

Delay- and Pressure-Dependent Neuromodulatory Effects of Transcranial Ultrasound Stimulation

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ABSTRACT

Objective: Despite the growing interest in transcranial focused ultrasound stimulation (TUS), our understanding of its underlying mechanisms remains limited. In this study, we aimed to investigate the effects of TUS on several functional magnetic resonance imaging metrics by considering their latency, duration, and relationship with applied acoustic pressure.

Materials and methods: We recruited 22 healthy volunteers and used a pre- vs post-TUS protocol. Half of the volunteers were stimulated in the right inferior frontal cortex and the other half in the right thalamus. The fractional amplitudes of low-frequency fluctuations, regional homogeneity, degree centrality, local functional connectivity density, and eigenvector centrality were considered. These metrics were compared before TUS and at three different time points in the first hour after TUS.

Results: Our results showed that 1) TUS primarily alters functional connectivity metrics at both the local and global levels; 2) stronger alterations are observed when the delay after TUS increases and 3) when the applied acoustic pressure is close to the maximum.

Conclusion: These results suggest that some consequences of TUS might not be immediate, inviting us to revise the premise that TUS consequences are immediate and will progressively disappear.

Keywords: fALFF, noninvasive neuromodulation, ReHo, voxel-based analyses, whole brain

INTRODUCTION

Transcranial focused ultrasound stimulation (TUS) is an emerging noninvasive neuromodulation technique that involves the application of acoustic pressure to the brain through the skull. This technique offers several advantages over other noninvasive neuromodulation approaches. Notably, TUS provides high spatial precision,¹ allowing targeted stimulation of specific brain regions, and it can reach deep brain structures^{2,3} that are often inaccessible by other noninvasive techniques, such as transcranial magnetic stimulation or transcranial direct current stimulation. These features make the TUS a promising tool for fundamental research and clinical applications.

Despite the increasing interest in TUS, the field faces significant challenges owing to a limited understanding of the underlying mechanisms through which TUS exerts its effects on the brain. For instance, current research has shown that TUS can induce various neurophysiological changes, including alterations in brain activity,^{4–10} functional connectivity,^{5,11–15} cortical excitability,^{4,11,16–19} and even neurotransmitter concentrations such as γ -amino butyric acid (GABA).¹²

Key areas of uncertainty include the effects of TUS on other functional magnetic resonance imaging (fMRI) metrics. Furthermore, the temporal dynamics of TUS-induced effects, specifically

the latency and duration, are not well understood. In addition, the relationship between the applied acoustic pressure and the resulting neuromodulatory effects requires further investigation to optimize the stimulation parameters and reliably predict outcomes.

In this study, we aimed to address some of these knowledge gaps by evaluating the effects of TUS on a range of fMRI metrics at the whole-brain level. We conducted this analysis in a group of 22

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healthy volunteers assessed before (control group) and after TUS in either the right inferior frontal cortex (IFC) or the right thalamus (11 in each group). These two targets were selected on the basis of their different depths and functions. If these two regions are distant, they are part of a shared corticosubcortical network. The thalamus plays the role of motor hub, receiving information from the cortex and other deep nuclei before sending motor commands to the primary motor cortex.²⁰ Conversely, the right IFC applies a motor top-down control (ie, inhibitory) through the cortico-subthalamic hyperdirect pathway.^{21,22} Therefore, we expect to observe both different and opposite results after either thalamus or IFC-TUS. We hypothesized that IFC-TUS could be related to changes within the associative and motor pathways, whereas thalamus-TUS would be more related to sensorimotor pathway alterations. In addition, if recruitment of the motor network is observed for both the IFC and thalamus TUS, we expect this recruitment to be in the opposite direction. Moreover, we focused on hypothesis-free fMRI metrics. This choice was based on three reasons: First, seed-based connectivity requires a strong hypothesis regarding seed placement. Although this may seem straightforward, it introduces considerable complexity because the seed will remain constant for all participants, whereas the ultrasound stimulation applied will differ for each participant. Second, seed-based connectivity provides information that always refers to seeds, whereas hypothesis-free metrics can be interpreted independently. Third, seed-based connectivity was addressed in a different study.¹⁵

By examining the effects of TUS at multiple time points after stimulation and exploring the relationship between these effects and the applied acoustic pressure, we sought to provide a more comprehensive understanding of the neuromodulatory capabilities of TUS.

MATERIALS AND METHODS

Participants

We enlisted 22 healthy, right-handed volunteers with no history of neurologic or psychiatric disorders, except those with a history of depression, who were in remission for $\geq$ one year (Table 1). Additional exclusion criteria included the following: having close relatives with a history of seizures, a predisposition to fainting, excessive hair that could impede transducer coupling, current or planned pregnancy, presence of any implanted metallic devices, skin diseases, claustrophobia, or anxiety related to magnetic resonance imaging (MRI), and tattoos near the head. The participants

received compensation of £40 on completion of their participation. This study was approved by the University of Nottingham Faculty of Psychology Ethics Committee (reference: F1298R) and adhered to the ethical principles outlined in the Declaration of Helsinki of 1978, as amended in 2008. Written informed consent was obtained from all participants before their participation in the study.

Study Design

The study was conducted in three phases (Fig. 1). Initially, the participants completed a 45-minute MRI session. Subsequently, the participants were pseudorandomly assigned to either the group that underwent TUS targeting the right IFC (IFC-TUS) or the group that underwent TUS targeting the right thalamus (Thal-TUS). This second step was necessary to individually plan the TUS (ie, to identify the target location on the native T1 and the optimal transducer position over the head). Finally, TUS was administered to the designated target, followed by another 45-minute MRI session (approximately 15 minutes between the TUS and the beginning of the MRI). The initial and final MRI sessions were conducted on separate days, with a minimum interval of one week between sessions.

