

# UPSIDE

## Deliverable D4.3

Grant Agreement No.	101070931
Start date of Project	1 September 2022
Duration of the Project	48 months
Deliverable name	<b>D4.3: eFUS chip: Biological/histological evidence of efficacy</b>
Partner Leader	UF
Dissemination Level	PU

<i>Status</i>	Draft
<i>Version</i>	V0.2
<i>Submission Date</i>	28-02-2025

<i>Author(s)</i>	Prof. Dr. Máté Döbrössy (UF)
<i>Co-author(s)</i>	Lisa Ratz (UF)





European  
Innovation  
Council



Funded by the European Union. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or EISMEA. Neither the European Union nor the granting authority can be held responsible for them

PU=Public, SEN=Confidential, only for members of the consortium (including the Commission Services), CI=Classified, as referred to in Commission Decision 2001/844/EC.

## House Style

	Red RGB	Green RGB	Blue RGB	HEX
				
Pink	247	181	192	#F7B5C0
Grey	161	161	161	#A1A1A1
Blue	41	171	226	#29ABE2
 <p>THE USE OF THE EU EMBLEM IN THE CONTEXT OF EU PROGRAMMES 2021-2027 <a href="#">LINK</a></p> <p>In addition to the obligations set out in <a href="#">Article 17</a>, communication and dissemination activities as well as infrastructure, equipment or major results funded under EIC actions must also display the following special logo:</p>				
 				
EU corporate blue	0	51	153	#003399
Yellow	255	204	0	#FFCC00

## Authors, Co-authors and contributors

Author	Organization	E-mail
Prof. Dr. Máté Döbrössy	UF	mate.dobrossy@uniklinik-freiburg.de
Lisa Ratz	UF	lisa.ratz@uniklinik-freiburg.de

## Quality Control

Author	Name	Date
WP leader	Prof. Dr. Máté Döbrössy	28-02-2025
Internal reviewer	Tiago Costa	28-02-2025
Coordinator	Tiago Costa	28-02-2025

## History of Changes

Version	Change made	Date
V0.1	First draft	28-02-2025
V0.2	Final version	28-02-2025

## *Table of Contents*

Executive Summary.....	3
Introduction .....	4
Results.....	6
2.1 Previous results with Fiber Photometry:.....	6
2.2 eFUS Chip design .....	7
2.3 In vivo acute (chronic) data using eFUS with FP monitoring/ parameter sweep .....	7
2.4 Confirmation of in vivo eFUS targeting and safety .....	10
Conclusions .....	12
References .....	14

## *List of Figures:*

Figure 1A-N. Fiber Photometry (FP) and DBS .....	6
Figure 2A-E. Evolution of the chip implantation .....	7
Figure 3A-E. Evaluating the biological effects of eFUS .....	8
Figure 4. Stimulation protocol .....	9
Figure 5A-E. Fiber Photometry and eFUS.....	11

## *List of Tables:*

Table 1. Overview of the stimulation conditions and the parameters .....	11
--------------------------------------------------------------------------	----

## *Abbreviations*

WP :	Work Package
mPFC:	medial pre-frontal cortex
NAC	nucleus accumbens
VTA	ventral tegmental area
mfb/ MFB	medial forebrain bundle (small letter refers to rats, capitals refers to humans)
DBS	Deep Brain Stimulation
eFUS	epidural Focused Ultrasound
FP	Fiber Photometry
PCB	Printed Circuit Board
LCP	Liquid Crystalline Polymer

## Executive Summary

D4.3 represents a key moment in the UPSIDE project as it is the first opportunity for the in vivo testing of the eFUS chip. It is the first stage where the eFUS device - constructed and tested in vitro by the micro-engineers - is transferred to the animal model team with the mandate to test its in vivo functionality. D4.3 covers the initial description of the biological and histological evidence of the chip's "efficacy". In the current context "efficacy" is defined as data that investigates whether the eFUS chip can induce physiological responses in a predictable and time-locked manner, i.e. whether and under what conditions the stimulation leads to specific biological responses, which can be reproduced in a regular fashion. There is fixed submission date for this document, however, the "deliverables" need to be perceived more as an on-going and evolving findings as the chip design and the in vivo testing protocols are likely to evolve based on incremental real-world testing data. Over the coming weeks and months new generation of chips will need to be tested to understand the potential of the eFUS device.

The testing of the in vivo biological efficacy of the eFUS chip began using Fiber Photometry (FP). FP is an innovative fluorescent-based technique used to monitor - with a high temporal and spatial resolution - general neuronal activity or the release of specific neurotransmitters in a single or multiple brain areas. The technique has been successfully employed for years in conjunction with DBS to shed light on the acute and chronic biological consequences and mechanisms of neurostimulation.

The data described in this document is sparse and allows only for partial conclusions. It represents the beginning of a learning process, and has already allowed to make improvements in the chip and testing protocol design. We have demonstrated that the surgical preparations (described in D4.1 and D4.2) were appropriate and the physical dimensions of the prototype eFUS chip (R2C6) was fine. The eFUS chip – targeting unilaterally the medial forebrain bundle - was placed and secured on the dura of the anesthetized animal, and connected up to the PCB and the computer. A series of stimulation parameters were tested using a defined stimulation protocol. Fiber photometry data monitored the pre-stimulation and post-stimulation neuronal activity in the mPFC. The first experience using the eFUS chip in vivo led to two observations: firstly, the analysis of the data did not identify any stimulation related changes in neuronal activity in the monitored brain region under any of the stimulation conditions; secondly, there is strong suspicion that the chip functioned only during conditions #1-4.

The possible interpretations are that either mfb eFUS stimulation did not alter neuronal activity in the mPFC or (and this is the more likely explanation in our opinion) the stimulation conditions (parameters, targeting) were not appropriately set. The Conclusion contains more detailed discussion of some of the key issues emerging from this first limited in vivo experience with the eFUS chip.

Additional data is required to demonstrate the physiological impact of eFUS stimulation in rats. Progress will require relentless testing, and constant dialogue between the engineers and in vivo testing team to make refinements in the device and adjustments in the testing parameters and conditions.

\*\*\*\*\*

D4.3 has been delayed due to the late supply of the eFUS chip. More chips will be needed for in vivo testing to comprehensively test its performance to modulate physiological process/network activity. Therefore, this submission is considered as a draft. The final version of D4.3 for publication is expected by M32 (April 2025). As a consequence of the eFUS chip supply delay, the next deliverable of WP4, D4.4 originally due M32 (April 2025), is expected to be also delayed and submitted by M36 (August 2025). The lateness of those submissions has no significant impact on the eventual future Milestones from WP4.

