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Safety of transcranial focused ultrasound stimulation: A systematic review of the state of knowledge from both human and animal studies

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ABSTRACT

Background: Low-intensity transcranial focused ultrasound stimulation (TFUS) holds great promise as a highly focal technique for transcranial stimulation even for deep brain areas. Yet, knowledge about the safety of this novel technique is still limited.
Objective: To systematically review safety related aspects of TFUS. The review covers the mechanisms-of-action by which TFUS may cause adverse effects and the available data on the possible occurrence of such effects in animal and human studies.
Methods: Initial screening used key term searches in PubMed and bioRxiv, and a review of the literature lists of relevant papers. We included only studies where safety assessment was performed, and this results in 33 studies, both in humans and animals.
Results: Adverse effects of TFUS were very rare. At high stimulation intensity and/or rate, TFUS may cause haemorrhage, cell death or damage, and unintentional blood-brain barrier (BBB) opening. TFUS may also unintentionally affect long-term neural activity and behaviour. A variety of methods was used mainly in rodents to evaluate these adverse effects, including tissue staining, magnetic resonance imaging, temperature measurements and monitoring of neural activity and behaviour. In 30 studies, adverse effects were absent, even though at least one Food and Drug Administration (FDA) safety index was frequently exceeded. Two studies reported microhaemorrhages after long or relatively intense stimulation above safety limits. Another study reported BBB opening and neuronal damage in a control condition, which intentionally and substantially exceeded the safety limits.
Conclusion: Most studies point towards a favourable safety profile of TFUS. Further investigations are warranted to establish a solid safety framework for the therapeutic window of TFUS to reliably avoid adverse effects while ensuring neural effectiveness. The comparability across studies should be improved by a more standardized reporting of TFUS parameters.

Introduction

Weak Transcranial Focused Ultrasound Stimulation (TFUS) aims to modulate neural activity by delivering a focused ultrasonic beam to a small target area in the brain. Currently, interest in TFUS is strongly increasing as it holds the promise of a far better spatial resolution than established non-invasive stimulation techniques and of the ability to reach deep brain areas [1]. This might open up intriguing new applications such as epilepsy treatment or pre-surgical diagnostics prior to electrode implantation for deep-brain stimulation [2,3]. TFUS is also attractive because it can be readily combined with neuroimaging modalities such as functional magnetic resonance imaging (fMRI) and electroencephalography

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(EEG) without interfering with the recordings, as it applies acoustic waves rather than electric or magnetic fields.

Firmly establishing its safety profile is a central requirement when aiming to move TFUS from initial pilot studies towards broader testing in humans in-vivo. Reviews on safety and bio-effects of ultrasound (US) in diagnostics [4] and therapy [5] as well as guidelines for the clearance of commercial diagnostic and therapeutic US systems as medical devices [6] are available and constitute a benchmark to avoid harmful effects also for TFUS. Relating the TFUS parameters to these guidelines, as done in many of the published studies, might be considered a conservative choice. However, several aspects put TFUS in a special position. TFUS usually employs lower frequency compared to diagnostic ultrasound (usually upper kHz range vs. MHz) and longer pulse bursts. TFUS has a static focus so that the total energy delivered at the focal point can be higher than the maximal local energy deposit for diagnostic US, as the latter uses scanning approaches. The mechanism-of-action of TFUS is still poorly understood, rendering it more difficult to principally exclude harmful effects. In addition, current findings about the dose-response curve of TFUS [7] suggest that future therapeutic applications might aim to use intensities above the safety limits for diagnostic US in order to increase the robustness of the neural effects. Such a choice requires solid knowledge about the safety margin of TFUS. Along similar lines, accurate dose control for human TFUS is complicated by the presence of the skull, which strongly attenuates the beam. The attenuation depends on the individual skull thickness and composition [8], which are difficult to account for and lead to conservative intensity choices with an increased risk of underdosing. If the safety margin of TFUS is not well established, the use of more lenient dosing strategies to mitigate this problem is not feasible.

There is a pressing need to establish specific safety guidelines for TFUS. Yet, the current knowledge about the risk-benefit ratio and the therapeutic window of TFUS is still rudimentary because TFUS is at an early stage of development. Indeed, no dedicated phase I safety human study has been performed so far, but the safety profile needs to be systematically investigated and monitored to ensure the patients' safety. However, relevant information is already available today, because some of the published studies on TFUS in animals or humans included safety-relevant tests. Here, we systematically summarize these findings to give an overview of the current state of knowledge about TFUS safety. We start by describing the relevant physical parameters used to characterize the TFUS stimulus. We then shortly describe the known physical mechanisms by which ultrasound can cause tissue damage and we introduce the established safety indices, based on the beam parameters. Finally, we introduce the methods that have so far been applied to test for adverse effects of TFUS, and list the corresponding results. In the discussion, we summarize the implications of the available findings for in-vivo human TFUS applications.

Material and methods

Literature review on the safety of TFUS

For this systematic review, we followed the PRISMA guidelines [9,10]. Details on the implementation of the PRISMA requirements in our review are stated in the Supplementary material (Table S1). Our review was based on searches in PubMed (www.ncbi.nlm.nih.gov/pubmed) and bioRxiv (https://www.biorxiv.org/) for published and pre-published studies, using the keywords 'tFUS', 'LIFUP', 'noninvasive brain stimulation focused ultrasound', 'neuromodulation brain transcranial ultrasound', 'focused ultrasound transcranial brain stimulation' and 'pulsed ultrasound brain stimulation'. The eligibility criteria were low intensity, low frequency TFUS in the brain of animals or humans with safety assessment, without use of microbubbles. Additional sources were reviews of the literature lists of relevant papers, and papers pointed out by the reviewers during the peer-review process. Fig. 1 shows details of the literature search. The last complete search was performed in January 2019 by one of the authors, and the last update was done in June 2019. From each paper, the sonication parameters and the methods used to assess safety and adverse effects were extracted as shown in Table 2 and Table 3 and categorized as described further below. Often, only some of the safety indices were reported. In that case, we give estimated values when possible.

Mechanism of ultrasound neuromodulation

Despite many hypotheses, the exact underlying mechanism of neuromodulation using low-intensity ultrasound is yet to be understood [11]. The initial hypotheses for the ultrasound neuromodulation were thermal effects and acoustic cavitation. While an increase in the tissue temperature could perturb neuronal activity levels, the temperature increase due to low-intensity ultrasound is often less than 0.1 °C. Thus, the thermal effects of low-intensity ultrasound are most likely negligible. The second hypothesis is based on acoustic cavitation. This hypothesis postulates that the ultrasound generates nanobubbles in the lipophilic zone of the plasma membrane, which then vibrates according to the pressure variations, alters the local curvature of the bilayer, and changes overall neuronal excitability [12]. However, since nanobubbles are formed at an intensity larger than 100 mW/cm^2, generation of micro or nanobubbles at the intensity used in standard neuromodulation protocols must be confirmed. The recent hypotheses now focus more on the effects of acoustic radiation forces on the permeability of the ion channels, such as mechanosensitive channels [13] and voltage-gated calcium, sodium, and potassium channels [14]. Another kind of hypotheses includes plasma deformation, which postulates that vibration of surrounding extra- and intracellular environment evokes mechanical changes in either the plasma membrane tension or the lipid bilayer and modulates neuronal activities [14].

Contrary to these works on the mechanisms involved with direct modulation of ion channels and membranes, an indirect in vivo ultrasound neuromodulation through auditory or cochlear pathways has been also recently proposed [15,16]. These studies demonstrated that ultrasound-induced activities were eliminated or reduced upon transection of the auditory nerves or removal of cochlear fluids. These results raised an important question of whether direct activation of neurons in the intact brain is possible. While more in-depth studies on the experimental protocols such as sharpness of the pulse, pulse repetition frequency, and bone transduction must be performed, these studies underscore the need for a solid understanding of the underlying mechanism of ultrasound neuromodulation [16].

