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RESEARCH REPORT

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Neuromodulation-dependent effect of gated high-frequency, LFMS-like electric field stimulation in mouse cortical slices

Ehsan Negahbani[1,2] | **Stephen L. Schmidt**[1,3] | **Nadia Mishal**[1] | **Flavio Fröhlich**[1,2,3,4,5,6]

[1]Department of Psychiatry, University of North Carolina, Chapel Hill, North Carolina

[2]Carolina Center for Neurostimulation, University of North Carolina, Chapel Hill, North Carolina

[3]Department of Biomedical Engineering, University of North Carolina, Chapel Hill, North Carolina

[4]Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, North Carolina

[5]Neuroscience Center, University of North Carolina, Chapel Hill, North Carolina

[6]Department of Neurology, University of North Carolina, Chapel Hill, North Carolina

Correspondence
Flavio Fröhlich, Department of Psychiatry, University of North Carolina, Chapel Hill, NC.
Email: flavio_frohlich@med.unc.edu

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Abstract

Low-field magnetic stimulation (LFMS) is a gated high-frequency non-invasive brain stimulation method (500 Hz gated at 2 Hz) with a proposed antidepressant effect. However, it has remained unknown how such stimulation paradigms modulate neuronal network activity and how the induced changes depend on network state. Here we examined the immediate and outlasting effects of the gated high-frequency electric field associated with LFMS on the cortical activity as a function of neuromodulatory tone that defines network state. We used a sham-controlled study design to investigate effects of stimulation (20 min of 0.5 s trains of 500 Hz charge-balanced pulse stimulation patterned at 0.5 Hz) on neural activity in mouse medial prefrontal cortex in vitro. Bath application of cholinergic and noradrenergic agents enabled us to examine the stimulation effects as a function of neuromodulatory tone. The stimulation attenuated the increase in firing rate of layer V cortical neurons during the post-stimulation period in the presence of cholinergic activation. The same stimulation had no significant immediate or outlasting effect in the absence of exogenous neuromodulators or in the presence of noradrenergic activation. These results provide electrophysiological insights into the neuromodulatory-dependent effects of gated high-frequency stimulation. More broadly, our results are the first to provide a mechanistic demonstration of how behavioral states and arousal levels may modify the effects of non-invasive brain stimulation.

KEYWORDS

cholinergic, noradrenergic, state dependence, transcranial electric stimulation

1 | INTRODUCTION

Low-field magnetic stimulation (LFMS) is a form of non-invasive brain stimulation in which a pattern of alternating trapezoidal magnetic pulses is delivered broadly across the cortex with low field strength. It is derived from echo-planar magnetic resonance spectroscopic imaging (EP-MRSI). A serendipitous observation by Rohan et al. (2004) suggested that LFMS can have rapid mood-enhancing effects during depressed phases in patients with bipolar disorder. This observation was subsequently confirmed in a randomized, controlled clinical trial (Rohan et al., 2014). Corresponding changes in depression-like symptoms were also found in rats (Carlezon et al., 2005). Interestingly, these effects mimicked the effects of drugs which act on the noradrenaline and

Abbreviations: aCSF, Artificial cerebrospinal fluid; CCh, Carbachol; EP-MRSI, Echo-planar magnetic resonance spectroscopic imaging; FR, Firing rate; LFMS, Low-field magnetic stimulation; MEA, Microelectrode array; mPFC, Medial prefrontal cortex; NE, Norepinephrine; rTMS, Repetitive transcranial magnetic stimulation; tDCS, Transcranial direct current stimulation; Thy1, Thymus cell antigen 1.

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serotonin systems (Aksoz et al., 2008; Carlezon et al., 2005). However, the effect of LFMS on neural activity has yet to be studied with the exception of an imaging study (Volkow et al., 2010) that assessed the effect of LFMS on brain glucose metabolism as an indicator of brain function in healthy human subjects. LFMS reduced glucose metabolism in the brain areas where the field strength was maximal.

The search for the neurophysiological mechanism of LFMS is inspired by the susceptibility of neuronal networks to exogenous electric fields. External periodic electric fields can interact with and influence ongoing neural oscillations (Deans, Powell, & Jefferys, 2007; Fröhlich & McCormick, 2010; Jackson et al., 2016; Radman, Su, An, Parra, & Bikson, 2007; Reato, Rahman, Bikson, & Parra, 2010, 2013; Schmidt, Iyengar, Foulser, Boyle, & Fröhlich, 2014). Importantly, these effects appear to depend on endogenous brain oscillations which is a functions of behavioral state (Alagapan et al., 2016; Neuling, Rach, & Herrmann, 2013). However, the effect of oscillating electric fields on the neural membrane potential drops exponentially as a function of stimulation frequency (Deans et al., 2007). It is thus unclear if and how the gated high-frequency electric field induced by LFMS changes neuronal spiking activity as a function of network state.