## Introduction

### D4.3. eFUS chip: Biological / histological evidence of efficacy (M27+4)

Our previous report (D4.2) used a commercial FUS transducer to test and evaluate stimulation parameters for safely stimulating the rat medial forebrain bundle (mfb). The use of a commercial transducer, while necessitating a larger craniotomy and limiting experiments to anaesthetized conditions, enabled the preliminary identification of safe parameters pressures between 0.5 and 1.2 MPa with tailored duty cycles to optimize stimulation effects. Importantly, the investigation confirmed the safety of the tested parameters (ISPTA up to 4662 mW/cm<sup>2</sup>), with no observed tissue damage, apoptosis or immune responses attributable to the stimulation. The data also suggested that the stimulation outcome could differ whether we are targeting predominantly grey-matter or white matter zones. In other words, 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. D4.2 successfully narrowed the range of parameters for effective mfb stimulation and demonstrated the safety of the approach, laying a robust foundation for subsequent investigations using the first-generation eFUS chips.

#### *Why is the mfb a stimulation target?*

The majority of information we have concerning the role of the mfb has been obtained via pre-clinical, experimental research, mainly using rodents. The mfb is composed of bi-directional, myelinated and unmyelinated projections of diverse neurotransmitter systems, including the monoamines, glutamate and GABAergic neurons[1]. The descending and ascending fibres between the mesencephalon and the forebrain connect with different regions associated with multiple functions such as motor, cognition and emotion/ mood processing. The fibres and hubs on the mfb also include components of the “reward” network such as the cingulate cortex, the mPFC, the NAC, and the VTA[2,3], structures which have been also implicated in depression.

Two cardinal symptoms for the clinical diagnosis of depression are depressed mood (reduced motivation) and the loss or reduction of interest in previously pleasurable activities (anhedonia). Experimental data strongly suggest that these functions are – in part – sub-served and modulated by dopaminergic projections of the frontal striatal “reward” network, originating in the midbrain and passing through the mfb. The rationale for targeting the mfb (in rodents) or the MFB (in humans in clinical studies) with DBS is to regulate the activity of the pathways implicated in the neurocircuitry of depression.

#### *What we expect following the stimulation? Behavioral and physiological consequences.*

The UF have both clinical and pre-clinical experience of medial forebrain stimulation. A series of clinical trials in treatment resistant depression patients have shown that bilateral, chronic, and high frequency MFB DBS results in rapid, sustained and enduring therapeutic effect in alleviating depressive symptoms[4–9]. The pre-clinical research group uses a wide variety of experimental approaches – and experimental models - to understand the mechanisms of neuromodulation at the behavioral, cellular, molecular, anatomical and physiological levels. Typically, we use the Flinders Sensitive Line rats (FSL) that spontaneously show “depressive-like” phenotype [10–12], or the chronic mild unpredictable stress (CMUS) paradigm, as the animal depression models in our work [13]. Studies from our group using mfb DBS or selective optogenetic stimulation of the midbrain dopamine projections, both showed significant anti-depressant effects in these models [12,14–16].

Stimulation of the mfb is also expected to regulate the neurotransmitter systems passing through the fiber bundles. As part of the UPSIDE project, we have started using Fiber Photometry (FP), an innovative neurotransmitter/ neuronal activity monitoring technique. FP is a state-of-the-art method measuring neuronal population bulk activity changes or the extracellular availability of key neurotransmitters associated with

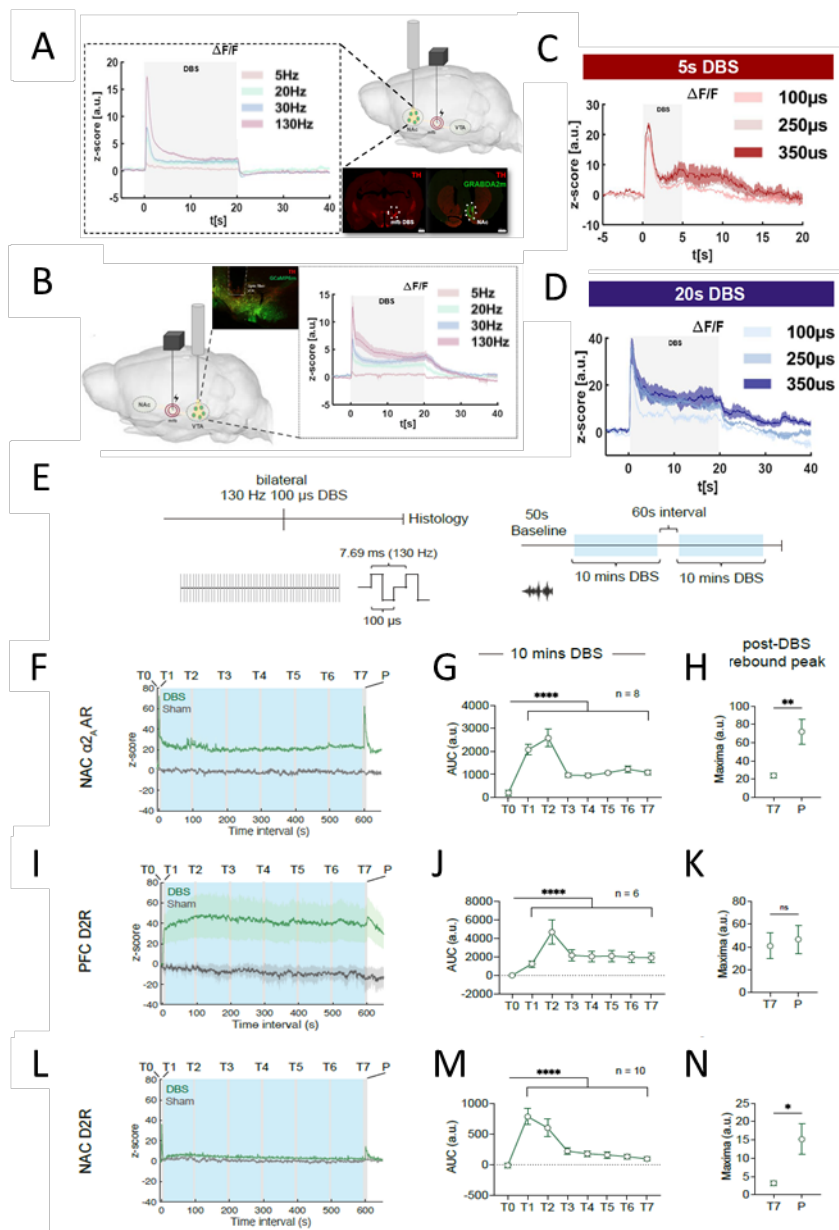
depression, like for example, dopamine, noradrenaline or glutamate [17–20]. FP offers several advantages over more traditional approaches such as dialysis or fast scanning cyclic voltammetry: i.) the neurotransmitters can be defined very specifically, and electrochemically inert substances like glutamate can also be detected; and ii.) chronic in vivo repeated measurements over a period of up to about 2 months can be performed without loss of signal. Using FP, we demonstrated that mfb DBS induces acute and enduring release of dopamine and noradrenalin in forebrain structures, e.g. prefrontal cortex and nucleus accumbens; that the release patterns are stimulation paradigm dependent; and that stimulation has differential effects on the depression model vs controls. The results further confirmed that modulation of monoamine release in key structures on the reward pathway are likely to be involved in the antidepressant mechanisms of mfb DBS[21–23].

