Neuronal responses to focused ultrasound are gated by pre-stimulation brain rhythms

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A B S T R A C T
Background: Owing to its high spatial resolution and penetration depth, transcranial focused ultrasound stimulation (tFUS) is one of the most promising approaches to non-invasive neuromodulation. Identifying the impact of endogenous neural activity on neuromodulation outcome is critical to harnessing the potential of tFUS.

Objective: Here we sought to identify the relationship between pre-stimulation neural activity and the neuronal response to tFUS.

Methods: We applied 3 min of continuous-wave tFUS to the hippocampal region of the rat while recording local field potentials (LFP) and multi-unit activity (MUA) from the target. We also tested the application of tFUS but with an air gap separating the transducer and the skull, as well as active stimulation of the contralateral olfactory bulb.

Results: We observed a modest but significant increase in firing rate during hippocampal tFUS, but not during stimulation of the olfactory bulb or when an air gap was present. Importantly, the observed firing rate increase was significantly modulated by the power of baseline oscillations in the LFP, with low levels of delta (1–3 Hz) and high levels of theta (4–10 Hz) and gamma (30–250 Hz) power producing significantly larger firing rate increases. Firing rate increases were also amplified by a factor of 7 × when stimulation was applied during periods of frequent sharp-wave ripple (SWR) activity.

Conclusion: Our findings suggest that baseline brain rhythms may effectively “gate” the response to tFUS.

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1. Introduction

Historically employed to image soft tissue, low-intensity focused ultrasound has more recently been shown to modulate brain activity [1–4] in models spanning cell cultures [5–8], rodents [9–17], primates [18–22], and humans [23–32]. Ultrasound overcomes the critical limitations of conventional (electromagnetic) non-invasive brain stimulation: it can be focused through the skull with millimeter precision [33] and penetrate deep brain regions [23]. This raises the tantalizing possibility of utilizing transcranial focused ultrasound stimulation (tFUS) to modulate mesoscale neural circuits without the need for surgery, potentially providing novel interventions for a host of neurological and psychiatric disorders associated with aberrant brain activity [34].

Substantial variability in neural and behavioral outcomes has been widely reported in non-invasive brain stimulation [35–37], including focused ultrasound [30]. Identifying the sources of this variability, whether it be exogenous (i.e., positioning of the transducer, anatomical differences) or endogenous (i.e., baseline neurophysiology) is essential to achieving robust and predictable outcomes with tFUS. The dynamics of the sonicated region leading up to stimulation, especially neural oscillations, may exert a causal influence on the subsequent response to stimulation [38]. Indeed, there is in vitro evidence that the level of synaptic input exerts an influence on the neuronal response to ultrasound [39], and findings from functional magnetic resonance imaging (fMRI) suggest that neuromodulation outcome is dependent on whether the stimulated region is active [40]. To our knowledge, however, the influence of baseline neural oscillations on the subsequent neuronal response to ultrasonic neuromodulation has not yet been investigated.
To evaluate the effect of endogenous activity on the neuronal response to tFUS, we stimulated the hippocampal region of the rat while simultaneously recording electrophysiological responses from the sonicated area. We conditioned the resulting changes in spiking on baseline population activity, considering both the power of oscillations in canonical frequency bands of the local field potential (LFP), as well as stereotyped markers of hippocampal excitability, namely sharp wave ripples (SWR) [41].

We found a modest but significant increase in firing rate during tFUS of the hippocampal region, but not when stimulating the contralateral olfactory bulb or when an air gap was present between the transducer and skull. Importantly, these changes were significantly more pronounced during periods of low power in the delta (1–3 Hz) and high power in the theta (4–10 Hz) and gamma (30–250) frequency bands. Moreover, tFUS produced much larger effects when stimulating during periods of frequent SWR.

2. Materials and methods

Data were obtained from 38 adult male Long Evans rats weighing at least 350 g (417.1 ± 38.1 g, mean ± sd). The relatively larger craniums of male rats facilitated concurrent tFUS and electrophysiological recording. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the City College of New York, City University of New York.

