

UPSIDE

Deliverable D4.2 in vivo targeting of FUS stimulation safety and precision

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Abbreviations

DAB : 3,3'-diaminobenzidine

DAPI : 4',6-Diamidin-2-phenylindol

DBS : deep brain stimulation

DC : duty cycle

eFUS : epidural focused ultrasound

ISI : inter stimulus interval

ISPPA : spatial peak pulse average intensity

ISPTA : spatial peak temporal average intensity

Mfb : medial forebrain bundle

MI : mechanical index

NAc : nucleus accumbens

PFC : prefrontal cortex

PNP : peak negative pressure

PRF : pulse repetition frequency

SD : sonication duration

VTA : ventral tegmental area

WP : work package

Executive Summary

To develop the epidural brain interface for focused ultrasound neuromodulation in an animal model of depression the optimal stimulation parameters have to be determined. These parameters must maximize the likelihood of alleviating depression-like symptoms while minimizing the risk of stimulation-related tissue damage. Deliverable 4.2 focused on narrowing down potential FUS parameters for stimulating the medial forebrain bundle (mfb) using a commercially available ultrasound transducer and assessing their safety.

The mfb, a fiber bundle with few cell bodies, connects key brain regions implicated in depression, including the prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA). Given the tissue-dependent nature of FUS's potential mechanisms of action and the transition to epidural stimulation, systematic testing of various parameter combinations was critical. The commercial transducer facilitated preliminary investigations despite requiring a larger craniotomy and limiting experiments to anesthetized conditions. These studies provided a foundation for parameter refinement pending the availability of the first-generation eFUS chip.

The commercial device reliably targeted distinct sections of the mfb, and stimulation-induced neuronal activation was assessed through c-Fos expression.

All animals including controls showed a strong cortical activation especially on the stimulated hemisphere, which can be partially attributed to the anaesthesia and craniotomy.

Although trends in c-Fos expression—such as downregulation in the ipsilateral prefrontal cortex or increased activation under specific conditions—align partially with expectations from DBS and optogenetic studies, statistical significance was not achieved. Future studies incorporating advanced readouts like fiber photometry will be essential for refining these parameters.

Trends indicate that pressures between 0.5 and 1.2 MPa with tailored duty cycles may optimize stimulation effects. Further optimization of parameters, including increased pulse repetition frequency, potentially duty cycle and parameter sweeps in awake animals using the finalized chip, could help the reported effects reach statistical significance. Additionally, effects slightly differed when targeting the ventral tegmental area (VTA), which may warrant a shift of the initial target towards the VTA. As the mfb is a fiber bundle, the mechanisms of ultrasound-induced stimulation may differ from those required for stimulating cell bodies, such as those in the VTA. This could alternatively help to enhance the robustness of the observed effects.

Importantly, the investigation confirmed the safety of the tested parameters (ISPTA up to 4662 mW/cm²), with no observed tissue damage, apoptosis or immune responses attributable to the stimulation. Minor bleeding associated with craniotomy was procedural and is unlikely to affect outcomes with the smaller surgical footprint and healing time after chip implantation.

In summary, the study has narrowed the range of parameters for effective mfb stimulation and demonstrated the safety of the approach, laying a foundation for subsequent investigations using the first-generation eFUS chip.

1. Introduction

The deliverable 4.2 aimed to narrow down potential focused ultrasound (FUS) parameters for the stimulation of the medial forebrain bundle (mfb) using a commercially available ultrasound transducer.

The commercial device operates at a frequency comparable to the eFUS chip; however, its larger size and focal spot dimensions necessitate a larger craniotomy to mitigate skull attenuation. This constraint restricts its application to anesthetized animals, thereby limiting the scope of potential readouts for parameter assessment. Nevertheless, a comprehensive parameter sweep will still be required with the finalized chip to optimize stimulation.

Additionally, the primary target of stimulation the medial forebrain bundle is a fiber bundle with little cell bodies. Therefore, the effect of ultrasound on the tissue could differ from reported results in preclinical depression studies with prefrontal cortex (PFC) and ventral tegmental area (VTA) stimulation and may rather resemble the stimulation of peripheral nerves.

Given the ongoing discussion around the various modes of action of focused ultrasound—which probably are also tissue-dependent—the systematic testing of different parameter combinations for mfb stimulation is critical. The different variables which can be adjusted are ranging over different time scales and are shown in figure 1.

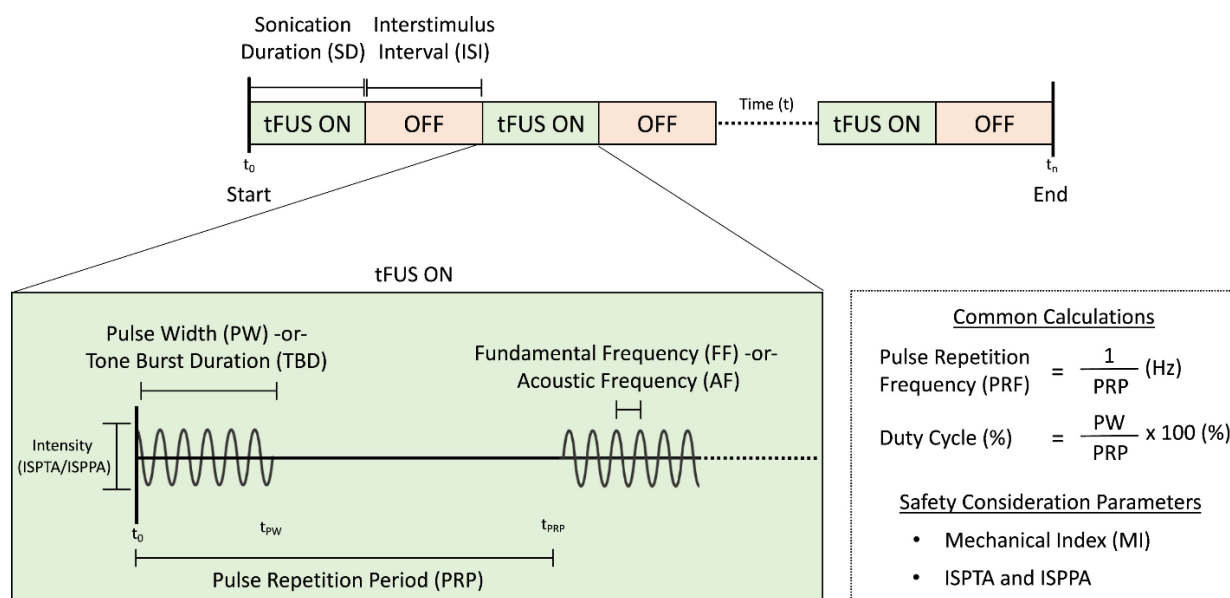


Figure 1. Focused ultrasound pulsed stimulation. Important parameters for safety considerations and stimulation effects include the spatial peak time averaged intensity (ISPTA) and the spatial peak pulse averaged intensity (ISPPA). Adjusted from Cox et. al, 2024.