Recent preclinical work from other labs using transcranial low-intensity FUS in acute study designs have shown that the intervention can reverse some of the “depressive-like phenotype”, and modulate the reward-pathway[24,25]. Typically other labs have targeted neural hubs, like the VTA or medial prefrontal cortex, as opposed to our target, the mfb, which are myelinated and unmyelinated fibers. However, our prediction is –based on our preclinical experience with DBS- that the eFUS chip will give qualitatively similar results when using physiological monitoring techniques such as FP.

#### *D4.3: A pivotal point in the project*

D4.3 represents a key moment in the UPSIDE project as it is the first opportunity for the in vivo testing of the eFUS chip. It is the first stage where the eFUS device - constructed and tested in vitro by the micro-engineers - is transferred to the animal model team to test its in vivo functionality. In other words, D4.3 covers the initial description of the biological and histological evidence of the chip’s “efficacy”. In the current context “efficacy” is defined as data that investigates whether the eFUS chip can induce physiological responses in a predictable and time-locked manner, i.e. whether and under what conditions the stimulation leads to specific biological responses, which can be reproduced in a regular fashion. The in vivo testing of the chip needs to be perceived as an on-going procedure that is expected to produce changes/ evolution in its design based on the in vivo, real-world testing data. It is likely that a new generation of chips will need to be tested to understand the potential of the eFUS device.

## Results



**Figure 1A-N. Fiber Photometry (FP) and DBS.** FP was established to monitor - in a high temporal and spatial resolution fashion - stimulation induced changes in transmitter release and neuronal activity levels. In these examples, FP was used in conjunction with mfb DBS. For explanation of the panels, see main text.

PFC following 10 minutes of continuous mfb DBS at 130Hz/ 100 $\mu$ s. The data showed strong and continuous - throughout the 10 min stimulation period - noradrenaline release in the NAC (Figure 1F), dopamine release in the PFC (Figure 1I), and lower (but significant) levels of dopamine release in the NAC (Figure 1L). A second 10 min stimulation period (Figures 1G, J, M) was preceded by a 5 seconds baseline recording (T0, -5-0s), followed by the 600 seconds chronic and continuous stimulation period broken down in the AUC analysis into six 100 seconds time-bins with 5 seconds of each time-bin used for the analysis (T1, 0-5s; T2, 95-100s; T3, 195-200s; T4, 295-300s;

### 2.1 Previous results with Fiber Photometry

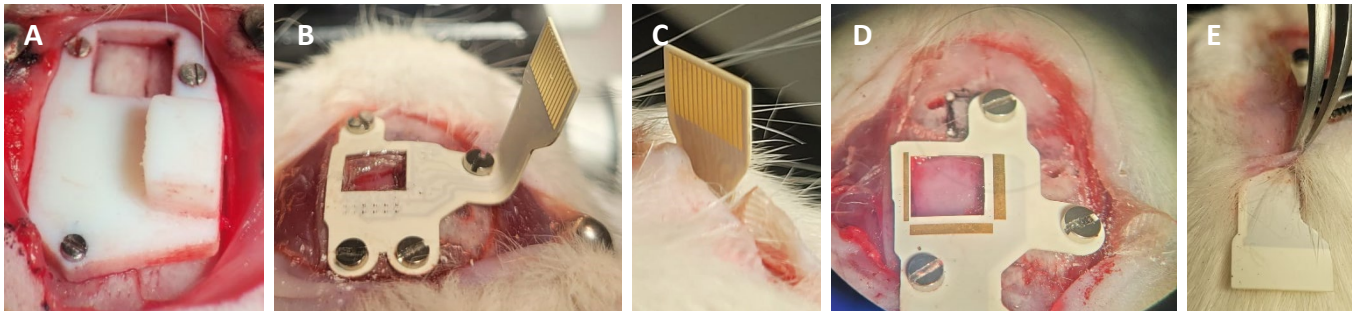
Fiber Photometry (FP) is an innovative fluorescent based technique used to monitor general neuronal activity or the release of specific neurotransmitters in a single or multiple brain areas. The monitoring can be done in in vivo behaving animals acutely or longitudinally/ repetitively overtime. The technique was established in the lab in conjunction with mfb-DBS and key results are summarized below. Initial work focused on varying the DBS parameters - such as frequency (5, 20, 30, 130Hz), pulse-width (100, 250, 350 $\mu$ s), and stimulation duration (5 or 20 seconds) - and investigated how accumbal dopamine or VTA neuronal activity (looking at changes in Ca<sup>2+</sup> concentrations) were impacted by mfb DBS in freely moving animals and longitudinally up to 8 weeks (Figure 1A-D)[22,23]. Long-term signal stability was confirmed, with no significant changes in the signals measured over 8 weeks for each stimulation condition. mfb DBS increased dopamine release in the NAC and Ca<sup>2+</sup> bulk activity in VTA in a frequency-dependent fashion (Figure 1A-B). Significant increase in accumbal dopamine release was also observed during 5s and 20s 130Hz mfb DBS across all pulse-widths, with increased release with the longer stimulation duration and the higher pulse width (Figure 1C-D). A follow-up study looked at noradrenaline release in the NAC, and dopamine release in the NAC and the

T5, 395-400s; T6, 495-500s; T7, 595-600s). After the 10 minutes of stimulation ended, dopamine and noradrenaline signals were continuously recorded post-DBS for another 55 seconds. The initial 5 seconds post-DBS (Figures 1H, K, N; P, 600-605s) were used for analysis and comparison with T7 to investigate the immediate consequence of the cessation of stimulation, such as “rebound” (burst release) effect. The recording showed that dopamine and noradrenaline signalling initiated with an acute peak, as we observed in the 5 seconds acute stimulation and followed with a sustained stable signalling until the end of stimulation. Interestingly, after the stimulation stopped, a rebounded post-stimulation peak was observed in the cases of both dopamine and noradrenaline.

