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Limited sensitivity of hippocampal synaptic function or network oscillations to unmodulated kilohertz electric fields

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Abstract:

Understanding the cellular mechanisms of kHz electrical stimulation is of broad interest in neuromodulation including forms of transcranial electrical stimulation (tES), interferential stimulation, and high-rate spinal cord stimulation (SCS). Yet, the well-established low-pass filtering by neuronal membranes suggests minimal neuronal polarization in respond to charge-balanced kHz stimulation. The hippocampal brain slice model is among the most studied systems in neuroscience and exhaustively characterized in screening the effects of electrical stimulation. High-frequency electric fields of varied amplitudes (1-150 V/m), waveforms (sinusoidal, symmetrical pulse, asymmetrical pulse) and frequencies (1 and 10 kHz) were tested. Changes in single or paired-pulse field excitatory post-synaptic potentials (fEPSP) in CA1 were measured in response to radial- and tangential-directed electric fields, with brief (30 s) or long (30 min) application times. The effects of kHz stimulation on ongoing endogenous network oscillations were studied.
activity were tested in carbachol-induced gamma oscillation of CA3a and CA3c. Across 23
conditions evaluated, no significant changes in fEPSP were resolved, while responses were
detected for within-slice control DC fields. 1 kHz sinusoidal and pulse stimulation (≥60 V/m), but
not 10 kHz induced changes in oscillating neuronal network. We thus report no responses to
low-amplitude 1 kHz or any 10 kHz fields, suggesting that any brain sensitivity to these fields is
via yet to be-determined mechanism(s) of action which were not identified in our experimental
preparation.

**Key words:** High-frequency stimulation, kilohertz electrical stimulation, neuronal excitability,
brain stimulation, gamma oscillation

**SIGNIFICANCE STATEMENT:**

There a large mismatch between enthusiasm for clinical treatments using kHz frequency
electrical stimulation and the understanding of kHz mechanisms of action. Indeed, the well-
established low-pass properties of cell membranes should attenuate any response to kHz
stimulation. This study presents the largest and broadest characterization of the cellular effects
of kHz stimulation using the most established animal model to detect CNS sensitivity to electric
fields: Our work systematically evaluated sensitivity of hippocampal synaptic function and
oscillatory network activity in response to kHz. Only at low kHz (1 kHz but not 10 kHz) with high
intensity and during oscillations responses were detected. This systematic and largely negative
experimental series suggest kHz neuromodulation operates via yet to be determined
mechanisms.
Electric fields at low frequencies (<100 Hz) are highly effective in changing firing rate and timing of neuronal population (Fröhlich and McCormick, 2010; McIntyre et al., 2004), including at very low (~1 V/m) intensities (Reato et al., 2010). However, as the frequency of electric field oscillations increases beyond a few hundred hertz, sensitivity to stimulation and brain responses diminishes (Deans et al., 2007). On the one hand, this is readily attributable to the low-pass filtering characteristics of cell membranes (Bikson et al., 2004; Deans et al., 2007; McIntyre and Grill, 1999; Ranck, 1975). Emerging neuromodulation techniques specifically using kHz frequency stimulation have been developed, in some cases with marked clinical efficacy. This includes transcranial Alternating Current Stimulation (tACS) with sinusoidal kHz waveforms (Chaieb et al., 2011), transcranial Random Noise Stimulation (tRNS) (Antal and Paulus, 2013; Laczo et al., 2014; Terney et al., 2008), kHz Spinal Cord Stimulation (SCS) (Bradley and Redwood City, 2017; Kapural et al., 2015), and recently, kHz Deep Brain Stimulation (DBS) (Harmsen et al., 2019; Khadka et al., 2020). Approaches using interferential or intersectional short pulse stimulation (Esmaeilpour et al., 2020; Grossman et al., 2017; Voroslakos and Takeuchi, 2018) are a special case underpinned by an assumption of sensitivity to amplitude modulated kHz field, but no responses to unmodulated kHz stimulation.

Across this proliferation of techniques and application of kHz neuromodulation, the cellular mechanisms of kHz electrical stimulation remain unclear (Dmochowski and Bikson, 2017; Pelot et al., 2017). While, at very high stimulation intensities, kHz stimulation may produce supra-physiological changes (e.g. conduction block (Crosby et al., 2017; Zhang et al., 2006), electroporation (Dowden et al., 2010)), for existing clinical applications these intensities are not expected at target tissue. Given that the response of neurons to kHz electrical stimulation is attenuated, the possibility of sub-threshold stimulation of baseline neuronal activity...
(where ongoing neuronal activity is modulated; (Bikson and Rahman, 2013)) is considered alongside supra-threshold stimulation (de novo generation of action potentials / pacing).