2.1. Transcranial focused ultrasound stimulation

Two separate tFUS systems were employed for the experiments. In the first, continuous-wave (CW) waveforms (carrier frequency 2.0 MHz, sinusoidal) were generated with a waveform generator (Keysight 33500B Series). The output of the waveform generator was fed into the input of an RF Amplifier (Electronics and Innovation, 40 W). The amplifier provided the drive voltage into a single-element ultrasonic transducer (Ultran KS25-2 immersion transducer, 2 MHz, 6.25 mm active diameter). Empirical measurements in a watertank indicated that the beampattern of this transducer had a full-width-half max (FWHM) of 1 mm laterally and 10 mm in depth. In the second, CW tFUS (carrier frequency 2.5 MHz, sinusoidal) was delivered with an integrated FUS system (Sonic Concepts TPO-201) feeding a dual-channel, axial steered ultrasonic transducer (Sonic Concepts SU-132, 9,625 mm active diameter). The FWHM of this transducer was empirically measured as 1 mm lateral and 1.5 mm in depth. The beampatterns of both transducers are shown in Fig. 1A. The transducers were mounted onto a micromanipulator arm of a stereotaxic frame (David Kopf Instruments) and coupled to the rat skull with ultrasonic coupling gel. The transducer was positioned over the desired anatomical target with the transducer face parallel to the skull (the right hippocampal region: −3.5 mm AP, +2.5 mm ML, targeted depth of 3.5 mm; the left olfactory bulb: +6 mm AP, −1.5 ML, targeted depth of 3.5 mm, coordinates relative to skull bregma and midline).

Two acoustic intensities were tested in this study: 13 mW/cm² and 52 mW/cm². These intensities were selected by conducting pilot experiments and observing electrophysiological changes online. The use of relatively low intensities, compared to the tFUS literature [2], partially reflects our employment of relatively long sonications (3 min).

2.2. Experimental design

In the primary cohort that received both active stimulation of the hippocampal region (referred to in the main text as “FUS HPC”) and stimulation of the contralateral olfactory bulb (“FUS OLF”), a within-subject design was employed where n = 18 animals received all combinations of the two intensities and two stimulation sites. The order of intensities was interleaved (e.g., 13 mW, 52 mW, 13 mW, 52 mW) and counterbalanced across the cohort. Within each intensity, hippocampal and olfactory sites were interleaved (and again balanced). An example condition ordering would then be: 13 mW olfactory, 13 mW hippocampal, 52 mW olfactory, 52 mW hippocampal. An interval of at least 1 h was introduced between the start of each condition in order to allow any outlasting effects from tFUS to dissipate.

In the secondary cohort that received “null” stimulation of the hippocampal region, achieved here by inserting an air gap of 1 cm between the transducer and the skull and termed “FUS AIR” in the main text, data was acquired from n = 20 animals. Three replicates from each animal were performed, spaced 2 h apart.

2.3. Acoustic intensity calibration

Ultrasound pressures were measured in a water tank with a calibrated hydrophone (Onda Corporation). Pressures were determined in both free field as well as with a model rat skull placed between the transducer and hydrophone. The values reported in the main text correspond to the intensities with the skull present. From the water tank calibrations, it was determined that the skull attenuates the acoustic pressure to a value that is 2/3 of the free-field pressure. For each transducer, the drive voltage required to produce acoustic intensities of 13 mW/cm² and 52 mW/cm² were determined and employed in the experiments. The pressure corresponding to 13 mW/cm² was 14 kPa, with an associated mechanical index (MI) of 0.01 at a 2 MHz center frequency. The pressure corresponding to 52 mW/cm² was 28 kPa, with an associated mechanical index of 0.02.

2.4. Anesthesia and surgery

Prior to the day of experimentation, selected animals weighing at least 350 g were fasted for 12–14 h to increase urethane absorption. On the day of the experiment, animals were placed into an induction chamber and induced with gaseous isoflurane at 3% L/min. Animals were removed from the induction chamber and a nose cone was attached so that the dorsal hair could be shaved in preparation for the craniotomy. Animals were then placed on a stereotaxic frame (David Kopf Instruments) with earbars securing the head. The isoflurane concentration was then reduced to 2%. A cross incision was performed over the dorsal skull to expose the cranium and skull landmarks. The distance from bregma to the interaural line was measured so that AP coordinates could be adjusted to account for differences in animal size. The center of the forthcoming craniotomy was marked at −7.5 AP and +2.5 ML (directly posterior of the target of hippocampal stimulation). This allowed sufficient clearance between the edge of the transducer and the border of the craniotomy. A 2 mm by 2 mm craniotomy was performed over the marked area, followed by the removal of the dura. A small titanium screw was implanted into the skull (left hemisphere) to provide an electrical ground for the electrophysiological recordings. The concentration of isoflurane was further reduced to 1% and a urethane cocktail (1.5 g/kg diluted with 2.5 ml/g saline, divided into 3–4 doses with one dose administered every 10 min) was administered via intraperitoneal injection. After the final urethane injection, isoflurane was lowered to 0.5% to allow for urethane absorption and anesthesia transition. After 30 min, isoflurane was discontinued and a 120 min period was allowed for complete expulsion of isoflurane and to achieve a stable anesthesia plane prior to the experiment.
Multi-unit activity (MUA) and local field potentials (LFP) were recorded with a linear 32-channel silicon electrode array (NeuroNexus A32, 100 μm spacing between adjacent contacts). Signals were recorded with a digital acquisition system (NeuroNexus SmartBox) at a sampling rate of 30 kHz. The probe was placed into the center of the craniotomy at an angle of 37° from horizontal (angled towards the posterior), and then advanced 6 mm so that the contacts sampled multiple subregions of the hippocampal formation, including CA1, CA3, and the dentate gyrus. Before recording proceeded, verification of correct probe location was performed by confirming the presence of sharp-wave ripples (SWR), the hallmark of hippocampal activity, in the captured electrophysiological traces [42]. Electrophysiological recording commenced 2 min before the onset of ultrasonic stimulation, continued throughout the 3-min stimulation period as well as an additional 5 min post-stimulation, resulting in 10 min data recordings for each experimental condition. A continuous trigger was outputted from either the waveform generator or integrated FUS system to the digital acquisition system throughout stimulation to mark tFUS onset.

