UNIVERSITÉ DE TOURS

ÉCOLE DOCTORALE : SSBCV

UMR 1253 – Imagerie & Cerveau

THÈSE en cotutelle avec l'université Libanaise présentée par :

Rasha NOUREDDINE

soutenue le : **15 Décembre 2021**

Pour obtenir le grade de : **Docteur de l'université de Tours et de l'université Libanaise**

Discipline/ Spécialité : Sciences de la Vie et de la Santé / Génie Biomédical

Impact of Focused Ultrasound Stimulation on Brain Activity in The Framework of Autism, In-Vivo Study in ''Autistic-Like'' Behavior Animal Model

THÈSE dirigée par :

M. Ayache Bouakaz	Professeur, Université de Tours
M. Jamal Charara	Professeur, Université Libanaise

RAPPORTEURS :

M. Jean-Louis Bossu	Professeur, Université de Strasbourg
M. Mohamad Diab	Professeur, Rafik Hariri University

JURY :

M. Jean-Louis Bossu	Professeur, Université de Strasbourg
M. Mohamad Diab	Professeur, Rafik Hariri University
Mme Emilie Franceschini	HDR, Aix-Marseille Université
M. Walid Hleihel	Professeur, Holy Spirit University Of Kaslik – President of The Jury
M. Alexandre Surget	Professeur Associé, Université de Tours
M. Ayache Bouakaz	Professeur, Université de Tours
M. Jamal Charara	Professeur, Université Libanaise
M. Mohamad Nassereddine	Professeur Associé, Université Libanaise

DEDICATION

I dedicate my dissertation work to my family. A special feeling of heart felt gratitude is to my loving parents and husband, who have provided me with continuous support, care, encouragement and love.

I also dedicate this dissertation to my directors, supervisors and all people who collaborated and supported me throughout the process.

I will always appreciate all they have done.

ACKNOWLEDGMENT

I would like to acknowledge the inspirational instruction and guidance of Dr. Ayache Bouakaz who have given me the support and feedback in my research project. I want to thank you for helping me out on numerous occasions. Thanks for your trust in me and for all your advice and support.

I want also to express my gratitude to Dr. Jamal Charara for his support and advice too. I have a special thought of Dr. Mohamad Nassereddine who helped me through to reach this position. I am so thankful for the trust you invested in me.

I also have to thank the members of my Ph.D. committee, Dr. Mohamad Diab and Dr. Jean-Louis Bossu, along with Dr. Walid Hleihel and Dr. Emilie Franceschini for being committe members and for their advice and suggestions in general.

I wish to thank Dr. Yassine Mofid who provided efforts, assistance and time to make this project succeed. I want also to thank the autism theme members. I think also of Dr. Frédérique Bonnet Brilhault, who supported our project and gave us some time, discussions and feedback.

I wish to thank all team members of the imaging and brain lab. Special gratitude for Mr. Tarik Iazourene for the time we spent together and for his cooperation in this project.

I wish to thank Dr. Alexandre Surget for his excellent advice, keeping up and our fruitful collaborations. I have a thought of his depression theme members, Dr. Arnaud Cressant, Mr. Bruno Brizard, Dr. Hoda Eliwa and Dr. Pascal Barone along with other lab colleagues who helped in making my project succeed and with whom we had good time. I want also to thank Miss Marie Audebrand for her cooperation.

I want to thank the Centre Universitaire de Pédopsychiatrie, CHRU de Tours and their members who allowed me to join them during inspection and treatment sessions for children with neurodevelopmental disorders.

I wish to express my heart felt gratitude to my family, who were always there when I needed. I wish to thank my mom for all she has done and her continuous support. I wish to express my gratitude to my dad who always provided me with backing and motivation. I

would also like to thank my sister who were always available for the scientific discussions, advice and emotional upholding. Thank you all for your ongoing support through all these years. I could not have completed this effort without your assistance, tolerance, and enthusiasm.

I wish to express my sincerest acknowledgments to my husband and partner, Liwaa Olabi. Thank you for all your love, care, support, advice, understanding and encouragements. The last months haven't been quite easy, but we'll make that up as soon as this manuscript is away. Thank you for all you did for me.

I have a very special thought for my little son, whose arrival changed completely my life. Thanks for all the love you gave and being patient while your mom was overloaded in writing her manuscript.

Finally, I wish to acknowledge the financial support of Walid Joumblatt Foundation for University Studies for my PhD grant.

ABSTRACT

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder with uncertain etiology, varying severity and deficits, increasing incidence and no effective therapies, making it a major public health problem. Additionally, its pathophysiology remains limited and unfortunately has not allowed the emergence of truly effective treatments. Therefore, it is very essential today to develop new therapeutic approaches. Recent advances in neurostimulation may permit the use of focal brain stimulation in ASD and test its therapeutic potential. Studies in the case of ASD have been recently conducted with several neurostimulation techniques involving vagus nerve stimulation, deep brain stimulation, electroconvulsive therapy, transcranial direct current stimulation and transcranial magnetic stimulation. However, these techniques face several limitations for ASD, either in terms of invasiveness, targeting and selectivity, or in terms of access to deep tissues; such limitations could be overcome in the case of transcranial focused ultrasound stimulation (tFUS). tFUS studies were recently performed on animals involving animal models of epilepsy and Alzheimer's disease, as well as on humans, resulting in positive outcomes and thus emphasizing the feasibility of this approach. However, no studies applying tFUS have been performed yet in autism or even tested in animal models of this disorder.

Thus, the objective of this thesis is to examine tFUS in an autistic-like valproate (VPA) animal model in C57BL/6J mice and assess its effects through behavioral phenotypic testing which encompasses different criteria associated with ASD. Implementation and optimization of the tFUS protocol in mice was needed as a first major step. For this purpose, the primary motor cortex was stimulated at various acoustic pressures using 500 kHz single element transducer. The success and reliability of the stimulations were evaluated through visual observation of the motor responses at the forelimbs validated with video recordings as well as electromyography (EMG). The results obtained showed that high success rates of stimulation (91% to 100%) could be achieved at 0 anesthesia level and acoustic pressures ranging from 330 to 550 kPa. We also found that the success rate of motor responses increased with increasing the acoustic pressure until the response saturated.

tFUS was also applied on the anterior cingulate cortex (ACC), a major brain structure implicated in various psychiatric diseases such as ASD and depression, in healthy mice for assessing the effect and safety of stimulation. The impact and safety of the ACC stimulations administered were evaluated on locomotor behavior and tissue integrity using open-field test and histological analysis respectively. No side effects on locomotor activity were reported and no lesions or injuries were detected in the brain sections.

Afterwards, determining the phenomenological validation of the VPA animal model used and the effects of tFUS was performed using set of behavioral tests involving three chamber test, free social interaction test, reversal learning test, marble burying test, and nesting test. Statistical analysis of the behavioral tests performed did not show significant differences between VPA and control mice. Variation was detected between these two groups only in the nesting test; suggesting that the VPA model used was not as successful as expected. However, lack of distinction between stimulated and non-stimulated mice was observed through almost all of the behavioral tests, confirming the safety of the protocol and methodology applied.

An effective and safe protocol for successful ultrasonic (US) neurostimulation in mice was developed and validated. The detailed and reliable methodology offered could be directed toward young students and researchers who are beginning in the brain stimulation field and facing practical challenges. Moreover, a simple and reliable non-invasive method for locating and targeting brain regions in mice was provided. It is a novel project that examines the application of tFUS in the case of ASD and would be the start of a long-term following research projects. Finally, this study presents a unique opportunity to initiate a new research activity with significant translational potential at the medical level: offering new prospects for autism therapy. Unfortunately, the implementation of the VPA mouse model turned to be unsuccessful which did not allow us to validate this innovative US neurostimulation approach for therapeutic purposes.

TABLE OF CONTENTS

DEDICATION ..... **I**
ACKNOWLEDGMENT ..... **II**
ABSTRACT ..... **IV**
TABLE OF CONTENTS ..... **VI**
LIST OF FIGURES ..... **VIII**
LIST OF TABLES ..... **X**
LIST OF ABBREVIATIONS ..... **XI**
CHAPTER 1 INTRODUCTION **3**
1.1 BACKGROUND: AUTISM SPECTRUM DISORDER 3
1.2 PROBLEM STATEMENT 5
1.3 OBJECTIVES 6
1.4 ORIGINALITY OF THE PROJECT 6
1.5 THESIS OUTLINE 7
CHAPTER 2 BACKGROUND AND DEFINITIONS **8**
2.1 ANIMAL MODELS OF ASD-LIKE 8
2.1.1 Key Features for A Successful Animal Model 8
2.1.2 Difficulties in Developing Animal Model for ASD 9
2.1.3 Rodents as Animal Models for ASD 9
2.1.4 Available Animal Models of ASD 9
2.1.5 Limitations of Current Animal Models of ASD 12
2.1.6 Behavioral Tests/Evaluation Measures for ASD Symptomatic Dimensions in Rodents 13
2.2 NEUROSTIMULATION TECHNIQUES 13
2.2.1 Vagus Nerve Stimulation 14
2.2.2 Deep Brain Stimulation 14
2.2.3 Electroconvulsive Therapy 15
2.2.4 Transcranial Direct Current Stimulation 16
2.2.5 Repetitive Transcranial Magnetic Stimulation 17
2.2.6 Transcranial Focused Ultrasound Stimulation 17
2.2.7 Advantages of tFUS over Other Techniques for ASD Neuromodulation 18
2.3 TRANSCRANIAL FOCUSED ULTRASOUND STIMULATION 19
2.3.1 Ultrasonic Neuromodulation in Animal Studies 19
2.3.2 Ultrasonic Neuromodulation in Human Studies 20
2.3.3 Mechanism Underlying Ultrasonic Neuromodulation 21
2.3.4 Safety Considerations 22
2.4 CONCLUSION 23
CHAPTER 3 NEUROSTIMULATION IN ASD: REVIEW OF PRECLINICAL LITERATURE **25**
CHAPTER 4 RESEARCH DESIGN AND METHODOLOGY **27**
4.1 INTRODUCTION 27
4.2 MOTOR CORTEX STIMULATION 27
4.2.1 Animal Preparation and Anesthesia 27
4.2.2 Brain Stimulation Setup and Ultrasonic Protocol 28
4.2.3 EMG Recordings/Electromyography 30
4.2.4 Identification of The Brain Regions of Interest for tFUS 31
a. Motor Cortex 31
b. Anterior Cingulate Cortex 31
4.2.5 tFUS Stimulation for Studying the Anesthesia Effect 34
4.2.6 tFUS Stimulation of the Primary Motor Cortex 34
4.3 ACC STIMULATION 34
4.4 VPA MODEL SETUP 37
4.4.1 VPA Model 37
4.4.2 Validation of VPA Animal Model and Effects of tFUS on VPA Mice 37

4.5 BEHAVIORAL MEASURES 39
4.6 CONCLUSION 46
CHAPTER 5 RESULTS AND DISCUSSION 47
5.1 ULTRASONIC NEUROSTIMULATION IMPLEMENTATION AND SAFETY 47
5.1.1 Success Rate of Motor Cortex Stimulation 47
5.1.2 Effect of Anesthesia on Motor Responses 53
5.1.3 Safety and Effect of ACC Stimulation 55
5.2 VPA MODEL AND EFFECT OF STIMULATION 57
5.2.1 3-Chamber Interaction Test 57
5.2.2 Free Social Interaction Test 62
5.2.3 Reversal Learning in Water Maze 65
5.2.4 Marble Burying Test 67
5.2.5 Nesting Test 68
CHAPTER 6 CONCLUSION AND FUTURE WORK 71
REFERENCES 74
APPENDIX A 92