Our goal was to systematically evaluate the sensitivity of hippocampal synaptic function and oscillatory network activity to kilohertz frequency extracellular electrical stimulation. For assessing the sub and supra-threshold effects of electric stimulation on brain excitability, the application of uniform electric fields across the rodent slice preparation is among the longest-standing and most exhaustively studied animal models (Bikson et al., 2004; Jackson et al., 2016; Jefferys, 1981). fEPSPs, including pair-pulse responses, are sensitive to modulation by electric fields through changes in axonal excitability (Kabakov et al., 2012; Rahman et al., 2017), synaptic activity (Rahman et al., 2013), dendritic activity (Bikson et al., 2004; Kronberg et al., 2017), and somatic activity (Fröhlich and McCormick, 2010; Radman et al., 2009), while generally providing a global index for excitatory and inhibitory synaptic efficacy (Jefferys, 1981), information processing (Gluckman et al., 1996; Lafon et al., 2017; Radman et al., 2007), and plasticity (Fritsch et al., 2010; Kronberg et al., 2017; Ranieri et al., 2012). Neuronal network oscillations, including those in the gamma frequency band, are highly sensitive to electric fields through well-characterized mechanisms of amplification (Deans et al., 2007; Fröhlich and McCormick, 2010; Reato et al., 2010).

Here, we use fEPSP and oscillations to test the effect of 1- and 10-kHz electrical stimulation using sinusoidal symmetric and asymmetric pulse waveforms. We used direct current (DC) electrical stimulation as a within-slice control to confirm the sensitivity to low-frequency stimulation. Our data suggest the presence of diminished neuronal sensitivity in response to kHz stimulation consistent with the dramatic low-pass filtering property of the neuronal membrane. Oscillatory networks (e.g. gamma oscillation) are more sensitive to electrical stimulation but only to 1 kHz stimulation at ≥60 V/m intensity. Thus, consistent with results using sub-kHz electric fields, the structure of ongoing network oscillations would
determine maximal sensitivity and effects of stimulation (Reato et al., 2013). If the brain is sensitive to high-kHz frequencies (i.e. 10 kHz) or lower-amplitude stimulation, it may be via mechanisms yet to be identified in the brain slice preparation (e.g. peculiarly sensitive neuronal elements, non-neuronal elements such as neuroglia, vascular response, heating), effects peculiar to non-uniform fields, and/or effects with a gradual (e.g. hours) onset.

Methods

All animal experiments were carried out in accordance with guidelines and protocols approved by the Institutional Animal Care and Use Committee at The City Collage of New York, CUNY.

Hippocampal slice preparation: Hippocampal brain slices were prepared from male Wistar rats aged 3–5 weeks old, which were deeply anaesthetized with ketamine (7.4 mg kg\(^{-1}\)) and xylazine (0.7 mg kg\(^{-1}\)) applied I.P. and sacrificed by cervical dislocation. The brain was quickly removed and immersed in chilled (2–6 °C) dissecting solution containing (in mM) 110 choline chloride, 3.2 KCl, 1.25 NaH2PO4, 26 NaHCO3, 0.5 CaCl2, 7 MgCl2, 2 sodium ascorbate, 3 sodium pyruvate, 10 D-glucose. Transverse hippocampal slices (400 μm thick) were cut using a vibrating microtome (Campden Instruments, Leicester, England) and transferred to a recovery chamber for 30 minutes at 34 °C with a modified artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3.2 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, 1.3 MgCl2, 2 sodium ascorbate, 3 sodium pyruvate, and 25 D-glucose. Slices were then transferred to a holding chamber for at least 30 minutes (or until needed) at 30 °C with ACSF containing (in mM) 124 NaCl, 3.2 KCl, 1.25 NaH2PO4, 26 NaHCO3, 2.5 CaCl2, 1.3 MgCl2, and 25 D-glucose. For fEPSP experimental recordings, slices were then transferred to a fluid–gas interface recording chamber (Hass top model, Harvard Apparatus, Holliston MA, USA) perfused...
with warmed ACSF (30.0 ± 0.1 °C) at 1.0 ml min⁻¹. For gamma oscillation experiments, slices were transferred to a fluid–gas interface recording at 34 °C. All solutions were saturated with a gas mixture of 95% O₂–5% CO₂. Gamma oscillations were induced by perfusing the slices with ACSF containing 20 μM carbachol (carbamoylcholine chloride). All reagents were purchased from Sigma Aldrich (St. Louis MO, USA).

**fEPSP recording (acute and long-term):** Recordings started 30 minutes after transfer to the recording chamber. fEPSPs were evoked in the Schaffer collateral pathway using a platinum–iridium bipolar stimulating electrode placed in stratum radiatum of CA1 approximately 300 μm from stratum pyramidale. Recording electrodes made from glass micropipettes (Aluminosilicate glass with 1.5 mm outer diameter, 1.0 mm inner diameter) pulled by a Sutter Instruments P-97 (Novato CA, USA) and filled with ACSF (resistance 0.5-2 MΩ) were placed in stratum radiatum of CA1, approximately 400 μm from the stimulating electrode and within 100 μm from stratum pyramidale (Figure 1). fEPSPs were quantified by the average initial slope, taken during the first 0.5 ms after the onset of the fEPSP. Stimulus intensity was set to evoke fEPSPs with 35-50% of the maximum slope, which was determined at the onset of recording.

For paired pulse facilitation (PPF) experiments, two fEPSPs were evoked at a 50 ms interval (Korte et al., 1995; Kronberg et al., 2017; Lessmann and Heumann, 1998). PPF was quantified as the ratio of the second to the first fEPSP slope in each condition.