Early results emerging from the first experience with the eFUS chip are described in section 2.3.

## 2.2 eFUS Chip design

The design of the PCB for the chip has gone through several permutations and has been constantly improved and changed based on our learning experience. The limitations in the fabrication at different stages required adaptation and the design required for chronic implantation has been re-tested at different stages of the project. This led to changes following the first implantations described in deliverable D4.1 (M18), and to different LCP designs, as seen in Figure 2A-E. Different options of connector orientation and fixation screws, allowed us to find the design for easiest implantation and secure fixation and comfort for the animal. The first acute in vivo testing in an anesthetized animal (see 2.3) again has inspired new changes that will be implemented and tested during the next implantation sessions. Changes will be needed to the system of connection to permit attachment/ detachment for chronic implantation and testing in freely moving behaving animals in studies that are expected to last several weeks.

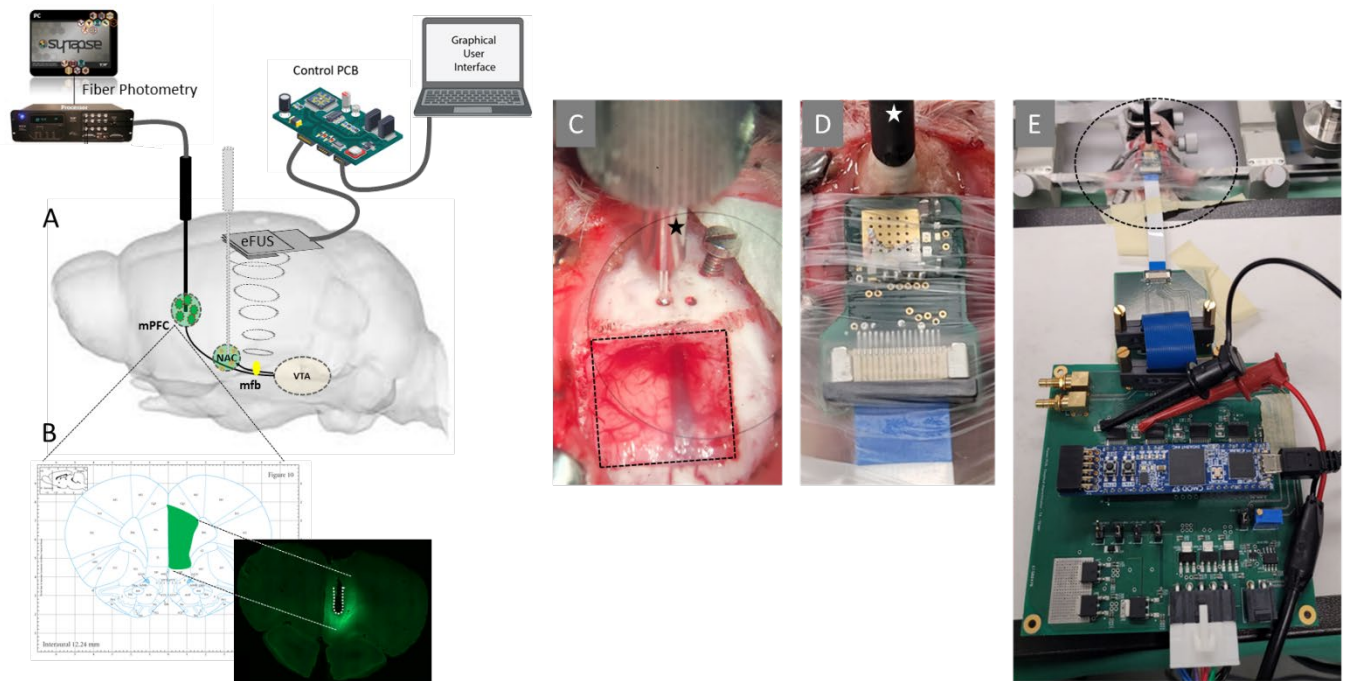


**Figure 2A-E. Evolution of the chip implantation. A.** First design for a rigid PCB, as presented in D4.1. **B. and C.** LCP design (#1) with a sideways facing connector. **D and E.** LCP design (#3) with a backwards facing connector, in this case the side usually facing the brain is facing upwards to test a chip implantation on the left hemisphere.

## 2.3 In vivo acute (chronic) data using eFUS with FP monitoring/ parameter sweep

The first set of eFUS chips designed by the consortium (D1.5) were made available to the Freiburg group in February and March 2025. More precisely, the first chip was delivered mid-February and a second delivery was made mid-March. Prior to in vivo use, each eFUS chip has been tested for functionality (data available from TUD group).

Figure 3A-E shows the preparatory work and the different components required to test the eFUS chips capacity to have biological/ physiological impact via mfb stimulation. The work was carried out over two surgical sessions separated by approximately 4 weeks. The first session involved the stereotactic injection of the genetically enhanced indicator used, the calcium sensor (AAV-5 CamK2a-GaMP6m) into the medial pre-frontal cortex (mPFC), a prerequisite for the Fiber Photometry (FP) technique to function. The fluorescent calcium sensor enables the detection in neuronal activity induced by stimulation. The second surgical session involved the placement of optic fiber, making of the craniotomy, the placement of the eFUS chip, and the testing of the physiological impact of eFUS stimulation of the medial forebrain bundle in the anaesthetized rat. The experimental setup used to test the eFUS (Figure 3A), the post-mortem tissue analysis confirming the placement of the optic fiber and the expression of the calcium sensor are illustrated below (Figure 3B).

