous Alternation Performance (SAP) indicates whether mice alternate between the three arms equally (**Figure 5.13Ai**), measured as $SAP = \frac{\text{alternation \#}}{\text{total entry \#} - 2}$,

where # = number. This is calculated as the number of sequence triplets with all arms entered, e.g. ABC, BAC, CAB. The chance level for this task is $6/27 = 22.2\%$ based on $3^3 = 27$ total combinations available and 6 possible alternation triplets. Previous literature suggests mice should alternate spontaneously above chance levels, at around 60-80% levels (d'Isa, Comi & Leocani, 2021), with reduced alternations linked to memory deficits (Sarnyai et al., 2000).

To determine whether any finer differences in behaviour could be detected, two more specialised measures were used. The Alternate Arm Returns (AAR) metric indicates whether mice alternated preferentially between a pair of arms (**Figure 5.13Bi**), e.g. ABA or CAC. This is defined as $AAR = \frac{\text{alternate arm return \#}}{\text{total entry \#} - 2}$. Finally, to assess whether mice returned to the same arm (**Figure 5.13Ci**; e.g. BB, CC), Same Arm Returns (SAR) metric was calculated as $SAR = \frac{\text{same arm return \#}}{\text{total entry \#} - 2}$.

Chapter 3. Network-level neuronal responses to temporally interfering electric fields

3.1 Introduction

Direct neuronal responses to Temporal Interference stimulation have so far been primarily investigated using electrophysiological recordings either at the single-cell level *in vivo* (Grossman et al., 2017) or at the network level but *ex vivo* (Esmaeilpour et al., 2021). While electrophysiological recordings have excellent temporal resolution, concurrent TI stimulation remains challenging due to the presence of nonlinear hardware artefacts (von Conta et al., 2022; Luff et al., 2024b). On the other hand, recent fMRI imaging studies in human subjects (Violante et al., 2023; Wessel et al., 2023) use changes in blood oxygenation flow to demonstrate spatial targeting of TI to deep brain structures. As such, however, they suffer from low temporal resolution and can only show indirect stimulation effects.

In order to bridge this experimental gap and demonstrate the functionally relevant, direct network-level dynamics evoked by TI stimulation, I developed a new experimental paradigm to perform cortical widefield calcium imaging and TI stimulation *in vivo* (**Figure 3.1**). This minimally invasive technique involved skull clearance and implantation of two stimulating electrodes in the skull, above the primary visual cortex, allowing to image the widefield cortical neuronal responses to various stimulation paradigms over multiple days. Full details on experimental methods specific to this chapter can be found in sections **2.7-2.10** of **Chapter 2**.

With this approach, I first verified that the neuronal responses to control visual and traditional, low frequency (LF) electrical stimulation can be reliably recorded (section **3.2.1**). I also measured the magnitude of electric fields induced by control LF, TI and amplitude-modulated kHz (AM condition) stimulation to map the relationship between current density and the resulting electric field between the two stimulating electrodes (section **3.2.2**). These measurements verify previous estimates of electric strengths needed with different stimulation modalities (Wang et al., 2022).

Subsequently, I compared the temporal and spatial dynamics evoked by LF and TI stimulation (sections **3.2.3-3.2.4**). Importantly, this characterisation included

comparison between the existing FEM models of expected electric field spread and the measured neuronal responses. Verification of existing models of TI evoked responses is critical for successful adoption of the technique. To further map the TI parameter space, I explored the effects of the TI carrier frequency on the spatiotemporal dynamics of the evoked neuronal responses (section 3.2.5).

Next, I investigated the differences between the AM stimulation, delivered via one electrode pair, and TI stimulation, delivered via two electrode pairs (section 3.2.6). AM kHz stimulation has been used as a proxy for TI in recent *ex vivo* studies (Esmaeilpour et al., 2021; Luff et al., 2024b). However, the different modalities were never directly contrasted. I also tested another modified TI method, pulse-width modulated TI (Acerbo et al., 2022; Luff et al., 2024a), which uses square rather than sinusoidal waveforms, to compare the efficacy of the two techniques (section 3.2.7).

Finally, I characterised the effects of pure kHz stimulation on ongoing neuronal activity, focusing on the quantification and mitigation of onset responses, and in the presence of other visual and electrical stimuli (section 3.2.8). These experiments aimed to experimentally address recent concerns that kHz fields used in TI may lead to depolarisation block of neighbouring neurons (Mirzakhilili et al., 2020).

This study is the first demonstration of the direct spatiotemporal dynamics evoked by the TI stimulation *in vivo* that sheds more light on the possible mechanisms of action behind it. It is also an important step in determining the effects of TI at clinically relevant spatial scales, crucial for further successful adoption of the technique.

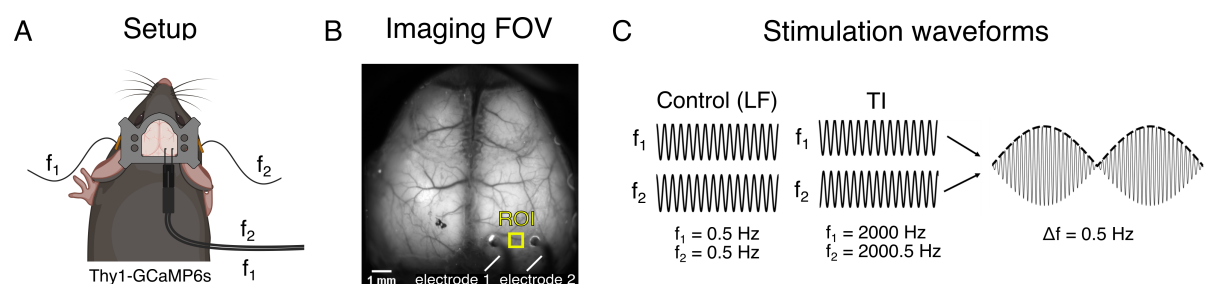


Figure 3.1 | Experimental protocol for simultaneous widefield cortical calcium imaging and Temporal Interference stimulation. **A.** Setup used to deliver TI stimulation and record cortical activity in *Thy1-GCaMP6s* mice. Stimulation at two frequencies, f_1 and f_2 , is applied through two electrode pairs, with stimulating electrodes implanted in the skull and returns placed on the animal cheeks. Schematic created with BioRender.com. **B.** Imaging Field of View (FOV), with electrodes implanted above the primary visual cortex (Configuration 1). Activity from the region of Interest (ROI) located in the visual

cortex, between the electrodes is used in most of the analysis. **C.** Waveforms used in the experiments include control, low frequency (LF) stimulation at 0.5 Hz and TI at 2kHz carrier frequency, with frequency difference Δf of 0.5 Hz.

3.2 Results

3.2.1 Control measurements of spatiotemporal responses to visual stimulation and frequency response range

Due to the presence of the two implanted electrodes above the right visual cortex, the signal underneath and around the electrodes, where the glue attaches the electrodes to the skull, is compromised. To ensure the neuronal activity can be detected despite the electrode presence, visual stimulation (flickering LED light, stimulation frequency $f_{\text{stim}} = 0.5$ Hz) was presented to mice on their left-hand side. First, a calcium trace was extracted from the ROI located between the two electrodes, where most TI activation is expected (as per FEM modelling done in **Figure 3.12B**). The visual stimulus evoked strong oscillatory activity which coincided with each stimulation pulse (**Figure 3.2A**). Power spectral density (PSD) of the calcium response revealed the square pulses of visual stimuli evoked not only activity at the stimulation frequency but also at its second and third harmonic ($2f_{\text{stim}} = 1$ and $3f_{\text{stim}} = 1.5$ Hz) (**Figure 3.2B**). To measure the strength of each of these frequency components, I bandpass filtered the neuronal activity around the frequency of interest (FOI) during the full stimulation period and the baseline period preceding it to obtain the amplitude of the signal at the specified FOI. There was a strong increase in activity present at all the three frequencies, particularly at 0.5 and 1.5 Hz (**Figure 3.2C-Ei**). I also tested whether the stimulation successfully entrained the neuronal oscillation, resulting in a phase-locked rather than a random pattern of activity. At 0.5 and 1.5 Hz, the phase responses were significantly unipolar during stimulation but not during baseline, while at 1 Hz the response was less locked ($p = 0.152$) (**Figure 3.2C-Eii**). The phase resultant at all three frequencies was significantly larger during stimulation than during baseline, further confirming strong entrainment (**Figure 3.2C-Eiii**). At the applied frequency of 0.5 Hz, there was a small phase delay between the applied waveform and the stimulation response (circular mean $\pm$ SD = $16.4 \pm 17.0^\circ$), equivalent to a ~0-200 ms delay, expected to arise due to both the slowness of calcium influx into the activated cells and the slow dynamics of the GCaMP6s indicator (Chen et al., 2013). The mean phases of responses at 1 and

1.5 Hz were $-128.6 \pm 60.3^\circ$ and $122. \pm 13.8^\circ$, respectively. Finally, the visual stimulus was confirmed to induce a strong response at the onset of the stimulation and to increase the steady-state, mean activity during the whole response window, compared to a spontaneous recording (**Figure 3.2F**). Full statistical details related to **Figure 3.2** can be found in **Table 3.1**.

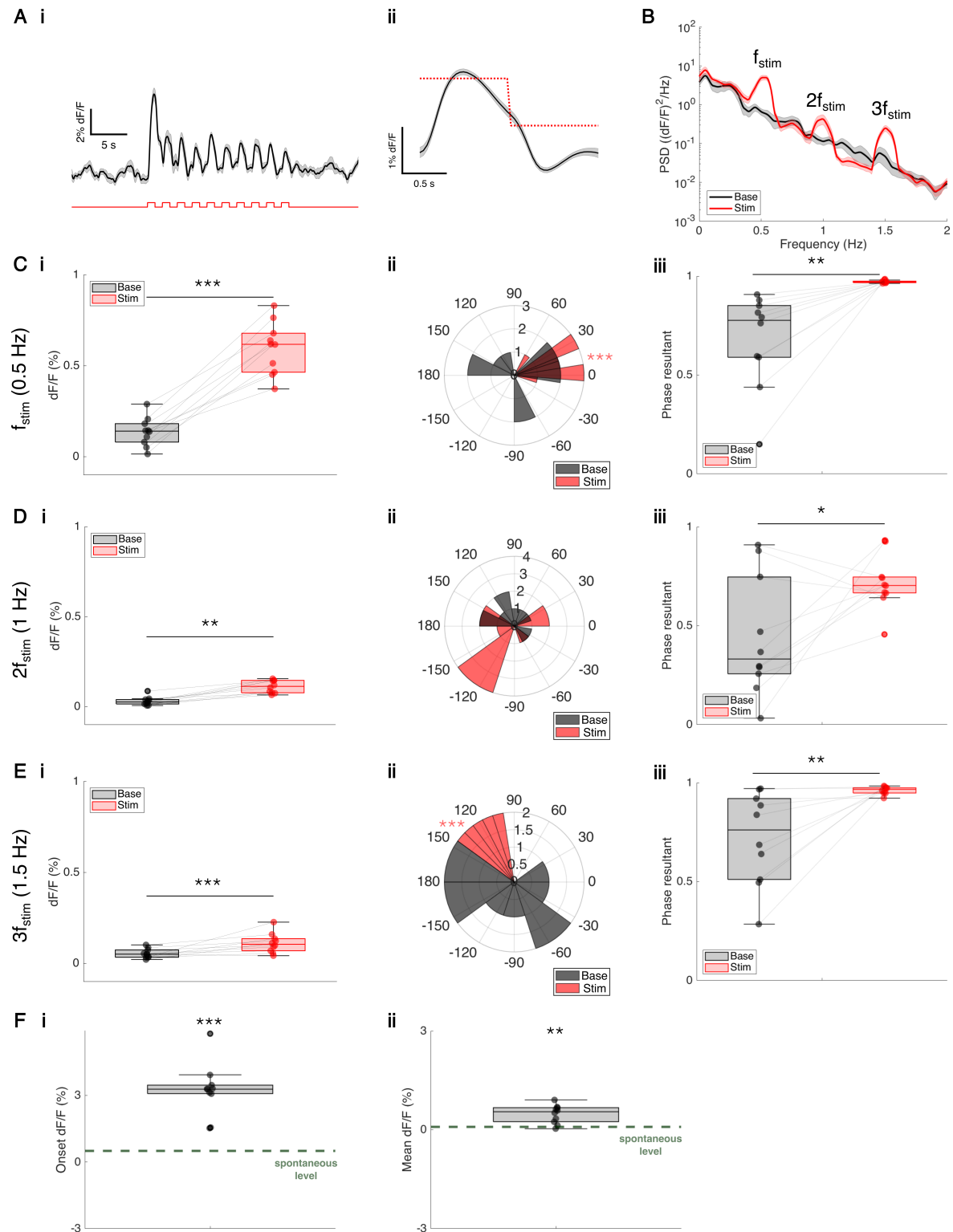


Figure 3.2 | Temporal neuronal responses evoked by visual stimulation at 0.5 Hz. **A. i.** Calcium activity trace extracted from the ROI located between the electrodes during visual stimulation at $f_{stim} = 0.5$ Hz. Mean $\pm$ SEM. $n = 10$. **ii.** Stimulus-triggered average (STA) of the visual response relative to the stimulus. Mean $\pm$ SEM. **B.** Power spectral density (PSD) of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. Visual stimulation evokes activity at f_{stim} but also its harmonics, $2f_{stim}$ and $3f_{stim}$. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at f_{stim} during stimulation was significantly higher than that during baseline. Neuronal activity was band passed filtered within a narrow window around f_{stim} . **ii.** Phase of the neuronal response at f_{stim} during baseline and stimulation periods. Phase of the stimulation response, unlike that of the baseline response was unipolarly distributed. **iii.** Phase resultant of the stimulation response was significantly higher than the resultant of the baseline period. Unipolar phase and significantly higher resultant indicate strong phase locking of the response to the stimulus. **D.** Same as in C but for $2f_{stim}$, the second harmonic of f_{stim} . Strong response, albeit less phase-locked (not unipolar), is present at 1 Hz. **E.** Same as in C but for $3f_{stim}$, the third harmonic of f_{stim} . Strong, phase-locked response is present at 1.5 Hz. **F. i.** Magnitude of the onset visual response is significantly higher than the spontaneous activity level. **ii.** Steady-state dF/F response during visual stimulation is significantly elevated above the spontaneous levels. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.1**.

Table 3.1 | Statistical analysis details related to Figure 3.2.

Measure	FOI (Hz)	Comparison	Test	p	Significance level	n (animals)
dF/F response	0.5	Base v Stim	Paired-sample t-test	2.E-06	***	10
Phase unipolarity		Base	Rayleigh test	0.768		
		Stim	Rayleigh test	4.E-06	***	
Phase resultant		Base v Stim	Paired-sample t-test	0.002	**	
dF/F response	1	Base v Stim	Paired-sample t-test	0.006	**	
Phase unipolarity		Base	Rayleigh test	0.152		
		Stim	Rayleigh test	0.137		
Phase resultant		Base v Stim	Paired-sample t-test	0.019	*	
dF/F response	1.5	Base v Stim	Paired-sample t-test	3.E-05	***	
Phase unipolarity		Base	Rayleigh test	0.186		
		Stim	Rayleigh test	2.E-06	***	
Phase resultant		Base v Stim	Paired-sample t-test	0.005	**	
Onset dF/F	-	Stim v Spont	Wilcoxon signed-rank test	0.001	***	
Mean dF/F		Stim v Spont	Paired-sample t-test	0.001	**	

To inspect the spatial profile of the visual response, I performed a similar analysis at the pixel level for the whole field of view (FOV). First, I checked whether the onset response was observed in the visual areas of the cortex. The onset activation areas, determined as significant using a cluster permutation approach, aligned well with the visual areas of both the hemispheres, delineated in the Allen brain atlas, and included mostly primary visual cortex (V1) but also secondary visual areas and retrosplenial areas adjacent to them (**Figure 3.3A**). Response at the stimulation frequency of 0.5 Hz was very similarly aligned, as was the response at 1.5 Hz (**Figure 3.3B**). The spatial profile of the 1 Hz response was smaller and concentrated in visual areas, suggesting that this component may be arising from local mixing of the frequencies within visual circuits. Spatial distributions of the mean phase of the responses for all FOIs (**Figure 3.3C**) match those previously reported in **Figure 3.2**.

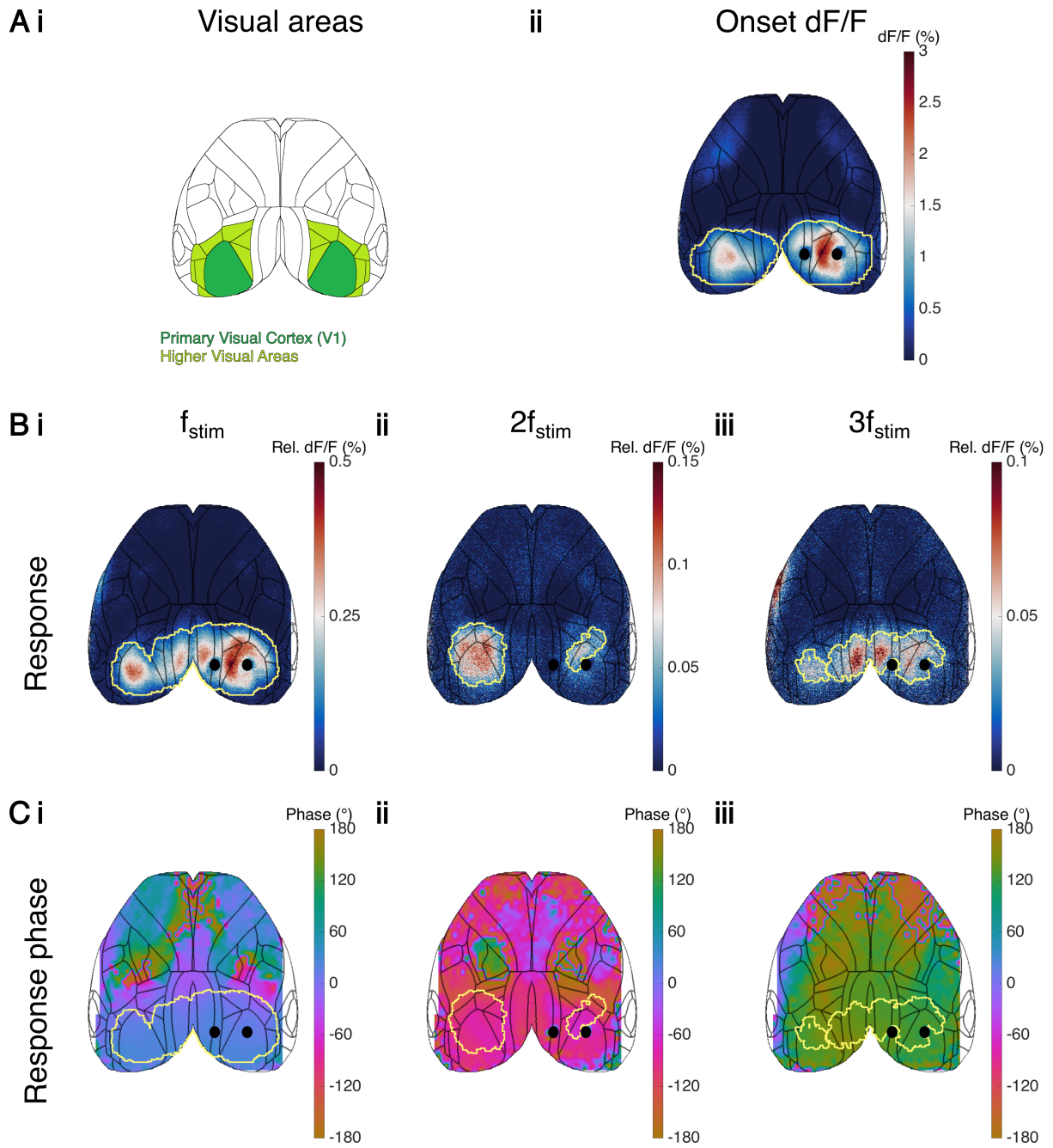


Figure 3.3 | Spatial neuronal responses evoked by visual stimulation at 0.5 Hz. **A. i.** Horizontal projection of cortical areas as delineated by Allen Brain Atlas, with visual areas highlighted. **ii.** Mean onset response map across all animals, with significantly activated areas (yellow outline). **B. i.** Mean f_{stim} response map, with activated areas highlighted. **ii.** Same as in **i** but for $2f_{stim}$. **iii.** Same as in **i** but for $3f_{stim}$. **C. i.** Mean f_{stim} phase map, with activated areas highlighted. **ii.** Same as in **i** but for $2f_{stim}$. **iii.** Same as in **i** but for $3f_{stim}$. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 10$ mice for all.

The calcium indicator GcaMP6s has relatively slow dynamics, with a rise time of ~ 0.5 s and a decay time on the order of ~ 1 s (Chen et al., 2013). Hence, its dynamics severely limit the frequency range and the frequency resolution of the oscillatory activity that it can reliably track. To map the range of frequencies that can be reliably resolved in subsequent experiments, I applied electrical stimulation in the 0.1 – 1.6 Hz range and measured the relative strength of the dF/F response within each frequency window. Given the indicator constraints, I hypothesised that stimuli at 0.5 Hz should be slow enough to allow for full rise and decay per each oscillation period (2 s) and that stimuli > 1 Hz would be too fast to be reliably tracked in this transgenic model. Relative dF/F response for each FOI was calculated as the difference between the stimulation and baseline dF/F response and compared to zero, for comparison equivalent to those in **Figure 3.2C-Ei**. Experimental results show that the responses above 0.3 Hz were reliably detected ($p < 0.01$ for all), even at frequencies above 1 Hz (**Figure 3.4A; Table 3.2**). For those frequencies, however, the value of the response at FOI was smaller in magnitude. Stimulation below 0.3 Hz could not be reliably detected in this setup, potentially due to both weaker effects of such slow stimuli and the detrending step of the pre-processing analysis.

In order to verify the minimum frequency difference between two stimuli that can be reliably detected, I applied three pairs of electrical stimuli with increasing frequency difference between them ($\Delta f = 0.1, 0.2, 0.3$ Hz, with first frequency f_1 set to 0.5 Hz, and second frequency $f_2 = f_1 + \Delta f$) (**Figure 3.4B**). Visual inspection of the resulting PSDs of the neuronal activity shows that stimuli with $\Delta f = 0.3$ Hz can be reliably distinguished as separate frequency peaks, with smaller Δf of 0.2 Hz resulting in incomplete separation of peaks, and Δf of 0.1 Hz leading to one merged peak.

Based on these frequency mappings, 0.5 Hz was established as a suitable stimulation frequency, with a high relative dF/F magnitude, to be used in further experiments, while 0.3 Hz was determined as the minimum frequency difference (Δf) that allows to recover the frequency content of the original stimuli.

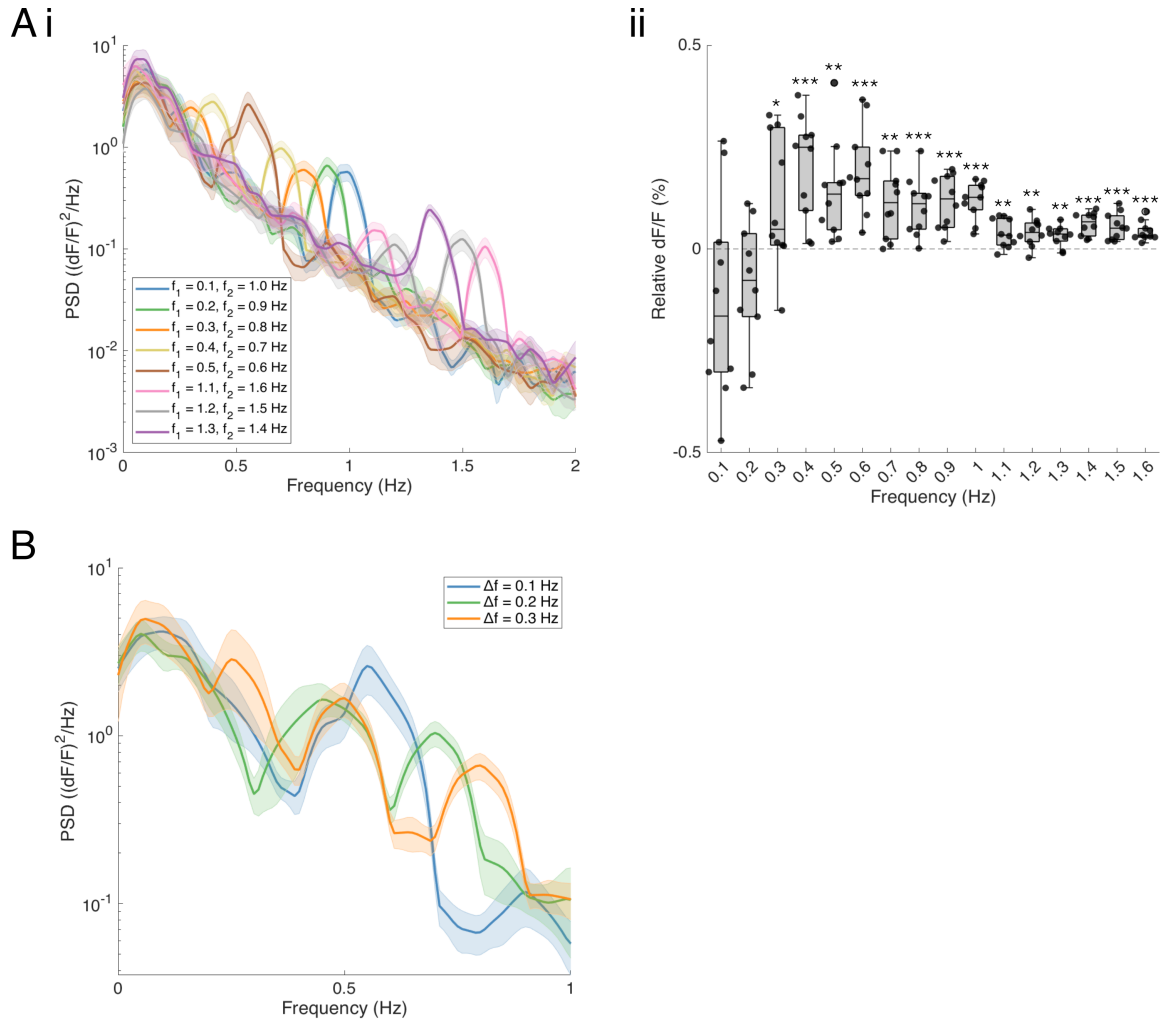


Figure 3.4 | Frequency response range captured in *Thy1-GCaMP6s* mice. A. i. PSD of all the applied electrical stimuli within the 0.1 – 1.6 Hz range. Control stimulation was applied in pairs of two stimuli at a time, through the two stimulating electrodes, to reduce the number of recordings. **ii.** Relative dF/F response at each frequency tested. All frequencies above 0.3 Hz can be reliably recorded, even at higher frequencies > 1 Hz. Mean $\pm$ SEM **B.** PSD of pairs of stimuli with increasing frequency difference, Δf , between them. Separation of at least 0.3 Hz is needed to fully resolve two separate frequency peaks. Mean $\pm$ SEM. $n = 10$ mice for all, for statistical details see **Table 3.2**.

Table 3.2 | Statistical analysis details related to Figure 3.4.

Measure	FOI (Hz)	Test	p	Significance level	n (animals)
Relative dF/F response	0.1	Paired-sample t-test	0.929		10
	0.2	Paired-sample t-test	0.948		
	0.3	Paired-sample t-test	0.028	*	
	0.4	Paired-sample t-test	4.E-04	***	
	0.5	Paired-sample t-test	0.002	**	
	0.6	Paired-sample t-test	2.E-04	***	
	0.7	Paired-sample t-test	0.001	**	
	0.8	Paired-sample t-test	0.001	***	
	0.9	Paired-sample t-test	2.E-04	***	
	1	Paired-sample t-test	1.E-05	***	
	1.1	Paired-sample t-test	0.003	**	
	1.2	Paired-sample t-test	0.003	**	
	1.3	Paired-sample t-test	0.002	**	
	1.4	Paired-sample t-test	1.E-05	***	
	1.5	Paired-sample t-test	3.E-04	***	
	1.6	Paired-sample t-test	3.E-04	***	

3.2.2 Relationship between electric field and current density applied

Electric field generated by applied electrical stimulation creates a potential difference across neurons. The strength of the electric field is thus the important quantity that drives the neuronal activation. Electric field strength depends on the current density applied, the distance from the electrodes but also the medium in which it spreads. To map the relationship between the current density applied and the resulting electric field strength in my experimental setup, I sampled the magnitude of electric potential between the two implanted electrodes (Configuration 2, with electrode separation of 4 mm) at various depths to calculate the resulting field magnitude at different cortical depths. I also measured the field strength in mediolateral (X-direction) and anteroposterior (Y-direction) axes to see if field amplitude depended on the direction

and calculate the average 3D field magnitude. I repeated the same sampling procedure for three experimental conditions: control, low frequency (LF) stimulation ($f_1 = f_2 = 0.5$ Hz, current density $a_1 = a_2 = 0.5$ mA/mm²), TI stimulation ($f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz, $a_1 = a_2 = 3.0$ mA/mm²), and AM stimulation through two cranial electrodes (combined TI waveform of $f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz, $a = 3.0$ mA/mm²).

For all three conditions (LF, TI and AM), the field magnitude remained relatively consistent at different depths (**Figure 3.5A-Ci**) and in all directions (**Figure 3.5A-Cii**; statistical details in **Table 3.3**). For TI stimulation, I additionally calculated the strength of the AM modulation (**Figure 3.5Biii-iv**), which was also consistent. This is equivalent to the electric field strength of the AM condition (**Figure 3.5C**), which by default is perfectly amplitude modulated.

Finally, to compare the three conditions and establish the mapping factor between current density and electric field, I calculated the average 3D field, normalising it to the same current density of 1 mA/mm² per channel (**Figure 3.6A**). The normalised median field was 36.9, 23.7 and 18.0 V/m² for LF, TI and AM conditions, respectively (average of all = 26.2 V/m). There was a small difference between the groups ($p = 0.039$, Friedman's test) but the LF amplitude was not statistically different from that of TI or AM (**Table 3.4**). In the AM condition, the spread of the electric field is different than for LF and TI, but the resulting electric field amplitude in the area between the electrodes was equivalent. The amplitude modulation strength for TI and AM conditions was also similar (**Figure 3.6B**), with median values of 20.8 and 17.5 V/m, respectively (average = 19.3 V/m).

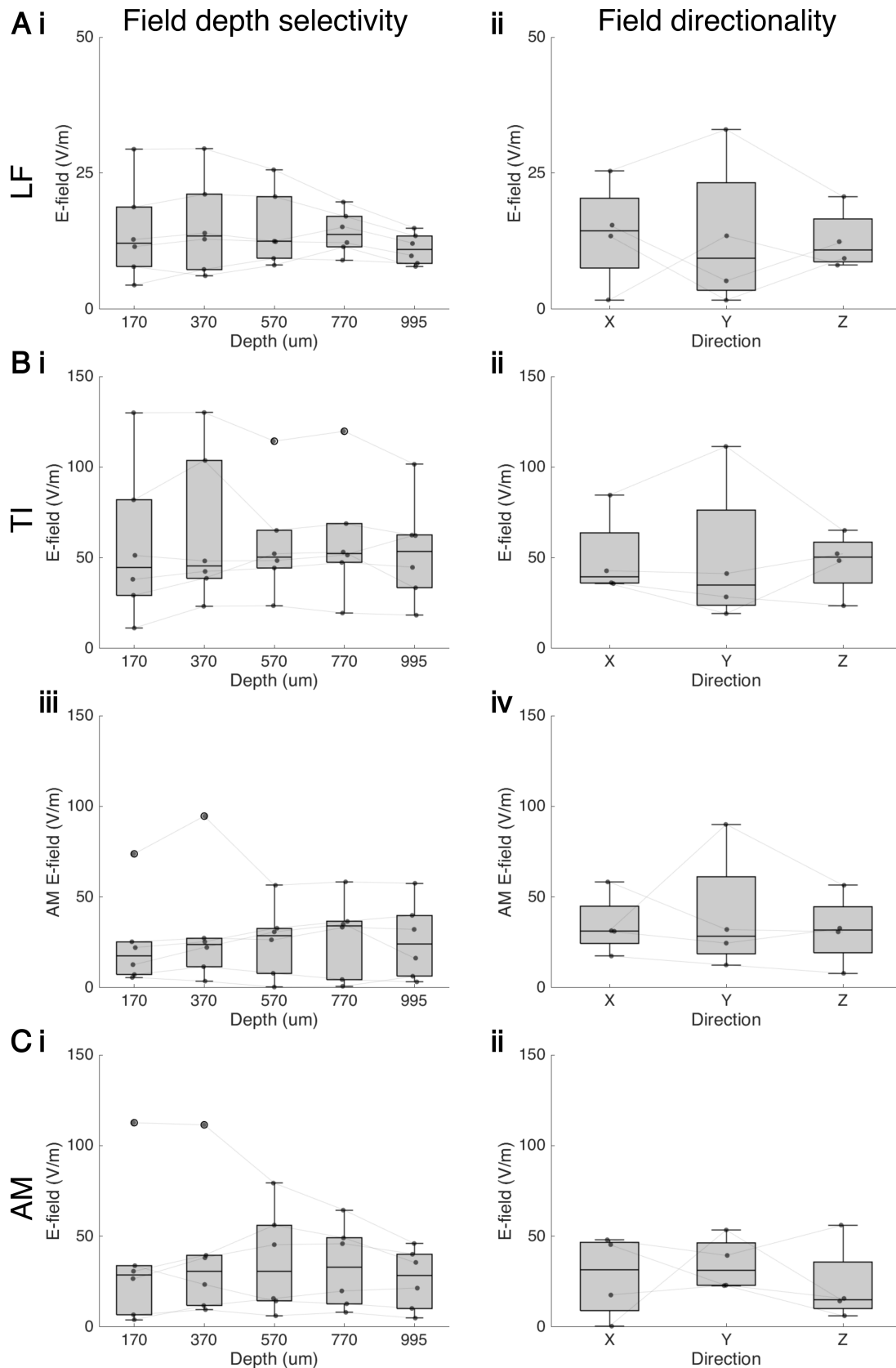


Figure 3.5 | Electric field strength generated by electrical stimulation is consistent at different depths and in all directions. A. i. Summary of the absolute electric field (E-field) amplitudes recorded at different depths in the low frequency (LF) condition ($f_1 = f_2 = 0.5$ Hz, current density $a_1 = a_2 = 0.5$

mA/mm²). **ii.** Summary of the absolute field amplitudes recorded in all 3 dimensions. **B. i-ii.** As in A but for TI condition ($f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz, $a_1 = a_2 = 3.0$ mA/mm²). **iii-iv.** As in **i-ii** but measuring AM modulation amplitude rather than absolute field amplitude. **C.** As in A but for AM condition ($f_1 = 2000$ Hz, $f_2 = 2000.5$ Hz, $a = 3.0$ mA/mm² applied through two cranial electrodes). $n = 4-6$ mice, for statistical details see **Table 3.3**.

Table 3.3 | Statistical analysis details related to Figure 3.5.

Measure	Condition	Comparison	Test	p	Significance level	n (animals)
E-field	LF	Depth selectivity	Friedman's test	0.107		6
		Directionality		0.779		4
	TI	Depth selectivity		0.463		6
		Directionality		0.779		4
	AM	Depth selectivity		0.443		6
		Directionality		0.779		4
AM E-field	TI	Depth selectivity		0.569		6
		Directionality		0.779		4

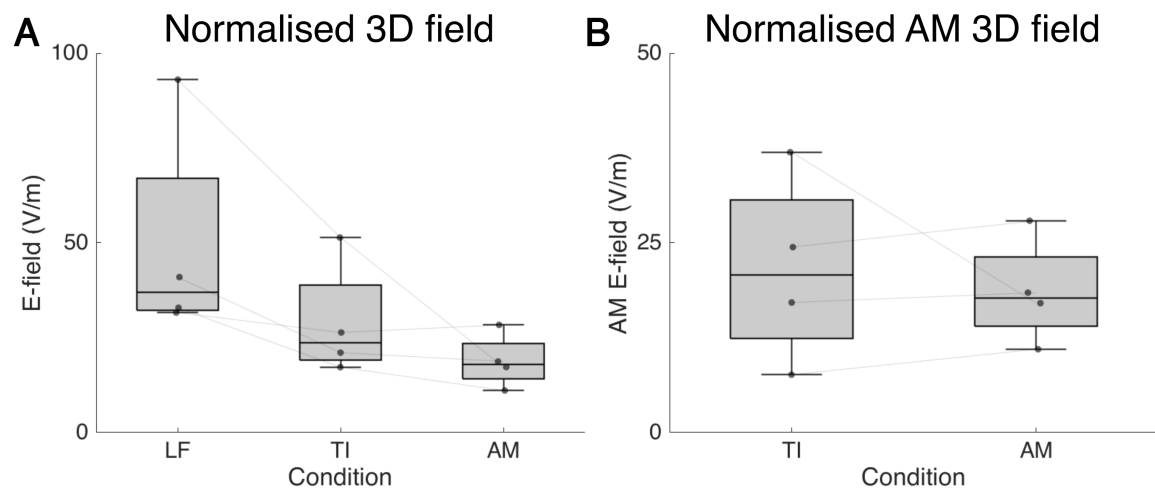


Figure 3.6 | Summary of normalised average 3D field strength for all conditions. A. Median field strength per 1 mA/mm² of current density applied was 36.9, 23.7 and 18.0 V/m for LF, TI and AM respectively. **B.** Median AM field strength per 1 mA/mm² of current density applied per channel was 20.8 and 17.5 V/m for TI and AM. $n = 4$ mice, for statistical details see **Table 3.4**.