Here, we asked if an exogenously applied electric field with the waveform replicating the LFMS-induced electric field alters neuronal network activity as a function of neuromodulatory tone. To answer this question, we performed experiments in acute slices of medial prefrontal cortex (mPFC) of the mouse. We used a placebo-controlled experimental design that included sham stimulation. In addition to record from the slices in the absence of neuromodulatory agents, we also used carbachol (CCh), a muscarinic cholinergic receptor agonist (Schmidt, Chew, Bennett, Hammad, & Fröhlich, 2013), and norepinephrine (NE), which both increase overall activity of cortical networks in vivo (Harris & Thiele, 2011; Lee & Dan, 2012), but yield differential effects on activity structure of cortical networks in vitro (Schmidt et al., 2013). Importantly, we used low concentrations of CCh and NE that are known to modulate network activity, and are similar to the corresponding in vivo levels in the extracellular space (Abercrombie, Keller, & Zigmond, 1988; Descarries, Gisiger, & Steriade, 1997; Tso, Blatchford, Callado, McLaughlin, & Stamford, 2004; Vizi, Fekete, Karoly, & Mike, 2010; Zhang, Coggan, & Berg, 1996).

2 | MATERIALS AND METHODS

All animal procedures were approved by the Institute of Animal Use and Care of the University of North Carolina at Chapel Hill, and were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publication No. 8023, revised 1978).

2.1 | Experimental procedure and data acquisition

Forty-nine (23 female) juvenile (p15-p30) Thy1-ChR2-eYFP mice (Jackson Laboratory, Bar Harbor, ME) were used in this study. The animal was first deeply anesthetized using Euthasol (0.5 ml/kg, Visrbac, Fort Worth, TX). When there was no response to the toe-pinch test, the animal was decapitated, the brain was extracted, and quickly placed in ice-cold sucrose solution (in mM: 83.0 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22.0 Dextrose Anhydrose, and 72.0 Sucrose). The 200 μm thick coronal slices of medial prefrontal cortex (4–6 slices per animal) were extracted and let recover for at least 2 hr at 34°C in incubation solution (in mM 119.0 NaCl, 4.5 KCl, 1 Na H_2PO_4, 26.2 $NaHCO_3$, 22.0 glucose, 2.0 $MgSO_4$, and 2.0 $CaCl_2$) before recording neural activity as described in (Schmidt, Dorsett, Iyengar, & Fröhlich, 2016). This mouse line expresses channelrhodopsin-2 and enhanced yellow fluorescent proteins in specific cell subtypes under the thymus cell antigen 1 (Thy1) promoter resulting in expression in LV pyramidal neurons (Arenkiel et al., 2007). Note that we briefly used fluorescent illumination to determine slice placement at the onset of the experiment as described below, but no optogenetic stimulation was used in this study. Slices were kept in low light conditions to reduce non-specific opsin activation during incubation. Slices were placed on a 6 × 10 perforated microelectrode array (MEA) of 30 μm electrodes, 100 μm spacing, and perfused with artificial cerebrospinal fluid (aCSF) (in mM 119.0 NaCl, 4.5 KCl, 1 Na H_2PO_4, 26.2 $NaHCO_3$, 22.0 glucose, 1.0 $MgSO_4$, and 1.0 $CaCl_2$) or aCSF with added neuromodulators at a rate of 7 ml/min and temperature of 36°C. The multiunit (MU) activity was monitored and recorded (MC_Rack, MultiChannel Systems, Germany) via 59 recording electrodes at the rate of 25 kHz (MEA2100-system, MultiChannel Systems). All recorded channels were hardware filtered (300–5,000 Hz) using a second order Bessel band-pass filter.

2.2 | Experiment design and electric field stimulation

We performed three sets of experiments using aCSF, aCSF with 5 μM CCh added (Sigma-Aldrich), or aCSF with 5 μM NE added (Sigma-Aldrich). For each aCSF condition, we randomly assigned the slices to one of the sham or active stimulation groups resulting in 57, 44, 32, 29, 43, and 42 slices, respectively, for sham aCSF, active aCSF, sham CCh, active CCh, sham NE, and active NE groups.

The stimulation electrodes were disconnected from the stimulation source for the sham groups. The recording started 10 min after positioning the slices on the MEA allowing slice recovery and activity emergence. Each recording session

consisted of pre-, during, and post-stimulation periods lasting 5, 20, and 10 min, respectively (Figure 1a). The stimulation waveform was composed of 240 μs pulses separated by 760 μs with alternating polarity, resulting in a 500 Hz charge-balanced pulsed waveform (Figure 1b). This stimulation waveform replicated the electric field waveform resulting from LFMS as described in (Rohan et al., 2004). During the stimulation, the electric field was applied to the slice for 500 ms every 2 s. The band-pass filtering protocol facilitated the immediate recovery of recording amplifiers after stimulation-induced amplifier saturation. This made it possible to record the neural activity over the brief 1.5 s between-stimulation periods.