MRI Data

The MRI scans were conducted for approximately 45 minutes using a General Electric 3 Tesla scanner (GE Healthcare, Milwaukee, WI) equipped with a 48-channel head coil. The following sequences were used for this study: T1-weighted magnetization-prepared rapid gradient echo (repetition time [TR] = 2.282 seconds; echo time [TE] = 2.96 milliseconds; inversion time = 800 milliseconds; flip angle [FA] = 8°; field of view [FOV] = 256 × 256 mm; 180 slices of 1-mm³ isotropic voxels); zero echo time (ZTE; TR = 0.531 seconds; TE = 0.016 milliseconds; FOV = 256 × 256 mm; 176 slices of 1.016 × 1.016 × 1-mm³ voxels); resting-state fMRI (rs-fMRI; 14 minutes during the first visit and 42 minutes during the second visit; TR = 1.4 seconds; TE = 35 milliseconds; FA = 68°; FOV = 212 × 212 mm; 88 interleaved slices of 2-mm³ isotropic voxels; no slice gap; multiband acceleration factor of 3). Cardiac and respiratory data were recorded during this sequence and were obtained with the participants' eyes open.

T1-weighted images were used to coregister other sequences, transform images into a template space, generate pseudocomputed tomography (PCT),²³ and conduct acoustic simulations.

Table 1. Demographic, Experimental, and Acoustic Properties of the Two Groups.

	IFC-TUS	Thal-TUS	<i>p</i> Values
Sex [F/M]	6/5	6/5	1
Age [y]	25 ± 5.7	22.8 ± 5.7	0.38
Delay TUS rs-fMRI [min.sec]	15.00 ± 2.16	15.04 ± 1.37	0.95
Target depth [mm]	31.1 ± 5	73.8 ± 3.7	< 0.001*
ISPTA [mW/cm ²]	83.3 ± 37.3	154.9 ± 69.2	0.008*
MI	0.224 ± 0.061	0.298 ± 0.067	0.013*
Maximum temperature increase [°C]	0.214 ± 0.09	0.393 ± 0.07	< 0.001*
Peak pressure [kPa]*	153.5 ± 34.9	134.8 ± 26.7	0.173

Values are given as mean ± SD.

F, female; M, male; MI, mechanical index.

*Significant differences.

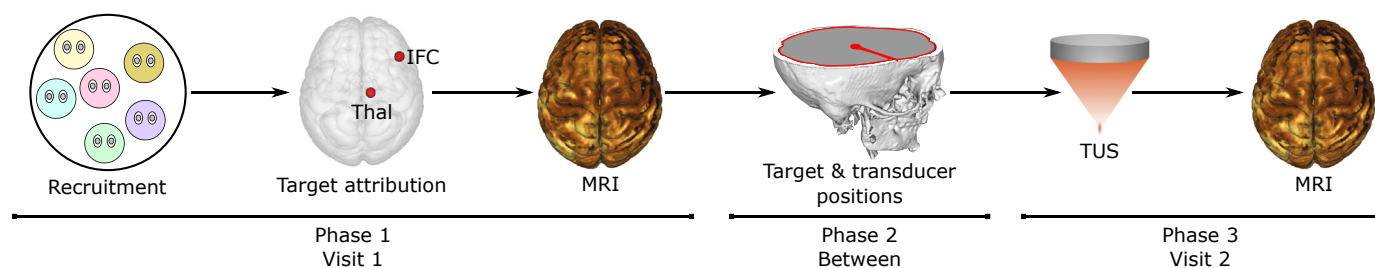


Figure 1. Illustration of the study design. [Color figure can be viewed at www.neuromodulationjournal.org]

ZTE sequences were used to extract the skull shape of each participant, calculate the skull thickness, and determine the optimal position for the TUS transducer on the head. This process involved enhancing the contrast of the T1 images, aligning the ZTE with the enhanced T1 images, and using the aligned T1 images to extract binary maps of the head and brain. The brain and background data were extracted. The skull was then estimated to fall above the mean + 1 SD of the ZTE values within the brain mask, and below ZTE – 1 SD of the ZTE values within the background mask. Manual adjustments were performed as required.

Preprocessing of rs-fMRI data involved removing signals from the ventricles and white matter (extracted using FreeSurfer; regressed by removing the first principal component), in addition to cardiac and respiratory artifacts (regressed using the AFNI 3 dretroicor function). The data were then despiked and processed using the `afni_proc.py` script, which included the following steps: discarding the first two volumes, time shifting, alignment with the minimum outlier volume, transformation to standard space, volume registration, masking, scaling (each volume mean was set to 100 with a voxel maximum value of 200), and regression (including motion correction by removing the mean and derivatives in a regression model). A 6-mm smoothing was applied after extracting the fMRI metrics. None of the participants reported falling asleep during the MRI, and visual examination of cardiac and respiratory recordings did not reveal any obvious sleep states.

Targets' Location and Transducer Position

The coordinates for the right IFC and thalamus were determined using the Montreal Neurological Institute (MNI) coordinates from the automated anatomical labeling atlas,²⁴ as follows: right IFC ($x = 46$, $y = 26$, $z = 8$); right thalamus ($x = 11$, $y = -18$, $z = 7$). For each participant, the target locations were transformed from the MNI space to the native space by applying an inverse warp map between the T1-weighted images and the MNI template. The right IFC was estimated to be 31.1 ± 5 mm deep (range: 24–42 mm), whereas the right thalamus was 73.8 ± 3.7 mm deep (range: 69–82 mm). Concurrently, the ZTE sequence was used to extract the skull of each participant as a binary map. A custom-built R code was then used to calculate both the distance from the brain target to the head and the skull thickness for all possible trajectories (approximately 250,000). This code, which has been validated and updated,²⁵ is available for free at https://github.com/CyrilAtkinson/TUS_entry. The transducer coordinate offering the minimum skull thickness was selected and integrated into the neuronavigation software (Visor2™ V2.7, AntNeuro, Hengelo, The Netherlands) to be used as the entry point during stimulation.