LIST OF FIGURES

Figure 2.1: Key features for a successful animal model of autism spectrum disorder. 8
Figure 2.2: Available animal models of autism. 10
Figure 2.3: Demonstration of vagus nerve stimulation [64]. 14
Figure 2.4: Demonstration of deep brain stimulation [66]. 15
Figure 2.5: Demonstration of electroconvulsive therapy [67]. 15
Figure 2.6: (a) Demonstration of transcranial direct current stimulation [70] (b) Anodal vs cathodal tDCS [71]. 16
Figure 2.7: Demonstration of repetitive transcranial magnetic stimulation [72]. 17
Figure 2.8: Demonstration of transcranial focused ultrasound stimulation [73]. 18
Figure 4.1: Mouse suited on a plate with its head settled in an anesthetic face mask for shaving its head to the left. Mouse rested on a heating pad with its head fixed with auxiliary ear bars and fitted in a built-in anesthetic face mask to the right. 28
Figure 4.2: Brain stimulation setup with EMG leads connected to the right forelimb for EMG recordings during tFUS stimulation of the primary motor cortex. 29
Figure 4.3: Schematic of ultrasound sequences and associated parameters [102]. 30
Figure 4.4: Anterior cingulate cortex in sagittal and coronal view of mouse brain [106]. 32
Figure 4.5: Schematic diagram for identifying hump and locating Bregma in a mouse head.33
Figure 4.6: Chronogram for evaluating the safety and effect of ACC stimulation by tFUS. ... 35
Figure 4.7: Chronogram for validating the VPA animal model and effects of tFUS on VPA mice. 38
Figure 4.8: Open-field test (a) Mouse placed in a surrounded arena (b) Ethovision software system for detecting the distance moved by the mouse. 39
Figure 4.9: Three-chambered apparatus for quantitating social approach behaviors in mice (a) Front view (b) Top view. 41
Figure 4.10: Illustration of free social interaction test. 42
Figure 4.11: Mice profiler tracker demonstration. 42
Figure 4.12: Example of ultrasonic vocalization calls emitted by a mouse. 43
Figure 4.13: Reversal learning test (a) Platform location during learning and retrieval phases (b) Platform location during reversal learning phase. 44
Figure 4.14: Marble burying test (a) Positioning of the marbles in the cage before the test (b) Example of the positioning of marbles after the test. 45
Figure 4.15: Nest building test (a) Nestlet at 5 hours from the beginning of the test with nest scoring of 3 (b) Nestlet at 24 hours from the beginning of the test with nest scoring of 5. .. 46
Figure 5.1: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials detected visually at different acoustic pressures for each mouse. 47
Figure 5.2: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials detected visually at different acoustic pressures for 10 tested mice. Results represent mean ± SEM. 48
Figure 5.3: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials obtained using EMG at different acoustic pressures for 10 tested mice. Results represent mean ± SEM. 49
Figure 5.4: Amplitude of EMG signals detected versus acoustic pressure. Results represent mean ± SEM. 50

Figure 5.5: Electromyogram of 6 randomly selected mice showing the diversity in shape, amplitude and duration of EMG signals obtained at 500 kPa. 51
Figure 5.6: Electromyograms obtained at various acoustic pressures from randomly selected mice showing the diversity in shape, amplitude and duration of elicited EMG signals. 52
Figure 5.7: tFUS stimulation targeting primary motor cortex induced motor response depending on anesthesia level. Motor responses following tFUS stimulation of the left primary motor cortex were estimated by electromyography through (a) Visual detection of a signal and (b) Evaluation of the averaged amplitude according to acoustic pressure and anesthesia level. Results in (b) represent mean ± SEM (n = 3 mice). 54
Figure 5.8: Distance moved by mice in the open-field test at three different intervals from tFUS. Results represent mean ± SEM. 55
Figure 5.9: tFUS targeting the anterior cingulate cortex showed no significant effect on tissue integrity over a 2-week post-tFUS period. (a) Representative images of Nissl staining of the cortical tissue (area corresponding to anterior cingulate cortex) at 90-minutes post-tFUS interval at x25 and x100 magnification. (b) Representative images of Nissl staining of the cortical tissue (area corresponding to anterior cingulate cortex) at 14-days post-tFUS interval at x25 and x100 magnification. 56
Figure 5.10: Distance covered by mice in 10-minutes sociability test period with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM. ... 58
Figure 5.11: Duration of time spent by mice in the social chamber with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM. 59
Figure 5.12: Duration of time spent by mice in the social contact zone with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM. ... 60
Figure 5.13: Frequency of entries in the social contact zone for (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM. 61
Figure 5.14: Different events evaluated during free social interaction test. A refers to the tested mouse and B indicates the visitor mouse. Results represent mean ± SEM. 63
Figure 5.15: Charts representing ultrasonic vocalizations emitted by VPA and control mice during the first 2 minutes of interaction with (a) Number of USVs emitted and (b) Their duration. Results represent mean ± SEM. 64
Figure 5.16: Percentage of ultrasonic vocalizations subtypes emitted by control mice to the left and VPA mice to the right. 64
Figure 5.17: The number of trails detected during (a) Learning phase for 4 mice groups, (b) Learning phase for 8 mice groups, (c) Retrieval phase for 4 mice groups, (d) Retrieval phase for 8 mice groups, (e) Reversal learning phase for 4 mice groups, (f) Reversal learning phase for 8 mice groups. Results represent mean ± SEM. 66
Figure 5.18: The number of marbles buried during the 30-minutes test duration versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM. 67
Figure 5.19: The rating scale calculated at 5 hours versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM. 68
Figure 5.20: The rating scale calculated at 24 hours versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM. 69

LIST OF TABLES

Table 2.1: Neurostimulation techniques used and status in ASD. 18
Table 2.2: Comparison between some neurostimulation methods and tFUS [8]. 19
Table 4.1: Mice classification for evaluating the safety and effect of ACC stimulation by tFUS. 36
Table 4.2: Mice classification for validating the VPA animal model and effects of tFUS on VPA mice. 38

LIST OF ABBREVIATIONS

ABA: Applied Behavior Analysis

ACC: Anterior Cingulate Cortex

ASD: Autism Spectrum Disorder

CTN: Central Thalamic Nucleus

DBS: Deep Brain Stimulation

DLPFC: Dorsolateral Prefrontal Cortex

DSM-5: Diagnostic and Statistical Manual of Mental Disorders – 5th Edition

ECS: Electroconvulsive Shocks

ECS: Electroconvulsive Seizure

ECT: Electroconvulsive Therapy

EDT: Exchange and Development Therapy

EEG: Electroencephalogram

E/I: Excitability and Inhibitory

EMG: Electromyography

EPM: Elevated Plus Maze

FDA: Food and Drug Administration

fMRI: Functional Magnetic Resonance Imaging

$GABA_AR$: γ-AminoButyric Acid Type A Receptors

GPi: Globus Pallidus Internus

ip: Intraperitoneal

Isppa: Spatial-Peak Pulse Average Intensity

Ispta:	Spatial-Peak Temporal-Average Intensity
LF rTMS:	Low frequency Repetitive Transcranial Magnetic Stimulation
MeCP2:	Methyl-CpG-Binding Protein
MI:	Mechanical Index
mPFC:	Medial Prefrontal Cortex
M1:	Primary Motor Cortex
PB:	Phosphate-Buffer
PBS:	Phosphate-Buffered Saline
PC:	Purkinje Cells
PF:	Platform
PFA:	Paraformaldehyde
PL:	Pulse Length
Poly(I:C):	Polyinosine:Cytosine
PRF:	Pulse Repetition Frequency
ROI:	Region of Interest
rTMS:	Repetitive Transcranial Magnetic Stimulation
STN:	Subthalamic Nucleus
tDCS:	Transcranial Direct Current Stimulation
tFUS:	Transcranial Focused Ultrasound Stimulation
TI:	Thermal Index
TSC:	Tuberous Sclerosis
TT:	Total Time
US:	Ultrasonic
VNS:	Vagus Nerve Stimulation

VPA: Valproate

VPA: Valproic Acid

8-OH-DPAT: 8-Hydroxy-2-(Di-N-PropylAmino) Tetralin

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND: AUTISM SPECTRUM DISORDER

Autism spectrum disorder (ASD) is a common and heterogeneous lifelong developmental disorder characterized by (i) social interaction and communication deficits, and (ii) restricted, repetitive patterns of behavior, interests, or activities as per the diagnostic and statistical manual of mental disorders (DSM-5) [1]. Autism spectrum disorder is related to a large spectrum of clinical expression from high functioning people to ASD children or adults with multiple comorbidities involving self injurious behaviors, epilepsy and obsessive compulsive behaviors. The precise etiology of autism remains unknown but it is now well admitted that it involves interaction of environmental and genetic factors as well as epigenetic factors [2] [3]. Mechanisms underlying ASD are hypothesized to involve excitation/inhibition imbalance and intense world theory. The prevalence of autism, which is approximately 1.6% in the general population, is increasing rapidly with a gender ratio of about 4 males for every female [3]. Despite the growing expansion of autism, its pathophysiology as well as its drug treatment remain controversial. Few drugs (bumetanide, memantine, ketamine, oxytocin, etc.) were proposed and useful in lessening some symptoms of ASD, such as irritability, aggression, and aberrant social behavior [4]. However, only two of them, particularly risperidone and aripiprazole, were approved by the Food and Drug Administration (FDA) only for the treatment of irritability in ASD patients [4]. Thus, for the time being, the pharmacological treatments that can treat or improve core symptoms of ASD have not been discovered [5]. Additionally, despite the existence of helpful rehabilitation and educative programs for ASD, such as the exchange and development therapy (EDT) and applied behavior analysis (ABA), some patients and their families are still facing challenges [6] [7]. Therefore, there is a big need for developing new complimentary interventional therapy in ASD.

Transcranial stimulation is based on a neurostimulation of a specific region in the brain, which directly or indirectly affects the electrical activity of the brain that successively prompts localized protracted neurochemical changes and is aiming at inducing beneficial therapeutic effects [5]. Currently, neurostimulation techniques are commonly studied in research and applied in clinical practices for the treatment of several neuropathological diseases. Neurostimulation techniques are classified as invasive techniques involving vagus nerve stimulation (VNS) and deep brain stimulation (DBS), and non-invasive methods including electroconvulsive therapy (ECT), transcranial direct current stimulation (tDCS), and single-pulse or repetitive transcranial magnetic stimulation (rTMS) [8]. These neuromodulation methods have been examined clinically in the case of ASD and resulted in moderately positive effects such as improving communication and social interactions, mood, language function, attention and concentration, and reducing autistic behaviors involving aggression, repetitive and stereotypic behaviors, self injurious behavior, anxiety, irritability, hyperactivity etc. [9] [10] [11] [12] [13] [14] [15] [16]. Although these neurostimulation techniques have been studied clinically in a trial to treat ASD and could cause positive effects, they have specific limitations for autism spectrum disorder. In fact, all these neurostimulation techniques are not yet approved by FDA for autism treatment and the majority of them offer low spatial resolution or necessitate invasive procedures [17] [18] [19]. Although DBS provides high spatial resolution, it is considered as an invasive technique that requires complex neurosurgery [18]. Additionally, tDCS provides poor spatial resolution with undetectable depth [18]. rTMS also offers low spatial resolution while targeting the region of interest (ROI) which is a superficial zone [18]. Thus, it is not adapted to stimulate deep regions in the brain such as anterior cingulate cortex and hippocampus. Furthermore, while it is significant to couple functional magnetic resonance imaging (fMRI) with neurostimulation as it helps in monitoring, visualizing and evaluating the effect of stimulation during and after the neurostimulation process, however, it is difficult to be implemented with tDCS [8] and rTMS [20] [21], and makes DBS not safe due to the dangerous risk of electrode heating during imaging that can cause permanent neurological injury [22] [23] [24] [25].

Therefore, there is a necessity for bringing a complimentary neurostimulation technique that can overcome the restrictions of the available techniques and target deep

brain regions implicated in ASD that can cause new behavioral impacts and enhance the outcome. As per recent findings, transcranial focused ultrasound stimulation (tFUS) is proposed to be the moderator that can overcome all the deficiencies of these neuromodulation techniques thanks to its noninvasiveness and ability to penetrate into deep brain tissues with high resolution [8] [26].

tFUS is a non-invasive stimulation technique that focuses ultrasound waves using single element or multi-element transducer into a small focal zone in the brain. tFUS has been studied in brain pathways as reported elsewhere [8]. The results showed that ultrasound waves activated the motor cortex and produced muscular contractions when applied to rodents [27] [28]. It also produced eyeball movement while stimulating the abducens nerve in rat, inhibited the primary visual cortex in rabbit, suppressed electroencephalogram (EEG) visual evoked potentials in rats, and modulated the latencies in antisaccade task when it targeted the left frontal eye field in monkey [29] [30] [31]. Moreover, tFUS showed neuroprotective effects against cerebral damages and caused memory retention in Alzheimer disease in rats, as well as reduction in the occurrence of epileptic EEG bursts in rat model of epilepsy [32] [33]. Lately, it has been tested and safely performed in sheep targeting the cortical and thalamic regions, and illustrating that sonication parameters influence stimulation efficiency [34]. Furthermore, tFUS has been examined in human by stimulating the primary somatosensory cortex and posterior frontal cortex; resulting in enhancing the performance on sensory discrimination tasks and improving mood/global affect respectively [35] [36]. Additionally, it modulated the excitability of the human motor cortex [37]. However, and to the best of our knowledge, no studies applying tFUS have been performed yet in the context of autism or even tested in animal models of this disorder.