The electric field was generated as in (Schmidt et al., 2014). Briefly, the calibrated output current from a stimulus-isolation unit was delivered via two parallel Ag-AgCl wires (1 mm diameter, 10 mm length) submerged in aCSF inside the recording chamber. The wires were spaced by 1 cm, centered on the MEA electrodes (Figure 1c). The stimulation current amplitude was calibrated to produce a 20 mV/mm electric field in the middle of recording chamber where the slice and recording electrodes were located. Slices were arranged such that the MEA electrodes covered the mPFC area (including infralimbic and prelimbic cortex) (Schmidt et al., 2016). Brief illumination of layer V neurons with ~470 nm blue light was used to ensure consistent and correct placement of the slices on the MEA across different recordings (Figure 1c), but where otherwise not stimulated with light during the experiment. We used 4% paraformaldehyde in PBS for tissue fixation at the end of the experiment.

2.3 | Preprocessing and data analysis

The band-pass filtered, multiunit activity from all channels (Figure 2a,b) and information about stimulation times were analyzed using custom MATLAB (MathWorks, Natick, MA) scripts as in (Schmidt et al., 2014). Broken recording channels on the MEA were detected visually and excluded from further analysis (Figure 2a,b, red asterisks). All remaining channels were included in the analysis. Figure 2a,b shows sample traces from active and sham groups, respectively. The stimulation artifacts are evident for the stimulation slice (Figure 2a, arrows). The spiking threshold (Figure 2c,d, horizontal dashed line) was set to negative four times of the standard deviation of the high-pass filtered data excluding the epochs contaminated by the stimulation artifact. We discarded the contaminated 0.5 s epochs in stimulated slices (Figure 2c, arrow) and extracted the spikes (green circles) only for the artifact-free periods. We did not extract spikes from sham slices during times that would have been contaminated with stimulation artifacts for stimulation slices (Figure 2d, double arrows and vertical dashed lines). Using this method, we recorded neural activities during the 20-min stimulation epoch in an interleaved manner between successive stimulation trains in stimulation groups and similarly for sham slices.

The median FR time course was computed for each slice using individual FR time courses based on the 4-s bins. The average FR of channels during each epoch (pre-, during, and post-stimulation) was computed. Then, the median FR across channels was determined resulting in three firing rate values

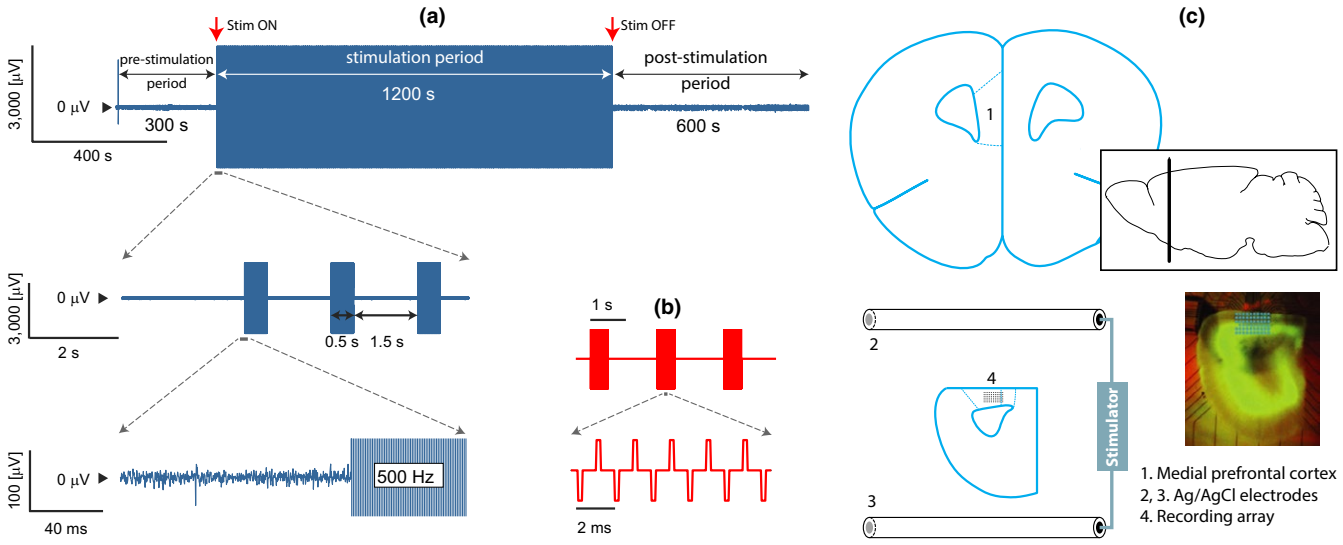

FIGURE 1 High-frequency, LFMS-like pulse stimulation of medial prefrontal cortical area. (a) A sample trace of 300-Hz high pass-filtered multi-unit activity during the course of experiment showing the stimulation artifact at different scales. (b) Induced electric field waveform of low-field magnetic stimulation (LFMS) is composed of blocks of 500 Hz charge-balanced pulses lasting 0.5 s and repeating every 2 s. (c) Placement of Coronal slices with medial prefrontal area, Ag/AgCl stimulation electrodes and multi-electrode recording array