Ultrasound Stimulation

For the application of ultrasound stimulation, we used the NeuroFUS PRO TPO-203 system equipped with the four-element CTX-500-4CH transducer (manufacturer: Sonic Concepts Inc, Bothell, WA; supplier: Brainbox Ltd, Cardiff, UK; number of elements: four; radius of curvature: 63.2 mm; aperture diameter: 45 mm; steering range: 30–70 mm). We used a theta-burst TUS protocol^{4,12} with the following parameters: fundamental frequency: 500 kHz, pulse duration: 20 milliseconds, pulse repetition interval: 200 milliseconds, pulse repetition frequency: 5 Hz, duty cycle: 10%, spatial-peak pulse-average intensity: 54.51 W/cm^2 , and total duration: 80 seconds. These settings adhere to the safety guidelines.^{26,27} To achieve proper coupling between the ultrasound transducer and the participant's head, an ultrasound transmission gel was applied to both the head and the transducer face, ensuring the manual removal of any air bubbles (using a syringe). The position of the transducer was based on the outputs provided by the code we developed,²⁵ allowing us to manually hold the transducer in the correct location and orientation during all stimulations.

Acoustic Simulations

Acoustic simulations were conducted using k-plan V1.0 (Brainbox Ltd). These simulations were performed after data acquisition, using both the T1-weighted scans and PCT scans, which were derived from the T1-weighted images for each participant.²³ The pressure estimation maps were subsequently transformed from the native space to the template space using a conversion map obtained during the MRI preprocessing phase. Figure 2 indicates the locations of the targets and sonicated regions for all participants. Considering a sphere of radius 6 mm, the center of which is in the middle of the target (ie, IFC: $x = 46$, $y = 26$, $z = 8$; right thalamus: $x = 11$, $y = -18$, $z = 7$), the acoustic simulations showed that $16.3\% \pm 11.72\%$ of the IFC received ≥ 50 kPa of acoustic pressure (spatial-peak temporal average intensity [ISPTA]) and $33.22\% \pm 11.92\%$ of the thalamus (see [Supplementary Table](#)). The difference between our two groups regarding the delivered acoustic pressure and size of the stimulated area was essentially due to the difference in the depth between the two targets. A deeper target led to a more pronounced cigar-shaped stimulated area and better acoustic wave penetration (Fig. 3).

fMRI Metrics

Five fMRI metrics were extracted using the AFNI. They were selected because they were not hypothesis-related, in contrast to seed-based functional connectivity, which requires the determination of region-of-interest location, size, and shape.