For acute experiments, fEPSPs were evoked every 30 s, alternating between control and kHz (or DCS) conditions. Waveforms were applied for 1 s and fEPSPs were evoked midway (0.5 s, mid-field, MF) through the stimulation (Figure 1). Where indicated, fEPSPs were also evoked 0.1 ms after the extracellular field was turned off (post-field, PF). For control conditions, fEPSPs were evoked alone (no kHz stimulation). Within a given slice, a single kHz waveform was tested at multiple intensities in a randomized order ranging from 1-80 V/m (1, 5, 10, 20, 40, 60, and 80 V/m) with each intensity repeated 3 to 15 times per slice. fEPSP slopes
during each kHz epoch were normalized to the average of the control fEPSP slopes immediately preceding and following it. Normalized fEPSP slopes were then averaged across the repeats for each intensity, producing one n per slice per waveform.

For long-term experiments, fEPSPs were evoked every 30 s and fEPSP slope was monitored online. After at least 30 minutes of stable baseline fEPSP recordings, 1 and 10 kHz waveforms were applied parallel to the somato-dendritic axis (radial) at 80 V/m for 30 minutes. fEPSPs were continuously evoked every 30 s throughout the kHz and for 60 minutes after kHz ended. To determine stability prior to stimulation, a least squares linear fit was applied to the baseline fEPSP slopes. The slope of the linear fit (mVms⁻¹min⁻¹) was required to be less than 0.33 % of the mean baseline fEPSP slopes (i.e. less than 20% drift expected over 60 minutes). For the control condition, the same stability criteria were used, but no stimulation was applied. To quantify long-term effects, fEPSP slopes were normalized to the mean of the 20 minutes immediately preceding high frequency stimulation. Sampling frequency was reduced to 10 kHz during long-term experiments in both 1 and 10 kHz stimulation due to technical limitations. The responses were compared between sham and control condition in three different times (immediately, 30 minutes and 60 minutes after termination of stimulation).

Data analysis: All data are reported as the mean ± standard error of the mean (SEM). Reported n values represent the number of slices used in each condition. Statistical analysis was performed using unpaired, one sample t-test for positive and negative DC control stimulation, after checking for normality in each group (Lilliefors test for normality, p > 0.05 in all cases) and one-way repeated measure ANOVA for different intensities used in kHz waveforms. Bonferroni correction was used for multiple comparison correction. All the analysis was performed in R (RStudio, Inc., Boston, MA).
Bayesian inference:

Difference across highest electric field intensity and baseline were analyzed using the Bayesian paired samples T-test as implemented in JASP v0.13.1.0 using default effect size prior (Cauchy 0.707) (Keysers et al., 2020). Results are reported using two tailed Bayes factor $BF_{+0}$ that represents $p(H_+ | 80 \text{ v/m} \neq \text{baseline}) / p(H_0 | 80 \text{ v/m} = \text{baseline})$. Effect size estimates are reported as median posterior Cohen's $\delta$ with 95% credibility interval using a two-tailed $H_1$ in order not to bias estimates in the expected direction. Bayesian ANOVAs were conducted using JASP with default priors, and effects are reported as Bayes factor for the inclusion of a particular effect, calculated as the ratio between the likelihood of the data given the model with vs the next simpler model without that effect.

Electrical filed stimulation: kHz and DCS extracellular electric fields were applied to slices via two parallel Ag–AgCl wires (1 mm diameter, 12 mm length, 10 mm apart) placed in the recording chamber on opposite sides of the brain slice with the recording site approximately equidistant from each wire. Slices were oriented so that the resulting electric field was either parallel (radial stimulation) or perpendicular (tangential stimulation) to the somato-dendritic axis of CA1 pyramidal neurons (Figure 1). In CA3 experiments, slices were oriented so that the resulting electric field was parallel to the main somato-dendritic axis of CA3a pyramidal neurons (perpendicular to pyramidal cell layer, figure 1 A.1). Field wires were connected to a custom high band-width voltage-controlled isolated current source. Before each recording, the applied current intensity was calibrated by measuring the electric field (voltage difference between two recording electrodes separated by 0.8 mm in the slice) in response to a 10 μA DC test pulse. This characterized the linear relationship between electric field magnitude and applied current, which was then used to determine the current intensity required for a desired electric field. Data acquisition and stimulation waveforms were controlled by Power1401-625 kHz hardware and Signal software Version 6.0 (Cambridge Electronic Design (CED), Cambridge, UK). Voltage
signals were amplified (10x), analog low pass filtered (20 kHz; Model 3000 differential amplifier, A-M systems, Carlsborg WA, USA) and digitized (200 kHz, Power1401-625 kHz and Signal, CED, Cambridge, UK). Prior to analyzing the fEPSP slope, all signals were digitally low pass filtered with Signal 6.0 (FIR filter, 2047 coefficients, 250 Hz transition gap, 1,099 -3 dB) or MATLAB to remove stimulation artifact (700 Hz cut-off for 1 kHz stimulation and 1 kHz cut-off for 10 kHz stimulation).