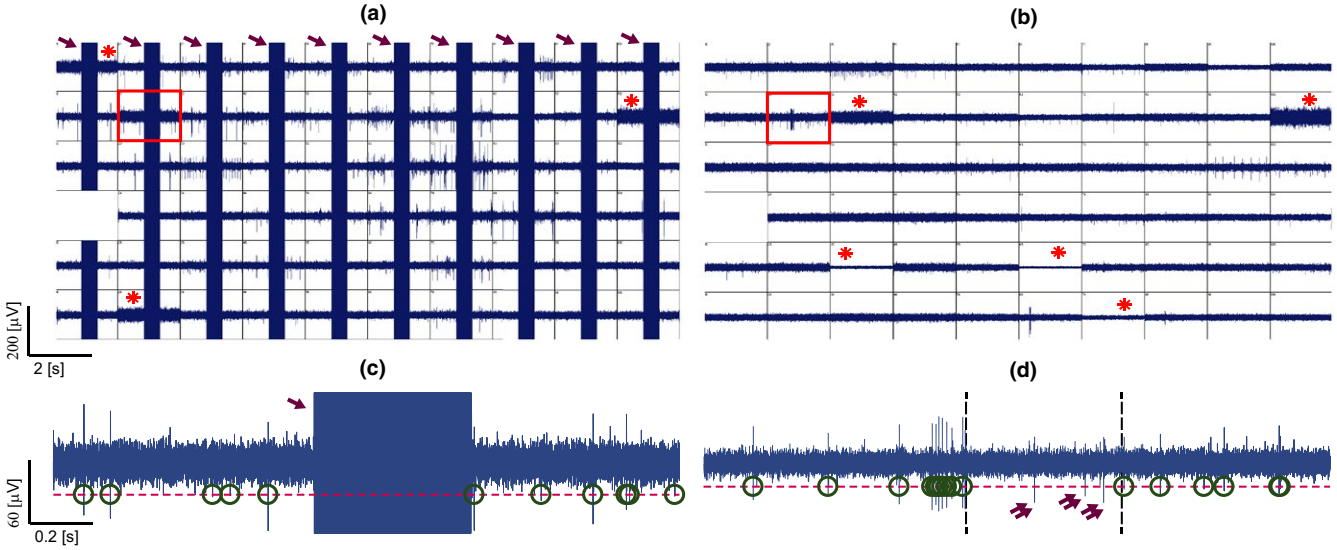

FIGURE 2 Multiunit (MU) activity, stimulation artifact, and spike extraction. (a) Highpass-filtered (300 Hz) MU activity from a stimulated slice and (b) a sham slice. (c) Detailed view of a recording channel (red box in a) showing spike extraction (green circles) in stimulation slices using the set spiking threshold (horizontal dashed line). The saturation of the amplifier due to stimulation is evident (arrow in a, c). (d) Detailed view of a recording channel (red box in b) showing spike extraction in sham slices. The spikes falling in the periods corresponding to stimulation epochs in stimulation slices (indicated by vertical dashed lines) were not analyzed (double arrows in d). The red asterisks indicated the broken channels

corresponding to before, during and after stimulation periods. This analysis was first performed regardless of which cortical layer are channels recording from. Then we also extracted layer specific firing rates per slice. Channels were assigned to one of three cortical layer groups (I-III, V, or VI) based on the relative location of illuminated layer V neurons in slice images. See Supporting Information (Figures S1–S6) for three median firing rate time course per slice for all experimental groups.

2.4 | Statistical analysis

The factorial 3×2×2 experimental design consisted of aCSF type (levels: aCSF, aCSF + CCh, aCSF + NE) and stimulation condition (levels: sham, active stimulation) as two between-slices factors and time (levels: during, and post-stimulation periods) as the repeated measures within-slices factor, and resulted in 12 experimental groups. We defined percentage gain score (GS) as two outcome measures per slice

$$GS_{\text{dur}} = 100 \times \frac{FR_{\text{dur}} - FR_{\text{before}}}{FR_{\text{before}}}, \quad GS_{\text{after}} = 100 \times \frac{FR_{\text{after}} - FR_{\text{before}}}{FR_{\text{before}}}$$

where FR_{before}, FR_{dur} and FR_{after} are the median firing rates before, during and after stimulation per slice.

Testing for underlying assumptions of ANOVA, Shapiro-Wilk test did not indicate normality of distribution for all experimental groups on neither original measures nor transformed (logarithmic or square-root) ones. A visual inspection indicated that the quartile-quartile (q-q) plot of each individual group is not distinguishable from eight q-q plots belonging to synthetic normal distributions with same mean and standard deviation as the experimental group. According to this evidence and considering the relatively large sample sizes in all groups and randomized group assignments, we used the parametric ANOVA method for statistical analysis. A three-way mixed ANOVA was used to identify the possible combined effect of stimulation and neuromodulation on neural activity as measured by percentage gain score during and after stimulation. Post-hoc analysis was performed using two-way ANOVA or robust two-sample trimmed t test (Yuen, 1974). We used corrected p-values (Hunh-Feldt) due to violations of compound symmetry of repeated measures if indicated by Mauchly's test of spherecity. The significance level was set to $p = 0.05$. All statistical analysis was performed using ezANOVA package in R (RDevelopment_CORE_TEAM, 2010).