Physical parameters and safety indices of US waves

A sketch of an experimental setup for TFUS is shown in Fig. 2A, using the stimulation of a rat as example. The main indices used to assess safety are:

- I_{spta} (spatial peak temporal average intensity) is the temporal average intensity, calculated at the position of the spatial maximum
- I_{sppa} (spatial peak pulse average intensity) is the pulse average intensity, calculated at the position of the spatial maximum
- *MI* (mechanical index) gives an estimation of the likelihood of inertial cavitation

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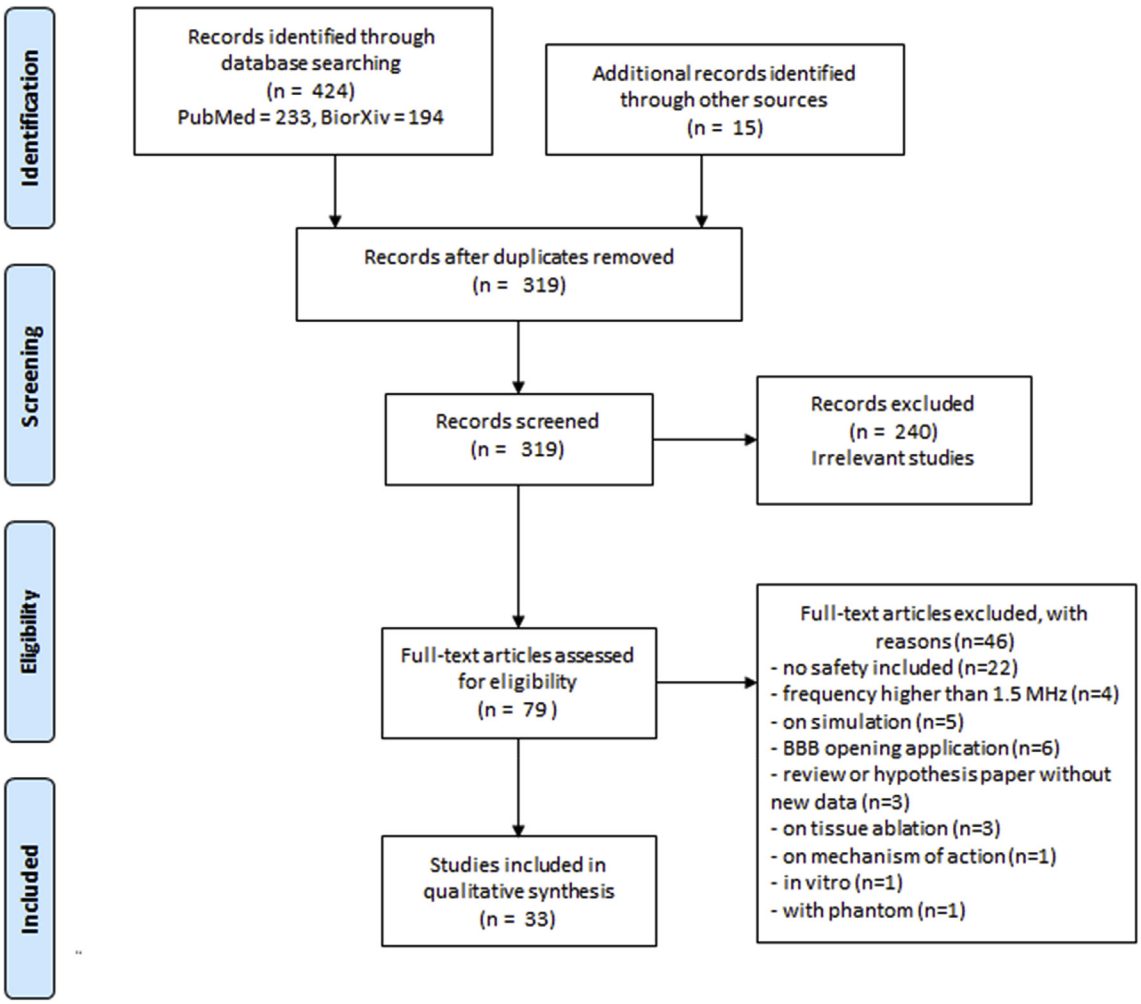

Fig. 1. Selection process for the studies included in this review. The scheme is from Refs. [9,10].

- *TI* (thermal index) is the steady-state temperature increase in soft tissue during ultrasound sonication
- *TIC* (thermal index for cranial bone) is a modification of TI, when the skull is close to the transducer face

I_{spta}, TI and TIC are related to the risk of thermal bio-effects, while I_{sppa} and MI are related to the risk of cavitation. The upper limits for these five indices allowed for diagnostic ultrasound are shown in Table 1. It should be noted that another guideline, IEC standard 60601-2-5 for physiotherapy US equipment, sets an upper limit for the "effective intensity", defined as the ratio of acoustic output power to effective radiating area, of 3 W/cm^2. The standard also states that this value should only be reached for short times to prevent substantial heating. The "effective intensity" of 3 W/cm^2 is usually interpreted as the upper limit for I_{spta} [17–19]. Lee and colleagues [17] compare the intensities used in their study against this limit rather than using the FDA guidelines for diagnostic US. Complementary to TI, the temperature increase at the target can be calculated as ΔT_{max} (equation 7 and 8 in Supplementary material) or through the bio-heat equation [20–22]. A more detailed explanation of these indices and formulae can be found in the Supplementary Material.

Table 1
Allowed limits for MI, TI, I_{spta} and I_{sppa} according to the FDA guidelines for diagnostic ultrasound. The limit for TI also applies to TIC when bone is close by.

I_{spta} (mW/cm^2)	I_{sppa} (W/cm^2)	MI	TI
720	190	1.9	6

Mechanisms underlying tissue damage by US

Ultrasound waves may cause harmful effects on tissues via two physical mechanisms, mechanical and thermal. The main mechanical effect is cavitation, in which vapor cavities (or "bubbles") form in the soft tissues during the periods of low pressure (i.e. the minima) of the acoustic wave cycles. Depending on intensity and center frequency, this can result in a stable oscillation (stable or non-inertial cavitation) or can result in violent bubble collapses (inertial cavitation) that create large forces in their neighborhood. The air bubbles can have an endogenous origin (for example in the lungs or intestine), or they can be created by the mechanical wave itself, if the peak rarefaction pressure (i.e. the pressure during the minima) is small enough to allow the liquid to reach vaporization. Alternatively, ultrasound contrast agents (UCA), which contain microbubbles, can be injected for, e.g. clinical purposes [23] or gene and drug delivery [24].

When a mechanical wave propagates linearly in a medium, its amplitude decreases exponentially starting from the source. The attenuation is caused by both scattering, i.e. the change in the

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Table 2

Overview of the parameters used in the reviewed studies. I_{spta} values very often exceeded the limits for diagnostic US. Cases where the I_{spta} values were higher than 3 W/cm^2, corresponding to the limit for physiotherapeutic US, are highlighted in bold. Also one case in which MI exceeded the limit of 1.9 is marked in bold. If needed, we calculated missing parameters from the available data stated in the paper, which we indicate by "*" in the table. When the peak pressure was reported, MI was calculated using its definition (eq. (2)), and I_{sppa} in water as indicated in Fig. 1 ($\rho = 1000$ kg/m^3, $c = 1500$ m/s). I_{spta} was finally determined as I_{sppa} x DC. 1) For [27], only the parameters employed in the safety tests of that study are listed here. 2) For [28], only the parameters for the main experiment are reported. 3) In Ref. [66], I_{spta} was determined by using ISI instead of PRP as the total pulse duration; this strongly reduces the value. 4) For [47], MI, I_{sppa} and I_{spta} are not stated, but authors asserted that they used the same waveform as [62]. 5) Not clear if it is in water or after cranial transmission. 6) A spatial average of intensity of 25–30 W/cm^2 is used. 6) Modulated focused ultrasound means that two transducer, one driven at 2.25 MHz and the other at 1.75 MHz, producing a difference frequency at 500 kHz at the focus, and a carrier frequency of 2 MHz.