Table 3.4 | Statistical analysis details related to Figure 3.6.

Measure	Comparison	Test	p	Significance level	n (animals)
3D E-field	LF v TI v AM	Friedman's test	0.039	*	4
	LF v TI	Wilcoxon signed-rank test	0.125		
	LF v AM		0.125		
	TI v AM		0.250		
AM 3D E-field	TI v AM	Wilcoxon signed-rank test	0.875		

3.2.3 Spatiotemporal responses to control, low frequency stimulation

Having established the frequency range that can be reliably tracked and the mapping between the current density and the resulting electric field magnitude, I tested the cortical responses to standard, control low frequency (LF) stimulation of 0.5 Hz. The response to this stimulus applied through both electrode pairs could be reliably tracked, as seen in the recorded calcium traces, with one clear peak in the PSD around f_{stim} (**Figure 3.7A-B**). To establish the group-level current threshold, i.e. the minimum amount of current density needed to evoke reliable increase in power at the stimulation frequency across all animals, I applied the stimulation at increasing current densities. For control electrical stimulation, 0.3 mA/mm² was found to induce significant increase in activity at 0.5 Hz, with the strength of the response increasing further for increasing current density (**Figure 3.7Ci**). The responses at ≥ 0.3 mA/mm² also resulted in strong phase-locked entrainment of the neuronal activity, as indicated by both the unipolarity of the phase responses and the significant increase in the phase resultant as compared to the baseline period. (**Figure 3.7Cii-iii**) The mean phase lag between the stimulus and the response at 0.3 mA/mm² was $45.0 \pm 11.6^\circ$ (~200-300 ms), with response lagging behind the stimulation waveform, as captured by the stimulus-triggered average (STA) plot (**Figure 3.7Aii**). Finally, the onset and mean responses were measured at all the current densities applied. Control low frequency stimulation did not cause an increase in activity at the stimulus onset or a sustained change of mean activity throughout the stimulation, even at the highest current density applied, with the resulting neuronal activity consistently oscillating around zero (**Figure 3.7D**).

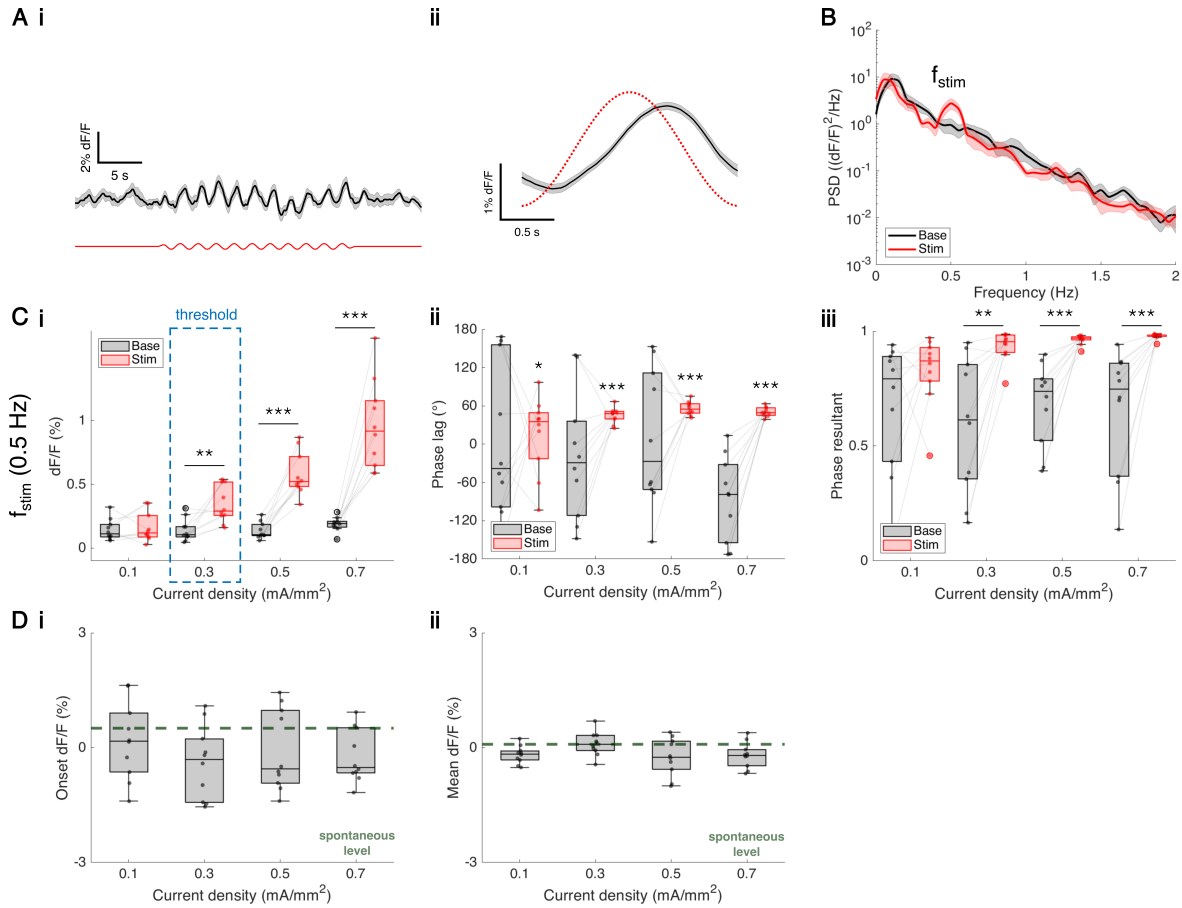


Table 3.5 | Statistical analysis details related to Figure 3.7.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0
dF/F response	0.5	Base v Stim	Wilcoxon signed-rank test	0.188	0.002	0.001	0.001	0.188	0.004	0.004	0.004		**	***	***	10	10	10	10
Phase unipolarity		Base	Rayleigh test	0.402	0.627	0.883	0.082	1.000	1.000	1.000	0.327								
		Stim	Rayleigh test	0.020	1.E-06	1.E-06	7.E-07	0.020	3.E-06	3.E-06	3.E-06	*	***	***	***				
Phase resultant		Base v Stim	Wilcoxon signed-rank test	0.065	0.002	0.001	0.001	0.065	0.004	0.004	0.004		**	***	***				
Onset dF/F	-	Stim v Spont	Paired-sample t-test	0.775	0.980	0.895	0.979	1.000	1.000	1.000	1.000								
Mean dF/F		Stim v Spont	Wilcoxon signed-rank test	0.968	0.500	0.976	0.968	1.000	1.000	1.000	1.000								

Following on the ROI analysis, I investigated the spatial profile of the responses to the control LF stimulation. **Figure 3.8** shows the evolution of the spread of the responses, with stronger currents activating more widespread regions spanning most of the imaged cortical area. At the threshold level of 0.3 mA/mm² the response was located around the two stimulating electrodes (**Figure 3.8Aii**), with the signal stronger around electrode 2. This was likely due to the reduced visibility of the calcium signal around the midline suture, close to electrode 1.

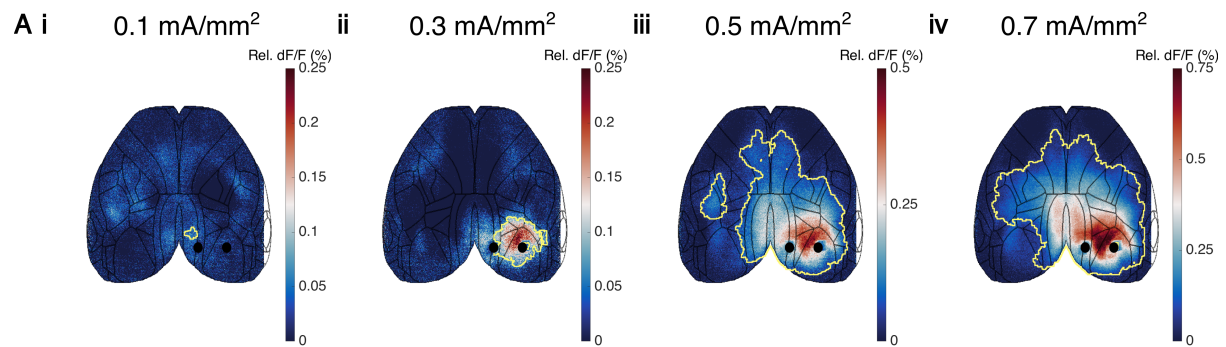


Figure 3.8 | Evolution of spatial neuronal responses evoked by control electrical stimulation of increasing strength. A. i. Mean f_{stim} response map at 0.1 mA/mm², with activated areas highlighted. **ii.** Same as in **i** but at 0.3 mA/mm². **iii.** Same as in **i** but at 0.5 mA/mm². **iv.** Same as in **i** but at 0.7 mA/mm². Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 10$ mice for all.

The absolute strength of the electric fields is expected to be the strongest just underneath the stimulating electrodes, as simulated using the Finite Element Method (FEM) model that implements the exact experimental configuration in a virtual mouse (**Figure 3.9B**). To compare the experimental results with the FEM model, I extracted a horizontal profile of the response within an ROI located at the electrode level (**Figure 3.9Ai-Bi**) and correlated the average experimental response with the model. While the Pearson correlation coefficient r is quite low ($r = 0.25$), this is due to the inability to

image just underneath the electrodes, i.e. the areas with the highest electric field strength. The shape of the experimental response, however, is similar to that of the modelled response (**Figure 3.9C**). Similarly, the response is expected to be quite superficial (**Figure 3.9Bii**), although this could not be measured with calcium imaging. Spatial maps of the response at the harmonic of f_{stim} , 1 Hz, and the onset dF/F both show no significant clusters in the activity, similar to the previous observation at the ROI level (**Figure 3.9D-E**).

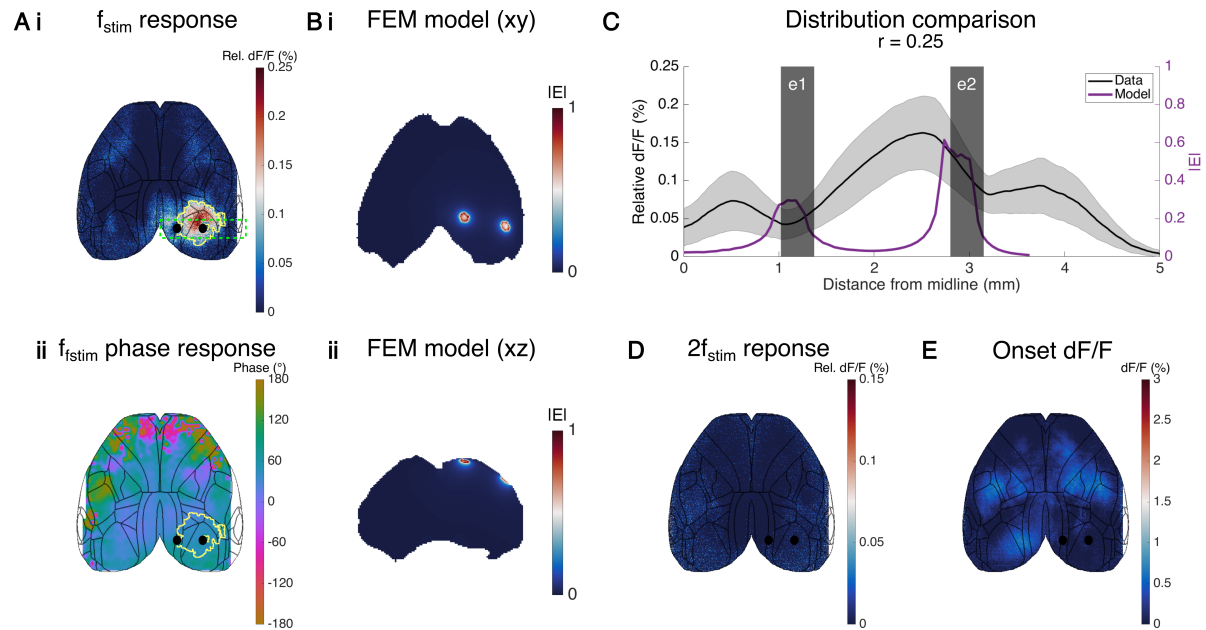


Figure 3.9 | Spatial neuronal responses evoked by control electrical stimulation. **A. i.** Mean f_{stim} response map at 0.3 mA/mm^2 , with activated areas highlighted and horizontal ROI used for analysis in **C** in green. **ii.** Corresponding mean f_{stim} phase map. **B. i.** Horizontal projection of maximum absolute electric field distribution estimated using Finite Element Methods (FEM) model. Field magnitude normalised to maximum value. **ii.** As in **i** but for a sagittal maximum projection. **C.** Comparison of the horizontal profile of the f_{stim} response (data) and the FEM distribution (model). Experimental measurements are affected by reduced visibility around stimulating electrodes, resulting in a relatively low correlation (Pearson correlation coefficient $r = 0.25$), but the response shape is similar to that of the model. Mean locations of implanted electrodes 1 (e1) and 2 (e2) indicated as grey bars. Electrode areas were excluded from the correlation fit. **D.** Mean $2f_{\text{stim}}$ response map at 0.3 mA/mm^2 confirms no activation is present at that frequency. **E.** Mean onset map confirms no onset response is present during control stimulation. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 10$ mice for all.

3.2.4 Spatiotemporal responses to TI stimulation

Similarly to the control LF test, temporal interference stimulation was applied through two electrode pairs, with frequencies $f_1 = 2000$ and $f_2 = 2000.5$ Hz, resulting in a signal that is amplitude modulated at $\Delta f = 0.5$ Hz. As in control condition, the neuronal activity measured in the ROI showed a clearly induced oscillation during TI (**Figure 3.10A**). However, the oscillation is not limited to Δf but also contains the second harmonic component ($2\Delta f$) at 1 Hz (**Figure 3.10B**), also captured by two peaks in the STA plot (Fig 3.8Aii). Applying increasing current density using the same stimulation parameters revealed that the current threshold for TI stimulation using a carrier frequency (f_{carrier}) of 2000 Hz was 2.5 mA/mm^2 , ~ 8 times higher than when using 0.5 Hz directly (**Figure 3.8C**). The mean phase of the evoked neuronal response at 2.5 mA/mm^2 was $-150.2 \pm 43.6^\circ$, seemingly preceding the amplitude modulation peak (**Figure 3.10Aii**), and was not as consistent in phase locking the neuronal response at the higher current of 3 mA/mm^2 (**Figure 3.10Cii**), despite relatively strong dF/F amplitude and phase resultant values at Δf . The current threshold to evoke activity at $2\Delta f$ was also 2.5 mA/mm^2 , indicating that both frequencies arise simultaneously when neurons respond to TI stimulation. Interestingly, the phase locking of the response at $2\Delta f$ was more consistent at the higher current amplitude of 3 mA/mm^2 , suggesting that this frequency component becomes even more dominant as the current is increased further, perhaps due to more nonlinear processes taking place at high current densities. Finally, I measured the onset and mean stimulation response magnitudes (**Figure 3.10E**). At the higher current amplitudes of $\geq 2.5 \text{ mA/mm}^2$, a significantly large onset response can be detected, and it increases with increasing current density. The mean dF/F amplitude, however, was only significantly different from the spontaneous level at the highest current density of 3 mA/mm^2 .

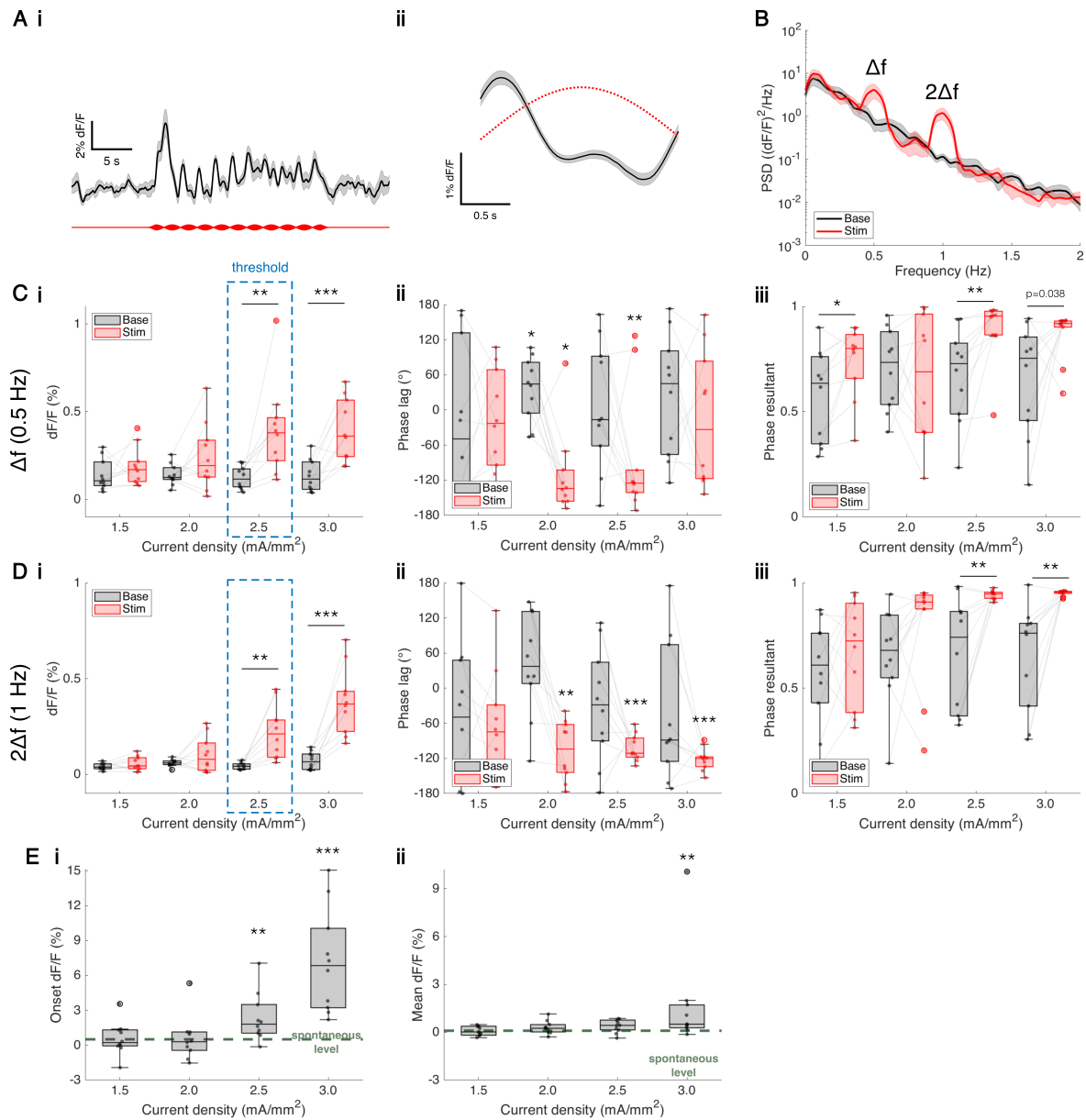


Figure 3.10 | Temporal neuronal responses evoked by TI stimulation with Δf of 0.5 Hz. **A. i.** Calcium activity trace extracted from the ROI located between the electrodes during TI stimulation of $f_1 = 2000$ and $f_2 = 2000.5$ Hz, $\Delta f = 0.5$ Hz, at 2.5 mA/mm^2 . Mean $\pm$ SEM. $n = 10$. **ii.** STA of the neuronal response relative to the stimulus. Mean $\pm$ SEM. **B.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. TI stimulation evokes activity at Δf and its harmonic, $2\Delta f$. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at Δf during stimulation and baseline for increasing current densities. Group-level threshold of activation was established as $2.5 \text{ mA/mm}^2/\text{channel}$. **ii.** Phase of the neuronal response at Δf during baseline and stimulation periods at all current amplitudes tested. **iii.** Phase resultant at Δf during baseline and stimulation periods at all current amplitudes tested. **D.** As in **C** but at $2\Delta f$. $2\Delta f$ group-level threshold was the same as the Δf one ($2.5 \text{ mA/mm}^2/\text{channel}$). The phase response at higher current amplitudes was more consistent for $2\Delta f$. **E. i.** TI stimulation induced onset responses at $\geq 2.5 \text{ mA/mm}^2$. **ii.** TI did not induce steady state dF/F responses, except at the highest current density of 3 mA/mm^2 . Spontaneous onset magnitude = $0.50 \pm$

0.81 %, spontaneous mean magnitude = 0.09 ± 0.22 %. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.6**

Table 3.6 | Statistical analysis details related to Figure 3.10.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0
dF/F response	0.5	Base v Stim	Paired-sample t-test	0.175	0.059	0.005	0.000	0.175	0.117	0.015	0.002			**	***	10	10	10	10
Phase unipolarity		Base	Rayleigh test	0.150	0.012	0.990	0.987	0.451	0.050	1.000	1.000		*						
		Stim	Rayleigh test	0.581	0.004	0.004	0.591	1.000	0.015	0.015	1.000								
Phase resultant		Base v Stim	Paired-sample t-test	0.011	0.653	0.005	0.038	0.033	0.653	0.019	0.076	*		**					
dF/F response	1	Base v Stim	Paired-sample t-test	0.116	0.094	0.001	0.000	0.188	0.188	0.003	0.000			**	***	10	10	10	10
Phase unipolarity		Base	Rayleigh test	0.789	0.356	0.813	0.176	1.000	1.000	1.000	0.705								
		Stim	Rayleigh test	0.216	0.006	1.E-05	6.E-06	0.216	0.012	4.E-05	2.E-05		**	***	***				
Phase resultant		Base v Stim	Paired-sample t-test	0.201	0.121	0.005	0.002	0.242	0.242	0.015	0.009			**	**				
Onset dF/F	-	Stim v Spont	Wilcoxon signed-rank test	0.500	0.862	0.007	0.001	1.000	1.000	0.021	0.004			**	***				
Mean dF/F		Stim v Spont	Wilcoxon signed-rank test	0.500	0.216	0.032	0.010	0.500	0.432	0.097	0.039				**				

Following the ROI analysis, I assessed the spatial distribution of the evoked responses at Δf and $2\Delta f$ at increasing current densities (**Figure 3.11**). In this case, the smallest current amplitude with significant clusters around the electrodes was 2 mA/mm². At this current density, a clear cluster of significant activity was located between the two stimulating electrodes and in the two smaller clusters located in the contralateral hemisphere (**Figure 3.11Aii**). Interestingly, no activation clusters at $2\Delta f$ for the same condition were yet present (**Figure 3.11Bii**). With the increasing current density, the stimulation area became much larger, especially in the other hemisphere. At 2.5 mA/mm², it spread much further in the contralateral hemisphere (**Figure 3.11Aiii**); and at 3 mA/mm², it covered most of the cortical surface (**Figure 3.11Aiv**), similarly to low frequency stimulation at high current amplitudes. The spatial profile of the $2\Delta f$ response (**Figure 3.11Biii**) at 2.5 mA/mm² is less focal than that of the Δf response (**Figure 3.11Aiii**), suggesting further mixing of the frequencies within the neuronal responses, resulting in creation of the second harmonic component, may be facilitated by the polysynaptic effects.

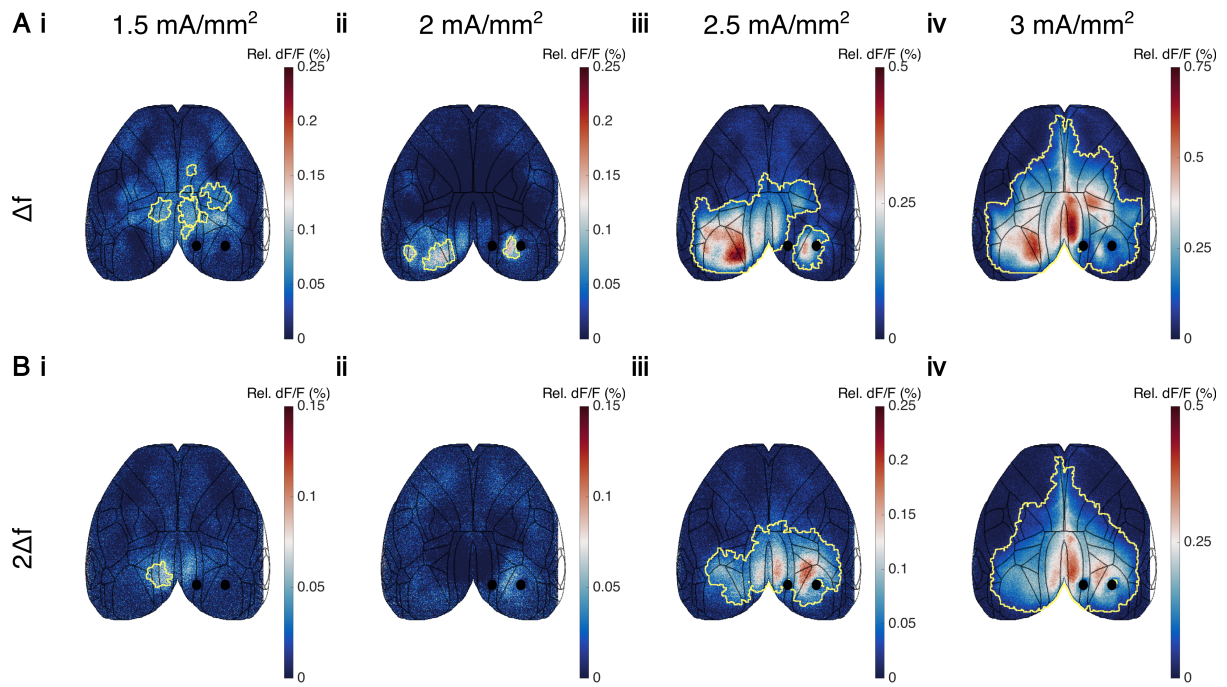


Figure 3.11 | Evolution of spatial neuronal responses evoked by TI stimulation of increasing strength. **A. i.** Mean Δf response map at 1.5 mA/mm^2 , with activated areas highlighted. **ii.** Same as in **i** but at 2 mA/mm^2 . **iii.** Same as in **i** but at 2.5 mA/mm^2 . **iv.** Same as in **i** but at 3 mA/mm^2 . **B.** Same as in **A** but for $2\Delta f$. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 10$ mice for all.

To investigate the spatial profile of the TI stimulation further, I compared the spatial response profile at 2 mA/mm^2 with the corresponding FEM model for TI. The FEM models, developed to determine the electrode configuration needed to reach a desired target, assume that the TI stimulation area corresponds to the area of the maximum amplitude modulation (AM), in the location where the two fields most overlap in space. However, this has not been directly verified up to date. **Figure 3.12Bi** demonstrates where the expected maximum AM area is located for present experimental configuration. Comparing the experimental response with the FEM model demonstrates that there is a good correlation between the data and the model ($r = 0.52$). The actual response peak was skewed closer to electrode 2 than that of the model, perhaps due to the curvature of the lateral cortex affecting the spread of the fields more than expected. The FEM model also indicates that the TI stimulation (**Figure 3.12Bii**) may be more penetrating than that of the LF control (**Figure 3.9Bii**), i.e. able to reach deeper cortical layers, some of which include neurons that have interhemispheric projections. Investigating the experimental phase response map

(Figure 3.12Aii) indicates that the clusters in the contralateral hemisphere have a different phase delay, suggesting this activity arises from polysynaptic activation rather than as a direct stimulation effect.

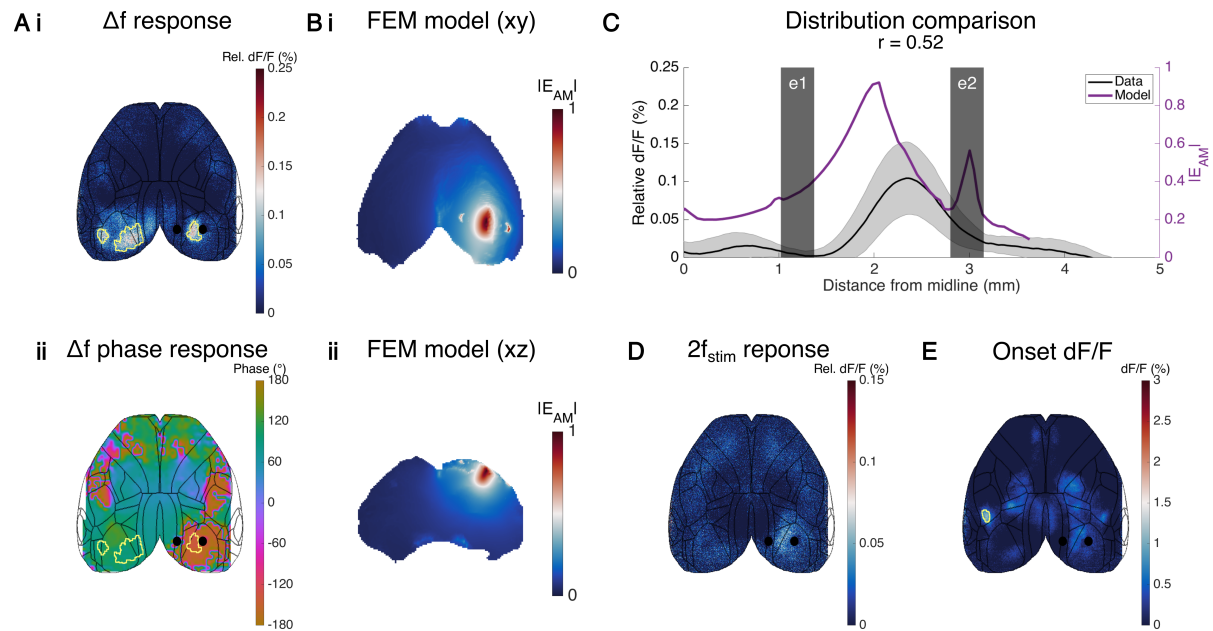


Figure 3.12 | Spatial neuronal responses evoked by TI stimulation. **A. i.** Mean Δf response map at 2 mA/mm², with activated areas highlighted. Horizontal ROI same as in **Figure 3.9. ii.** Corresponding mean Δf phase map. **B. i.** Horizontal projection of maximum amplitude modulation (AM) of the electric field distribution estimated using the Finite Element Methods (FEM) model. Field magnitude normalised to maximum value. **ii.** As in **i** but for a sagittal maximum projection. **C.** Comparison of the horizontal profile of the Δf response (data) and the FEM distribution (model). Experimental measurements correlate with the predicted model (Pearson correlation coefficient $r = 0.52$). Mean locations of implanted electrodes 1 (e1) and 2 (e2) indicated as grey bars. **D.** Mean $2\Delta f$ response map at 2 mA/mm² shows activation is yet present at that frequency, indicating response at Δf is generated before that at $2\Delta f$. **E.** Mean onset map shows no major onset response is present during TI stimulation at 2 mA/mm². Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 10$ mice for all.

To further investigate the nature of this secondary activation, the neuronal response in the contralateral ROI, in a location mirrored across the midline for each mouse, was measured at 2.5 mA/mm² (**Figure 3.13A**). While the contralateral activity also shows clear frequency peaks at both Δf and $2\Delta f$, the phase delay between stimulus and the response at Δf is different from that seen in the ipsilateral hemisphere (**Figure 3.13Di**), with the phase of $104.5 \pm 20.7^\circ$. Interestingly, the $2\Delta f$ phases of both contralateral and ipsilateral responses were not significantly different from each other (**Figure 3.13Dii**).

Despite no significant increase in dF/F amplitude detected at $3\Delta f$ (relative dF/F = 0.0094 ± 0.0391 ; $p = 0.2326$, paired-sample t-test), the response was entrained to that frequency, as indicated by a significantly unipolar phase of the resulting signal (with increase in phase resultant, stimulation vs baseline, 0.86 ± 0.17 , 0.61 ± 0.32 ; $p = 0.0363$, paired-sample t-test), suggesting further mixing of frequencies may be taking place in the area receiving TI input polysynaptically. Inspecting the ipsilateral and contralateral neuronal traces suggests that the ipsilateral activation results in suppression in activity in the contralateral hemisphere, with ipsilateral peaks leading to contralateral signal suppression (**Figure 3.13C**). The very first peak seen at the stimulus onset for both ipsilateral and contralateral responses most likely arises due to the onset of kHz stimulation, as kHz stimulation alone is able to elicit a simultaneous onset response in both hemispheres (**Figure 3.13E**).

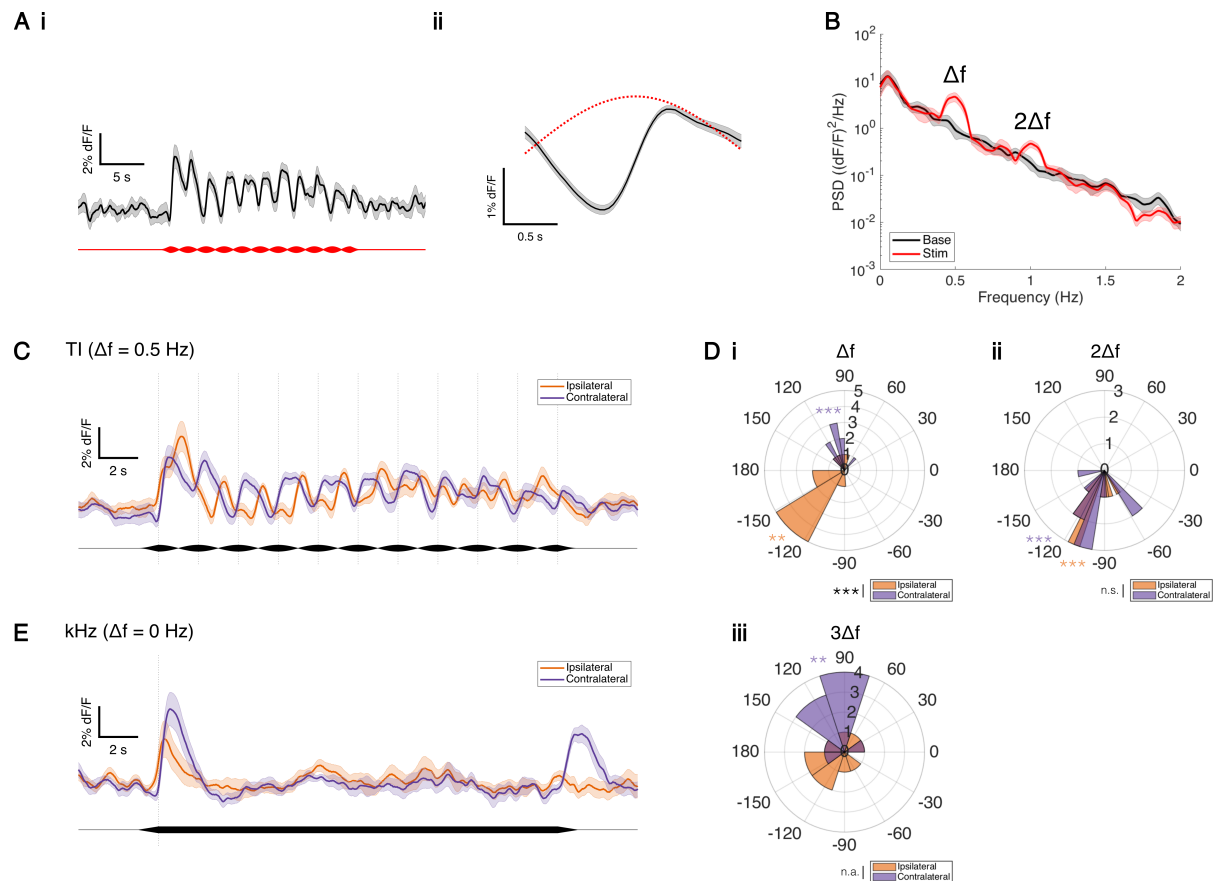


Figure 3.13 | Contralateral cortical response evoked by TI stimulation. **A. i.** Calcium activity trace extracted from the contralateral ROI located during TI stimulation of $f_1 = 2000$ and $f_2 = 2000.5$ Hz, $\Delta f = 0.5$ Hz, at 2.5 mA/mm^2 . Mean $\pm$ SEM. $n = 10$. **ii.** STA of the neuronal response relative to the stimulus. Mean $\pm$ SEM. **B.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. In the contralateral hemisphere, TI stimulation evokes activity at both Δf and $2\Delta f$. Mean $\pm$

SEM. **C.** Contralateral activity (purple) seems to be inhibited by the direct ipsilateral activity (orange). Current density of 2.5 mA/mm². Mean $\pm$ SEM. **D. i.** Phase of the neuronal response at Δf is different in the contralateral hemisphere than in the ipsilateral one. **ii.** Contralateral $2\Delta f$ phase matches the ipsilateral one. **iii.** Unlike ipsilateral one, contralateral activity shows unipolar phase distribution at the third harmonic, $3\Delta f$. **E.** kHz stimulation ($f_1 = f_2 = 2000$ Hz) generates the first simultaneous onset peak in both hemispheres, visible also in TI traces in **C.** Current density of 2.5 mA/mm². Mean $\pm$ SEM. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.7** and **Table 3.8**.

Table 3.7 | Statistical analysis details related to Figure 3.13; phase unipolarity.

Measure	FOI (Hz)	Comparison	Test	p		Bonferroni-Holm p		Significance level		n (animals)	
				Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
Phase unipolarity	0.5	Stim	Rayleigh test	0.004	0.000	0.004	0.000	**	***	10	10
	1		Rayleigh test	0.000	0.000	0.000	0.000	***	***		
	1.5		Rayleigh test	0.588	0.005	0.588	0.009		**		

Table 3.8 | Statistical analysis details related to Figure 3.13; phase comparison.