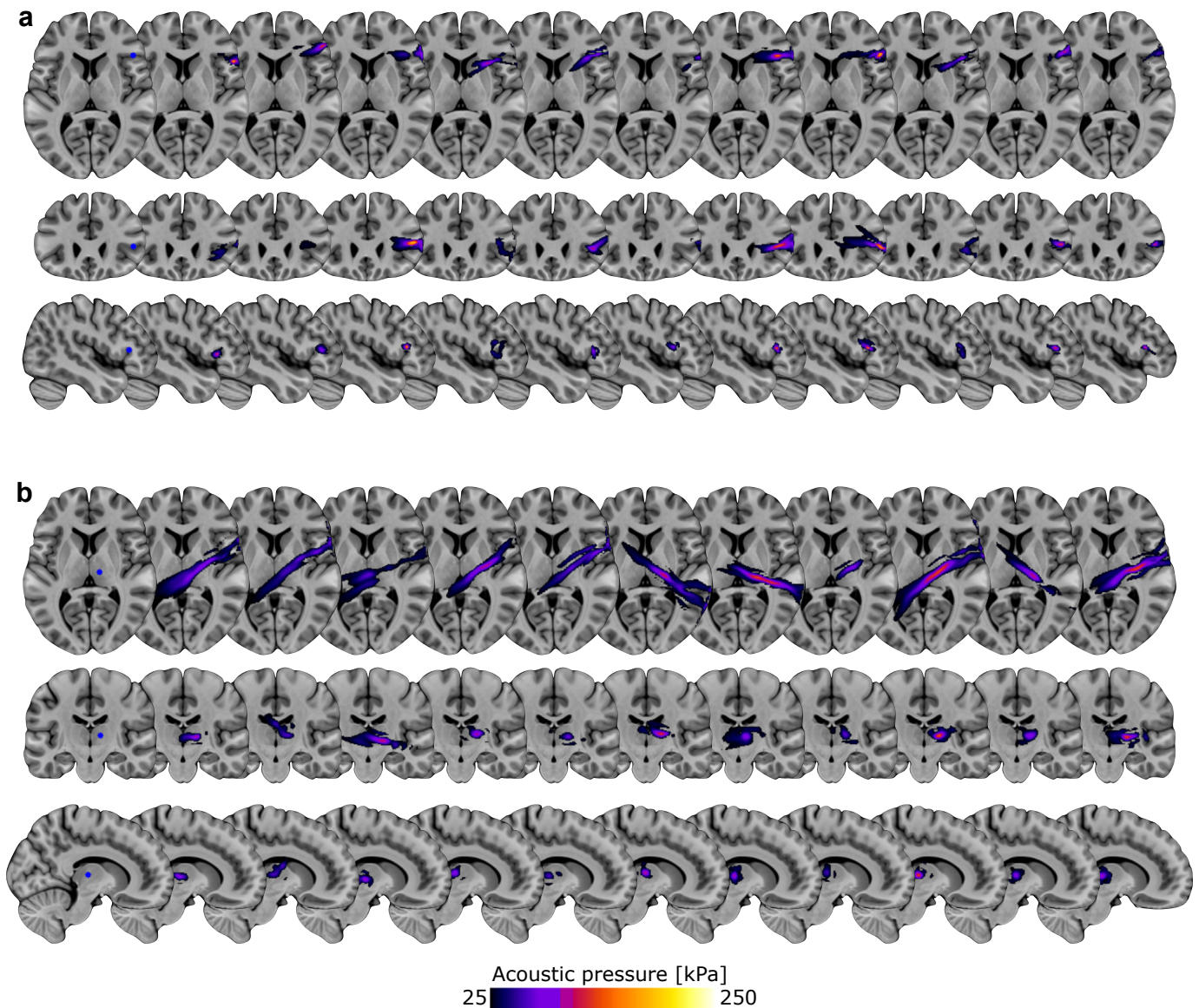


Figure 2. Applied acoustic pressure for the IFC (panel a) and the thalamus (panel b). The figure highlights the estimated applied acoustic pressure based on the acoustic simulations produced using the k-plan. The first column shows the targets in blue, whereas the other columns show the results for all participants (11 per group). The slices are centered on the target middle (IFC: $x = 46$, $y = 26$, $z = 8$; Thal: $x = 11$, $y = -18$, $z = 7$). [Color figure can be viewed at www.neuromodulationjournal.org]

The fractional amplitude of low-frequency fluctuations (fALFF) metric corresponds to the ratio of the low-frequency power spectrum (0.01–0.1 Hz) and reflects the spontaneous brain activity intensity.²⁸

Regional homogeneity (ReHo) is a calculation of the similarity between the time series of one voxel and its neighborhoods (calculated using Kendall's coefficient concordance).²⁹ It reflects local functional connectivity.

Degree centrality (DC) corresponds to the number of connections a voxel has (threshold: $R \geq 0.6$). This metric is based on graph theory and is often used to assess whole-brain neural network alterations.

Local functional connectivity density (LFCD) is a local variant of the (general) DC. After calculating the connections between every

pair of voxels, LFCD identifies spatially connected clusters of connected voxels (threshold: $R \geq 0.6$). Therefore, LFCD reflects the strength of the connectivity between one voxel and its neighborhood.³⁰ In contrast to ReHo, LFCD is not restricted to direct neighbors because it considers clusters in any direction and size if they reach a predefined threshold.

Eigenvector centrality (EC) first calculates the correlations between each pair of voxels. This allowed us to determine the importance of every voxel by considering its connectivity with others and their connectivity (ie, whether a voxel is connected to central voxels).³¹

To obtain the same number of volumes among all conditions (ie, 598), we decomposed the 42 minutes fMRI obtained after TUS into three. This splitting approach allowed us to generate three time

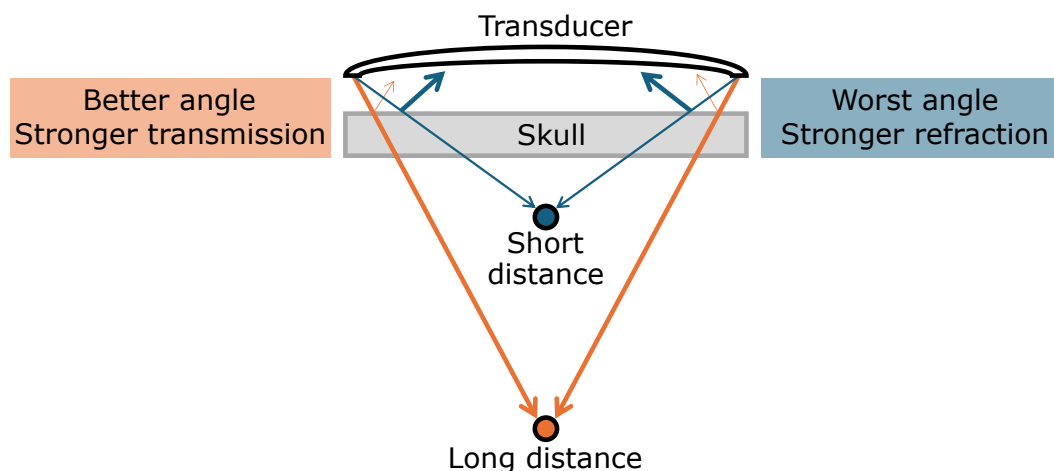


Figure 3. Schematic illustration explaining the higher delivered acoustic pressure in deeper targets. [Color figure can be viewed at www.neuromodulationjournal.org]

points after TUS for the IFC (T1: volumes acquired between 15.01 and 28.96 minutes after TUS [SD = 2.26 minutes]; T2: between 28.96 and 42.91 minutes after TUS; T3: between 42.91 and 56.87 minutes after TUS) and the thalamus groups (T1: between 15.06 and 29.01 minutes after TUS [SD = 1.62]; T2: between 29.01 and 42.97 minutes after TUS; T3: between 42.97 and 56.92 minutes after TUS). All fMRI metrics were calculated using nonsmoothed pre-processed fMRI, after which 6-mm smoothing was applied. No framewise displacement difference was found between the pre-TUS, T1, T2, and T3 conditions, after IFC-TUS ($F_{(3,40)} = 1.3119$, $p = 0.2839$) and Thal-TUS ($F_{(3,40)} = 2.135$, $p = 0.111$).

Statistical Analyses

Statistical analyses were performed on R in two steps.

First, we evaluated the spatial differences in fMRI metrics (fALFF, ReHo, DC, LFCD, and EC) after TUS. To do so, we ran *t*-tests between the fMRI of the 22 controls (ie, obtained before TUS) and of 11 of each group (IFC or Thal), and the three different moments after TUS (T1, T2, and T3). Owing to the high number of comparisons, the threshold for significance was set at $p \leq 0.05$ after Bonferroni correction (applied per metric, on the 235,685 voxels present in the mask). On the basis of post hoc power analyses, only effect sizes ≥ 0.3 could be observed.