While multiple factors collectively impact the potential effects of the ultrasound stimulation, the stimulation frequency, duty cycle (% of time for which the ultrasound was turned on), pulse repetition

frequency and intensity seem to be most critical. Table 1 shows the commonly used ranges for the key parameters according to the literature.

To adjust for skull attenuation and prevent heating during transcranial stimulation, frequencies below 1 MHz are most common. Since the developed FUS chip will stimulate epidurally, higher frequencies are used (5-12 MHz) to reach a higher focal spot resolution.

To assess safety of low intensity transcranial FUS, currently FDA guidelines for transcranial doppler ultrasound are referenced (Table 2). But as Lee and colleagues (2021) suggest, unlike in imaging, for neuromodulation the pulsed signal leads to a smaller ON time of ultrasound and thus less tissue heating. Therefore, not only the duty cycle but also the sonication duration has to be taken into account. This is also shown by multiple studies which do not report any damage in spite of higher intensities.

Table 1. Commonly used parameters for transcranial FUS stimulation.

Frequency [MHz]	Pulse repetition frequency [kHz]	Burst duty cycle [%]	Intensity _{SPPA} [W/cm ²]	sonication duration [ms]
<1	0.1-1.5	3-100 majority 30-50	0.02-50 majority 1.5-18	5-6000 majority 60-500

Table 2. FDA safety requirements for transcranial doppler ultrasound according to Lee et. al, 2021. Maximal

Intensity _{SPPA} [W/cm ²]	Intensity _{SPTA} [mW/cm ²]	MI	Max. temp increase
≤ 190	≤ 720	≤ 1.9	1.5 -2.5 °C (1h+)

2. mfb FUS stimulation parameters

2.1 Targeting

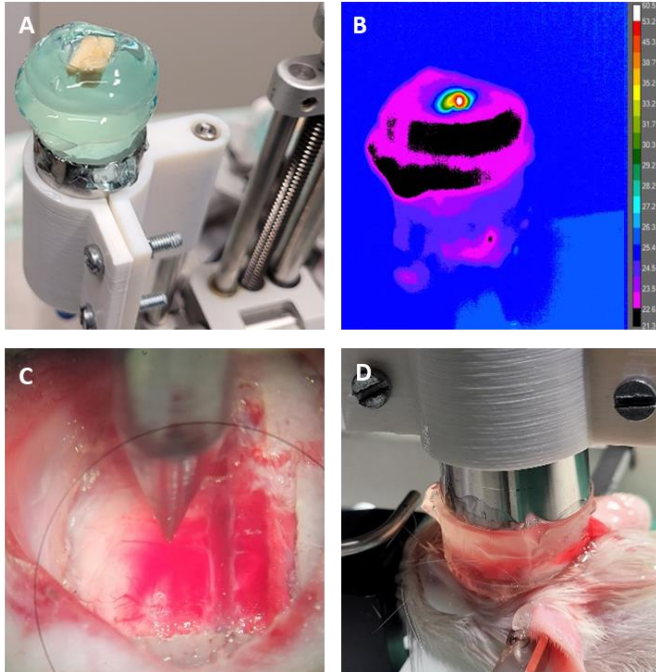


Figure 2. Temperature measurement and stimulation set up. **A** Set up for measuring the temperature increase in fixated brain tissue during the stimulation with a continuous sinusoidal waveform and different pressures. **B** Recordings with a thermal imaging camera 5 s after the begin of stimulation. **C** 9x9 mm large craniotomy above the left mfb and positioning of the commercial transducer. **D** FUS stimulation of the left mfb using the commercially available FUS transducer.

The targeting of the medial forebrain bundle (mfb) with the commercially available FUS transducer was already the focus in the previous report. Further tests using temperature measurements in brain tissue showed, the desired increase for tissue damage to 57-60 °C, could be detected after only 5 s of stimulation using a continuous sinusoidal waveform and pressures above 1.4 MPa (see fig. 2 A, B).

When the same parameters were applied to the brain of recently dead animals, the focal spot could be visualized using DAPI (4',6-diamidino-2-phenylindole). Showing different sections of the mfb could be targeted reliably (see fig. 3 A, C-E). Of course, animal specific differences could lead to slight variations in targeting, as can occur even when applying stereotactic surgery approaches (fig. 3 B). Since the delivered energy during FUS stimulation is not high enough to see the focal spot, the correct targeting could not be validated in each of the experimental animals. Consistency in methodology should ensure reliable stimulation of the desired target, although