Measure	FOI (Hz)	Comparison	Test	p	Significance level	n (animals)
Phase comparison	0.5	Ipsi v Contra	Watson-Williams test	1.E-05	***	10
	1			0.854		
	1.5			n.a.		

3.2.5 Effects of the TI carrier frequency on the neuronal response

The original TI study tested the effect of varying carrier frequency (f_{carrier}), in the range of 1 to 4 kHz, on the amount of current needed to evoke reliable motor response and found that the motor threshold increased with higher carrier frequency (Grossman et al., 2017). To verify that this is reflected in the network-level neuronal responses and also to test whether TI stimulation can be achieved with lower carrier frequencies in the endogenous frequency range (≤ 100 Hz), I performed a TI threshold finding procedure at different carrier frequencies: 10, 100 and 5000 Hz (**Figure 3.14**). The respective current thresholds were found to be 0.7, 0.7 and 4 mA/mm². In all these conditions, the phase of the response at Δf relative to AM waveform applied was, unlike in LF stimulation, negative, with the mean phase of $-85.4 \pm 32.1^\circ$, $-36.1 \pm 48.3^\circ$ and $-148.6 \pm 45.9^\circ$, respectively (**Figure 3.14A-Cii-iv**). The phases of f_{carrier} of 10 and 100 Hz were different than those of f_{carrier} of 2000 Hz reported previously in **Figure 3.10** ($p = 0.0038$ and $p = 0.0002$, respectively; Watson-Williams test), while that of

5000 Hz matched it ($p = 0.9460$). Inspecting the PSD of resulting neuronal oscillations revealed that only the carrier frequency of 5000 Hz had a secondary peak component of $2\Delta f$ (**Figure 3.14A-Cv**), suggesting the presence of the kHz frequencies contributes to the increased mixing of signals present, generating the additional frequency component. Similarly to the threshold for f_{carrier} of 2000 Hz, $2\Delta f$ threshold for f_{carrier} of 5000 Hz was the same as the one for Δf , at 4 mA/mm^2 .

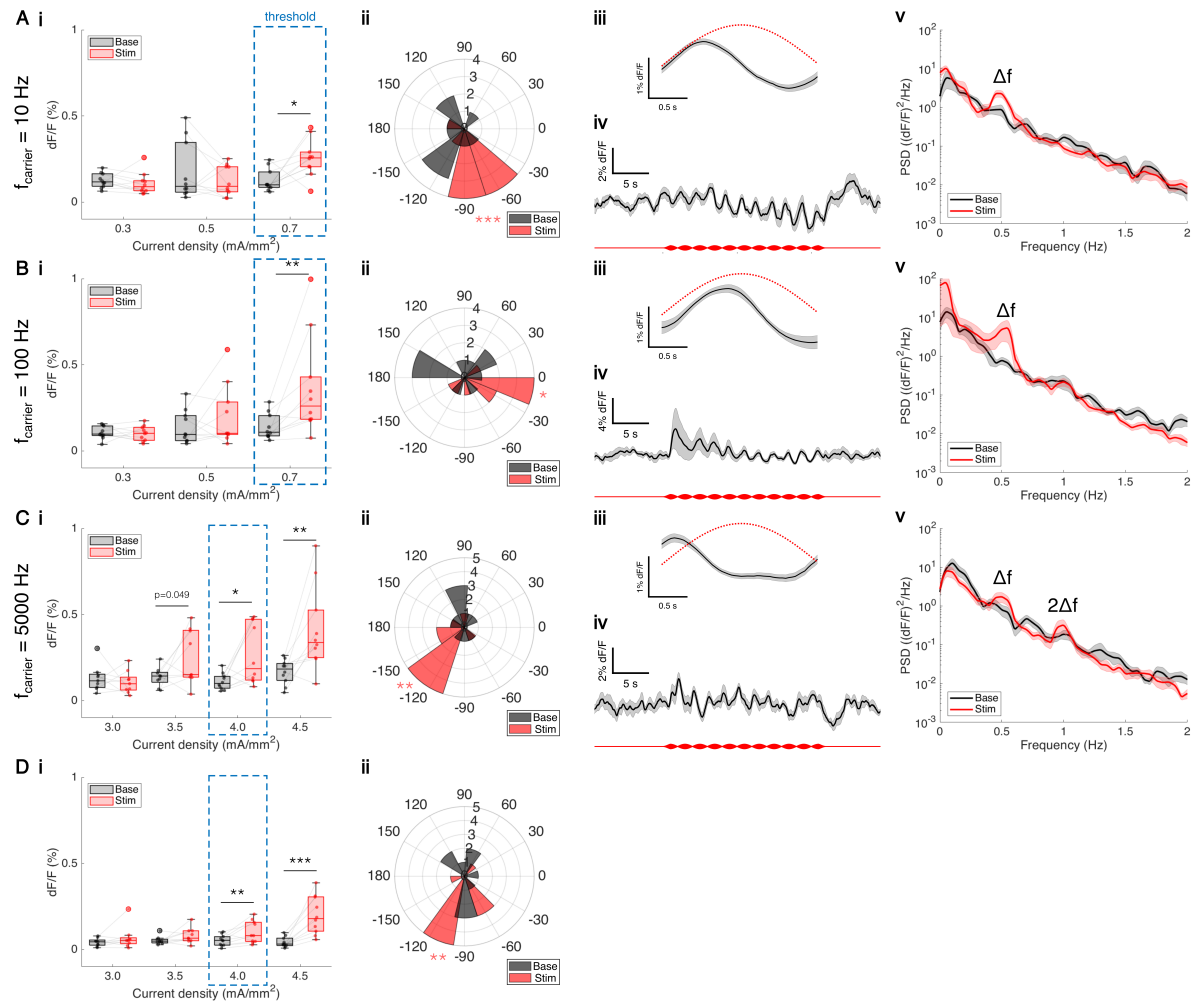


Figure 3.14 | TI stimulation can be achieved with carrier frequencies in both endogenous and kHz range. Additional carrier frequencies of 10 (A), 100 (B) and 5000 (C) Hz were tested. **A. i.** Amplitude of the neuronal response for $f_{\text{carrier}} = 10 \text{ Hz}$ at Δf during stimulation and baseline for increasing current densities. Group-level threshold of activation was established as $0.7 \text{ mA/mm}^2/\text{channel}$. **ii.** Phase response at Δf during baseline and stimulation periods. **iii.** STA of the neuronal response relative to the stimulus corresponding to the trace in **iv**. Mean $\pm$ SEM. $n = x$. **iv.** Calcium activity trace during TI stimulation of $f_1 = 10$ and $f_2 = 10.5 \text{ Hz}$, $\Delta f = 0.5 \text{ Hz}$. Mean $\pm$ SEM. **v.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **iv**. **B.** As in **A** but for $f_{\text{carrier}} = 100 \text{ Hz}$. Group-level threshold of activation was $0.7 \text{ mA/mm}^2/\text{channel}$. **C.** As in **A** but for $f_{\text{carrier}} = 5000 \text{ Hz}$. Group-level threshold of activation was $4 \text{ mA/mm}^2/\text{channel}$. Only the carrier frequency in the kHz frequency range

evoked neuronal response at $2\Delta f$. **D. i-ii.** As in C but at $2\Delta f$. $2\Delta f$ group-level threshold was also 4 mA/mm²/channel. Plots **ii-v** corresponded to threshold current densities of 0.7, 0.7 and 4 mA/mm² for carrier frequencies of 10, 100 and 5000 Hz, respectively. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.9**, **Table 3.10**, and **Table 3.11**.

Table 3.9 | Statistical analysis details related to Figure 3.14; $f_{\text{carrier}} = 10$ Hz.

Measure	FOI (Hz)	Comparison	Test	p			Bonferonni-Holm p			Significance level			n (animals)		
				Current density (mA/mm ²)			Current density (mA/mm ²)			Current density (mA/mm ²)			Current density (mA/mm ²)		
				0.3	0.5	0.7	0.3	0.5	0.7	0.3	0.5	0.7	0.3	0.5	0.7
dF/F response	0.5	Base v Stim	Paired-sample t-test	0.784	0.837	0.010	1.000	1.000	0.031			*	10	10	10
Phase unipolarity		Base	Rayleigh test	0.316	0.849	0.163	0.632	0.849	0.489						
		Stim	Rayleigh test	0.130	0.331	2.E-04	0.259	0.331	0.001			***			

Table 3.10 | Statistical analysis details related to Figure 3.14; $f_{\text{carrier}} = 100$ Hz.

Measure	FOI (Hz)	Comparison	Test	p			Bonferroni-Holm p			Significance level			n (animals)		
				Current density (mA/mm ²)			Current density (mA/mm ²)			Current density (mA/mm ²)			Current density (mA/mm ²)		
				0.3	0.5	0.7	0.3	0.5	0.7	0.3	0.5	0.7	0.3	0.5	0.7
dF/F response	0.5	Base v Stim	Paired-sample t-test	0.586	0.136	0.010	0.586	0.271	0.029			**	10	10	10
Phase unipolarity		Base	Rayleigh test	0.101	0.104	0.475	0.303	0.303	0.475						
		Stim	Rayleigh test	0.982	0.045	0.012	0.982	0.089	0.036			*			

Table 3.11 | Statistical analysis details related to Figure 3.14; $f_{\text{carrier}} = 5000$ Hz.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5
dF/F response	0.5	Base v Stim	Paired-sample t-test	0.721	0.049	0.012	0.004	0.721	0.098	0.036	0.017			*	**	9	9	10	10
Phase unipolarity		Base	Rayleigh test	0.325	0.198	0.426	0.027	0.649	0.595	0.649	0.109								
		Stim	Rayleigh test	0.008	0.013	0.007	0.112	0.027	0.027	0.027	0.112	**	*	**					
dF/F response	1	Base v Stim	Paired-sample t-test	0.103	0.077	0.010	4.E-04	0.155	0.155	0.029	0.002			**	***				
Phase unipolarity		Base	Rayleigh test	0.426	0.588	0.952	0.367	1.000	1.000	1.000	1.000								
		Stim	Rayleigh test	0.155	0.064	0.005	5.E-04	0.155	0.128	0.016	0.002			**	***				

Next, I verified the spatial activation profile for the varying carrier frequencies (**Figure 3.15**). When applying TI at 10 and 100 Hz, the resulting spatial response at Δf was much broader than when applying TI at 2000 or 5000 Hz, where, in alignment with the FEM model, the stimulation was located just between the stimulating electrodes. This suggests that the response to temporal interference using frequencies that neurons can follow may arise, at least partially, from synaptic mixing of inputs each neuron may receive individually as f_1 and f_2 . 100 Hz stimulation resulted in a particularly wide activation area, with somatosensory activation present. On the other hand, TI at kHz frequencies does not rely on receiving inputs at 5000 and 5000.5 Hz from neighbouring neurons and thus must be able to extract these components at the single-cell level. Above the threshold amplitude, the spatial profile of the response at

f_{carrier} of 5000 Hz matched that seen at the 2000 Hz TI, with contralateral activation also present.

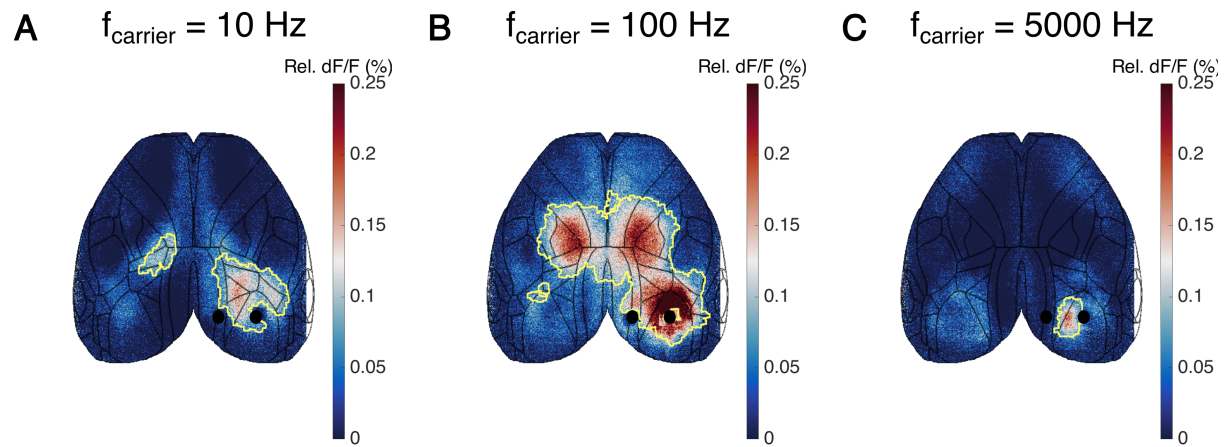


Figure 3.15 | Spatial neuronal responses for varying carrier frequencies. i. Mean Δf response map for carrier frequency of 10 Hz, at 0.7 mA/mm^2 , with activated areas highlighted. ii. As in i but for carrier frequency of 100 Hz, at 0.7 mA/mm^2 . iii. As in i but for carrier frequency of 5000 Hz, at 4 mA/mm^2 . TI stimulation in the endogenous frequency range results in activation of widespread areas, while kHz TI leads to more focal stimulation between the electrodes.

The summary of the current thresholds required to observe the response at the frequency difference (Δf) and its harmonic ($2\Delta f$) for all stimulation conditions tested is shown in **Figure 3.16**. The higher the carrier frequency, the higher the stimulation threshold, in a highly non-linear relationship. The strong response at $2\Delta f$ is generated only in the presence of kHz frequencies (**Figure 3.16B**).

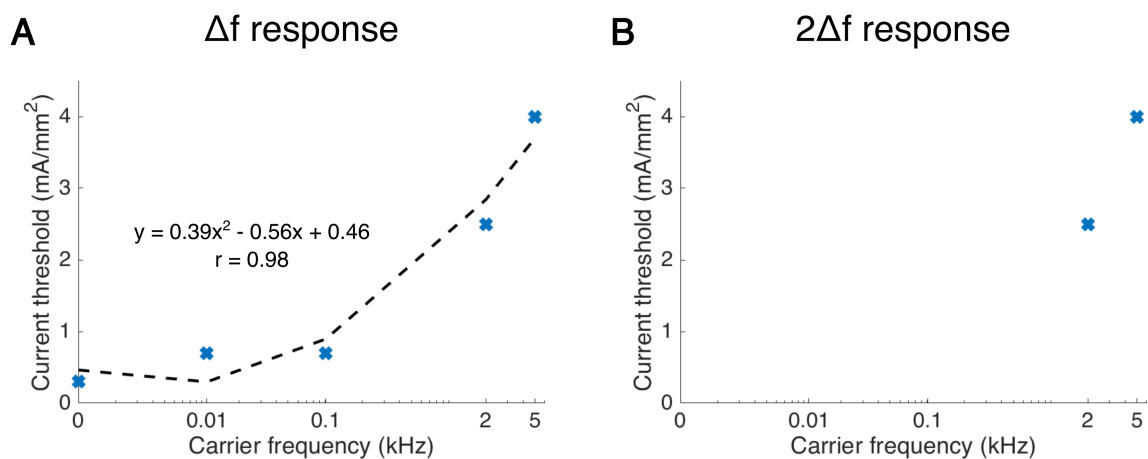


Figure 3.16 | TI current threshold increases non-linearly with increasing carrier frequency. Summary of the current thresholds for Δf (A) and $2\Delta f$ (B) activation for all the stimulation conditions

tested. f_{carrier} of 0 Hz corresponds to the control stimulation. Generation of the harmonic component of $2\Delta f$ was seen only in the kHz frequency range (**B**).

3.2.6 Differences between AM and TI stimulation

In TI, the two fields interfere to create a perfect amplitude modulated signal in the area where the fields are of equal amplitude and orientation meet (as in **Figure 3.1C**). However, in the 3D brain space, the interference at different points will have varied AM envelope magnitude and field rotation (Wang et al., 2022). Many experimental (Esmailpour et al., 2021; Luff et al., 2024b) and modelling (Mirzakhilili et al., 2020; Luff et al., 2024a) studies up to date assumed that, at least at the single-neuron level, the responses to TI, applied as two currents I_1 and I_2 via two electrode pairs, and to perfectly amplitude modulated (AM) signal, applied as a combined current I_1+I_2 via a single electrode pair, are equivalent.

To test this assumption, I applied AM stimulation via two cranial electrodes (Configuration 2; larger separation between the electrodes) and compared it with standard TI stimulation through two electrode pairs, as reported previously. I applied both stimuli at multiple current amplitudes (2, 2.5, 3, 3.5 mA/mm²/channel) and found that, at the spatial response level, both AM and TI showed initial activation at 3 mA/mm² (**Figure 3.17**). For AM, the total current applied was 0.3 mA (corresponding to 3 mA/mm²) and for TI it was 0.6 mA (3 mA/mm² for two channels). This, however, matches the similar electric field strengths despite different absolute current amplitudes for both conditions as reported in section **3.2.2**.

In both conditions, the resulting activation was widespread, likely including polysynaptic responses due the relatively large current density steps; the spatial profile of activation, however, was different between the conditions. Similar to previous results, TI activated the area between the two stimulating electrodes and the contralateral hemisphere at Δf (**Figure 3.17Ai**), with different phases of the responses in the two hemispheres (**Figure 3.17Aiii**). In this case, the resulting response at $2\Delta f$ was located only between the electrodes, indicating second order mixing in the area of field overlap. In the AM stimulation, the spatial spread of response was widespread between the two electrodes and similar for both Δf and $2\Delta f$ (**Figure 3.14Bi-ii**). There was no contralateral activation in the AM condition and the mean AM phase response at Δf (**Figure 3.17Biii**) but not $2\Delta f$ (**Figure 3.17Biv**) was different than for TI condition.

To investigate these differences in the phase of the responses, I analysed the calcium activity in response to AM stimulation measured in the ROI next to electrode 2 (1 mm medial; indicated in **Figure 3.17Bi**) (**Figure 3.18A-B**). For AM, unlike for TI, the neuronal response peak followed the AM amplitude (**Figure 3.18Aii**) but, like for TI, a clear secondary frequency peak at $2\Delta f$ could also be seen in the corresponding PSD (**Figure 3.18B**). Comparing the AM and TI calcium traces revealed obvious phase differences in the peak responses (**Figure 3.18C**), with mean AM phase at Δf , $61.8 \pm 27.0^\circ$, different from mean Δf TI phase, $-172.6 \pm 32.1^\circ$ (**Table 3.13**). Interestingly, the $2\Delta f$ phase response was the same for AM ($-104.0 \pm 16.4^\circ$) and TI ($-125.8 \pm 66.1^\circ$).

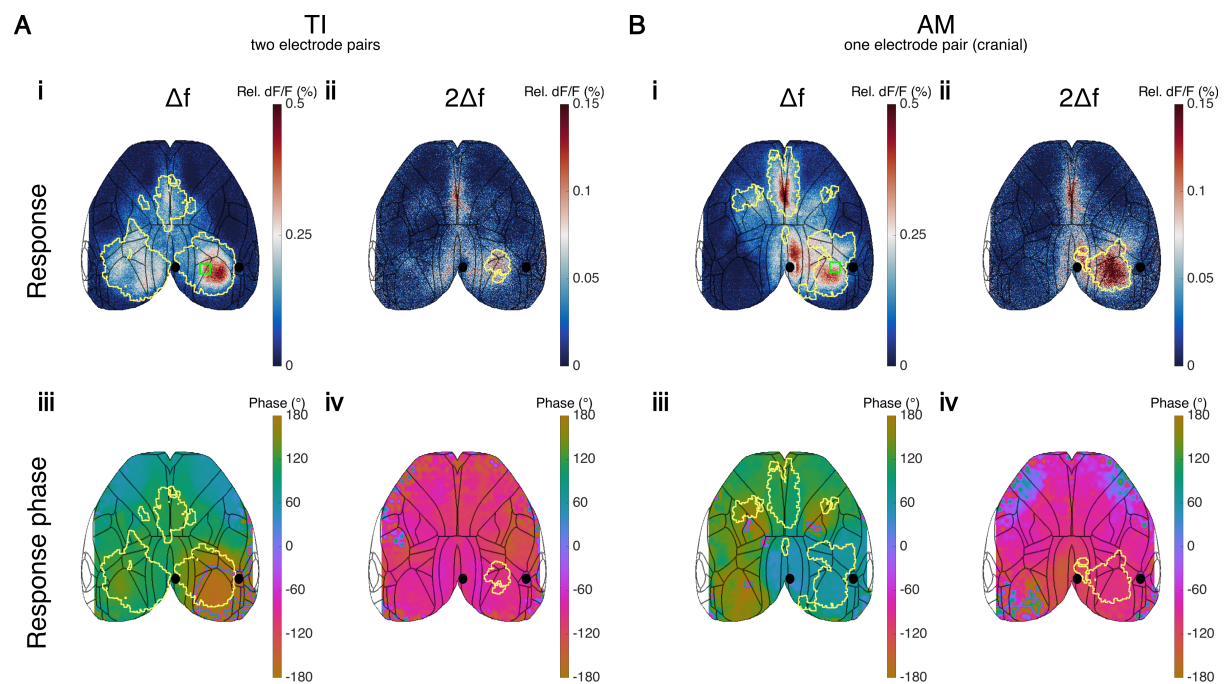


Figure 3.17 | Spatial neuronal responses evoked by TI versus AM stimulation. **i.** Mean Δf phase map during TI stimulation ($f_1 = 2000$, $f_2 = 2000.5$ Hz; $a_1 = a_2 = 3$ mA/mm²), with activated areas highlighted. Mean ROI used for subsequent analysis in green. **ii.** Same as in **i** but for $2\Delta f$. **iii.** Corresponding mean phase map for the response map from **i**. **iv.** Corresponding mean phase map for the response map from **ii**. **B.** As in **A** but for the AM stimulation delivered through the cranial electrode pair ($a = 3$ mA/mm²). ROI close to electrode 2 used for subsequent analysis in green. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 9$ mice for all.

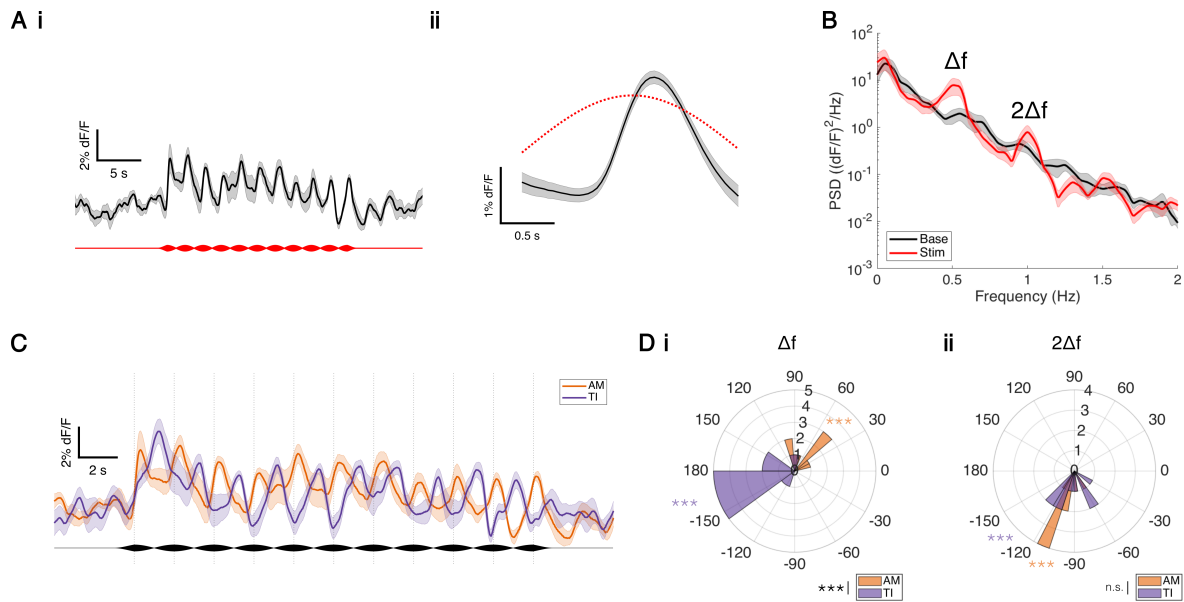


Figure 3.18 | Neuronal response evoked by single-pair AM has a different phase compared to response evoked by two-pair TI. **A. i.** Calcium activity trace extracted from the ROI located next to electrode 2 during AM stimulation of $f_1 = 2000$ and $f_2 = 2000.5$ Hz, $\Delta f = 0.5$ Hz, at 3 mA/mm^2 through the cranial electrode pair. Mean $\pm$ SEM. $n = 9$. **ii.** STA of the neuronal response relative to the stimulus. Mean $\pm$ SEM. **B.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. Similarly to TI, AM stimulation evokes activity at both Δf and $2\Delta f$. Mean $\pm$ SEM. **C.** Peaks in AM-evoked activity (orange) are more in phase with the AM of the signal applied than those of TI stimulation. Current density of 3 mA/mm^2 . Mean $\pm$ SEM. **D. i.** Phase of the neuronal response at Δf is different in the AM stimulation than in the TI stimulation. **ii.** $2\Delta f$ phases are the same for both AM and TI stimulation. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.12** and **Table 3.13**.

Table 3.12 | Statistical analysis details related to Figure 3.18; phase unipolarity.

Measure	FOI (Hz)	Comparison	Test	p		Bonferroni-Holm p		Significance level		n (animals)	
				AM	TI	AM	TI	AM	TI	AM	TI
Phase unipolarity	0.5	Stim	Rayleigh test	2.E-05	3.E-04	3.E-04	0.001	***	***	9	9
	1		Rayleigh test	2.E-05	3.E-04	3.E-05	3.E-04	***	***		

Table 3.13 | Statistical analysis details related to Figure 3.18; phase comparison.

Measure	FOI (Hz)	Comparison	Test	p	Significance level	n (animals)
Phase comparison	0.5	AM v TI	Watson-Williams test	7.E-07	***	9
	1			0.447		

3.2.7 Pulse-width modulated TI

Conventionally, Temporal Interference uses sinusoidal fields I_1 and I_2 with frequencies f_1 and $f_2 = f_1 + \Delta f$ to create an amplitude modulated signal in the area of their interference (**Figure 3.19Ai**). However, recent studies (Missey et al., 2021; Acerbo et al., 2022) proposed the use of biphasic square kHz fields with 50% duty cycle for TI stimulation in contexts where the hardware output is limited to square waveforms. The resulting interference pattern of biphasic square fields is not amplitude modulated; instead, it is the pulse width of the combined field that is modulated periodically at Δf (**Figure 3.19Aii**). The pulses created when the combined signal is fully constructive have the maximum pulse width corresponding to that of current I_1 ($0.5/f_1$, corresponding to 0.25 ms when $f_1 = 2000$ Hz) (**Figure 3.19Aii**, in green). Then, the pulse width gets progressively smaller (**Figure 3.19Aii**, in yellow and red) until it reaches the width of 0 ms, when the combined signal is fully destructive. Following on that, the pulse width gets progressively longer until it reaches maximum pulse width again. This pulse width modulation cycle is repeated at the frequency of Δf . Similarly to conventional TI, this modulation is strongest in the area of maximum overlap of the two fields (**Figure 3.19B-C**).

Initial studies using the PWM-TI approach did not directly verify whether such stimulation paradigm, without amplitude modulation, can stimulate neurons at Δf . Additionally, these studies did not test the efficacy of this modified TI method compared to standard TI.

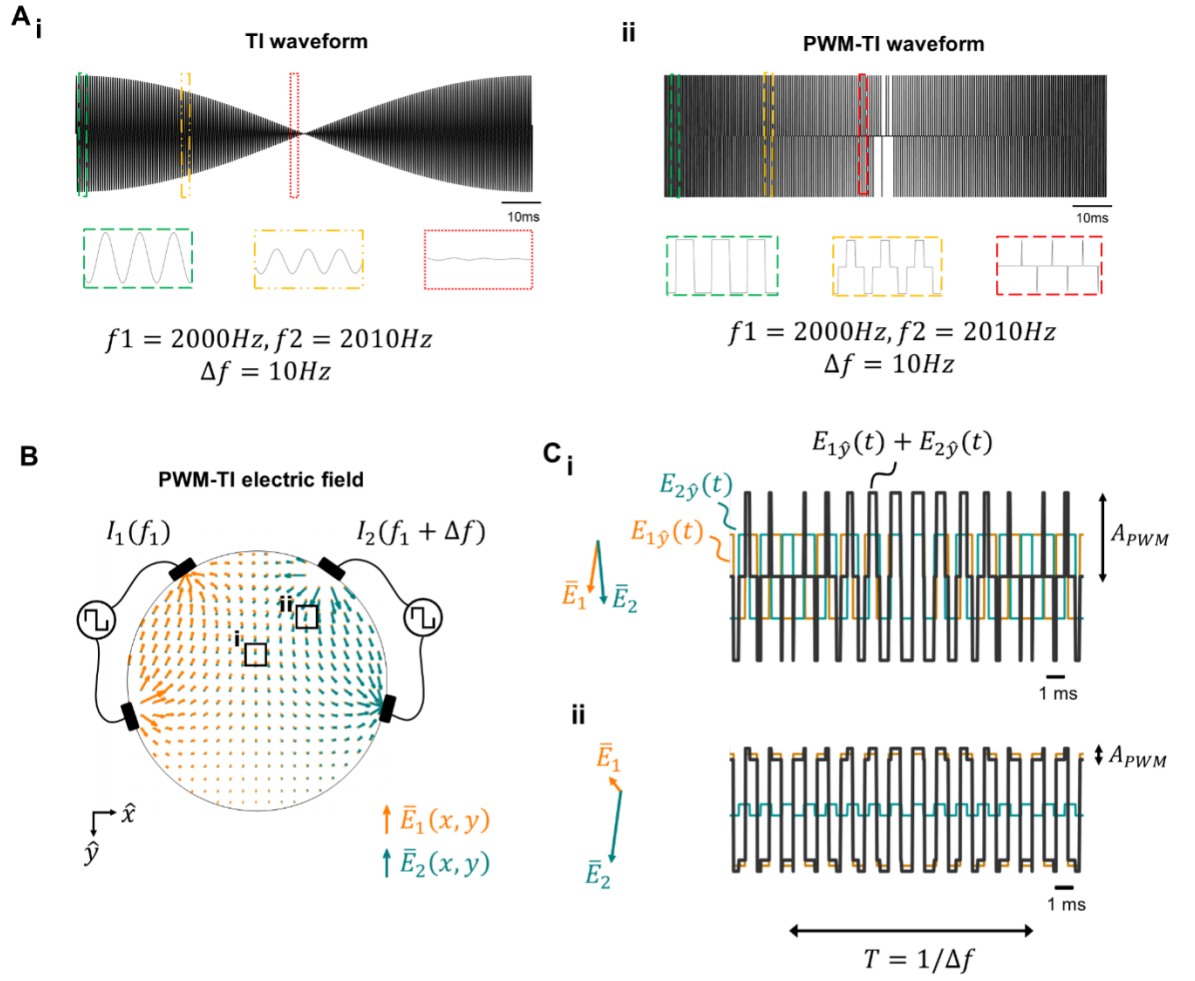


Figure 3.19 | Principle of pulse-width modulated TI (PWM-TI) stimulation. **A. i.** Standard TI stimulation produces combined TI waveform that is amplitude modulated at Δf . **ii.** PWM-TI waveform has constant amplitude but is pulse-width modulated at Δf . **B.** Electric field (E_1 , E_2) distributions produced by two applied biphasic square currents (I_1 , I_2) of frequencies f_1 and $f_2 = f_1 + \Delta f$. **C. i.** In the area of the overlap of the two fields, the interference signal is pulse-width modulated at Δf . **ii.** Closer to the second electrode pair, the interference is dominated by the second current, I_2 . Figure reproduced from (Luff et al., 2024a) under Creative Commons CC-BY 4.0 license.

To address these questions at the network-level, I applied the PWM-TI stimulation at increasing current densities to measure the neuronal dynamics evoked by PWM-TI and its activation threshold. PWM-TI evoked similar neuronal responses as TI, including frequency responses at both Δf and $2\Delta f$ (**Figure 3.20**). The current density threshold was determined as 2 mA/mm^2 for Δf (**Figure 3.20Ci**) and 1.5 mA/mm^2 for $2\Delta f$ (**Figure 3.20Di**). These were smaller than corresponding thresholds for TI (2.5 and 2.5 mA/mm^2 , respectively), suggesting PWM-TI may be slightly stronger than TI (20% and 40% decrease in threshold at Δf and $2\Delta f$). This was also reflected in several

significantly stronger dF/F (**Figure 3.20C-Di**), onset (**Figure 3.20Ei**) and steady-state (**Figure 3.20Eii**) responses at multiple current densities (**Table 3.15**). Fitting linear mixed-effect models on the dF/F responses further confirmed that PWM-TI had an overall stronger effect at both Δf (current density: $F = 11.44$, $df = 3$, $p = 4.4e-06$; condition: $F = 6.79$, $df = 1$, $p = 0.011$; interaction: $F = 5.40$, $df = 3$, $p = 0.0023$) and $2\Delta f$ (current density: $F = 22.19$, $df = 3$, $p = 4.77e-10$; condition, $F = 10.13$, $df = 1$, $p = 0.0022$; interaction term did not improve the model fit: $\chi^2 = 1.06$, $p = 0.79$).

Phases of neuronal responses to PWM-TI (**Figure 3.20C-Dii-iii**) followed similar trends to those seen in TI (**Figure 3.10**), with the phase difference between PWM-TI- and TI-evoked activity at the current density of 2 mA/mm^2 equal to zero (**Figure 3.20F**). Interestingly, at the highest current density of 3 mA/mm^2 , the phase of the PWM-TI response at Δf changed its value from $-123.1 \pm 39.1^\circ$ (at 2 mA/mm^2) to $51.2 \pm 28.9^\circ$, making it more in phase with the AM waveform and similar to the phase lag observed in the control LF stimulation. As seen previously with TI, phase responses at $2\Delta f$ were more consistent at all current densities than those at Δf .

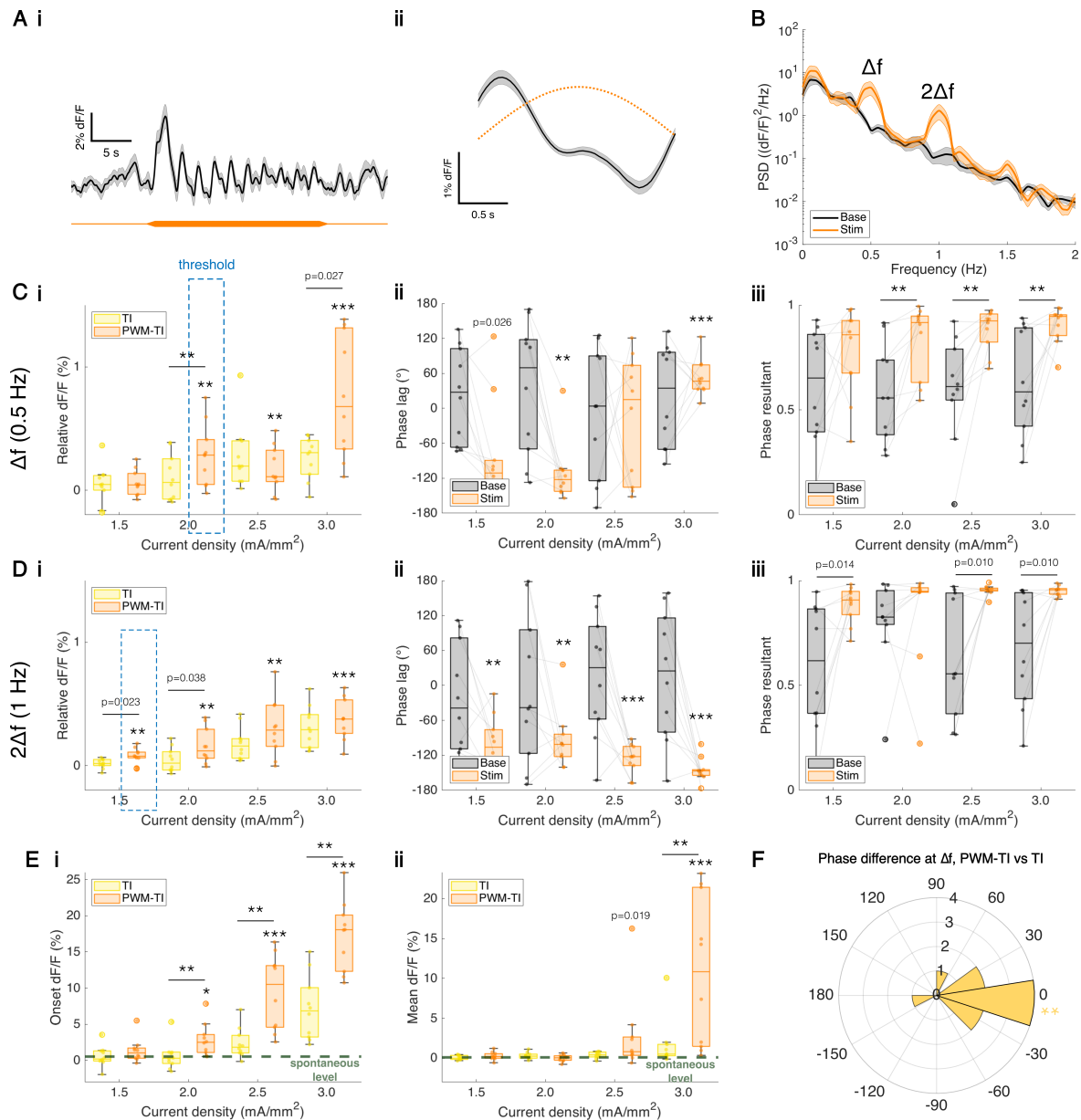


Figure 3.20 | Temporal neuronal responses evoked by PWM-TI stimulation with Δf of 0.5 Hz.