2.6. Spike sorting

We employed the Kilosort 2 [43] software running on Matlab (Mathworks, Release 2019a) to perform semi-automated spike detection and sorting. This technique forms a generative model of the extracellular voltage, learns a spatiotemporal template of each spike waveform based on the singular value decomposition, and employs multiple passes through the data to yield clusters of spikes. We employed most default parameters provided by the developers, as described in the standard configuration file provided at github.com/MouseLand/Kilosort. We employed a high pass filter cutoff of 400 Hz to exclude slow activity and in particular SWR, lowered the detection threshold from 6 to 5 standard deviations, and doubled the default batch size to improve the learning algorithm for the spatiotemporal template. Spike detection and sorting was performed separately for each animal, with recordings chronologically assembled into a single data record prior to processing. The results of the automated procedure were imported into the Phy software [44], an open source Python library and graphical user interface for manual curation of large-scale electrophysiological data. Following the procedures recommended by the
developers of Kilosort [45], curation of the automated sorting results was performed to discard units deemed to be non-biological. Based on the probe’s 100 μm spacing, any units whose spikes appeared at more than three contacts were rejected. Units with grossly non-biological mean waveforms were rejected. Units whose spike amplitude distributions were strongly non-Gaussian were rejected. Within each retained unit, spikes violating the refractory period (2 ms) were removed by examining the auto- and cross-correlograms. Spikes appearing as clear outliers in feature space were also removed. After completing the manual curation procedure, n = 392 units were retained for MUA analysis in the primary cohort (FUS HPC and FUS OLF), and n = 260 units were obtained from the secondary cohort (FUS AIR).

2.7. Time resolved firing rate

In order to investigate the dynamics of hippocampal firing, we binned time in non-overlapping 5 s intervals (120 windows during the 10 min recording period). The mean firing rate was measured within each 5 s window. To account for the varying baseline rates across the sample of units, we performed baseline correction of the firing rate time series. This was achieved by computing the average firing rate of the first 24 windows for each unit (the baseline period was 120 s), and then subtracting the resulting basal rate from all windows.

2.8. Baseline LFP measurement

In order to investigate the role of baseline brain state on neurope-modulation outcome, we computed the mean LFP power in the following frequency regions: delta (1–3 Hz), theta (4–10 Hz), and gamma (30–250 Hz). For each MUA unit, we determined the electrode contact best expressing the unit’s spikes by searching for the channel with largest spike amplitude. We then measured the logaritically transformed LFP power during the 2 min pre-stimulation period at the identified contact. This formed the independent variable whose influence on firing rate was probed throughout the main text. When delineating “low” and “high” baseline LFP power, we partitioned the units into two groups with assignment based on the LFP power relative to the median power across all units. This assignment was performed separately for each frequency band.

2.9. Sharp wave ripple detection

In order to measure the prevalence of sharp wave ripples (SWRs) during the pre-stimulation period, we followed the detection algorithm described previously by Levenstein et al. [46]. Sharp waves were detected when the power of the contact-averaged LFP in the 2–50 Hz band exceeded 2.5 standard deviations of the mean power. Only segments exceeding 20 ms were retained. Ripples were detected when the contact-averaged LFP power in the 80–249 Hz band exceeded 2.5 standard deviations of the mean power, with a minimum segment length of 25 ms. Samples that passed both the sharp wave and ripple detectors were then designated as SWRs. The proportion of time “spent” in SWR then served as the independent variable in the subsequent analysis of the gating of firing rate by the prevalence of SWR. In order to define periods of rare and frequent SWR, we performed a median split on the percentage of samples passing the SWR detector. Units that did not show any SWRs were excluded from the analysis.