kHz was applied at 1 and 10 kHz using the following kHz waveforms (leading polarity pulse width - interphase interval - opposite polarity pulse width): sinusoid, pulse (40-10-40 μs for 1 kHz and 10 kHz), and an asymmetric pulse waveform with the shorter duration pulse at 2x the amplitude of the longer duration pulse (25-15-50 μs for 10 kHz) (Figure 1). Reported magnitude for the asymmetric pulse waveform is the electric field during the leading (shorter) pulse. For each slice, DCS at 40 V/m was applied with alternating polarity before kHz waveforms as a basis for comparing effect sizes. Here positive, radial +DCS refers to uniform DC electric fields that are parallel to the somato-dendritic axis of CA1 pyramidal neurons, with the positive terminal closer to the apical dendrites (as opposed to basal dendrites). Positive, tangential DCS refers to uniform DC electric fields that are parallel to Schaffer collaterals in CA1 with DCS current flow in the same direction as orthodromic action potential propagation (Figure 1). Unless otherwise stated, the electric field reported throughout the manuscript is the peak electric field for each waveform.
Figure 1: Experimental design of hippocampal slice recordings. *Acute experiments:* Direct current stimulation as within-slice control condition before high frequency stimulation paradigm. fEPSP was evoked and recorded in 4 different conditions: Mid-field, Mid-filed PPF, Post-field and Post-field PPF. Bipolar stimulation and glass recording electrodes depicted in CA1 stratum radiatum along with a pyramidal neuron and Schaffer collateral (gray).

Stimulation: field wires were placed on opposite sides across the slice and connected to a current source. In radial configuration electric fields were applied parallel to the CA1 pyramidal somato-dendritic axis and in tangential configuration, electric fields were applied perpendicular to the CA1 pyramidal somato-dendritic axis. 

Waveform: Direct current and various electric field waveforms for kHz stimulation. The duration of each waveform component is given in μs for 1 kHz and 10 kHz stimulation. Alternating control and kHz (or direct current) epochs were repeated every 30 s. Raw data were low pass filtered to obtain fEPSPs for analysis. fEPSP obtained during kHz/DCS (mid-field) or 0.1 ms after kHz/DCS (post-field) were normalized to the average of proceeding and following fEPSP. 

Long-term Experiment: fEPSP was evoked every 30 seconds. Stimulation was applied for 30 min after a 20 min stable baseline. Field EPSP recording was continued 1 hour after the end of stimulation.
**Extracellular recordings (Gamma oscillation):** Recordings of extracellular field potentials in the pyramidal layer of CA3a and CA3c region of hippocampus were obtained using glass micropipettes (15 MΩ pulled on a P-97, Sutter instruments) field with ACSF. Data acquisition and electrical stimulation were controlled by Power1401-625 kHz hardware and Signal software Version 6.0 (Cambridge Electronic Design (CED), Cambridge, UK). Voltage signals were amplified (10x), analog low pass filtered (20 kHz; Model 3000 differential amplifier, A-M systems, Carlsberg WA, USA) and digitized (20 kHz, Power1401-625 kHz and Signal, CED, Cambridge, UK). To reduce noise and stimulation artifacts, the voltage recordings were always performed relative to an iso-potential electrode placed in bath (Figure 6, A.1). Field recordings overcome potential limitations of intracellular recording during kHz field such as current collection by the capacitive-walled microelectrode leading to artifactual intracellular stimulation (FallahRad et al., 2019) or possible amplifier distortion (Lesperance et al., 2018).

**Power analysis and statistics:** Signals were recorded in frames of 5 s (1.5 s before and 1.5 s after stimulation) and stimulation was applied for 2 s. Stimulation artifacts were minimized by subtracting the voltage in an iso-potential reference electrode from the recording electrode in the slice (Figure 6). Spectrograms were computed (200 ms hamming window, 90% overlap) on individual 5 s frames and averaged over 100 frames for each stimulation condition (i.e. frequency, waveform and amplitude). Normalized power was measured as a power ratio normalized by pre-stimulation power in the frequency band of the endogenous oscillation. Mean gamma power was calculated in the center frequency of oscillation (5 Hz window). To quantify the slope of post-stimulation, a line was fitted within a 300 ms window immediately after stimulation turned off using the “polyfit” function in MATLAB 2016b (MathWorks Inc, Natick, MA, USA). All the results are reported as mean ± SEM; n= number of slices. For statistical analysis paired t-test was used to compare post and pre stimulation in each electric field intensity and
significance level (p) was corrected using Bonferroni for multiple (e.g. for four comparisons made in each experiment p<0.0125 was considered significant). All the analysis was performed in R (RStudio, Inc., Boston, MA).

Results

Effect of kHz stimulation on hippocampal field potentials in CA1

Field excitatory post-synaptic potentials (fEPSP) measured at dendrites reflect the aggregate post-synaptic current entering to a population of neurons, which is a measure of synaptic input. Field EPSPs are sensitive to low-frequency electric fields (Bikson et al., 2004; Lafon et al., 2017). Using rat hippocampal slice preparation, we tested the acute and long-term effects of uniform unmodulated kHz electric fields on synaptic efficacy with electric field direction in parallel or perpendicular to primary somato-dendritic axis (Bikson et al., 2004). The effects of DC electric field were also assessed as within-slice positive controls. Field EPSPs were evoked in CA1 region of rat hippocampus by activating the Schaffer collateral pathway. Unless otherwise stated, changes in fEPSP slope from electric field application were calculated as a ratio of slope during electric field application versus control (i.e. no stimulation). Paired pulse facilitation (PPF) which is a measure of short-term synaptic plasticity was used in our recording and was calculated as the ratio of the second fEPSP slope to the first (50 ms inter-pulse interval) in each condition. Unless otherwise stated, results are reported as mean ± SEM and stimulation were applied for 1 s in all acute experiments and 30 min in long-term experiments.