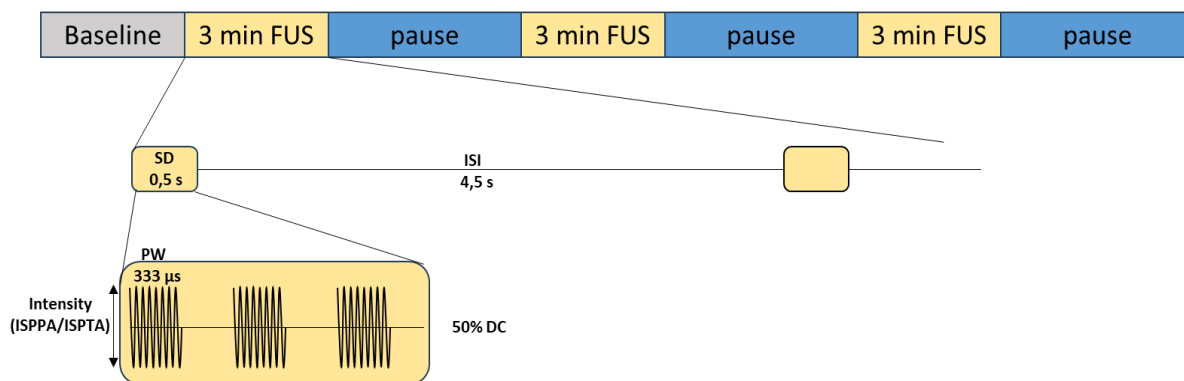


**Figure 3A-E. Evaluating the biological effects of eFUS.** **A.** Setup for the in vivo testing of the eFUS chip. Animals have optic fiber implanted into either the medial Pre-frontal cortex (mPFC) or nucleus accumbens (NAC). The eFUS chip was placed on the dura via the craniotomy. The eFUS chip was used to target the medial forebrain bundle (mfb) which projects to mPFC and NAC. Stimulation induced changes in neuronal activity in the target regions were monitored using Fiber Photometry. **B.** The mPFC and histological evidence of the fluorescent calcium sensor. The position where the optic fiber was placed is visible (delineated by the white dots). **C.** Craniotomy (black dashed line) for the eFUS chip and implantation of optic fiber for fiber photometry (black star) and anchor screw (white star). **D.** Connected fiber optic (white star) and eFUS chip placed on the dura via the craniotomy, fixed with parafilm. **E.** PCB steering the eFUS chip (black dashed line) and experimental set up.

The initial tests were done in anesthetized animals. The optic fiber was implanted in the left mPFC and fixed with dental cement; the eFUS chip was carefully placed on the dura via the craniotomy and coupled to the brain using sterile ultrasound gel. The chip was held in place with parafilm (Figure 3C-D). The PCB was connected up with the eFUS and the user interface that permitted the programming of the stimulation parameters (Figure 3E).

Stimulation protocol and parameters

The stimulation protocol is shown in Figure 4. It consisted of 3 minutes Baseline recording and three times 3 minutes of FUS stimulation followed by a 5 min pause between each stimulation blocks. For example (Condition #1), the 3 min stimulation block was made up of 36 bursts, with each burst lasting 0.5s (sonication duration), and with 4.5s between bursts (inter-stimulation interval). Each burst consisted of 750 stimuli, resulting in 3 x 36 x 750 = 581000 “events”.



**Figure 4. Stimulation protocol.** Stimulation and fiber photometry monitoring of neuronal activity was done initially using anesthetized animals. The protocol (3 min Baseline recording/ three x 3 minutes FUS/ 5 min pause between each FUS stimulation block) was repeated multiple of times with different stimulation parameters varying the amplitude, pulse repetition frequency, etc. The example above is for Condition #1. SD, sonication duration; ISI, inter-stimulation interval; PW, pulse-width; DC, duty cycle.

The conditions and parameters used in the first testing round are summarized in Table 1. For the estimation of some of the variables, two different conditions were taken into account: the theoretical ultrasound parameter calculations in water and the probable ultrasound parameter values achieved in the brain medium.

Test conditions	Target	Total stim. time (s)	Freq. (MHz)	Amplitude (Vpp)	Peak negative pressure (Kpa)		Mechanical index MI (Mpa/MHz <sup>1/2</sup> )		ISPPA (W/cm <sup>2</sup> )		ISPTA (mW/cm <sup>2</sup> )		Pulse rep. frequency (Hz)	Duty cycle (%)	PW (μs)	SD (ms)	Bur st (s)	ISI (s)	Isoflurane (%)
					H <sub>2</sub> O	brain	H <sub>2</sub> O	brain	H <sub>2</sub> O	brain	H <sub>2</sub> O	brain							
1	mfb	3x180	4	5	225	180	0.11	0.09	1.58	1.01	29.6	18.92	1500	50	333	500	5	4.5	1.6
2	mfb	3x180	4	10	450	360	0.23	0.18	6.32	4.05	23.7	15.19	1500	10	67	500	5	4.5	1.6
3	mfb	3x180	4	15	675	540	0.34	0.27	14.22	9.10	26.7	17.09	1500	5	33	500	5	4.5	1.6
4	mfb	3x180	4	20	900	720	0.45	0.36	25.29	16.18	19.0	12.16	1500	2	13	500	5	4.5	1.6
5	mfb	3x180	4	5	225	180	0.11	0.09	1.58	1.01	26.7	17.06	1500	45	300	200	2	1.8	1.5
6	mfb	3x180	4	5	225	180	0.11	0.09	1.58	1.01	26.7	17.06	1500	45	300	200	2	1.8	1.5
7	mfb	3x180	4	10	450	360	0.23	0.18	6.32	4.05	23.7	15.18	200	4	200	1000	4	3.0	1.5
8	mfb	3x180	4	15	675	540	0.34	0.27	14.22	9.1	16.02	10.25	130	3	500	500	5	4.5	1.5

**Table 1. Overview of the stimulation conditions and the parameters.** Test Conditions #1-4 (red frame) were used for the data analysis as there is reasonable doubt that the chip stopped emitting ultrasound after that. See text for explanation.

During the test, the eFUS chip and the connector were subjected to physiological fluids originating from the freshly craniotomized brain and surrounding tissue, as well as from the ultrasound gel which was introduced between the brain and the chip. It is suspected that i.) this led to a short circuit in the connector, potentially subjecting the chip to higher voltages than intended; and ii.) most likely caused the chip to work inconsistently towards the end of the test. It is suspected that this occurred following Condition #4, approximately 90 minutes into the testing. For this reason, the analysis includes only data collected during the first initial conditions. The better isolation of the eFUS chip and the connector will be ensured for the next generation of chips.

#### Data analysis (conditions #1-4)

Fiber Photometry data monitoring changes in  $\text{Ca}^{2+}$  dynamics in the mPFC was collected according to the previously described protocol and stimulation conditions. Changes in  $\text{Ca}^{2+}$  dynamics is taken as a biological correlate for changes in neuronal activity in the mPFC.

#### *Subject characteristics:*

Animal: FSL female # 977  
GEI/ virus: AAV5 CAmK2a GCaMP6m injection made on 12.01.25, left PFC  
eFUS chip: R2C6, implanted on 14.02.25 for acute testing/ FP measurements  
Craniotomy dimensions: 9.3 x 9.0 mm  
Stimulation aimed at mfb (AP= -2.8; ML= +1.7; DV= -8.0)

#### *Data treatment:*

Demodulated fiber photometry data ( $\Delta F/F$ ) were extracted from the TDT system using pMAT[26], an open-source software for data analysis. TTL signals, which synchronize with the stimulation protocol, were also recorded. The extracted data were subsequently down sampled from 1000 Hz to 10 Hz and de-trended to eliminate low-frequency noise.  $\Delta F/F$  is the change in fluorescence signal divided by the baseline signal level. The data can also be normalized across sessions (conditions in our case) by calculating the Z score by subtracting the mean and dividing by the standard deviation.