2.10. Analysis of tFUS effects on LFP power

To probe the effects of tFUS on LFP power in the stimulated region, signals were analyzed offline using custom Matlab scripts (Matworks, Release 2019a). Data was bandpass filtered to the 1–250 Hz band with a second-order Butterworth filter and then downsamped to 500 Hz. A series of notch filters were then applied to remove 60 Hz noise and its first four harmonics. Robust principal components analysis (robust PCA) [47] was employed to remove gross artifacts by decomposing the observed data matrix into low-rank and sparse components. Due to the smoothness of volume conducted signals, the sparse component is expected to be artifactual and was thus removed from the data. Data were then transformed into the frequency domain by performing Thomson multi-taper spectral analysis with a time-bandwidth product of 4. All spectra were normalized by the total spectral power measured in the pre-stimulation period of each animal’s first condition to account for varying power levels between animals. Spectra were further resampled in the frequency domain to 1000 samples between 1 and 250 Hz via linear interpolation. Spectral powers for any frequency greater than 57 and less than 63 Hz were marked as missing data due to possible contamination from line noise. Spectral powers were then logarithmically transformed, averaged across contacts within the hippocampal region (two contacts outside of the region were excluded), and then averaged across the appropriate segment of time (baseline stimulation, post-stimulation). The dependent variable was formed as the difference in LFP power between the stimulation (or post-stimulation) and baseline segments, measured frequency-wise. Note that averaging of the LFP signal across contacts was performed when probing tFUS induced changes in LFP power (to minimize the number of statistical comparisons), but not when examining gating of firing rate by the baseline LFP.

2.11. Statistical testing

Unless otherwise specified, testing for statistical significance was carried out by comparing the dependent variable measured under active stimulation (either FUS HPC or FUS OLF) against that observed with null stimulation (FUS AIR). When testing for significant differences in time-averaged firing rate change (e.g. Fig. 2D and E), we employed two-sample, two-tailed t-tests. To increase statistical power when performing the multiple tests of time-resolved firing rate (e.g. Fig. 2B and C; one test for each time window), we employed a permutation test where the assignment of the “treatment” (either FUS OLF or FUS HPC) and “control” (FUS AIR) was randomly shuffled. By repeating the randomization over 1000 iterations, a mock distribution of the instantaneous firing rate was formed at each time sample. The p-value was then computed as the proportion of the 1000 resamples whose absolute firing rate exceeded the true absolute firing rate (i.e., the one measured with the correct assignment of condition). The resulting p-values were then corrected for multiple comparisons by controlling the FDR at 0.05. Baseline correction of each unit’s time series was performed prior to testing, as described above.

When probing significant discrimination of firing rate change by the baseline LFP power (Fig. 3), we measured Spearman rank correlation between the baseline LFP power and associated firing rate change, and obtained the p-value from the theoretical distribution for the rank correlation coefficient under zero correlation. When probing the influence of SWR prevalence on firing rate across tFUS periods (Fig. 5), we performed two-tailed, two-sample t-tests on the firing rate change distributions of the low and high SWR groups.

When probing tFUS-induced changes to the LFP power spectrum during and after stimulation (Fig. 7), a non-parametric test
scrambling the assignment of treatment and control groups was employed, with 1000 permutations employed to estimate the mock distribution of LFP power spectral changes at each frequency (1–250 Hz with a resolution of 0.25 Hz). Cluster correction was then employed to correct for multiple comparisons [48]. Namely, clusters of $n_{\text{C}} \geq 7$ consecutive significant frequency samples (approximately 2 Hz) were retained for second-level testing. For each retained cluster, the change in LFP power was averaged over the cluster’s frequency range, and a final non-parametric test ($n = 1000$ permutations estimating the mock change at that cluster) was employed to test for corrected significance.

3. Results

In order to determine the effects of tFUS on hippocampal neuron spiking, as well as its relationship to baseline neural activity, we recorded multi-unit activity (MUA) and local field potentials (LFP) from the hippocampal region before, during, and after the application of 180 s of continuous-wave (CW) tFUS at two intensities (13 mW/cm$^2$, 52 mW/cm$^2$). For each intensity, we stimulated both the contralateral olfactory bulb (herein referred to as “FUS OLF”) as well as the hippocampal region (“FUS HPC”). To account for spontaneous firing rate changes in the absence of exogenous stimulation, we also recorded the LFP and MUA from a separate cohort of animals where an air gap was introduced between the transducer and the skull, thus preventing any sonication of the brain (“FUS AIR”). Two separate transducers, whose beampatterns are depicted in Fig. 1A, were used throughout the experiments. Concurrent electrophysiological recording and tFUS was achieved by inserting the electrode at an angle (Fig. 1B).