Study	Target	Parameters									Observed neural effect and adverse effect (if any)
		f_c [kHz]	TBD	PRF	SD	Number of sonica-tions	ISI	MI	I_{sppa}	I_{spta}	
Legon et al. preprint [55]	Human thalamus or M1	Follow-up questionnaire of 7 experiments, only 3 are published so far ([62,65] and one preprint [47])									This work presents results on safety assessment.
Verhagen et al. [22]	Non-human primate SMA, FPC and pre-SMA	250	30 ms	10 Hz	40 s	1	–	2.4 in water * 1.68 after cranial transmission (estimated from pressure peak) *	48 W/cm^2 in water * 23.52 W/cm^2 after cranial tx *	14.4 W/cm^2 in water * **7.056 W/cm^2** after cranial tx *	Reversible change in brain connectivity, that last up to 2 h after treatment.
Fisher et al., 2018 [31]	Mice primary somatosensory cortex	510	500 μs	1 kHz	1 s	1	–	0.24 in water *	0.69 W/cm^2 in water	345 mW/cm^2 in water *	Early sensory-evoked cortical responses (3.0 ± 0.7 ms earlier) and alteration of Ca^{2+} responses.
		510		continuous	?	?	?	?	280 W/cm^2 in water	–	Parameter tested as control. BBB intentionally opened. An increased number of astrocytes was found.
Tufail et al., 2010 [27] 1)	Mouse motor cortex	500	0.45 ms	1.5 kHz	67* (53) ms	180*	10 s	0.13 after cranial tx	211.72 mW/cm^2 after cranial tx *	142.2 mW/cm^2 after cranial tx	Neuron's spike frequency and c-fos$^+$ cell density increase and the activity of endogenous brain-derived neurotrophic factor (BDNF) were stimulated. Low frequency (250 KHz) and low intensities (up to around $I_{spta} = 80$ mW/cm^2) result in more robust EMG response. The EMG failure probability increased with shorter ISI (200 ms), but decrease with multiple stimuli. BBB intentionally opened with the use of microbubbles.
Kim et al., 2012 [28] 2)	Rat abduncens nerve	350	0.36 ms	1.5 kHz	200 ms	10	1 s	0.9 after cranial tx (estimated)	8.6 W/cm^2 after cranial tx (estimated)	**4.6 W/cm^2** after cranial tx (estimated)	$f_c = 650$ kHz and I_{sppa} in the range 0.5–20 W/cm^2 did not elicit eye movement in any animals. Movements observed when $f_c = 350$ KHz for an I_{sppa} of 8.6 W/cm^2.
Lee et al., 2015 [34]	Sheep SM1 and V1	250	1 ms	500 Hz	300 ms	100 (groups of sonications repeated up to 8 times per animal)	5 s (motor cortex) or 1 s (visual cortex)	in the range 0.5 –1.4 after cranial tx	Up to 11.8 W/cm^2 after cranial tx –SM1 Up to 14.3 W/cm^2 after cranial tx -V1	Up to **5.9 W/cm^2** * after cranial tx –SM1 Up to **7.15 W/cm^2** * after cranial tx -V1	MEP or VEPs were detected over a certain intensity threshold, which varied across sheep and was always above diagnostic limits, and in some cases also above the physiotherapy limit. In both cases, higher I_{sppa} result in stronger response amplitude. Four animals which underwent 600 sonications at $I_{sppa} = 6.6$–10.5 W/cm^2 showed micro-hemorrhages in the primary visual cortex.
Yoo et al., 2011 [29]	Rabbit (after craniotomy), SM and visual area (the bottom line is only for temperature increase study)	690	0.05, 0.5, 10 and 50 ms	10, 20, 100 and 1000 Hz	0.5, 1, 1.5, 2, 9 s	1	–	<0.5 in water (for an $I_{sppa} = 3.3$ W/cm^2, resulting in clear BOLD activity)	3.3, 6.4, 9,5, 12.6 W/cm^2 in water	1.6 W/cm^2 in water (for $I_{sppa} = 3.3$ W/cm^2)	The BOLD activation was observed at a much lower acoustic intensity ($I_{sppa} = 3.3$ W/cm^2, $I_{spta} = 1.6$ W/cm^2) compared to the intensity that resulted in forepaw movement ($I_{sppa} = 12.6$ W/cm^2, $I_{spta} = 6.3$ W/cm^2)
		690	0.5 ms	100 Hz	27 s	1	–	?	23 W/cm^2	1.15 W/cm^2	Parameter tested as control for temperature increase

Lee et al., 2015 [51]	Human S1	250	1 ms	500 Hz*	300 ms	Around 200	3 s	0.62 after cranial tx (maximal simulated value across N = 12 subjects)	3 W/cm^2 in water 2.5 W/cm^2 after cranial tx (maximal simulated value)	1.5 W/cm^2 in water 1.25 W/cm^2 * after cranial tx (maximal simulated value)	Tactile sensations were not the same among subjects, but mostly at the hand area contralateral to the sonicated hemisphere. 1 out of 12 subjects did not report any sensation. Different peak amplitudes of EEG recording of SEP with and without stimulation.
Lee et al., 2016 [54]	Human S1+S2	210	1 ms	500 Hz	500 ms	20	7 s	?	35 W/cm^2 in water <8.8 W/cm^2 after cranial tx (estimated)	17.5 W/cm^2 in water **< 4.4 W/cm^2** after cranial tx (estimated)	Response rates of elicited sensations during the FUS procedures were different among subjects (68 ± 28% S1, 59 ± 22% S2, 61 ± 26% S1+S2, average ± sd across subjects).
Kim et al., 2014 [35]	Rats somatomotor area	350 and 650	0.25, 0.5, 1, 2, 3 or 5 ms	in the range [0.06, 2.8] kHz and continous wave	150, 200, 300 or 400 ms	?	2 or 3 s	1.38 (value for animal with signs of bleeding)	22.4 W/cm^2 after cranial tx (max value reported, corresponding to animal with signs of bleeding)	**11.2 W/cm^2** after cranial tx (max value reported)	Motor responses were observed at minimum threshold (I_{sppa} = 4.9−5.6 W/cm^2, I_{spta} = 2.5 −2.8 W/cm^2) in a limited range of sonication parameters (TBS = 1−5 ms, 50% of duty cycle, and SD = 300 ms, at fc = 350 kHz). Pulsed sonication elicited motor responses at lower acoustic intensities than its equivalent continuous sonication (I_{sppa} = 7.73 W/cm^2). One animal which underwent a sonication of I_{spta} = 11.2 W/cm^2 for a short period of time (<9 s using 1 ms TBD, 50% duty cycle and 300 ms SD) showed signs of local bleeding.
Kim et al., 2013 [32]	Rats	350	0.5 ms	1 kHz	300 ms	1200 *	2 s	0.74 after cranial tx	6 W/cm^2 * after cranial tx	**3 W/cm^2** after cranial tx	Changes in glucose metabolism for up to more than 1 h after sonication.
Lee et al., 2016 [17]	Human V1	270	1 ms	500 Hz	300 ms	50	13 s (fMRI) or 2.5 s (EEG)	2.8 * in water 1.2 after cranial tx (maximal simulated value across N = 19 subjects)	16.6 W/cm^2 in water 11.6 W/cm^2 after cranial tx (maximal simulated value)	8.3 W/cm^2 in water * **5.8 W/cm^2** * after cranial tx (maximal simulated value)	fMRI: 11 out of 19 participants reported the perception of phosphenes, and a clear fMRI response. EEG: 10/10 subjects reported phosphene sensation. Changes in VEP EEG peak.
Yoo et al., 2011 [56]	Rats thalamus	650	0.5 ms	100 Hz	20 min	1	–	0.61 after cranial tx	6 W/cm^2 after cranial tx	300 mW/cm^2 after cranial tx	The sonication reduced the time to emergence of voluntary movement from intraperitoneal ketamine-xylazine anesthesia. A preliminary test showed that a I_{sppa} = 3.3 W/cm^2 failed to decrease the duration of the anesthetic state.
Deffieux et al., 2013 [66]	Monkey frontal eye field	320	1 ms	1 kHz	100 ms	40	≥30 s	1.06 in water * 0.6 after cranial tx (average across several skull positions)	12 W/cm^2 in water* 4 W/cm^2 after cranial tx	6 W/cm^2 in water* 13.5 mW/cm^2 after cranial tx [3)] 2 W/cm^2 after cranial tx (using standard formula) *	Ultrasound increased antisaccade latencies in two monkeys.
Mueller et al., 2014 [67]	Human somatosensory cortex	500	0.36 ms	1 kHz	500 ms	120	6 s	1.13 in water	23.9 W/cm^2 in water	8.6 W/cm^2 in water*	The phase distribution of beta frequencies was altered, together with a change in phase rate of beta and gamma frequencies.
Legon et al., 2014 [62]	Human S1	500	0.36 ms	1 kHz	500 ms	?	?	1.13 in water	23.9 W/cm^2 in water	8.6 W/cm^2 in water*	Amplitudes of SEPs (recorded by EEG) elicited by median nerve stimulation were significantly attenuated. The spectral content of sensory-evoked brain oscillations were significantly modulated by tFUS.
Legon et al. preprint [47]	Human M1	500	0.36 ms	1 kHz	500 ms	1	–	[4)]	[4)]	[4)]	The amplitude of single-pulse TMS MEPs was decreased; the intracortical facilitation was attenuated; no effect on intracortical inhibition. Ultrasound reduces reaction time on a simple stimulus response task

