Journal of Neuroscience Research 00:00–00 (2016)

Prolonged Stimulation With Low-Intensity Ultrasound Induces Delayed Increases in Spontaneous Hippocampal Culture Spiking Activity

Hyun-Bum Kim,[1] Kelley M. Swanberg,[2] Hee-Sok Han,[3] Jung-Chae Kim,[4] Jun-Woo Kim,[5] Sungon Lee,[6] C. Justin Lee,[7] Sungho Maeng,[2] Tae-Seong Kim,[3] and Ji-Ho Park[2]⋆

[1]Department of East-West Medical Science, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea
[2]Department of East-West Medicine, Graduate School of East-West Medical Science, Kyung Hee University, Yongin, Republic of Korea
[3]Department of Biomedical Engineering, Kyung Hee University, Yongin, Republic of Korea
[4]Biometrics Team, CTO Future IT Laboratory, LG Electronics Umyeon R&D Campus, Seocho-gu, Republic of Korea
[5]Division of Polar Logistics, Korea Polar Research Institute, Incheon, Republic of Korea
[6]School of Electrical Engineering, Hanyang University, Ansan, Republic of Korea
[7]Center for Neuroscience and Functional Connectomics, Brain Science Institute, Korea Institute of Science and Technology, Seoul, Republic of Korea

Ultrasound is a promising neural stimulation modality, but an incomplete understanding of its range and mechanism of effect limits its therapeutic application. We investigated the modulation of spontaneous hippocampal spike activity by ultrasound at a lower acoustic intensity and longer time scale than has been previously attempted, hypothesizing that spiking would change conditionally upon the availability of glutamate receptors. Using a 60-channel multielectrode array (MEA), we measured spontaneous spiking across organotypic rat hippocampal slice cultures (N = 28) for 3 min each before, during, and after stimulation with low-intensity unfocused pulsed or sham ultrasound (spatial-peak pulse average intensity 780 $\mu W/cm^2$) preperfused with artificial cerebrospinal fluid, 300 μM kynurenic acid (KA), or 0.5 μM tetrodotoxin (TTX) at 3 ml/min. Spike rates were normalized and compared across stimulation type and period, subregion, threshold level, and/or perfusion condition using repeated-measures ANOVA and generalized linear mixed models. Normalized 3-min spike counts for large but not midsized, small, or total spikes increased after but not during ultrasound relative to sham stimulation. This result was recapitulated in subregions CA1 and dentate gyrus and replicated in a separate experiment for all spike size groups in slices pretreated with aCSF but not KA or TTX. Increases in normalized 18-sec total, midsized, and large spike counts peaked predominantly 1.5 min following ultrasound stimulation. Our low-intensity ultrasound setup exerted delayed glutamate receptor-dependent, amplitude- and possibly region-specific influences on spontaneous spike rates across the hippocampus, expanding the range of known parameters at which ultrasound may be used for neural activity modulation.

SIGNIFICANCE

Ultrasound is a promising, noninvasive brain stimulation modality with millimeter-level spatial resolution, millisecond-level temporal resolution, and the option for focused subcortical penetration. Its safe application to human patients, however, depends on our thorough understanding of the range of parameters at which it may exert physiological effects. Here we show that unfocused ultrasonic stimulation of cultured rat hippocampal slices enhances spontaneous spike activity at a lower intensity (spatial peak pulse average 780 $\mu W/cm^2$) and over a longer time course (3 min poststimulation) than previously reported, suggesting the need for further study of a range of stimulation parameters wider than those examined to date.

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: National Research Foundation of Korea (NRF); Contract grant number: MEST 2014R1A2A2A09052449; Contract grant sponsor: Gachon University of Medicine and Science Gil Medical Center; Contract grant number: 2013-36

⋆Correspondence to: Ji-Ho Park, PhD, Department of East-West Medicine, Graduate School of East-West Medical Science, Kyung Hee University, 732 Deogyeong-daero, Giheung-gu, Yongin 446-702, Republic of Korea. E-mail: jihopark@khu.ac.kr

Received 18 October 2015; Revised 3 June 2016; Accepted 3 July 2016

Published online 00 Month 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23845

Key words: unfocused pulsed ultrasound; spontaneous activity; spike detection; hippocampus; multielectrode array; electrophysiology

In 1942, 835-kHz "supersound" focused into the cortex was shown to disrupt neural function in the living mammal (Lynn et al., 1942); by 1958, it had been demonstrated that these effects could be both focused subcortically and reversed (Fry, 1958). Further investigations of frequencies as low as 250 kHz (Tufail et al., 2011) have impelled recognition that ultrasound, with its millimeter-level spatial resolution, millisecond-level temporal resolution (Muratore, 2012), and deep but noninvasive penetration of brain tissues (Tyler, 2011; Yoo et al., 2011), may be an attractive neurostimulatory device. Consequently, in addition to its use in thrombolysis (Hitchcock and Holland, 2010) and ablative neurosurgery (Martin et al., 2009; Lipsman et al., 2013), ultrasound has been proposed as a means to modulate neural activity therapeutically in epilepsy (Yang et al., 2011), traumatic brain injury (Demirtas-Tatlidede et al., 2012; Villamar et al., 2012), and Parkinson's disease (Strauss et al., 2014).

Although the mechanisms behind ultrasound-mediated neural stimulation are still unclear, a few mutually compatible factors have been suggested, including tissue hyperthermia (Bachtold et al., 1998), membrane potential alteration by pericellular fluid mechanics (Tyler, 2011), mechanosensitive ion channel stimulation (Tyler, 2011), neurotransmitter release (Tyler et al., 2008), and tissue cavitation (Muratore, 2012). In contrast to the variety of hypotheses about the possible mechanisms of neural stimulation by ultrasound, the body of existing research in this field has examined the phenomenon within only a narrow range of possible parameters. For example, although ultrasound's effects on evoked potentials have been previously examined on time scales on the order of several minutes (Bachtold et al., 1998), investigations of spontaneous activity have been confined to postexposure times on the order of milliseconds (Tyler et al., 2008; Tufail et al., 2010; Muratore, 2012) or a maximum of 1 min (Khraiche et al., 2008). Furthermore, the lowest-intensity reported systematic investigation of ultrasonic stimulation of neural activity reported a minimum I_{SPPA} of 75 mW/cm^2 (Tyler et al., 2008; Tufail et al., 2010, 2011), despite previous preliminary evidence showing neurophysiological effects at intensities less than 0.3% of this value (Han, 2014). It is important to experiment with ultrasound on a greater variety of time scales and intensities, for investigations of ultrasound at a broader variety of parameters than those currently published may provide insights into mechanisms and possible applications that continue to elude our grasp.

In this study, we used a 60-channel multielectrode array (MEA) to measure spontaneous population spike activity in dentate gyrus (DG), CA3, CA2, and CA1 in organotypic hippocampal slice cultures from 7-day-old Sprague-Dawley rats before, during, and after 3 min of stimulation with centered unfocused pulsed ultrasound (center frequency 0.5 MHz, pulse duration 2.097 μsec, pulse repetition frequency 1.16 kHz) or sham. We hypothesized that, even at the low power of our setup (780 $\mu W/cm^2$ spatial-peak pulse-average intensity), physiological influences of stimulation would manifest as time-dependent alterations in the rate of spontaneous spiking activity in tissues exposed to ultrasound but not sham stimulation. These changes in spike rate would, in turn, be abolished by kynurenic acid (KA)-mediated glutamate receptor inhibition, suggesting the possible involvement of intracellular responses to glutamatergic activity.

MATERIALS AND METHODS

Ultrasound Wave Intensity Analysis

The ultrasound-induced acoustic field intensity profile was obtained by using a calibrated hydrophone (HNR-500, ONDA Corporation, Sunnyvale, CA) linked with a three-axis linear motorized stage (T-XYZ-LSM150A; Zaber Technologies, N. Vancouver, British Columbia, Canada) in degassed water. Hydrophone signals were read by oscilloscope (U2701A; Agilent Technologies, Santa Clara, CA). The ultrasound transducer was placed in the tank facing the hydrophone at 5 mm. Using a custom LabView (National Instruments, Austin, TX) program, we synchronized stage movement, transducer excitation, and data acquisition to scan a $20 \times 20 \times 8$ mm region at 500-μm resolution.

Spatial peak pressure amplitude was measured to be 11.52 kPa; spatial-peak pulse-average intensity (I_{SPPA}) was calculated from pressure signals per industry standards (NEMA, 2004). The x and y dimensions of focus of the half-pressure maximum was approximately 8.5×8.5 mm, within which the I_{SPPA} came to 780 $\mu W/cm^2$ (Fig. 1).