3 | RESULTS

The cortical slices maintained in aCSF on a microelectrode array (MEA) exhibited only sparse firing patterns in a subset of slice locations. Bath exposure to cholinergic (CCh) and noradrenergic (NE) neuromodulators increased the firing activity in slices. Despite the fact that the two neuromodulators has a qualitatively similar effect on network activity, we considered the CCh and NE neuromodulation as two distinct states. Thus, we were able to study if neural activity associated

with different states can be altered by LFMS-related gated high-frequency stimulation. In addition, the multi-electrode array recording enabled us to contrast our results between different cortical layers.

The median firing rate (FR) of mPFC neurons across all channels were extracted for different periods of the experiment for each slice: baseline (pre), concurrent with stimulation (during), and following stimulation (post). Before performing further analysis to answer our main question about the effect of stimulation, we first asked if the baseline FR was the same for sham and stimulation slices within each aCSF type. The pre-stimulation firing rate is shown in Figure 3a. The baseline firing rates of sham and stimulation slices were not significantly different from each other within each aCSF type (for aCSF sham and stimulation: $t(53.33) = 1.06$, $p = 0.29$, effect size of $r = 0.23$, for CCh sham and stimulation: $t(34.82) = 0.11$, $p = 0.91$, effect size of $r = 0.14$, and for NE sham and stimulation: $t(48.84) = 0.57$, $p = 0.56$, effect size of $r = 0.05$). After combining the sham and stimulation slices within each aCSF type, the post-hoc analysis indicated that the firing rate was different for at least one aCSF type ($F_{2,244} = 57.98, p < 0.05$ one-way ANOVA). We found that on average the CCh and NE slices showed higher baseline firing rate ($FR_{(\text{mean, standard error})} = (6.07, 3.83), (5.01, 3.17)$ Hz, respectively) than the ones in the aCSF group ($FR_{(\text{mean, standard error})} = (1.72, 1.01)$ Hz). The difference was significant $(39.58) = 8.40$, $p =< 0.05$ with an effect size of $r = 1.75$ when comparing aCSF with CCh, was also significant $t(56.85) = 7.82$, $p < 0.05$ with an effect size of $r = 1.44$ when comparing aCSF with NE. The mean firing rate of baseline activity was not significantly different between CCh and NE slices $(77.62) = 1.32$, $p = 0.18$ with an effect size of $r = 0.31$. These results indicated that both sham and stimulation slices within each aCSF type had the same firing rate at the beginning of the recording session. Figure 3b,c show the raw firing rates during and after stimulation across different experimental groups.

As the next step, we computed the percentage gain score (percentage change in firing rate when compared to pre-stimulation baseline) during and after application of stimulation to study the immediate and after-effects of stimulation. We did not find a significant three-way interaction between the study factors, e.g., aCSF type, time and stimulation condition ($F_{2,183} = 2.53$, $p = 0.08$, three-way mixed ANOVA). The significant two-way interaction between ACSF type and time from same ANOVA analysis ($F_{2,183} = 6.02, p < 0.05$) could be further analyzed using its simple effects, however, this post-hoc analysis did not provide stimulation-related insights. The other two non-significant two-way interactions oriented us to interpretation of two significant main effects. The significant main effect of aCSF type ($F_{2,183} = 8.30$, $p < 0.05$) suggested that ignoring the stimulation and time effect, the change in firing rate depends on aCSF type. The significant main effect of time factor ($F_{1,183} = 236, p < 0.05$) suggested that ignoring the stimulation and neuromodulator effects, the change in firing rate is a function of time. Altogether, these results did not indicate any significant change in firing rate due to LFMS stimulation in any group. We next performed layer-specific analysis to find out the possible stimulation effect on neurons in specific cortical layers. We did not consider layers as the fourth factor in our study design due to the lack of independence between firing rate of different layers.