Second, we were interested in the relationship between the observed effects (ie, the T-score, corresponding to the normalized difference of the post- minus pre-TUS conditions) and the applied acoustic pressure, regardless of brain anatomy or location. Therefore, we first extracted a whole-brain Neuroimaging Informatics Technology Initiative map of the applied acoustic pressure for each participant and averaged it (11 per target) to create a single representation of the average acoustic pressure. The averaged images were scaled (minimum value = 0, maximum = 100). To counterbalance the low number of voxels with a high applied acoustic pressure, we calculated the average T-score of all voxels for all acoustic pressure percentage values (limiting our analysis to a maximum of 101 data points instead of approximately 235,000). Specifically, this produced one value for each percentage point of applied acoustic pressure. Subsequently, a generalized additive model (GAM) was fitted with the applied acoustic pressure (as a percentage) as a predictor and the T-score (effect size) as the

predicted variable. This allowed us to determine whether increasing the acoustic pressure was related to a change in the average T-score regardless of its location. A GAM is a type of generalized linear model that allows the automatic smoothing of predictor variables, rendering it more flexible and less hypothesis-driven than a linear model (linear assumption), leading to a better fit with the data. In this study, the degree of smoothing of the predictor variables was determined using a restricted maximum likelihood approach. We therefore assessed whether the estimated T-scores were different from 0 (ie, no effect) and deemed significant any effects with $p \leq 0.05$ after Bonferroni correction.

RESULTS

Group Comparison

There were no significant differences between the two groups in terms of sex (six women and five men in each group; Table 1) or age (IFC: 25 ± 5.7 years; Thalamus: 22.8 ± 5.7 years; $p = 0.38$). The time interval between the TUS procedure and initiation of rs-fMRI did not differ between the groups ($p = 0.95$). The acoustic simulations revealed no significant differences in the applied peak pressure ($p = 0.173$) or peak pressure at the target ($p = 0.272$). However, significant differences were observed in the activated volume ($p < 0.001$), ISPTA ($p = 0.008$), mechanical index ($p = 0.013$), and maximum temperature increase ($p < 0.001$). These variations were primarily attributed to the differences in depth between the two target regions. Acoustic simulations also revealed that different parts of the thalamus were reached. Averaged for the 11 participants, the ventral posterolateral (right) was the most reached, representing 11.5% of the activated volume (mean), followed by the ventral lateral (right, 5.4%), the pulvinar medial (right, 4.8%), the pulvinar anterior (right, 2.9%), the lateral posterior (right, 2.4%), the mediodorsal medial (right, 1.3%), the intralaminar (right, 0.9%), the mediodorsal lateral parvocellular (left, 0.8%), the pulvinar lateral (right, 0.7%), and the anteroventral (right, 0.4%).

Spatial Consequences of TUS

We observed significant alterations in LFCD after IFC-TUS and Thal-TUS, in addition to in DC after Thal-TUS (Fig. 4 and Table 2). Specifically, we observed that IFC-TUS led to increased LFCD in the

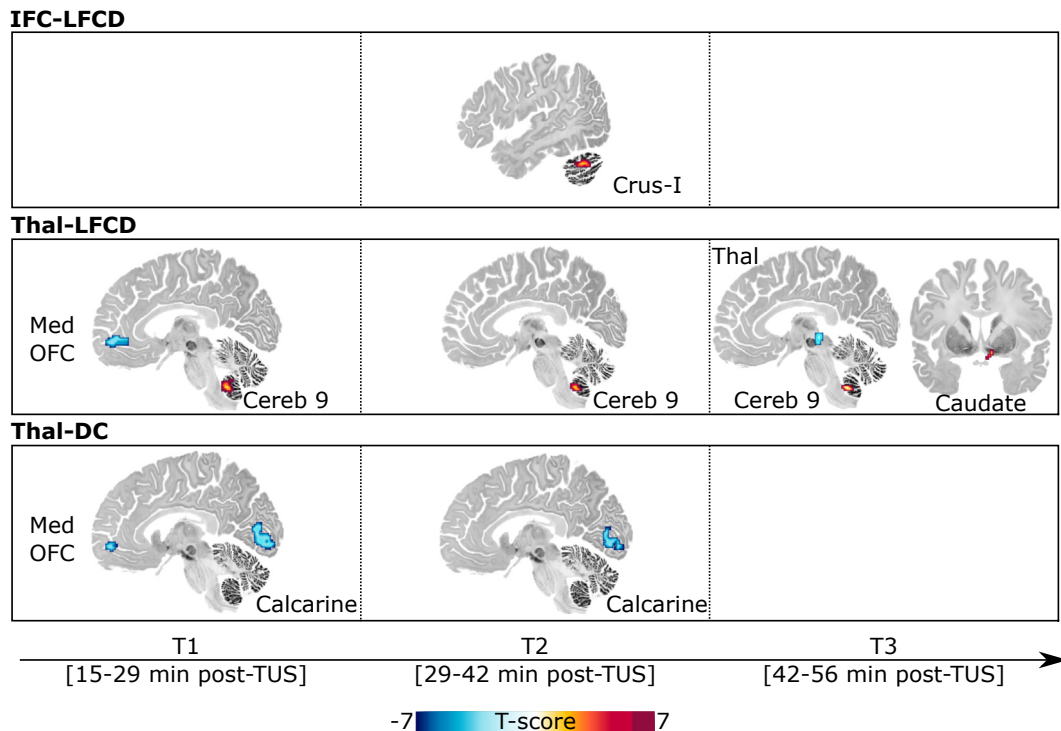


Figure 4. Spatial functional changes after TUS. The figure highlights the significant increases (red) and decreases (blue) observed after IFC and Thal-TUS at three different time points. The results are significant at $p \leq 0.05$ after Bonferroni correction, but owing to the small size of the clusters, we extended them to include neighbors' voxels significant at $p \leq 0.001$ uncorrected. Cereb, cerebellum; Med OFC, medial orbitofrontal cortex. [Color figure can be viewed at www.neuromodulationjournal.org]

right cerebellum Crus-I part, only present at T2. After Thal-TUS, we observed increased LFCD in the cerebellum part 9 left, which was significant at T1, T2, and T3. LFCD also was altered in the right medial orbitofrontal cortex (T1), left thalamus (T3), and right caudate nucleus (T3). DC was decreased in the left calcarine cortex (at T1 and T2) and in the right medial orbitofrontal cortex (T1). No significant effects were observed for the other metrics: fALFF, ReHo, and EC.