1.2 PROBLEM STATEMENT

Although of the prevalence of ASD and its rapid expansion, its pathophysiology and drug discovery remain poor. Few drugs are available and beneficial in decreasing some symptoms of ASD, however, only two of them (risperidone and aripiprazole) were FDA approved for the treatment of irritability in autistic patients. Therefore, there is no obtainable pharmacological treatments that can treat or improve core symptoms of ASD yet. Moreover,

despite the presence of useful rehabilitation and educative programs for ASD, such as EDT and ABA, some patients and their families are still encountering challenges [6] [7]. Thus, it is essential to develop new therapeutic approaches and bring a complimentary non-invasive physical therapy for treating ASD that can be based on transcranial neuromodulation.

Furthermore, in spite of the existence of several well-known common neurostimulation techniques that have been widely used in neuroscience with the aim to treat ASD; these techniques face several limitations for autism spectrum disorder, either in terms of targeting and selectivity, or in terms of access to deep brain zones. Available neuromodulation methods are not yet approved by FDA for autism treatment and the majority of them offer low spatial resolution or necessitate invasive procedure. Over and above, some of these neuromodulation methods are not adapted to stimulate deep regions in the brain. Besides, it is difficult to couple the commonly used neuromodulation methods such as tDCS, rTMS and DBS with fMRI, which is important to be coupled during neurostimulation for monitoring, visualizing and evaluating the effect of stimulation during and after the neurostimulation process. All these previously mentioned limitations of the current existing neurostimulation methods, could be overcome in the case of tFUS. tFUS studies have been examined in humans and rodents involving animal models for epilepsy, alzheimer and depression; resulting in positive outcomes and thus emphasizing the feasibility of this approach [32] [33] [38]. However, no studies applying tFUS have been implemented yet in the situation of ASD or even evaluated in animal models of this disorder.

1.3 OBJECTIVES

The aim of our project is thus to study and evaluate the effects of tFUS in an “autistic-like” behavior animal model in C57BL/6J mice, particularly the valproate animal model (VPA), through behavioral phenotypic monitoring encompassing different dimensions associated with ASD.

1.4 ORIGINALITY OF THE PROJECT

The thesis is characterized by its originality as it is the first study that examines the application of tFUS in the case of autism spectrum disorder. It is a novel project through which

an in-vivo tFUS system is optimized in an “autistic-like” behavior animal model and a major deep brain region implicated in ASD, particularly the anterior cingulate cortex, is targeted.

1.5 THESIS OUTLINE

This thesis is allocated into several parts. In the second chapter, animal models of ASD-like and neurostimulation techniques in comparison with tFUS, are defined and described. In the third chapter, our review paper dealing with a survey of neurostimulation studies in ASD in preclinical scenario is presented. The fourth chapter includes the proposed design and methodology of tFUS implementation and optimization through motor cortex stimulation, ACC stimulation, setting up VPA model, and behavioral measures for evaluating the safety and effect of stimulation. At the end, the results are shown and discussed in the fifth chapter and a conclusion of the work is presented in the sixth chapter in addition to a general review of the future possible work to be done to further advance the proposed project.

CHAPTER 2

BACKGROUND AND DEFINITIONS

2.1 ANIMAL MODELS OF ASD-LIKE

2.1.1 Key Features for A Successful Animal Model

ASD, a complex neurodevelopmental disorder with unrevealed reasons and varied symptoms, is firmly restricted to human. An animal model pertinent to psychiatric condition in human such as ASD should suit some conditions involving construct, face and predictive validity as shown in Fig. 2.1 [3] [39] [40] [41]. Construct validity refers to the similarity of causation between the animal phenotype and the human disease. Face validity indicates that the phenomenological features in the animal and in patients are identical. Predictive validity implies that the treatments that diminish and inhibit the symptoms of the disease in human can also decrease the symptoms existing in the animal model. Moreover, it is essential to study the molecular parameters along with the behavioral parameters for having an ideal model. Furthermore, the model should be reliable and reproducible.

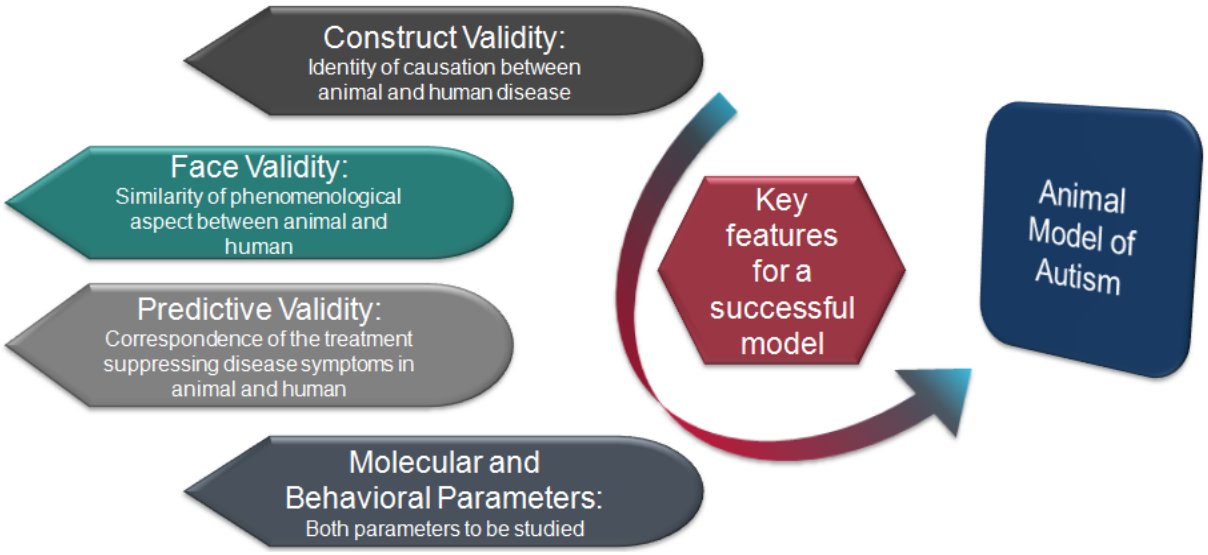


Figure 2.1: Key features for a successful animal model of autism spectrum disorder.

2.1.2 Difficulties in Developing Animal Model for ASD

Several difficulties exist in developing “autistic-like” animal models consisting of: (i) the autism spectrum disorder is determined by a set of core behavioral abnormalities rather than by objective biomarkers; (ii) two of the core symptoms of this disorder which are the social interaction and communication deficits, can be approached in rodent studies; (iii) autism spectrum disorder is defined by a series of behaviorally different disorders, with various causes and varying pathogenesis; (iv) the usage of the wide definition which is ASD enhances this issue more, (v) the genetics of autism is complicated, compromising many candidate genes, copy number variations, and monogenic, syndromic disorders which exhibit autistic features; (vi) there is no pathognomonic feature of autism observed in animals which can be used for easy differentiation, such as distinguishing an autistic mouse from a schizophrenic mouse [42]. Furthermore, the theory of mind which is the ability of human to empathize and intuit the feelings and intentions of others, might be impossible in rodents [41].

2.1.3 Rodents as Animal Models for ASD

Rodents have been chosen as model species for ASD because their biology and behavior are well known and have been studied extensively [43]. Rodents are social species. They are considered as popular subjects for social tests because of their high levels of social interaction and exploration, sexual behaviors, and parenting and territorial aggressive behaviors [44]. Noting that social interaction is considered as an important quantitative measure for animal model of autism spectrum disorder [41]. In addition, these species are easier to use than monkeys, from a practical point of view (development time, costs) [43].

2.1.4 Available Animal Models of ASD

The development of validated animal models imitating ASD origins and symptoms would be of high value for studying autism, deciphering its neurobiological underpinning and probing new therapeutic strategies. However, ASD is a complex neurodevelopmental disorder with unrevealed reasons and varied symptoms, and firmly restricted to human, making it difficult to develop models capable of recapitulating this disorder in its entirety. However, researchers have developed strategies focusing on specific environmental or genetic risk

factors, or on specific endophenotypes allowing to assess some hypothesis about etiology and pathophysiology of ASD. Attempts to develop such models have been made in species like mice and rats principally, but also in guinea pigs, Microtus, zebrafish, and fly [45] [46] [47]. It exists therefore two main different types of ASD animal models: the genetic models and the environmental risk factors models as shown in Fig. 2.2. Genetic models involve chromosome engineering or loss of function mutations [39]. On the other hand, models based on environmental risk factors have involved prenatal and neonatal environmental challenges including maternal valproic acid (VPA) exposure, maternal bacterial and viral infections, neonatal isolation, and stress experiences during gestation [3].

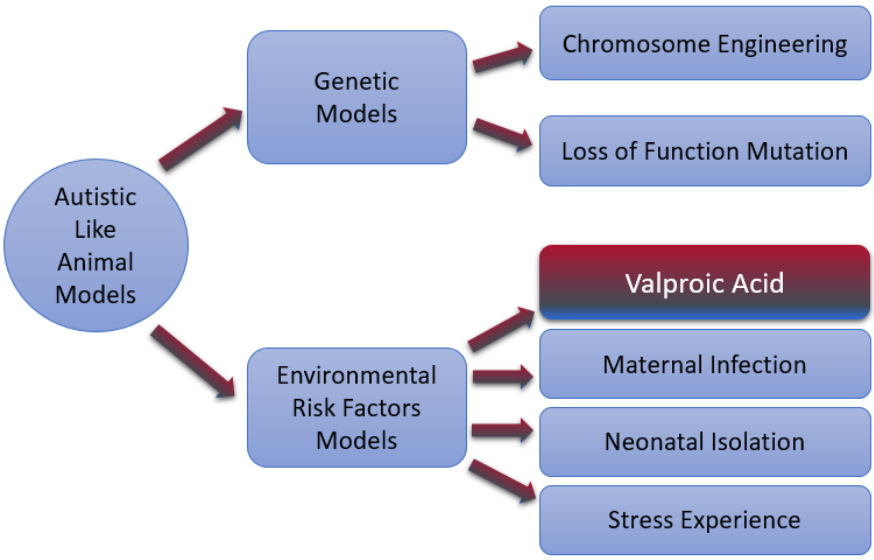


Figure 2.2: Available animal models of autism.

- **Genetic Models**

There are many genetic models for autism involving the monogenic syndromes which displays core autistic features as well as other serious problems such as tumors and physical malformations that do not exist in ASD [42]. Another genetic models include Mendelian mutations and de novo chromosomal deletions or duplications which generate autistic symptoms while causing a broader phenotype than that existing in ASD [42]. Fragile X syndrome is one of the genetic models that is caused by mutations in the FMR1 gene on the X chromosome. FMR1 mice show core features of autism including impaired social interaction and repetitive behavior as well as other ASD symptoms such as anxiety and hyperactivity.

Another genetic model for autism is the Rett syndrome which is caused by mutations in the methyl-CpG-binding protein (MeCP2). This disorder presents lot of symptoms, some of which are similar to those found in autism spectrum disorder involving aberrant social interactions and enhanced anxiety [42]. Tuberous sclerosis (TSC) is another genetic disease which displays autistic symptoms. It is caused by mutations in one of two TSC genes that create benign tumors in several tissues comprising the brain. Other ASD genetic model is the BTBR mouse strain which displays low levels of sociability, abnormal social learning, repetitive grooming, and resistance to change in routine [42].