(*continued on next page*)

Table 2 (*continued*)

Study	Target	Parameters									Observed neural effect and adverse effect (if any)
		f_c [kHz]	TBD	PRF	SD	Number of sonica-tions	ISI	MI	I_{sppa}	I_{spta}	
Lee et al., 2018 [30]	Rats (anesthetized and awake) motor cortex	600	1 ms	500 Hz	300 ms	10	5–10 s	1.38	Minimum value: 2.1 W/cm^2; incremented by 1 W/cm^2; maximum value: 14.9 W/cm^2 [5)]	7.5 W/cm^2 [5)]	Different thresholds to evoke observed motor response: $I_{sppa} = 3.4 \pm 1.8$ W/cm^2 for the awake condition (grand mean response rate 76.2%) $I_{sppa} = 10.2 \pm 2.4$ W/cm^2 (grand mean response rate 68.6%) or 12.4 ± 2.8 W/cm^2 (grand mean response rate 38.6%) for 2 different types of anesthetics[5)]
Yoo et al., 2017 [48]	Rats somatosensory cortex	650	0.5 ms	100 Hz	10 min	1	–	?	4.2 W/cm^2 [5)]	210 mW/cm^2 [5)]	Different SEP features compared to controls were evident and persisted beyond 35 min after the administration of FUS.
Yang et al., 2018 [52]	Monkey S1	250	0.252 ms	2 kHz	300 ms	10 *	3 s	1.87 in water 1.08 after cranial tx (estimated after measurement on skull attenuation)	29.5 W/cm^2 in water 9.9 W/cm^2 after cranial tx	1.34 W/cm^2 in water 0.452 W/cm^2 after cranial tx	Excitation effects with BOLD fMRI not only at the target but also off-target somatosensory and associated brain regions as a cause of modulation in downstream brain regions.
Daniels et al., 2018 [39]	Pigs (after craniotomy) auditory and rats inferior colliculus	230 (1000 element transducer)	100 ms	0.333 Hz *	52 s	1	–	Rats: 0.08 * Pigs and rats: 0.17 * [5)]	Rats: 2.3 W/cm^2 Pigs and rats: 4.6 W/cm^2 [5)]	Rats: 765.9 mW/cm^2 * Pigs and rats: 1.53 W/cm^2 * [5)]	AEP decreases by 59.8 ± 3.3% (with $I_{sppa} = 2.3$ W/cm^2) and by 36.9 ± 7.5% (with $I_{sppa} = 4.6$ W/cm^2) of the baseline value in rats. AEP amplitudes decreased to an average of 27.7 ± 5.9% of baseline in pigs. This effect lasted between 30 min and 1 month in most treated animals.
Kim et al., 2018 [33]	Mice motor cortex	183 (CMUT 32 elements array)	4.5 ms	200 Hz	200 ms	25	Around 9.6 s	0.12 * before cranial tx	Up to 61.5 mW/cm^2 before cranial tx	Up to 55.4 mW/cm^2 before cranial tx	At an intensity of $I_{spta} = 34.1$ mW/cm^2, the average stimulation success rate of four mice was over 70%.
Dallapiazza et al., 2018 [21]	Swine thalamic regions	1.145 MHz (single element) 650 and 220 kHz (multi element phased array transducer)	43.7 ms	10 Hz	40 s	1	–	0.53 [5)]	? [6)]	?	Suppression of SSEP amplitude
Kim et al., 2015 [49]	Rats visual cortex	350	0.5 ms	20, 100, 166 Hz	150 s	1	–	Max 0.75 [5)]	1, 3, 5 W/cm^2 [5)]	Max 250 mW/cm^2 [5)]	$I_{sppa} = 1$ W/cm^2, TBD = 0.5 at PRF = 100 Hz and $I_{sppa} = 3$W/cm^2, TBD = 0.5 ms, PRF = 20 Hz, corresponding to 50 and 30 mW/cm^2 I_{spta} did not change VEP. $I_{sppa} = 3$ W/cm^2 with TBD = 0.5 ms and PRF = 100 Hz (5% duty cycle) successfully suppressed the VEP. Higher duty cycle (8.3%) increased the VEP. The same effect was observed at $I_{sppa} = 5$ W/cm^2 and 5% duty cycle.
Min et al., 2011 [2]	Rats thalamus	690	0.5 ms	100 Hz	180 s	1	–	0.33 after cranial transmission	2.6 W/cm^2 after cranial transmission	130 mW/cm^2 after cranial transmission	Suppression of the number of epileptic signal bursts. Average among all 9 rats that underwent treatment.
Baek et al., 2018 [19]	Mice lateral cerebellar nucleus (LCN)	350	0.5 ms	1 kHz	300 ms	600	2 s	0.54 in water	2.5 W/cm^2 in water	1.25 W/cm^2 in water	Enhancement of sensorimotor recovery after stroke. Decreased level of brain edema and tissue swelling in the affected hemisphere 3 days after the stroke.