A. i. Calcium activity trace extracted from the ROI located between the electrodes during PWM-TI stimulation of $f_1 = 2000$ and $f_2 = 2000.5$ Hz, $\Delta f = 0.5$ Hz, at 2 mA/mm^2 . Mean $\pm$ SEM. $n = 10$. **ii.** STA of the neuronal response relative to the stimulation. Stimulation peak plotted corresponds to equivalent maximum modulated pulse width. Mean $\pm$ SEM. **B.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. Similarly to conventional TI, PWM-TI stimulation evokes activity at Δf and its harmonic, $2\Delta f$. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at Δf during stimulation and baseline for increasing current densities. Group-level threshold of activation was established as $2 \text{ mA/mm}^2/\text{channel}$. **ii.** Phase of the neuronal response at Δf during baseline and stimulation periods at all current amplitudes tested. **iii.** Phase resultant at Δf during baseline and stimulation periods at all current amplitudes tested. **D.** As in **C** but at $2\Delta f$. $2\Delta f$ group-level threshold was $1.5 \text{ mA/mm}^2/\text{channel}$, lower than the threshold for Δf . The phase response at higher current amplitudes was again more consistent for $2\Delta f$. **E. i.** PWM-TI stimulation induced onset responses at $\geq 2 \text{ mA/mm}^2$. **ii.** Mean dF/F (%) for PWM-TI stimulation. **F.** Phase difference at Δf , PWM-TI vs TI.

ii. PWM-TI did not induce steady state dF/F responses, except at the highest current density of 3 mA/mm², with smaller non-significant increase observed at 2.5 2 mA/mm². **F.** Phase between neuronal responses evoked with PWM-TI versus with TI, at the current density of 2 mA/mm², was unipolar (p = 0.0098, Rayleigh test, n = 10) and the same (h = 0, 95% μ = 4.5, CI [-33.9, 42.90], one-sample test for the mean angle). Spontaneous onset magnitude = 0.50 $\pm$ 0.81 %, spontaneous mean magnitude = 0.09 $\pm$ 0.22 %. Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001. n = 10 mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.16** and **Table 3.17**. Figure modified from (Luff et al., 2024a) under Creative Commons CC-BY 4.0 license, with data reanalysed and extra panels added.

Table 3.14 | Statistical analysis details related to Figure 3.20; PWM-TI.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0
Relative dF/F response	0.5	Stim v 0	Paired-sample t-test	0.054	0.003	0.009	5.E-04	0.054	0.008	0.017	0.002		**	**	***	10	10	10	10
Phase unipolarity		Base	Rayleigh test	0.497	0.632	0.804	0.749	1.000	1.000	1.000	1.000								
		Stim	Rayleigh test	0.026	0.001	0.814	9.E-05	0.052	0.004	0.814	4.E-04	**			***				
Phase resultant		Base v Stim	Paired-sample t-test	0.090	0.002	0.003	0.007	0.180	0.018	0.018	0.037		**	**	**				
Relative dF/F response	1	Stim v 0	Paired-sample t-test	0.002	0.003	0.001	3.E-05	0.005	0.005	0.004	0.000	**	**	**	***				
Phase unipolarity		Base	Rayleigh test	0.772	0.572	0.889	0.928	1.000	1.000	1.000	1.000								
		Stim	Rayleigh test	0.002	0.002	2.E-05	7.E-06	0.003	0.003	7.E-05	3.E-05	**	**	***	***				
Phase resultant		Base v Stim	Wilcoxon signed-rank test	0.014	0.161	0.010	0.010	0.059	0.413	0.059	0.059								
Onset dF/F	-	Stim v Spont	Paired-sample t-test	0.074	0.013	3.E-04	1.E-06	0.074	0.026	0.001	4.E-06		*	***	***				
Mean dF/F		Stim v Spont	Wilcoxon signed-rank test	0.216	0.754	0.019	0.001	0.432	0.754	0.056	0.004				***				

Table 3.15 | Statistical analysis details related to Figure 3.20; comparison between TI and PWM-TI.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0
Relative dF/F response	0.5	TI v PWM-TI	Paired-sample t-test	0.863	0.004	0.208	0.027	0.863	0.017	0.416	0.082		**			10	10	10	10
Relative dF/F response	1		Paired-sample t-test	0.023	0.038	0.142	0.385	0.091	0.115	0.284	0.385								
Onset dF/F	-		Wilcoxon signed-rank test	0.275	0.002	0.002	0.002	0.275	0.008	0.008	0.008		**	**	**				
Mean dF/F			Wilcoxon signed-rank test	0.275	0.084	0.105	0.006	0.275	0.252	0.252	0.023				**				

3.2.8 Effects of pure kHz stimulation on ongoing neuronal activity

In TI, neurons directly underneath stimulating electrodes are exposed to unmodulated kHz electric fields. It is therefore essential to understand whether pure kHz fields affect ongoing neuronal activity.

To investigate this question, I first applied kHz stimulation, at 2000 and 5000 Hz, through two electrode pairs at the same current densities as previously used with TI protocols. First, I validated that the stimulation did not result in an increase in activity at 0.5 Hz (Δf used previously) (**Figure 3.21C-Di**). Subsequently, I computed the transient onset and mean steady-state responses to kHz stimulation. Similarly to TI

with f_{carrier} of 2 kHz (**Figure 3.10**), pure 2 kHz fields induced significant onset response and an almost significant steady-state response ($p = 0.014$; did not pass multiple comparisons correction) at the highest current density amplitude of 3 mA/mm². This amplitude corresponds to widespread TI activation through most cortical surface (**Figure 3.11Aiv**) and further highlights the need to dose the current density appropriately to avoid stimulation of too large areas. At 2.5 mA/mm², the current density threshold for 2 kHz TI, the onset response was significantly elevated for TI (**Figure 3.10**) but not kHz stimulation (**Table 3.16**). In a few mice, however, an increased response can be observed (**Figure 3.21Cii**), leading to an onset peak visible in the mean calcium trace (**Figure 3.21A**). For 5 kHz stimulation, no onset nor steady-state responses can be seen at any of the amplitudes tested (**Figure 3.21Dii-iii**), even above the 5 kHz TI threshold of 4 mA/mm². This suggests that one way of reducing the amplitude of the kHz onset responses is to use higher carrier frequency.

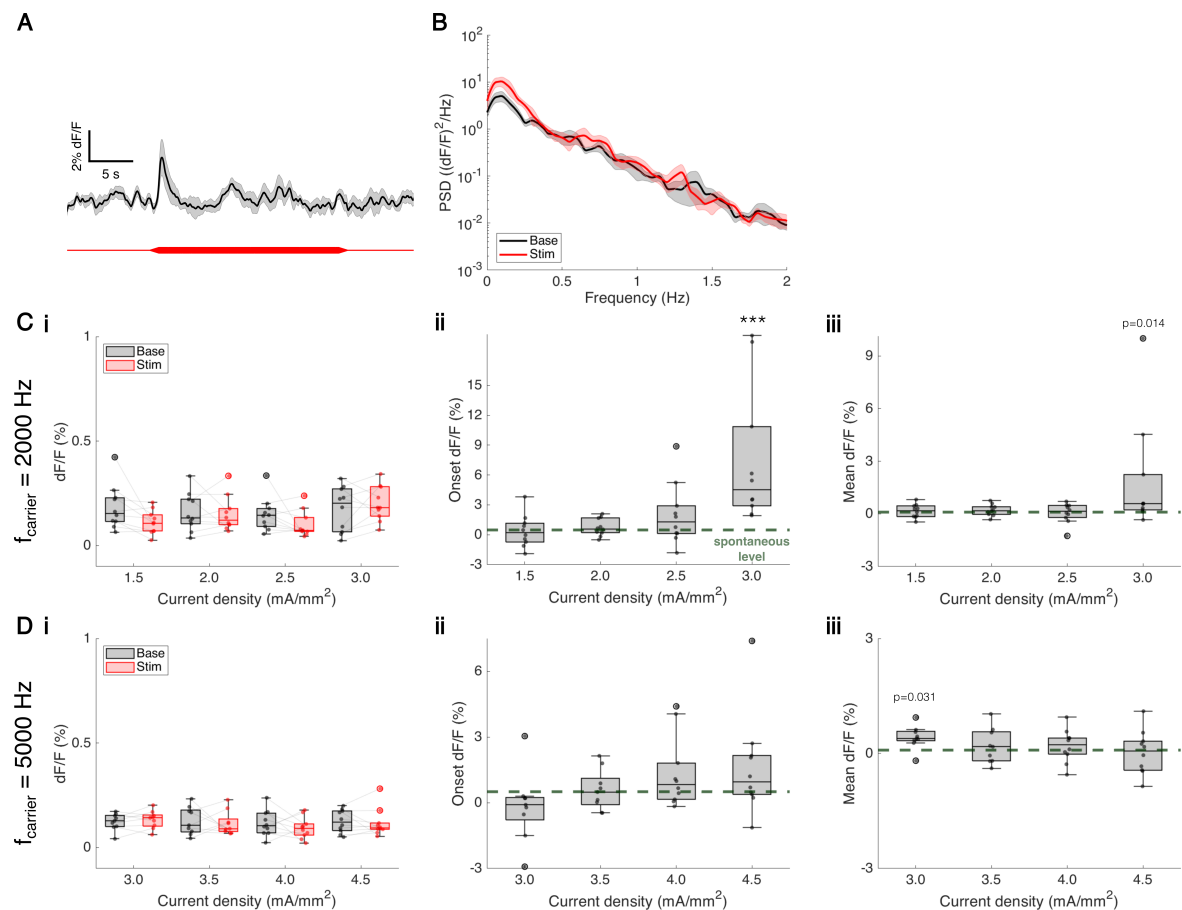


Figure 3.21 | Temporal neuronal responses evoked by kHz stimulation. **A.** Calcium activity trace extracted from the ROI located between the electrodes during kHz stimulation of $f_1 = f_2 = 2000$ Hz, at 2.5 mA/mm². Mean $\pm$ SEM. $n = 10$. **B.** PSD of the full baseline (Base; in black) and stimulation period (Stim; in red) from trace in **Ai**. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at 0.5 Hz during

stimulation and baseline for increasing current densities for carrier frequency of 2000 Hz. No increase in activation at FOI was detected. **ii.** Magnitude of the induced onset response with kHz stimulation was significant at 3 mA/mm². **iii.** Steady-state response to kHz stimulation was slightly but non-significantly elevated only at 3 mA/mm². **D.** As in C but for the carrier frequency of 5000 Hz. No activation at 0.5 Hz, onset or steady-state effects were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 10$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.16** and **Table 3.17**.

Table 3.16 | Statistical analysis details related to Figure 3.21; $f_{\text{carrier}} = 2000$ Hz.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0	1.5	2.0	2.5	3.0
dF/F response	0.5	Base v Stim	Wilcoxon signed-rank test	0.993	0.577	0.947	0.216	1.000	1.000	1.000	0.863					10	10	10	10
Onset dF/F		Stim v Spont	Wilcoxon signed-rank test	0.577	0.461	0.188	0.001	0.922	0.922	0.563	0.004				***				
Mean dF/F		Stim v Spont	Wilcoxon signed-rank test	0.278	0.161	0.423	0.014	0.557	0.483	0.557	0.055								

Table 3.17 | Statistical analysis details related to Figure 3.21; $f_{\text{carrier}} = 5000$ Hz.

Measure	FOI (Hz)	Comparison	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)				Current density (mA/mm ²)			
				3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5	3.0	3.5	4.0	4.5
dF/F response	0.5	Base v Stim	Paired-sample t-test	0.220	0.653	0.804	0.668	0.879	1.000	1.000	1.000					9	9	10	10
Onset dF/F		Stim v Spont	Wilcoxon signed-rank test	0.875	0.410	0.138	0.348	1.000	1.000	0.551	1.000								
Mean dF/F		Stim v Spont	Paired-sample t-test	0.031	0.256	0.220	0.630	0.123	0.660	0.660	0.660								

Another approach to reduce the onset amplitudes, investigated previously in the original TI study (Grossman 2017), is to increase the rate of the current density ramp up by increasing the ramping duration. I tested this approach by applying strong kHz stimulation (3.5 mA/mm²) through one cranial electrode pair (Configuration 2) with varying ramp up times of 0, 1, 3, 5 and 7 s (**Figure 3.22A**). Longer ramp up period of 5 seconds successfully reduced the onset response by ~40 %, compared to when no ramp was applied (**Figure 3.22B**).

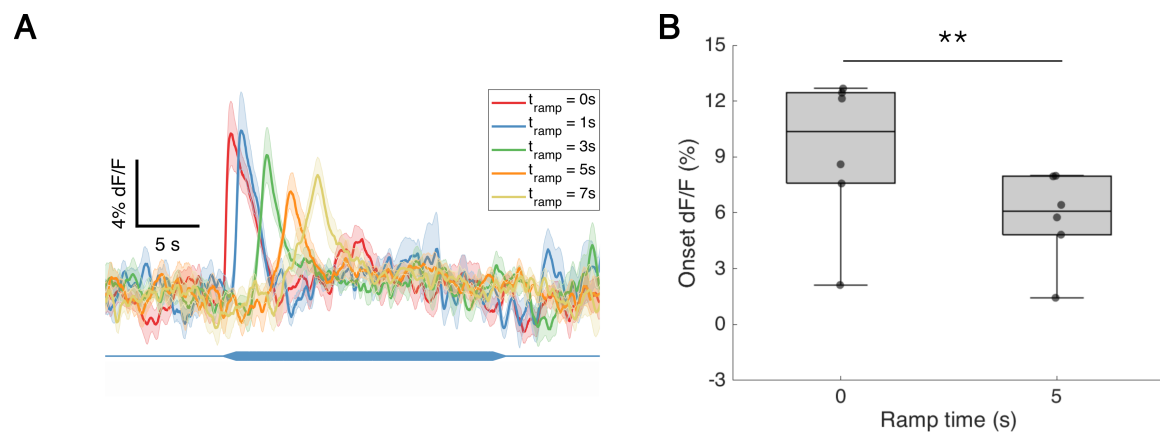


Figure 3.22 | Onset responses evoked by kHz stimulation with varying current ramp up rates. A. Neuronal responses to strong kHz stimulation at $f = 2000$ Hz at current density of 3.5 mA/mm², applied

through the cranial electrode pair, in the ROI between the two electrodes. B. Longer ramp up time of 5 s significantly reduced the magnitude of the evoked kHz onset response ($p = 0.004$, right-tailed paired t-test, $n = 6$).

Finally, I investigated whether kHz stimulation affects neuronal responses to other stimuli, such as visual or other electrical inputs. A recent modelling study (Mirzakhaili et al., 2020) suggested kHz fields can lead to a neuronal depolarisation block. As cells directly underneath the TI electrodes are exposed to such fields, this question needs to be addressed and verified experimentally.

I applied visual stimulation at f_{stim} of 0.5 Hz alone and in the presence of kHz electric fields ($f_1 = f_2 = 2000$ Hz, $a_1 = a_2 = 3$ mA/mm²; Configuration 2) to inspect the resulting response distribution at f_{stim} but also $2f_{\text{stim}}$ and $3f_{\text{stim}}$. In the absence of kHz fields, the harmonic responses to visual stimulation were less widespread than reported in previous control experiments (**Figure 3.3A**), likely due to smaller sample size ($n = 5$ versus previous $n = 10$) and single recording per mouse used (versus previous average of three recordings). This highlights that some of the mixing effects seen, especially now absent at $2f_{\text{stim}}$ (**Figure 3.23Aii**) and much smaller at $3f_{\text{stim}}$ (**Figure 3.23Aiii**), are likely to arise due to repeated, averaged stimulation. In the presence of kHz fields, the resulting response at f_{stim} (**Figure 3.23Bii**) closely matched the standard one (**Figure 3.23Aii**). Meanwhile, the response at $2f_{\text{stim}}$ was enhanced while the response at $3f_{\text{stim}}$ was reduced.

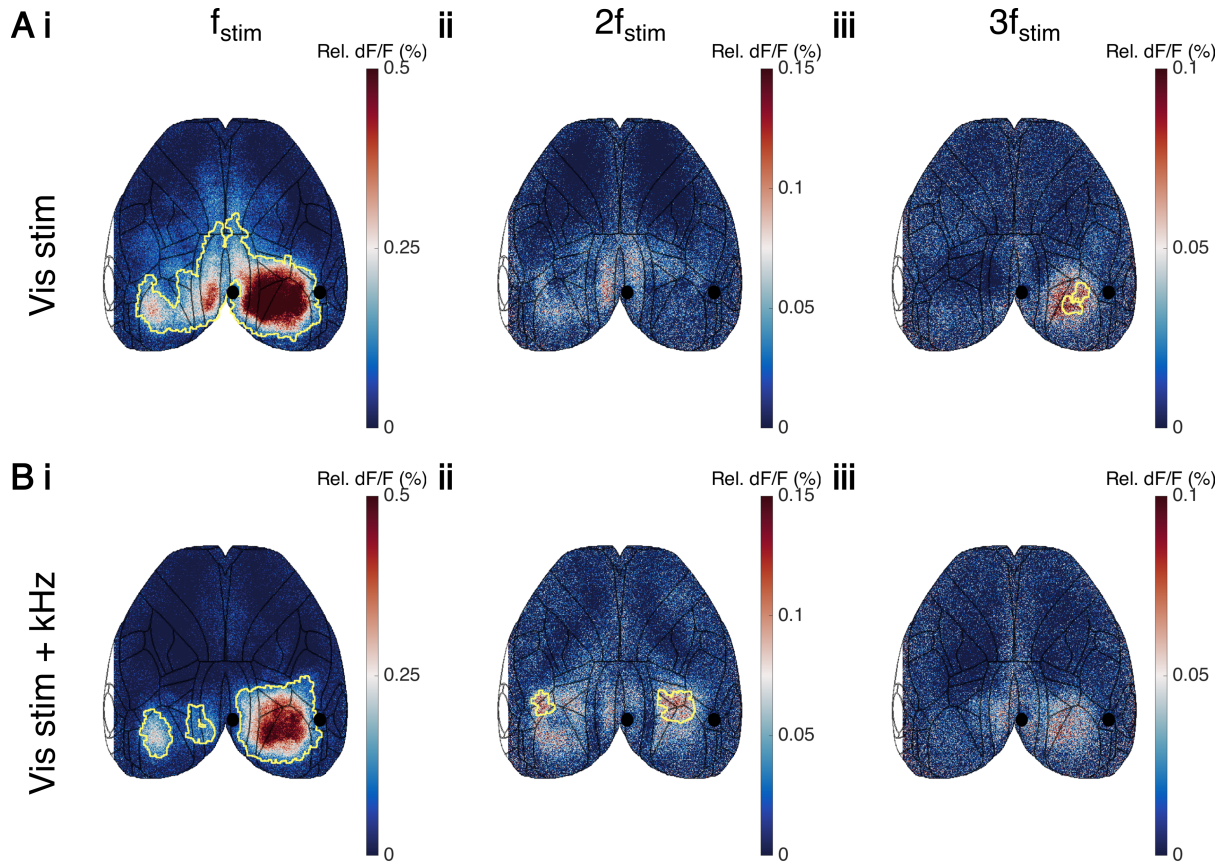


Figure 3.23 | Spatial neuronal responses evoked by visual stimulation alone and in the presence of kHz electric fields. A. i. Mean f_{stim} response map for visual stimulation at 0.5 Hz, with activated areas highlighted. **ii.** Same as in **i** but for $2f_{\text{stim}}$. **iii.** Same as in **i** but for $3f_{\text{stim}}$. **B.** As in **A** but for visual stimulation at 0.5 Hz combined with kHz stimulation ($f_1 = f_2 = 2000$ Hz, $a_1 = a_2 = 3$ mA/mm²). The distributions are similar, with enhanced response at $2f_{\text{stim}}$ and diminished response at $2f_{\text{stim}}$ when kHz fields are present. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 5$ mice for all.

To examine these effects closer, I extracted the neuronal activity traces in the ROI between the two electrodes for the two conditions (**Figure 3.24A**). The resulting PSD spectra showed enhanced $2f_{\text{stim}}$ peak in the presence of kHz fields but similar peaks at f_{stim} and $3f_{\text{stim}}$ (**Figure 3.24B**). This was further confirmed with magnitudes of relative responses at all the three frequencies. Importantly, both conditions evoked equally strong response at f_{stim} (**Figure 3.24C**; **Table 3.19**). The responses for $2f_{\text{stim}}$ and $3f_{\text{stim}}$ between the two conditions were also not different, although only visual stimulation combined with kHz fields induced significantly strong response at $2f_{\text{stim}}$, indicated by both magnitude and phase of the response (**Table 3.18**). For all the frequencies tested, the phase response was the same in both conditions.

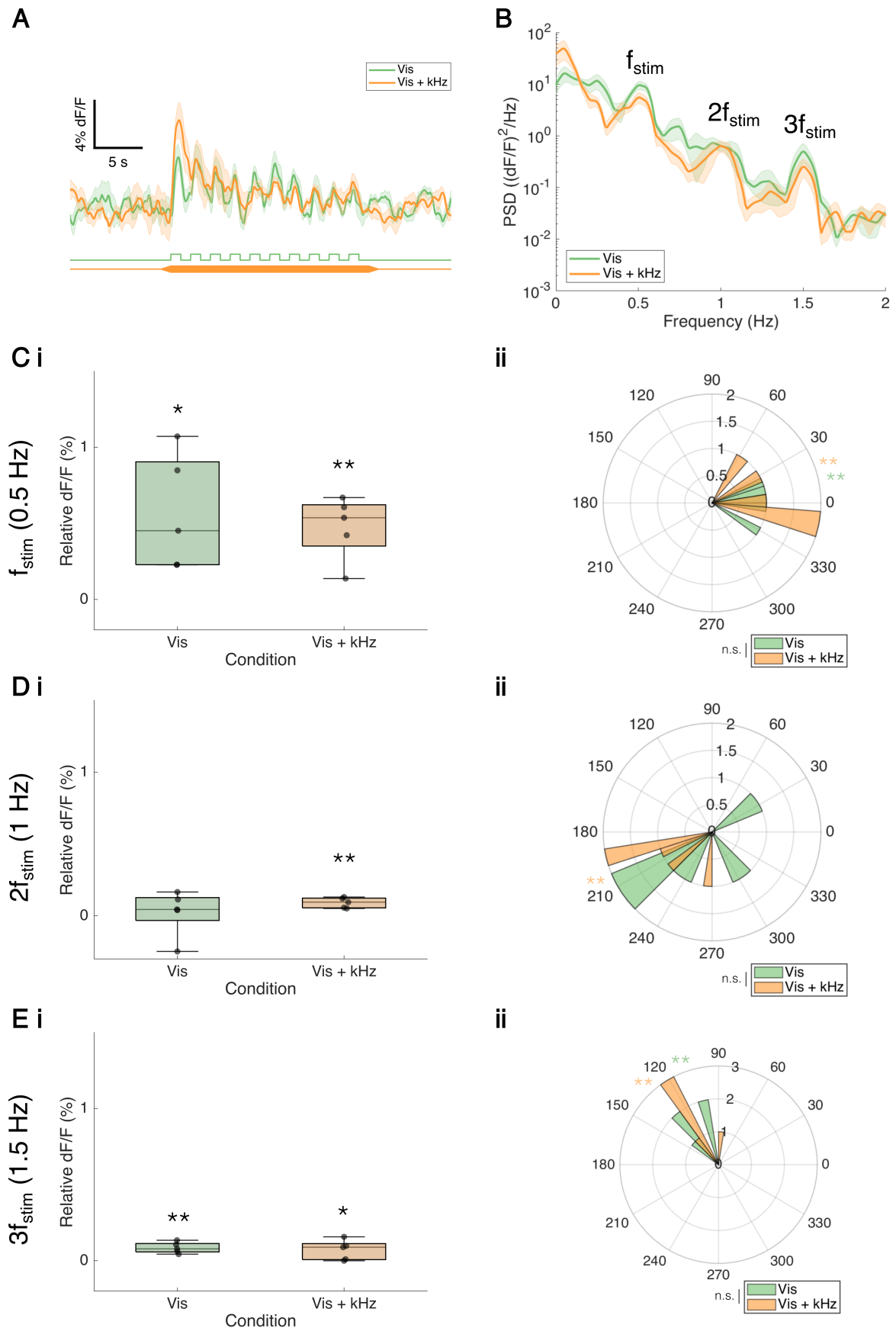


Figure 3.24 | Temporal effects of kHz fields on visually evoked responses. A. i. Calcium activity trace extracted from the ROI located between the electrodes during visual stimulation at $f_{\text{stim}} = 0.5$ Hz

with (Vis + kHz; orange) and without (Vis; green) the presence of kHz stimulation ($f_1 = f_2 = 2000$ Hz, $a_1 = a_2 = 3$ mA/mm²). Mean $\pm$ SEM. $n = 5$. **B.** PSD of the stimulation period from traces in **Ai**. Visual stimulation evokes activity at f_{stim} but also its harmonics, $2f_{stim}$ and $3f_{stim}$. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at f_{stim} was equally high in both conditions. **ii.** Phase of the neuronal response at f_{stim} was the same for both conditions. **D.** Same as in **C** but for $2f_{stim}$. **i.** Significant dF/F response at $2f_{stim}$ was detected in the presence of kHz fields but the responses of the two conditions were not different. **ii.** Phase response at $2f_{stim}$ was significantly unipolar only when kHz fields were present; however, the phases of the two conditions were not different. **E.** Same as in **C** but for $3f_{stim}$. Amplitude and phase of the neuronal response at $3f_{stim}$ did not differ between the two conditions. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.18** and **Table 3.19**.

Table 3.18 | Statistical analysis details related to Figure 3.24; test of each condition (Vis, Vis + kHz).

Measure	FOI (Hz)	Comparison	Test	p		Bonferroni-Holm p		Significance level		n (animals)	
				Vis	Vis + kHz	Vis	Vis + kHz	Vis	Vis + kHz	Vis	Vis + kHz
Relative dF/F response	0.5	Stim v 0	Paired-sample t-test	0.015	0.004	0.015	0.007	*	**	5	5
Phase unipolarity		Stim	Rayleigh test	0.004	0.008	0.008	0.009	**	**		
Relative dF/F response	1	Stim v 0	Paired-sample t-test	0.382	0.003	0.382	0.005		**		
Phase unipolarity		Stim	Rayleigh test	0.329	0.010	0.329	0.020		**		
Relative dF/F response	1.5	Stim v 0	Paired-sample t-test	0.003	0.038	0.006	0.038	**	*		
Phase unipolarity		Stim	Rayleigh test	0.004	0.003	0.007	0.007	**	**		

Table 3.19 | Statistical analysis details related to Figure 3.24; comparison between the two conditions (Vis, Vis + kHz).

Measure	FOI (Hz)	Comparison	Test	p	Significance level	n (animals)
Relative dF/F response	0.5	Vis v Vis + kHz	Paired-sample t-test	0.567		5
Phase comparison			Watson-Williams test	0.397		
Relative dF/F response	1		Paired-sample t-test	0.472		
Phase comparison			Watson-Williams test	0.433		
Relative dF/F response	1.5		Paired-sample t-test	0.389		
Phase comparison			Watson-Williams test	0.710		

Next, I repeated the experiment but instead of visual stimulation, I applied low frequency electrical stimulation ($f_1 = f_2 = 0.5$ Hz, $a_1 = a_2 = 0.1$ mA/mm²) alongside kHz fields (Configuration 2). The LF stimulation alone was strong, leading to widespread cortical activation at f_{stim} only (**Figure 3.25A**). When kHz fields were present, the

resulting response at f_{stim} was less widespread but additional response at the harmonic, $2f_{\text{stim}}$, was also observed (**Figure 3.25B**).

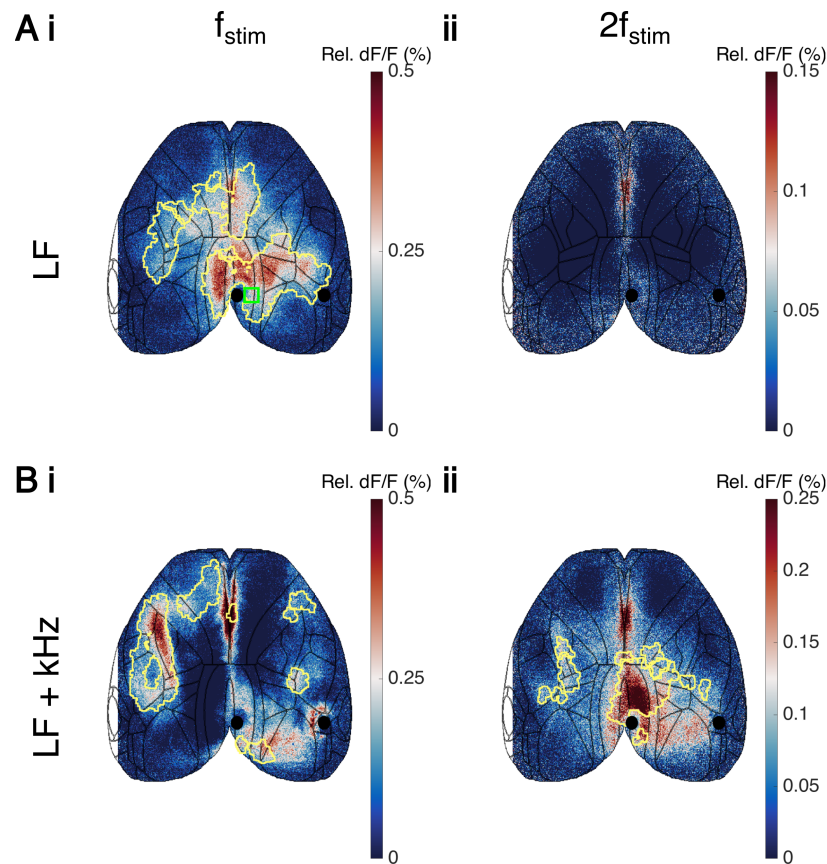


Figure 3.25 | Spatial neuronal responses evoked by low frequency stimulation alone and in the presence of kHz electric fields. A. i. Mean f_{stim} response map for electrical stimulation at 0.5 Hz ($f_1 = f_2 = 0.5$ Hz, $a_1 = a_2 = 1$ mA/mm²), with activated areas highlighted and ROI close to electrode 1 used for subsequent analysis in green. **ii.** Same as in i but for $2f_{\text{stim}}$. **B.** As in **A** but for electrical stimulation at 0.5 Hz combined with kHz stimulation ($f_1 = f_2 = 2000$ Hz, $a_1 = a_2 = 3$ mA/mm²). In the presence of kHz fields, additional response at $2f_{\text{stim}}$ emerges. Statistical significance: $p < 0.05$ for activated clusters of activity, cluster permutation test. $n = 5$ mice for all.

Further analysis of the responses at the ROI-level was done on the traces extracted from the ROI directly next to electrode 1 (0.5 mm lateral; indicated in **Figure 3.25Ai**). Both stimulation conditions led to similar oscillation at f_{stim} (**Table 3.21**), though none of them significant, likely due to the small sample size (**Figure 3.26Ci**; **Table 3.20**). However, the phase of the two responses was significantly unipolar at f_{stim} and different between the two conditions (LF: $37.5 \pm 29.7^\circ$, LF + kHz: $79.0 \pm 16.2^\circ$; **Table 3.20**). Presence of the kHz frequencies also resulted in stronger activation at 1 Hz, i.e. $2f_{\text{stim}}$, as reflected in a potentially larger, but non-significant ($p = 0.049$; did not survive

multiple comparisons correction), dF/F response (**Figure 3.26Di**) and significantly unipolar response phase at $2f_{\text{stim}}$ for LF + kHz but not for LF alone (**Figure 3.26Dii**). PSD plots (**Figure 3.26B**) reveal both conditions display a secondary PSD peak around 1 Hz, but this frequency component is amplified when kHz fields are present. This harmonic frequency can also be seen in the neuronal traces and the STA plot (**Figure 3.26A**) as the secondary peak in the response, the creation of which may have led to a small shift in phase at f_{stim} .

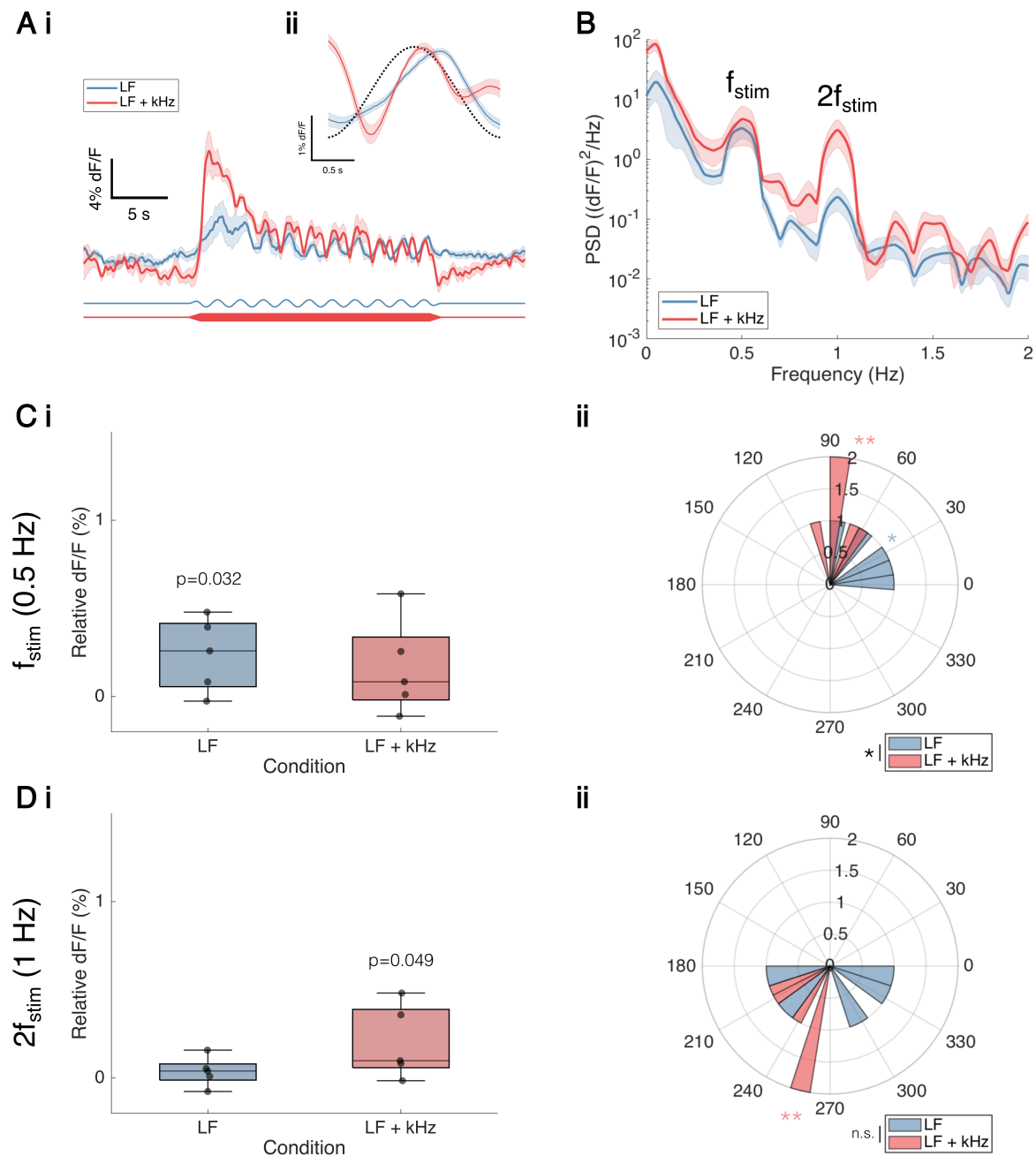


Figure 3.26 | Temporal effects of kHz fields on electrically evoked responses. A. i. Calcium activity trace extracted from the ROI located next to electrode 1 during electrical stimulation at 0.5 Hz ($f_1 = f_2 =$

0.5 Hz, $a_1 = a_2 = 1 \text{ mA/mm}^2$) with (LF + kHz; pink) and without (LF; blue) the presence of kHz stimulation ($f_1 = f_2 = 2000 \text{ Hz}$, $a_1 = a_2 = 3 \text{ mA/mm}^2$). Mean $\pm$ SEM. $n = 5$. **ii.** STA of the responses from **Ai** relative to the stimulus (black). Mean $\pm$ SEM. **B.** PSD of the stimulation period from traces in **Ai**. Electrical stimulation evokes activity at f_{stim} but also its harmonic, $2f_{\text{stim}}$, especially strongly in the presence of kHz fields. Mean $\pm$ SEM. **C. i.** Amplitude of the neuronal response at f_{stim} was equally high, and not significantly large, in both conditions. **ii.** Phase of the neuronal response at f_{stim} was significantly shifted by $\sim 30^\circ$ in the presence of kHz fields. **D.** Same as in **C** but for $2f_{\text{stim}}$. **i.** An almost significant dF/F response at $2f_{\text{stim}}$ was detected in the presence of kHz fields but the responses of the two conditions were not different. **ii.** Phase response at $2f_{\text{stim}}$ was not different between the two conditions but only the phase at $2f_{\text{stim}}$ was significantly unipolar. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5$ mice, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 3.20** and **Table 3.21**.