Semi-automated spike sorting identified a total of $n = 392$ MUA units from the primary tFUS cohort (FUS OLF and FUS HPC), and $n = 260$ units from the secondary cohort (FUS AIR). Average firing rates were reproducible across the three main experimental conditions: $1.98 \pm 0.23$ Hz for FUS AIR, $2.12 \pm 0.14$ Hz for FUS OLF, and $1.96 \pm 0.12$ Hz for FUS HPC (means $\pm$ sems across units, all pairwise comparisons between average firing rates failed to reject the null hypothesis, $p > 0.39$, two-sample t-tests.)
3.1. Significant firing rate changes at 52 mW/cm²

The time course of firing rate was measured in non-overlapping 5 s windows, and is shown for FUS AIR in Fig. 2C. Notice that in the absence of exogenous stimulation, a slight decreasing trend was observed, likely stemming from the changing anesthesia plane. At the lower acoustic intensity of 13 mW/cm², we did not resolve significant changes in firing rate for FUS OLF relative to FUS AIR (Fig. 2B, light red). During FUS HPC at 13 mW/cm², only a few windows of significantly increased firing rate were observed near the middle segment of the sonication (Fig. 2C, light red, significance indicated by diamonds near horizontal axis).

Stimulation of the contralateral olfactory bulb at 52 mW/cm² also did not yield any significant changes in firing rate during sonication (Fig. 2B, dark red). On the other hand, 52 mW/cm² FUS HPC produced a significant increase in firing rate that was resolved throughout the majority of the stimulation period (p < 0.05, $n_{	ext{hpc}} = 392$, $n_{	ext{air}} = 260$, permutation test scrambling the assignment of units between treatment and control groups, corrected for multiple comparisons by controlling the FDR to 0.05; Fig. 2C, dark red). Notably, significance was observed immediately at stimulation onset (120 s), and the magnitude of the increase waned towards the end of the 3-min stimulation. The firing rate also tended to decrease following sonication, perhaps reflecting a compensatory response following tFUS.

Averaged over the 3-min sonication period, the effect of FUS OLF (13 mW/cm²: $-0.025 \pm 0.033$ Hz; 52 mW/cm²: $-0.21 \pm 0.04$ Hz) did not differ significantly from FUS AIR ($-0.13 \pm 0.05$ Hz) at either intensity ($p = 0.17$ and $p = 0.29$ at 13 and 52 mW/cm², respectively, two-sample t-test; Fig. 2D). A significant time-averaged firing rate increase during FUS HPC was resolved for both intensities, although the effect was much larger at 52 mW/cm² (13 mW/...
cm²; \( +0.054 \pm 0.064 \text{ Hz, } p = 0.031 \); 52 mW: \( +0.21 \pm 0.05 \text{ Hz, } p = 2 \times 10^{-3} \) (Fig. 2E).

From the shape of the firing rate change distribution in Fig. 2E (FUS HPC), tFUS produced an “all-or-nothing” phenomenon, where many units were unperturbed (tall narrow peak near 0), while others experienced a large increase in firing (long fat tail). Note the presence of the inflection in the histogram above 2 Hz, indicating that these units doubled their firing rate (baseline rate was approximately 2 Hz). Thus, even though the average change was modest, the change in the affected units was pronounced.

Two transducers were employed in the data collection (see Fig. 1A). To determine whether the nature of the transducer had a qualitative effect on the observed neuromodulation, we separated the units according to the identity of the transducer employed in the respective experiment. We found no significant differences in firing rate change for both FUS HPC (mean firing rate change with transducer 1 at 32 mW/cm²: \( +0.20 \pm 0.064 \text{ Hz, } n = 220 \), transducer 2: \( +0.23 \pm 0.063 \text{ Hz, } n = 172 \), \( p = 0.44 \)) or FUS OLF (\( -0.25 \pm 0.051 \text{ Hz, } -0.16 \pm 0.052 \text{ Hz, } p = 0.83 \)).

Having established significant firing rate modulations in the hippocampal region during 52 mW/cm² tFUS, we proceeded to determine whether the modulations were “gated” by pre-stimulation neural activity.

### 3.2. tFUS neuromodulation is gated by baseline LFP power

We suspected that the effect of tFUS on spiking is influenced by the state of the neuronal population prior to stimulation, in particular the level of synaptic activity. To test this hypothesis, we measured the LFP power spectrum during the 2-min baseline period leading up to stimulation, calculating the strength of neural oscillations at the contact that most strongly registered the spikes of each unit (see Methods). We separately considered the delta (1–3 Hz), theta (4–10 Hz), and gamma (30–250) frequency bands. To test whether baseline rhythms modulate the neuronal response to tFUS, we calculated the Spearman rank correlation between baseline LFP power and the corresponding change in firing rate (Fig. 3).