Organotypic Hippocampal Slice Culture Preparation

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP[SU]-13-03). Organotypic hippocampal slice cultures were prepared based on the work of Stoppini et al. (1991) in a closed room with autoclaved equipment. Immediately following decapitation, the brains of 7-day-old Sprague-Dawley rats (N = 28) were removed and soaked in ice-cold HBSS with 20 mM HEPES (Sigma, St. Louis, MO). The frontal cortex and cerebellum were excised, and the hippocampi were isolated and tissue was chopped (Mickle Laboratory Engineering Co., Surrey, United Kingdom) every 350 μm. Each slice was placed onto a 4.0-μm polytetrafluoroethylene membrane insert (Millicell-CM; Millipore Co., Bedford, MA) in a six-well plate of 1 ml 50% minimum essential medium, 25% horse serum, 25% Hanks balanced salt solution (JBI, Daegu, Republic of Korea), 6 g/L D-glucose, 1 mM L-glutamine, 20 mM HEPES (Sigma), and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY) titrated to pH 7.1 with NaOH and HCl. The medium was changed every second day, and cultured slices, incubated at 36 °C in 5% CO_2 and 95% humidity, were used after 14 days.

Microelectrode Array Setup

The 8×8 microelectrode array (MEA; Multi Channel Systems GmbH, Reutlingen, Germany) included an amplifier, four-channel stimulus generator, and two temperature-control units maintaining solution and baseplate at 33 °C within

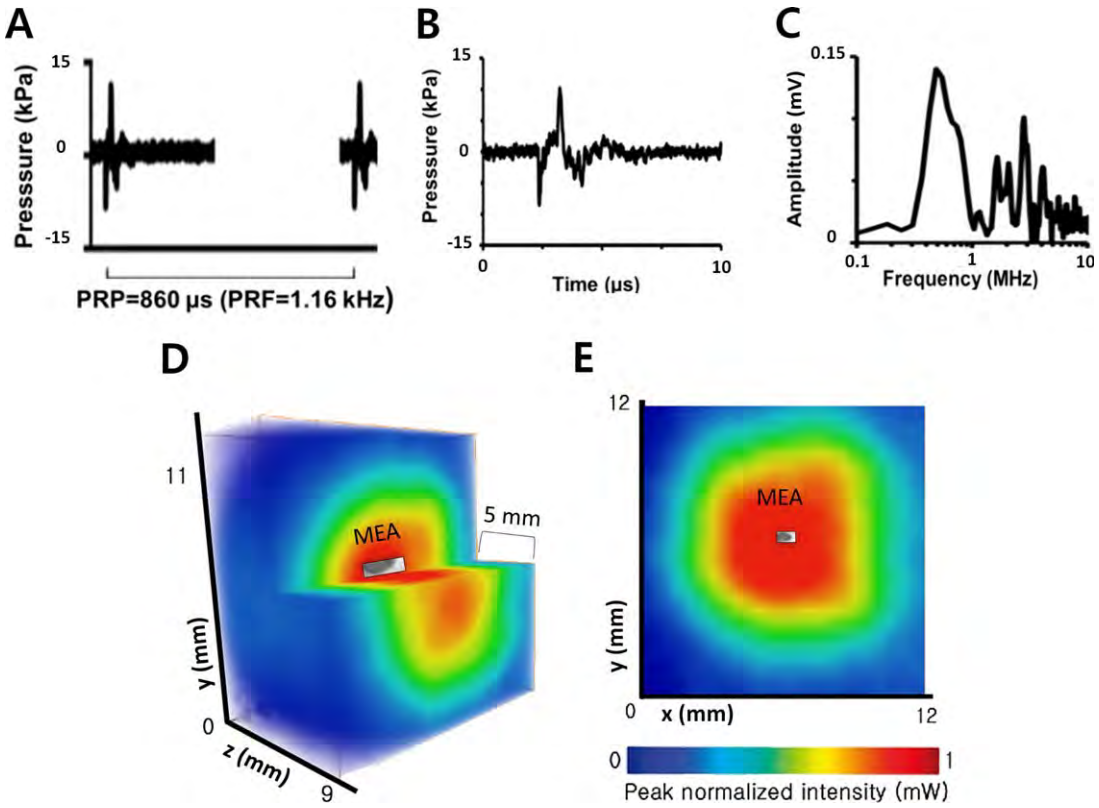

Fig. 1. Ultrasound waveform properties. **A**: The ultrasound stimulus was generated with a pulse repetition period of 860 µsec. **B,C**: Acoustic pressure wave generated by our transducer (B) and fast fourier transform thereof (C) showing 0.5-MHz center frequency and residuals. **D**: Three-dimensional intensity map of peak normalized ultrasound wave intensity. The transducer was placed 5 mm from the sample along the z-axis. **E**: Two-dimensional intensity map of wave intensity in transverse plane. ⋆Pairwise comparison vs. sham $P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

0.01 °C (Egert et al., 1998). The MEA was bathed for 1 hr in 2% ultrasonol 7 (Carl Roth GmbH, Karlsruhe, Germany), brushed, coated with 0.1% polyethylenimine (PEI; Sigma), and UV sterilized for at least 3 hr. Between experiments, probes were cleaned with 2% ultrasonol 7 in distilled water for 30 min, then rinsed and kept in room-temperature distilled water.

One representative slice per rat was presoaked in artificial cerebrospinal fluid (NaCl 124 mM, $NaHCO_3$ 26 mM, glucose 10 mM, KCl 3 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, pH 7.4), centered on the MEA, and perfused in 3 ml/min aCSF. Slices were stabilized for 1 hr at 33 °C, and the slice and MEA array were transferred onto an MEA1060 amplifier interface (1,200 dB gain) and grounded with an Ag/AgCl pellet. Sixty channels of data were sampled at 25 kHz and processed in MC_Rack and MC_Data Tool (Multi Channel Systems GmbH) by desktop computer (Fig. 2A).

Regional Spike Detection

Raw MEA signals were four-order bandpass filtered (300-3,000 Hz) and parsed into spikes with a custom Matlab program (R2011b, Mathworks, Nattick, MA). The threshold for spike detection was set using the formula k⋆median($|X|/0.6745$), where X is the bandpass-filtered signal (adapted from Quiroga et al., 2004) and k represents one of three factors (1, 4.8, and 6) applied to distinguish three threshold ranges of interest (level 1 $1 < k \leq 4.8$, level 2 $4.8 < k \leq 6$, level 3 $k > 6$). Spikes were cut 800 µsec from either side of threshold. Prior to statistical analysis, spikes were summed over one to eight representative channels defined for dentate gyrus, CA3, CA2, and CA1 according to location and baseline spike activity, and level-specific spike counts over 18-sec intervals from experiments 1 and 2 were examined for sudden and unsustained increases of 500% or more over baseline spiking simultaneously across all four subregions, representing the artefactual recording of MEA contact with bubbles. Such cases were replaced with the average of remaining nonartefactual spike counts within that recording period (before, during, or after stimulation) and subtracted accordingly from prestimulation, during-stimulation, and poststimulation totals at all levels. Because of limitations in data collection, this artifact removal process was not applied to the KA treatment group or to one member each of the vehicle-treated and tetrodotoxin (TTX)-treated sham and US groups of experiment 2.