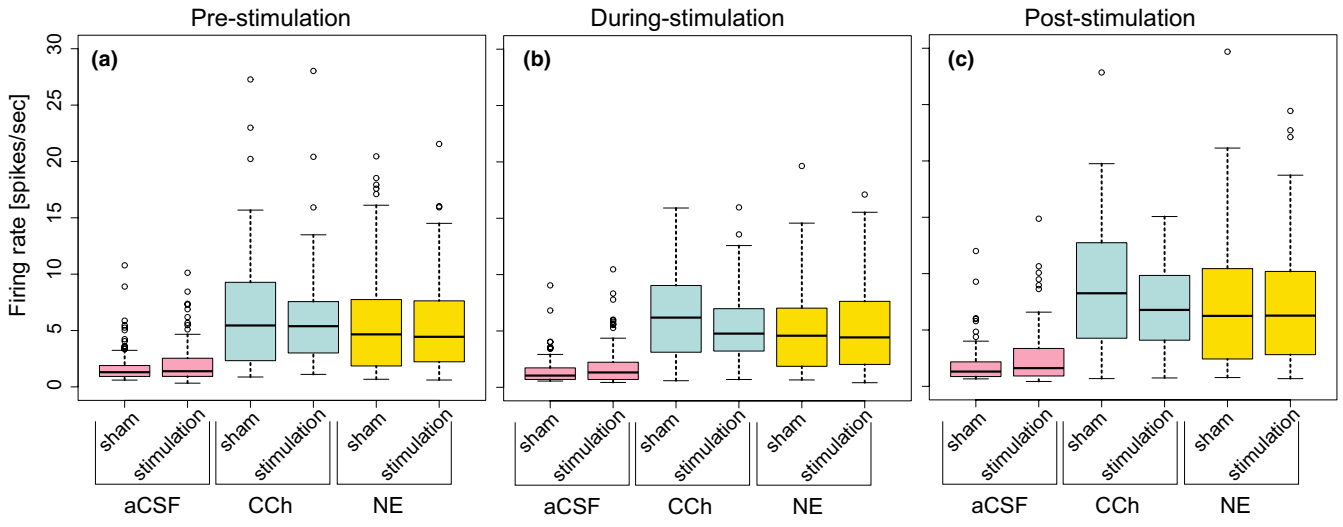

FIGURE 3 Firing rates (FR) across all groups. (a) Comparison of pre-stimulation FR between sham and stimulation groups for all artificial cerebrospinal fluid (aCSF) types. There was no significant difference in the pre-stimulation FR between sham and stimulation slices within aCSF, aCSF with 5 μM carbachol (CCh), and aCSF with 5 μM norepinephrine (NE) slices. (b) FR across all groups during the stimulation period and (c) after the stimulation offset

3.1 | Stimulation effect on layer V cortical neurons

Looking at data recorded from layer V cortical neurons (Figure 4a–c) we found a significant three-way interaction between study factors ($F_{2,183} = 4.12$, $p = 0.01$). Post-hoc analysis on simple effect of interaction between stimulation condition and aCSF type did not result to a significant interaction during stimulation period ($F_{2,183} = 1.74$, $p = 0.17$, two-way ANOVA). In addition, during the stimulation application, the two main effects (aCSF type and stimulation condition) were not significant. We also did not find any significant difference between stimulation effects on sham and active slices within each aCSF type. These results suggest that LFMS stimulation (with or without neuromodulatory intervention) did not have any significant immediate effect on firing rate of layer V neurons. Post-hoc analysis of post-stimulation period showed significant interaction between stimulation condition and neuromodulatory tone ($F_{2,183} = 4.74$, $p < 0.05$, two-way ANOVA). This suggests the way LFMS alters the post-stimulation firing rate of layer V neurons is dependent on neuromodulatory tone. Figure 4b shows how LFMS stimulation reduced post-stimulation gain score of Layer V neurons only in cholinergic group ($t(22.31) = 2.41$, $p = 0.02$ with an effect size of $r = 0.69$). This indicates the significant after-effect of the LFMS stimulation in suppressing the increase in the firing rate of CCh slices compared to NE and aCSF groups.

3.2 | Stimulation effect on layer VI and layers I-III cortical neurons

The interaction graphs of percentage gain scores for layer I-III (Figure 4d–f) and layer VI (Figure 4g–i) neurons showed similar results for each layer. The three-way interaction between study factors (aCSF type, time, and stimulation condition) was not significant for layer VI neurons ($F_{2,183} = 0.71$, $p = 0.50$, three-way ANOVA). The post-hoc analysis did not indicate any type of effect involving the LFMS stimulation. Same non-significant results were also obtained for layers I-III neurons ($F_{2,183} = 1.16$, $p = 0.32$, three-way ANOVA).

3.3 | Stimulation effect when considering the last 300 s of stimulation period instead of its entire period

Since we did not find an immediate stimulation effect, but a significant after-effect, we asked if LFMS modulates the firing rate on a slow time course. To answer this question we re-analyzed the entire data considering only the last 300 s of stimulation period instead of its entire period. Following the same statistical analysis approach as before, the three-way interaction was not significant when considering all three layers together ($F_{2,183} = 2.24$, $p = 0.11$, three-way mixed ANOVA). None of the following post-hoc analyses indicated a significant LFMS effect. We next performed three separate ANAOVA analysis for each layer. Layers I-III and layer VI neurons did not show significant three-way interactions ($F_{2,183} = 0.49$, $p = 0.61$, $F_{2,183} = 0.67$, $p = 0.41$, three-way mixed ANOVA, respectively, for Layers I-III and VI neurons). The corresponding post-hoc analyses also did not show any significant stimulation effect for these layers. Layer V neurons also did not show any significant three-way interaction between study factors ($F_{2,183} = 1.34$, $p = 0.27$, three-way mixed ANOVA). The same ANOVA indicated a significant two-way interaction between stimulation