- **Environmental Risk Factors Models**

  i. Valproic Acid Animal Model

One of the most widely used animal model in the field of ASD is the maternal valproic acid experience [40]. Women consuming the valproic acid as a medication for mental illness, epilepsy, mania, and migraine prophylaxis are at high risk of delivering offspring with ASD [42] [3]. Offspring of VPA rat model display autistic symptoms including impaired social interaction, increased stereotypic/repetitive behavior, altered sensitivity to sensory stimuli, enhanced anxiety and eye blinking, impaired reversal learning and increased fear memory [3]. Male VPA rats display these autistic-like features, while female VPA rats show only increased stereotypic/repetitive-like activity; thus making these VPA behavioral alterations gender-specific [3]. Additionally, VPA rats show disturbed immune function similar to autistic patients. VPA animal model offers construct, face and predictive validity as per a review study [40]. Several brain regions have been affected by VPA model in rodents including prefrontal cortex, medial prefrontal cortex, motor cortex, amygdala, cerebral cortex, cerebellum, somatosensory cortex, anterior cingulate cortex, temporal lobe, striatum, hippocampus, and even the whole brain [48] [49] [50] [51] [52] [53] [54] [55] [56] [57]. VPA model offers a platform for performing preclinical studies to better study ASD, understand it and develop appropriate treatments. VPA model provides valuable tools for investigating the brain region specific neurodevelopmental and cellular defects, and thus assisting in analyzing the mechanisms underlying this pathology. Moreover, it provides a platform for testing potential drug treatments such as bumetanide and oxytocin, as well as, other new potential therapies including neurostimulation techniques [58] [59].

ii. Maternal Infection

Another model for autism spectrum disorder is the maternal infection. Maternal infection is considered as a risk factor for ASD in offspring. It has been shown that more than 10,000 autism cases were related to maternal viral infection in the first trimester [60]. Animal models of the maternal infection risk factor involve maternal respiratory infection with influenza virus and maternal immune activation with either polyinosine:cytosine [poly(I:C)] which is a synthetic double-stranded RNA that evokes an antiviral-like immune reaction, or lipopolysaccharide which evokes an antibacterial-like immune reaction [42]. Maternal immune activation with poly(I:C) offspring display symptoms similar to core autistic features including social interaction and communication deficits and increased repetitive/stereotypic motor behaviors, as well as other autistic symptoms involving anxiety and eye blinking. Maternal immune activation with lipopolysaccharide generates offspring with some of the same symptoms, and little abnormal behaviors which can be reversed by antipsychotic drug treatment. Maternal infection models match face and construct validity and may display predictive validity too.

iii. Stress Experience

Maternal stress experience is also related to enhancing the risk of ASD incidence in offspring. Undergoing stress during gestation generates rat male-specific decreased social interaction, impaired spatial learning and reversal learning, while causing increased social interaction and spatial learning in rat females; highlighting male: female ratio in ASD [3].

2.1.5 Limitations of Current Animal Models of ASD

Genetic and environmental risk factor models have been used increasingly to evaluate and understand the features and pathophysiology of ASD. But, it is essential to realize the strengths and limitations of these models in targeting autism spectrum disorder which is genotypic and phenotypically heterogeneous complex disorder [44]. Many of the previously discussed genetic and environmental risk factor models display some or all the core behavioral features of ASD. However, the analysis of neuropathology and electrophysiology is at a more advanced stage in the environmental models [42]. As previously mentioned, a network of candidate genes is implicated in ASD. Thus, creating a genetically modified animal

model by using only one chemical or one mutation in a particular gene do not attain the construct validity of the model. Furthermore, the face validity of the animal model of ASD which is only based on behavioral assays would be incomplete. The construct, face and predictive validity must be satisfied by a successful animal model of autism to reproduce a combination of the behavioral, neuropathological, immunological, biochemical and genetic basis for this disorder [39]. The use of greater number of causative agents in the formation of the animal model can create more realistic animal model of ASD [39]. It is thought that lot of additional work should be done concerning the immune status of the models as well as the state of the gastrointestinal tract that can be disrupted in autism [42].

2.1.6 Behavioral Tests/Evaluation Measures for ASD Symptomatic Dimensions in Rodents

The diagnostic measures for autism spectrum disorder are atypical reverse social interactions, impaired communication and stereotyped repetitive behaviors with narrow restricted interests [41]. These core features of autism in rodents can be evaluated through various behavioral tests. Social interaction deficits can be measured through using three-chamber social interaction apparatus or analysis of videotapes, while communication deficits can be assessed using ultrasonic vocalizations or scent marking. Repetitive and stereotypic motor behaviors can be estimated by self-grooming or marble burying, and insistence on sameness and restricted interests or activities can be determined through detecting neophobia or perseveration in the T maze or water maze. Mice and rats can also be examined for another autistic features including enhanced anxiety, eye blink conditioning, and deficit in sensorimotor gating [42].

2.2 NEUROSTIMULATION TECHNIQUES

Nowadays, neurostimulation techniques are widely studied in research and applied in clinical practices for the treatment of several neuropathological diseases such as depression, epilepsy, schizophrenia, and Parkinson disease [5]. Neurostimulation techniques are categorized as invasive techniques involving VNS and DBS, and non-invasive methods including ECT, tDCS, rTMS, and tFUS [8].

2.2.1 Vagus Nerve Stimulation

Vagus nerve stimulation (VNS) is an invasive neurostimulation technique that necessitates the implantation of a neurostimulator in the chest and electrodes around the vagus nerve as represented in Fig. 2.3. This technique is based on stimulating the left vagus nerve through electrical pulses at regular intervals [5]. Vagus nerve is linked to many significant regions in the brain involving amygdala, hippocampus and others that are important in emotional and cognitive processes. VNS tends to cause suppression of particular focal cortical actions [61] and was approved by FDA for treatment-resistant epilepsy and depression [5]. However, it has many adverse effects including surgical complications, dyspnea, paresthesia, headache, pain, pharyngitis, hoarseness, cough, throat discomfort and poor spatial resolution [62] [63].

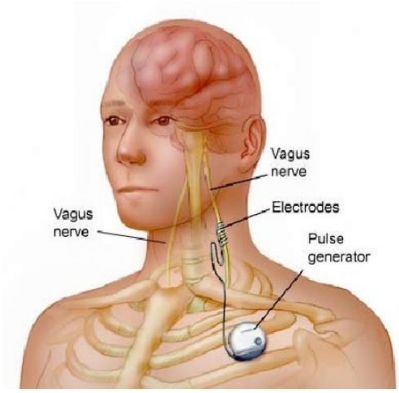


Figure 2.3: Demonstration of vagus nerve stimulation [64].

2.2.2 Deep Brain Stimulation

Another invasive brain stimulation technique is the deep brain stimulation (DBS). DBS requires the implantation of stimulated generator and electrodes in specific areas of the brain as shown in Fig. 2.4. It consists in sending the electrical stimulus through the implanted electrodes to prompt an electrical field that alters the activity in the neuronal circuits. DBS was approved by FDA for essential tremor particularly thalamic DBS in 1997, Parkinson's disease through stimulation of globus pallidus internus (GPi) and subthalamic nucleus (STN) by 2003, dystonia by stimulation of GPi and STN in 2003 and obsessive-compulsive disorder through stimulation of the anterior limb of the internal capsule in 2009 [5] [65]. Its untoward effects include motor control problems, mood changes or feelings of depression, and surgical

complications [5]. Since DBS requires surgical procedure, it can also increase the risks of infections and neural immune system reactions to external materials. Additionally, it requires cumbersome maintenance and frequent replacement of the battery [17] [18].

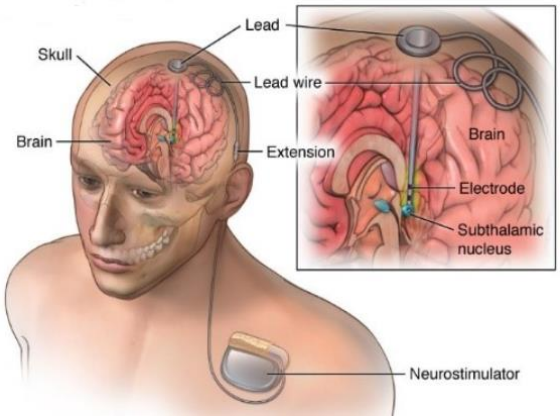


Figure 2.4: Demonstration of deep brain stimulation [66].

2.2.3 Electroconvulsive Therapy

Electroconvulsive therapy (ECT), displayed in Fig. 2.5, is the oldest brain stimulation technique developed in 1930s [61]. ECT is a non-invasive technique that entails general anesthesia. It provides an electric stimulus to the surface of the head in which widespread seizure is induced with neurotransmitters liberation. ECT is FDA approved for depression, bipolar manic (and mixed) states, schizophrenia, schizoaffective disorder, schizophreniform disorder and catatonia [5]. On the contrary, it has adverse effects involving confusion, delirium, memory impairment; similar to the general physical risks of general anesthesia [5].



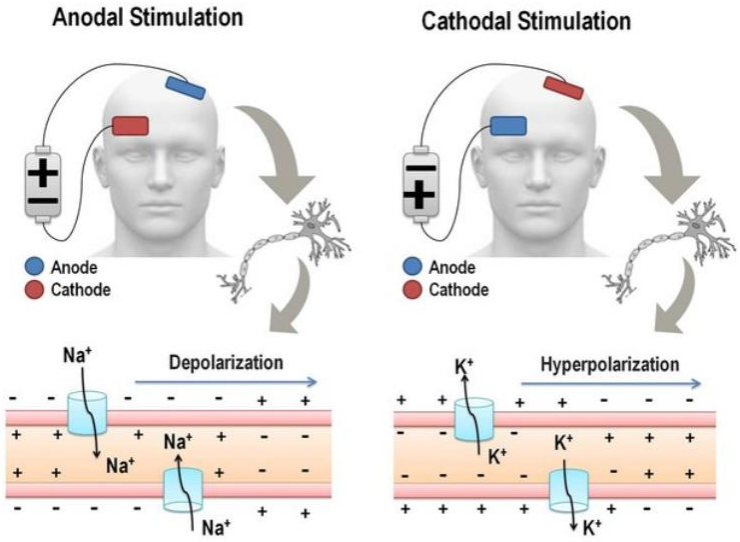
Figure 2.5: Demonstration of electroconvulsive therapy [67].

2.2.4 Transcranial Direct Current Stimulation

Transcranial direct current stimulation (tDCS) is another non-invasive technique that involves the delivery of low voltage stimulation through electrodes to the scalp to alter the neuronal activity in the brain as shown in Fig. 2.6 (a). Depolarization can be produced in tDCS when the electrical stimulus traverses from the anode to cathode causing cortical excitability, and its reverse creates hyperpolarization leading to suppression of cortical excitability that ends in therapeutic effect as displayed in Fig. 2.6 (b). It was found to be effective in obsessive-compulsive disorder, posttraumatic stress disorder, and generalized anxiety disorder [61]. tDCS has minor adverse effects involving itching, mild nausea, numbness at the electrode site, transient redness in the stimulation region, and persistent effects including skin lesions similar to burns [68] [69].



(a)



(b)

Figure 2.6: (a) Demonstration of transcranial direct current stimulation [70] (b) Anodal vs cathodal tDCS [71].

2.2.5 Repetitive Transcranial Magnetic Stimulation

The repetitive transcranial magnetic stimulation (rTMS), shown in Fig. 2.7, is based on the application of magnetic fields to the brain. These fields, generated via a stimulating coil, pass through skull and induce an electrical current in the brain to modulate its activity. rTMS is FDA approved for resistant depression [5]. However, it causes headache, neck pain, discomfort at the site of stimulation, transient increases in auditory thresholds, changes in cognition or mood, and presyncopal reaction [5].

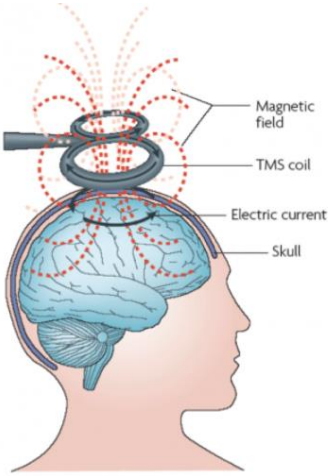


Figure 2.7: Demonstration of repetitive transcranial magnetic stimulation [72].

2.2.6 Transcranial Focused Ultrasound Stimulation

Transcranial focused ultrasound stimulation (tFUS) is an emerging neurostimulation technology that is a non-invasive stimulation technique and directs low intensity ultrasound waves through specialized transducer into a small focus on the head, as displayed in Fig. 2.8. These focused waves penetrate through skull and modulate the brain function by causing neuronal excitation and/or inhibition [18]. It modulates the neuronal functions in the brain through non-thermal mechanical pressure effect on cellular membranes and ion channels [8] [35]. Recent studies have demonstrated the ability of tFUS to activate voltage-gated sodium and calcium channels, and thus triggering action potentials and synaptic transmission [8]. tFUS has been recently examined in neurological disorders involving Alzheimer and epilepsy diseases.