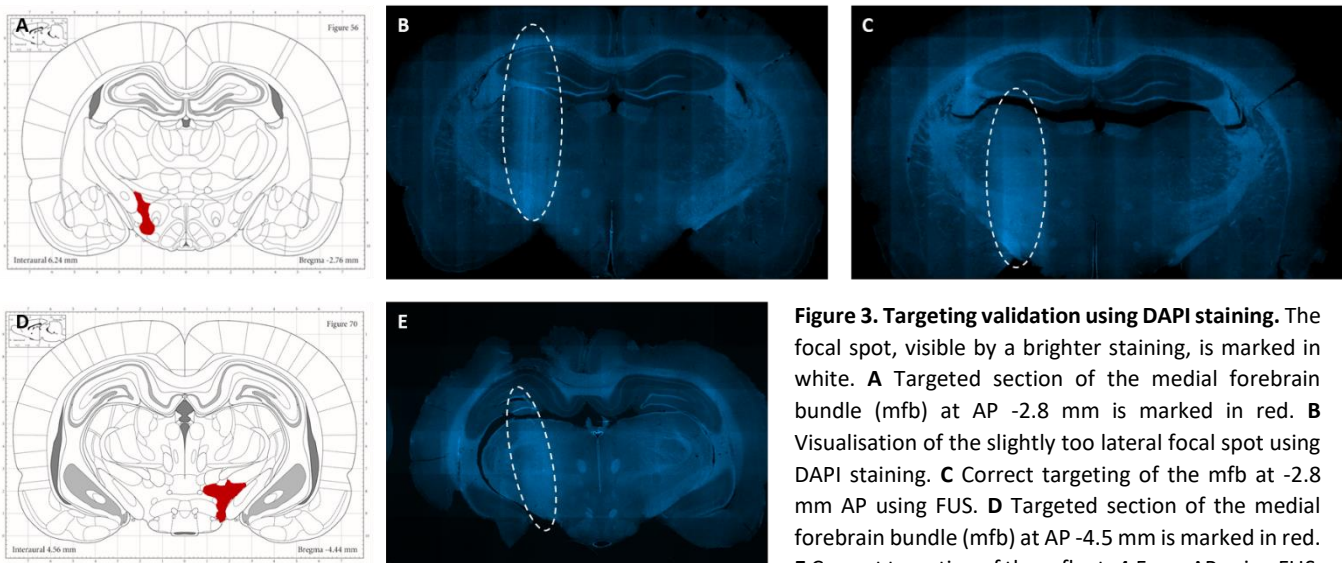


Figure 3. Targeting validation using DAPI staining. The focal spot, visible by a brighter staining, is marked in white. **A** Targeted section of the medial forebrain bundle (mfb) at AP -2.8 mm is marked in red. **B** Visualisation of the slightly too lateral focal spot using DAPI staining. **C** Correct targeting of the mfb at -2.8 mm AP using FUS. **D** Targeted section of the medial forebrain bundle (mfb) at AP -4.5 mm is marked in red. **E** Correct targeting of the mfb at -4.5mm AP using FUS.

targeting accuracy and the focal spot size, which extends above the mfb, remain confounders in this study.

2.2 Neuronal activation after FUS stimulation

To analyse the effects of different stimulation parameters, the animals were put under isoflurane anaesthesia and a 9x9 mm sized craniotomy was drilled above the mfb to ensure the ultrasound could propagate without absorption by the skull (comparable to the chip). Animals were stimulated for half an hour (or up to 2 h) and left under anaesthesia for another hour to ensure peak protein expression after stimulation. The animals were perfused with 4 % paraformaldehyde and the brains were histologically stained for several markers.

The tested stimulation parameters and conditions are shown in Table 3. Among several parameters used in the literature on different animal models of depression (conditions 1 (Zhang et al. 2021), 2 (Wang et al. 2024; Hou et al. 2024), 9 (Hou et al. 2024)), we tried to test the most common duty cycles and intensities. Since the centre frequency of the used transducer was 5.24 MHz, it was kept the same in all conditions similar to the pulse repetition frequency (PRF), where 130 Hz was selected in parallel with common mfb-DBS stimulation settings.

Table 3. Overview of tested parameter combinations with the commercially available FUS transducer. PNP= Peak negative pressure; MI= mechanical index; ISPPA= spatial peak-pulse average-intensity; ISPTA= spatial peak temporal average intensity; PRF= pulse repetition frequency; DC= duty cycle; SD= sonication duration; ISI= inter stimulus interval

test condition #	target	total stimulation time [h]	frequency [MHz]	drive amplitude [Vpp]	PNP [Mpa]	MI	ISPPA [W/cm ²]	ISPTA [mW/cm ²]	PRF [Hz]	DC [%]	pulsewidth [μs]	SD [ms]	burstperiod [s]	ISI [s]	# animals	strain
sham A	mfb	0.5	-	-	-	-	-	-	-	-	-	-	-	-	2	FSL
sham B	mfb	2	-	-	-	-	-	-	-	-	-	-	-	-	1	FSL
1	mfb	2	5.24	1.5	0.1	0.02	0.1	47	1500	45	300	200	2	1.8	1	FSL
2	mfb	2	5.24	5.4	0.2	0.07	0.8	31	200	4	200	1000	4	3	1	FSL
3	mfb	0.5	5.24	9.5	0.3	0.12	2.6	78	130	3	231	500	5	4.5	1	FSL
4	mfb	0.5	5.24	9.5	0.3	0.12	2.6	1292	130	50	231	500	5	4.5	1	FSL
5	mfb	0.5	5.24	18.4	0.5	0.24	9.3	280	130	3	231	500	5	4.5	2	FSL
6	mfb	0.5	5.24	18.4	0.5	0.24	9.3	4662	130	50	231	500	5	4.5	2	FSL
7	mfb	0.5	5.24	41.4	1.2	0.52	45.5	1366	130	3	231	500	5	4.5	2	FSL
8	VTA	0.5	5.24	18.4	0.5	0.24	9.3	280	130	3	231	500	5	4.5	2	FSL
9	hippocampus	2	5.24	1.5	0.1	0.02	0.1	10	1500	10	300	200	2	1.8	1	FSL

To study the neuronal activation in response to FUS stimulation, several immediate early genes were tested as potential markers. These genes, often transcription factors, are expressed within the first