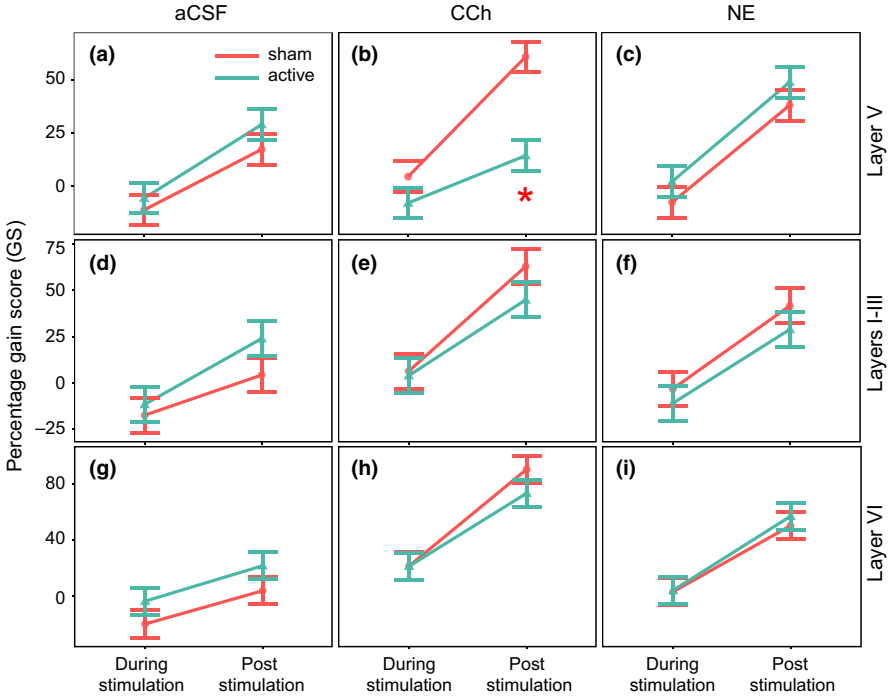

FIGURE 4 Interaction plots between sham and active stimulation slices corresponding to During-stimulation and Post-stimulation periods for (a, b, c) layer V, (d, e, f) layers I-III, and (g, h, i) layer VI neurons. The mean percentage gain score of firing rate for each period of experiment is plotted for sham (red) and active stimulation (blue) slices for three aCSF types: pure aCSF (left column), aCSF with added 5 μM Carbachol (CCh, middle column) and aCSF with added 5 μM norepinephrine (NE, right column). Error bars indicate the 95% confidence intervals of the means. The significant difference is indicated by asterisk symbol

condition (sham vs. active) and aCSF type ($F_{2,183} = 3.67$, $p = 0.02$, three-way mixed ANOVA). Looking at the simple effects of this interaction we did not find any significant stimulation effect (interaction or main effect) during stimulation period ($F_{2,183} = 1.81$, $p = 0.16$, two-way ANOVA). Looking at the after-stimulation period, we found a significant two-way interaction between stimulation condition and aCSF type ($F_{2,183} = 4.74$, $p < 0.05$, two-way ANOVA), and a significant difference between sham and active group only for CCh group ($t(22.31) = 2.31$, $p = 0.02$ with an effect size of $r = 0.62$). In summary, we did not find a significant immediate effect of LFMS even during the last 300 s of stimulation period.

4 | DISCUSSION

Our study investigated the immediate and post-stimulation effects of LFMS-related gated high-frequency electric field stimulation on the network activity in mPFC slices as a function of neuromodulatory tone. We found that stimulation altered the neural firing rates only in Layer V for slices exposed to a low dose of CCh, a cholinergic agonist (Figure 4b). More specifically, the stimulation reduced the percentage increase of firing rate (resulting in smaller growth of FR compared to its pre-stimulation value) when an exogenously induced cholinergic tone was present. While this effect was significant after the stimulation offset, the acute effect was not significant. We did not find any significant acute or outlasting effect of stimulation on FR of layer V neurons for the slices without exogenous neuromodulation (aCSF group, Figure 4a) or with bath application of 5μM NE (Figure 4c). We also did not find any acute or outlasting stimulation effect for layers I-II (Figure 4d–f) or layer VI (Figure 4g–i) neurons regardless of type of exogenous neuromodulator.

The state-dependent neural response to externally induced sensory or electrical stimulation is a known property of neural systems (Harris & Thiele, 2011; Hasenstaub, Sachdev, & McCormick, 2007; Leuchter, Cook, Jin, & Phillips, 2013; Marder, O'Leary, & Shruti, 2014; Steriade, 2000). We have previously shown that the effect of weak electric current stimulation is also constrained by network endogenous oscillations in a biophysical model of thalamus (Li, Henriquez, & Fröhlich, 2017), brain slices (Schmidt et al., 2014) and human cortex (Alagapan et al., 2016). Despite the similar effect of the NE and CCh neuromodulators in increasing the network activity relative to the aCSF condition (Figure 3a, comparable increased firing rate in CCh and NE groups regardless of LFMS application), only the CCh condition responded to gated-high frequency stimulation. This observed neuromodulatory-dependent effect of electric stimulation also confirms that the intrinsic state of the network has a major influence on the responsiveness to the stimulation. Interestingly, we found the evidence for the modulation of neuronal activity by the stimulation when comparing the FR levels before and after stimulation (Figure 4b). This suggests that the gated high-frequency stimulation can cause network reorganization that outlasts the stimulation (often referred as offline effects). Other forms of non-invasive brain stimulation such as repetitive transcranial magnetic stimulation (rTMS) have been suggested to alter synaptic connections via long-term potentiation or long-term depression mechanisms (Pell, Roth, & Zangen, 2011). It is unknown if similar mechanisms explain our results. Unlike rTMS, we used a weaker stimulation amplitude that is unlikely to induce action potentials from rest, but may influence patterns of ongoing, synaptically mediated activity. The possible role of long-lasting changes of synaptic activity in mediating the effects we observed in our experiments should be addressed in future experiments. In addition, the interpretation is complicated by the fact that our main result is a decrease in the increase in FR by stimulation. It is notable, however, that imaging findings in humans show results consistent with LFMS-induced suppression of neural activity. A PET study in healthy volunteers (Volkow et al., 2010) observed that glucose uptake, a measure of neural metabolism, was decreased in regions receiving the highest LFMS field strength. Similarly, resting state fMRI analysis suggests that LFMS treatment yields reductions in functional connectivity in patients with depression (Marc Dubin, personal communication).