Folloni et al. [46]	Monkey amygdala and anterior cingulate cortex (ACC)	250	30 ms	10 Hz	40 s	1	–	Maximum 2.64 in amygdala and 1.64 in ACC * (from estimation after cranial transmission)	Maximum 51 W/cm^2 in amygdala and 17 W/cm^2 in ACC (estimation after cranial transmission)	Maximum **15.3 W/cm^2** in amygdala and **5.3 W/cm^2** in ACC (estimation after cranial transmission)	After TFUS, the functional coupling of the stimulated areas, but not of control areas, was selectively reduced. This effect was measured by fMRI and lasted for more than 1 h after stimulation.
Li et al., 2016 [36]	Mice motor cortex	1 MHz (and high frequency, 5 MHz)	0.5 ms	1 kHz	300 ms	20 * 600 * (for safety assessment)	3 s	?	260–460 mW/cm^2 after cranial transmission *	From 130 to 230 mW/cm^2 after cranial transmission	The peak EEG amplitude increased with increasing I_{spta}.
Yang et al., 2012 [37]	Rats thalamus	650	0.5 ms	100 Hz	20 min	1	–	0.2	3.5 W/cm^2 after cranial transmission	175 mW/cm^2 after cranial transmission	Extracellular GABA level started to decrease upon sonication and remained reduced compared to control group up to 100 min after the end of sonication. The same effect was not observed for the extracellular glutamate level.
Han et al., 2017 [38]	Mice motor cortex	350	0.23 ms	1.5 kHz	66.67 ms	600 * (for safety assessment)	2 s	0.1–1.16 *	3.38–39.5 W/cm^2 * 10 W/cm^2 * (for safety assessment) -all after cranial transmission-	1.16–**13.55 W/cm^2** **3.46 W/cm^2** (for safety assessment) -all after cranial transmission-	The robustness of the visual observed responses increased and the latency of the response decreased with increasing I_{spta}. I_{spta} = 3.46 w/cm^2 was sufficient to induce strong motor response; no response was observed for I_{spta}<1.16 W/cm^2. Ultrasound-induced motor responses were inhibited more than 20 min after ketamine injection. This was confirmed in in vitro cortical neuron sample by fluorescence calcium imaging, showing a dose-dependent effect.
Gulick et al., 2017 [45]	Rat motor cortex (after craniotomy)	200	0.5 ms	1 kHz	300 ms or 3 ms	?	2 s or 10 s	**Max 3.1**	9 W/cm^2 * or 30 W/cm^2 *	**4.5** W/cm^2 or 9 mW/cm^2	US directly evokes hindlimb movement, even at short burst (3 ms) and had short latency (10 ms) and long refractory (3 s) periods. US modulation significantly suppressed forelimb and hindlimb responses following ECS for several minutes after the stimulation, but shows no short-term effect.
Younan et al., 2012 [50]	Rat cortex (target to elicit motor response, not corresponding to motor cortex)	320	0.23 ms	2 kHz	250 ms	?	10 s	From 0.7 to 1.77 *	I_{sppa} of 7.5 W/cm^2 (to have 50% response) in water. Via computer stimulation, it corresponds to 17.5 W/cm^2 after cranial transmission due to reverberation	**3.75 W/cm^2** in water and **8.75 W/cm^2** after cranial transmission * (to have 50% response)	A pressure threshold of 0.79 and 0.59 MPa was required to reach 50% of responsiveness, for deep or light anesthesia stage, respectively, and the sigmoid respond was less sharp in the light anesthesia stage. These pressures corresponded to an average I_{sppa} of 7.5 W/cm^2.
Mehić et al., 2014 [40]	Different locations in mice cortex	500 (from unfocused ultrasound or modulated focused ultrasound [6], mFUS)	0.2 ms	1.5 kHz	10 s	1	–	?	0.45–16 W/cm^2 for unfocused US * 3–33 W/cm^2 for mFUS *	**0.15–5.25 W/cm^2** for unfocused US 1–**10 W/cm^2** for mFUS	Increasing the I_{spta} increase the motor movement robustness, assessed by visual assessment with unfocused US and mFUS, and the normalized success rate in mFUS.

Table 3
Overview of the safety assessments included in the reviewed studies. The involved methods are: fluorescein isothiocyanate-dextran (FITC−Dextran), trypan blue dye (T.b.), Evans blue dye (E.B.), magnetic resonance contrast agent (MR c.a.) to assess the BBB opening; antibodies to Caspase-3, quantitative transmission electron microscopy (e.m.), hematoxylin and eosin (H&E), terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay, cresyl violet (c.v.), GFAP (glial fibrillary acidic protein), VAF (Vanadium acid fuchsin) and luxol fast blue dye (LFB) to monitor cell death, damage, brain ultrastructure and hemorrhage; Sensors (thermo-couple [27,28,33,45,48] or optical fiber based thermal sensor [36]), maximum temperature increase, (equation (7) to estimate ΔT_{max}), magnetic resonance thermometry (MR th.) and the bioheat equation for the temperature increase; motor task (m.t.) or other for behavioral assessments. 1) safety assessed in rats that did not undergo pentylenetetrazol (PTZ) injection to induce epileptic activity [2] or photothrombosis procedure to induce ischemic stroke [19].

	Target	BBB integrity				Cell death, damage, brain ultrastructure and hemorrhage									Thermal effect				Behaviour	
		FITC −Dextran	T.b.	E.B.	MR c.a.	Caspase-3	E.m.	H&E	TUNEL	C.v.	GFAP	VAF	MRI	LFB	Sensors	ΔT_{max}	MR th.	Bioheat eq.	M.t.	Other
Legon et al. preprint [55]	Human thalamus or M1																			X
Verhagen et al. [22]	Non-human primate SMA, FPC and pre-SMA							X		X								X		
Fisher et al., 2018 [31]	Mice primary somatosensory cortex			X							X									
Tufail et al., 2010 [27]	Mouse motor cortex	X				X	X								X	X			X	
Kim et al., 2012 [28]	Rat abduncens nerve		X					X							X					X
Lee et al., 2015 [34]	Sheep SM1 and V1							X								X				X
Yoo et al., 2011 [29]	Rabbit (after craniotomy), SM and visual area		X		X			X	X								X			X
Lee et al., 2015 [51]	Human S1															X				X
Lee et al., 2016 [54]	Human S1+S2																			X
Kim et al., 2014 [35]	Rats somatomotor area							X								X				
Kim et al., 2013 [32]	Rats							X												X
Lee et al., 2016 [17]	Human V1															X				X
Lee et al., 2018 [30]	Rats (anesthetized and awake) motor cortex		X			X		X			X	X				X				X
Yoo et al., 2017 [48]	Rats somatosensory cortex														X	X				
Yang et al., 2018 [52]	Monkey S1																X			
Daniels et al., 2018 [39]	Pigs (after craniotomy) auditory and rats inferior colliculus							X			X		X				X			
Kim et al., 2018 [33]	Mice							X							X					X
Dallapiazza et al. [21]	Swine thalamic regions							X						X			X	X		
Min et al., 2011 [2]	Rats thalamus [1)]							X	X											
Baek et al., 2018 [19]	Mice lateral cerebellar nucleus (LCN)							X [1)]								X			X	
Li et al., 2016 [36]	Mice motor cortex							X							X					
Yang et al., 2012 [37]	Rats thalamus							X												
Han et al., 2017 [38]	Mice motor cortex							X												
Gulick et al., 2017 [45]	Rat motor cortex (after craniotomy)														X					X
Younan et al., 2012 [50]	Rat cortex (target to elicit motor response, not corresponding to motor cortex)																			X
Mehić et al., 2014 [40]	Different locations in mice cortex							X												