Table 3.20 | Statistical analysis details related to Figure 3.26; test of each condition (LF, LF + kHz).

Measure	FOI (Hz)	Comparison	Test	p		Bonferroni-Holm p		Significance level		n (animals)	
				Vis	Vis + kHz	Vis	Vis + kHz	Vis	Vis + kHz	Vis	Vis + kHz
Relative dF/F response	0.5	Stim v 0	Paired-sample t-test	0.032	0.122	0.064	0.122			5	5
Phase unipolarity		Stim	Rayleigh test	0.015	0.004	0.015	0.007	*	**		
Relative dF/F response	1	Stim v 0	Paired-sample t-test	0.192	0.049	0.192	0.098				
Phase unipolarity		Stim	Rayleigh test	0.313	0.006	0.313	0.013		**		

Table 3.21 | Statistical analysis details related to Figure 3.26; comparison between the two conditions (LF, LF + kHz).

Measure	FOI (Hz)	Comparison	Test	p	Significance level	n (animals)
Relative dF/F response	0.5	LF v LF + kHz	Wilcoxon signed-rank test	1.000		5
Phase comparison			Watson-Williams test	0.043	*	
Relative dF/F response	1		Paired-sample t-test	0.122		
Phase comparison			Watson-Williams test	0.184		

3.3 Discussion

The study reported here is the first direct network-level evidence of neuronal dynamics evoked by Temporal Interference *in vivo*. As such, it sheds more light on possible mechanisms of action of TI, especially concerning the network-level interactions involved. It is also an important step in determining the effects of TI at clinically relevant spatial scales, which is crucial for further successful translational adoption of the technique.

To enable these experiments, I developed an experimental setup allowing for simultaneous widefield calcium imaging and TI stimulation in transgenic *Thy1-GCaMP6s* mice. First, I characterised the method by ensuring that visual responses can be reliably recorded in the presence of implanted stimulating electrodes and mapping the frequency range of the responses evoked by standard electrical stimulation. I then measured and compared the electric field strengths induced by the different stimulation protocols (LF, TI, AM). These recordings were done in Configuration 2, with larger separation between the two stimulating electrodes; the order of magnitude of the evoked fields was assumed to be equivalent for Configuration 1. I found that the generated fields were homogeneous across axial and lateral dimensions. The normalised average field values induced by all stimulation types were not statistically different from each other. The sample size of these measurements was, however, very low ($n = 4$) and the median value per 1 mA/mm² of current density per channel was non-significantly larger for low frequency, LF, than for TI (36.9 versus 23.7 V/m). This could potentially be attributed to the variations in the dielectric properties of the brain tissue in different frequency ranges (Gabriel, Lau & Gabriel, 1996) and should be further validated for low and kHz frequencies in future experiments. The stability of the field amplitudes evoked at different depths is unexpected based on the FEM models, especially for the LF condition. This result may potentially be attributed to the recording setup not being optimised for electric field but LFP recordings, leading to low resolution measurements. Further validation of depth selectivity in future experiments would be beneficial.

The widefield imaging approach, unlike electrophysiological techniques, allowed me to characterise the spatiotemporal responses of widespread cortical regions to both control, low frequency stimulation and TI. Both stimulation types induced reliable network-level oscillations, at f_{stim} and Δf respectively, in the ROI located between the two stimulating electrodes. However, there were crucial differences in the responses observed in the two conditions.

Importantly, I found that the group-level threshold, i.e. current density required for evoking a significant oscillatory activity, was ~ 7 -8 higher for TI at the carrier frequency of 2000 Hz than for the control stimulation. This corresponds to electric fields of ~ 50 -60 V/m (current density of 2-2.5 mA/mm²/channel) for TI and ~ 10 V/m (current density of 0.3 mA/mm²/channel) for LF, indicating a need for a few folds larger electric field

strength for TI stimulation. This matches the order of magnitude of some of the previous modelling and experimental results, in terms of the absolute electric field strengths (similar to AM field strengths used *ex vivo* in (Esmailpour et al., 2021) and lower range of that hypothesised in (Wang et al., 2022)) and the ratio between the LF and TI threshold (Luff et al., 2024a). On the other hand, previous *ex vivo* single-cell neuronal recordings (Luff et al., 2024b) reported the threshold required for TI was ~1.5 times larger than that for LF stimulation. These differences in the threshold ratios could potentially be attributed to different experimental configurations used. This includes factors such as a more homogeneous field distribution delivered with AM stimulation *ex vivo*, potential peak of TI stimulation located in deeper cortical layers *in vivo*, and different sensitivities of the two recording approaches measuring neuronal response at largely different spatial scales.

Control LF stimulation induced activation around both stimulating electrodes, while the direct spatial response to TI was located between the two electrodes, matching the modelled area of maximum amplitude modulation. With higher stimulus amplitude, the TI response spread to the contralateral hemisphere, an effect not observed in the control low frequency or the AM conditions. Contralateral TI response has a different phase profile, suggesting it could be driven polysynaptically. Cortical layers 5a (Naka & Adesnik, 2016) and 6 (Tasic et al., 2018; Liang et al., 2021) contain intratelencephalic (IT) excitatory neurons that project to the contralateral hemisphere. These callosal projections can have net inhibitory effects, a mechanism mediated by local layer 1 circuitry (Palmer et al., 2012). Activation of ipsilateral IT neurons suggests that TI is able to stimulate deeper layers than LF, as expected by the FEM models, or preferentially targets these cell types, or both.

The spatial profile of the induced neuronal responses was in relative alignment with the FEM models. Due to the limited visibility underneath and just around the stimulation electrodes, the evaluation of the LF model was impaired. The overall LF response shape matched the model prediction, spreading around both electrodes, with a larger peak visible between the electrodes, likely due to best visibility in that area. The direct TI response was located between the two stimulating electrodes only, although it was also biased towards electrode 2. Further experimental verification of the FEM model's validity using different electrode configurations would be beneficial. Careful characterisation of neuronal responses at different depths (when targeting

deep structures such as hippocampus) as well as verifying the degree of steerability of the TI stimulation assumed in this model will be crucial.

Control LF stimulation induced a reliable oscillation at f_{stim} alone. In contrast, TI evoked a neuronal response at the difference frequency, Δf , but also its second harmonic, $2\Delta f$. At the ROI level, the $2\Delta f$ component was generated along with the Δf component, both emerging at the same current density. In fact, with increasing stimulation amplitude, the response at the $2\Delta f$ remained more stable than the response at Δf , as evidenced by more consistent phase locking. This interesting effect may be related to the network-level mechanism of TI, as it has not been observed previously at the single-cell level (Luff et al., 2024b).

One explanation could be that TI, unlike LF, is able to stimulate both inhibitory and excitatory cell populations. This could result in two peaks visible within each AM cycle. Dominance of inhibitory activation could lead to the seemingly out-of-phase responses observed in TI, as only the activity of a subset of excitatory cells is recorded in this setup. At high enough current densities (3 mA/mm^2 for PWM-TI), the phase of the response shifts ($\sim 50^\circ$) and becomes more in phase with the envelope of the TI field, suggesting a possible change in the E/I balance of the stimulation. A recent *ex vivo* study suggested that L2/3 pyramidal cells are more likely to respond to AM stimulation than L2/3 PV interneurons (Caldas-Martinez et al., 2021); however, the same study also reported that the activation threshold was ~ 3 times smaller for PV neurons. Depending on the applied current density, the balance between the activity in the two populations could be shifted. An alternative, or complimentary, explanation relates to the mechanism of frequency mixing proposed previously to explain TI (Luff et al., 2024b). Harmonic components can arise from nonlinear processing of the input frequencies in systems such as nonlinear mixers. At the first level of mixing, the difference, sum and harmonics of original frequencies are expected to emerge (Haufler & Paré, 2019; Luff et al., 2024b); however, further mixing could lead to generation of additional frequency components such as harmonics of existing frequencies.

This spatial activation profile of TI was preserved with kHz carrier frequencies (2000, 5000 Hz) but not when TI was applied in the endogenous neuronal range (10, 100 Hz). In the low frequency range, the evoked response at Δf was much more

widespread around the electrodes. This matches previous single-cell observations suggesting that synaptic inputs amplify frequency mixing in the low frequency but not in the kHz range (Luff et al., 2024b). The phases of the response in the low frequency TI were also different to those observed in the kHz TI, possibly due to this increased influence of synaptic inputs. The contralateral activation of V1 and the generation of the harmonic of Δf , $2\Delta f$, was also only observed in the kHz range. Again, this might indicate that different cell types are more sensitive to kHz stimulation or that the cells become primed to nonlinearly mix the incoming signals in the presence of kHz fields.

Previous studies (Esmaeilpour et al., 2021; Mirzakhilili et al., 2020; Luff et al., 2024a, 2024b) assumed the response of neurons to the AM kHz field would be the same as to the TI fields. In this study, the response profile of AM stimulation, applied through a single electrode pair, versus TI stimulation, applied through two pairs, varied in two important aspects. Δf phase of the AM response was different than that of the TI response ($\sim 60^\circ$ versus $\sim -170^\circ$). Higher firing of pyramidal versus inhibitory PV cells in response to AM, compared with TI, could explain the discrepancy between the phases observed (Caldas-Martinez et al., 2021). Additionally, AM did not induce contralateral activation, despite similar electric field amplitudes achieved at the current densities tested. The lack of contralateral activation could be related to the different spread of the electric field or could be due to different cell types being activated. Another key difference between these two modalities is that in TI, neurons are continuously exposed to full amplitude kHz electric fields.

PWM-TI is another TI modality tested and compared to conventional TI in these experiments. Here, PWM-TI, was found to be a suitable alternative to TI, with similar response dynamics and a slightly increased stimulation strength. This difference in strength can potentially be attributed to the higher power of square versus sinusoidal waveforms. The power delivered is proportional to the RMS amplitude, rather than peak-to-peak amplitude, which is 40% larger when using square waves. Consequently, when using PWM-TI, current densities applied should be scaled appropriately compared to those used with standard TI. Lower current threshold for $2\Delta f$ than for Δf suggests the generation of the frequencies in PWM-TI may rely on non-linear frequency mixing mechanisms even more than in standard TI. Despite no amplitude modulation of the signal present, neurons seem capable of extracting Δf . This raises interesting questions on the mechanism of action of PWM-TI. Further

computational modelling suggested neuronal cell membrane may be able to convert PWM signal to AM signal due to its low-pass filtering properties (Luff et al., 2024a).

Testing the effects of kHz electric fields on network-level activity showed that such frequencies can induce responses at stimulation onset but are unlikely to affect steady-state responses at the relevant current densities. The observed onset response could be eliminated when using a higher carrier frequency of 5000 Hz or attenuated with increased current ramp rates. Stimulation with kHz fields was not found to induce depolarisation block as previously hypothesised (Mirzakhilili et al., 2020). Other modelling study (Wang et al., 2022) showed that strong electric fields of >1700 V/m would be needed to induce such blocks with kHz stimulation, which is far beyond the fields used in this study.

Here, the kHz effects on ongoing neuronal activity were also tested by simultaneous application of visual and electrical low frequency stimuli in the presence of kHz electric fields. kHz stimulation did not affect visual responses at f_{stim} applied. Small differences for the responses at the harmonics, $2f_{\text{stim}}$ and $3f_{\text{stim}}$ were observed but these could have arisen due to variation between mice related to small sample size ($n = 5$). Application of kHz fields during LF stimulation did not significantly change the responses at f_{stim} measured at the ROI level. Overall, electrical low frequency stimulation seemed, however, modulated by the presence of kHz fields. kHz stimulation led to potential generation of responses at $2f_{\text{stim}}$, the harmonic of f_{stim} , that was not present otherwise. It also led to a small phase shift of the response at f_{stim} .

While kHz stimulation did not affect ongoing spontaneous or visual activity beyond the transient onset effects and did not cause a depolarisation block, it may have modulated the neuronal response to other electrical stimuli. This observation could be mediated by the differential activation of cell types (such as recruitment of inhibitory neurons), leading to different activity patterns on the network level, or affecting the sensitivity of other neuronal cells to additional electrical stimuli. It is also possible that the nonlinear effects observed here are caused by the sheer strength of the two signals applied, i.e. high current density, rather than by the properties of kHz stimulation. The results reported suffer from a small sample size ($n = 5$), leading to statistically non-significant increases in activity at frequencies tested for both conditions, and thus should be further validated. Additionally, both LF and kHz fields

are applied simultaneously, leading to a large onset effect. A better experimental design could involve applying the LF stimulation once the kHz stimulation reaches a steady-state response. Finally, further confirmation of the lack of kHz effects on ongoing neuronal activity in the form of behavioural outcome would be valuable.

While informative, the study presented here has its limitations and weaknesses. While the widefield imaging approach captures large cortical areas, it is not possible to measure neuronal activity directly underneath the stimulating electrodes to evaluate the effects of the stimulation in the area of the highest absolute current density. The quality of preparation also affects the variability of the responses, with the glue used to secure the electrodes to the skull affecting how much signal is visible between the electrodes. The decision to implant the two stimulating electrodes in the skull was motivated by the need to image animals over multiple days, through a skull covered in non-conductive glue and nail polish. This involved drilling two holes in the skull down to dura, making the procedure more invasive than simple cranial electrode placement. However, this procedure ensured that the impedance across the electrode pairs was low enough to deliver reliable stimulation across many animals. The return electrodes were placed on the animal cheeks which could induce sensory confounds at higher current amplitudes. Placing all the electrodes on the skull would avoid that issue but, on the other hand, would limit the imaging FOV.

In future experiments, monitoring animal behaviour by video recording would help to determine whether any of the neuronal effects observed can be related to spontaneous or stimulation evoked motor responses or potential somatosensory responses. *Thy1*-GCaMP6s mice were used in this study due to their good signal-to-noise ratio and the well-established status as the Tg model. Using improved, faster indicators of neuronal activity, either calcium (Zhang et al., 2023) or voltage based (Piatkevich et al., 2019; Fan et al., 2023), would allow for tracking higher frequencies, both when applying higher Δf and when tracking the polysynaptic stimulation effects on existing, functionally relevant neuronal circuits. An interesting extension of this study could involve using widefield 2-photon imaging, such as (Sofroniew et al., 2016), to track finer single-cell rather than bulk responses to TI. While more technically challenging, such studies would also allow targeted genetic expression of indicators to specific cell types to reveal any cell-type specificity effects of TI. Finally, anaesthesia (0.5% isoflurane in oxygen) was used in this study to minimise the recorded motor

responses. This design was based on previous studies shown to reliably record visual responses and spontaneous activity (Doostdar et al., 2021; Radulescu et al., 2023). While the isoflurane levels used were low and mice were still responsive, this may have affected the electrically evoked responses observed. Future studies could combine behaviour monitoring, prolonged setup habituation and no anaesthetic application for better confound-free recordings.

Chapter 4. Targeting cortical hyperexcitability in an early-stage Alzheimer's Disease model with TI

4.1 Introduction

Neuromodulatory interventions have the potential to offer new promising therapies by targeting aberrant circuit activity that underpins a disease. One of the key features of early-stage Alzheimer's Disease is cortical and hippocampal circuit hyperactivity caused by, but also amplified by, amyloid- β accumulation (Busche & Konnerth, 2016). Early-stage hyperactivity reduction may stop this positive feedback loop, slowing or even halting the disease progression.

In **Chapter 3**, I verified that TI can successfully drive neuronal activity at Δf at the network level. In this set of experiments (**Figure 4.1**), I tested whether TI can thus be used to transiently suppress hyperactive cortical networks in an early-stage AD mouse model. Here, I used the experimental setup developed previously (**Figure 3.1**) to measure cortical activity following the application of the 1 Hz TI stimulation protocol in the App^{NL-G-F} mice. This new KI mouse model, which does not cause APP overexpression associated with previous transgenic disease models, has shown promise in better preclinical modelling of the disease progression (Saito et al., 2014). Similarly to previous transgenic models, App^{NL-G-F} mice have been shown to display non-convulsive epileptiform activity associated with elevated A β and APP (E. C. B. Johnson et al., 2020). They also show increased glutamate release probability prior to plaque formation (Benitez et al., 2021), suggested to initiate the neuronal hyperactivity in AD (Zott et al., 2019)

In this study, I delivered a 15-minute-long 1 Hz TI stimulation protocol to the visual cortex of ~5-month-old wild type (WT) and homozygous App^{NL-G-F} (APP) mice. The protocol was delivered in three blocks of 5-minute stimulation on three consecutive days. The slow stimulation paradigm was inspired by the existing long-term depression (LTD) protocols commonly used in brain slices to cause transient synaptic depression (e.g. Yang et al., 2011). This necessitated the use of shorter stimulation pulses of 200 ms applied every second. Delivering such stimulation by continuously switching the

currents on and off could result in potential onset effects, as shown in **Chapter 3**, at the start of each pulse. To avoid these confounds, I delivered the stimulation using my recently proposed pulsed TI approach instead (**Figure 4.1B**; details described in section **2.4.6**).

I tested the TI stimulation effects by measuring the magnitude of electrically evoked responses (EERs), visually evoked responses (VERs) and spontaneous activity prior to and post each 5-minute stimulation block (**Figure 4.1C**). To simplify the analysis, I compared the responses before and after the full 15 minutes of stimulation on each day. Full details on experimental methods specific to this chapter can be found in sections **2.7** and **2.11** of **Chapter 2**.

This pilot study is the first application of TI stimulation in App^{NL-G-F} mice, the next-generation model of the Alzheimer’s Disease, to test the potential of TI to transiently reduce the AD hyperexcitability.

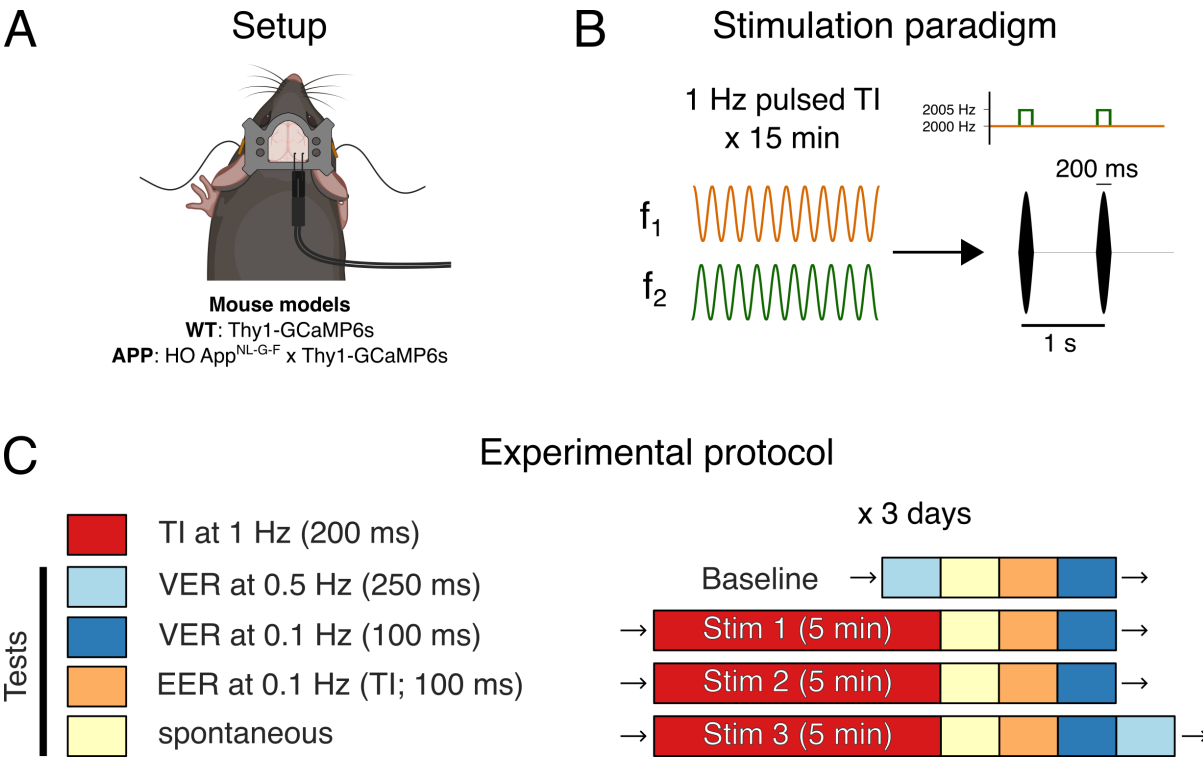


Figure 4.1 | Experimental protocol for suppression of excitability in cortical neurons of 6-month-old App^{NL-G-F} mice. **A.** Setup used to deliver TI stimulation and record cortical activity in WT and APP mice was the same as in **Chapter 3** experiments. For each mouse, activity from the ROI between the two stimulating electrodes was used for the analysis. Homozygous App^{NL-G-F} Thy1-GCaMP6s mice and control WT Thy1-GCaMP6s mice were used in this study. Schematic created with BioRender.com. **B.** 1 Hz stimulation, delivered as pulses of 200 ms, was delivered using frequency modulation approach

of pulsed TI. 15 minutes of stimulation was applied in three blocks of 5 minutes each. Details on pulsed waveform generation can be found in section **2.4.6. C**. Testing protocol consisted of recording spontaneous neuronal activity as well as evoked neuronal responses to visual and electrical stimuli prior to and after each 5-minute stimulation block on three consecutive days.

4.2. Results

4.2.1 Spontaneous and evoked responses do not differ between the WT and APP mice

First, I sought to establish whether the pathology in the App^{NL-G-F} mice at ~5 months of age had a marked impact on spontaneous neuronal activity or the magnitude of the evoked responses, compared to the WT mice. Similarly to **Chapter 3** analysis, calcium activity traces used in this chapter come from the ROI located between the two stimulating electrodes for each mouse. To determine whether there are any differences between the WT and APP groups in any of the tests used, I compared the responses of WT and App^{NL-G-F} mice to the test stimuli before the stimulation protocol started on the first day (Day 1) (**Figure 4.2**).

These responses were quantified as the mean area under the curve (AUC), within a test-specific response window, of the calcium traces recorded in the ROI between the two stimulating electrodes. In none of the four tests performed were there any significant differences between the two groups (**Table 4.1**). Notably, in the 0.1 Hz EER test, three of the six mice displayed much larger responses than the rest of the group and the WT group.

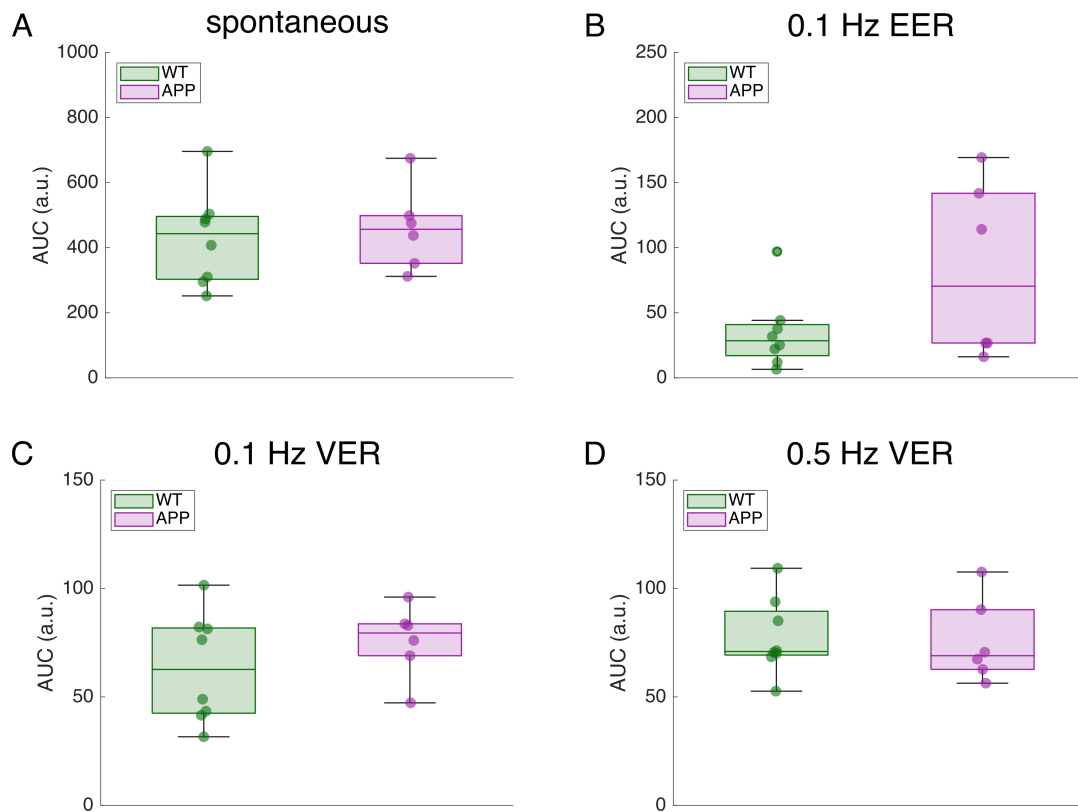


Figure 4.2 | Baseline responses to tests applied do not differ between the WT and APP mice. A. Spontaneous activity levels are the same for both WT and APP mice on Day 1 of the experiments, before the TI stimulation protocol was applied. Average value per mouse reported. AUC = area under the curve. **B.** As in B but for electrically evoked responses (EER) delivered at the frequency of 0.1 Hz. Some mice in the APP group show much higher amplitude of responses but the effect is not consistent for all mice. **C.** As in A but for visually evoked responses (VER) delivered at the frequency of 0.1 Hz. **D.** As in C but for VER delivered at the frequency of 0.5 Hz. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 6-8$ mice per group, for statistical details see **Table 4.1**.

Table 4.1 | Statistical analysis details related to Figure 4.2.

Condition	Comparison	Test	p	Significance level	n (animals)
Spontaneous	WT v APP	Two-sample t-test	0.700		WT: 8 APP: 6
0.1 Hz EER	WT v APP	Two-sample t-test	0.092		WT: 8 APP: 6
0.1 Hz VER	WT v APP	Two-sample t-test	0.314		WT: 8 APP: 6
0.5 Hz VER	WT v APP	Two-sample t-test	0.853		WT: 8 APP: 6

4.2.2 Effects of 1 Hz TI protocol on spontaneous neuronal activity are inconclusive

Spontaneous activity levels are often used as a neuronal hyperactivity indicator. Although the two experimental groups at this age point did not display any differences in spontaneous levels (**Figure 4.2**), I tested whether this endogenous activity could be reduced following the 1 Hz TI stimulation.

I compared the spontaneous activity levels before (baseline; Base) and after the full 15 minutes of the 1 Hz TI protocol (post stimulation; Post Stim) on each experimental day ('Between' comparison). All data was normalised to baseline of Day 1 to investigate whether any carry-over effects could be seen on subsequent stimulation days ('Base v 0') and to ensure the post-stimulation effect is consistent ('Stim v 0').

In both WT and APP groups, significant reduction in spontaneous activity levels could be detected on Day 3 (**Figure 4.3A**), as measured by calculating mean AUC for each animal during undisturbed recordings. This could indicate that the stimulation causes accumulated carry-over effects on spontaneous activity levels, noticeable only after repeated stimulation over three days.

In order to account for other experimental factors that might contribute to these effects, such as habituation to the imaging setup or prolonged anaesthesia effects, I repeated the experiments in a sham control configuration. The sham animals underwent the same surgical and experimental procedure, but they did not receive the 15-minute 1 Hz TI stimulation.

The sham control experiments showed a significant reduction in activity levels on all experimental days, relative to either baseline of Day 1 or baseline of each day, for both experimental groups (**Figure 4.3B**). This effect was much stronger than in the active experimental group, despite smaller group sizes. Given that the dampening effect was much more persistent for the sham group than the active group, it is likely that the spontaneous procedure differed for the sham group in some crucial way, confounding the overall trend over the experimental day. This is confirmed by comparing the baseline spontaneous levels of sham versus stimulated mice (**Figure 4.4**). The sham groups had larger baseline activity levels than the stimulated groups, especially in the

APP group but also in the WT group (close to significance at $p = 0.057$). This makes the overall interpretation of the active spontaneous results difficult.

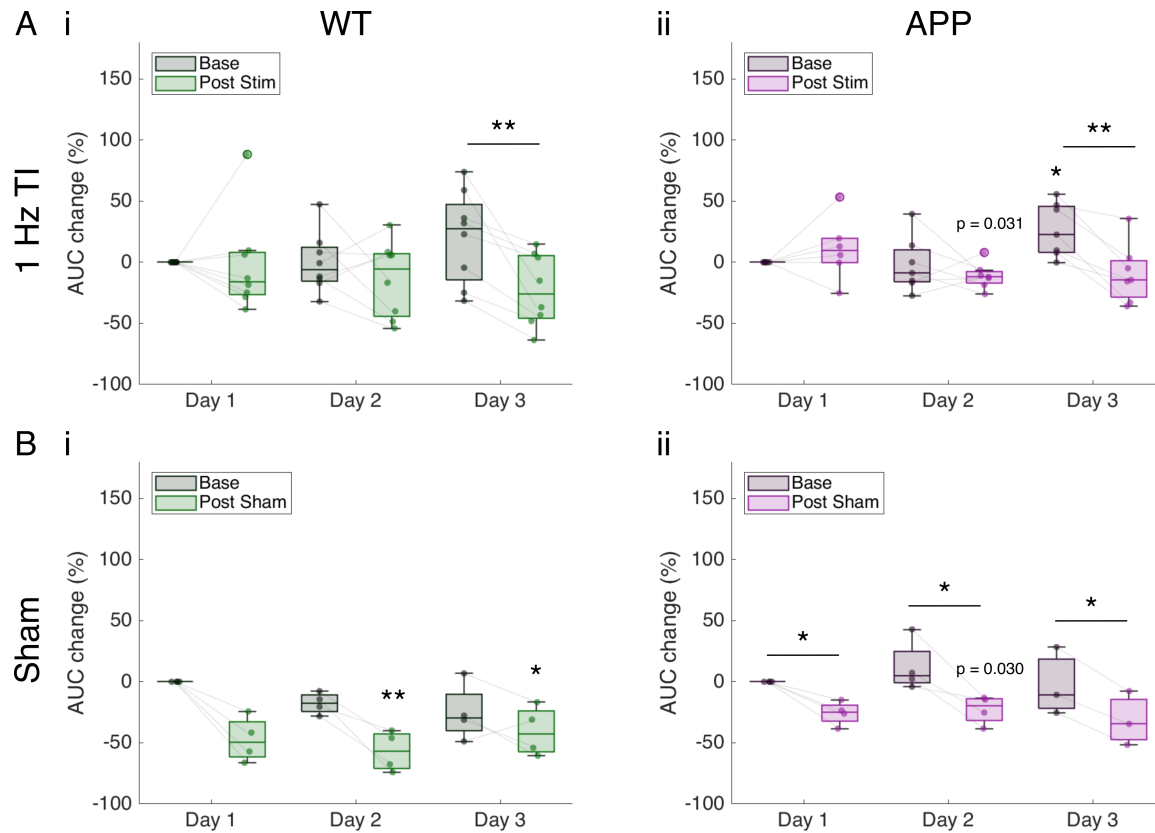


Figure 4.3 | The effects of 1 Hz TI stimulation on spontaneous activity levels are unclear. A. i. Change in AUC for all experimental days in the WT group, normalised to baseline of Day 1. 1 Hz TI protocol may have reduced the spontaneous activity levels after three full days of stimulation. **ii.** As in **Ai** but for the APP group. Similar trends are observed as in the WT group. **B. i.** As in **Ai** but for the WT sham group. Reduction in AUC levels compared to baseline of Day 1 can be observed after each daily experimental session. **ii.** As in **Bi** but for the APP sham group. Reduction in AUC levels post sham relative to its daily baseline is maintained on all experimental days. Stronger effect for both groups during sham than during active stimulation may indicate changes in spontaneous protocols between the two experiments. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3-8$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 4.2**.

Table 4.2 | Statistical analysis details related to Figure 4.3.

Condition	Comparison	Test	p			Bonferroni-Holm p			Significance level			n (animals)		
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
WT, 1 Hz TI	Between	Wilcoxon signed-rank test	0.383	0.383	0.008	0.766	0.766	0.023			**	8	8	8
	Base v 0	Paired-sample t-test	-	0.963	0.173	-	0.963	0.346	-					
	Stim v 0	Paired-sample t-test	-	0.253	0.065	-	0.253	0.129	-					
APP, 1 Hz TI	Between	Paired-sample t-test	0.344	0.376	0.003	0.687	0.687	0.008			**	6	7	7
	Base v 0	Paired-sample t-test	-	0.815	0.019	-	0.815	0.037	-		*			
	Stim v 0	Paired-sample t-test	-	0.031	0.351	-	0.061	0.351	-					
WT, Sham	Between	Wilcoxon signed-rank test	0.125	0.125	0.250	0.375	0.375	0.375				4	4	4
	Base v 0	Paired-sample t-test	-	0.027	0.119	-	0.055	0.119	-					
	Stim v 0	Paired-sample t-test	-	0.006	0.028	-	0.013	0.028	-	**	*			
APP, Sham	Between	Paired-sample t-test	0.013	0.035	0.017	0.040	0.040	0.040	*	*	*	4	4	3
	Base v 0	Paired-sample t-test	-	0.331	0.888	-	0.662	0.888	-					
	Stim v 0	Paired-sample t-test	-	0.030	0.135	-	0.059	0.135	-					

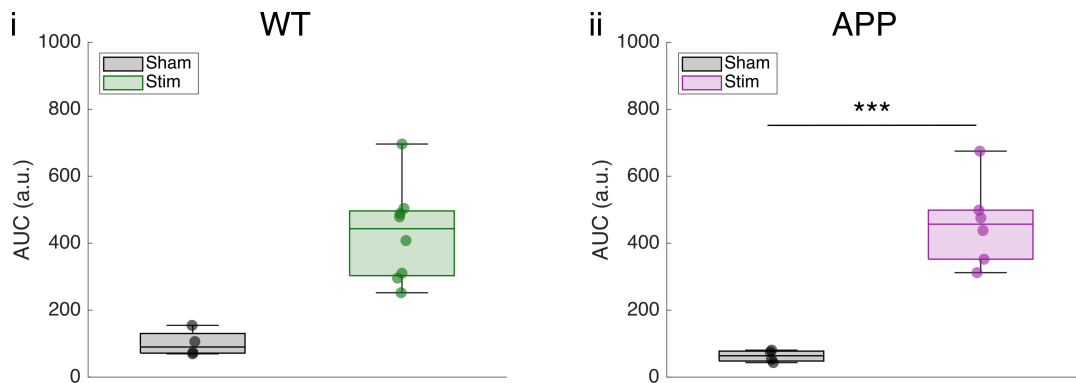


Figure 4.4 | Baseline spontaneous activity on Day 1 is different for sham and active groups. i Magnitude of spontaneous activity of sham WT group is almost higher ($p = 0.057$) than that of the stimulated WT group. **ii** Magnitude of spontaneous activity of APP sham group is higher than that of the stimulated APP group. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4-8$ mice per group, for statistical details see **Table 4.3**.

Table 4.3 | Statistical analysis details related to Figure 4.4. Paired comparison was done as the sham group was used for subsequent stimulation experiments.

Condition	Comparison	Test	p	Significance level	n (animals)
0.1 Hz EER	WT Sham v WT Stim	Paired-sample t-test	0.057		Sham:4 Active: 8
0.1 Hz EER	APP Sham v APP Stim	Paired-sample t-test	3.E-04	***	Sham: 4 Active: 6

4.2.3 1 Hz TI protocol reliably reduces electrically evoked responses

To test whether the 1 Hz TI stimulation can reduce neuronal excitability, I compared the neuronal responses evoked by short electrical stimuli repeated every 10 s (0.1 Hz repetition frequency) applied before and after the TI stimulation. Both the WT and APP groups displayed strong reduction in the EER magnitude following the full stimulation protocol (**Figure 4.5A-B**). This effect was achieved on all the three experimental days, indicating that the slow 1 Hz TI protocol reliably suppresses stimulated neurons. The APP group showed even more pronounced reduction in the EER amplitudes and less variability, relative to the baseline of Day 1, than the WT group. The APP group also showed a trend towards a potential sustained carry-over effect, with small reduction of the baseline response on Day 2 and Day 3 normalised to baseline of Day 1 (Day 2 Base: mean $\pm$ SD = -15.9 ± 18.2 %, $p = 0.031$; Day 3 Base: mean $\pm$ SD = -17.6 ± 18.7 %, $p = 0.078$). The sham APP group did not display reduced EERs, while the WT group showed significant EER reduction on Day 3 only (**Figure 4.5C**), suggesting a potential minor contribution of the experimental procedure to the final result for this group. Interestingly, the baseline response on this day was also significantly larger than baseline of Day 1, perhaps indicating differences in the experimental procedure, amplified by the small sample size ($n = 4$).

Altogether, these results demonstrate that the 1 Hz TI stimulation successfully dampened the neuronal activity evoked by repeated electrical stimuli, suggesting the protocol can reduce excitability of stimulated neurons.