Stimulation Experiments

We used an ultrasound pulser (center frequency 0.5 MHz, pulse repetition frequency 1.16 kHz, pulse duration 2.097 µsec;

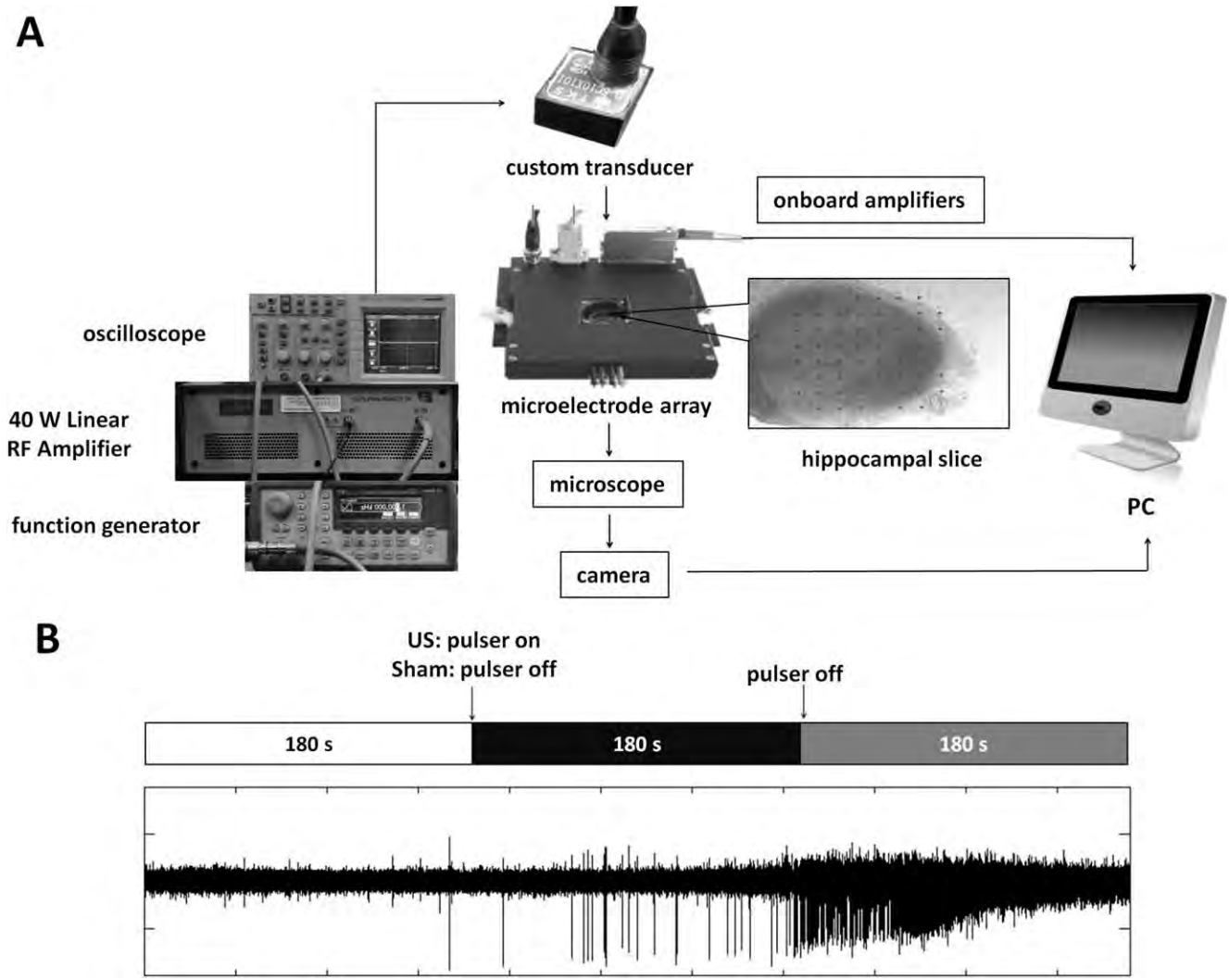

Fig. 2. Ultrasound and sham stimulation experimental setup. **A**: Each hippocampal slice was placed on a 60-channel microelectrode array perfused with artificial cerebrospinal fluid. An ultrasound pulser and custom-made square transducer (100 mm^2) were centered on the fluid surface and transmitted either sound wave pulses of 0.5 MHz center frequency and 1.16 kHz pulse repetition frequency (ultrasound) or no signal (sham). **B**: In each experiment, local slice voltage potentials were recorded via 60-channel microelectrode array for 3 min each before and after 3 min of ultrasound (pulser turned on) or sham (pulser kept off) stimulation.

MKPR-1025; MKC Korea) and water-immersion transducer (10 × 10 mm square crystal element; TKS Co.) centered approximately 5 mm over the MEA in contact with the perfusing aCSF. Parameters were optimized to yield a spatial peak pressure of 11.52 kPa, associated with the smaller of two effective pressures among three previously tested for influence on primary hippocampal neuronal activity (Han, 2014). Additional tests demonstrated that our setup dose dependently influenced spike activity at intensities beyond that applied here (see Supporting Information).

Experiment 1 explored the sustained or delayed modulation of spontaneous spiking by our low-intensity ultrasound setup. After 3 min of baseline recording, ultrasound (n = 4; pulser on) or sham (n = 4; pulser off) stimulation was applied for 3 min, followed by 3 min of recording (Fig. 2B). Experiment 2 examined the glutamate receptor contribution to the ultrasound effect investigated in experiment 1. It was identical in schedule to the prior experiment except that slices were perfused with 300 μM KA as a nonspecific antagonist of glutamate receptor activity, 0.5 μM TTX as a blocker of voltage-gated sodium channels and consequent positive control for attenuated spiking, or aCSF at 3 ml/min for 10 min between baseline measurement and ultrasound or sham stimulation (n = 6 per group). Each trial used cultures from a different rat.

Temperature measurements were made via manual readout of the aforementioned two built-in MEA temperature control units for device and perfusing liquid during a separate trial identical to experiment I without slice culture placement.

Statistical Analysis

Spike totals from dentate gyrus, CA3, CA2, and CA1 were binned by 3-min or 18-sec periods (one or 10 each

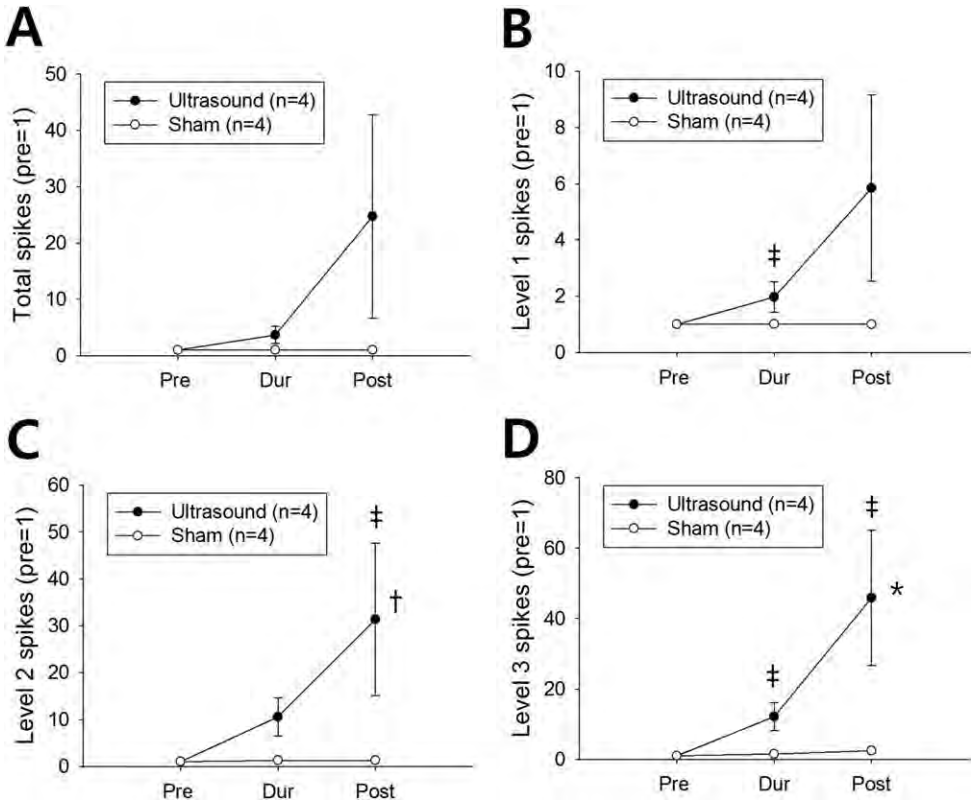

Fig. 3. **A–D**: Ultrasound stimulation alters spontaneous spiking in a threshold-dependent manner. Spikes binned by threshold (levels 1, 2, 3 ascending) and normalized to pre-stimulation baselines in four regions (dentate gyrus, CA3, CA2, CA1) of rat organotypic hippocampal slices measured by microelectrode array for 3 min before ("Pre"), during ("Dur"), and after ("Post") 3 min of unfocused pulsed ultrasound or sham stimulation. Repeated-measures ANOVA for each bin demonstrated a significant treatment effect in level 3 spikes only ($F_{1,6} = 9.735$, $P = 0.021$) during and after stimulation. Treatment effect $\star P < 0.05$, $\dagger P < 0.1$. ‡Pairwise comparison vs. sham $P < 0.1$.

before, during, and after stimulation) and normalized to prestimulation values. When zero baselines precluded division, zero values were normalized as 1 and nonzero values imputed as is. Factor effects and interactions were calculated by using generalized linear mixed models (GLMM) with identity linking unless otherwise noted (Laird and Ware, 1982) or repeated-measures ANOVA with Huynh-Feldt correction (Huynh, 1976) and planned within-subject comparisons relative to baseline. Significance within sets of fewer than three planned comparisons per omnibus test was corrected by Fisher's least significant difference test as recommended previously (Levin et al., 1994), and sets of more than three were corrected for false discovery rates using the step-down Benjamini-Hochberg procedure (Benjamini, 2006). Statistical analyses were performed in the Statistical Package for the Social Sciences 20 (IBM, Armonk, NY). Alpha for significance was set to 0.05 aand, for reported trends, to 0.1.