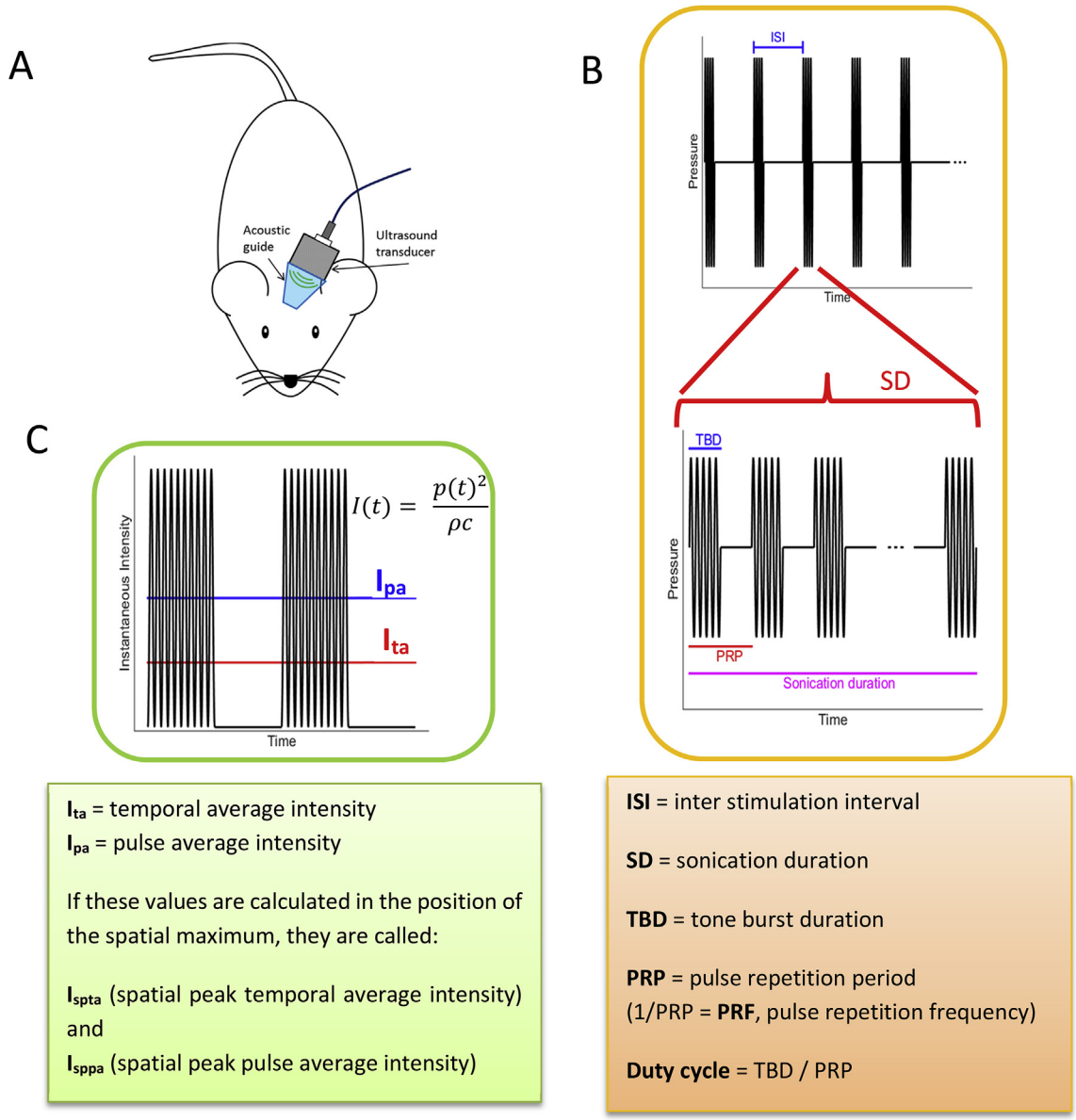

Fig. 2. Overview of the TFUS setup and parameters. A) The ultrasound pressure wave is generated by a transducer and delivered to the target through a guide filled with acoustic gel. B) The *pressure* stimulus over time is shown to indicate the main parameters. C) The main *intensity* values are shown for a fixed space position, together with their relationship with the pressure signal.

direction of wave propagation due to the presence of microscopic obstacles along the beam, and absorption. Absorption is the process by which the wave energy is converted into heat, and therefore the medium is heated. Several ways to model or monitor the resulting temperature increase in the medium exist, and they will be further discussed below.

Types of adverse and side effects caused by TFUS

In this section, we summarize the potential adverse and side effects, which have so far been tested in TFUS studies, and briefly outline the employed techniques to assess the occurrence of these effects. The majority of results were obtained in animal studies, which tested for the following effects:

- *Blood-brain barrier (BBB) opening*: The BBB is a semi-permeable membrane formed by endothelial cells which separates the vessels and the central nervous system (CNS) [25]. Air bubbles subjected to cavitation can break the BBB. Exploiting this effect, TFUS combined with US contrast agents is tested as a method for targeted drug delivery [26]. However, BBB opening is undesired for normal TFUS. Assessing BBB integrity is usually based on the intravenous injection of a substance, which cannot cross the barrier under normal conditions, prior to sonication. It is then tested whether TFUS causes the substance to diffuse into brain tissue. The dyes fluorescein isothiocyanate-dextran (FITC-dextran) [27], trypan blue [28–30] or Evans blue [31] have been used for this purpose, and their presence inside the brain was investigated in post-mortem microscopy analyses of brain slices. Alternatively, an MRI contrast agent (a gadolinium chelate) was injected before the stimulation and its penetration into brain tissue was tested by assessing the MRI signal change due to the contrast agent [29].

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- *Bleeding*: The occurrence of bleeding has been investigated using tissue staining, in particular hematoxylin and eosin (H&E) staining [2,19,22,28,32–38], which reliably stain blood cells. Yet, H&E staining is not specific to blood cells and thus requires experience to correctly interpret the results.
- *Cell death and damage*: A general approach to qualitatively analyze the presence of cell death and damage is through H&E staining [2,19,21,22,28–30,32–40], as described above, cresyl violet Nissl staining [22], or luxol fast blue dye (LFB) [21], used to identify myelin in nervous tissue. Cell death can be of two types, apoptosis and necrosis. While apoptosis is part of the normal life cycle of the cells, necrosis is harmful and triggered by external factors or disease. It is possible to differentiate between both types of cell death based on morphological criteria, but this requires experience [41]. Additional techniques specifically label apoptotic cells and have therefore been used to distinguish between apoptosis and necrosis. For example, the presence of fragmented DNA is a sign of apoptosis, and not necrosis, and it can be labeled by terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay [2,29]. Alternatively, since apoptosis is mediated by caspase [42], standard immunocytochemistry techniques with antibodies against cleaved caspase-3 can be used [27,30].

  An alternative approach to detect cell damage is staining for acidophilic cells, for example with VAF (vanadium acid fuchsin) [30,43]. Acidophilia refers to the property of cells of staining readily with an acid dye and occurs after acute neuronal damage and death in brain ischemia.

  Finally, also transmission electron microscopy has been used to quantitatively observe the effect of ultrasound on brain ultrastructure (postsynaptic density, docked vesicles, etc.) [27]. It has been shown that neural trauma causes an abnormal increase in the number of astrocytes [44] that can be detected by the expression of GFAP (glial fibrillary acidic protein) [30,31,39]. One study assessed possible permanent tissue damage after sonication in rats using MRI [39].
- *Irreversible changes of neural activity:* Recordings after the sonication can determine whether changes in neural activity are reversible and characterize the duration of recovery. The effects on local neural activity in the TFUS target region can be detected directly via invasive recordings or voltage sensitive dyes [31] or Ca^{2+} imaging in transgenic mice that express the green fluorescent calcium indicator [31]. TFUS-related changes in extracellular concentrations of excitatory and inhibitory neurotransmitters such as glutamate and λ-aminobutyric acid (GABA) can be measured via microdialysis techniques [37]. TFUS has also been combined with measurements of the forelimb and hindlimb responses to epidural cortical stimulation (ECS) to assess the cortical excitability changes after sonication [45]. Alternatively, electroencephalography (EEG) [29], functional MRI [22,46], PET [32], measurements of peripheral muscle evoked potentials (MEP) [47], sensory evoked potentials (SEP) [48], somatosensory evoked potentials (SSEP) [21], visual evoked potentials (VEP) [49] or auditory evoked potentials (AEP) [39] give non-invasive but less specific measurements of neural activity changes.
- *Undesired changes in animal behavior*: TFUS may affect normal behavior in unintended ways. In animals, this is controlled by monitoring of every day behavior, like food uptake, defecation and movement behavior and checking for signals of pain and distress or change in weight [28–30,32–34,45,50]. In addition, tasks such as the rotorod task and wire-hanging task allow for quantitatively assessing the impact of TFUS on specific aspects of behavior [27]. One study induced ischemic stroke in mice and compared the behavioral changes of the mice which were treated with TFUS via a balance test and an adhesive removal test [19].

Adverse effects can be caused by cavitation or tissue heating. As outlined above, cavitation is prevented by controlling the pressure levels. While the temperature increase in the brain can be roughly estimated using Equations (7) and (8) in the Supplemetary Material [17,19,27,30,34,35,48,51], some studies inserted a thermocouple [27,28,33,45,48] or an optical fiber based thermal sensor [36] in the brain of the animal after craniotomy to track the temperature change in real time during sonication. A non-invasive alternative to this approach is measuring the temperature increase with thermocouples in a phantom [33], or MR thermometry [21,29,39,52], which exploits temperature sensitive MR parameters such as the water proton resonance frequency, or T1 and T2 relaxation times [53].