Relations With the Applied Acoustic Pressure

When examining the relationship between the observed effects (T-scores) and the applied acoustic pressure, we found some interesting results (Fig. 5). First, it seems that most effects tend to occur with higher acoustic pressure and increased delay with TUS (more at T3 than at T2 or at T1). Specifically, we observed that fALFF was abnormally high after TUS at T3 for both IFC-TUS (when the applied acoustic pressure was between 58% and 96% of the maximum) and Thal-TUS (regardless of the acoustic), in addition to at T2 for Thal-TUS (acoustic pressure between 9% and 96% of the maximum). For ReHo, changes were only found after IFC-TUS at T3 if the acoustic pressure was between 35% and 97% of the maximum. As for DC changes, these also were only observed after IFC-TUS, with changes occurring at T2 when the acoustic pressure was between 52% and 89% of the maximum and at T3 when it was between 47% and 92% of the maximum. LFCD decreased after Thal-TUS, only at the short term (T1) and for an acoustic pressure between 54% and 98% of the maximum. The pattern was more varied for EC, which decreased at T1 after IFC-TUS (between 18%

and 33% of the maximum) and at T2 (between 76% and 92% of the maximum).

DISCUSSION

In this study, we provide new evidence regarding the consequences of TUS in healthy human brains using whole-brain and hypothesis-free fMRI metrics. First, we showed that TUS mostly alters functional connectivity metrics at both the local (LFCD) and global (DC) levels. Second, we identified strong relationships between metric alterations and applied acoustic pressure, with stronger alterations observed when the delay after TUS increased, suggesting that some TUS consequences may not be immediate.

This study is particularly relevant regarding the variety of brain metrics considered. This allowed us to provide an accurate picture of the consequences of TUS. In addition, the duration of fMRI obtained after TUS was significantly long, making it possible to observe both short-term (~15 minutes after TUS) and longer-term changes (~45 minutes after TUS). We also reached two different targets; therefore, we can conclude that the changes were both target specific and nonspecific. Last, we used a dedicated tool to determine the optimal transducer position on the participants' heads. The advantages of this tool are twofold: It allowed us to be accurate and to systematically reach our targets, and the transducer location was then used in k-plan with the same position as that used during the actual stimulation. This aspect is particularly relevant because most published studies based on ultrasound neuromodulation did not use this tool or an equivalent, making

Table 2. Details of the Significant Brain Changes After IFC- and Thal-TUS.

Group	Label	t	x	y	z	k
LFCD						
IFC-TUS (T1)	No suprathreshold clusters					
IFC-TUS (T2)	Crus-I R	7.49	47	-59	33	3
IFC-TUS (T3)	No suprathreshold clusters					
Thal-TUS (T1)	Medial OFC R	-7.62	1	47	-7	3
	Cereb 9 L	6.83	-5	-49	-47	2
Thal-TUS (T2)	Cereb 9 L	6.98	-1	-51	-51	1
Thal-TUS (T3)	Thalamus L	-7.00	-3	-25	-3	7
	Cereb 9 L	6.84	-3	-49	-49	3
	Caudate R	6.63	11	5	-11	1
DC						
IFC-TUS (T1)	No suprathreshold clusters					
IFC-TUS (T2)	No suprathreshold clusters					
IFC-TUS (T3)	No suprathreshold clusters					
Thal-TUS (T1)	Calcarine L	-7.71	-9	-91	-5	3
	Medial OFC R	-6.74	11	51	-7	2
Thal-TUS (T2)	Calcarine L	-7.01	-9	-91	-5	2
Thal-TUS (T3)	No suprathreshold clusters					
fALFF / ReHo / EC						
IFC-TUS (T1)	No suprathreshold clusters					
IFC-TUS (T2)	No suprathreshold clusters					
IFC-TUS (T3)	No suprathreshold clusters					
Thal-TUS (T1)	No suprathreshold clusters					
Thal-TUS (T2)	No suprathreshold clusters					
Thal-TUS (T3)	No suprathreshold clusters					
Thal-TUS (T3)	No suprathreshold clusters					

k, number of voxels within the cluster; L, left; OFC, orbitofrontal cortex; R, right.

their stimulation more likely to miss the target and their acoustic simulations less reliable.

Nevertheless, it is important to acknowledge the limitations of this study. Specifically, we did not compare our results with those of real sham conditions. If this is a significant weakness, it is relevant to specify that there is no valid sham model for TUS studies. Keeping the TUS transducer off is unsatisfactory because it lacks associated sensations (sounds and vibrations). Flipping a transducer over can damage it. Targeting other regions of the brain could lead to similar outcomes. Delivering noninvasive sensations to hide auditory and vibrational confounds is not satisfactory.³² However, our results could be deemed as controlled by an active sham, given we did not find identical outcomes after IFC and thalamus-TUS. To minimize the risk of false positive results, we rigorously corrected our analyses using the Bonferroni method. Hence, we have a high level of confidence that our findings accurately reflect the real effects of TUS. However, owing to the small sample size, it is likely that our Bonferroni correction led to false negatives. Another limitation is the approximately 15-minute delay between stimulation and the fMRI, which renders us blind to the immediate effects. Future studies will have to assess the immediate effects by applying TUS within the scanner. It also is possible that ultrasound stimulation affects unintended regions of the brain adjacent to the primary targets (Fig. 2). Currently, most TUS devices are relatively compact, producing stimulation of a broad cigar-shaped area of the brain. Therefore, we cannot completely exclude the possibility that some of our findings stem from stimulation of brain areas other than our targets. However, this is less likely, considering that the targets were the only regions consistently reached across all participants. Finally, other

limitations could be mentioned, such as the possibility that some participants fell asleep during MRI, order effects (TUS was always applied during the second visit), the small sample size (two groups of 11 individuals), and the use of a PCT based on T1, which did not provide skull information.