RESULTS

Artifact Removal and Undefined Value Imputation

In experiment 1 one outlier (one US rat during) and in experiment 2 13 outliers (two TTX sham rats before and during, three TTX US rats before, and four vehicle US rats after and during) were recalculated for all hippocampal subregion and spike levels as described under Regional spike detection. In experiment 1 30 undefined cases of 384 total 3-min and 300 undefined cases of 3,840 total 18-sec spike counts (three sham; three US rats) and in experiment 2 114 undefined cases of 1,728 total 3-min spike counts (five sham vehicle; four sham TTX; four US vehicle; five US TTX; two sham KA; two US KA rats) were imputed as described under Statistical analysis.

Effect of Ultrasound on Total Spike Activity

GLMM with repeated measures region (DG, CA3, CA2, CA1) testing for an effect of between-subject factor stimulation type (ultrasound, sham) and a treatment × region interaction on total regional normalized spike counts showed neither during or after stimulation (Fig. 3A).

Amplitude and Regional Distribution of Ultrasound Effect

Repeated measures ANOVAs with repeated within-subject factor region (DG, CA3, CA2, CA1) and period (during, after stimulation) with between-subject factor stimulation type (ultrasound, sham) on level spike totals demonstrated no effect of treatment on level 1 spikes

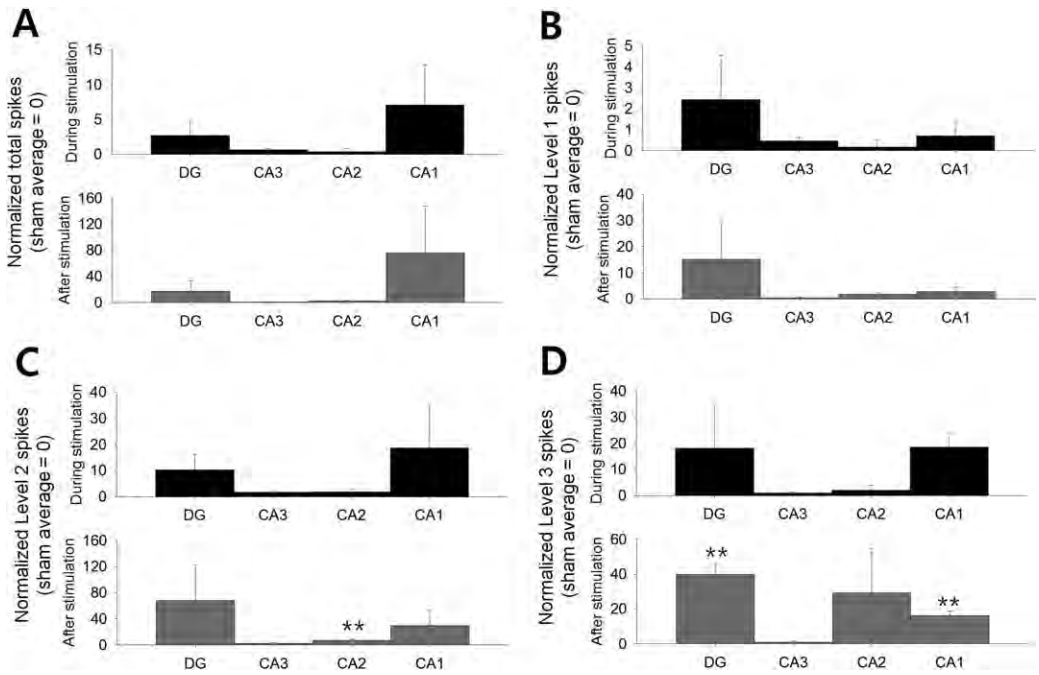

Fig. 4. **A–D**: Ultrasound stimulation alters spontaneous spiking in a region- and threshold-specific manner. Corrected pairwise comparisons following repeated-measures ANOVA omnibus (Fig. 3) demonstrated significantly enhanced normalized spike counts following ultrasound relative to sham stimulation in CA2 for level 2 spikes (mean difference = 11.127 ± 2.045, $P = 0.002$; C) and in DG (mean difference = 32.764 ± 4.954, $P = 0.001$) and CA1 (mean difference = 49.031 ± 7.987, $P = 0.001$) for level 3 spikes (D). **Pairwise comparison vs. sham $P < 0.01$.

($F_{1,6} = 2.506$, $P = 0.164$), a marginal effect of treatment on level 2 spikes ($F_{1,6} = 4.888$, $P = 0.069$), and a significant effect of treatment on level 3 spikes ($F_{1,6} = 9.735$, $P = 0.021$) but no treatment × period or region × treatment × period interactions.

Corrected planned comparisons between ultrasound and sham groups showed no treatment difference in level 1 spiking (ultrasound 3.909 ± 1.691, sham 1.005 ± 0.019; mean difference [MD] = 2.904 ± 1.834, $P = 0.164$), a trend toward higher level 2 spiking (ultrasound 20.967 ± 8.457, sham 1.284 ± 0.142; MD = 19.684 ± 8.903, $P = 0.069$), and significantly higher level 3 spiking (ultrasound 29.018 ± 10.138, sham 1.986 ± 10.339; MD = 27.032 ± 8.664, $P = 0.021$) in ultrasound relative to sham groups during and after stimulation.

Additionally, separate trends to increased spiking in ultrasound relative to sham groups were seen in level 1 during stimulation (ultrasound 5.846 ± 3.319, sham 1.009 ± 0.026; MD = 0.964 ± 0.488, $P = 0.096$; Fig. 3B), level 2 after stimulation (ultrasound 31.368 ± 16.259, sham 1.293 ± 0.156; MD = 30.075 ± 14.375, $P = 0.081$; Fig. 3C), and level 3 spiking both during (ultrasound 12.143 ± 3.930, sham 1.531 ± 0.248; MD = 10.613 ± 5.188, $P = 0.087$) and after (ultrasound 45.893 ± 19.272, sham 2.441 ± 0.622; MD = 43.452 ± 20.023, $P = 0.073$) stimulation (Fig. 3D).

Finally, corrected planned comparisons by treatment within each level, period, and subregion showed a significant increase in level 2 spiking after stimulation in CA2 (ultrasound 12.650 ± 2.009, sham 1.523 ± 0.382; MD = 11.127 ± 2.045, $P = 0.002$) as well as in level 3 spiking after stimulation in DG (ultrasound 33.586 ± 4.949, sham 0.822 ± 0.229; MD = 32.764 ± 4.954, $P = 0.001$) and CA1 (ultrasound 52.045 ± 7.864, sham 3.014 ± 1.396; MD = 49.031 ± 7.987, $P = 0.001$; Fig. 4).

Ultrasound Effect Time Course

GLMMs with between-subject factor stimulation (ultrasound or sham) and repeated within-subject factors region (DG, CA3, CA2, CA1) and 18-sec interval (11–30 during and after stimulation) indicated a trend toward a treatment effect ($F_{1,6} = 5.542$, $P = 0.058$) and significant treatment × period interaction ($F_{38,16} = 2.531$, $P = 0.024$) for total regional normalized 18-sec spikes counts a trend toward a significant treatment effect ($F_{1,6} = 4.634$, $P = 0.077$) and significant treatment × period interaction ($F_{38,14} = 2.669$, $P = 0.026$) for level 1 spikes, a significant treatment effect ($F_{1,6} = 7.242$, $P = 0.035$) and treatment × period interaction ($F_{38,12} = 3.857$, $P = 0.008$) for level 2 spikes, and a significant treatment effect ($F_{1,23} = 52.459$, $P < 0.001$) and treatment × period interaction ($F_{38,14} = 6.915$, $P < 0.001$) for level 3 spikes.