So far, most TFUS studies used animal models. Tests for adverse effects in the few human studies were based on neurological examinations and/or structural MR imaging before and at one or several time points after the experiment [17,51,54]. In some of the studies, the participants were additionally contacted by telephone 2 months after the experiment and interviewed about any changes in their mental and physical health status, including experiences of any discomfort [17,51]. A pre-print manuscript [55] presents results of phone interviews based on a 'Participant report of symptoms questionnaire' of 64 participants who had participated in one or more of seven human TFUS experiments before.

Results

Studies screened in this review

This systematic review follows PRISMA guidelines [9,10], and the PRISMA checklist can be found in the Supplementary Methods (Table S1). The reviewing process shown in the PRISMA diagram Flow (Fig. 1) resulted in the selection of 31 peer-reviewed and 2 pre-published studies included in this review. From each of those papers (a complete list with citations is shown in Table 2), the sonication parameters (Table 2) and the methods used to assess safety and adverse effects (Table 3) were extracted and categorized as described further below. Often, only some of the safety indices were reported. In that case, we give estimated values when possible. The risk of bias was assessed and is reported in a separate section in the Supplementary Material.

BBB opening

BBB opening did not occur in any of the included studies [28–30], except for two cases where it was intentionally provoked in control conditions [27,31], using a high I_{sppa} of 280 W/cm^2 [31] or an ultrasound contrast agent [27].

Bleeding

Several studies [2,19,22,28,32,33,36–38] tested for bleeding, without finding evidence for it. A further study [34] tested different sonication parameters on eight sheep in total, and reported four animals with micro-hemorrhages in the primary visual cortex after undergoing 600 sonications at 6.6 W/cm^2 I_{sppa} (6 repetitions of 100 sonications, with 30 s gaps). While the reported value for I_{sppa} is within FDA limits, our calculated value for I_{spta} of 3.3 W/cm^2 is exceeding the diagnostic limit, and is also slightly higher than the limit for physiotherapeutic US of 3 W/cm^2. Interestingly, a sheep undergoing a single sonication at an I_{sppa} of 13.4 W/cm^2 did not present micro-hemorrhages. In another study [35], 1 of 37 rats was

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exposed to a high intensity (11.2 W/cm^2 I_{spta}) for a short period of time (<9 s using 1 ms TBD, 50% duty cycle and 300 ms SD). It exhibited several areas containing hemosiderin, which indicate the potential of local bleeding, while none of the other animals showed any sign of bleeding.

Cell damage or death

Most of the studies testing for cell damage or death [2,19,21,22,27–30,32–40] did not observe harmful effects of TFUS. One recent study [31] observed no differential GFAP expression between the control and sonicated hemisphere for $I_{sppa} = 0.69$ W/cm^2, suggesting the absence of neural trauma. However, an increased number of astrocytes was observed for a control condition with $I_{sppa} = 280$ W/cm^2 (~1.5 times above the FDA limit). Interestingly, no damage was observed even when AEP was not fully recovered after one month [39] in rats.

Long-term change of neural activity

Yoo et al. [29] tested parameter ranges for excitatory and inhibitory TFUS effects in craniotomized rats. While excitatory effects were very short-termed, suppression effects lasted several minutes. A reduction in the EEG response of up to 80% and a corresponding reduction of the BOLD signal that both lasted up to 10 min were reported for a long sonication duration of 9 s. Dallapiazza et al. [21] observed peak electrophysiological suppression in SSEP 5 min post-treatment, and the values returned to near baseline within 20 min. A further study [31] tested the facilitatory effects of ultrasound on somatosensory evoked potentials by measuring the changes in fractional fluorescence in the brains of mice dyed with voltage sensitive dyes. The TFUS-related changes disappeared within 20 min after ultrasound stimulation. Yang et al. [37] observed a decreased extracellular GABA level (approximately 20% below baseline) compared to a control group that lasted up to 100 min after the sonication ended. The same effect was not observed for glutamate. Gulick et al. [45] showed that TFUS significantly suppressed forelimb and hindlimb responses to ECS for several minutes after the stimulation blocks, even though effects immediately after single, short TFUS trials were absent. Kim et al. [32] observed a local increase in glucose metabolism induced by FUS to rat brain. This effect was demonstrated via PET imaging, which was started 20 min after the sonication and performed for 1 h. After that time, the metabolism had still not returned to baseline. In a work [22] on primates, the authors observed change in functional connectivity after a long sonication of 40 s at $I_{spta} = 7$ W/cm^2. The change lasted for more than 1 h after sonication. A similar effect was observed in a related work [46], where they used a sonication of 40 s at a maximum I_{spta} of 15.3 W/cm^2. Yoo et al. [48] observed that the SEP signals after 10 min sonication were distinctively different compared to the control condition, even 35 min after the sonication. Daniels et al. [39] observed a full recovery of AEP amplitudes in rats within maximum 1 week post-treatment with $I_{sppa} = 2.3$ W/cm^2, while the signal from 5 out of 10 rats recovered up to one month post-treatment for an $I_{sppa} = 4.6$ W/cm^2. In the same study, 1 out of 5 pigs showed a fully recovered signal 1 h post-treatment while the other did not show any recovery 3 h post-treatment (in all 5 cases, $I_{sppa} = 4.6$ W/cm^2). Kim et al. [49] observed an increase in VEP in rats up to 5 min post-treatment with $I_{sppa} = 5$ W/cm^2 and a slight increase of VEP 150 s after treatment when $I_{sppa} = 3$W/cm^2. One study [19] induced ischemic stroke in mice and found a better sensorimotor performance in mice that underwent 20 min TFUS session via a balance test and an adhesive removal test. These improvements lasted for 4 weeks after treatment, suggesting an enhancement in brain plasticity. In the same study, the TFUS treatment in cerebellar LCN significantly lowered the percentage change in increased water content and tissue swelling in the ipsilateral hemisphere to the stroke.

Animal behavior

Several studies tested for changes from normal daily behavior after the sonication studies [28–30,32–34,45,50], but did not find any abnormalities. A single study also employed behavioral tasks [29] (rotorod running task and wire-hanging task), without revealing differences in motor performance.

Temperature

Theoretical calculations based on Equations (7) and (8) in the Supplementary Methods suggest that “typical” TFUS parameters used so far in most studies cause negligible temperature increases in brain tissue [17,19,27,30,34,35,48,51]. In a recent study [22], this was partly confirmed using the more realistic bio-heat equation to estimate the temperature increase after 40 s of TFUS through a 3 mm thick skull, with an $I_{spta} = 7$ W/cm^2 in the brain. The maximal increase in the brain was less than 0.2 °C. Interestingly, however, they found rather strong increases in the skull (2.8 °C). Also experimental results show mostly only small temperature increases due to sonication [27,28,33,36,48]. However, it is important to note that the overall temperature increase depends on the combination of several TFUS parameters. For example, one study [36] reported a measured peak temperature increase of 0.2 °C for an extended stimulation (~30 min) at a low $I_{spta} \leq 230$ mW/cm^2 at 1 MHz (1.6 °C at 5 MHz for otherwise same parameters). In contrast, another study [45] reported a temperature increase up to 3 °C after two blocks of 5 min stimulation at 200 kHz, separated by a 2 min break, at $I_{spta} = 4.5$ W/cm^2 and a MI = 3.1 (higher than the allowed limit). Both studies applied longer durations than used in most other TFUS studies so far, but the combination with the higher I_{spta} caused noticeable temperature rises in the second study.