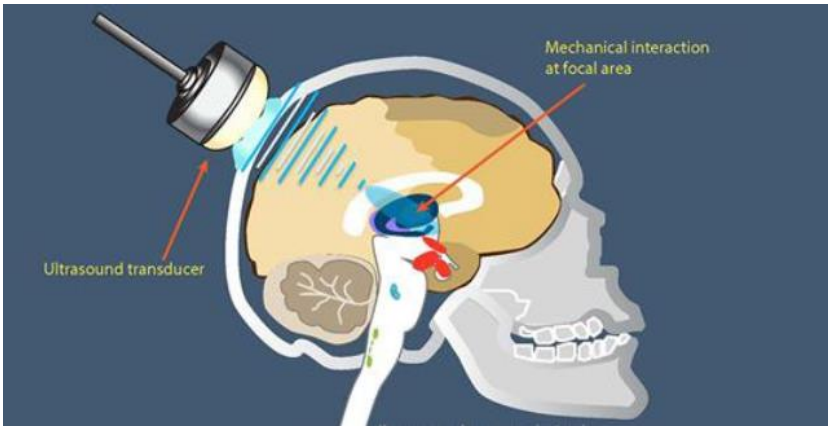


Figure 2.8: Demonstration of transcranial focused ultrasound stimulation [73].

All of the above-mentioned techniques are still studied in clinical research in the case of ASD, except for tFUS. Table 2.1 summarizes the neurostimulation techniques and their status in ASD.

Table 2.1: Neurostimulation techniques used and status in ASD.

Neurostimulation Technique	Abbreviation	Year of Development	Physical Basic	Invasive/ Non-invasive	Status in ASD
Vagus Nerve Stimulation	VNS	1980s	Electrical	Invasive	Clinical Research
Deep Brain Stimulation	DBS	1990s	Electrical	Invasive	Preclinical & Clinical Research
Electroconvulsive Therapy	ECT	1930s	Electrical	Non-invasive	Preclinical & Clinical Research
Transcranial Direct Current Stimulation	tDCS	1960s	Electrical	Non-invasive	Preclinical & Clinical Research
Repetitive Transcranial Magnetic stimulation	rTMS	1990s	Magnetic	Non-invasive	Preclinical & Clinical Research
Transcranial Focused Ultrasound Stimulation	tFUS	1950s	Mechanical	Non-invasive	No studies exist

2.2.7 Advantages of tFUS over Other Techniques for ASD Neuromodulation

Despite the fact that the previously mentioned well-known neurostimulation techniques including VNS, DBS, ECT, tDCS, and rTMS have been widely used in neuroscience and in the treatment of mental and motor disorders, they have their own limitations for ASD. The majority of these neurostimulation techniques offer low spatial resolution or necessitate

invasive procedure [17] [18] [19]. However, as per recent findings, transcranial focused ultrasound stimulation offers several advantages over the previously discussed neuromodulation techniques for ASD. tFUS is proposed to be the moderator that can overcome all the limitations of the commonly used neurostimulation techniques due to its noninvasiveness and ability to penetrate into deep brain tissues at 10 to 15 cm and even more with high resolution of 1 to 5 mm [8] [26]. tFUS modulates the brain activity in the ROI without causing tissue damage and can target deep regions in the brain without affecting surrounding tissues [18] [35]. Moreover, while it is difficult to couple fMRI with rTMS, tDCS and DBS; it is easily possible to use it with tFUS [8].

Table 2.2 recaps the advantages and disadvantages of the commonly used neurostimulation techniques along with tFUS.

Table 2.2: Comparison between some neurostimulation methods and tFUS [8].

Specification	DBS	tDCS	rTMS	tFUS
Invasiveness	Invasive	Non-invasive	Non-invasive	Non-invasive
Spatial Resolution	~1 mm	Undetectable	~3-5 cm	1-5 mm
Stimulation Depth	Unlimited	Undetectable	~1-1.5 cm unless H-coil is used	10-15 cm or more
fMRI Brain Mapping	Difficult	Difficult	Very difficult	Easily possible

2.3 TRANSCRANIAL FOCUSED ULTRASOUND STIMULATION

Although of the previously mentioned well characteristics of tFUS, its application to neuroscience remains astonishingly not discovered.

2.3.1 Ultrasonic Neuromodulation in Animal Studies

tFUS has been studied in brain pathways as reported elsewhere [8]. tFUS activated the motor cortex and produced muscular contractions when applied to rodents (mice, rat and rabbits) [27] [29] [28]. It also induced non-invasive neuromodulation in freely moving mice by stimulating the primary somatosensory cortex [74]. Additionally, it resulted in visible changes in the blood oxygenation-level dependent signal [29]. It also produced eyeball movement

while stimulating the abducens nerve in rat [75], inhibited the primary visual cortex in rabbit [29], suppressed EEG visual-evoked potentials in rats [30] and auditory evoked potentials in pigs and rats [76], caused widespread activation via an indirect auditory mechanism in mice [77], increased cortico-muscular coupling between motor cortex and tail muscle in mice [78], altered the anesthesia time in rats when applied to the thalamus [79], induced long-term changes in somatosensory evoked potentials during stimulation to anesthetized rats [80], caused local increase in glucose metabolism in the rat brain [81], caused reversible reduction of essential tremor in rats [82], selectively stimulated the motor brain area in rats at an acoustic intensity that is compatible with regulatory safety limits on biological tissue [83], produced extensive brain activation via a cochlear pathway in pigs [84], and modulated the single-neuron discharge [85] and latencies in antisaccade task when it targeted the left frontal eye field in monkeys [31]. MRI guided tFUS modulated sensory networks in monkeys [86]. Also, excitability followed by reduction of action potential in frogs' sciatic nerve was observed using short bursts of tFUS [87]. Furthermore, tFUS increased the extracellular levels of serotonin and dopamine in the frontal lobe [88], increased the glucose metabolic activity [81], and reduced the time took the animals got anesthetized [79], during thalamus stimulation in rats. tFUS was shown to have an antidepressant-like effect in rats [89] and to be neuroprotective when applied immediately after the ischemic brain injury in rats [90]. It was also proven that brain electrophysiological response following tFUS and imaging spatiotemporal distributions of brain activation induced by tFUS is feasible in rats [91]. Moreover, tFUS showed neuroprotective effects against cerebral damages and caused memory retention in Alzheimer disease in rats [32], as well as reduction in the occurrence of epileptic EEG bursts in rat model of epilepsy [33]. Besides, tFUS at the mice hippocampus activated voltage-gated sodium channels, voltage-dependent calcium transients, synaptic vesicle exocytosis, and synaptic transmission, while inhibited triggered local field potential in hippocampal slice cultures and increased spike frequency [27] [92]. Lately, it has been tested and safely achieved in sheep [34] [93].

2.3.2 Ultrasonic Neuromodulation in Human Studies

Furthermore, tFUS has been examined in human by stimulating the primary somatosensory cortex [35] [94] and posterior frontal cortex [36]; resulting in enhancing the

performance on sensory discrimination tasks and improving mood/global affect respectively. It was also tested by stimulating the primary visual cortex [95] and by being paired with the blood oxygen level dependent fMRI in humans [96]. It was demonstrated that tFUS paired with high field MRI increased the activation volumes of the targeted finger representation which is spatially restricted within the primary motor cortex while not extending to functionally connected motor regions [97]. Additionally, it modulated the activity of unilateral thalamus in humans [98], increased the excitability in the primary motor cortex [99], inhibited the amplitude of motor evoked potentials and reduced the reaction time on a simple stimulus response task when targeted the primary motor cortex [37]. tFUS showed the ability to simultaneously stimulate multiple brain areas, primary and secondary somatosensory areas, in a spatially restricted fashion [100]. It was also examined by stimulating the thalamus in disorders of consciousness after severe brain injury [101]. These outcomes, along with the enormous potential of tFUS, suggest a promising tool in the treatment of neuropsychiatric disorders [8] [18], including ASD. However, this has not been yet tested neither on animal nor on human.

2.3.3 Mechanism Underlying Ultrasonic Neuromodulation

As per recent review, nerve impulses are now considered as a combination of electrical, mechanical, chemical and conformational changes in the excited cells rather than being historically recognized as only electrical signals that are caused by depolarization of the membrane beyond a threshold potential that leads to excitation [102]. tFUS has reversible effect on neurons and can cause neuronal excitation and inhibition [18] [26]. Stimulation at low intensities and for short exposure times does not cause tissue heating; so it is thought that tFUS modulates the neuronal functions in the brain through nonthermal mechanical effect on cellular membranes and ion channels [8] [35]. It was suggested that ultrasound triggers action potential through a combination of several mechanisms involving: the generation of capacitive currents due to membrane displacements, activation of mechanosensitive channels, opening of pores in the lipid bilayer (a phenomenon named sonoporation), and coupling into membrane waves along the axon [102]. Prieto et al. demonstrated that ultrasound induces capacitive currents in pure lipid membranes through flexoelectric effects or conformational changes [103]. Tyler et al. [104] proposed that tFUS

might alter neuronal excitability through mechanical stress which might modify the viscoelastic properties of lipid bilayers and mechanosensitive transmembrane proteins that might adjust receptor channel gating kinetics. Although the mechanisms underlying how tFUS modulates neuronal functions are poorly understood, recent reviews have indicated the ability of tFUS to mechanically alter the state of mechanosensitive ion channels (transmembrane proteins which are sensitive to mechanical forces) existing within the cellular membranes. Compression, tension and shear forces generated by ultrasound can enhance the possibility of opening/activating voltage-gated ion channels including sodium, calcium, and potassium channels, and thus triggering action potentials and synaptic transmission [8] [26]. Dalecki [105] and O'Brien [106] linked the impact of ultrasound waves to acoustic radiation forces generated as a result of the momentum travelling from the acoustic field to the medium. Such forces cause the creation of radiation torque and acoustic streaming, and are able to displace small ions, molecules, and organelles or induce movement of the fluid along and around cell membranes. However, these forces are thought to play an important function in endothelial nitric oxide synthesis that organizes the functions of ion channels [107] [108] [109]. Furthermore, it is supposed that nerve impulses are linked to elastic interface waves which propagate along the wall of the axon or the plasma membrane [110]. Nerve impulses are supposed to include mechanical, thermal, chemical and conformational changes in the plasma membrane. Ultrasound/acoustic perturbations are likely to couple to these several features of cellular excitability and adjust the conformational or thermodynamic state of the plasma membranes of cells, which could result in sufficient depolarization to trigger a nerve impulse or to suppress depolarization and inhibit nerve firing. The excitatory or inhibitory actions can be through changes in membrane capacitance, changes to ion channels, opening of pores and coupling to interfacial elastic waves.

2.3.4 Safety Considerations

No sign of heat, cavitation, or tissue damage at the stimulated region or surrounding tissues was reported in tFUS research as per recent reviews [8] [102]. Many studies searched for damage evidence caused by tFUS, however they did not report any potential damage [92] [111] [112] [113]. In a study, temperature was monitored in the focus of tFUS during stimulation through which researchers did not report any increase in the temperature even

during prolonged stimulation [29]. More than 30 tFUS studies achieved on human could cause biological effect without causing tissue damage [18]. The parameters applied during tFUS studies were below the standard level/safety guidelines for diagnostic ultrasonic examinations set by FDA, American Institute of Ultrasound in Medicine, and the National Electrical Manufacturers Association [8] [26]. These regulations/parameters involve the mechanical index (MI), thermal index (TI) for soft tissue and cranial bone, spatial-peak temporal-average intensity (Ispta), and spatial-peak pulse average intensity (Isppa). Mechanical index is an estimate of the maximum amplitude of the pressure pulse in a tissue. It is defined as the ratio of acoustic pressure and the square root of frequency. Thermal index estimates the temperature increase in soft tissue and cranial bone. Spatial-peak temporal-average intensity is the maximum intensity in the beam averaged over the pulse repetition period. Ispta is the best measure of the amount of heat delivered to a tissue by ultrasound. Spatial-peak pulse average intensity is the pulse average intensity, calculated at the position of the spatial maximum. FDA specified the maximum allowed acoustic limits of diagnostic ultrasound system involving TI = 6.0, MI = 1.9, I_{spta} = 720 mW/cm^2, and I_{sppa} = 190 W/cm^2. These parameters should be treated as minimum requirements for ultrasonic neuromodulation treatment protocols [26]. However, 2 out of 54 studies reported damage linked to ultrasonic stimulation where several brain areas containing hemosiderin indicating the potential of local bleeding were reported in a rat animal, as well as, micro-hemorrhages were detected in sheep [102]. Therefore, further investigations and long-term follow-up studies on the impacts of ultrasound on the brain should be warranted to avoid adverse impacts while ensuring neural effectiveness.