We did not find significant correlation between baseline LFP power and the spontaneous firing rate changes during FUS AIR (delta: \( p = -0.02 \), \( p = 0.52 \), \( n = 780 \); theta: \( p = -0.04 \), \( p = 0.27 \); gamma: \( p = 0 \), \( p = 0.91 \)) (Fig. 3A,B,C). To our surprise, we did detect significant correlation between baseline LFP power and firing rate change during FUS OLF, with delta (\( p = 0.11 \), \( p = 0.02 \), \( n = 392 \); Fig. 3D), theta (\( p = -0.13 \), \( p = 0.01 \); Fig. 3E), and gamma oscillations (\( p = -0.18 \), \( p = 3 \times 10^{-4} \); Fig. 3F) all producing mild but significant modulation of the firing rate change during stimulation. We speculate that stimulation of the olfactory region, which is strongly connected to the hippocampus, may have facilitated this effect (see Discussion).

The strongest influence of baseline LFP power was found during FUS HPC, where significant correlations were observed for all three bands: (delta: \( p = -0.20 \), \( p = 5 \times 10^{-5} \); theta: \( p = 0.17 \), \( p = 8 \times 10^{-4} \); gamma: \( p = 0.22 \), \( p = 1 \times 10^{-5} \)) (Fig. 3G,H,I). From the sign of the correlations, successful neuromodulation was promoted by low levels of delta power, high levels of theta power, and high levels of gamma power, with the strongest correlation observed for gamma power.

In order to determine the LFP frequencies most conducive to successful tFUS, we measured the rank correlation between LFP power and firing rate change in increments of 1 Hz. As expected, the level of gating for FUS AIR was near 0 across the entire spectrum (Fig. 4A). An interesting pattern emerged for FUS OLF and FUS HPC, where the shape of the rank correlation curves tended to mirror each other. Both curves showed broad minima/maxima over the gamma frequency range (Fig. 4A). For FUS HPC, the largest gating effects were found near 130 Hz (62 Hz for FUS OLF) as assessed by squaring the rank correlation (Fig. 4B). Notably, at high gamma frequencies (>130 Hz), there was a pronounced increase in the gating effect for FUS HPC, leading to a substantial increase in the overall area under the curve relative to both FUS AIR and FUS OLF (Fig. 4C).

### 3.3. tFUS neuromodulation is enhanced during periods of SWR

Under conditions of urethane anesthesia and natural sleep, the hippocampus alternates between periods of spiking and inactivity. The active periods manifest in the LFP by sharp wave ripples (SWRs), short bursts of high-frequency activity [41,46]. Given that SWRs denote an excitabile state, we suspected that tFUS would result in stronger responses when applied during periods of frequent SWR. To test this, we conditioned firing rate change on the prevalence of SWR during the 2 min leading up to stimulation, dividing units into two groups depending on the amount of SWR observed during their baseline periods (i.e., a median split).

The prevalence of SWR did not have a significant effect on the spontaneous changes measured during FUS AIR (\( p = 0.21 \), t-test, \( n_{\text{low}} = 369 \), \( n_{\text{high}} = 160 \); Fig. 5A). Similarly, the outcome of FUS OLF was not significantly modulated by SWR prevalence (\( p = 0.34 \), \( n_{\text{low}} = 173 \), \( n_{\text{high}} = 106 \); Fig. 5B). On the other hand, the effect of FUS HPC on firing rate varied significantly with the amount of SWR in the pre-stimulation period: the firing rate increase was \( 0.54 \pm 0.10 \text{ Hz when preceded by frequent SWR, and only } 0.075 \pm 0.049 \text{ Hz when preceded by infrequent SWR (} p = 4 \times 10^{-6} \), \( n_{\text{low}} = 169 \), \( n_{\text{high}} = 101 \); Fig. 5C), representing a seven–fold increase. This suggests that the success of tFUS is linked to the level of excitability in the stimulated region.