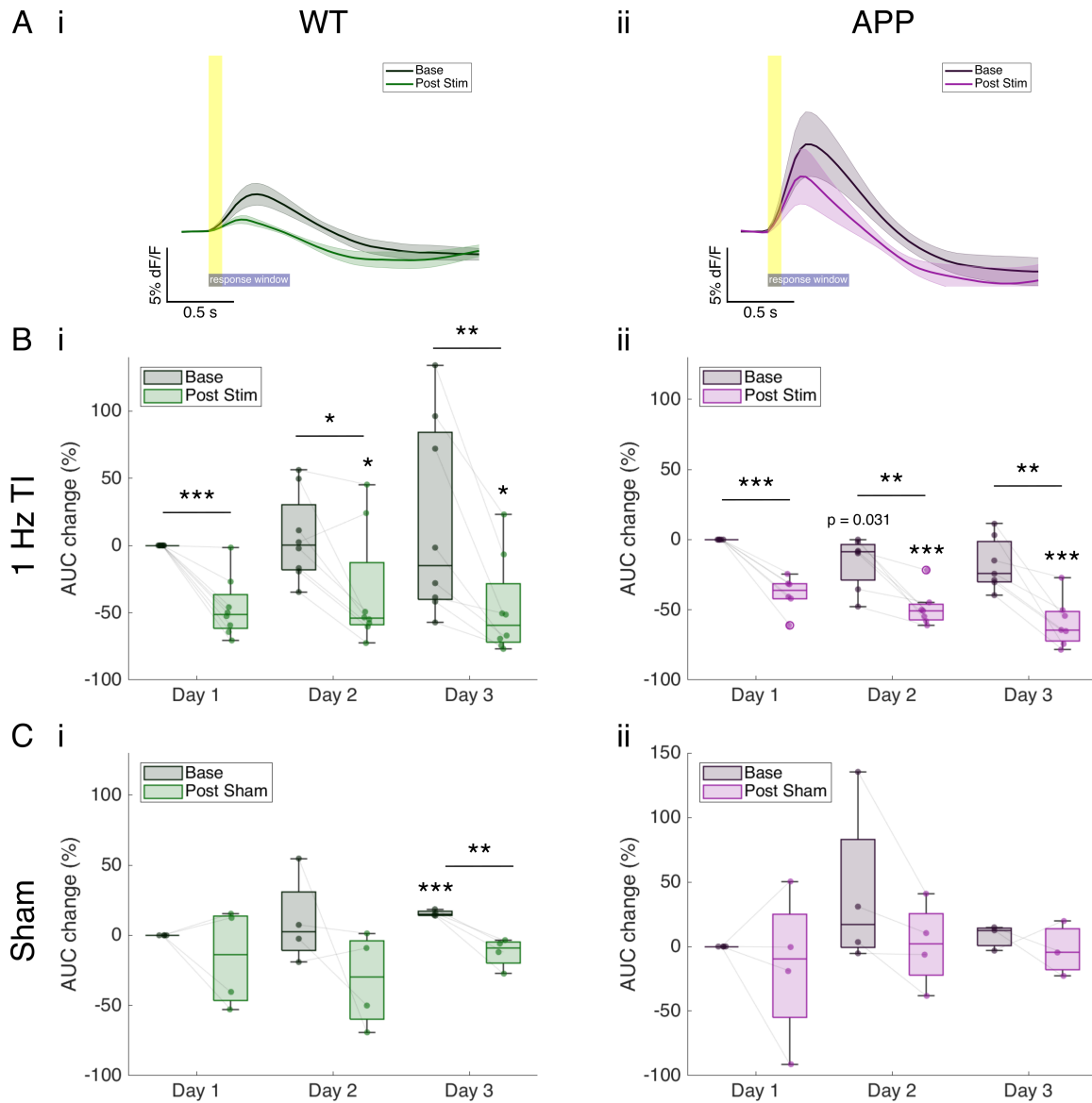


Figure 4.5 | 1 Hz TI stimulation protocol significantly reduces the amplitude of electrically evoked events (0.1 Hz EER). **A. i.** Stimulus-triggered average (STA) for WT mice prior to (Base) and post stimulation (Post Stim) on Day 1. Electrical stimulus consisted of 100 ms long electrical pulses applied at the repetition frequency of 0.1 Hz. Each individual stimulus response was normalised by subtracting mean dF/F of 0.2 s preceding the stimulus. Response window used to calculate the AUC was 0.6 s long, starting at the stimulus onset. Mean $\pm$ SEM, $n = 8$ animals. **ii.** As **Ai** but for APP mice. $n = 6$. **B. i.** Change in AUC response for all experimental days in the WT group, normalised to baseline of Day 1. 1 Hz TI protocol successfully reduced the EER amplitudes on all experimental days. **ii.** As in **Bi** but for the APP group. Strong reduction in EER amplitudes per day, with a potential trend towards baseline reduction across days. **C. i.** As in **Bi** but for the WT sham group. Reduction in EER amplitude on Day 3 can be observed but the effect is not sustained on all experimental days. **ii.** As in **Ci** but for the APP sham group. No difference was observed between Base and Post Sham responses. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3-8$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 4.4**.

Table 4.4 | Statistical analysis details related to Figure 4.5.

Condition	Comparison	Test	p			Bonferroni-Holm p			Significance level			n (animals)		
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
WT, 1 Hz TI	Between	Paired-sample t-test	0.001	0.016	0.007	0.002	0.016	0.014	***	*	**	8	8	8
	Base v 0	Paired-sample t-test	-	0.627	0.535	-	1.000	1.000	-					
	Stim v 0	Wilcoxon signed-rank test	-	0.039	0.023	-	0.047	0.047	-	*	*			
APP, 1 Hz TI	Between	Paired-sample t-test	7.E-04	0.005	3.E-03	0.002	0.006	0.006	***	**	**	6	7	7
	Base v 0	Wilcoxon signed-rank test	-	0.031	0.078	-	0.063	0.078	-					
	Stim v 0	Paired-sample t-test	-	6.E-05	1.E-04	-	1.E-04	1.E-04	-	***	***			
WT, Sham	Between	Paired-sample t-test	0.423	0.256	0.010	0.511	0.511	0.029			**	4	4	4
	Base v 0	Paired-sample t-test	-	0.566	0.001	-	0.566	0.001	-		***			
	Stim v 0	Paired-sample t-test	-	0.154	0.109	-	0.217	0.217	-					
APP, Sham	Between	Paired-sample t-test	0.643	0.145	0.604	1.000	0.435	1.000				4	4	3
	Base v 0	Paired-sample t-test	-	0.293	0.291	-	0.582	0.582	-					
	Stim v 0	Paired-sample t-test	-	0.924	0.856	-	1.000	1.000	-					

4.2.4 1 Hz TI protocol unlikely to affect visually evoked responses

Electrical stimulation pulses delivered during the EER test are likely to activate the same neuronal populations that are directly activated during the 15-minute 1 Hz stimulation. To test whether the TI protocol implemented could also affect the responses to other types of stimuli, I next tested its effects on visually evoked responses (VERs).

When applying visual test stimuli equivalent to prior electrical test stimuli (100 ms duration, 0.1 Hz repetition frequency), the magnitude of the evoked responses before and after the stimulation period did not differ (**Figure 4.6A-B**). There was no significant difference in VER amplitudes for either WT or APP groups. Similarly, sham control groups showed no differences in amplitudes before and after the procedure (**Figure 4.6C**), indicating the dampening effect of electrical stimulation did not extend to visual stimuli.

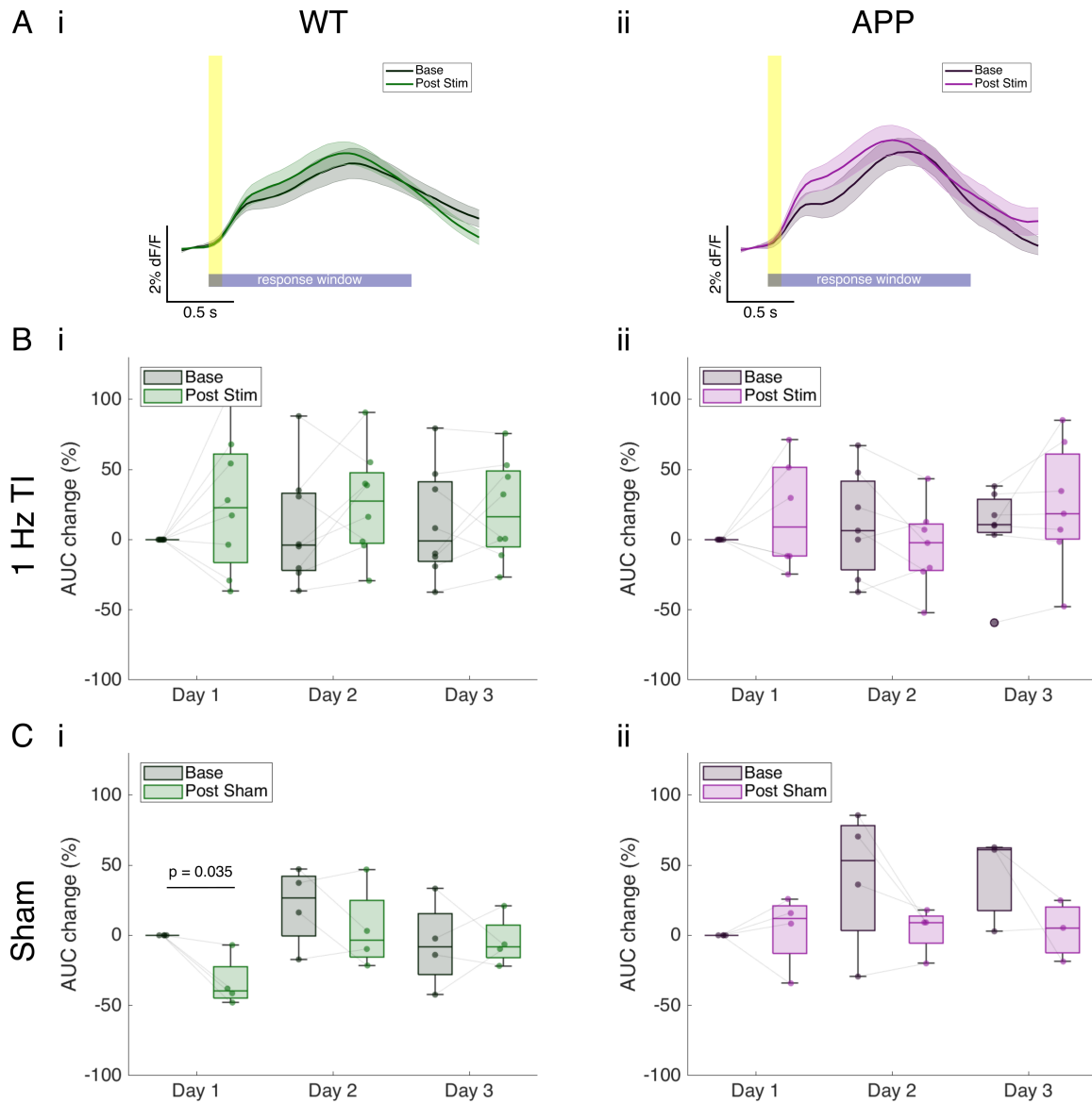


Figure 4.6 | 1 Hz TI stimulation did not affect the amplitude of the visually evoked responses to short and infrequent visual stimuli (0.1 Hz VER). **A. i.** STA for WT mice prior to and post stimulation on Day 1. Visual stimulus consisted of 100 ms long pulses of flashing LED applied at the repetition frequency of 0.1 Hz. Each individual stimulus response was normalised by subtracting mean $\Delta F/F$ of 0.2 s preceding the stimulus. Response window used to calculate the AUC was 1.5 s long, starting at the stimulus onset. Mean $\pm$ SEM, $n = 8$ animals. **ii.** As **Ai** but for APP mice. $n = 6$. **B. i.** Change in AUC response for all experimental days in the WT group, normalised to baseline of Day 1. 1 Hz TI protocol did not affect the VER amplitudes for short visual stimuli delivered at 1 Hz repetition frequency. **ii.** As in **Bi** but for the APP group. Similarly, no effect post stimulation was observed. **C. i.** As in **Bi** but for the WT sham group. No significant differences were observed. **ii.** As in **Ci** but for the APP sham group. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3$ -8 mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 4.5**.

Table 4.5 | Statistical analysis details related to Figure 4.6.

Condition	Comparison	Test	p			Bonferroni-Holm p			Significance level			n (animals)		
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
WT, 1 Hz TI	Between	Paired-sample t-test	0.186	0.207	0.397	0.557	0.557	0.557				8	8	8
	Base v 0	Paired-sample t-test	-	0.590	0.436	-	0.872	0.872	-					
	Stim v 0	Paired-sample t-test	-	0.098	0.137	-	0.196	0.196	-					
APP, 1 Hz TI	Between	Paired-sample t-test	0.327	0.123	0.186	0.371	0.369	0.371				6	7	7
	Base v 0	Wilcoxon signed-rank test	-	0.563	0.297	-	0.594	0.594	-					
	Stim v 0	Paired-sample t-test	-	0.686	0.211	-	0.686	0.422	-					
WT, Sham	Between	Paired-sample t-test	0.035	0.340	0.909	0.104	0.681	0.909				4	4	4
	Base v 0	Paired-sample t-test	-	0.238	0.714	-	0.477	0.714	-					
	Stim v 0	Paired-sample t-test	-	0.774	0.669	-	1.000	1.000	-					
APP, Sham	Between	Paired-sample t-test	0.784	0.159	0.247	0.784	0.478	0.495				4	4	3
	Base v 0	Paired-sample t-test	-	0.209	0.165	-	0.331	0.331	-					
	Stim v 0	Paired-sample t-test	-	0.657	0.789	-	1.000	1.000	-					

The second visual stimulation test consisted of applying longer visual stimuli more frequently (250 ms duration, 0.5 Hz repetition frequency). Interestingly, the VERs for this test were reduced post stimulation on every experimental day in the WT but not in the APP group (**Figure 4.7A-B**). Why this effect was different between the two groups remains unclear. However, the WT sham group showed a similar dampening trend in the VER responses post the sham treatment (**Figure 4.7Ci**), suggesting this effect may be a confound due to the experimental procedure rather than the TI stimulation itself.

Combined, these results suggest that the implemented 1 Hz TI stimulation protocol was unlikely to affect visually evoked responses in either of the two experimental groups.

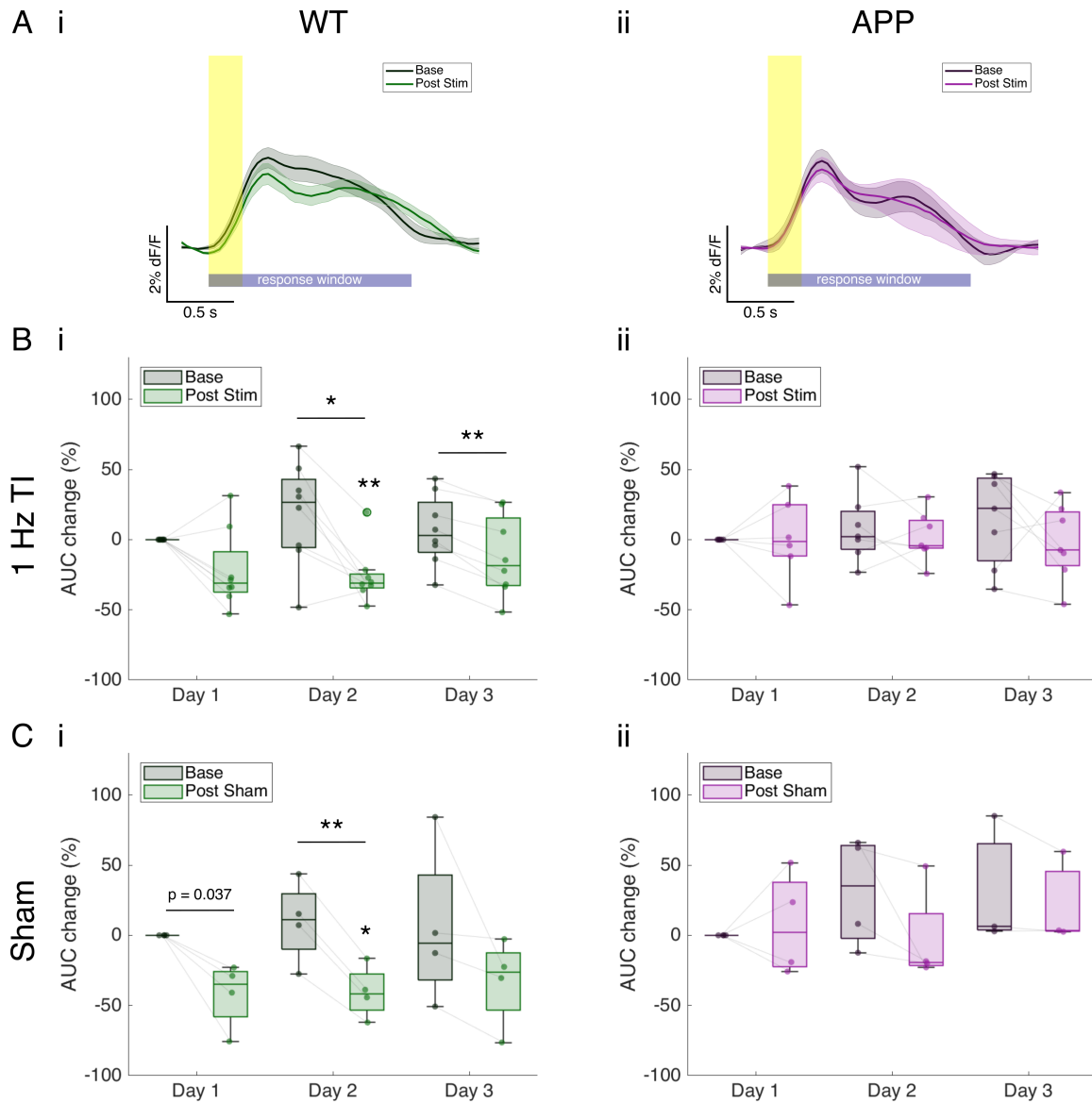


Figure 4.7 | 1 Hz TI stimulation unlikely to affect the amplitude of the visually evoked responses to more frequent and longer visual stimuli (0.5 Hz VER). **A. i.** STA for WT mice prior to and post stimulation on Day 1. Visual stimulus consisted of 250 ms long pulses of flashing LED applied at the repetition frequency of 0.5 Hz. Each individual stimulus response was normalised by subtracting mean dF/F of 0.2 s preceding the stimulus. Response window used to calculate the AUC was 1.5 s long, starting at the stimulus onset. Mean $\pm$ SEM, $n = 8$ animals. **ii.** As **Ai** but for APP mice. $n = 6$. **B. i.** Change in AUC response for all experimental days in the WT group, normalised to baseline of Day 1. 1 Hz TI protocol reduced the VER amplitudes in the WT group. **ii.** As in **Bi** but for the APP group. No effect post stimulation was observed in this group. **C. i.** As in **Bi** but for the WT sham group. Reduction in VERs was observed on Days 1 and 2, suggesting the effect may be explained by other experimental confounds. **ii.** As in **Ci** but for the APP sham group. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 3-8$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 4.6**.

Table 4.6 | Statistical analysis details related to Figure 4.7.

Condition	Comparison	Test	p			Bonferroni-Holm p			Significance level			n (animals)		
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
WT, 1 Hz TI	Between	Wilcoxon signed-rank test	0.078	0.016	0.008	0.078	0.031	0.023		*	**	8	8	8
	Base v 0	Paired-sample t-test	-	0.203	0.482	-	0.406	0.482	-					
	Stim v 0	Paired-sample t-test	-	0.008	0.272	-	0.015	0.272	-	**				
APP, 1 Hz TI	Between	Paired-sample t-test	0.980	0.526	0.310	1.000	1.000	0.931				6	7	7
	Base v 0	Paired-sample t-test	-	0.422	0.290	-	0.580	0.580	-					
	Stim v 0	Paired-sample t-test	-	0.769	0.839	-	1.000	1.000	-					
WT, Sham	Between	Paired-sample t-test	0.037	0.003	0.193	0.075	0.008	0.193		**		4	4	4
	Base v 0	Paired-sample t-test	-	0.556	0.855	-	1.000	1.000	-					
	Stim v 0	Paired-sample t-test	-	0.023	0.124	-	0.046	0.124	-	*				
APP, Sham	Between	Paired-sample t-test	0.706	0.167	0.359	0.718	0.500	0.718				4	4	3
	Base v 0	Paired-sample t-test	-	0.212	0.363	-	0.423	0.423	-					
	Stim v 0	Wilcoxon signed-rank test	-	0.875	0.250	-	0.875	0.500	-					

4.3. Discussion

This pilot study explored the potential of using Temporal Interference as a therapeutic treatment to mitigate the neuronal hyperexcitability caused by the presence of amyloid beta in the early-stage Alzheimer's Disease model *in vivo*. It is the first demonstration of the application of TI in App^{NL-G-F} mice, a new generation knock-in model which, unlike previous transgenic models, does not suffer from the side effects associated with overexpression of APP (Saito et al., 2014).

Inspired by the well-established LTD stimulation protocol commonly used *ex vivo* to induce synaptic weakening (Mockett, Coussens & Abraham, 2002; Yang et al., 2011), I applied the 15-minute-long 1 Hz (delta) TI stimulation protocol on three consecutive days to the primary visual cortex of wild type and App^{NL-G-F} mice. A novel stimulation technique, pulsed TI, was used to shape the pulse duration to a desired width (200 ms), thus allowing for continuous electrical stimulation, without the need to change the current amplitudes. This improved stimulation technique avoids the stimulation confounds caused by the onset of kHz frequencies, paving the way for fully customisable stimulation paradigms, often required for effective plasticity induction protocols (Albeni et al., 2007).

First, I tested whether the two experimental groups differed in their responses to various test stimuli at a baseline level, i.e. prior to stimulation on Day 1. I found no

significant differences between the two groups. Interestingly, a subset of mice in the APP group displayed elevated responses to electrical stimuli (EER test). Overall, this suggests that at 5 months of age, the functional pathology in the App^{NL-G-F} mice is still in the early stages, with hyperexcitability potentially starting to emerge. However, the effects observed here were restricted to a small ROI and further analysis of the spatial profile of the neuronal responses should be explored. This could potentially reveal more baseline differences between the WT and APP groups at this age.

Following the 15 minutes of stimulation, I found that the 1 Hz TI protocol was able to reliably reduce the magnitude of electrically evoked responses (EERs) in both WT and APP groups but most likely did not affect the magnitude of visually evoked responses (VERs). This may be explained by the fact that it is likely that exactly the same neuronal circuits were probed during the EER test as the ones that underwent the 1 Hz TI stimulation. Visual circuits encoding the visual stimuli, however, could have included unstimulated neurons and thus perhaps were not directly affected by the stimulation protocol.

The EER reduction was particularly strong in the APP group which also exhibited larger (but not significantly so) baseline responses to the electrical test stimulus than the WT group. This is a promising result as it suggests that the hyperexcitability of neurons in the vicinity of amyloid beta may be successfully dampened by a short, 15-minute-long protocol. The extension of this silencing effect to spontaneous activity, one of the earliest dysfunctions present in the Alzheimer's Disease pathology (Busche & Konnerth, 2016), would be even more relevant. In the experiments so far, a potential reduction in spontaneous activity was observed only on the third day of the experiments (Day 3). While promising, it is unclear whether this effect was truly caused by the stimulation itself or simply was a confound of the experimental procedure. Sham animals that underwent the same surgical and experimental treatment (except for the 1 Hz TI protocol) displayed a strong reduction in spontaneous activity levels on most experimental days. Surprisingly, this spontaneous effect was stronger in mice that underwent sham rather than active stimulation. It is likely that there was some crucial experimental difference in the baseline recordings of the sham mice, as the baseline spontaneous activity was found to be larger in that group. These measurements were recorded at the very beginning of each imaging session and hence were sensitive to

factors such as varying preparation time, potentially resulting in inconsistent anaesthesia levels.

These preliminary results show that 1 Hz TI stimulation is a promising treatment to reduce the neuronal hyperexcitability in the presence of A β . However, further work is needed to establish the mechanism of action of the effect observed and verify its clinical usefulness. The reliability of these results should be further confirmed with additional experiments to increase the sample size. The sham experiments, especially, are underpowered (n = 3-4) which may have an impact on their interpretability. Recordings in the next sham experiments should be performed carefully to verify the true source of changes in the spontaneous activity observed in this study. Further increase of the sample size would also allow to determine whether any of the effects were sex dependent.

Future work for this project will focus on exploring the mechanism behind the reduction in functional responses to electrical stimuli. The plasticity effect may have its origin in the Hebbian plasticity and be mediated by NMDA receptors (Caporale & Dan, 2008). Alternatively, it can be a homeostatic plasticity mechanism instead, caused by changes in inhibition or synaptic scaling (Fox & Stryker, 2017) via regulation of AMPA receptors (Styr & Slutsky, 2018). Examination of the brain tissue collected following these experiments is ongoing and will include investigating changes in the synaptic plasticity to better understand the molecular mechanisms involved. It will also be used to assess the finer stimulation effects present at the level of the AD pathology and brain autoimmune response, such as density and distribution of amyloid- β plaques, microglia and astrocytes.

Elucidating the mechanism of action behind the effects observed will enable the optimisation of stimulation protocols for the most effective therapeutic outcome. A control experiment testing whether a different stimulation frequency has the same effects could also guide as to whether synaptic depression or homeostatic plasticity (via general overstimulation) is responsible for the reduction in the responses observed.

Further experiments should investigate the effects of longitudinal stimulation with a TI protocol found the most effective in reducing hyperexcitability in the model of amyloidosis. The stimulation will only be beneficial if it can bring long-lasting, sustained

effects on the neuronal circuits. This should be explored via behavioural testing of functions most heavily affected in the disease, such as spatial memory (Zott et al., 2018). Ideally, the stimulation should be targeted directly to the hippocampus, where the pathology originates (Panza et al., 2019). It should also be tested at different time points of the disease to see whether it can effectively halt or slow down the disease progression, offering a real clinical benefit.

Chapter 5. Targeting hippocampal pathology in an early-stage Alzheimer's Disease model with TI

5.1 Introduction

Early accumulation of amyloid in AD starts in the hippocampus and the entorhinal cortex (Panza et al., 2019). Hippocampal hyperactivity, believed to be dependent on but also further aggravated by presence of A β (Zott et al., 2019), can appear even before the symptom onset (Zott et al., 2018). Correcting the imbalance between hippocampal excitation and inhibition resulting in the pathological firing patterns could potentially prevent further disease progression that would ultimately result in cell loss and cognitive impairment. Pharmacological means that correct the E/I balance by e.g. increasing inhibition are one strategy shown to improve behavioural performance in transgenic AD models (Busche et al., 2015). Restoring disturbed hippocampal gamma rhythms with optogenetic or somatosensory stimulation at 40 Hz has also been proposed as a therapeutic treatment for AD and shown to reduce amyloid- β load, increase neuroimmune response (Iaccarino et al., 2016; Adaikkan et al., 2019) and improve memory (Martorell et al., 2019) following 1 h of stimulation per day for as little as 7 days.

Both pharmacological and somatosensory approaches are untargeted and affect large brain regions. This may lead to undesirable side effects or be less effective than a targeted stimulation to hippocampus. Direct stimulation of deep brain structures such as hippocampus normally requires an invasive surgery to gain access to it. Temporal Interference is a unique stimulation technique capable of reaching deeper brain structures non-invasively, thus capable of direct targeting of affected regions.

Based on the previous encouraging results of stimulation in the delta (1 Hz protocol developed in **Chapter 4**) and gamma (Iaccarino et al., 2016) range in AD studies, and theta range to enhance learning in human studies (Violante et al., 2023; Wessel et al., 2023), I investigated whether similar longitudinal stimulation of hippocampus with TI can affect the AD pathology and behavioural performance.

For this purpose, I developed a novel experimental setup where App^{NL-G-F} mice were stimulated while freely moving in a cage-like environment for 1 h/day over a period of 8 days (**Figure 5.1A**). In this pilot study, I applied the same 1 Hz TI protocol as in **Chapter 4** (delta) as well as functionally relevant theta (8 Hz) and gamma (40 Hz) frequencies. The target stimulation area was the right hippocampus. Following the stimulation treatment, the animal behaviour was tested with two spatial memory tasks. At the end of the experiment, brain tissue was collected and stained for relevant disease biomarkers (**Figure 5.2**) present in hippocampus and cortex. Full details on experimental methods specific to this chapter can be found in section **2.12** of **Chapter 2**.

This early study is the first longitudinal application of the TI stimulation in the App^{NL-G-F} model of AD, testing its feasibility to affect the AD pathology at the preclinical stage.

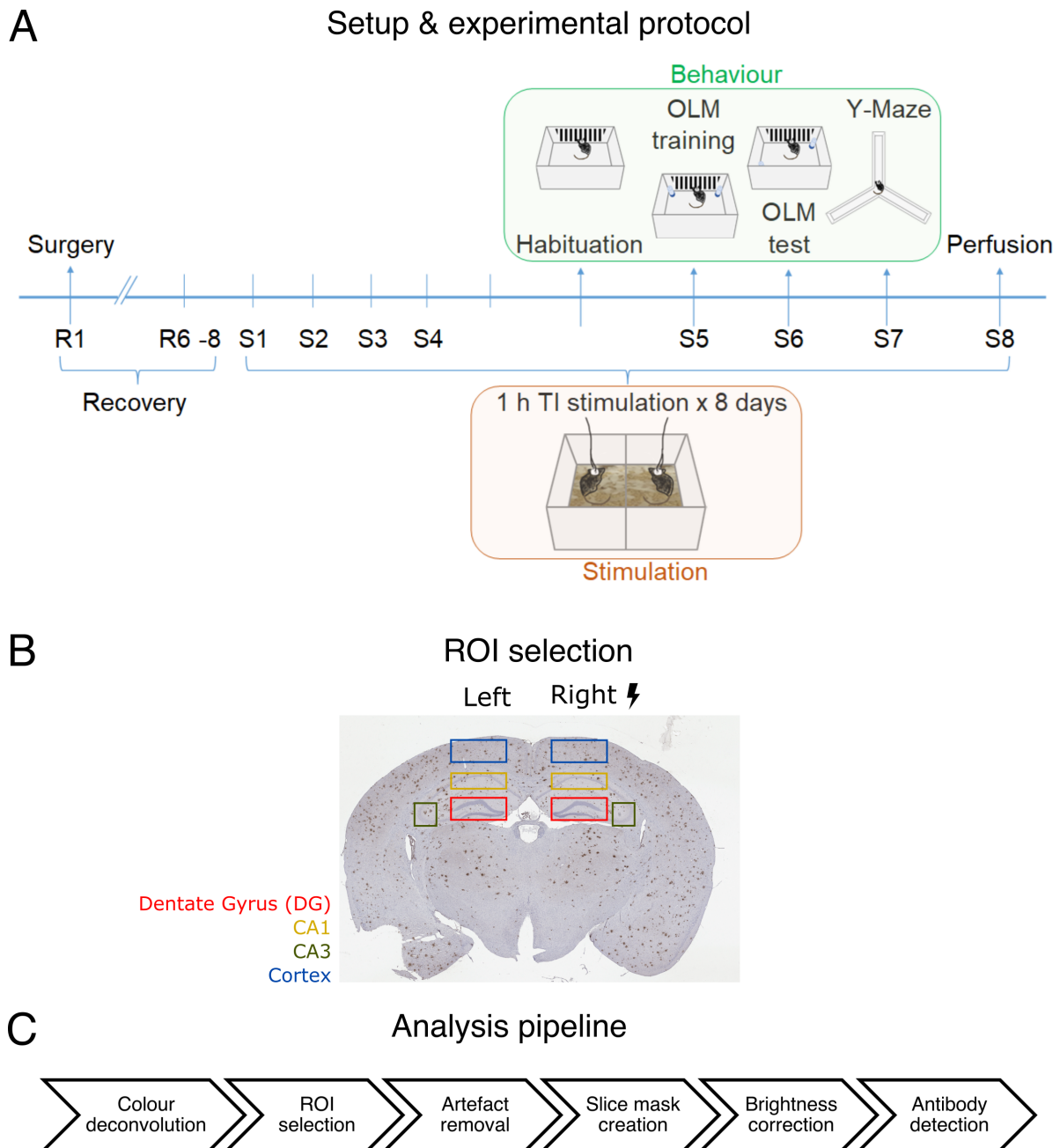


Figure 5.1 | Experimental protocol for longitudinal hippocampal TI stimulation followed by behavioural testing and pathology assessment. Experimental protocol. Following the recovery from the surgical implantation of the stimulating electrodes, the App^{NL-G-F} mice underwent 8 TI stimulation sessions, 1 h / day over a period of two weeks. On the stimulation days 5-8 (S5-8), they were tested in the Open Location Memory (OLM) test and Y-maze. Finally, mice were perfused 60 minutes after the last stimulation day (S8). **B.** Example of a mOC23 image recorded from a slice containing hippocampus. Eight regions of interest (ROI), including hippocampal and cortical areas, were selected from each image for subsequent analysis of the stimulation effects. **C.** Analysis of the immunohistological images consisted of several sequential steps to ensure unbiased processing of the data.

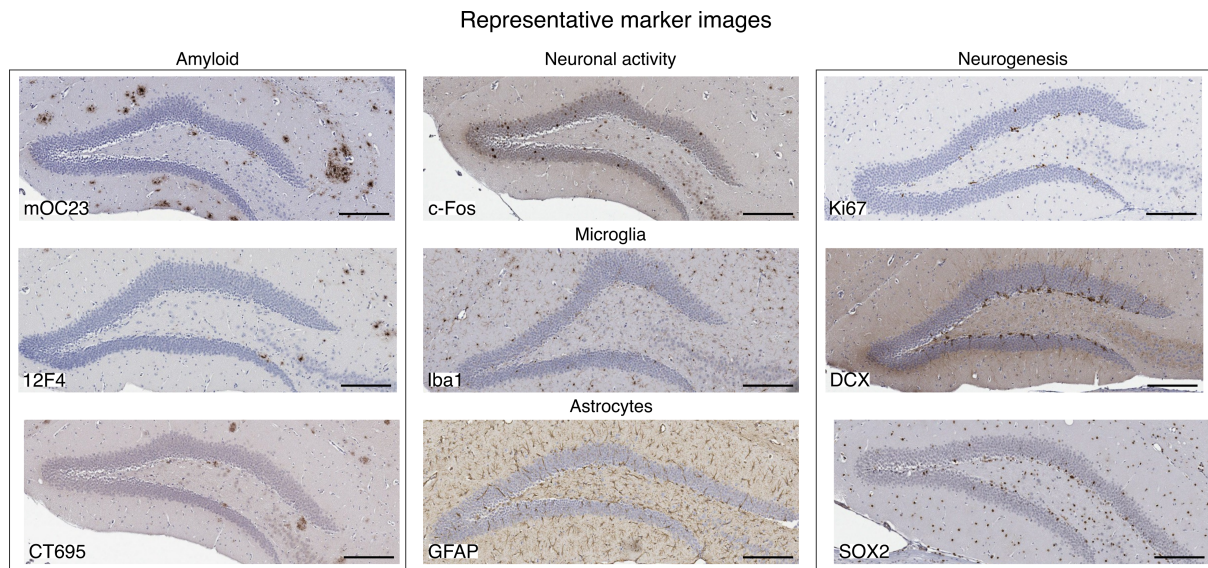


Figure 5.2 | Representative images of the biomarkers used to assess the TI stimulation effects. The markers used consisted of four main types capturing the levels of amyloid (mOC23, 12F4, CT695), neuronal activity (c-Fos), glia (Iba1, GFAP) and neurogenesis (Ki67, DCX, SOX2) observed. Scalebar = 200 μ m.

5.2 Results

To quantify the effects of the longitudinal TI protocol on the amyloidosis model of the AD pathology, I analysed the brain tissue of stimulated mice to determine the load of relevant functional biomarkers, as previously assessed in similar studies (Iaccarino et al., 2016; Martorell et al., 2019). These consisted of nine different antibodies (**Figure 5.2**) that measured four classes of effects: 1) amyloid- β levels (antibodies: mOC23, 12F4, CT695), 2) neuronal activation (c-Fos), 3) glial cell presence (Iba1, GFAP) and 4) neurogenesis markers (Ki67, DCX, SOX2).

I conducted a comprehensive analysis of the presence of all the markers in multiple relevant brain areas: hippocampal dentate gyrus (DG), CA1 and CA3, as well as the cortex directly above each hippocampus (**Figure 5.1C**). Data from both right (stimulated) and left (unstimulated) hemispheres was assessed to see whether the stimulation may have interhemispheric effects (as seen in the TI imaging data in **Chapter 3**) or whether the left hemisphere can be used as a control region.

5.2.1 Effects of longitudinal TI stimulation on amyloid levels

First, I investigated the effects of different TI stimulation protocols on amyloid levels, the most prominent marker of the pathology present in the App^{NL-G-F} mice.

Encouragingly, (Iaccarino et al., 2016) have previously found that prolonged gamma stimulation was able to reduce the number and the size of A β plaques, as well as the levels of A β_{40} and A β_{42} .

The levels of mOC23, which stains a subset of A β plaques, preferentially targeting aggregated A β , were measured as the percentage of the area covered by the staining. While no significant differences between sham and other stimulation groups were observed (**Figure 5.3**), there was a trend in the reduction of amyloid area for the delta group in the right (stimulated) DG (mean $\pm$ SD: 3.17 $\pm$ 0.69 % for Sham vs 2.38 $\pm$ 0.79 % for Delta), CA1 (4.08 $\pm$ 0.49 % vs 3.59 $\pm$ 0.35 %) and CA3 (3.88 $\pm$ 0.53 % vs 3.22 $\pm$ 0.64 %) and the left DG (3.01 $\pm$ 0.75 % vs 2.45 $\pm$ 0.97 %) relative to the sham group (statistics in **Table 5.1**).