As any scientific study, the work presented here has limitations. First, our findings are based on the recordings from acute brain slices of the rodent mPFC. The influence of stimulation on other cortical areas with different cytoarchitecture remains unknown. More importantly, the effect of gated high-frequency stimulation on the interactions between spatially segregated, but functionally connected, networks in the intact brain remains to be fully investigated. Second, although we observed similar effects of NE and CCh in increasing the overall spontaneous firing rate (Figure 3a), only the layer V neurons of slices in CCh group responded to stimulation. We did not investigate why the stimulation had a significant effect only in the presence of cholinergic and not the noradrenergic neuromodulator. Mixed findings for the cellular effects of cholinergic neuromodulation have been reported including decreased (Favero, Varghese, & Castro-Alamancos, 2012), increased (Eggermann & Feldmeyer, 2009; McCormick, 1992), and both decreased and increased (McCormick & Prince, 1986) excitability of excitatory cells and reduced γ-aminobutiric acid (GABA) neurotransmitter release from fast-spiking inhibitory neurons (Kruglikov & Rudy, 2008). For NE both excitatory and inhibitory effects on excitatory neurons (Favero et al., 2012; O'Donnell, Zeppenfeld, McConnell, Pena, & Nedergaard, 2012), and enhanced inhibitory network activity has been reported (O'Donnell et al., 2012). In addition, we have recently shown

how the effect of high-frequency electric field stimulation is modulated by specific ion channels and morphology of neural structures (Toloza, Negahbani, & Frohlich, 2017). These diverse effects suggest that the interaction between neuromodulators and externally induced electric fields cooperatively influences the neural dynamics. Follow-up studies are required to understand what different aspects of cholinergic and noradrenergic activations results in modified effect of electric stimulation on neural activity. These experiments may help in dissecting the primary neural targets of gated high-frequency stimulation. Third, while we did not observe an acute effect of stimulation in CCh slices, the effect was significant after the stimulation offset. This finding suggests follow-up experiments with longer stimulation time that may result in significant acute effects. Fourth, we used a relatively high field strength of 20 V/m to find effects in a study with a practical sample size. We note that our choice of field strength is similar with the field strength used in in vitro examination of effect of transcranial direct current stimulation (tDCS) (Kronberg, Bridi, Abel, Bikson, & Parra, 2017; Ranieri et al., 2012). The resulting electric field of transcranial stimulation in humans is estimated to be <1 mV/mm (Fröhlich, 2016), which is at the lower side of the effective field strength in animal studies (Reato et al., 2013). Further studies are required to expand our results to weaker field strength. Finally, while it is a standard experimental design practice to record baseline activity before an intervention, we did record the baseline activity before LFMS application but not before the application of the neuromodulators. The limitations in viability of slices in prolonged periods prevented us from recording the firing rates before application of neuromodulators. We made the assumption of similarity of baseline activity for different neuromodulatory groups based on two facts: (a) randomized assignment of animals and corresponding slices to experimental groups, and (b) following the exact same slice preparation protocol for all groups. It is unlikely that this assumption biases our results, but follow-up experiments for further delineation of neuromodulatory-dependent effect of electric stimulation should address the requirements for all baseline recordings.

To our knowledge, the study presented here is the first to demonstrate that the state-dependent effects of electric field stimulation may be mediated by neuromodulatory tone. Our results thus suggest that tracking and controlling brain state during human studies of non-invasive brain stimulation is paramount to reduce variability in outcome and thereby accelerate the successful development of novel clinical brain stimulation paradigms.

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CONFLICT OF INTEREST

FF is the founder, chief scientific officer, and majority owner of Pulvinar Neuro LLC.

DATA ACCESSIBILITY

All pre-processed firing-rate data recorded during this study are available from the corresponding author on request.

AUTHORS' CONTRIBUTION

EN, SLS, and FF designed the study. EN and NM conducted the experiments. EN and SLS analyzed the data. EN and FF interpreted the results. EN prepared the figures and wrote the manuscript. All authors reviewed the manuscript.

ORCID

Flavio Fröhlich https://orcid.org/0000-0002-3724-5621

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