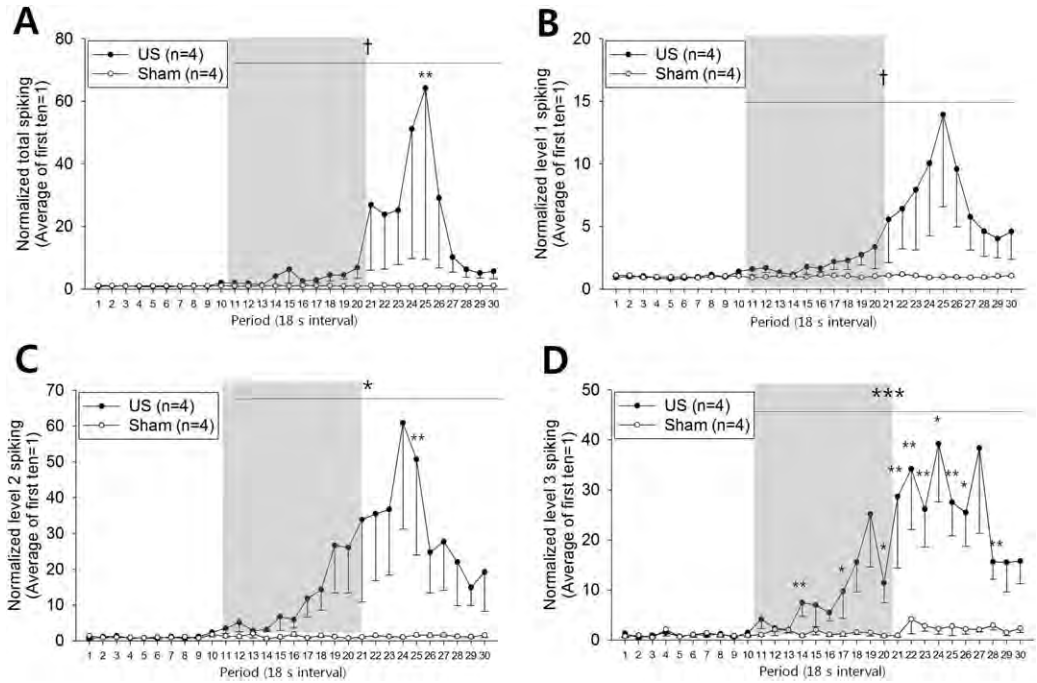

Fig. 5. Ultrasound stimulation induces delayed enhancements in spontaneous spiking that increase with spike threshold level. GLMM demonstrated a trend to stimulation treatment effect in normalized regional 18-sec spike counts for total (**A**) and level 1 (**B**) spikes and a significant treatment effect in level 2 ($F_{1,6} = 7.242$, $P = 0.035$; **C**) and level 3 ($F_{1,23} = 52.459$, $P < 0.001$; **D**) spikes. Note the gradual rise in ultrasound effect with significant peak at time point 25 following stimulation in total spikes, level 2, and level 3, in addition to several other points of significant increase in level 3 spikes only. Ultrasound or sham stimulation indicated by shaded box. F-test or pairwise comparison vs. sham $\star P < 0.05$, $\star\star P < 0.01$, $\star\star\star P < 0.001$.

Planned comparisons by treatment showed a trend tosard increased normalized 18-sec spiking in ultrasound relative to sham groups in total spikes (ultrasound 14.204 ± 3.974, sham 1.021 ± 0.017; $t(6) = 2.354$, $P = 0.058$) and level 1 (ultrasound 4.606 ± 0.673, sham 1.015 ± 0.017; $t(6) = 2.153$, $P = 0.077$) and significant increases in level 2 (ultrasound 21.573 ± 3.134, sham 1.248 ± 0.0996; $t(6) = 2.691$, $P = 0.035$) and level 3 (ultrasound 17.827 ± 1.811, sham 1.892 ± 0.230; $t(23) = 7.243$, $P < 0.001$). Planned comparisons by treatment from individual time points 11–30 demonstrated significant increases relative to sham in total and level 2 spike counts after ultrasound at time point 25 and in level 3 spike counts during ultrasound at time points 14, 17, and 20 and after ultrasound at time points 21-26 and 28 (Fig. 5).

Dependence of Ultrasound Effect on Glutamate Receptor Activity

Gamma-regression GLMMs for interactions between between-subject factors stimulation type (ultrasound, sham) and blocker (KA, TTX, vehicle) as well as with repeated within-subject measure region (DG, CA3, CA2, CA1) on total normalized regional spiking demonstrated a significant stimulation × blocker interaction both during ($F_{5,30} = 7.760$, $P < 0.001$) and after ($F_{5,35} = 6.220$, $P < 0.001$) stimulation. No region × stimulation type-× blocker interaction was found either during or after stimulation. Planned comparisons between ultrasound and sham groups showed significant differences in normalized spiking in vehicle but not KA or TTX groups both during (KA ultrasound 0.7325 ± 0.0420, sham 0.5213 ± 0.0382; $t(30) = 1.684$, $P = 0.103$; TTX ultrasound 0.9096 ± 0.0457, sham 0.7161 ± 0.0738, $t(30) = 1.507$, $P = 0.142$; vehicle ultrasound 1.8973 ± 0.3097, sham 1.0290 ± 0.0316; $t(30) = 2.095$, $P = 0.045$) and after (KA ultrasound 0.6200 ± 0.0341, sham 0.5842 ± 0.0402; $t(35) = 0.395$, $P = 0.695$; TTX ultrasound 0.8742 ± 0.0459, sham 0.6996 ± 0.0701; $t(35) = 1.227$, $P = 0.228$; vehicle ultrasound 2.0717 ± 0.5002, sham 0.9930 ± 0.0288; $t(35) = 2.034$, $P = 0.05$) stimulation (Fig. 6A).

GLMMs for interactions between between-subject factor stimulation (ultrasound, sham) and blocker (TTX, vehicle) and with repeated within-subject factors spike level (1, 2, 3) or region (DG, CA3, CA2, CA1), or both, showed a significant stimulation × blocker interaction during ($F_{5,161} = 16.644$, $P < 0.001$) and after ($F_{5,115} = 16.277$, $P < 0.001$) stimulation. Planned comparisons by stimulation within each blocker showed greater normalized spiking in ultrasound than sham conditions