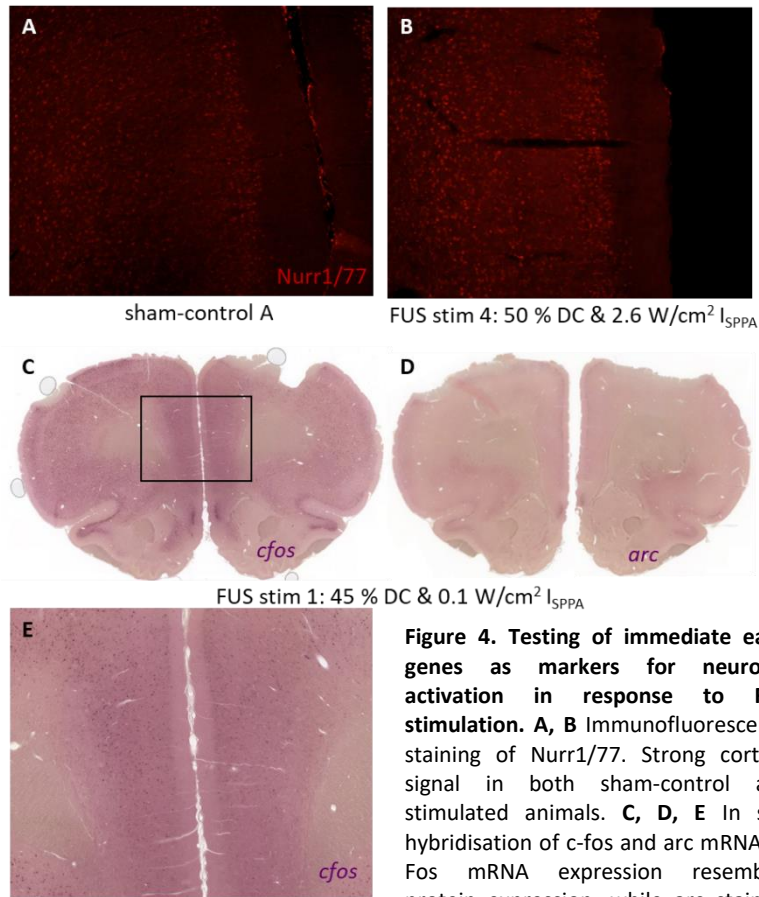


Figure 4. Testing of immediate early genes as markers for neuronal activation in response to FUS stimulation. A, B Immunofluorescence staining of Nurr1/77. Strong cortical signal in both sham-control and stimulated animals. C, D, E In situ hybridisation of c-fos and arc mRNA. c-Fos mRNA expression resembles protein expression, while arc staining was not successful.

minutes after certain stimuli and can indicate in some cases neuronal activation. The most commonly used marker c-Fos, can be expressed in response to several stimuli, including FUS and DBS stimulation, but also anaesthesia e.g. with isoflurane. Levels of c-Fos expression can indicate an up or downregulation of neuronal activity in response to certain stimuli, but it cannot distinguish, if neurons are less active or if inhibitory pathways have been activated.

Stainings for c-Fos showed strong cortical expression in all animals, including unstimulated sham controls. Subjectively the left hemisphere, with the craniotomy, often showed stronger c-Fos signals than the unstimulated side. Although a certain baseline c-Fos expression was expected, especially

due to anaesthesia, the lateralisation of the signal is most likely due to the exposure of the brain through the craniotomy. Other tested markers like Nurr1/77 or c-Fos and arc mRNA levels (in situ hybridisation; see fig. 4) showed similar trends but would have required further staining optimisation. Therefore c-Fos DAB staining was used for further analysis.

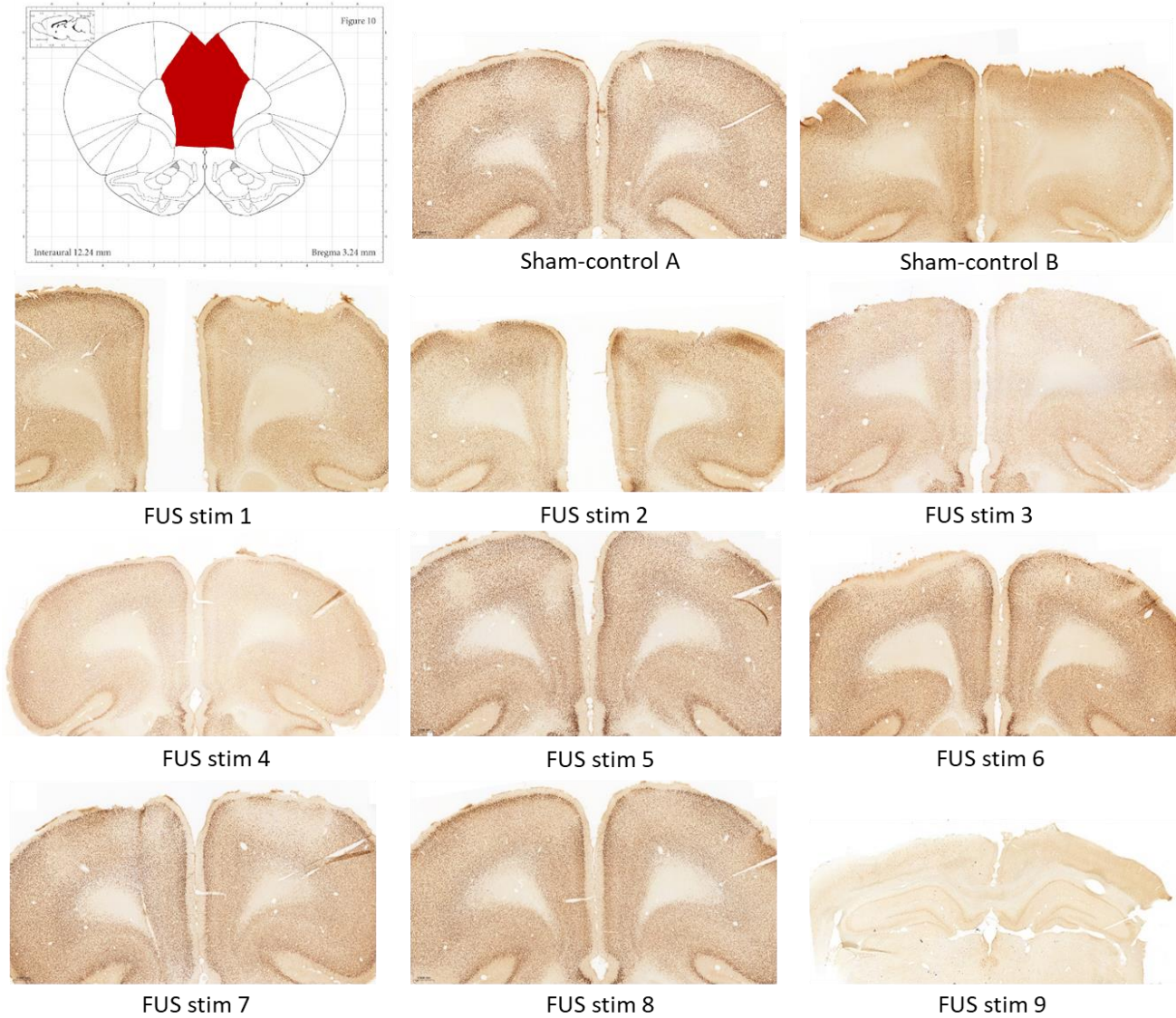


Figure 5. Overview of c-Fos expression in the prefrontal cortex after mfb eFUS stimulation with different parameters.