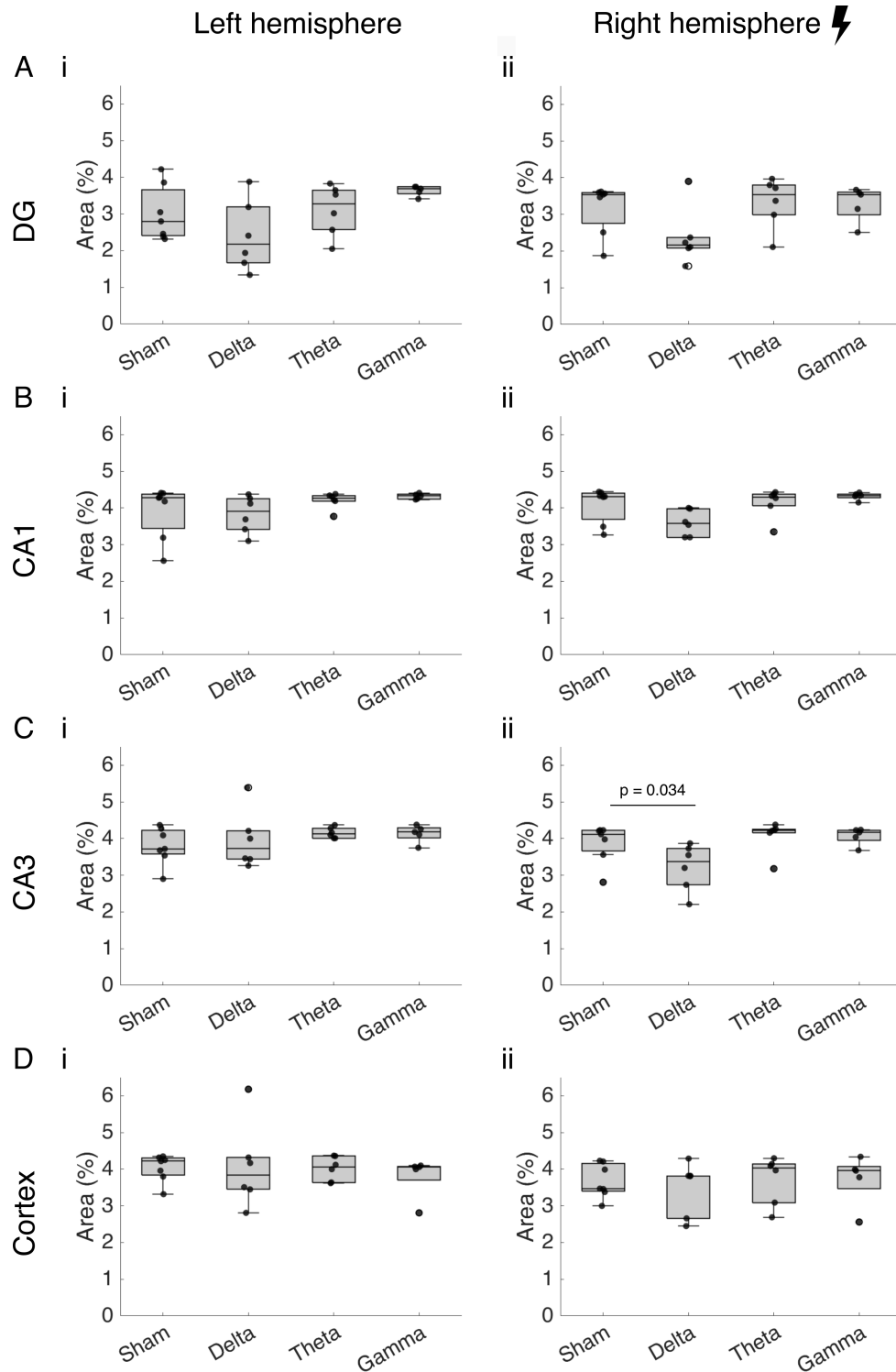


Figure 5.3 | Stimulation effects on the amyloid-β plaque density, measured by % of ROI area covered by the mOC23 staining. Delta stimulation shows potential trends in the reduction of levels of Aβ plaques in left and right dentate gyrus (DG), right CA1, and right CA3, although the effects are not significant. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.1**.

Table 5.1 | Statistical analysis details related to Figure 5.3.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.228	0.820	0.102	0.455	0.820	0.305				7	6	6	5
Right DG	Wilcoxon rank-sum test	0.181	0.534	0.848	0.542	1.000	1.000				7	6	6	5
Left CA1	Wilcoxon rank-sum test	0.445	0.836	0.465	1.000	1.000	1.000				7	6	6	5
Right CA1	Wilcoxon rank-sum test	0.070	0.976	0.563	0.210	1.000	1.000				7	6	6	5
Left CA3	Two-sample t-test	0.658	0.122	0.195	0.658	0.366	0.391				7	6	6	5
Right CA3	Wilcoxon rank-sum test	0.034	0.246	0.528	0.101	0.492	0.528				7	6	6	5
Left Cortex	Wilcoxon rank-sum test	0.655	0.945	0.432	1.000	1.000	1.000				7	6	6	5
Right Cortex	Wilcoxon rank-sum test	0.808	0.945	0.788	1.000	1.000	1.000				7	6	6	5

12F4 antibody, which binds to the A β ₄₂ peptide species but not the A β ₄₀ one, showed similar but non-significant trends between sham and delta groups, suggesting a potential reduction in the 12F4 area covered in the left DG (mean $\pm$ SD: 2.27 $\pm$ 0.77 % for Sham vs 1.48 $\pm$ 0.52 % for Delta), right DG (2.14 $\pm$ 0.78 % vs 1.39 $\pm$ 0.77 %) and right CA3 (2.69 $\pm$ 0.81 % vs 2.02 $\pm$ 0.67 %) (**Figure 5.4**; statistics in **Table 5.2**).

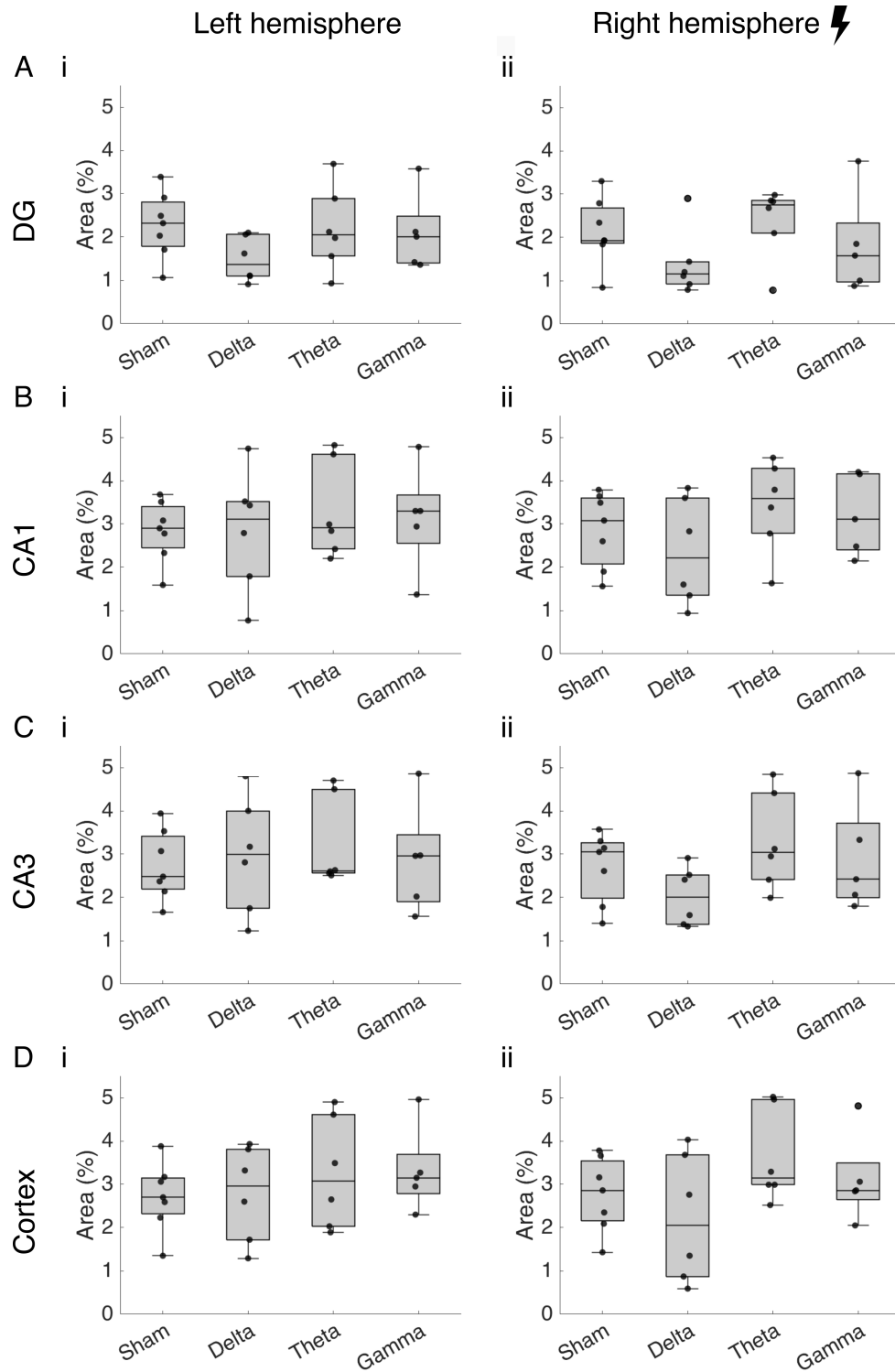


Figure 5.4 | Stimulation effects on the Aβ₄₂ density, measured by % of ROI area covered by the 12F4 staining. Delta stimulation shows potential trends in the reduction of levels of Aβ₄₂ in left and right dentate gyrus, and right CA3, although the effects are not significant. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.2**.

Table 5.2 | Statistical analysis details related to Figure 5.4.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.057	0.875	0.724	0.171	1.000	1.000				7	6	6	5
Right DG	Two-sample t-test	0.113	0.617	0.573	0.339	1.000	1.000				7	6	6	5
Left CA1	Two-sample t-test	0.998	0.375	0.601	1.000	1.000	1.000				7	6	6	5
Right CA1	Two-sample t-test	0.404	0.344	0.520	1.000	1.000	1.000				7	6	6	5
Left CA3	Wilcoxon rank-sum test	0.731	0.234	0.876	1.000	0.703	1.000				7	6	6	5
Right CA3	Two-sample t-test	0.138	0.292	0.738	0.415	0.585	0.738				7	6	6	5
Left Cortex	Two-sample t-test	0.901	0.366	0.259	0.901	0.777	0.777				7	6	6	5
Right Cortex	Two-sample t-test	0.422	0.135	0.518	0.844	0.406	0.844				7	6	6	5

Finally, I inspected the levels of CT695, the antibody that detects intracellular amyloid precursor protein, APP. No significant differences between the groups were observed apart from the increase in the theta group in the right DG that did not pass the multiple comparisons correction (mean $\pm$ SD: 0.1 $\pm$ 0.05 % for Sham vs 0.18 $\pm$ 0.07 % for Theta) (**Figure 5.5**; statistics in **Table 5.3**).

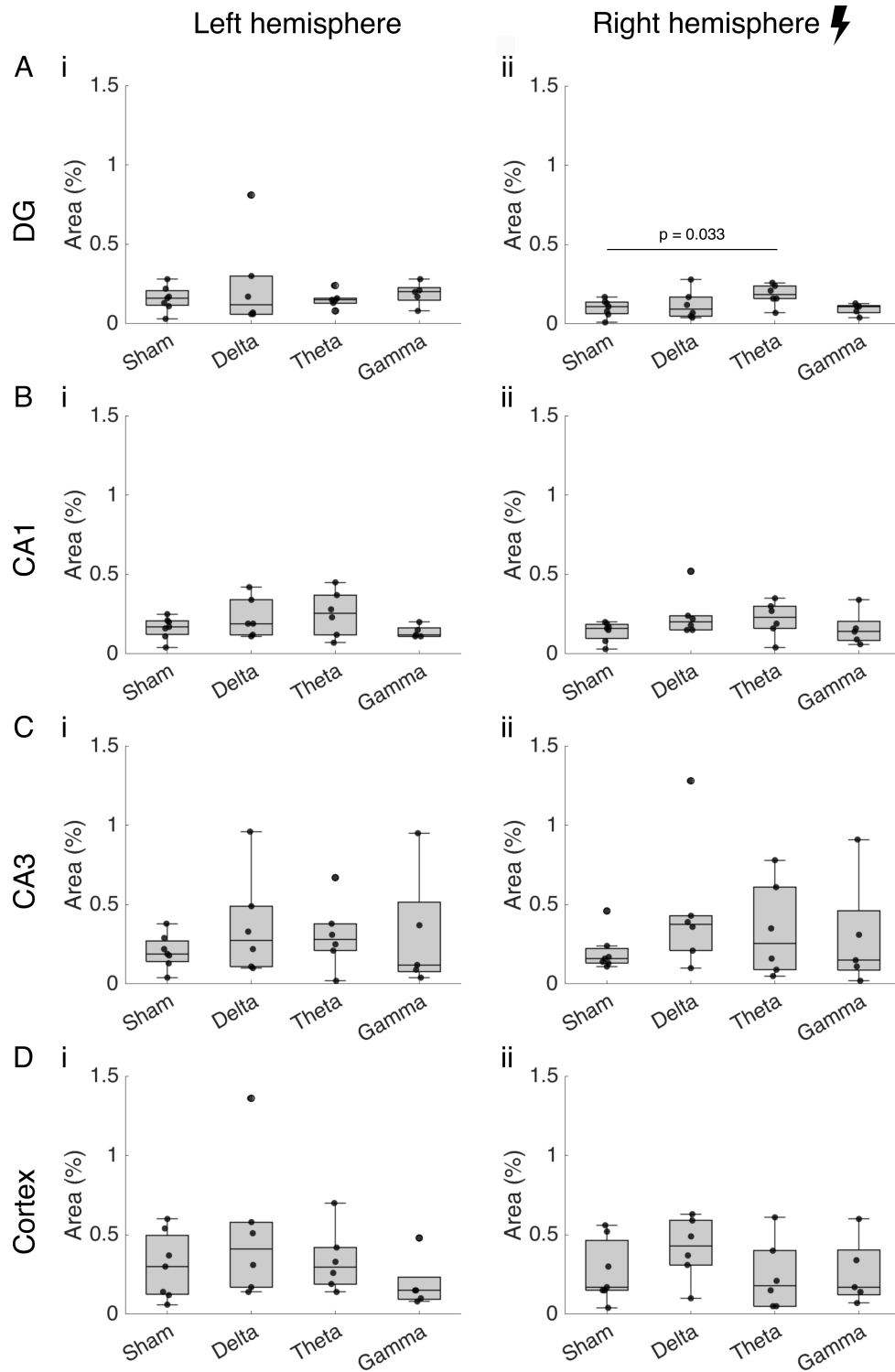


Figure 5.5 | Stimulation effects on the APP density, measured by % of ROI area covered by the CT695 staining. Theta stimulation shows potential trend in the increase of APP in the right dentate gyrus, although the effects are not significant. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.3**.

Table 5.3 | Statistical analysis details related to Figure 5.5.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.459	0.889	0.510	1.000	1.000	1.000				7	6	6	5
Right DG	Two-sample t-test	0.607	0.033	0.833	1.000	0.098	1.000				7	6	6	5
Left CA1	Two-sample t-test	0.258	0.169	0.490	0.516	0.506	0.516				7	6	6	5
Right CA1	Wilcoxon rank-sum test	0.178	0.169	0.783	0.507	0.507	0.783				7	6	6	5
Left CA3	Two-sample t-test	0.232	0.292	0.477	0.697	0.697	0.697				7	6	6	5
Right CA3	Wilcoxon rank-sum test	0.234	0.761	0.912	0.703	1.000	1.000				7	6	6	5
Left Cortex	Wilcoxon rank-sum test	0.386	0.655	0.508	1.000	1.000	1.000				7	6	6	5
Right Cortex	Two-sample t-test	0.216	0.834	0.961	0.649	1.000	1.000				7	6	6	5

5.2.2 Effects of longitudinal TI stimulation on neuronal activity levels

To assess whether the TI stimulation had lasting effect on neuronal activity levels, I quantified the levels of c-Fos, an immediate early gene marker induced by a broad range of stimuli and commonly used as a marker for neuronal activity (Joo et al., 2015). Mice that underwent the prolonged Delta stimulation paradigm displayed a marked decrease in their c-Fos levels (**Figure 5.6**) in almost all the analysed ROIs, with significantly reduced responses observed in the left and right DG, left CA1, and both cortices; right CA1 and left CA3 did not pass multiple comparisons correction (statistics in **Table 5.3**). This matches the previously observed reduction in hyperexcitability as measured by the magnitude of the visually evoked responses (**Figure 4.5**) and a potential trend towards decrease in spontaneous activity (**Figure 4.3**) in the App^{NL-G-F} mice reported in **Chapter 4**. The spatial results reported here indicate that the possible dampening effect may include widespread brain regions rather than just the stimulation area, or that the stimulation area was much larger than expected.

Additionally, two mice in the theta group and one mouse in the gamma group showed much higher levels of the c-Fos staining, especially in the DG areas, than the rest of their respective groups.

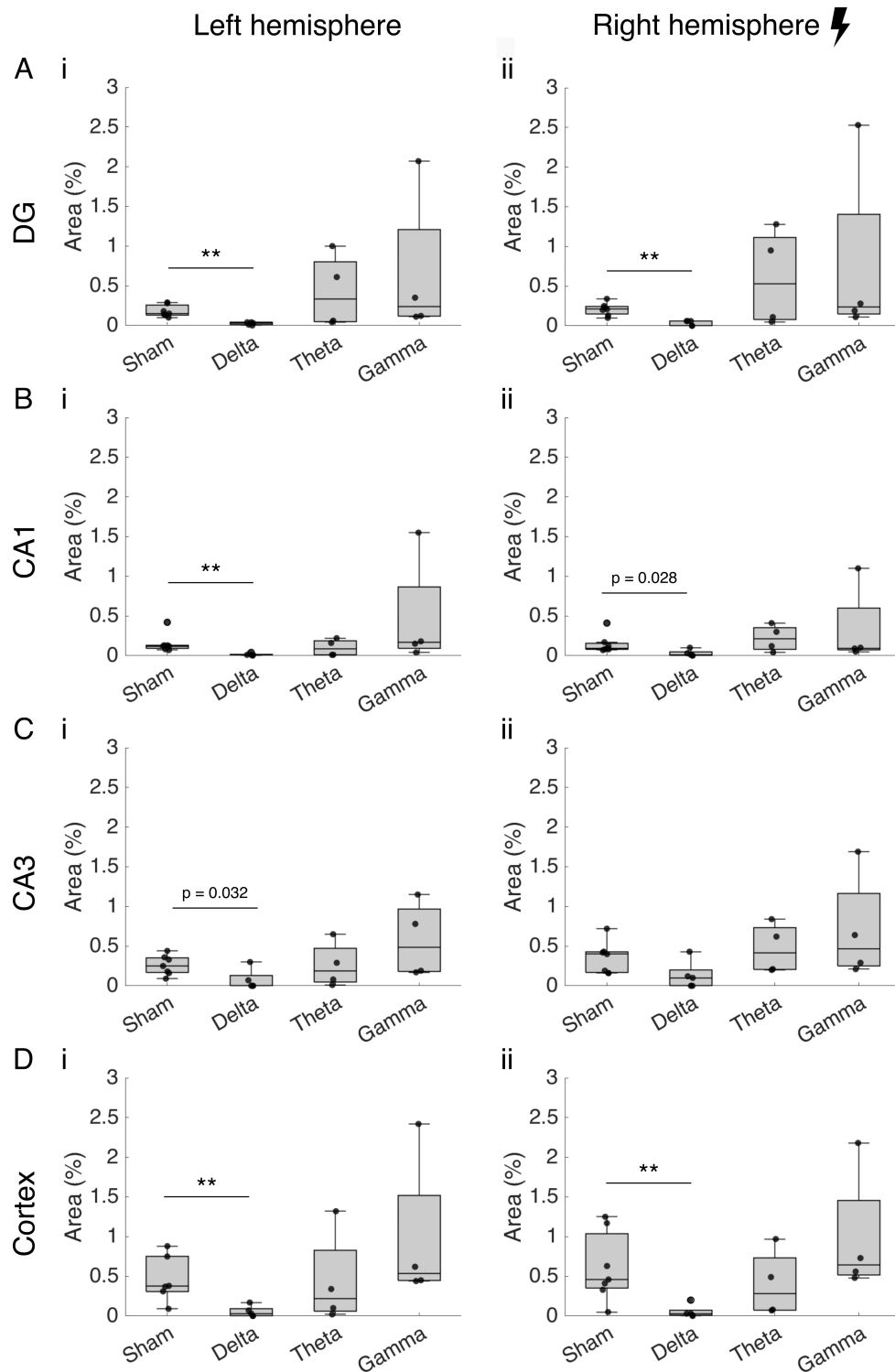


Figure 5.6 | Stimulation effects on the neuronal activation density, measured by % of ROI area covered by the c-Fos staining. Delta stimulation induced reduction in neuronal activity levels as measured with the c-Fos marker in almost all ROIs. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 4-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.4**.

Table 5.4 | Statistical analysis details related to Figure 5.6.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Wilcoxon rank-sum test	0.003	1.000	0.788	0.008	1.000	1.000	**			7	5	4	4
Right DG	Wilcoxon rank-sum test	0.003	0.927	0.788	0.008	1.000	1.000	**			7	5	4	4
Left CA1	Wilcoxon rank-sum test	0.003	0.776	0.376	0.008	0.776	0.752	**			7	5	4	4
Right CA1	Wilcoxon rank-sum test	0.028	0.648	0.976	0.083	1.000	1.000				7	5	4	4
Left CA3	Two-sample t-test	0.032	0.993	0.123	0.096	0.993	0.245				7	5	4	4
Right CA3	Two-sample t-test	0.076	0.483	0.219	0.227	0.483	0.439				7	5	4	4
Left Cortex	Wilcoxon rank-sum test	0.009	0.610	0.257	0.026	0.610	0.514	**			6	5	4	4
Right Cortex	Wilcoxon rank-sum test	0.005	0.648	0.315	0.015	0.648	0.630	**			7	5	4	4

5.2.3 Effects of longitudinal TI stimulation on glial cells

Glial cells support the functioning of neurons, with microglia serving important immune response functions in the brain and astrocytes involved in the inflammatory responses following injury and the regulation of blood flow in cerebral vasculature (Freeman & Rowitch, 2013). Previous gamma neuromodulation research showed that reduction in amyloid- β load following the stimulation protocol was also associated with increased microglia reactivity as measured by reduced microglia process length, increased levels of microglia colocalization with A β plaques and increased microglia cell body diameter, suggestive of plaque engulfment (Iaccarino et al., 2016). Gamma stimulation was also linked to increase in the number of astrocytes, potentially related to vascular clearance of A β (Martorell et al., 2019).

The longitudinal TI stimulation protocol resulted in the significant increase in the microglia density, as captured with the Iba1 marker, in the right DG of the theta group and an almost significant ($p = 0.045$; did not pass multiple comparisons correction) increase in the same region of the gamma group (**Figure 5.7**). Similar, although weaker, trends can be seen in the left DG (full statistics in **Table 5.5**).

Size of the microglia was measured as an approximate diameter of each particle detected (**Figure 5.1**) but no significant differences were found between sham and the stimulated groups. Only a weak potential increase in microglial size ($p = 0.048$; did not pass multiple comparisons correction) was detected in the left DG of the gamma group.

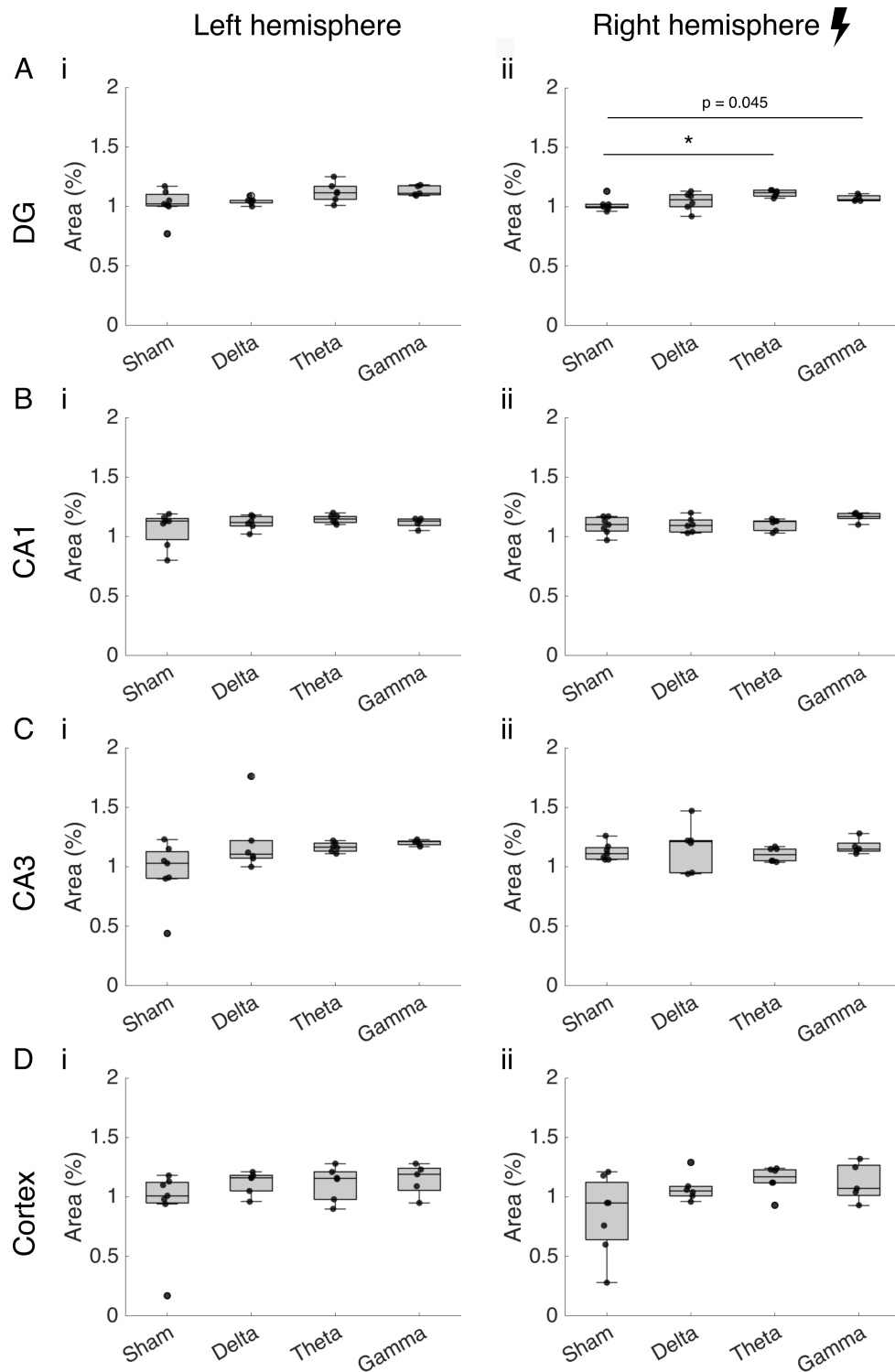


Figure 5.7 | Stimulation effects on the microglia density, measured by % of ROI area covered by the Iba1 staining. Theta stimulation induced increased microglia levels in the right dentate gyrus and gamma stimulation shows the same potential trend, although the effect is not significant. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.5**.

Table 5.5 | Statistical analysis details related to Figure 5.7.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.648	0.129	0.094	0.648	0.283	0.283				7	6	6	5
Right DG	Wilcoxon rank-sum test	0.381	0.010	0.045	0.381	0.031	0.091		*		7	6	6	5
Left CA1	Wilcoxon rank-sum test	0.867	0.341	0.909	1.000	1.000	1.000				7	6	6	5
Right CA1	Two-sample t-test	0.855	0.807	0.070	1.000	1.000	0.209				7	6	6	5
Left CA3	Two-sample t-test	0.119	0.080	0.065	0.194	0.194	0.194				7	6	6	5
Right CA3	Two-sample t-test	0.619	0.533	0.301	1.000	1.000	0.904				7	6	6	5
Left Cortex	Wilcoxon rank-sum test	0.146	0.248	0.149	0.437	0.437	0.437				7	6	6	5
Right Cortex	Two-sample t-test	0.138	0.062	0.119	0.237	0.186	0.237				7	6	6	5

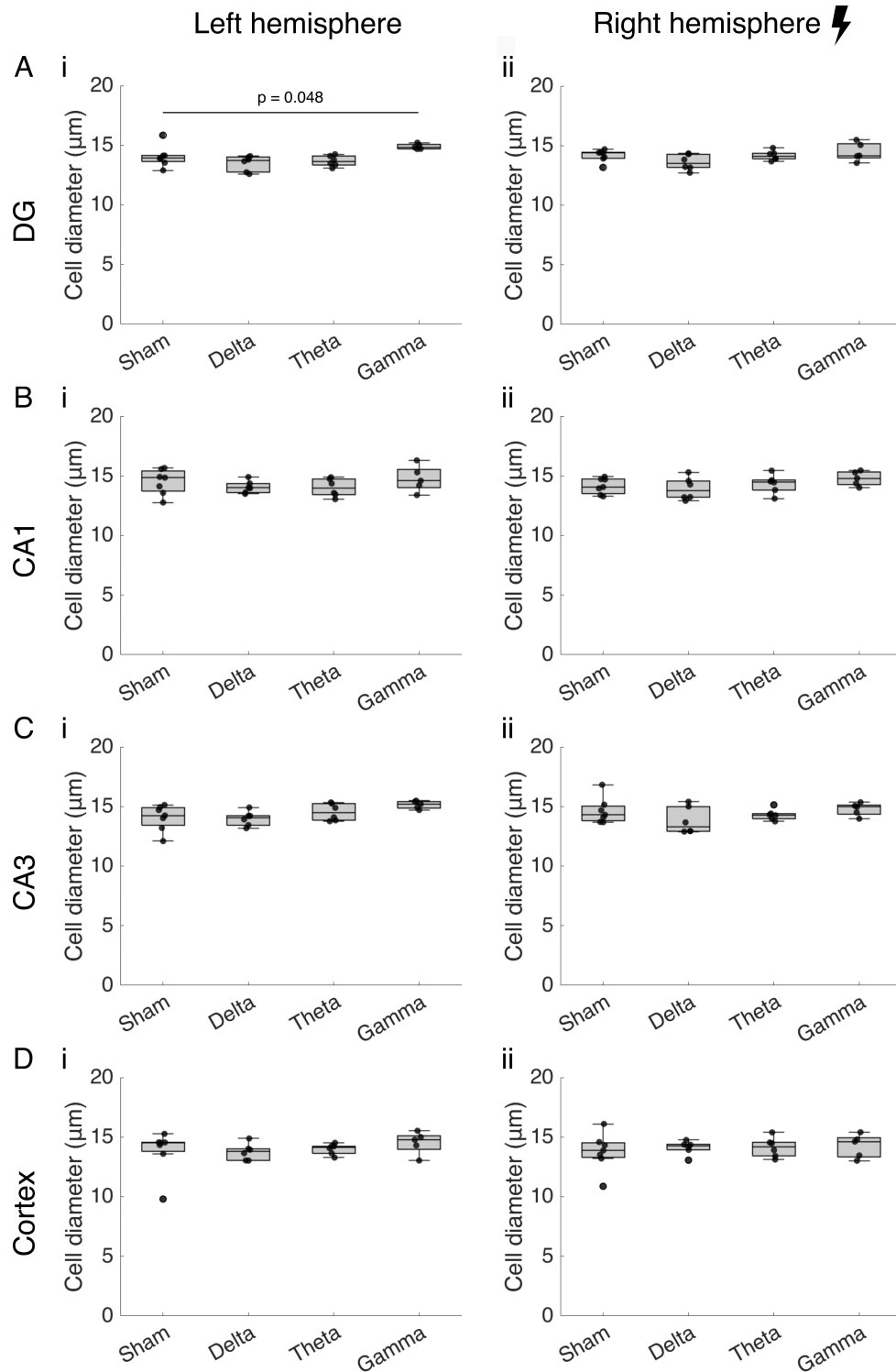


Figure 5.8 | Stimulation effects on the microglial size, measured as cell diameter of the Iba1 staining. No significant differences in the microglia size of the stimulated groups were observed although there is a potential trend in the left dentate gyrus of the gamma group. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.6**.

Table 5.6 | Statistical analysis details related to Figure 5.8.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Wilcoxon rank-sum test	0.234	0.445	0.048	0.469	0.469	0.144				7	6	6	5
Right DG	Two-sample t-test	0.115	0.967	0.398	0.344	0.967	0.797				7	6	6	5
Left CA1	Two-sample t-test	0.383	0.369	0.687	1.000	1.000	1.000				7	6	6	5
Right CA1	Two-sample t-test	0.614	0.663	0.121	1.000	1.000	0.364				7	6	6	5
Left CA3	Two-sample t-test	0.892	0.373	0.055	0.892	0.746	0.164				7	6	6	5
Right CA3	Two-sample t-test	0.209	0.517	0.798	0.626	1.000	1.000				7	6	6	5
Left Cortex	Wilcoxon rank-sum test	0.366	0.234	0.530	0.732	0.703	0.732				7	6	6	5
Right Cortex	Two-sample t-test	0.644	0.628	0.569	1.000	1.000	1.000				7	6	6	5

Similar quantification of the astrocytic presence, measured by the GFAP marker, showed no differences between the different stimulation groups (**Figure 5.9**).

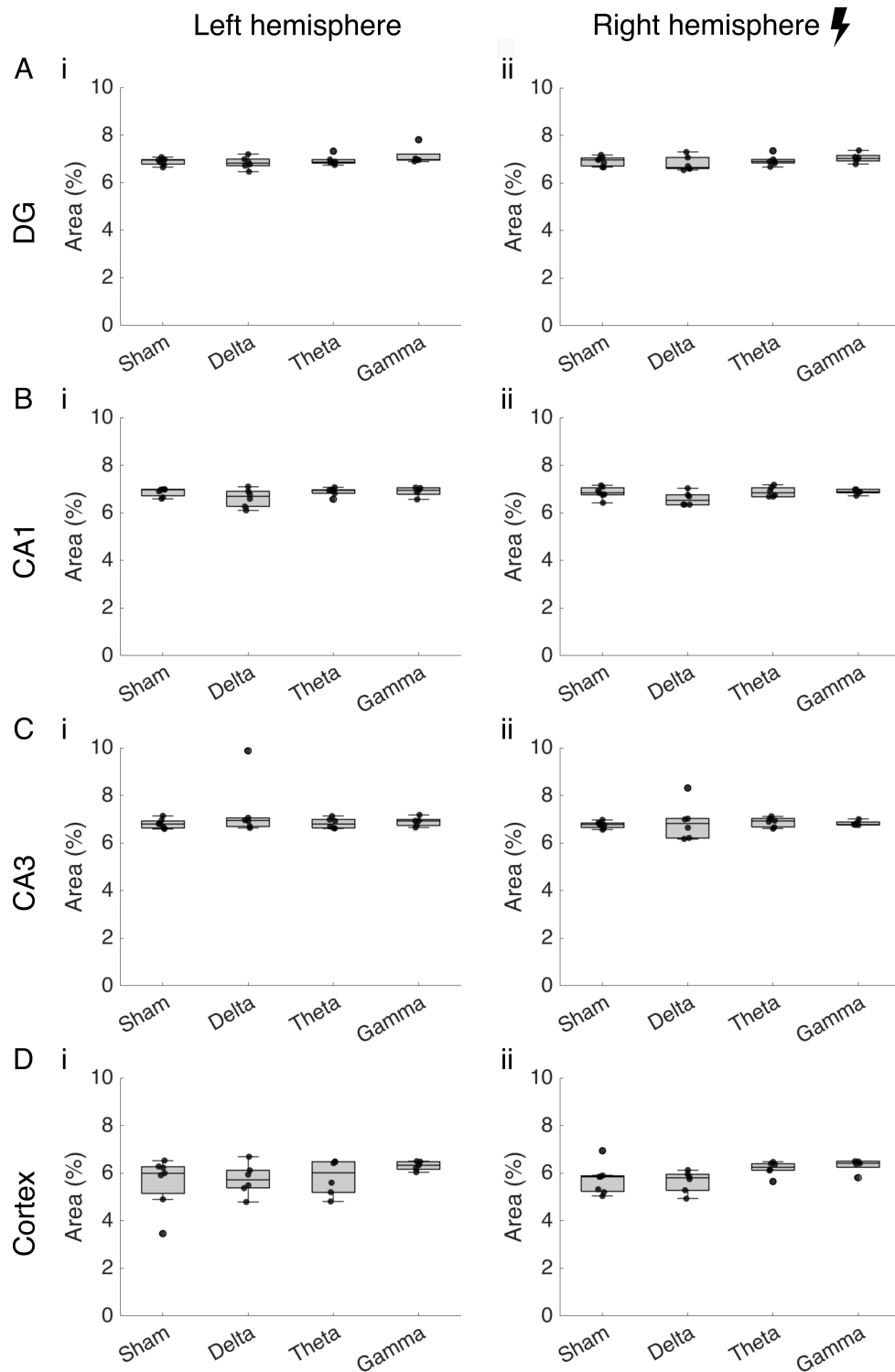


Figure 5.9 | Stimulation effects on the astrocyte density, measured by % of ROI area covered by the GFAP staining. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG (A), CA1 (B), CA3 (C) and Cortex (D) ROIs. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.7**.