When electric fields were applied in the radial direction (electric field parallel to the somato-dendritic axis of CA1 pyramidal neurons), sinusoidal stimulation with 1 kHz did not produce significant effects (F(6, 75)=0.5835, ns) in any of intensities tested (1, 5, 10, 20, 40, 60, 80 V/m). However, DC stimulation significantly modulated fEPSP slope (-DC (1.06 ± 0.014,
Neither DC nor 1 kHz sinusoidal stimulation affected PPF. Increasing stimulation frequency from 1 kHz to 10 kHz (fEPSP, 10 kHz: \( F(6,160)=0.86, \text{ns} \), PPF, 10 kHz: \( F(6,55)=2.8, \text{ns} \)), or changing recording time from during stimulation to immediately after the field was turned off (fEPSP, 1 kHz \( F(6,66)=1.21, \text{ns} \); PPF \( F(6,66)=0.88, \text{ns} \); fEPSP, 10 kHz \( F(7,175)=2.2, \text{ns} \), PPF \( F(7,47)=1.316, \text{ns} \)) did not modulate fEPSP over the range of electric field intensities tested (Figure 2.B, C).

Symmetric and asymmetric charge-balanced waveforms are ubiquitous in implanted stimulators including DBS and SCS. Stimulation with radially-directed symmetric pulse waveforms at 1 kHz and 10 kHz electric fields did not modulate fEPSP (1kHz, \( F(6,73)=0.788, \text{ns} \); 10kHz, \( F(6,50)=1.03, \text{ns} \)) or PPF (1kHz, \( F(6,72)=1.30, \text{ns} \); 10kHz, \( F(6,61)=0.68, \text{ns} \)) (Figure 2.E, F). Radially directed electric fields with asymmetric pulse waveform also did not modulate fEPSP or PPF regardless of frequency (Figure 2.G, H) (fEPSP: 1kHz, \( F(6,15)=0.63, \text{ns} \); 10kHz, \( F(6,84)=1.022, \text{ns} \); PPF: 1kHz, \( F(2,9)=0.72, \text{ns} \); 10kHz, \( F(2,32)=0.86, \text{ns} \)).
Figure 2: Acute effect of direct current and high frequency electrical stimulation in radial electric field. (A) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 1 kHz sinusoidal stimulation. (B) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 10 kHz sinusoidal stimulation. (C) Normalized slope of fEPSP and paired-pulse facilitation (PPF) immediately after 1 kHz sinusoidal stimulation (post-field). (D) Normalized slope of fEPSP and paired-pulse facilitation (PPF) immediately after positive and negative 40 V/m DC and 10 kHz sinusoidal stimulation (post-field). (E) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 1 kHz symmetric pulse waveform stimulation. (F) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 10 kHz symmetric pulse waveform stimulation. (G) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 1 kHz Asymmetric pulse waveform stimulation. (H) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 10 kHz Asymmetric pulse waveform stimulation. Black circles indicate each data point. Recording frame was 30 s long in all the acute experiments. Stimulation was applied for 1 s in the middle of the recording frame (14.5 -15.5 s). Each data point represents average of 3-15 repetition. N, the number of hippocampal slices in each intensity. EF: Electric Field. \( ^* p<0.05 \).

When electric field was applied in tangential direction (i.e. perpendicular to somato-dendritic axis of CA1 pyramidal neurons), sinusoidal waveform (1kHz: fEPSP, \( F(6,105)=0.231, \text{ns} \), PPF, \( F(5,90)=0.58, \text{ns} \); 10 kHz: fEPSP \( F(7,83)=1.52, \text{ns} \), PPF, \( F(6,96)=0.52, \text{ns} \)) (Figure 3.A, D), symmetric (1 kHz: fEPSP, \( F(6,96)=0.08, \text{ns} \), PPF, \( F(6,96)=0.52, \text{ns} \) and asymmetric waveforms (10 kHz: fEPSP, \( F(6,36)=1.71, \text{ns} \), PPF, \( F(6,41)=1.30, \text{ns} \)), at 1 kHz or 10 kHz, did not modulate fEPSPs.
Figure 3: Acute effect of direct current and high frequency stimulation in tangential direction. (A) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 1 kHz sinusoidal stimulation. (B) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 1 kHz symmetric pulse waveform. (C) Normalized slope of fEPSP and paired-pulse facilitation (PPF) during positive and negative 40 V/m DC and 10 kHz asymmetric pulse waveform. (D) Normalized slope of fEPSP during positive and negative 40 V/m DC and 10 kHz asymmetric sine waveform. Colored circles indicate different data points. Black line: mean, light grey box: standard deviation and dark grey boxes demonstrate SEM for each experiment. N, the number of hippocampal slices. EF: Electric Field \( * p<0.05. \)