Since no prior studies have investigated focused ultrasound (FUS) stimulation of the mfb, the expected effects were hypothesized to resemble those of mfb deep brain stimulation (DBS) or optogenetic stimulation. Given that the mfb is a fiber bundle connecting the prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA), stimulation effects are typically observed in these connected regions rather than the mfb itself.

Previous studies from our group demonstrated a downregulation of c-Fos in the PFC following DBS, whereas optogenetic stimulation resulted in hemispheric differences, with fewer c-Fos-positive cells ipsilaterally in the PFC. However, FUS stimulation under conditions derived from published studies did not replicate these effects or produce significant c-Fos activation after hippocampal stimulation. The most promising FUS stimulation conditions, using higher intensities and peak negative pressures (PNP), were analysed for c-Fos positive cell density (fig.6 C, D). Here we see not the expected downregulation but a slight trend for an upregulation when using either 0.5MHz PNP with a DC of 50% or higher pressures (1.2MPa) with lower DC (3%) similar to the effects of VTA stimulation (3% DC; 0.5MPa PNP). Differences

between hemispheres were less pronounced under stimulation than in sham controls, though lower DCs (3%) at 0.5 MPa produced patterns more similar to optogenetic results, with reduced ipsilateral c-Fos positive cell density. Models suggest that lower DCs may favor inhibitory effects, while higher DCs may promote excitation.

The inconclusive results may stem from high baseline cortical activation masking potential differences. The limited parameter variations tested, particularly pulse repetition frequencies, further constrain interpretations.

For further optimisation it seems advisable to use pressures between 0.5 and 1.2 MPa and higher duty cycles the lower the pressure is. Alternatively, a shift of the target closer to the VTA might yield more robust results.

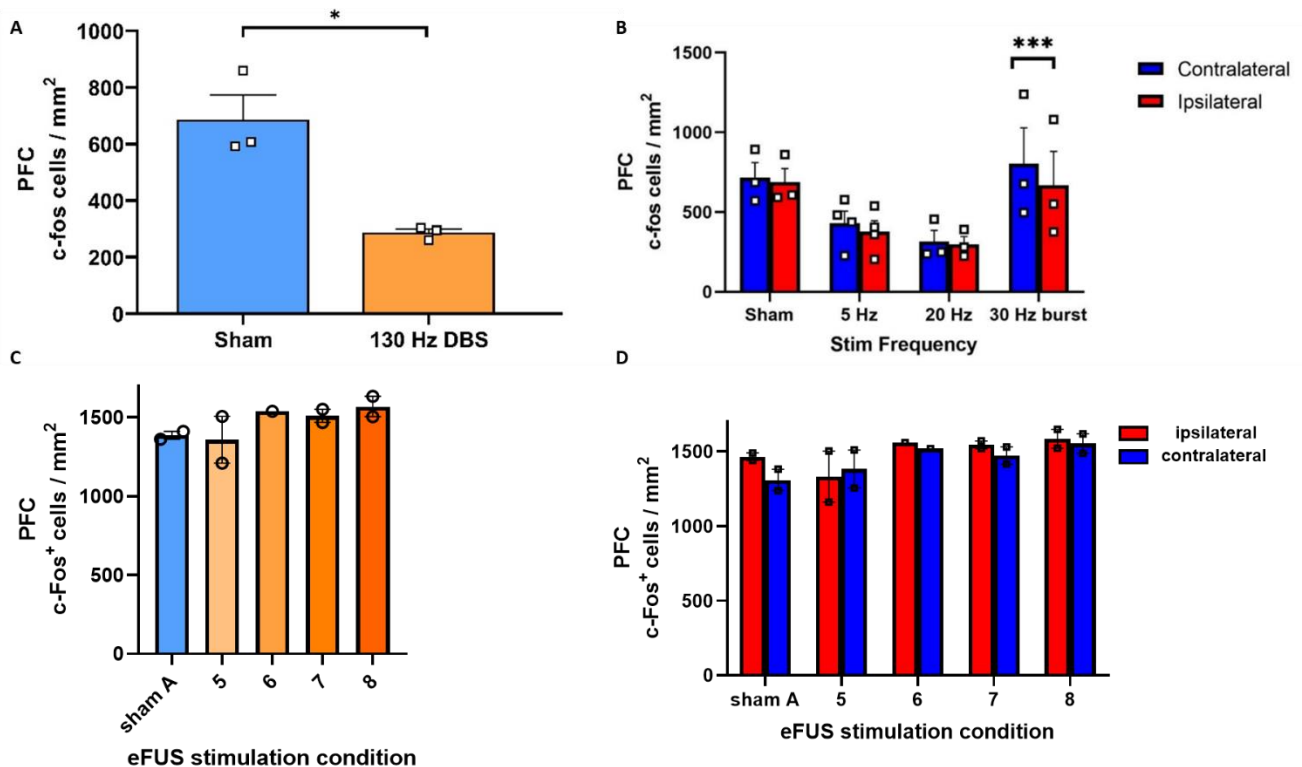


Figure 6. A-D. Density of cFos positive cells in the prefrontal cortex (PFC) after A DBS stimulation with 130 Hz B optogenetic stimulation with 5/20/30 Hz C,D Cell volume density after FUS stimulation with stimulation conditions: sham -control A ; 5: 3% DC 9,3 W/cm² ISPPA; 6: 50 %DC 9,3 W/cm² 7: 3%DC 45.5 W/cm² ISPPA; 8: VTA 3%DC 9.3 W/cm² ISPPA. (Data generated by Tong et al., manuscript in preparation).

2.3 Safety

As all of the used parameters range within the FDA safety guidelines for doppler ultrasound, no safety concerns were expected. The only exception being the ISPTA in condition 4, 6 and 7 which are higher and could lead to potential thermal damage.