The results are spatially consistent. After the IFC-TUS, we observed an increase in cerebellar LFCD, which is relevant because this area of the cerebellum is functionally connected to the IFC and plays a crucial role in executive processes,³³ such as the IFC itself.³⁴ After Thal-TUS, we interestingly found a decreased LFCD in the contralateral thalamus and an increased LFCD in the right caudate nucleus and the medial cerebellar lobule 9. These results could reflect an alteration in the sensorimotor/basal ganglia network, for which the thalamic local disconnection is compensated by the increased local connectivity of the caudate nucleus and cerebellum. We also observed that Thal-TUS led to a decreased local and global connectivity of the medial orbitofrontal cortex. The relationship between the thalamus and orbitofrontal cortex has already been described as important for cognitive functioning and as impaired in numerous psychiatric conditions when disconnected.³⁵ The relationship between the thalamus and the calcarine cortex was found to decrease. Although the calcarine cortex is primarily known as part of the visual cortex, it also has been identified as being functionally connected to the thalamus,³⁶ with both structures involved in pain processing.^{37,38} Overall, these results suggest that alterations after TUS vary depending on specific brain regions. Although the cortical areas showed decreased connectivity, deeper brain areas exhibited increased connectivity after Thal-TUS, except in the contralateral thalamus.

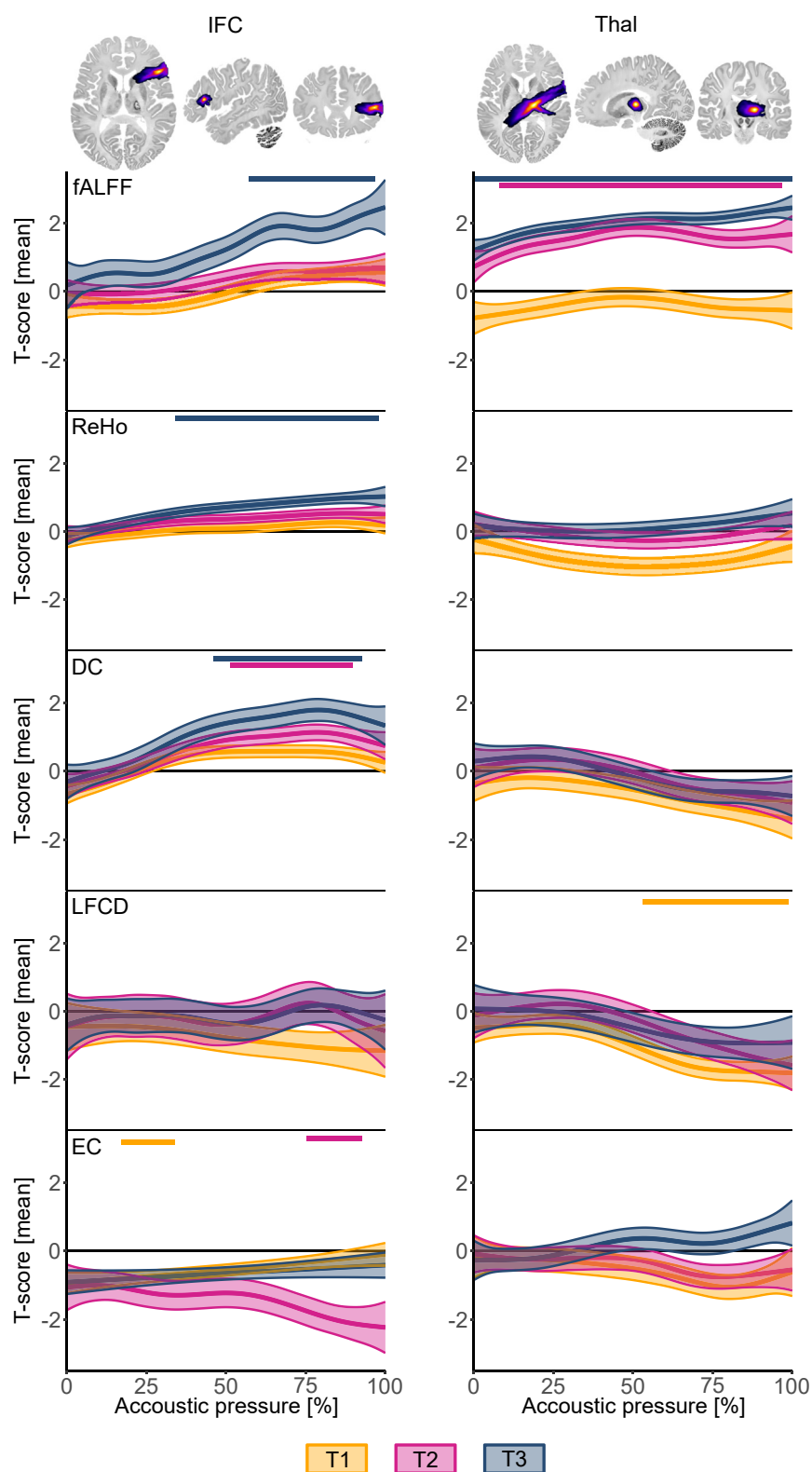


Figure 5. Relationship between functional changes and acoustic pressure after TUS. The figure shows ways the average effects (T-scores) are related to acoustic pressure. A value >0 reflects an increase in the metrics, whereas a value <0 reflects a decrease. The horizontal bars at the top of each element indicate significant effects. The three different time points are represented in yellow (T1—volumes acquired from 15.01–28.96 minutes after TUS, and from 15.06–29.01 minutes after Thalamus-TUS), pink (T2—volumes acquired from 28.96–42.91 minutes after IFC-TUS, and from 29.01–42.97 minutes after Thalamus-TUS), and blue (T3—volumes acquired from 42.91–56.87 minutes after TUS, and from 42.97–56.92 minutes after Thalamus-TUS). The top images represent the mean applied acoustic pressure threshold of 20%. k, number of voxels within the cluster; L, left; OFC, orbitofrontal cortex; R, right. [Color figure can be viewed at www.neuromodulationjournal.org]