Table 5.7 | Statistical analysis details related to Figure 5.9.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Wilcoxon rank-sum test	0.758	0.944	0.318	1.000	1.000	0.955				7	6	6	5
Right DG	Two-sample t-test	0.457	0.800	0.264	0.913	0.913	0.791				7	6	6	5
Left CA1	Two-sample t-test	0.156	0.831	0.799	0.467	1.000	1.000				7	6	6	5
Right CA1	Two-sample t-test	0.098	0.830	0.756	0.294	1.000	1.000				7	6	6	5
Left CA3	Wilcoxon rank-sum test	0.295	0.836	0.503	0.885	1.000	1.000				7	6	6	5
Right CA3	Two-sample t-test	0.667	0.254	0.384	0.768	0.763	0.768				7	6	6	5
Left Cortex	Wilcoxon rank-sum test	0.836	0.836	0.202	1.000	1.000	0.606				7	6	6	5
Right Cortex	Wilcoxon rank-sum test	0.915	0.094	0.139	0.915	0.283	0.283				7	6	6	5

5.2.4 Effects of longitudinal TI stimulation on neurogenesis

Dentate gyrus of the hippocampal structure is one of the two brain structures where the unique process of adult neurogenesis happens, resulting in the generation of new granule cells from stem cells (Seib & Martin-Villalba, 2015). Evidence suggests that some neurological diseases, including Alzheimer's Disease, impair adult hippocampal neurogenesis which may contribute to the disease symptoms (Toda et al., 2019; Moreno-Jiménez et al., 2019). Hence, a rescue of the neurogenesis processes may lead to improvements in the disease progression.

To test whether any of the stimulation paradigms can induce changes in the neurogenesis in DG, I tested the tissue for the presence of three neurogenesis antibodies: Ki67, a neural stem cell proliferation marker, DCX, a neural stem cell differentiation marker, and Sox2, a less specific stem cell pluripotency renewal marker (Hagihara et al., 2019). Neurogenesis is limited to the DG area of the hippocampus; therefore, this analysis was limited to the DG regions.

Ki67 was increased in both right and left dentate gyri of the theta group only. The effect was significant only in the right DG (mean $\pm$ SD: 0.016 ± 0.018 % for Sham vs 0.090 ± 0.110 % for Theta), which was the one directly targeted (**Figure 5.10**). This effect may have been particularly strong due to a presence of one highly activated mouse, but similar trend is also visible in the left DG (0.019 ± 0.019 % vs 0.067 ± 0.055 ; statistics in **Table 5.8**).

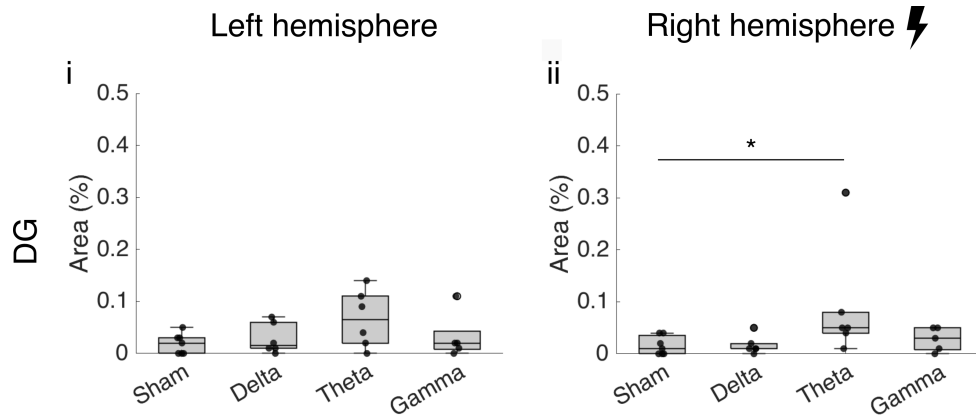


Figure 5.10 | Stimulation effects on the neural stem cell proliferation density, measured by % of ROI area covered by the Ki67 staining. Theta stimulation induced increase in cell proliferation in the right dentate gyrus and smaller, non-significant increase in the left DG. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG ROIs. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.8**.

Table 5.8 | Statistical analysis details related to Figure 5.10.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Wilcoxon rank-sum test	0.598	0.124	0.924	1.000	0.371	1.000				7	6	6	5
Right DG	Wilcoxon rank-sum test	0.744	0.016	0.288	0.744	0.049	0.576		*		7	6	6	5

Further confirmation of potential neurogenesis effects was also observed in the DCX staining which showed a clear increase in the level of staining in both the right and the left DG in the theta group (**Figure 5.11**).

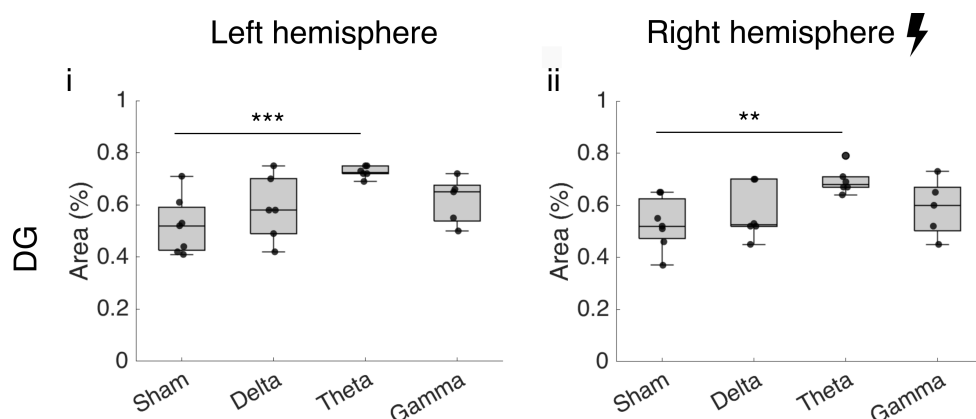


Figure 5.11 | Stimulation effects on the neural stem cell differentiation density, measured by % of ROI area covered by the DCX staining. Theta stimulation induced increase in cell differentiation in both right and left dentate gyri. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG ROIs. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per

group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.9**.

Table 5.9 | Statistical analysis details related to Figure 5.11.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.326	0.001	0.140	0.326	0.003	0.280		***		7	6	6	5
Right DG	Two-sample t-test	0.497	0.004	0.347	0.695	0.012	0.695		**		7	6	6	5

Assessing the last, and least specific (Hagihara et al., 2019), neurogenesis marker, SOX2, showed no differences in the levels detected in any of the stimulation versus the sham group (**Figure 5.12**). This may suggest that, while there was an increase in stem cell proliferation and differentiation, the overall number of stem cells present remained consistent within the stimulation period (8 stimulation days within the total period of 11 days).

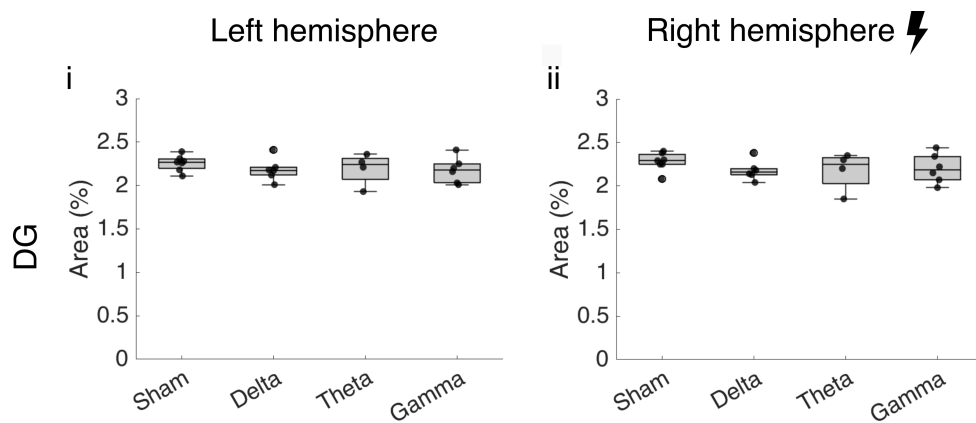


Figure 5.12 | Stimulation effects on the neural stem cell pluripotency renewal density, measured by % of ROI area covered by the SOX2 staining. Theta stimulation induced increase in cell differentiation in both right and left dentate gyri. Summary of all data for ROIs in the left (i) and the right, stimulated, (ii) hemisphere for DG ROIs. No significant differences were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.10**.

Table 5.10 | Statistical analysis details related to Figure 5.12.

ROI	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Left DG	Two-sample t-test	0.382	0.448	0.431	1.000	1.000	1.000				7	5	4	4
Right DG	Two-sample t-test	0.201	0.317	0.735	0.604	0.635	0.735				7	5	4	4

5.2.5 6-month-old App^{NL-G-F} mice did not display spatial memory deficits

The second assessment of the stimulation efficacy involved behavioural testing of spatial memory. Object Location Memory (OLM) task and Y-maze were performed to test the long-term spatial memory and spatial working memory, respectively. They were chosen as simple pilot tasks, based on the previous studies (Etter et al., 2019; Saito et al., 2014) reporting the decreased performance of J20 and App^{NL-G-F} mice at 6 months of age. The aim of the behavioural testing reported here was to assess whether prolonged hippocampal TI stimulation can improve spatial memory in the App^{NL-G-F} mice.

The OLM tasks consisted of the habituation, training and test phase completed on three consecutive days (**Figure 5.13A**). First, I measured the time the mice explored the two objects during the training day to ensure all mice were sufficiently engaged in the task. I found that all mice explored the objects for longer than the minimum acceptable time (20 s) and that there were no differences in the baseline behaviour between the groups (**Figure 5.13B**). The Discrimination Index (DI) was used to assess the preference of mice to explore object A versus object B during the training and the test days. During the training day, the DI for all stimulated groups was the same as for the sham group and not different from chance level, indicating mice explored both objects equally (**Figure 5.13C**). During the test day, the cognitively unaffected mice are expected to show preference for the object that was moved, i.e. object A. No clear difference in performance was detected between the sham vs the stimulated groups (**Figure 5.13D**). The gamma group displayed a potentially worse performance than the sham group (non-significant; did not pass multiple comparisons correction: $p = 0.024$). Additionally, only the sham group performed above the chance level in the test (statistics in **Table 5.11** and **Table 5.12**).

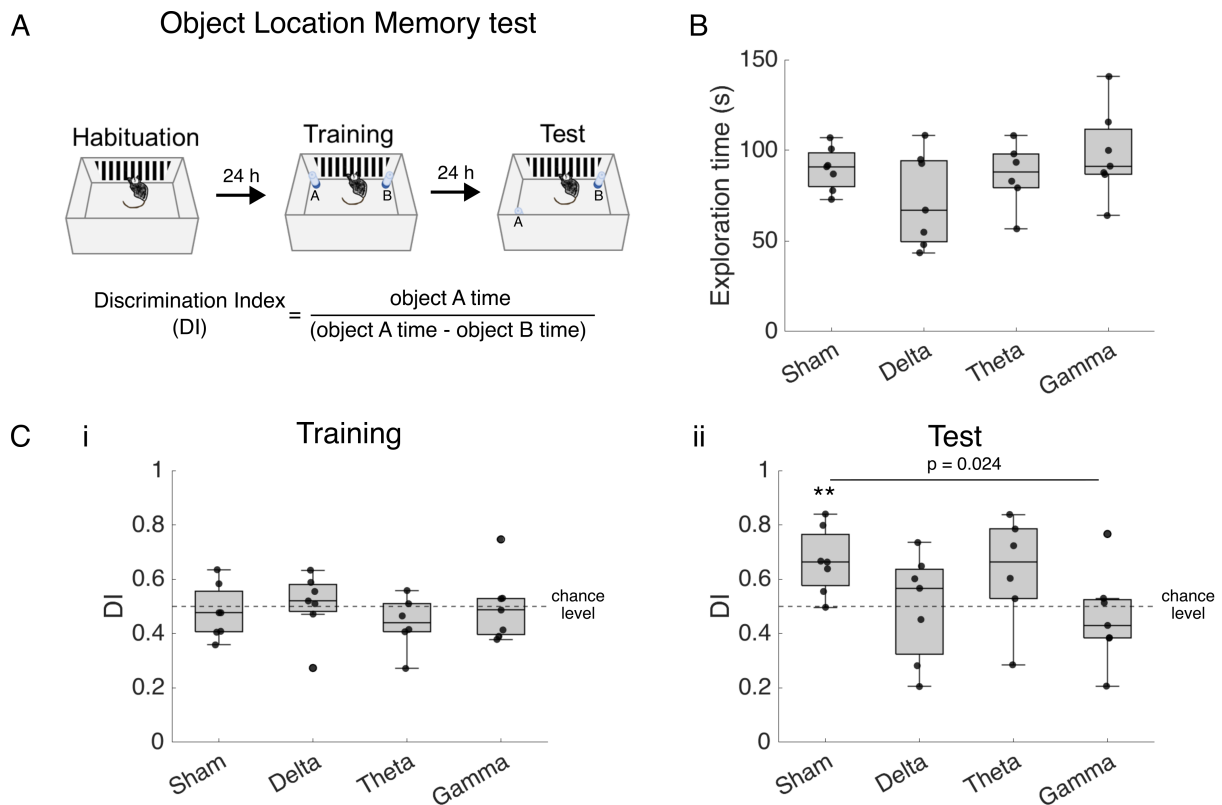


Figure 5.13 | 6-month-old App^{NL-G-F} mice showed no decrease in the performance in the OLM task. A. Schematic of the Object Location Memory (OLM) test. **B.** All experimental groups explored the open field equally. **C.** All experimental groups visited the objects equally in the training phase of the task. **D.** All stimulated groups performed similarly to the sham group in the test phase. Gamma stimulation shows a trend in worsening of the performance, although the effect is not significant. Stimulated groups (Delta, Theta, Gamma) did not perform above the chance level. No significant differences between the groups were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.11** and **Table 5.12**.

Table 5.11 | Statistical analysis details related to Figure 5.13; comparison to Sham (S).

Measure	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
Expected time	Two-sample t-test	0.140	0.699	0.436	0.419	0.871	0.871				7	7	6	7
Training DI	Two-sample t-test	0.617	0.488	0.767	1.000	1.000	1.000				7	7	6	7
Test DI	Two-sample t-test	0.079	0.683	0.024	0.159	0.683	0.072				7	7	6	7

Table 5.12 | Statistical analysis details related to Figure 5.13; comparison to chance level.

Measure	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
		Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma
Training DI	One-sample t-test	0.712	0.436	0.907	0.530	1.000	1.000	1.000	1.000					7	7	6	7
Test DI	One-sample t-test	0.006	0.507	0.093	0.722	0.023	1.000	0.278	1.000	**				7	7	6	7

Y-Maze task focuses on the short-term spatial working memory, testing whether the animal remembers the sequence of previous behaviours. The common metrics quantify whether the mouse evenly alternates the arms it enters (Spontaneous Alternation Performance, SAP, **Figure 5.14Ai**), keeps alternating between two of the three arms (Alternate Arm Returns, AAR, **Figure 5.14Bi**) or keeps returning back to the same arm (Same Arm Returns, SAR, **Figure 5.14Ci**). All experimental groups performed above the chance level in the SAP metric, showing no deficit in the performance (**Figure 5.14Aii**) from expected level. There was also no difference in performance observed between sham and other experimental groups in all of the metrics quantified, apart from a small, non-significant decrease in the SAR measure for the theta group ($p = 0.019$; did not pass multiple comparisons correction).

Overall, the performance of the sham group suggests that the 6-month-old App^{NL-G-F} mice do not have a deficit in the long-term spatial memory (OLM test) or the spatial working memory (Y-maze). As such, the tests were not best suited to assess whether hippocampal stimulation can rescue memory deficits.

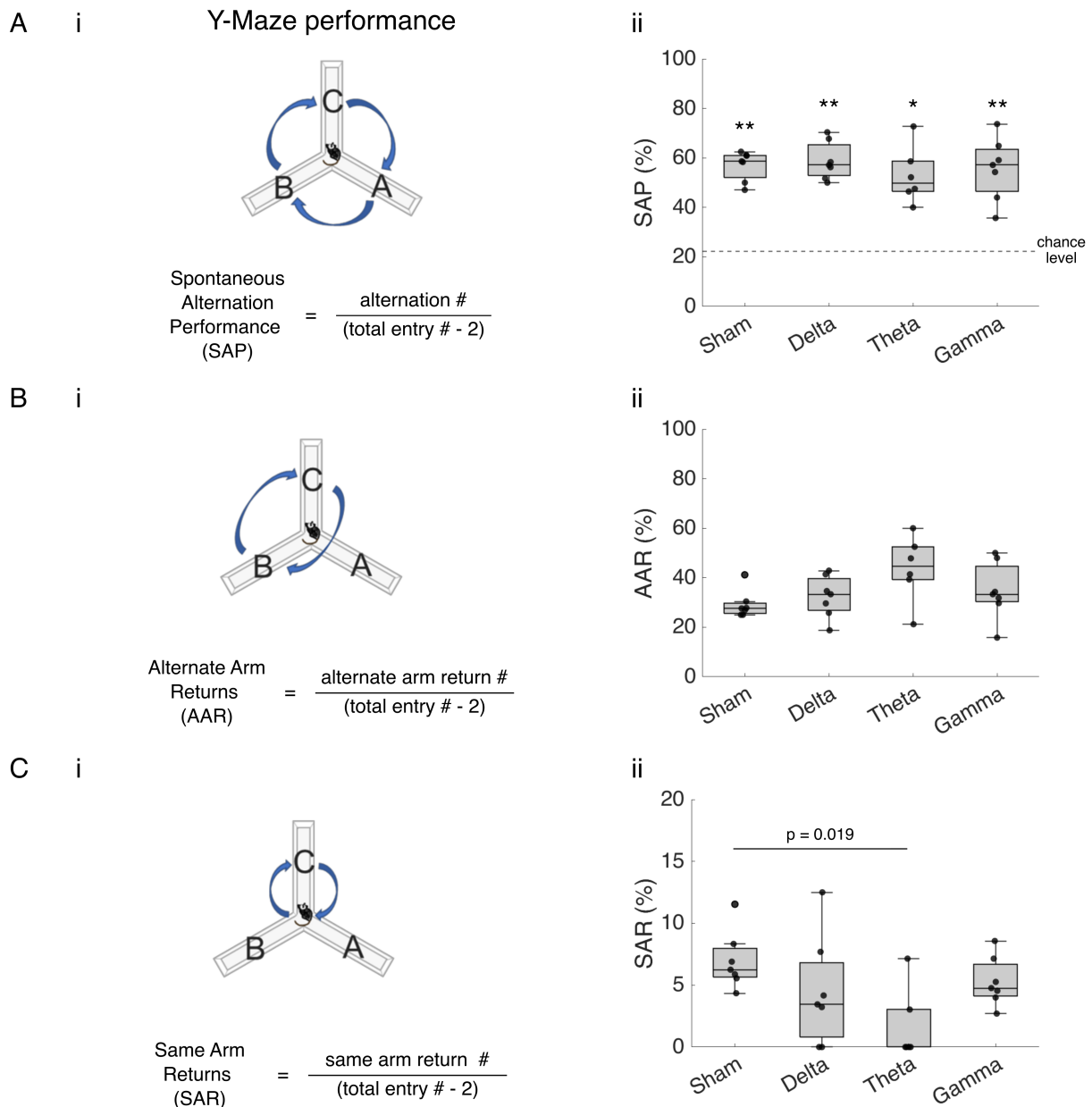


Figure 5.14 | 6-month-old $\text{App}^{\text{NL-G-F}}$ mice showed no decrease in the performance in the Y-maze task. **A. i.** Schematic of the Y-maze and the quantification of the SAP performance metric. **ii.** All experimental groups alternated between the three arms equally and above the chance level. **B. i.** Quantification of the AAR performance metric. **ii.** All experimental groups alternated between pairs of arms equally. **C. i.** Quantification of the SAR performance metric. **ii.** All experimental groups returned to the same arm at equal rates. Theta group shows a potential reduction in the SAR compared to sham, although the effect is not significant. No significant differences between the groups were observed. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. $n = 5-7$ mice per group, all tests corrected for multiple comparisons (Bonferroni-Holm), for statistical details see **Table 5.13** and **Table 5.14**.

Table 5.13 | Statistical analysis details related to Figure 5.14; comparison to Sham (S).

Measure	Test	p			Bonferroni-Holm p			Significance level			n (animals)			
		Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Delta v S	Theta v S	Gamma v S	Sham	Delta	Theta	Gamma
SAP	Wilcoxon rank-sum test	0.977	0.314	0.805	1.000	0.941	1.000				7	7	6	7
AAR	Wilcoxon rank-sum test	0.301	0.070	0.121	0.301	0.210	0.242				7	7	6	7
SAR	Wilcoxon rank-sum test	0.122	0.019	0.209	0.244	0.056	0.244				7	7	6	7

Table 5.14 | Statistical analysis details related to Figure 5.14; comparison to chance level.

Measure	Test	p				Bonferroni-Holm p				Significance level				n (animals)			
		Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma	Sham	Delta	Theta	Gamma
SAP	Wilcoxon signed-rank test	0.008	0.008	0.016	0.008	0.031	0.031	0.031	0.031	**	**	*	**	7	7	6	7

5.3 Discussion

This early-evidence study is the first one to investigate the effects of longitudinal, 8-day-long, hippocampal TI stimulation at the disease pathology in the App^{NL-G-F} Alzheimer's Disease model. For this set of experiments, I developed a new stimulation setup where mice were stimulated while freely moving in a cage-like environment, with the stimulation target set as the right hippocampus. Multiple stimulation frequencies were tested to verify previously hypothesised effects: the delta (1 Hz) frequency, shown to reduce hyperexcitability in **Chapter 4**, the theta frequency (8 Hz), previously demonstrated to enhance learning (Violante et al., 2023; Wessel et al., 2023), and the gamma frequency (40 Hz), reported to and engage microglial immune response and decrease the A β density in AD models (Iaccarino et al., 2016; Martorell et al., 2019; Adaikkan et al., 2019).

In this study, I found that only the delta stimulation showed potential trends in the reduction of A β plaques and A β_{42} levels. The results were not significant; however, given the small sample size (n = 5-7 per group), the fact that most of the effect was localised in the right, stimulated hippocampus, and the two related antibodies showed similar trend, it is likely that the effect is underpowered and could be confirmed by a larger sample size. The levels of APP remained unchanged in all groups, except for a slight elevation present in the right DG in the theta group. Further increase in the sample size and additional correlational analysis with other increased biomarkers, such as microglia or neurogenesis markers, would help determine whether this effect is sufficiently strong and caused by the stimulation.

The neuronal activation, as measured by a proxy of the level of c-Fos expressed 60 minutes after the last stimulation session, indicated a strong reduction in overall activity levels in almost all ROIs in the delta group alone. This effect confirms previous results reported in **Chapter 4**, showing that the delta stimulation protocol is able to reduce hyperexcitability and perhaps might even reduce spontaneous activity levels in the App^{NL-G-F} model. The number of mice in the delta group for this staining is relatively low (n = 5) and 2 out of these belonged to the cohort that had some potential issues with the tissue processing (details in section **2.12.1**). While the tissue of the mice included in this chapter seemed to be stained properly, it is possible that the resulting staining was affected. Further increase in the sample size, and comparison to the tissue collected in **Chapter 4** experiments, will be crucial to verify the validity of this result.

The effect of the TI stimulation on the microglia was small, with no differences observed in the astrocytic density, and an increase in microglial density observed in the right DG in the theta group and potentially in the gamma group (although not significant). Larger group size and further, more detailed analysis would help verify these findings. For example, (Iaccarino et al., 2016) found that microglial cell body size was increased, the microglial process length was lower, and microglial colocalization with A β plaques was higher following the gamma stimulation. Higher resolution imaging at multiple depths should be performed for reliable quantification of such biomarkers.

Interestingly, the theta stimulation group was the only group to display a strong increase in neurogenesis, as evidenced by the higher rate of neural stem cell proliferation and differentiation in the DG hippocampal regions. This is potentially a highly relevant result, as Alzheimer's Disease can impair adult neurogenesis which might lead to some of its symptoms (Toda et al., 2019; Moreno-Jiménez et al., 2019). However, abnormal neurogenesis has also been linked to hippocampal seizures, which ultimately lead to neuronal stem cell death (Fu et al., 2019). Similarly to human AD patients (Vessel et al., 2016), animal models of amyloidosis are known to exhibit increased epileptic activity (Minkeviciene et al., 2009; E. C. B. Johnson et al., 2020), with the origin linked to hyperactivity caused by soluble A β (Busche et al., 2012; Minkeviciene et al., 2009; Zott et al., 2019) or APP (Born et al., 2014; E. C. B. Johnson et al., 2020). Increased c-Fos expression in two mice of the theta and one from the

gamma group could be an indication of such subclinical epileptic activity. Further investigation into the nature of the neurogenesis effects observed is necessary to ensure the TI theta stimulation is not actually detrimental to the disease progression.

Both of the behavioural tests chosen, the object location memory and the Y-maze, turned out to be inadequate to test the effects of TI stimulation. Despite some previous literature suggesting mice models of AD may show behavioural deficits in these tests (Saito et al., 2014; Etter et al., 2019), I found that the sham mice performed above chance levels in both tasks. The stimulation had no effect on the performance of the other groups when compared with the sham group, except for slightly worse performance in the OLM test, especially in the gamma group (not significant but also not above the chance level). While the two tests were chosen based on their simplicity, they were not adequate to capture the AD pathology effects at this age point in the App^{NL-G-F} model. Behavioural tests are notoriously difficult to replicate, with subtle effect sizes (Latif-Hernandez et al., 2019), leading to conflicting studies reported. For example, contrary to other studies, (Whyte et al., 2018) showed no decrease in the performance in the Y-maze, a non-significant decrease in the novel object recognition and a reduction in the open field exploration in App^{NL-G-F} mice. (Kundu et al., 2021), on the other hand, demonstrated reduced performance of only female App^{NL-G-F} mice in the Y-maze. Small sample group (n=6-7) and mixed sex of mice used in this study were both additional sources of variability in the results reported here. Finding robust behavioural tests for this relatively new AD model will be crucial to examining the behavioural effects of TI stimulation in future experiments, with emerging literature starting to identify stronger behavioural deficits (Mehla et al., 2019; Kundu et al., 2021; Saifullah et al., 2020).

These results do not confirm the effect of the gamma stimulation reported previously (Iaccarino et al., 2016). No significant decrease in any of the amyloid markers was observed in this study and the microglial effect detected was only minor. Differences in the design and execution of the two studies could perhaps account for some of these discrepancies. One key difference is the AD models used in the two studies, with transgenic 5XFAD and APP/S1 mice used in (Iaccarino et al., 2016). Other differences pertained to tissue processing and subsequent analysis. Here, I analysed brightfield images of large (1300 x 500, 1300 x 400 or 800 x 600 μ m) ROIs as opposed to much smaller ROIs (~250 x 250 μ m). This approach has the advantage of being

more unbiased in the quantification of the effects, not being limited to a small, arbitrarily chosen region within each structure. However, it comes at the cost of reduced resolution, with the images used in this study acquired at 20x rather than 40x magnification, and a much smaller slice width of 7 μm imaged at a single plane versus a 40 μm slice imaged as a stack. This limits the potential to analyse the morphology of the cells such as microglia, as many cells cannot be fully captured within a single imaging plane. This also informed the choice of % area covered by the antibody as the main quantification metric used, as opposed to standard counting of the number of cells; this is because counting is more likely to be biased if multiple pieces of the same cell are visible in a single imaging plane. Further ongoing work for this project includes immunofluorescent co-staining of amyloid and microglia to quantify the co-localisation of the two cell types and further optimisation of both the imaging and analysis pipelines to make it more sensitive to subtler effects.

Despite these limitations, it was the delta and not the gamma group that showed a potential trend in the decrease of the amyloid levels. This, along with the reduction in neuronal activation levels, is a promising result that needs to be further investigated. Combined with the imaging results reported in **Chapter 4**, it suggests the 1 Hz delta stimulation may have beneficial effects on the amyloidosis model of AD.

Another promising stimulation paradigm was the theta frequency which displayed increased presence of microglia in the stimulated DG, and, most notably, increased neurogenesis effects in both hippocampi. Future experiments will investigate the mechanism behind these effects to ensure they are beneficial rather than detrimental to the disease progression. One possibility will be to test the time-course of the neuronal stem cell proliferation with BrdU, a marker of stage S of the proliferation process only, at different time points throughout the stimulation protocol. Co-staining BrdU with mature neuronal markers (e.g. NeuN+) and cell death markers (e.g. CC3) should help establish the source of the neurogenesis effects observed. Establishing a behavioural paradigm that reliably captures memory deficits related to neurogenesis at the relevant age point is another essential step to test the relevance of the effects observed.

Surprisingly, the results from this study suggest that the delta and theta frequencies may have higher clinical relevance on the AD pathology than the gamma stimulation.

Further, more detailed analysis and experimental assessment is needed to verify these findings.

Finally, it is essential that follow-up studies investigate the transient effect of the stimulation on the neuronal activity in multiple brain regions. The electrode locations for hippocampal targeting here were chosen based on the previous studies using TI to target hippocampus (Miskey et al., 2020; Acerbo et al., 2022). However, future work must verify the focality of the stimulation obtained in the exact experimental setup used and correlate the neuronal activity with the spatial distribution of the biomarkers observed. Tracking neuronal activity will be essential to monitor the stimulated mice for any subclinical epileptic activity, to detect deficits in the neuronal activity in the specific frequency bands, and to demonstrate the direct relationship between the stimulation frequency, its influence on the neuronal activity and the resulting changes in the behavioural outcomes.

Chapter 6. General discussion

6.1 Overview

In this thesis, I present the first evidence of direct network-level effects of Temporal Interference stimulation *in vivo* (**Chapter 3**) and investigate the potential of applying TI to target aberrant neuronal activity in an Alzheimer's Disease model (**Chapter 4 and 5**).

Using widefield calcium imaging, I found that TI is able to drive neuronal activity at the difference frequency, Δf , of the applied fields (**Chapter 3**). The spatial distribution of the direct evoked TI response, unlike that of the control low frequency stimulation, was concentrated between the two stimulating electrodes, in agreement with the existing FEM models. This suggests that both targeting of deep brain structures and steerability could be possible. TI also induced a response in the contralateral hemisphere, suggesting the stimulation was able to recruit IT neurons located in deeper cortical layers.

The temporal profile of the TI-evoked dynamics revealed further differences from standard LF stimulation, potentially related to the mechanism of action of TI. Based on the presence of the harmonic frequency, $2\Delta f$, and the phase of the response profile, it is likely that TI, at the network level, involves additional nonlinear processes not seen at a single-cell level. This could involve the differential engagement of both excitatory and inhibitory circuits, with their relative balance depending on the stimulation modality and the applied strength. It could also be caused by the amplification of existing frequency mixing processes in the presence of kHz fields.

Stimulation with pure kHz fields induced onset responses which were attenuated by the use of higher kHz frequencies and longer current ramp up rates. No evidence of depolarisation block was observed. kHz fields were not found to affect sensory stimulation responses but possibly modulated responses to other electrical stimuli. Understanding the mechanism underlying this modulatory effect could shed light on the mechanism of action of TI.

Having established that TI can reliably drive neuronal activity at Δf , I explored its therapeutic potential in the next-generation App^{NL-G-F} model of Alzheimer's Disease

(**Chapter 4**). Short-term low frequency stimulation in the delta frequency range dampened neuronal excitability in App^{NL-G-F} mice at the early disease stage. The effects of the stimulation protocol on the spontaneous activity levels and the mechanism of action involved must be further investigated. However, this pilot study demonstrates that TI is a promising neuromodulation tool potentially capable of counteracting neural circuit dysfunctions present in AD.

Further testing of TI in longitudinal stimulation protocols targeting the hippocampus, the area earliest affected in AD, showed encouraging results as well (**Chapter 5**). Theta stimulation protocol was found to induce neurogenesis processes and increase the presence of microglia in the stimulated hippocampus. Additionally, the delta stimulation reduced the presence of neuronal activity markers, in the form of c-Fos expression, and led to non-significant but potential decreases in amyloid- β markers that need further investigation.

6.2 Key advances

The experiments presented in this thesis establish TI as a potent neuromodulation technique.

The first direct demonstration of network-level neuronal dynamics evoked by TI *in vivo* (**Chapter 3**) is an essential first step to determine the feasibility of using the technique to affect specific neuronal circuits. Importantly, calcium imaging was used to verify these effects, with the approach having multiple benefits over electrophysiological recordings. Imaging of neuronal activity leads to stimulation artefact-free recordings, overcoming one of the key challenges of the prior work. With electrophysiology, careful and often non-trivial signal filtering is needed to ensure the effects observed can be attributed to neuronal activity. On the other hand, other imaging approaches such as fMRI do not have sufficient temporal resolution to capture the dynamics of the response. This study is thus a crucial confirmation and extension of the previous work.

Similar characterisation of TI stimulation parameters was performed previously at the single-cell level (Luff et al., 2024b). This study demonstrated that individual neurons can oscillate at Δf in response to TI, even at the subthreshold level, linking it to the mechanism of frequency mixing. Based on the results reported here, including the emergence of the harmonic response, it is clear that the network-level interactions

may have more complex dynamics. Understanding the additional mechanisms emerging from these interactions is vital for successful application of TI to target circuit dysfunction.

Lastly, the comparison between spatial responses evoked by TI and the existing FEM models is another key outcome. The models are based on the assumption that the neurons respond most strongly in the area of maximum amplitude modulation, AM, of the two fields. This should allow for direct targeting of deeper brain structures by adjusting the relative position of the two electrode pairs on the scalp, and for steering of the stimulated area by adjusting the current ratio between them. Importantly, these FEM models have been employed to optimise the electrode positioning in recent studies in human participants (Wessel et al., 2023; Violante et al., 2023); establishing validity of these models is thus essential.

The two studies reported here (**Chapter 4** and **5**) are the first demonstration of testing the feasibility of using TI as a treatment intervention in the early-stage Alzheimer's Disease. In contrast to studies in human patients, the use of an AD mouse model allows for systematic characterisation of TI stimulation parameters. Importantly, this work allows to investigate the mechanism of action behind the stimulation effects in specific frequency bands in much more detail than would be possible in human studies. Thus, these experiments can inform ongoing and future experiments of the use of TI in AD patients. Additionally, the next generation KI App^{NL-G-F} mouse model was used, considered to be a better representation of the early stages of the disease than the earlier Tg models.

For all the studies reported here, I developed novel experimental protocols. This included software and hardware to deliver modified stimulation paradigms (pulsed TI, PWM-TI), developing two recovery surgery procedures and setting up new experimental methodologies. These approaches have since been adopted by many colleagues, allowing for other studies exploring the neuromodulatory potential of TI in both basic and translational contexts. The experiments reported here are the stepping stone to further, more detailed investigations, with two of the ongoing projects (**Chapter 4** and **5**) aiming to elucidate the mechanisms behind the effects observed.

6.3 Implications and future directions

One of the key differences between animal and human studies is the magnitude of the applied electric fields. In the experiments reported in this thesis, fields in the order of 10 - 100 V/m were applied. Meanwhile, in recent TI study in human subjects (Violante et al., 2023), approximate fields of 0.5 V/m were reported. While this is consistent with previous human reports, the effects evoked by much smaller fields are much more likely to cause entrainment, i.e. synchronisation of the neuronal spiking, rather than impose an activity pattern (Krause et al., 2019; L. Johnson et al., 2020). This may affect the range of neuronal mechanisms involved in human studies, but it also highlights that possible cell selectivity effects may contribute even more substantially under such conditions.

Further investigation into the modulatory effects of kHz fields on the neuronal response to electrical stimuli is crucial. Simultaneous imaging of excitatory and inhibitory cell populations would be the first step to establish whether the E-I balance can explain the network-level effects observed and how these interactions can be explored with different stimulation paradigms or further development of TI modalities.

The imaging study reported here demonstrated the first direct evidence of the FEM models accurately capturing the spatial distribution of the TI-evoked neuronal responses. However, the visibility around the electrodes in this setup was limited and only the simplest TI configuration was tested. Further confirmation of the FEM models, most importantly the possibility to steer the stimulation site and to target deeper structures such as hippocampus, should be performed to ensure the models match the neuronal response profile in a variety of applications.

The early investigation into the effects of TI stimulation of the amyloidosis pathology reported here is important but its clinical translation must be further investigated. Most treatments that led to pathology reduction in the preclinical studies subsequently failed in the clinical trials, possibly due to the differences between sporadic and familial AD. Finding treatment paradigms that target the common core of AD and generalise well is thus of utmost importance. Additionally, amyloidosis is only one facet of AD. Other disease characteristics, such as tau accumulation, have been implicated in the AD phenotype and disease progression. Exploring the therapeutic potential of TI in other

AD models, for example in the presence of tau, could establish the efficacy of the technique at various disease stages. Ultimately, establishing behavioural improvements in AD models is essential as it is most clinically relevant. Future work should focus on establishing which behavioural deficits can be reliably detected in the AD model used and investigating whether different stimulation paradigms can rescue these.

Correcting circuit dysfunction, rather than solely reducing the A β load, as commonly trialled with pharmacological means, may potentially lead to better outcomes. The use of such functional treatment approaches may also be extended to disorders beyond Alzheimer's Disease. Further testing and development of more efficient pulsed TI paradigms, such as theta burst (Huang et al., 2005) (recently trialled in healthy subjects (Wessel et al., 2023)) or closed-loop stimulation (phase-locked to relevant ongoing neuronal oscillations), should also be explored in the future. Ultimately, understanding the mechanisms behind the stimulation effects observed, in frequency ranges such as delta and theta, will be crucial to successful development of translational applications for use in human subjects.

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Appendix

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