Whereas all the prior results used brief application of electric fields, we further tested if stimulation for a longer period (i.e. 30 min) can induce lasting effects on fEPSP under the hypothesis that small effects could be amplified with longer stimulation duration. Stable baseline...
fEPSP was recorded every 30 s for over 20 min before stimulation and 60 min after stimulation. Electrical stimulation was done using sinusoidal 1 and 10 kHz stimulation with 80 V/m electric field intensity (Figure 4) and effect on fEPSP was analyzed for condition (i.e. sham, stimulation) and time (i.e. immediately, 30 and 60 minutes after termination of stimulation). A repeated measure ANOVA revealed no significant effects for stimulation condition (1 kHz: F(1,27)=0.113, p=0.739; 10 kHz: F(1,23)=0.09, p=0.767), time (1 kHz: F(2,54)=0.024, p=0.97; 10 kHz: F(2,46)=1.01, p=0.375) and no interactions (1 kHz: F(2,54)=1.01, p=0.37; 10 kHz: F(2,46)=1.92, p=0.158).

Figure 4: Long-term effect of kHz stimulation on synaptic efficacy. (A) Normalized field EPSP slope in response to 30 minutes stimulation (between 0 to 30) 1 kHz sine waveform, 80 V/m in radial direction after at least 20 minutes stable baseline. Follow up recording continued for 60 minutes after stimulation. (B) Normalized field EPSP slope in response to 30 minutes 10 kHz sine waveform, 80 V/m in radial direction. Error bars indicates standard error of mean. N, number of slices. Blue (control), red (stimulation).

Bayesian analysis for supporting null hypothesis:

Since these negative results may support either evidence of absence (provide support for null hypothesis) or absence of evidence due to lack of statistical power, we performed Bayes factor hypothesis testing for fEPSP evoked during 80 V/m stimulation applied in radial direction.
(parallel to somato-dendritic axis of pyramidal neurons) for 1 and 10 kHz sinusoidal, symmetric and asymmetric waveforms. Moderate evidence was found for the absence of effect using 80 V/m, 10 kHz sinusoidal waveform, meaning that the observed data was ~ 3x more likely to be under the null hypothesis (BF=0.34 with median posterior $\delta = 0.187$, 95% CL=[-0.177,0.560]), and anecdotal evidence for absence of effect in 1 kHz sinusoidal stimulation, meaning that the observed data was 1.67x more likely to be under the null hypothesis than the alternative (BF=0.63 with median posterior $\delta = -0.334$, 95% CL=[-0.924,0.210]).

Using Bayes factor in symmetric pulse waveforms showed that data observed in 10 kHz is ~ 3x more likely to be under the null hypothesis; providing moderate evidence for null (BF=0.33 with median posterior $\delta = -0.122$, 95% CL=[-0.665,0.402]) whereas observed data in 1 kHz the data provided anecdotal evidence for null hypothesis: data was 1.33x more likely to be under the null hypothesis (BF=0.75 with median posterior $\delta = 0.369$, 95% CL=[-0.162,0.943]). The data observed in during asymmetric pulse stimulation provided anecdotal evidence for both 1 and 10 kHz stimulation, meaning the observed data was 2.13x and 1.23x more likely to be under the null hypothesis, respectively (1 kHz: BF=0.47 with median posterior $\delta = -0.081$, 95% CL=[-1.004,0.789], 10 kHz: BF=0.81 with median posterior $\delta = -0.42$, 95% CL=[-0.216,1.143]).

Bayesian repeated measure ANOVA revealed strong evidence (1kHz: BF=0.1; 10 kHz: BF=0.3) in support of the null hypothesis regarding effect of time (effect on EPSP immediate, 30 min or 60 min after stimulation) and moderate evidence (1 kHz: BF=0.4, 10 kHz: BF=0.3) in support of the null hypothesis regarding effect of stimulation condition (i.e. sham vs stimulation on). Regarding interactions, Bayesian analysis revealed moderate and anecdotal evidence in support of the null hypothesis for 1 kHz and 10 kHz, respectively (1 kHz: BF=0.35, 10 kHz: BF=0.8).
Effect of kHz stimulation on hippocampal gamma oscillations

Uniform unmodulated 1 and 10 kHz electric fields were applied across hippocampal slices exhibiting gamma oscillations under carbachol perfusion (Figure 5.A.1). Oscillations were typically stable over ~3 hours and experiments started after verifying stabilization of gamma oscillation power. We evaluated the sensitivity of gamma network activity to stimulation with kHz electric fields. Each stimulation was 2 s long and signals were recorded in frames of 5 s (acute effect, 5 s frame length (1.5 s pre, 2 s stim, 1.5 post), 80-100 frames per slice). Gamma oscillation was recorded from both CA3a and CA3c region of hippocampus. There was no significant difference in baseline gamma power between the two recording locations (CA3a, N=14; CA3c, N=12, ns) (Figure 5.A.2).