To control for safety of the used parameters, selected conditions were tested with a TUNEL staining for apoptotic cells (fig. 7). At the stimulation site, the mfb, there were no signs of apoptosis. Only when there was bleeding due to the craniotomy (see fig. 7 C, D) some apoptotic cells were detected. A marker for microglia and macrophage homeostasis, Iba 1, could not detect any accumulation of microglia around the stimulation site indicating no immune response due to the stimulation (see fig. 8).

A haematoxylin & eosin (H&E) staining of the conditions with the highest ISPTA also did not indicate any tissue damage (fig. 9), except for minimal subcortical blood traces, which are most likely due to the craniotomy or brain preparation.

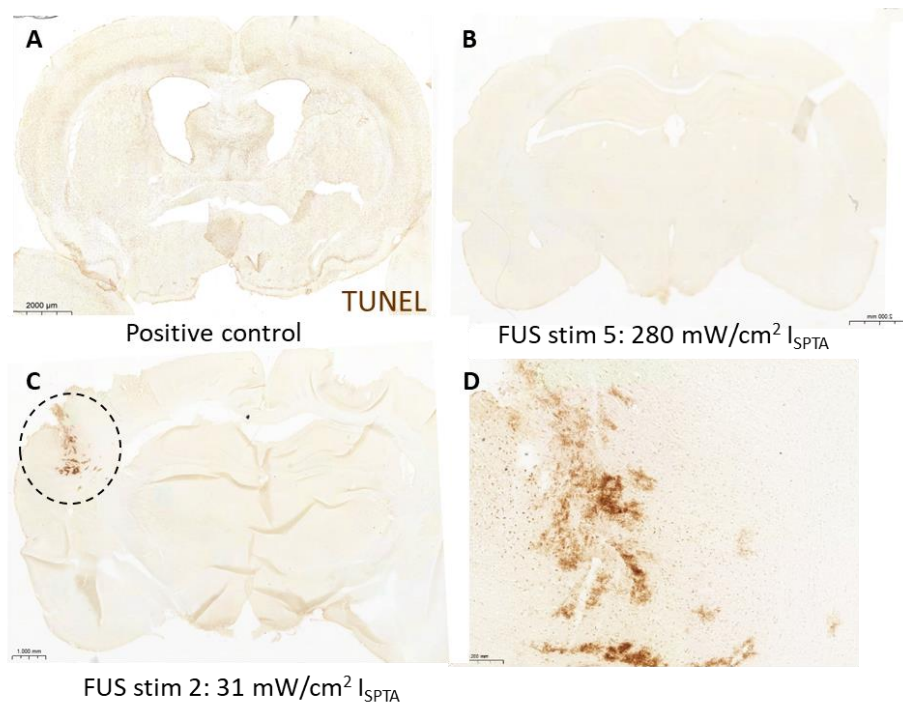


Figure 7. TUNEL staining for apoptotic cells. A Positive control B mfb stimulated with parameters from condition 5 (3%DC 9,3 W/cm²ISPPA) didn't show signs of apoptosis. C Condition 2 only showed some apoptotic cell in areas with some bleeding due to the drilling of the craniotomy. D Area marked in C with some apoptotic cells.