To examine the gating effect of SWR further, we measured firing rate time series separately for low and high baseline SWR (Fig. 6). As expected, firing rate during FUS AIR was not modulated by baseline SWR, with the time series exhibiting a large amount of overlap (Fig. 6A). Although punctate regions of time did show significant differences between low and high SWR for FUS OLF, many of these were observed prior to stimulation (Fig. 6B, diamonds on the horizontal axes depict significant differences between low and high SWR, assessed by a non-parametric test scrambling the assignment of low and high SWR, corrected for multiple comparisons by controlling the FDR at 0.05). In contrast, the level of baseline SWR exhibited a large modulation of time-resolved firing rate during FUS HPC, where a marked elevation of firing was observed shortly after sonication onset, and was sustained for 7 windows of the stimulation window, with all occurring during the first half of tFUS (Fig. 6C).

### 3.4. tFUS modulates LFP power

The LFP provides a complementary measure of neural activity, reflecting the overall synaptic activity at the recorded region. We probed changes in LFP power spectrum during and after sonication. Sample traces of the LFP are shown in Fig. 7A for FUS OLF and Fig. 7B for FUS HPC. As expected, we did not find significant changes in LFP power at any frequency during 13 mW/cm² stimulation, either during or after stimulation (Fig. 7C). Consistent with the findings of the MUA analysis, tFUS produced significant changes in LFP power at 52 mW/cm². Compared to FUS AIR, FUS HPC increased power in the theta band during and after stimulation (significant cluster between 3 and 10 Hz during stimulation, \( 8–13 \text{ Hz after stimulation, } n_{\text{base}} = 18 \), \( n_{\text{post}} = 20 \), frequency wise t-tests, cluster corrected for multiple comparisons using a permutation test; Fig. 7D). Interestingly, FUS OLF led to a significant reduction in gamma power during
stimulation (significant clusters at 71–77 Hz, 165–166 Hz, and 228–230 Hz; Fig. 7D).

4. Discussion

Neuromodulation techniques capable of directly evoking neuronal firing are referred to as “super-threshold”. Examples of these are transcranial magnetic stimulation, deep brain stimulation, and optogenetics. On the other hand, sub-threshold techniques such as transcranial direct current stimulation (tDCS) do not directly evoke firing but rather bring the membrane potential closer to (or further from) the threshold for action potential initiation. Our findings suggest that, at the intensities tested here (13–52 mW/cm²), tFUS belongs to the subthreshold category. Only a subset of the measured units were modulated, with a key determinant of response being the state of the unit prior to stimulation.

The significant influence of pre-stimulation rhythms on the resulting neuromodulation outcome suggest that concurrent synaptic input is a key ingredient of successful neuromodulation with focused ultrasound. LFP power reflects the amount of coherent synaptic input into the region [49], while SWRs are highly synchronous events marked by coordinated firing across many neurons [41]. Notably, SWRs are associated with a transient increase in hippocampal excitability [50], consistent with our finding of an enhanced response to tFUS during periods of frequent SWR. tFUS led to significantly larger effects on spiking in the presence of strong gamma rhythms and frequent SWRs, with the latter effect being a seven-fold increase. The term “gating” denotes that without a sufficient level of synaptic drive into the stimulated cell, tFUS may not produce a change in spiking rate. Provided a sufficient level of baseline excitability, however, ultrasonic stimulation is capable of...
Fig. 6. Time course of firing rate is modulated by SWR prevalence. The baseline corrected firing rate time series are shown separately for units whose baseline periods exhibited low (black) and high (red) SWR prevalence, as assessed by performing a median split. (A) The firing rate time series of FUS AIR was not modulated by the prevalence of SWR. (B) Punctate regions of time showed significantly different firing rates for low and high SWR during FUS OLF, although the mean rates were largely overlapping across time. (C) During FUS HPC, a large elevation in firing rate was observed near onset of sonication, but only when preceded by frequent SWR. This increase was most prominent in the first half of the stimulation period. In all plots, diamonds on the horizontal axes depict significant differences in firing rate between low and high SWR, assessed by a non-parametric test scrambling the assignment of low and high SWR, corrected for multiple comparisons by controlling the FDR at 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 7. tFUS modulates LFP power. (A) Traces of wide band (1–250 Hz) LFP before, during, and after FUS OLF at 52 mW/cm² for a sample animal (grey area indicates sonication). (B) Same as (A) but now shown for FUS HPC, where elevated power is apparent during tFUS. (C) Vertical axes depict the change in LFP power across frequency (horizontal axes). Relative to FUS AIR (black), no significant changes were found at 13 mW/cm² with either FUS OLF (black) or FUS HPC (red), either during (top panel) or after stimulation (bottom). (D) Compared to FUS AIR, FUS HPC increased power in the theta band during and after stimulation (significant cluster between 8 and 10 Hz during stimulation, 8–13 Hz after stimulation, n_{hpc} = 18, n_{air} = 20, frequency wise permutation test, cluster corrected for multiple comparisons using a permutation test). We also found decreased gamma power during FUS OLF (71–77 Hz, 165–166 Hz, and 228–230 Hz). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
modifying firing rates of the stimulated neurons, and as a consequence, downstream neurons to which these cells project.