Consistent with previous reports (Esmaeilpour et al., 2020; Reato et al., 2010), low kHz stimulation generated transient effect at the onset of stimulation as well as a sustained effect in CA3a region (Figure 5.B.1). This muted sustained effect is presumably reflecting homeostatic network regulation to bring the network back toward equilibrium (e.g. baseline oscillatory level). Moreover, stimulation produced a post-stimulation suppression of oscillation (see below) which is a marker of network rebound from homoeostatic adaptation (Reato et al., 2010). Gamma oscillation recorded from CA3c region was not modulated during stimulation (Figure 5.B.2), highlighting the importance of electric field direction relative to somato-dendritic axis of pyramidal neurons for somatic polarization (Radman et al., 2009).
Figure 5: Sensitivity of hippocampal gamma oscillations during application of 1 and 10 kHz sinusoidal and square waveform stimulation. (A) Rat in vitro model of gamma oscillation. A.1, Experimental setup: spatially uniform electric field was applied across hippocampal slices in an interface chamber. Recording of gamma oscillation from CA3a and CA3c relative to bath electrode to minimize stimulation noise. A.2, Mean (±SEM) of baseline gamma power (in dB) for CA3a and CA3c across slices. (B) Mean (±SEM) of normalized gamma power across slices for 2 seconds of stimulation (between 1.5 and 3.5 s) using 1 kHz sinusoidal waveform with different field intensities recorded from CA3a (B.1) and CA3c (B.2). (C) Mean (±SEM) of normalized gamma power across slices for 2 seconds of stimulation...
(between 1.5 and 3.5 s) using 10 kHz sinusoidal waveform with different field intensities recorded from CA3a (C.1) and CA3c (C.2). (D) Mean (±SEM) of normalized gamma power across slices for 2 seconds of stimulation (between 1.5 and 3.5 s) using 1 kHz symmetric pulse waveform with different field intensities (D.1) and 10 kHz symmetric pulse waveform with different field intensities (D.2) recorded from CA3a region of rat hippocampus. N, number of slices.

Due to technical concerns of reliably removing stimulation artifact during 10 kHz sinusoidal stimulation and symmetric pulse waveforms, oscillation data was analyzed comparing only the pre and post stimulation time windows (Figure 5.C, D). We defined slope of average gamma power (see methods) measured in 300 ms window immediately after termination of stimulation as a metric to quantify post-stimulation suppression (Figure 6).

Significant post-stimulation suppression was detected using 1 kHz sinusoidal waveform with field intensities ≥ 60 V/m in CA3a region (gamma power slope: 60 V/m, Post: 0.62 ± 0.010, Pre: 8.5*10^-4 ± 0.11, N=15, p<0.001; 80 V/m Post: 0.83 ± 0.09, Pre: 0.15 ± 0.074, N=14, p<0.001) (Figure 6.A.1), however in CA3c region, no change was detected in slope of gamma power immediately after stimulation (Figure 6.A.2). Similarly, symmetric pulse 1 kHz stimulation using intensities ≥ 60 V/m induced significant rebound after stimulation (gamma power slope: 60 V/m, Post: 0.58 ± 0.06, Pre: -0.15 ± 0.23, N=7, p<0.01; 80 V/m, Post: 0.77 ± 0.11, Pre: 0.13 ± 0.64, N=7, p<0.01) (Figure 6.A.3). Increasing stimulation frequency from 1 to 10 kHz abolished the effect. No effect was observed in 10 kHz symmetric pulse and sinusoidal stimulation using post-stimulation suppressions as an index even when testing still higher electric field strength (i.e. 100, 120 and 150 V/m) (Figure 6.B).
Figure 6: Post-stimulation suppression of average gamma oscillation power. (A) Slope of mean gamma oscillation power (illustrated in figure 5) measured from 300 ms window immediately before and after 2 s of stimulation using 1 kHz sine waveform recorded from CA3a (A.1) and CA3c (A.2) and symmetric pulse waveform electrical stimulation recorded from CA3a region (A.3). (B) Slope of gamma oscillation immediately before and after 10 kHz stimulation recorded from CA3a (B.1) and CA3c (B.2) using sinusoidal and symmetric pulse waveform recorded from CA3c region (B.3). Red, post stimulation gamma slope. Blue, pre stimulation gamma slope. Black line: mean, light grey box: standard deviation and dark grey boxes demonstrate standard error of mean for each experiment.
Discussion