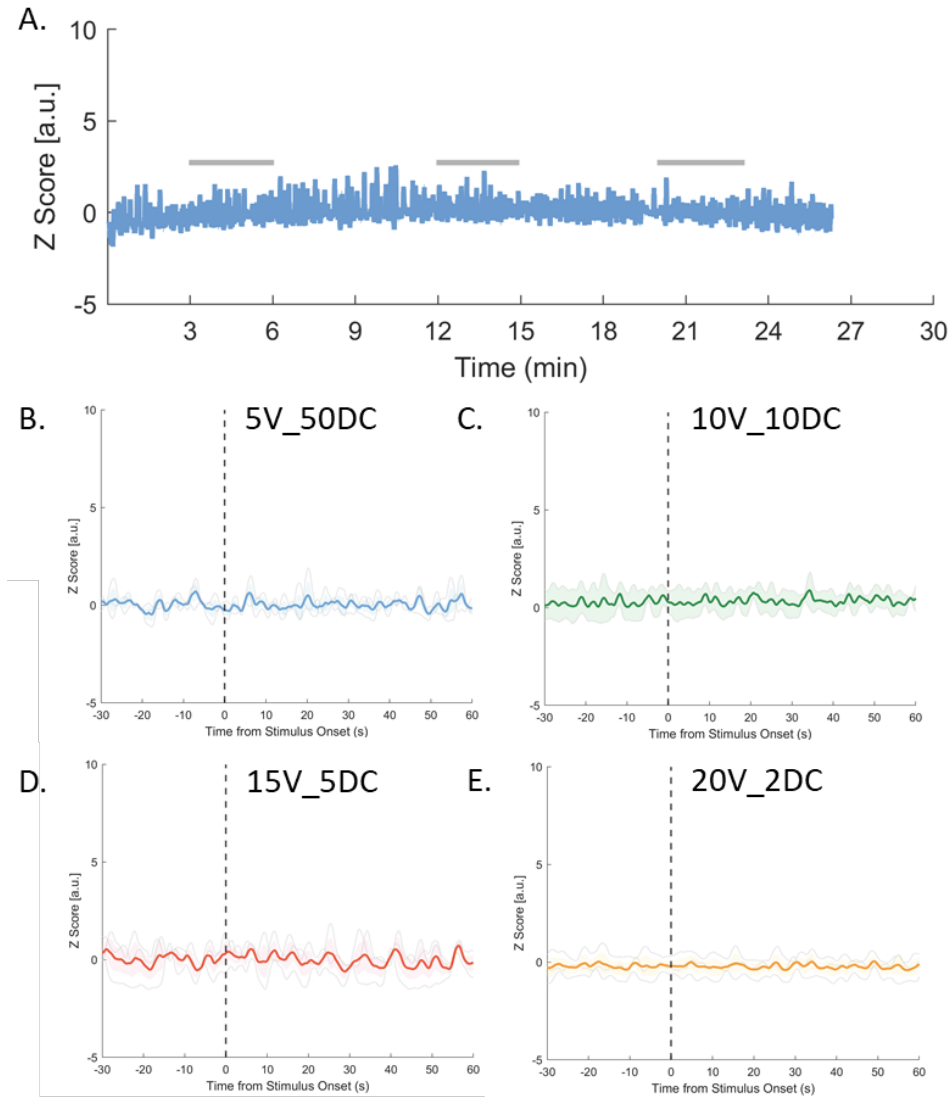
#### *Results:*

The results across the four stimulation conditions are presented in Figure 5A-E. No clearly identifiable stimulation effects were observed in this first test. Oscillation/ spiking in the data suggests that biological signal was recorded. The data is discussed in more detail in the Conclusion, section 3.

### **2.4 Confirmation of in vivo eFUS targeting and safety**

Confirmation of appropriate targeting and steerability of the eFUS chip will be demonstrated using intravenous injection of Evans blue dye with microbubbles (SonoVue®). The circulating blue dye is normally excluded from penetrating the brain due to its large size which is not permissible through the blood brain barrier (BBB). SonoVue microbubbles are composed of sulfur hexafluoride gas packaged by a phospholipid monolayer shell and in conjunction with FUS have cavitation properties that promote the opening up of the BBB. In specific targeted areas corresponding to the focal point of the US, the circulating blue dye will diffuse and mark up the local interstitial brain tissue.

Safety of the stimulation will be confirmed using H&E and TUNNEL staining. The former will indicate any disruptions in the targeted areas cytoarchitecture and the latter is a more refined method to detect apoptotic neuronal death.



**Figure 5A-E. Fiber Photometry and eFUS.** The Z-score represents the normalized  $\Delta F/F$  value, which is the change in fluorescence signal divided by the baseline signal level. **A.** The protocol included 3 minutes Baseline recording and three times 3 minutes of FUS stimulation (gray bars) followed by a 5 min pause between each stimulation blocks. The protocol was repeated over multiple conditions including **B.** Condition #1 (5V, 50% DC); **C.** Condition #2 (10V, 10% DV); **D.** Condition #3 (15V, 5% DC) and **E.** Condition #4 (20V, 2% DC). The results were similar across all conditions with no stimulation effect observed. The dashed line represents the onset of stimulation. Mean scores for the three pre-stimulation periods 30s prior to the onset and the initial 60s of the three post-stimulation periods are shown.

## Conclusions

Deliverable D4.3 is committed to initiate – and report on – the investigation of the biological/ histological evidence of efficacy of the eFUS chip. The completion of this deliverable hinges on two essential requirements and components: *firstly*, an experimental in vivo platform sensitive to monitor stimulation induced physiological changes; and *secondly*, the availability of a constant flow of functioning eFUS devices that can be tested in the in vivo experimental platform.

The current document reports on the successful tried and tested experimental platform capable of in vivo monitoring of stimulation-evoked changes in network activity, including neurotransmitter release. Data included in this report gave examples of our efforts demonstrating the integration of Fiber Photometry with DBS in freely moving and behaving rats, including experimental models of depression[22,23]. The approach has been used to test how varying stimulation parameters can affect neuronal activity, or regulate dopamine and noradrenalin release in key neuronal hubs associated with the neurocircuitry of depression such as the mPFC or NAC. The second condition to complete D4.3 - the availability of eFUS chips to test in vivo – has been met but the number of devices tested to date has been limited.