A temperature increase of 0.5 °C was reported through MR thermometry after 30 s sonication at $I_{sppa} = 9.9$ W/cm^2 [52]. Another study [39] reported temperature variation within the measurement noise level of the baseline temperatures (±2 °C) with MR thermography. The strongest effect was reported by a study using MR thermometry (sensitivity 0.3 ± 0.06 °C [29]), demonstrating an increase of ~0.7 °C in the sonicated area [29], using an $I_{sppa} = 23$ W/cm^2 for 27 s. Dallapiazza et al. [21] showed a negligible temperature increase during treatment using both MR thermometry and estimations based on the bio-heat equation.

Findings from human studies

In a recent preprint work [47], the authors tested the effects of ultrasound stimulation on motor cortex excitability measured by single-pulse transcranial magnetic stimulation (TMS). They report significant changes in the recorded muscle responses to TMS only when it was applied during, but not after, sonication. Follow-up neurological exams and anatomical MRIs after the TFUS experiment did not reveal any abnormalities or changes in the mental or physical status, nor any discomfort associated with the procedure [17,51,54]. Follow-up interviews at later time points confirmed those observations. A recent study published as preprint [55] presents results from a follow-up questionnaire after TFUS that could be obtained from 64 out of in total 120 participants. Seven subjects reported mild or moderate symptoms (mild neck pain, scalp tingling, headache, difficulty paying attention, muscle twitches and anxiety) that they felt were possibly or probably related to the

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experiment. These initial symptoms disappeared upon follow-up. The authors found a linear correlation ($r = 0.797$, $p = 0.0319$) between I_{sppa} and the occurrence of observed symptoms among the 7 subjects who reported mild to moderate symptoms that were perceived as 'possibly' or 'probably' related to participation in TFUS experiments.

Discussion and conclusions

Harmful effects of TFUS were absent in the majority of the 33 studies reviewed here. In two cases, microhemorrhages occurred in a subset of the tested animals when using a high I_{spta} of 11.2 W/cm^2 for a short duration [35] or an I_{spta} of $\geq$3.3 W/cm^2 for a high number of sonications ($\geq$500) given at a relatively short ISI of 1s [34]. Both doses are clearly above the safety limits of the FDA guidelines for diagnostic US and above the IEC standard 60601-2-5 for physiotherapy US equipment. However, this also holds for several other included studies, where no adverse effects were reported. While the parameters chosen in one of the studies [35] did not result in substantial heating, as also pointed out by the authors, the high I_{spta} of 11.2 W/cm^2 differentiates it from many other TFUS studies. That might indicate that mechanical effects caused the microhemorrhages, even though the limits for MI and I_{sppa} were not exceeded. However, as this was observed in only one of the tested animals, this conclusion remains very speculative and a replication including sham controls would be favorable to ensure that the microhemorrhages were indeed related to TFUS. In the second study [34], the chosen parameter combination might have led to a high total energy deposit, opening the possibility that a thermal mechanism underlay the adverse effects that occurred in four animals. For example, Gulick et al. [45] observed a temperature increase of 3 °C for a less intense protocol using an $I_{spta} = 4.5$ W/cm^2 and in total 180 sonications in a time period of 13 min. It seems reasonable to assume that heating might have been even higher in the four animals that showed microhemorrhages in Ref. [34] and indicates that calculating the temperature increase for a single sonication, as done in Ref. [34], can strongly underestimate the real increase.

While I_{sppa} stayed below the safety limit in all studies, MI exceeded the limit in two studies [45,46] and I_{spta} exceeded the FDA limits for diagnostic US in soft tissue in 14 out of the 20 studies in which I_{spta} was reported or could be calculated post hoc (values after cranial transmission or for craniotomized animals). I_{spta} was also above the physiotherapeutic limit in 11 of the 20 studies. This suggests that I_{spta} is the most sensitive safety index in case of TFUS and, unlike current practice, should be reported so that it can be followed up by a more detailed estimation of the thermal effects when its limits are exceeded. We consider this relevant as the current studies indicate that TFUS parameters within the FDA limits for diagnostic US might often lack neural stimulation effectiveness. For example, an I_{spta} of around 2 W/cm^2 for pulsed waves and 4 W/cm^2 for continuous waves was necessary to reach a 50% success rate for stimulation at 500 kHz [7]. Similarly, while many studies included in this review reported neural effects for parameters within the safety limits [2,19,27,31,33,48,49,52,56], several studies found stable effects only when exceeding at least one of the safety indices ([28–30,34,35] and Table 2). In addition, recent studies show that heating of the skull (potentially causing indirect heating of soft tissue) and/or brain tissue can reach several degrees for more intense and long protocols [22,45]. The systematic assessment of heating will thus be relevant in future studies that might aim at extending the parameter envelope of TFUS and should be part of any safety test of new sonication regimes in particular for human TFUS.

It is worth noting that the safety limit of 720 mW/cm^2 for I_{spta}, which was generally used in TFUS studies so far and which we also applied here, was introduced to limit the heating in soft tissue. In case of transcranial US, the FDA limits for diagnostic US actually apply an even stricter limit of 94 mW/cm^2 for I_{spta} to prevent excessive heating of the skull, which absorbs most of the beam energy. It seems that almost none of the studies published so far reported neural effects for intensities below this threshold. However, it is important to stress that both limits are based on worst-case scenarios and exceeding them does not necessarily mean that strong heating occurs. Rather, the FDA standard for diagnostic US requires a case-by-case estimation of the maximum temperature rise in soft tissue and skull once they are exceeded, specific for the used ultrasound parameters and setup. Simulations of the propagations of the TFUS beam through the skull, combined with evaluations of the bio-heat equation for TFUS [22], might be valuable tools that allow realistic estimates of the amount of heating for new sonication regimes on a more standard basis.

The neural aftereffects can exceed 1 h [22,32,46], making TFUS a potent neuromodulation modality. This is encouraging for therapeutic applications. In contrast to diagnostic US, future TFUS applications might resort to repeated sessions over extended time periods to achieve and maintain therapeutic efficacy. As such, a safety framework will also need to cover these more intense settings (see, e.g. Refs. [57,58] for a related example of adverse effects that only occurred after repeated applications in case of transcranial direct current stimulation) or combinations of TFUS with other brain stimulation techniques. This will require safety studies that specifically test this parameter space in order to inform an international consensus on accepted settings and procedures, similar to established non-invasive brain stimulation methods [59]. Along similar lines, in the few TFUS studies performed in humans so far, the type and extent of follow up exams differed strongly [17,51,54,55]. This suggests a need for guidelines that provide a secure framework for experimental settings and practical procedures, including mandatory safety screening and appropriate follow-up procedures. For example, the importance of establishing best practices also for apparently simple procedures was highlighted in a recent review [60] of low-intensity low-frequency US (20–100 kHz), showing that US can cause skin damage due to inertial effect cavitation in the coupling gel if non-degassed gel is employed.

Along similar lines, guidelines are important to prevent intensity hotspots that can occur due to unintended standing waves and focusing effects of the skull. While these effects more likely emerge in small animals [50], they have been shown to be also relevant in non-human primates for targets close to the skull base such as the amygdala [46]. Moreover, a retrospective modeling study [61] suggests that unwanted secondary hotspots might have been the cause of intracerebral hemorrhages that occurred in a clinical trial on transcranial low frequency ultrasound for sonothrombolysis [68] and that resulted in the early termination of the trial. Finally, the reviewed studies differed in regards to the choice of the stated safety-relevant parameters and the way those were assessed. A more standardized reporting of the relevant pulse parameters and of all safety indices of the FDA guidelines is a prerequisite for the development of future TFUS guidelines for human applications. Accurate estimation of the TFUS intensity after cranial transmission is particularly challenging in humans, as it has to rely on hydrophone measurements based on "representative" skull samples or computer simulations [17,51,62]. The uncertainty range of the intensity estimates obtained by these procedures seems still unclear [63,64], and contributes to variations in the values reported across studies. As such, it seems useful that future studies

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additionally state intensity values for a pure water background to ensure good comparability of the baseline TFUS parameters.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

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