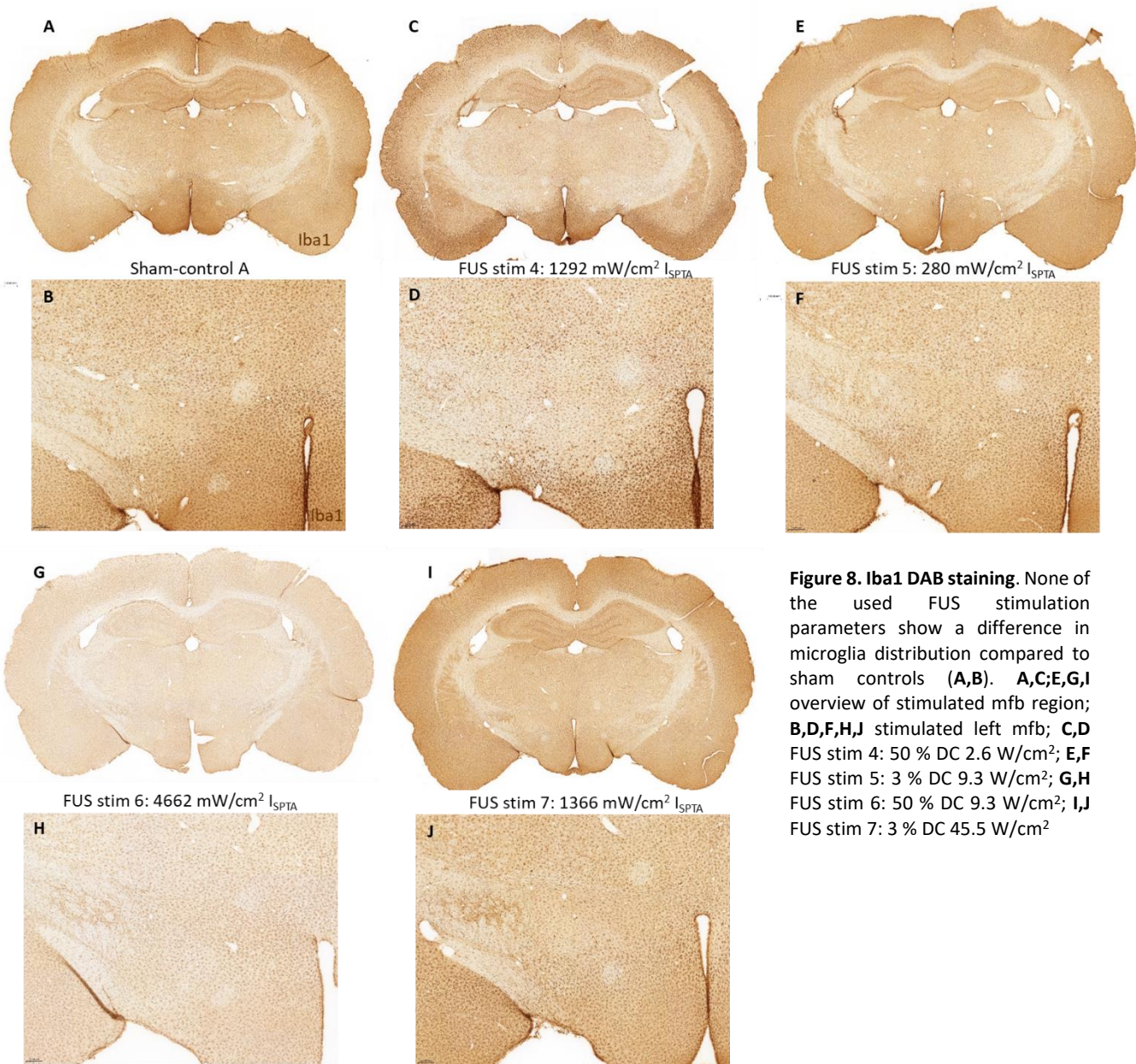


Figure 8. Iba1 DAB staining. None of the used FUS stimulation parameters show a difference in microglia distribution compared to sham controls (A,B). A,C,E,G,I overview of stimulated mfb region; B,D,F,H,J stimulated left mfb; C,D FUS stim 4: 50 % DC 2.6 W/cm²; E,F FUS stim 5: 3 % DC 9.3 W/cm²; G,H FUS stim 6: 50 % DC 9.3 W/cm²; I,J FUS stim 7: 3 % DC 45.5 W/cm²

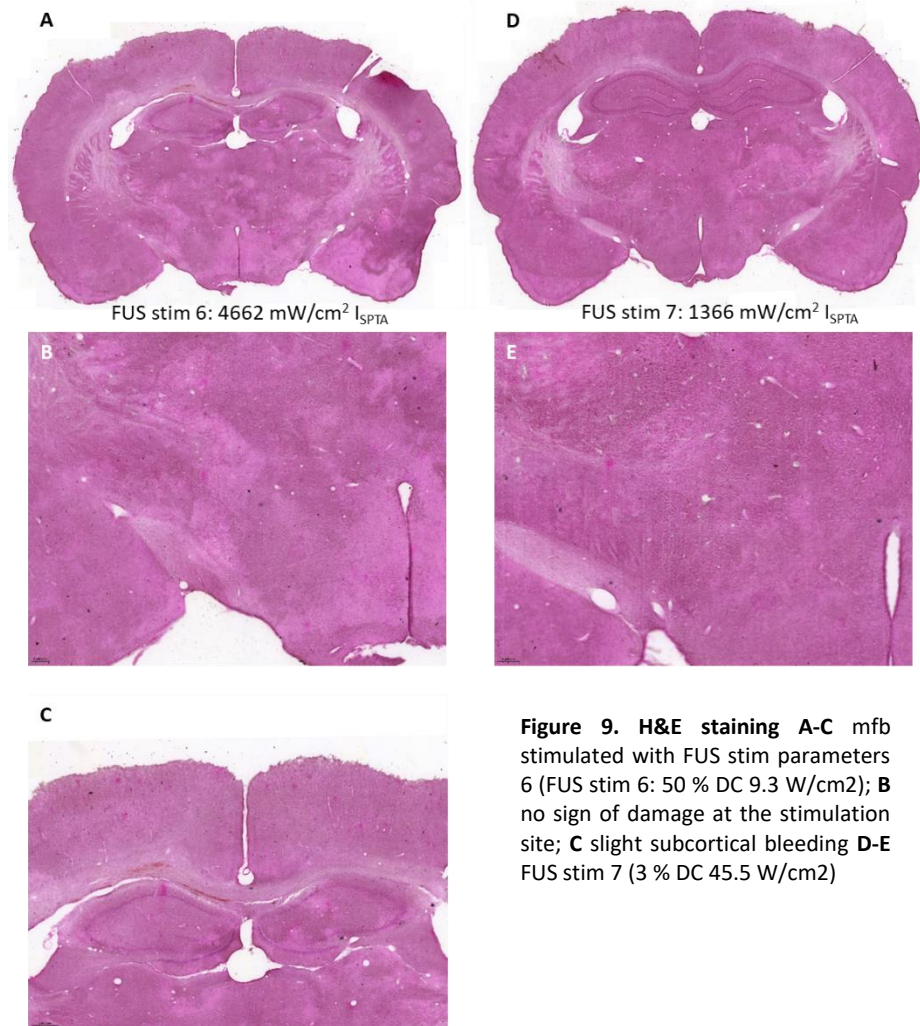


Figure 9. H&E staining A-C mfb stimulated with FUS stim parameters 6 (FUS stim 6: 50 % DC 9.3 W/cm²); **B** no sign of damage at the stimulation site; **C** slight subcortical bleeding **D-E** FUS stim 7 (3 % DC 45.5 W/cm²)

2.3 Alternative methods/ outlook

Since the histological assessment of the effect of different stimulation parameters only allows the evaluation of one parameter combination at a time, different other methods of parameter evaluation have been explored (fig. 10). The assessment of neurotransmitter release after mfb stimulation using fiber photometry would allow for an easy sweep of parameters and our group has extensive experience using fiber photometry in combination with mfb-DBS (Miguel Telega et al., 2022, 2024). Unfortunately, due to the diameter of the commercial transducer it is not possible to place the optic fiber in the PFC (fig.10 B) and lower the transducer enough to stimulate the mfb (fig. 10 C, D). Alternatively, we have attempted to culture sagittal brain slices with access but the mfb or VTA. But the tested stimulation did not cause any c-Fos expression (fig. 10 E-H). Similarly, the electrophysiological recording from a stimulated sciatic nerve (fig. 10 I, J) did not show any effects, most likely due to the higher pressures needed to stimulate explanted peripheral nerves.

While brain slice culture and peripheral nerve stimulation were potentially interesting methods, the most promising approach will be to use fiber photometry in combination with the eFUS chip. Pilot studies are planned for January/ February 2025. This approach will be used to obtain immediate real-time

physiological feedback by sweeping parameters with the eFUS chip within the indicated range suggested from this report to determine the optimal parameters for epidural mfb FUS stimulation.