2.4 CONCLUSION

The establishment of adequate animal models that mimic ASD reasons and symptoms is highly relevant for studying autism spectrum disorder, investigating its pathophysiology and finding new therapeutic approaches. However, it is difficult to develop such models due to the complexity of this disorder, unknown etiology, diversity of symptoms, and being strictly limited to human. Despite these obstacles, researchers have developed two main different types of ASD animal models, the genetic models and the environmental risk factors models,

using rodents, guinea pigs, Microtus, zebrafish, and fly. In spite of the various existing animal models, there is no ideal model available yet. The major reason behind that is that a perfect animal model linked to human psychiatric disorder should fit several criteria involving matching the causation, symptoms, and treatment between animal and human disorder. However, it is not the case for available autism models as animal models did not clarify the male bias existing in autism, some models generated by genetic modifications can create some serious conditions such as tumors and physical malformation, and some models which are produced by mutations can produce a broader phenotype than that obtained in ASD.

Several neurostimulation techniques exist including invasive methods such as VNS and DBS, and non-invasive techniques including ECT, tDCS, and rTMS. These commonly used neurostimulation techniques face some limitations for ASD. However, it is proposed that tFUS might be the moderator which can overcome the limitations of these neurostimulation techniques with its noninvasiveness, ability to penetrate into deep brain tissues with high resolution, and safety. tFUS has reversible effect on neurons and can cause neuronal excitation and inhibition. Although the mechanisms underlying how tFUS modulates neuronal functions are poorly understood, it is thought that it modulates the neuronal activity in the brain through nonthermal but mechanical effect on cellular membranes and ion channels. tFUS has been studied in animals including rodents, rabbit, monkey and sheep, and resulted in positive impacts. It has also been tested in animal models of epilepsy and Alzheimer. Moreover, it has been examined in humans and reached favorable outcomes in enhancing performance and mood. However, it has never been yet tested on human with ASD or animal models of this disorder.

CHAPTER 3

NEUROSTIMULATION IN ASD: REVIEW OF PRECLINICAL LITERATURE

ABSTRACT

Background: Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder with uncertain etiology, varying severity and deficits, and an increasing incidence making it a major public health problem. Additionally, its pathophysiology remains limited and unfortunately has not allowed the emergence of effective therapies.

Objective: The objective of the survey study is to review the preclinical research using neurostimulation methods applied to autism spectrum disorder and their therapeutic potential. There is a huge need to characterize the relationship between the symptomatic dimensions and pathophysiology of this disorder, and to develop new therapeutic and targeted approaches.

Methods: We surveyed human studies to document the Human Brain Regions hypothesized to be involved in ASD. We then described studies conducted with the various neurostimulation techniques, including deep brain stimulation, electroconvulsive therapy, and repetitive transcranial magnetic stimulation, in preclinical scenarios for autism that were based on findings from human studies. We also sought to investigate the biological mechanisms involved in brain stimulation.

Results: The review study shows that the used neurostimulation techniques face various limitations, either in terms of invasiveness, targeting and selectivity, or in terms of accessibility to deep brain zones. We also introduce a novel neurostimulation technique using ultrasound waves. This emerging technology offers real therapeutic opportunity as it might overcome the limitations of currently available neurostimulation approaches.

Conclusions: This review summarizes the human brain regions implicated in ASD, goes over the currently available neurostimulation approaches tested in the field of ASD in preclinical research, and interprets the underlying mechanisms of each technique. Finally, we introduce

a novel neurostimulation approach using ultrasound. We believe that in the future low intensity focused ultrasound stimulation could be a promising therapeutic strategy as it overcomes the limitations in terms of resolution and spatial targeting.

Keywords: Autism Spectrum Disorder; Brain regions; Neurostimulation; Preclinical research, Ultrasound.

PAPER REFERENCE

Title: Neurostimulation for Autism Spectrum Disorder: An Overview of Preclinical Studies

Journal: CNS Neuroscience & Therapeutics

Authors: Rasha Noureddine[a,b], Yassine Mofid[a,e], Mohamad Nassereddine[c], Alexander Surget[a], Jamal Charara[c], Ayache Bouakaz[a*], Frédérique Bonnet Brilhault[a,d,e]

Affiliations:

[a]UMR 1253, iBrain, Université de Tours, Inserm, Tours, France.

[b]Lebanese University, Doctoral School of Science & Technology, Hadath, Lebanon.

[c]Lebanese University, Faculty of Sciences I - Department of Physics – Electronics, Hadath, Lebanon.

[d]Centre Universitaire de Pédopsychiatrie, CHRU de Tours, Tours, France.

[e]EXcellence Center in Autism and Neurodevelopmental Disorders - Tours ExAC-T, Tours, France.

[*]To whom correspondence should be addressed. Dr. Ayache Bouakaz: UMR 1253, iBrain, Université de Tours, Inserm, 10 boulevard Tonnellé, 37032 Tours, France. E-mail: **ayache.bouakaz@univ-tours.fr**

CHAPTER 4

RESEARCH DESIGN AND METHODOLOGY

4.1 INTRODUCTION

The main objective of this project is to study and assess transcranial focused ultrasound stimulation technique as a therapeutic tool in a VPA animal model of ASD. For this purpose, it is essential to determine a detailed methodology and ultrasonic parameters needed for implementing tFUS. The implementation and optimization of tFUS stimulation requires targeting the primary motor cortex for determining the stimulation parameters from which the activation of the neuronal population of the targeted area is effective. Once the required parameters and procedure get identified, another main goal is to study the impact and safety of stimulation on the anterior cingulate cortex, a deep major brain structure implicated in various psychiatric diseases such as ASD and major depression. Following, developing VPA model and examining tFUS on the VPA model are to be achieved.

This chapter explains a detailed procedure for motor cortex stimulation in mice involving animal preparation, anesthesia, stimulation setup and protocol, eliciting EMG signals, and identifying brain regions. In addition, it covers ACC stimulation and setting up the VPA model. Moreover, this chapter describes in details the behavioral tests performed for validating the VPA animal model used and effects of tFUS.

4.2 MOTOR CORTEX STIMULATION

4.2.1 Animal Preparation and Anesthesia

C57BL/6J mice were obtained from Janvier Labs SAS (Le Genêt Saint-Isle, France) and were housed in standard conditions (12:12 light-dark cycle). All experiments were compliant with Directive 2010/63/EU guidelines on animal ethics. At the beginning of each tFUS experiment, the mouse was anesthetized in a closed transparent chamber with 3% isoflurane induction delivered with oxygen at a rate of 2 liters per minute for about 2 minutes. When

anesthetized, the mouse was taken out from the chamber and suited on a plate with its head settled in a face mask that delivered 2% isoflurane with 2 L per min oxygen rate for shaving its head (refer to Fig. 4.1 to the left). Subsequently, it was transferred to the stereotactic frame for head fixation. The mouse was rested on a heating pad to maintain its normal body temperature. Its head was fixed with auxiliary ear bars and fitted in a built-in face mask through which it received oxygen continuously at a rate of 2 liters per minute and isoflurane at 2% induction that maintained it fully anesthetized throughout the preparation phase of the experiment and placement of EMG electrodes (Fig. 4.1 to the right). Ophthalmic ointment was placed on the eyes to keep them wet. After locating the coordinates of the region of interest in the brain (details given in section 4.2.4), US gel was applied to the shaved head and ultrasound transducer was positioned approximately at 0.5 mm over the region to be stimulated. Then, isoflurane was reduced to 0% and the mouse was allowed to habituate for 2 minutes until a toe pinch-response was detected signifying that a light anesthesia level had been reached. At that point, tFUS stimulation was performed. Throughout the experiments, respiration rate of the mouse was monitored during mouse preparation to ensure good state of the tested mouse.



Figure 4.1: Mouse suited on a plate with its head settled in an anesthetic face mask for shaving its head to the left. Mouse rested on a heating pad with its head fixed with auxiliary ear bars and fitted in a built-in anesthetic face mask to the right.

4.2.2 Brain Stimulation Setup and Ultrasonic Protocol

Ultrasound stimuli were generated using a single-element transducer (Imasonic, Besançon, France) with a central frequency of 500 kHz, focal length of 57.5 mm and an active diameter of 41 mm. The transducer was driven by an electrical signal consisting of bursts of sine waves generated from a function generator (Agilent 33250A, Santa Clara, CA, USA) and

then amplified using a power amplifier (500 W ADECE, Artannes sur Indre, France) as shown in Fig. 4.2. The corresponding acoustic pressure profiles were measured in a degassed water tank using a calibrated hydrophone (ONDA, HGL-0200, Sunnyvale, CA, USA) connected to an oscilloscope (TDS 3044B, Tektronix, Beaverton, OR, USA). The function generator was triggered by the PowerLab (ADInstrument, Australia) that was in turn controlled by the LabChart 8 software on a PC. A plastic column filled with degassed water was attached to the transducer and sealed with polyethylene to insure a colocation of the ultrasound focus and the targeted brain zone. The column was coupled with ultrasound gel placed on the shaved head of the animal. Ultrasound gel was also placed on the attached surface of the transducer and cone to prevent air bubbles from entering during assembly of the stimulation setup. The transducer setup was settled to a 3-axis positioning system that allowed free movement of the transducer over the fixed head of the animal for precise aiming. For all experiments the transducer was positioned approximately 0.5 mm from the surface of the animal's head while having the head fixed with auxiliary ear bars. Fig. 4.2 shows the brain stimulation setup used.

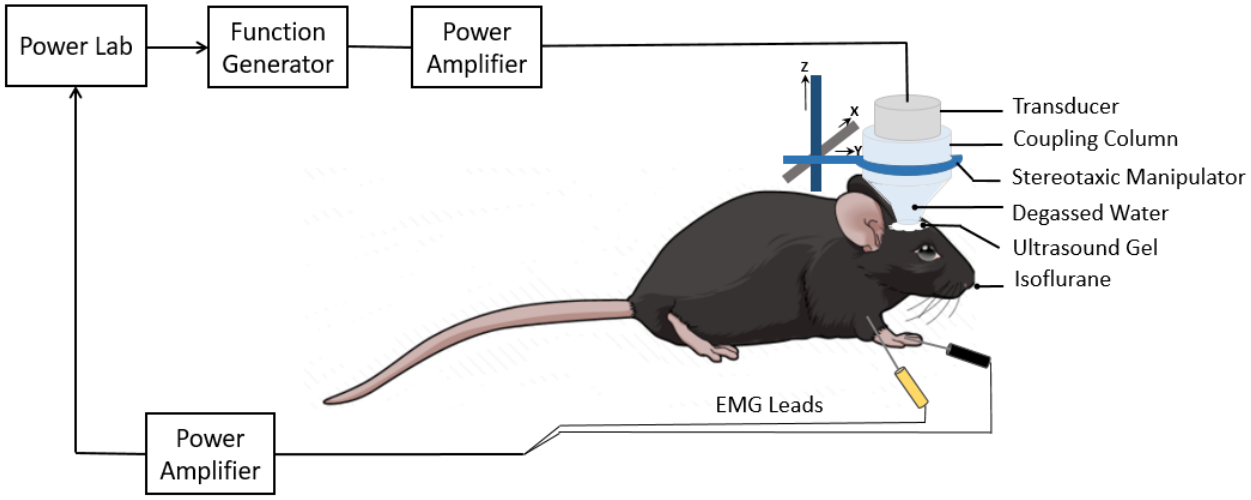


Figure 4.2: Brain stimulation setup with EMG leads connected to the right forelimb for EMG recordings during tFUS stimulation of the primary motor cortex.