Temporally, our results were unexpected because most TUS-related alterations were observed at T3 (~45 minutes after TUS), suggesting a progressive impact of TUS rather than an abrupt one. In a short time (T1, ~15–30 minutes after TUS), we found a decreased LFCD related to higher acoustic pressure (after Thal-TUS), which was not significant at T2 and T3. This outcome suggests that the TUS parameters induced prompt disconnection of the targeted region for only a short period. Given no change was found for DC, we also could argue that the effects were mostly spatially restricted. In the longer term, we observed that IFC-TUS was associated with increased DC (from T2), ReHo (from T3), and fALFF (from T3), whereas Thal-TUS was associated with increased fALFF (from T2). Stronger effects (mean T-score >2) were observed for fALFF. This suggests increased brain activity near the target after IFC-TUS, and for the whole brain after Thal-TUS (significantly higher than zero for all acoustic pressure values). Taken together, these results are twofold.

First, there is the question regarding the direct impact of TUS on a specific part of the brain. It is estimated that TUS has the potential to either increase or decrease brain activity, depending on certain parameters. However, there is no direct evidence of this potential in humans. Several studies have suggested that adjusting the duty cycle and intensity parameters can activate or suppress brain activity.^{39,40} However, our results indicate that the induced changes could be more complex. First, we observed LFCD at T1, followed by increased functioning in the longer term (increased fALFF, ReHo, and DC). Recent *in vitro* and animal studies have revealed interesting and comparable findings regarding the dynamics of brain changes after TUS. First, the *in vitro* study⁴¹ highlighted an increased neuron excitability that was higher four to six hours after 40-second TUS than 0 to two hours thereafter. Second, a nonhuman primate study⁴² identified six different time courses of brain connectivity alterations, some of which were transient and could occur hours after TUS. Therefore, different or even opposing outcomes could be observed, depending on the time delay after stimulation.

Second, there is a question regarding the delay required to induce an effect. Most research on TUS is based on the premise that its effects are immediate and will progressively decrease. However, similar to most well-known therapeutic approaches, it seems plausible that changes will progressively appear (with an unknown dynamic) and subsequently disappear. This is the case in pharmacology, for which pharmacodynamics are always studied, but also in deep brain stimulation, for which some effects are almost immediate (eg, tremor and rigidity in Parkinson's disease), whereas others require a significant latency of minutes to days (eg, cognitive signs in Parkinson's disease and tics in Tourette syndrome). Although the exact mechanisms of TUS remain a matter of debate, there is a consensus regarding induced homeostatic plasticity⁴³ and neural activity stabilisation. On this point, one specific study focused on the impact of different transcranial direct current stimulations (tDCS) on human brain plasticity.⁴⁴ The authors identified that if two 5-minute tDCS are performed 30 minutes apart, the second one has the same effects as the first. However, if the delay between the two tDCS is only 3 minutes, the second tDCS has the opposite effects to the first. This indicates that the consequences of brain stimulation depend on the brain states, which could differ depending on the stimulation parameters and delay.

Finally, although our findings help enhance our understanding of the effects of TUS on human brain function, they also may lead to further uncertainty. TUS is promising and therefore attracts

significant attention; however, the high variability among TUS studies complicates interstudy comparisons. Studies can differ in various aspects such as the protocols used, targeted areas, metrics used, conditions under which TUS was applied (whether at rest or during tasks), populations studied (healthy individuals versus patients), and the timing between TUS application and outcome measures (online versus offline studies). For instance, the TUS protocol we used is typically deemed excitatory owing to previous findings showing increased cortical excitability^{16,17} or seed-based connectivity.¹² However, interpreting these results is complex given the observed increased excitability was linked to a decrease in GABA concentration rather than an increase in glutamate levels, which was significant in only one of the two TUS targets.¹² Moreover, this increase in cortical excitability has not been replicated in a concurrent double-blind study⁴⁵ and was found to decrease in another study with a close TUS protocol.⁴⁶ In addition, theoretical models and literature reviews mostly suggest that this protocol might be more suppressive than excitatory.^{39,40} Hopefully, the accumulation of evidence will progressively lead to obtaining enough information to disentangle these heterogeneous findings.

CONCLUSIONS

Our study highlights ways TUS alters brain functioning, with which delay, and with which relationship with the acoustic pressure we applied. If we mostly observed local and global functional connectivity alterations, we also discovered that most of the TUS effects appeared approximately 45 minutes after the stimulation and in different directions, depending on the considered metric. Altogether, our findings invite us to reconsider the premise of the delay and direction of TUS effects.

Data Availability

All the data and the original code used to analyze the data in this study are available from the corresponding contacts upon request.

Authorship Statements

Cyril Atkinson-Clement was responsible for the conceptualization, methods, formal analysis, investigation, data curation, visualization of the study, and the writing of the original draft. Mohammad Alkhawashki, Marilyn Gatica, and Stefanos Alexandros Kontogouris were responsible for the study investigation, writing, reviewing, and editing. Marcus Kaiser was responsible for conceptualization, methods, writing—review and editing, supervision, project administration, and funding acquisition. All the authors approved the final version of the manuscript.

Conflict of Interest

The authors reported no conflict of interest.

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SUPPLEMENTARY DATA

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COMMENT

In this study, the authors applied transcranial focused ultrasound stimulation (tFUS or TUS) in a small cohort of healthy human participants who received stimulation in either the thalamus or the right inferior frontal cortex. The authors report several metrics of local and

global changes in resting-state neuroimaging data measured at different delays after offline application of TUS. This study is an important contribution to the growing interest in the neuro-modulatory effects of TUS.

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