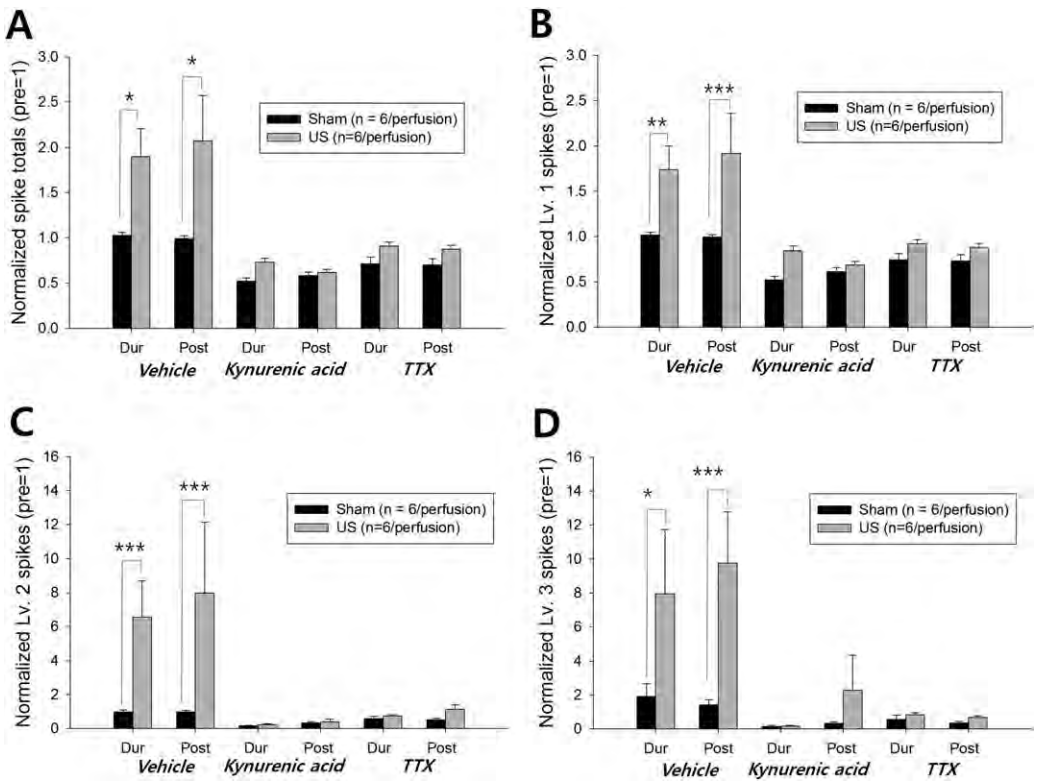

Fig. 6. **A–D**: Ultrasound-mediated enhancements in spontaneous spiking depend on glutamate receptor activity. GLMM demonstrated a significant treatment × blocker (vehicle, KA, TTX) interaction on normalized regional spike counts during ($F_{5,30} = 7.760$, $P < 0.001$) and after ($F_{5,35} = 6.220$, $P < 0.001$) stimulation. A significant treatment × vehicle × level interaction during ($F_{8,26} = 4.004$, $P = 0.003$) and after ($F_{8,40} = 5.540$, $P < 0.001$) stimulation and significantly higher regional normalized spiking only in vehicle-treated ultrasound vs. sham groups in level 1 spikes during ($t(40) = 2.866$, $P = 0.007$) and after ($t(88) = 3.632$, $P < 0.001$) stimulation (B), level 2 during ($t(111) = 6.250$, $P < 0.001$) and after ($t(141) = 6.896$, $P < 0.001$) stimulation (C), and level 3 (D) spikes during ($t(81) = 2.433$, $P = 0.017$) and after ($t(67) = 4.262$, $P < 0.001$) stimulation were also seen. Pairwise comparison vs. sham $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$.

in vehicle-treated but not KA- or TTX-treated subjects both during (KA ultrasound 0.4084 ± 0.0492, sham 0.2876 ± 0.0346; $t(161) = 0.123$, $P = 0.902$; TTX ultrasound 0.8318 ± 0.0491, sham 0.6275 ± 0.0966; $t(161) = 0.022$, $P = 0.982$; vehicle ultrasound 5.4212 ± 1.4580, sham 1.3007 ± 0.2576; $t(161) = 5.565$, $P < 0.001$) and after (KA ultrasound 1.1118 ± 0.6915, sham 0.4240 ± 0.0403; $t(161) = 0.123$, $P = 0.902$; TTX ultrasound 0.9039 ± 0.0872, sham 0.5268 ± 0.0561; $t(161) = 0.022$, $P = 0.982$; vehicle ultrasound 6.5489 ± 1.7410, sham 1.1281 ± 0.1031; $t(161) = 5.565$, $P < 0.001$) stimulation. Significant stimulation × blocker × level interactions on normalized spike counts during ($F_{12,107} = 6.147$, $P < 0.001$) and after ($F_{12,69} = 7.649$, $P < 0.001$) stimulation were also found. Planned comparisons showed increased normalized spiking in the ultrasound relative to sham condition in vehicle-treated, but not KA- or TTX-treated, subjects during stimulation in level 1 (KA ultrasound 0.8415 ± 0.0534, sham 0.5213 ± 0.0382; $t(40) = 1.101$, $P = 0.278$; TTX ultrasound 0.9208 ± 0.0437, sham 0.7419 ± 0.0723; $t(40) = 0.676$, $P = 0.503$; vehicle ultrasound 1.7367 ± 0.2603, sham 1.0172 ± 0.0301; $t(40) = 2.866$, $P = 0.007$), level 2 (KA ultrasound 0.229 ± 0.0614, sham 0.1572 ± 0.0315; $t(111) = 0.034$, $P = 0.973$; TTX ultrasound 0.7407 ± 0.0856, sham 0.5807 ± 0.1290; $t(111) = 0.155$, $P = 0.877$; vehicle ultrasound 6.5705 ± 2.111, sham 0.9823 ± 0.0991; $t(111) = 6.250$, $P < 0.001$), and level 3 (KA ultrasound 0.1538 ± 0.0587, sham 0.1505 ± 0.0631; $t(81) = -0.161$, $P = 0.872$; TTX ultrasound 0.8339 ± 0.1115, sham 0.5600 ± 0.2525; $t(81) = -0.292$, $P = 0.771$; vehicle ultrasound 7.9565 ± 3.7729, sham 1.903 ± 0.7617 $t(81) = 2.433$, $P = 0.017$) as well as after stimulation in level 1 (KA ultrasound 0.6861 ± 0.0379, sham 0.6152 ± 0.0403; $t(88) = 0.348$, $P = 0.729$; TTX ultrasound 0.8841 ± 0.0434, sham 0.7300 ± 0.0684; $t(88) = -0.588$, $P = 0.558$; vehicle ultrasound 1.918 ± 0.4394, sham 0.9933 ± 0.0291; $t(88) = 3.632$, $P < 0.001$), level 2 (KA ultrasound 0.3853 ± 0.1634, sham 0.3247 ± 0.0618; $t(141) = -0.236$, $P = 0.814$; TTX ultrasound 1.1512 ± 0.2343, sham 0.5062 ± 0.1073; $t(141) = 1.438$, $P = 0.153$; vehicle ultrasound 7.9654 ± 4.1696, sham 0.9666 ± 0.0989; $t(141) = 6.896$, $P < 0.001$), and level 3 (KA ultrasound 2.2638 ± 2.0765, sham 0.3322 ± 0.0848; $t(67) =$

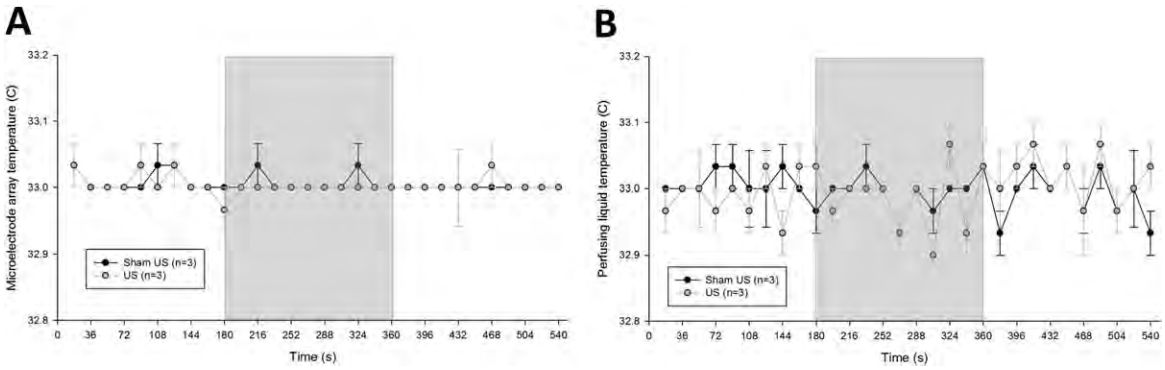

Fig. 7. Ultrasound does not alter temperature of recording setup. Temperature recorded by microelectrode array probes for microelectrode array (**A**) and perfusing artificial cerebrospinal fluid (**B**) for 3 min before, during, and after ultrasound and sham stimulation. GLMM demonstrated no significant treatment effect on either outcome.

0.030, $P = 0.977$; TTX ultrasound 0.6765 ± 0.0933, sham 0.3441 ± 0.0985; $t(67) = 0.234$, $P = 0.816$; vehicle ultrasound 9.7627 ± 3.0039, sham 1.4243 ± 0.2864; $t(67) = 4.262$, $P < 0.001$).

Temperature Effects of Ultrasound Setup

Corrected pairwise comparisons of simulated ultrasound (n = 3) and sham ultrasound (n = 3) experiments demonstrated no significant differences between either multielectrode array or perfusion temperatures at any time point (Fig. 7).

DISCUSSION

We investigated the effect of 3 min of unfocused pulsed ultrasound (frequency 0.5 MHz, I_{SPPA} 780 $\mu W/cm^2$) on spontaneous subregional spiking in postnatal rat organotypic hippocampal slices. We hypothesized that ultrasound relative to sham stimulation would induce measurable changes in spontaneous spiking from prestimulation baseline in spike rates during and/or after exposure, but whether these effects would show any regional or spike threshold specificity was unclear.

Despite the absence of a general treatment effect across the hippocampus, a significant treatment effect and trend to increase were demonstrated for spikes extracted using the highest threshold (level 3) both during and after stimulation. Furthermore, when 3-min spike counts were separated by region and threshold level, level 2 spikes in CA2 and level 3 spikes in dentate gyrus and CA1 demonstrated significant increases from baseline after but, notably, not during ultrasound relative to sham stimulation. Similarly, significant increases in 18-sec spike counts were found predominantly following stimulation in levels 2 and 3, in particular, at time point 25 after stimulation in level 2 and at three time points during stimulation but seven time points after stimulation in level 3. These trends were largely replicated under vehicle administration but abolished under TTX administration in experiment 2 and encompass a number of novel observations.