Optimization of the ultrasonic protocol/parameters is the essential key for inducing successful ultrasonic neuromodulation. US protocol consists of sequences determined over multiple time scales, as represented in Fig. 4.3. The individual US pulses exist at the shortest time scales. These pulses have a pulse repetition frequency (PRF) and are repeated at this frequency for a length of time defined by the burst duration (BD). Each burst comprises one

distinct trial, and these bursts are delivered at a burst repetition frequency (BRF). The time between each burst is defined as the inter-stimulus interval (ISI). The total time (TT) of the experiment has a total duty cycle (TDC) that refers to the duty cycle over the whole experiment, accounting for the ISI between bursts [102].

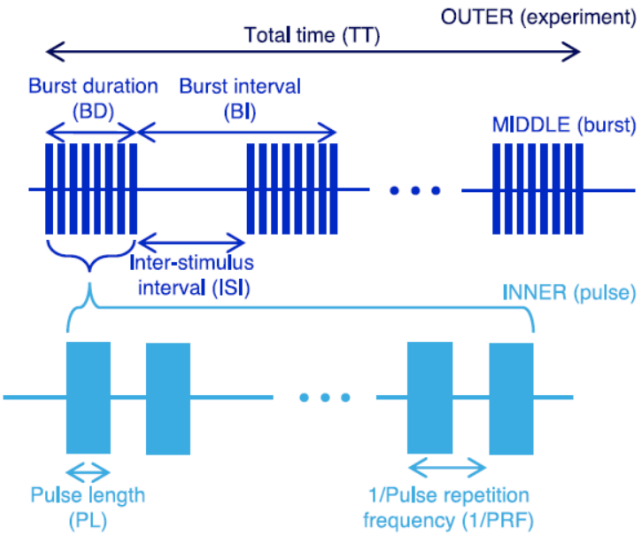


Figure 4.3: Schematic of ultrasound sequences and associated parameters [102].

In our protocol, we used the single element transducer of 500 kHz frequency (each individual cycle has a period of T= 2µs). Each pulse consisted of 80,000 cycles forming a pulse duration (or length) of PL = 160 ms. The pulse was repeated every 10 seconds (PRF = 0.1 Hz) for a total duration of TT = 10 minutes, producing a total of 60 pulses. This stimulation was applied for 5 consecutive days forming the treatment tFUS protocol. The choice of the selected parameters was based on a previous study performed in our Inserm unit and used for treating depression disorder in depressive-like mice [38]. Our measurements showed that the values of MI and I_{spta} used were 0.78 and 155 mW/cm^2 respectively, far below the limits fixed by the FDA for the use of ultrasound on the brain.

4.2.3 EMG Recordings/Electromyography

Two subdermal electromyography (EMG) electrodes were inserted in the right upper limb, particularly an electrode was implanted in the brachioradialis muscle group of the right forelimb and another electrode was embedded between the 3rd and 4th carpometacarpal joints to obtain the EMG recordings during tFUS stimulation as shown in Fig. 4.2 [114]. EMG

electrodes were fed into a bio amplifier of the PowerLab (ADInstrument, Australia). EMG signals were acquired and post-processed using LabChart 8 (ADInstrument, Australia). A band pass digital filter, between 300 Hz and 1 kHz, was applied to the signal [38]. Then the absolute value with a gain of 1000 was taken [38]. Smoothing using triangular (Bartlett) window was done on 301 samples [38]. A single-event motor evoked potential (MEP) was monitored up to a 590-msec. Averaged MEP amplitudes were then obtained for each individual regardless of motor response success.

4.2.4 Identification of The Brain Regions of Interest for tFUS

a. Motor Cortex

The establishment and optimization of the tFUS protocol recommended determining the minimum thresholds of stimulation from which the activation of the neuronal population of the targeted area is effective. For this purpose, the primary motor cortex (M1) was chosen for stimulation. Locating the region M1 to be stimulated in the brain was achieved using an initial identification of a hump existing at the back of the head which could be easily recognized by eye and by touch. Bregma was reached reliably by moving 8 mm anteriorly from this elevation with a precision of ±0.5 mm. This average distance was confirmed by surgical opening of 5 mice heads and validated by the success rates of our experimentation. The left primary motor cortex of the forelimbs targeted was positioned at Bregma -0.25 mm, 1.5 mm laterally to the left [115].

b. Anterior Cingulate Cortex

A preliminary step for this project was to define a target brain area for tFUS. ASD heavily affects the brain and the neural circuits globally. But certain areas of the brain seem particularly critical in the autism spectrum disorder, including the prefrontal cortex, cerebellum, parietal lobe, temporal lobe, **anterior cingulate cortex (ACC)**, temporo-parietal junction, occipito-temporal region, thalamus, amygdala and precentral sulcus as described in chapter 3. Several studies have shown that the ACC which is linked to attention, emotion, value attribution, personality patterns, social functioning, and cognitive control is extensively implicated in ASD. Researchers reported decrease in cell size, abnormal overgrowth, lack of activity, reduced activation, and neuroglial activation and neuroinflammation of this region

[116] [117] [118] [119] [120]. Furthermore, hypo connectivity as well as hyper connectivity between ACC and associated brain regions have been implicated in ASD. Thus, the ACC is selected as our region of interest as it is one of the most important regions linked to autism as per the literature review [121] [122]. Furthermore, the choice of this region was motivated by the fact that its equivalent area is well identified in the brain of the mouse (red contoured in Fig. 4.4) and that it represents a deep zone in the human brain. ACC was located by moving anteriorly from Bregma by 0.5 mm [123].

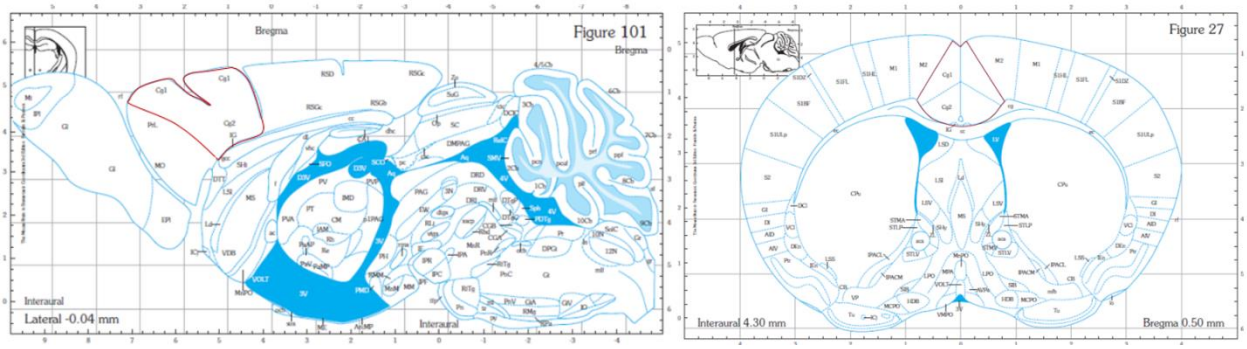


Figure 4.4: Anterior cingulate cortex in sagittal and coronal view of mouse brain [106].

Detailed Procedure:

- Make sure that head of the mouse is totally fixed with the auxiliary ear bars in the stereotactic frame, and its nose and mouth are fitted inside the face mask.
- Use a small ruler and place it softly, laterally at the middle of the shaved head to form a temporary line on the skin which serves as an approximate reference for the midline of the brain.
- Attach a needle to the collimator. Move the needle laterally and in the anterior-posterior directions using the corresponding knobs within the 3-axis positioning system and place it above any point of the drawn reference. Move the needle down using the knob which correlates to the up-down direction until it softly contact the reference for further visual validation. Record the lateral coordinate. Make sure to move the needle up at this stage after noting the coordinate to avoid harming the skin during further movements of the needle.
- Identify visually and through touching the location of the hump shown in Fig. 4.5. Move the needle in the posterior direction and place it on the hump. Move the needle

down until it softly contact the hump for further visual validation that the needle is placed at the point of intersection of the hump and reference line. Write down the coordinates in three directions.

- Move the needle up until it appears clearly that the needle end is totally above the

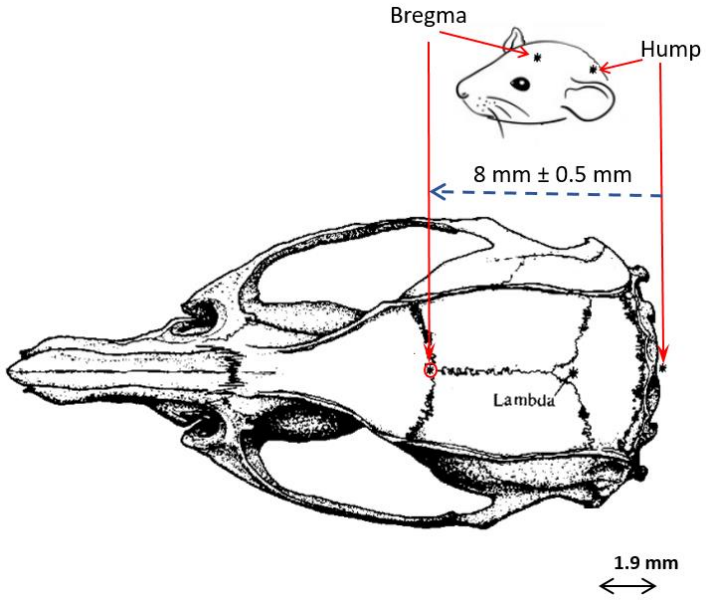


Figure 4.5: Schematic diagram for identifying hump and locating Bregma in a mouse head.

head and will not come in contact with the skin surface during movement. Use the knob that correlates with the anterior direction, and move the needle 8 mm anteriorly to reach Bregma. Refer to Fig. 4.5.

- From Bregma, move posteriorly by 0.25 mm and laterally by 1.5 mm to the left to reach the left M1. However, for locating the ACC, move anteriorly from Bregma by 0.5 mm.
- After locating the region of interest, move the needle down until it softly comes in contact with the skin to make a temporary soft mark on the skin.
- Using the same knob that corresponds to the up-down direction, move the needle up enough to be able to disconnect it from the collimator.
- Place degassed US gel on the area to be stimulated. It is better to put gel enough to totally cover the shaved area. Using the same knob, move the transducer with the collimator down until the collimator is approximately at 0.5 mm over the region to be stimulated.

4.2.5 tFUS Stimulation for Studying the Anesthesia Effect

Male mice aged 4 weeks received tFUS at the left M1 (Bregma -0.25 mm, 1.5 mm laterally) at three different acoustic pressures (220, 330 and 440 kPa) at 0.5 and 0% anesthesia. 3 pulses, 0.1 Hz PRF, were delivered at each acoustic pressure step. EMG signals elicited from the right forelimb were used to evaluate the impact of anesthesia level on the motor responses.

4.2.6 tFUS Stimulation of the Primary Motor Cortex

10 healthy male mice aged 4 weeks were stimulated at M1 at 0% anesthesia. Some measures consisting of success and reliability of the stimulation across subjects were evaluated. Success and reliability of tFUS stimulation was assessed by real time visual observation of muscular responses at the left upper limb and through EMG recordings elicited from the right upper limb. A video camera was connected to the computer and programmed using the LabChart 8 software for recording the muscular responses of the left upper limb during tFUS stimulation. These video recordings were studied in post-treatment for validating the real time visual observation of the detected muscular movements. The applied peak negative acoustic pressure was varied from 55 kPa to 550 kPa with a step of 55 kPa. 10 pulses, 10 seconds apart, were delivered at each acoustic pressure step. The inter-stimulation interval of 10 seconds was used to avoid blunting of the motor responses [27].

4.3 ACC STIMULATION

Evaluation of the safety and effect of ACC stimulation by tFUS required stimulating the anterior cingulate cortex in healthy mice followed by behavioral test analysis, the open-field test (described in section 4.5), for two weeks and finally receipt of a new ACC stimulation or sham stimulation session followed by obtaining the brain for measuring and confirming the impact and safety of stimulation through histological analysis. In this phase, the same stimulation setup and ultrasonic protocol described in section 4.2.2 were used. Stimulation was applied for 10 minutes with a total of 60 pulses. The ACC was located at 0.5 mm anteriorly from Bregma [123]. Please refer to section 4.2.4 for the detailed procedure of identifying ACC.

ACC tFUS stimulation was applied at day 28 followed by open-field test that was performed at days 29, 35 and 41 (stimulation day +1/ stimulation day +7/ stimulation day +13) as displayed in Fig. 4.6. Open-field test was performed for evaluating locomotion and exploration. Then at day 42 (stimulation day +14), new tFUS stimulation or sham stimulation session was administered to the ACC followed by perfusion and acquiring the brain for histological analysis.