The emerging data allows only for partial conclusions. We have demonstrated that the surgical preparations (described in our previous deliverables D4.1 and D4.2) were appropriate and the physical dimensions of the prototype eFUS chip tested (R2C6) were fine. The animal was prepared, the eFUS chip was placed on the dura and secured temporarily and the chip connected to the PCB and the computer as planned. The experimenters started applying the predetermined protocol, and the conditions were repeated three times. As described in section 2.3, the testing of chip R2C6 ended after having tested eight conditions, although the strong suspicion is that the chip functioned as intended only during Conditions #1-4. The four conditions differed in the Amplitude (5, 10, 15, 20 Vpp) and the Duty Cycle (50, 10, 5, 2%), resulting in the predicted peak negative pressure in the brain tissue (180, 360, 540, 720 Kpa). Fiber photometry data monitored the change of fluorescence in the mPFC, and this is taken as a biological correlate indicating the change in the neuronal activity reflecting calcium dynamics[20].

*According to the data, the pre-stimulation and post-stimulation neuronal activity in the mPFC were identical, and this was observed across the four stimulation conditions.* The similar calcium dynamics (neuronal activity) during the monitoring period would suggest that either mfb eFUS stimulation did not alter neuronal activity in the mPFC or (and this is the more likely explanation in our opinion) *the stimulation conditions (parameters, targeting) were probably not appropriately set.* Re-evaluation of the stimulation parameters used during Conditions #1-4 suggest that focal pressure set was too low, as was the duty cycle. Additional parameters were planned, but the R2C6 chip stopped functioning from Condition #5 on. *Future testing sessions will use higher pressures and duty cycles.*

The group has many years of experience with DBS in rodent models of depression, and we trust that this platform is appropriate: with time and effort, it can be adapted to the use of eFUS as well. eFUS represents novel challenges for both the engineers and those testing it in the animal models: many of the challenges can be anticipated and be built into the device. However, many of the challenges will be encountered only during the in vivo testing and will require reflection from the engineers and the in vivo team for them to be corrected. Some of the key issues (the examples below are not exhaustive) to resolve are:

i.) *eFUS chip related issues.* The in vivo testing team will require approximately 5-10 functioning eFUS chips per month to make headways. The early testing will be acute and involve anesthetized animals. However, later the eFUS chips will be implanted to perform longitudinal testing. The chips will need to have hermetic circuitry and

equipped with reliable connectors to the PCB. The connectivity needs to take into account repetitive connection/disconnection that will be necessary for studies lasting over several weeks.

ii.) *in vivo application related issues*. The limited data collected suggests that monitoring acutely from anesthetized animals might not be ideal for two reasons. Firstly, freshly implanted optic fiber causes acute tissue damage in the local area where the monitoring takes place; secondly, isoflurane anaesthesia reduces brain oscillation and network activity[27]. Both of these factors could interfere with the Fiber Photometry measurements. Performing acute studies will continue for now, but these limitations need to be considered. We will also perform chronic measurements where – following the optic fiber implantation and the placement and fixation of the eFUS chip – the animals recovers, and the Fiber Photometry measurements are performed after a post-surgical recovery period. This condition is more akin to our experience with DBS. Another *in vivo* application related issue is targeting. Targeting will be looked at using the blue dye/ microbubbles (SonoVue®) method described in section 2.4. We are also certain that targeting can be confirmed by combining the eFUS's steerability with the real-time feedback using Fiber Photometry: as observed from the pilot data with DBS, stimulation of the mfb produces immediate changes in neuronal activity/ transmitter release in the distal regions such as the NAC and mPFC. Once the setup is running, we will also use the eFUS chip to stimulate directly the VTA, where the cell bodies of the midbrain dopaminergic neurons originate from. It is possible that targeting gray matter will yield better results than targeting the mfb that contains both myelinated and unmyelinated fibers.

In conclusion, additional data is required to demonstrate the physiological impact of eFUS stimulation in rats. The GUI will also need to be developed. For example, additional functions will be added to do automatic scans in “search” of the mfb. Future versions of the GUI, the investigator will be able to program (by entering values) the chip to focus on a location defined according coordinates  $x/y/z$  and for  $t$  seconds, and then follow a search pattern in a 3D grid with a given step size of  $\Delta x$ ,  $\Delta y$  or  $\Delta z$ , and stay  $t$  seconds in each location. Ideally, once the beam happens to be in the mfb, we will be able detect the change in the Fiber Photometry. Also, having a built-in “self-testing” mode that could be activated via the user-interface would be desirable. This would allow the *in vivo* testing person to regularly (e.g. each day before use) verify the proper functioning of the eFUS chip, which will be essential when doing repetitive behavioural testing (D4.5, M44). Progress will require relentless testing, and constant dialogue between the engineers and the *in vivo* team to make refinements in the device and the testing conditions.