There is a long-standing interest in explaining neuronal responses to kHz range electrical stimulation (Katz, 1939; Ward, 2009) with many results still inconclusive or without satisfactory theoretical treatment. Various forms of kHz neuromodulation techniques have shown promise in managing chronic pain (Al-Kaisy et al., 2015; Thomson et al., 2018) improving motor function in Parkinson’s disease (Harmsen et al., 2019) and modulating excitability of human motor cortex (Antal and Paulus, 2013; Chaieb et al., 2011; Terney et al., 2008). Variations of kHz stimulation (electrode position, pulsed/sinusoidal waveforms) has been characterized in a broad range of applications including physiotherapy (Medeiros et al., 2017; Ward, 2009), ceasing abnormal neuronal activity (Kilgore and Bhadra, 2014; Lempka et al., 2015; Pelot and Grill, 2020) or generating spontaneous or asynchronous firing (Crosby et al., 2017; Litvak et al., 2003; Rubinstein et al., 1999). In contrast, it is a fundamental property of cells that the parallel leak conductance and capacitance of outer membrane forms an equivalent of a filter that attenuates neuronal responses to inputs with high frequency components. This intrinsic low pass filtering property of neuronal membrane explains various electrophysiological finding at the cellular and neuronal network level on limited sensitivity to kHz electric fields (Deans et al., 2007; Reato et al., 2010) - though once polarized, ions channel have some kinetics with sub-ms time constants (Zhang et al., 2006; Zhao et al., 2014). At the same time, some application using Amplitude-Modulated (AM) kHz stimulations are based on the assumption neurons are insensitive to the unmodulated kilohertz component (Goats, 1990; Grossman et al., 2017; Ward, 2009). We therefore set out to clarify the sensitivity of the brain to unmodulated, uniform, 1 or 10 kHz sinusoidal (e.g. single frequency band) fields between 1 and 150 V/m.
The acute brain slice model has been extensively used as a model system to screen for effects of a broad range of stimulation waveform and intensities, including sub-threshold fields (Bikson et al., 2004; Jackson et al., 2016; Rahman et al., 2017; Rahman et al., 2013) and is generally among the most characterized experimental system in neuroscience (Ranieri et al., 2012). Consistent with screening for a broad range of possible effects, single and paired fEPSPs are sensitive to changes either in pre- or post-synaptic excitability. Oscillations are similarly highly sensitive to changes in excitatory and inhibitory cellular function through mechanism of amplification specific to network’s architecture and level of activity (Jackson et al., 2016; Reato et al., 2010, 2013). Furthermore, field measures are insensitive to intracellular artifacts specific to kHz fields (FallahRad et al., 2019; Lesperance et al., 2018). A change in fEPSP or oscillations in response to kHz electric fields are thus robust and broad indicators of changes in brain function – which, if positive, can then be followed by more specific testing to identify cellular targets.

We systematically evaluated responses to a range of waveforms (sinusoidal, symmetric, asymmetric pulses), intensities, 1 kHz and 10 kHz frequencies, electric field direction (radial, tangential), stimulation duration (30 s typical, 30 min), and during and post-field effects. While impractical to test all combinations, our overall experimental strategy was intended to identify responses. We focused (number of slices) on 80 V/m but tested a range of intensities in case responses are not monotonic. Given established sensitivity to DC fields of slice prep neurons (Bikson et al., 2004; Jackson et al., 2016), we conducted within-slice positive controls for general sensitivity to electric fields. By any measure, field EPSPs were not modulated by kHz waveform tested, regardless of intensity (up to 80 V/m), waveform, direction, or timing. 1 kHz but not 10 kHz electric field modulated ongoing network oscillations. The intensity required for 1 kHz electric fields to modulate gamma oscillation was substantially higher than for low-frequency (e.g. ~100 Hz) fields (Esmaeilpour et al., 2020). This overall lack of sensitivity is
consistent with prior kHz-stimulation mechanistic studies (Couto and Grill, 2016; Esmaeilpour et al., 2020; Lempka et al., 2015; Negahbani et al., 2018) and the established low-pass filtering characteristics of neuronal membranes to electrical stimulation (Deans et al., 2007; Reato et al., 2013).

Our results are limited by several factors. It is never possible to exclude beta errors, though our use of a high SNR experimental system, with multiple slices and numerous repetitions per condition per slice, as well as within slice positive DC controls, together suggest such undetected effects would be variable or small in any case. Alternative mechanisms of electric fields such as ion concentration changes (Bikson et al., 2001; Shapiro et al., 2020; Wang et al., 2020), fiber block (Patel and Butera, 2015; Shapiro et al., 2020; Zhang et al., 2006; Zhao et al., 2014) and transverse axonal polarization (Wang et al., 2018) are suggested for kilohertz stimulation at very high intensities. However these very high intensities are not expected in existing clinical applications, such as SCS, with targeted tissue some mm away from the electrode (Idlett et al., 2019; Lempka et al., 2015). As emphasized throughout this paper, these results are limited by any biophysical features absent from our experimental model system. Effective kHz stimulation with intensities comparable to these clinical applications would require a transduction mechanism with an especially fast time constant that is absent in acute rodent brain slice.

Following the quasi-uniform assumption (Bikson et al., 2013; Bikson et al., 2015; Khadka et al., 2019), we applied uniform fields, leaving open the possibility that geometry-sensitive effects were missed (Idlett et al., 2019). Our results are limited to the intensities and specific waveforms tested, though a range of pulse-shapes were considered. We cannot consider possible mechanisms not captured by the hippocampal brain slice, such as a highly sensitive subtype of neurons (Lee et al., 2020; Litvak et al., 2003; Rubinstein et al., 1999), vascular
responses (Cancel et al., 2018) or temperature (Zannou et al., 2019a; Zannou et al., 2019b); the latter in fact increases with kHz frequency.

Disclosures

The City University of New York holds patents on brain stimulation with MB as inventor. MB has equity in Soterix Medical Inc. MB consults, received grants, assigned inventions, and/or serves on the SAB of Boston Scientific, GlaxoSmithKline, Mecta, Halo Neuroscience, X.

Other authors reported no conflict of interest.

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