First, our results suggest that ultrasound may affect neural physiology at intensities much lower than those previously documented. The lowest-intensity unfocused pulsed ultrasound setup shown to modulate neuronal activity in vitro had a pulse average intensity of 2.9 W/cm^2, which was shown to induce sodium and calcium ion transients in both neurons and glial cells of organotypic hippocampal slice cultures (Tyler et al., 2008). Successful modulation of spontaneous neural activity in noncraniectomized animals by ultrasound on the order of hundreds of milliwatts as measured outside the skin and skull (Tufail et al., 2010; King et al., 2013) was ostensibly associated with lower-than-reported effective intensities at the site of neural tissue contact, but, to our knowledge, no explicit claim has been made to date of neuromodulation by ultrasound of an intensity lower than ours.

Second, our data suggest that ultrasound-mediated effects on spontaneous electrical activity may encompass time scales longer than those observed to date in central nervous circuits. Previously published results suggest long-term influences of sonication on evoked neural activity, such as alteration of extracellular evoked potentials in the rat dentate gyrus for up to 10 min (Bachtold et al., 1998) and in CA1 for up to 25 min postexposure (Rinaldi et al., 1991), inhibition of evoked action potential activity in frog sciatic nerves for 90 min postexposure (Colucci et al., 2009), and increases in BDNF protein expression in mouse CA1 and CA3 recorded 45 min after exposure in vivo (Tufail et al., 2010). Given these findings, it is plausible that ultrasound would also continue to influence spontaneous neural activity even after stimulation has ceased, but our results are the first to demonstrate that this is the case for longer than 1 min (Khraiche et al., 2008). Experiment 1 data suggest that these influences may, indeed, peak past the minute mark—approximately 90 sec postexposure for our setup, as seen in normalized 18-sec rates for total and level 2 spiking—and continue to be manifested even past that, as in level 3 18-sec spike rates, which exhibited significant increases above baseline

for more than 2 min following cessation of exposure. Furthermore, although the fine-grained time course of the effects seen in experiment 2 was not examined, our finding of increased spiking following the cessation of stimulation was recapitulated at all amplitude levels in the larger sample sizes of the second experiment, implying a robust effect replicable across slightly different protocols. Taken together, these data suggest that continued study of ultrasound's sustained or delayed effects is important to investigating its full potential. Along the same vein, that region-specific 3-min spiking increased after but not during ultrasound application and that normalized 18-second total and level 2 rates peaked approximately 1.5 min after stimulation suggest that our ultrasound setup touched off one or more gradual or delayed mechanisms of effect.

It is not likely that hyperthermia was one of these mechanisms. Our ultrasound setup was associated with a 2.097-μsec pulse duration and estimated peak pressure of 11.52 kPa, far less than the 50-msec duration and 100-kPa pressure previously reported as threshold parameters for even small (0.02 °C) changes in temperature at average intensities thousands of times higher than our own (Tufail et al., 2010). Second, although prolonged focused ultrasound stimulation has been associated with temperature increases themselves sufficient for a neurophysiological effect, these increases, and their associated changes in neural field potential, peaked during, not after, ultrasound application (Bachtold et al., 1998). Finally, empirical examination of our own setup revealed no ultrasound-mediated temperature alterations in either the microelectrode array or the perfusing liquid during or after stimulation (Fig. 7).

Although our estimated peak pressure of 11.52 kPa falls well below the previously reported 40 MPa threshold for significant cavitation-induced tissue damage in soft tissues naturally containing little gas (Dalecki, 2004), the appearance of arraywide bubble artifacts during and after stimulation was indeed greater in the ultrasound than in the sham condition in both experiments 1 (one US-exposed rat during vs. no sham-exposed rats) and 2 (two US-exposed rats during and two after vs. one sham-exposed rat during). These data suggest that our hippocampal slices were exposed to stronger cavitation-mediated mechanical perturbations under the ultrasound than the sham condition, although whether this influence extends beyond the scale associated with the obvious arraywide bubble artifacts is unclear. This postulated mechanism of effect may explain our observation that the influences of ultrasound increased with spike threshold because, depending on their size, sonically disturbed bubbles might influence the activity of cell groups only above a minimum number. It does not, however, explain why ultrasound-mediated increases in normalized spike count peaked after and not during exposure, as explained above.

It is thus also probable that one or more other mechanisms, including noncavitational fluid mechanical influences on membrane potential (Tyler, 2011), stimulation of mechanosensitive Na^+, K^+, or Ca^{2+} channels (Tufail et al., 2010), or SNARE-mediated induction of vesicular neurotransmitter release (Tyler et al., 2008), might have contributed to the delayed increases in spike rate demonstrated. Our results are possibly consistent with the involvement of signal cascades, particularly those sensitive to ionic second messengers such as Ca^{2+}, resulting in sustained alterations to ionic transport across the membrane. It has, indeed, been previously shown that ultrasound may alter the expression of various proteins in some cell types (Reher et al., 1999; Naruse et al., 2003; Ebisawa et al., 2004; Mukai et al., 2005). It is also possible that modulation of neurotransmission by nearby glial cells, such as by the astrocytic release of glutamate and GABA, contributed to the observed delay in ultrasound-mediated changes in normalized spike activity. Experiment 2 findings that ultrasound-mediated increases in spike activity were abolished by perfusion of the nonspecific glutamate receptor blocker KA to a level comparable to that of positive control TTX strengthens the case for glutamate receptor transmission, as well as the calcium signaling with which it is associated, as a potential mediator of effect. Further research is needed to pinpoint the particular receptor and cell types involved in these observations.

Finally, our data suggest that the ultrasonic modulation of spontaneous neural activity is not robust or generalized, depending instead on a number of conditions worthy of systematic study. Contrary to previously published results suggesting robust ultrasound-mediated activity increases across treatments ranging from hippocampal slices (Khraiche et al., 2008) to the somatosensory cortices of living mice (Tufail et al., 2010), we found no treatment effect on total spike activity when data from four subregions (DG, CA3, CA2, and CA1) were pooled. Although this disparity with the work of other groups may be attributable to our smaller samples, longer stimulation time, comparatively low associated spatial peak-pulse average intensity, different transducer and waveform parameters, and numerous other procedural differences, we noted two factors in particular, spike threshold definition and cell type, that appeared to influence the appearance of an ultrasound effect.

In experiment 1, ultrasound relative to sham stimulation engendered a treatment effect on level 3 spike activity postbaseline and significant enhancements in level 2 and 3 spike activity poststimulation. This trend was replicated in experiment 2, with stimulation × blocker × level interactions on spike activity coupled with higher normalized spiking in US-treated levels 2 and 3 relative to level 1 spikes, suggesting that our ultrasound-mediated effect in higher threshold levels is not merely a byproduct of low statistical power in experiment 1 (Fig. 6). Taken together, these results suggest that the effects of ultrasound increased with spike thresholds as measured by our extraction algorithm. Notably, spikes qualifying for higher levels were subtracted from those in lower ones, meaning that spike counts in the latter represented only those with maxima that did not extend beyond subsequent thresholds. The resultant increased voltage maxima of higher levels represent a number of possible phenomena within the context of extracellular microelectrode array recording, including higher firing rates, larger active cell populations, increased synchrony, and greater ionic

throughput across nonaxonal or even nonneuronal membranes, the differentiation among which with regard to the mechanisms of ultrasound awaits further investigation.

Our ultrasound effect appeared to be dependent on not only spike threshold but also, more tentatively, hippocampal subregion. Though it must be noted that effect interactions with the within-subject factor region did not reach significance in experiment 1 and these data thus must be interpreted with caution pending replication in experiments with larger groups, ultrasound-mediated influences in experiment 1 reached significance in level 2 after stimulation in CA2 only and in level 3 after stimulation in DG and CA1 only. Examination of the reasons for this particular pattern of effect, be they reflective of structural differences on the tissue or cell level resulting in inequalities in effective wave pressures, differences in the relative concentrations of receptor types affected by ultrasound, emergent properties of prolonged network activity, or some combination of these and other possible factors, is beyond the scope of the present investigation, which aimed only to determine whether spontaneous spiking could be influenced at all by an ultrasound setup of much lower intensity than that applied to date and is instead a topic of further experimentation currently underway. Although it remains to be seen whether similar trends will be replicated in experiments of higher statistical power, the potential of subregional selectivity suggests that ultrasonic effects may not be as nonspecific as the wide range of documented influences in preparations as varied as those from in vitro cultures of mouse CA1 pyramidal neurons (Tyler et al., 2008) and the excised sciatic nerves of frogs (Colucci et al., 2009) to the somatosensory cortex of humans (Mueller et al., 2014) may otherwise imply. The effective application of ultrasonic stimulation to specific desired endpoints in local tissue may thus depend on a broader understanding of its particular mechanisms than we currently possess.