## References

- [1] Döbrössy MD, Ramanathan C, Ashouri Vajari D, Tong Y, Schlaepfer T, Coenen VA. Neuromodulation in Psychiatric disorders: Experimental and Clinical evidence for reward and motivation network Deep Brain Stimulation: Focus on the medial forebrain bundle. *Eur J Neurosci* 2021;53:89–113. <https://doi.org/10.1111/ejn.14975>.
- [2] Russo SJ, Nestler EJ. The brain reward circuitry in mood disorders. *Nat Rev Neurosci* 2013;14:609–25. <https://doi.org/10.1038/nrn3381>.
- [3] Dean J, Keshavan M. The neurobiology of depression: An integrated view. *Asian J Psychiatr* 2017;27:101–11. <https://doi.org/10.1016/j.ajp.2017.01.025>.
- [4] Bewernick BH, Kayser S, Gippert SM, Coenen VA, Schlaepfer TE. Acute antidepressant effects of deep brain stimulation – Review and data from sIMFB-stimulation. *Personalized Medicine in Psychiatry* 2017;3:1–7. <https://doi.org/10.1016/j.pmip.2017.01.002>.
- [5] Bewernick BH, Kilian HM, Schmidt K, Reinfeldt RE, Kayser S, Coenen VA, et al. Deep brain stimulation of the supero-lateral branch of the medial forebrain bundle does not lead to changes in personality in patients suffering from severe depression. *Psychol Med* 2018:1–9. <https://doi.org/10.1017/S0033291718000296>.
- [6] Coenen VA, Bewernick BH, Kayser S, Kilian H, Boström J, Greschus S, et al. Superolateral medial forebrain bundle deep brain stimulation in major depression: a gateway trial. *Neuropsychopharmacology* 2019;44:1224–32. <https://doi.org/10.1038/s41386-019-0369-9>.
- [7] Fenoy AJ, Schulz P, Selvaraj S, Burrows C, Spiker D, Cao B, et al. Deep brain stimulation of the medial forebrain bundle: Distinctive responses in resistant depression. *J Affect Disord* 2016;203:143–51. <https://doi.org/10.1016/j.jad.2016.05.064>.
- [8] Fenoy AJ, Schulz PE, Selvaraj S, Burrows CL, Zunta-Soares G, Durkin K, et al. A longitudinal study on deep brain stimulation of the medial forebrain bundle for treatment-resistant depression. *Transl Psychiatry* 2018;8:111. <https://doi.org/10.1038/s41398-018-0160-4>.
- [9] Schlaepfer TE, Bewernick BH, Kayser S, Mädler B, Coenen VA. Rapid effects of deep brain stimulation for treatment-resistant major depression. *Biol Psychiatry* 2013;73:1204–12. <https://doi.org/10.1016/j.biopsych.2013.01.034>.
- [10] Overstreet DH, Wegener G. The flinders sensitive line rat model of depression--25 years and still producing. *Pharmacol Rev* 2013;65:143–55. <https://doi.org/10.1124/pr.111.005397>.
- [11] Steyn SF. An Updated Bio-Behavioral Profile of the Flinders Sensitive Line Rat: Reviewing the Findings of the Past Decade. *Pharmacol Res Perspect* 2025;13:e70058. <https://doi.org/10.1002/prp2.70058>.
- [12] Thiele S, Spehl TS, Frings L, Braun F, Ferch M, Rezvani AH, et al. Long-term characterization of the Flinders Sensitive Line rodent model of human depression: Behavioral and PET evidence of a dysfunctional entorhinal cortex. *Behav Brain Res* 2016;300:11–24. <https://doi.org/10.1016/j.bbr.2015.11.026>.
- [13] Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* 2005;52:90–110. <https://doi.org/10.1159/000087097>.
- [14] Gardner W, Fuchs F, Durieux L, Bourgin P, Coenen VA, Döbrössy M, et al. Slow Wave Sleep Deficits in the Flinders Sensitive Line Rodent Model of Depression: Effects of Medial Forebrain Bundle Deep-Brain Stimulation. *Neuroscience* 2022;498:31–49. <https://doi.org/10.1016/j.neuroscience.2022.06.023>.
- [15] Thiele S, Sörensen A, Weis J, Braun F, Meyer PT, Coenen VA, et al. Deep Brain Stimulation of the Medial Forebrain Bundle in a Rodent Model of Depression: Exploring Dopaminergic Mechanisms

- with Raclopride and Micro-PET. *Stereotact Funct Neurosurg* 2020;98:8–20.  
<https://doi.org/10.1159/000504860>.
- [16] Tong Y, Pfeiffer L, Serchov T, Coenen VA, Döbrössy MD. Optogenetic stimulation of ventral tegmental area dopaminergic neurons in a female rodent model of depression: The effect of different stimulation patterns. *Journal of Neuroscience Research* 2022.  
<https://doi.org/10.1002/jnr.25014>.
- [17] Sabatini BL, Tian L. Imaging Neurotransmitter and Neuromodulator Dynamics In Vivo with Genetically Encoded Indicators. *Neuron* 2020;108:17–32.  
<https://doi.org/10.1016/j.neuron.2020.09.036>.
- [18] Wang Y, DeMarco EM, Witzel LS, Keighron JD. A selected review of recent advances in the study of neuronal circuits using fiber photometry. *Pharmacol Biochem Behav* 2021;201:173113.  
<https://doi.org/10.1016/j.pbb.2021.173113>.
- [19] Wu Z, Lin D, Li Y. Pushing the frontiers: tools for monitoring neurotransmitters and neuromodulators. *Nat Rev Neurosci* 2022;23:257–74. <https://doi.org/10.1038/s41583-022-00577-6>.
- [20] Simpson EH, Akam T, Patriarchi T, Blanco-Pozo M, Burgeno LM, Mohebi A, et al. Lights, fiber, action! A primer on in vivo fiber photometry. *Neuron* 2024;112:718–39.  
<https://doi.org/10.1016/j.neuron.2023.11.016>.
- [21] Ashouri Vajari D, Ramanathan C, Tong Y, Stieglitz T, Coenen VA, Döbrössy MD. Medial forebrain bundle DBS differentially modulates dopamine release in the nucleus accumbens in a rodent model of depression. *Exp Neurol* 2020;327:113224.  
<https://doi.org/10.1016/j.expneurol.2020.113224>.
- [22] Miguel Telega L, Ashouri Vajari D, Ramanathan C, Coenen VA, Döbrössy MD. Chronic in vivo sequelae of repetitive acute mfb-DBS on accumbal dopamine and midbrain neuronal activity. *J Neurochem* 2024. <https://doi.org/10.1111/jnc.16223>.
- [23] Miguel Telega L, Ashouri Vajari D, Stieglitz T, Coenen VA, Döbrössy MD. New Insights into In Vivo Dopamine Physiology and Neurostimulation: A Fiber Photometry Study Highlighting the Impact of Medial Forebrain Bundle Deep Brain Stimulation on the Nucleus Accumbens. *Brain Sci* 2022;12:1105. <https://doi.org/10.3390/brainsci12081105>.
- [24] Wang L, Wang S, Mo W, Li Y, Yang Q, Tian Y, et al. Ultrasound Stimulation Attenuates CRS-Induced Depressive Behavior by Modulating Dopamine Release in the Prefrontal Cortex. *IEEE Trans Neural Syst Rehabil Eng* 2024;32:1314–23. <https://doi.org/10.1109/TNSRE.2024.3378976>.
- [25] Olaitan G, Ganesana M, Strohmman A, Lynch WJ, Legon W, Venton BJ. Focused Ultrasound Modulates Dopamine in a Mesolimbic Reward Circuit. *Journal of Neurochemistry* 2025;169:e70001. <https://doi.org/10.1111/jnc.70001>.
- [26] Bruno CA, O'Brien C, Bryant S, Mejaes JI, Estrin DJ, Pizzano C, et al. pMAT: An open-source software suite for the analysis of fiber photometry data. *Pharmacol Biochem Behav* 2021;201:173093. <https://doi.org/10.1016/j.pbb.2020.173093>.
- [27] Sitdikova G, Zakharov A, Janackova S, Gerasimova E, Lebedeva J, Inacio AR, et al. Isoflurane suppresses early cortical activity. *Ann Clin Transl Neurol* 2014;1:15–26.  
<https://doi.org/10.1002/acn3.16>.