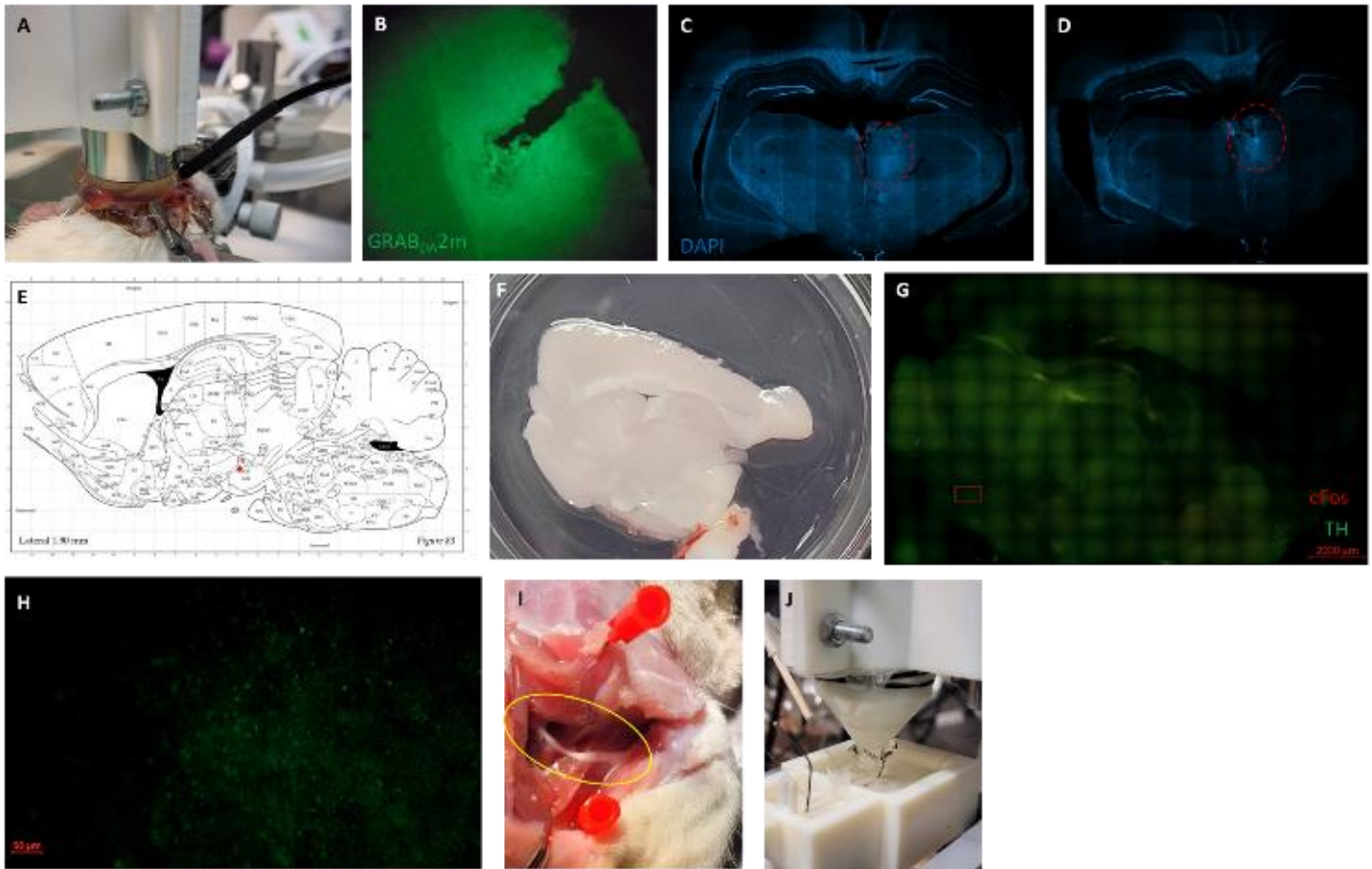


Figure 10. Exploration of alternative read outs for the effects of different FUS stimulation parameters. A-D Measurement of dopamine release after mfb FUS stimulation was unsuccessful, because the focal spot did not reach the mfb due to the physical restrictions of the transducer and the optic fiber. A Placement of optic fiber at an angle with commercial transducer. B successful expression of GRAB_{D_A2m} virus to detect dopamine release and correct placement of optic fiber in the PFC at an 50 °angle. C,D The transducer could not be lowered enough to reach the mfb with the focal spot. E Rat brain atlas picture showing the FUS stimulation side with a red dot F exemplary 300 um brain slice culture G,H brain slice stained with c-Fos (red) and TH (green), location of cells shown in H are indicated in red in G. No detectable c-Fos expression compared to positive control. I,J FUS stimulation of explanted rat sciatic nerve.

3. Conclusions

The aim of deliverable D4 was to identify optimal parameters for stimulating the medial forebrain bundle (mfb) in a rat brain using epidural focused ultrasound (eFUS) and to evaluate their safety.

The use of a commercial transducer, while necessitating a larger craniotomy and limiting experiments to anaesthetized conditions, enabled the preliminary identification of stimulation parameters pending validation with the first-generation eFUS chip.

The commercial device reliably targeted distinct sections of the mfb, and stimulation-induced neuronal activation was assessed through c-Fos expression.

All animals including controls showed a strong cortical activation especially on the stimulated hemisphere, which can be partially attributed to the anaesthesia and craniotomy.

Although trends in c-Fos expression—such as downregulation in the ipsilateral prefrontal cortex or increased activation under specific conditions—align partially with expectations from DBS and optogenetic studies, statistical significance was not achieved. Future studies incorporating advanced readouts like fiber photometry will be essential for refining these parameters.

Trends indicate that pressures between 0.5 and 1.2 MPa with tailored duty cycles may optimize stimulation effects. Further optimization of parameters, including increased pulse repetition frequency, potentially duty cycle and parameter sweeps in awake animals using the finalized chip, could help the reported effects reach statistical significance. Additionally, effects slightly differed when targeting the ventral tegmental area (VTA), which may warrant a shift of the initial target towards the VTA. As the mfb is a fiber bundle, the mechanisms of ultrasound-induced stimulation may differ from those required for stimulating cell bodies, such as those in the VTA. This could alternatively help to enhance the robustness of the observed effects.

Importantly, the investigation confirmed the safety of the tested parameters (ISPTA up to 4662 mW/cm²), with no observed tissue damage, apoptosis or immune responses attributable to the stimulation. Minor bleeding associated with craniotomy was procedural and is unlikely to affect outcomes with the smaller surgical footprint and healing time after chip implantation.

In summary, the study has narrowed the range of parameters for effective mfb stimulation and demonstrated the safety of the approach, laying a foundation for subsequent investigations using the first-generation eFUS chip.

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