We observed threshold- and region-specific increased normalized spike activity in organotypic hippocampal slices both during and after 3 min of stimulation with unfocused pulsed low-intensity ultrasound. These findings suggest that the threshold of intensity required for meaningful neurophysiological effect by ultrasound stimulation may be lower than previously reported, expand upon previous evidence suggesting that ultrasound's effect on spontaneous activity may extend beyond its immediate application, implicate glutamate receptor activity in possible mechanisms of effect, and demonstrate that the ultrasonic mediation of neural electrophysiology may vary with qualities such as activity threshold and region. Although the confident generalization of our conclusions to the more physiologically relevant in vivo condition awaits further experimentation, our data demonstrate the need for more systematic exploration of the range of parameters by which ultrasound-mediated neural stimulation may be applied to therapeutic benefit or unintended harm.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: H-BK, H-SH, KMS. Acquisition of data: H-BK. Analysis and interpretation of data: KMS, H-BK, J-CK. Drafting of the manuscript: KMS, H-BK. Critical revision of the manuscript for important intellectual content: KMS, H-BK, SL, CJL. Statistical analysis: KMS. Obtained funding: J-HP, T-SK. Administrative, technical, and material support: J-WK, SL, CJL, SM, T-SK, J-HP. Study supervision: T-SK, J-HP.

REFERENCES

Bachtold MR, Rinaldi PC, Jones JP, Reines F, Price LR. 1998. Focused ultrasound modifications of neural circuit activity in a mammalian brain. Ultrasound Med Biol 24:557–565.

Benjamini Y, Krieger AM, Yekutieli D. 2006. Adaptive linear step-up procedures that control the false discovery rate. Biometrika 93:491–507.

Colucci V, Strichartz G, Jolesz F, Vykhodtseva N, Hynynen K. 2009. Focused ultrasound effects on nerve action potential in vitro. Ultrasound Med Biol 35:1737–1747.

Dalecki D. 2004. Mechanical bioeffects of ultrasound. Annu Rev Biomed Eng 6:229–248.

Demirtas-Tatlidede A, Vahabzadeh-Hagh AM, Bernabeu M, Tormos JM, Pascual-Leone A. 2012. Noninvasive brain stimulation in traumatic brain injury. J Head Trauma Rehabil 27:274–292.

Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, Watanabe H. 2004. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng 10:921–929.

Egert U, Schlosshauer B, Fennrich S, Nisch W, Fejtl M, Knott T, Muller T, Hammerle H. 1998. A novel organotypic long-term culture of the rat hippocampus on substrate-integrated multielectrode arrays. Brain Res Brain Res Protoc 2:229–242.

Fry WJ. 1958. Intense ultrasound in investigations of the central nervous system. Adv Biol Med Phys 6:281–348.

Han HS, Hwang SY, Akram F, Jeon HJ, Nam SB, Jun SB, Kim JT, Kim ST. 2014. Neural activity modulation via ultrasound stimulation measured on multi-channel electrodes. Proceedings of the World Congress on Engineering, vol 1, London.

Hitchcock KE, Holland CK. 2010. Ultrasound-assisted thrombolysis for stroke therapy: better thrombus break-up with bubbles. Stroke 41(Suppl):S50–S53.

Huynh HaLF. 1976. Estimation of the Box correction for degrees of freedom from sample data in randomized block and split-plot designs. J Educ Behav Statist 1:69–82.

Khraiche ML, Phillips WB, Jackson N, Muthuswamy J. 2008. Ultrasound induced increase in excitability of single neurons. Annual International Conference of the IEEE Engineering in Medicine and Biology Society 2008:4246–4249.

King RL, Brown JR, Newsome WT, Pauly KB. 2013. Effective parameters for ultrasound-induced in vivo neurostimulation. Ultrasound Med Biol 39:312–331.

Laird NM, Ware JH. 1982. Random-effects models for longitudinal data. Biometrics 38:963–974.

Levin JR, Serlin RC, Seaman MA. 1994. A controlled, powerful multiple-comparison strategy for several situations. Psychol Bull 115: 153–159.

Lipsman N, Schwartz ML, Huang Y, Lee L, Sankar T, Chapman M, Hynynen K, Lozano AM. 2013. MR-guided focused ultrasound thalamotomy for essential tremor: a proof-of-concept study. Lancet Neurol 12:462–468.

Lynn JG, Zwemer RL, Chick AJ, Miller AE. 1942. A new method for the generation and use of focused ultrasound in experimental biology. J Gen Physiol 26:179–193.

Martin E, Jeanmonod D, Morel A, Zadicario E, Werner B. 2009. High-intensity focused ultrasound for noninvasive functional neurosurgery. Ann Neurol 66:858–861.

Mueller J, Legon W, Opitz A, Sato TF, Tyler WJ. 2014. Transcranial focused ultrasound modulates intrinsic and evoked EEG dynamics. Brain Stim 7:900–908.

Mukai S, Ito H, Nakagawa Y, Akiyama H, Miyamoto M, Nakamura T. 2005. Transforming growth factor-beta1 mediates the effects of low-intensity pulsed ultrasound in chondrocytes. Ultrasound Med Biol 31: 1713–1721.

Muratore R, LaManna JK, Lamprecht MR, Morrison B III. 2012. Hippocampal culture stimulus with 4-megahertz ultrasound. AIP Conference Proceedings 1481(254).

Naruse K, Miyauchi A, Itoman M, Mikuni-Takagaki Y. 2003. Distinct anabolic response of osteoblast to low-intensity pulsed ultrasound. J Bone Mineral Res 18:360–369.

Quiroga RQ, Nadasdy Z, Ben-Shaul Y. 2004. Unsupervised spike detection and sorting with wavelets and superparamagnetic clustering. Neural Comput 16:1661–1687.

Reher P, Doan N, Bradnock B, Meghji S, Harris M. 1999. Effect of ultrasound on the production of IL-8, basic FGF and VEGF. Cytokine 11:416–423.

Rinaldi PC, Jones JP, Reines F, Price LR. 1991. Modification by focused ultrasound pulses of electrically evoked responses from an in vitro hippocampal preparation. Brain Res 558:36–42.

Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nervous tissue. J Neurosc Methods 37:173–182.

Strauss I, Kalia SK, Lozano AM. 2014. Where are we with surgical therapies for Parkinson's disease? Parkinsonism Rel Disorders 20(Suppl 1): S187–S191.

Tufail Y, Matyushov A, Baldwin N, Tauchmann ML, Georges J, Yoshihiro A, Tillery SI, Tyler WJ. 2010. Transcranial pulsed ultrasound stimulates intact brain circuits. Neuron 66:681–694.

Tufail Y, Yoshihiro A, Pati S, Li MM, Tyler WJ. 2011. Ultrasonic neuromodulation by brain stimulation with transcranial ultrasound. Nat Protoc 6:1453–1470.

Tyler WJ. 2011. Noninvasive neuromodulation with ultrasound? A continuum mechanics hypothesis. Neuroscientist 17:25–36.

Tyler WJ, Tufail Y, Finsterwald M, Tauchmann ML, Olson EJ, Majestic C. 2008. Remote excitation of neuronal circuits using low-intensity, low-frequency ultrasound. PloS One 3:e3511.

Villamar MF, Santos Portilla A, Fregni F, Zafonte R. 2012. Noninvasive brain stimulation to modulate neuroplasticity in traumatic brain injury. Neuromodulation 15:326–338.

Yang T, Chen J, Yan B, Zhou D. 2011. Transcranial ultrasound stimulation: a possible therapeutic approach to epilepsy. Med Hypoth 76:381–383.

Yoo SS, Bystritsky A, Lee JH, Zhang Y, Fischer K, Min BK, McDannold NJ, Pascual-Leone A, Jolesz FA. 2011. Focused ultrasound modulates region-specific brain activity. Neuroimage 56: 1267–1275.