Our finding that high baseline theta and gamma power promote firing rate increases are aligned with a recent in vitro study reporting that ultrasound potentiates firing during periods of high synaptic currents [39]. The same study also found that tFUS reduced firing during low input periods—we did not observe an analogous observation in the present data, as low baseline theta and gamma powers generally resulted in an absence of neuromodulation (Fig. 3). The drastically different ultrasonic parameters between the two studies (i.e., a three order of magnitude difference in intensity) is one possible explanation for this discrepancy. Motivated by similar hypotheses in electrical stimulation, one approach that has been employed in tDCS is to pair the stimulation with a task that engages the stimulated area [51]. Indeed, a recent combined MRI-FUS study found that the effect of stimulation on the blood-oxygenation-level dependent (BOLD) signal varied depending on whether the region was task-active [40]. Future tFUS studies that combine behavioral interventions with stimulation may further solidify the role of concurrent input on neural outcomes. Furthermore, it is possible that at higher acoustic intensities, the effect of tFUS may become super-threshold, and especially if a local temperature increase is produced at the sonicated region.

One surprising finding of this study was the presence of gating effects during tFUS of the contralateral olfactory bulb. In particular, we found stronger hippocampal firing rate decreases when stimulating the olfactory bulb during periods of reduced delta and increased theta and gamma power. Despite the off-target stimulation, it is possible that some amount of neuromodulation was produced as a result of the rodent brain’s connectivity. Namely, the olfactory bulb is strongly connected to the hippocampus [52,53]. The level of LFP power at the hippocampal region could have mediated indirect effects from stimulating off-target regions. Note that overall, olfactory tFUS did not produce a significant change in hippocampal firing rate (Fig. 2D). Furthermore, whereas elevated theta and gamma power led to larger firing rate increases during FUS HPC, the gating effect during FUS OLF manifested as a larger decrease in firing rate.

The majority of recent tFUS investigations have employed brief sonications, typically in the tens to hundreds of milliseconds [2], with stimulation applied between relatively long intertrial intervals. An advantage of this approach is that it affords an increase in statistical power, as the evoked response may be time locked and averaged over many repeated trials. Nevertheless, previous investigations that have instead employed single sonications with long duration have shown promising effects. For example, 40 s of tFUS to the primate brain was found to produce a long-lasting effect on functional connectivity [18]. A 20-min application of tFUS in the rat was shown to shorten the time required to recover from ketamine-xylazine anesthesia [54]. Three minutes of tFUS was shown to reduce epileptic discharges when applied after the onset of chemically-induced seizures [55]. Here we were able to resolve modest but significant neuronal responses during 3 min of tFUS, showing that the state of the neurons leading up to stimulation is a key determinant of successful neuromodulation.

Whereas this study employed long sonication periods, our ultrasonic intensities (up to 52 mW) fall into the low end of the intensities used in tFUS investigations [2]. In combination with a recent report showing tFUS effects at intensities of 53 mW [56], it appears that ultrasonic neuromodulation is feasible at very modest acoustic power. Such findings promote the safety of tFUS in human applications. Indeed, the acoustic intensities employed in this study are well below the FDA safety guidelines for ultrasonic imaging: 720 mW/cm². Furthermore, the mechanical index employed here (<0.02) is also an order of magnitude lower than the 0.3 suggested to be the upper limit of diagnostic imaging. Given the excellent safety profile of low-intensity ultrasound in imaging, it is very likely that the stimulation investigated here is safe. Moreover, recent studies that have tested tFUS intensities much higher than here (up to 25.8 W/cm²) reported no tissue damage as assessed by histological assessment of post-mortem brain tissue [57]. The fact that neuromodulation was observed here at such low acoustic intensities is encouraging, as it implies that testing the effects of tFUS in humans may be carried out at power levels that are currently used in human ultrasound imaging practice, and thus unlikely to produce tissue damage.

Finally, a limitation of this study is the exclusive use of anesthetized animals. The state changes inherent to sleep and anesthesia are well-suited to investigating the gating of tFUS effects by baseline brain activity. Nevertheless, future studies are needed to understand the role of endogenous rhythms in the awake state on neuronal responses to focused ultrasound.

Author contributions

DN collected data, analyzed data, and wrote the paper.
DB collected data.
EK designed the experiments.
JPD designed the experiments, collected data, analyzed data, and wrote the paper.

Declaration of competing interest

The authors declare no financial or personal relationships with any individuals or organizations that could inappropriately influence this work.

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