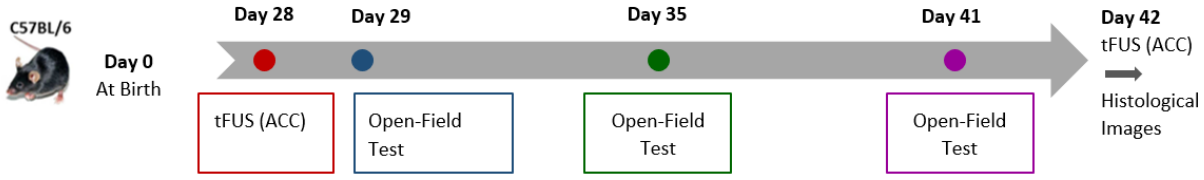


Figure 4.6: Chronogram for evaluating the safety and effect of ACC stimulation by tFUS.

In this phase, 28 healthy mice were used and categorized into stimulated groups (mice that received ACC stimulation) and control groups (mice that received no stimulation/sham stimulation) for studying the impact and safety of ACC stimulation by tFUS. Non-stimulated mice received same conditions as stimulated mice i.e. non-stimulated mouse was similarly anesthetized, head shaved, placed on the stimulation platform and prepared for the sham stimulation simultaneously with the stimulated mouse, except that non-stimulated mouse received no tFUS stimulation. Two separate but nearly placed platforms were used to perform the experiment on two mice, one stimulated and other non-stimulated, simultaneously under the same conditions. Mice of stimulated groups received ACC stimulation at 330 kPa at Day 28 [124]; 12 mice received this stimulation for only 1 session while another group receiving chronic stimulation consisting of 4 mice received the stimulation on 5 daily consecutive sessions as shown in Table 4.1. However, 12 mice of the control group received sham stimulation. Mice were classified in this way to allow the investigation of the effect and safety of one tFUS stimulation session as well as for several consecutive sessions for treatment purposes. As mentioned previously, open-field test followed tFUS or sham stimulation and was performed for 2 weeks, particularly at days 29, 35 and 41 for evaluating locomotion and exploration. Then, at day 42, mice were classified again into stimulated and non-stimulated groups (refer to Table 4.1), through which stimulated mice received ACC tFUS stimulation at 330 kPa. Perfusion followed tFUS or sham stimulation for histological study.

- Perfusion, Brain Sectioning and Nissl Staining

Within 90-minutes following tFUS or sham stimulation, animals were deeply anesthetized using sodium pentobarbital, 40 mg/kg intraperitoneal (ip), and transcranially perfused with 50 ml heparinized saline (0.9% sodium chloride, 1000 IU heparin; 10 mg/ml) for 4 minutes, followed by 100 ml 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PB, pH 7.4) [125]. Brains were collected and post-fixed for 24 h in 4% PFA/ 0.1 M PB and then cryoprotected in 20 % sucrose/0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4 °C till sectioning [125]. Coronal sections (40 µm) were cut with a cryostat (Leica CM 3050 S) and successive sections were sequentially collected in six tubes (5ml) filled with a freezing solution (glycerol, ethylene glycol, and 1X PBS; 1:1:1) and stored at 4°C for histopathological examination [125]. Nissl staining of the cortical tissue corresponding to the anterior cingulate cortex was accomplished for assessing the impact and safety of stimulation on tissue integrity. It was performed at 90-minutes and 14 days post-tFUS interval for identifying the acute effect and distant effect of stimulation respectively. One out of six series of brain sections was mounted onto charged glass slides (Superfrost Plus, Thermo Scientific) and dried for 30 min at 37 °C. Tissue slices were rehydrated in a descending series of ethyl alcohol, stained in 0.5% Cresyl violet stain for 5-10 min, washed in distilled water till removing excess stain and observing a good level of staining and differentiation, dehydrated in ascending series of ethyl alcohol [125], then cleared in Claral, mounted, and covered with cover slip. Sections were examined for capturing photos using a light microscope (Leica DM 2000).

Table 4.1: Mice classification for evaluating the safety and effect of ACC stimulation by tFUS.

Groups	Number of mice per group	tFUS (ACC) D28	Open-Field D+1/D+7/D+13	tFUS (ACC) D+14	Perfusion D+14
Group 1	4	No	Yes	No	Yes
Group 2	8	No	Yes	ACC	Yes
Group 3	4	1 session	Yes	No	Yes
Group 4	8	1 session	Yes	ACC	Yes
Group 5	4	5 sessions	Yes	ACC	Yes

4.4 VPA MODEL SETUP

4.4.1 VPA Model

The use of animal models is an essential step in the implementation and characterization of new therapeutic strategies. In this project, the valproate animal model has been used as the animal model of autism because it has been shown that the exposure to VPA in utero increases the risk of autism in humans and induces behavioral phenotypes in rodents reproducing several symptomatic dimensions of ASD [126] [127]. In this study, 30 pregnant female mice were obtained from Janvier Labs SAS (Le Genêt Saint-Isle, France). 15 females were injected at gestation day 12.5 with VPA (600 mg/kg, ip injection), while other 15 females received saline ip injection at gestation day 12.5 [126]. These injected female mice were used for delivering newborns needed for validating the VPA model and effects of tFUS.

4.4.2 Validation of VPA Animal Model and Effects of tFUS on VPA Mice

ASD is an eminently human and complex disorder affecting high cognitive functions; so it is not possible to summarize all the dimensions of ASD among rodents or autistic-like mice. On the other hand, there is a wide variety of tests to measure the behavioral deficits presenting conceptual analogies with several ASD symptoms at the cognitive and social levels (measuring the phenomenological validity).

In this phase, 22 VPA and 24 healthy mice, aged 4 weeks, received ACC stimulation or control conditions (no stimulation) for 5 days, as shown in Fig. 4.7, followed by set of several behavioral tests (listed below and described in details in section 4.5) for evaluating the model and effects of tFUS on different diagnostic criteria involving:

- ✓ Communication and social interaction deficits: free social interaction test with analysis of ultrasonic vocalizations and three-chamber test.
- ✓ Cognitive rigidity and resistance to change in routine: reversal learning test.
- ✓ Anxiety and impaired interest toward new objects: marble burying test and nesting test.

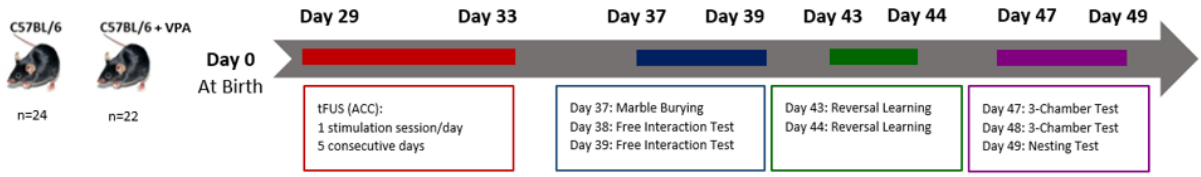


Figure 4.7: Chronogram for validating the VPA animal model and effects of tFUS on VPA mice.

8 groups of mice were formed and categorized according to their model (healthy or VPA), gender (female or male) and stimulation (stimulated or non-stimulated) as shown in Table 4.2. Stimulated groups received ACC tFUS stimulation at 330 kPa for 5 consecutive days while non-stimulated groups received no stimulation/sham stimulation for 5 consecutive days starting from day 29. Non-stimulated mice received same conditions as stimulated mice i.e. non-stimulated mouse was similarly anesthetized, head shaved, placed on the stimulation platform and prepared for stimulation in parallel with the stimulated mouse, except that non-stimulated mouse received no tFUS stimulation. Two separate nearly placed platforms were used for experimenting two mice, one stimulated and other non-stimulated, simultaneously. Several behavioral tests were then performed starting from day 37 at which mice were tested initially using the marble burying test. Please refer to Fig. 4.7. Then, free interaction test was done on days 38 and 39. After that, reversal learning was made at days 43 and 44, followed by the 3-chamber test which was achieved at days 47 and 48. Nesting test came finally after, at day 49.

Table 4.2: Mice classification for validating the VPA animal model and effects of tFUS on VPA mice.

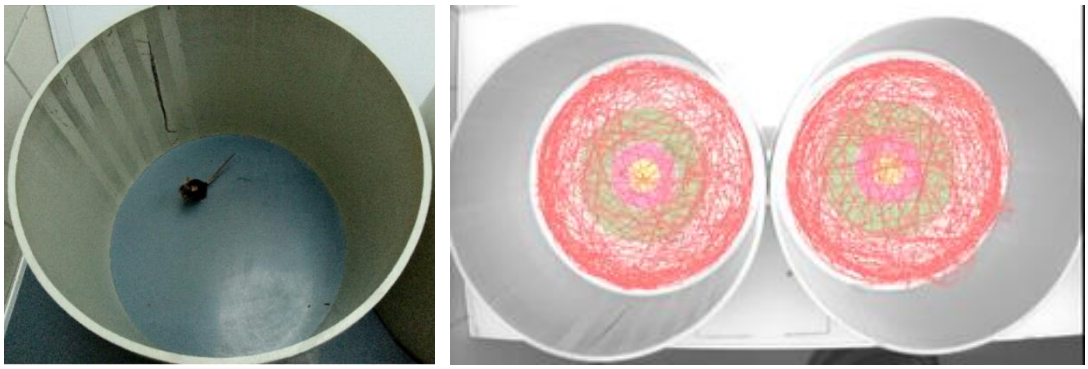
Groups	Number of mice per group	Model	Gender	tFUS (ACC) P29 -P33	Behavioral tests
Group 1	6	Control	Male	Non-stim	Yes
Group 2	6	Control	Female	Non-stim	Yes
Group 3	6	Control	Male	tFUS	Yes
Group 4	6	Control	Female	tFUS	Yes
Group 5	5	VPA	Male	Non-stim	Yes
Group 6	6	VPA	Female	Non-stim	Yes
Group 7	5	VPA	Male	tFUS	Yes
Group 8	6	VPA	Female	tFUS	Yes

4.5 BEHAVIORAL MEASURES

Mice behaviors were evaluated during the dark phase of the light-dark cycle, active and awake stage. Mice underwent open-field test for evaluating the impact and safety of ACC stimulations. Additionally, mice were assessed for impairments in social interactions, impaired verbal and nonverbal communication, and restricted range of interests; the core clinical features of ASD [44]. This was achieved for validating the VPA model performed and the effects of tFUS.

- Open-Field Test

Open-field test was used to provide measurement of locomotion and exploration in a new environment. During the test, the subject was placed in the center of a circular arena surrounded by high walls, as shown in Fig. 4.8 (a). The mouse was tested for 5 minutes and video tracked using Ethovision (Noldus, Netherlands) for determining the total distance covered by the mouse (refer to Fig. 4.8 (b)).



(a) **(b)**

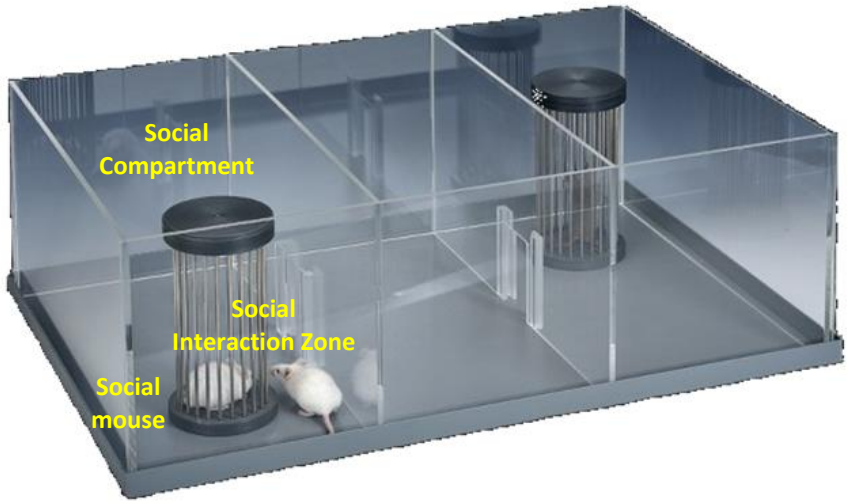
Figure 4.8: Open-field test (a) Mouse placed in a surrounded arena (b) Ethovision software system for detecting the distance moved by the mouse.

- Deficits in Social Interaction and Communication
  - 3-Chamber Interaction Test

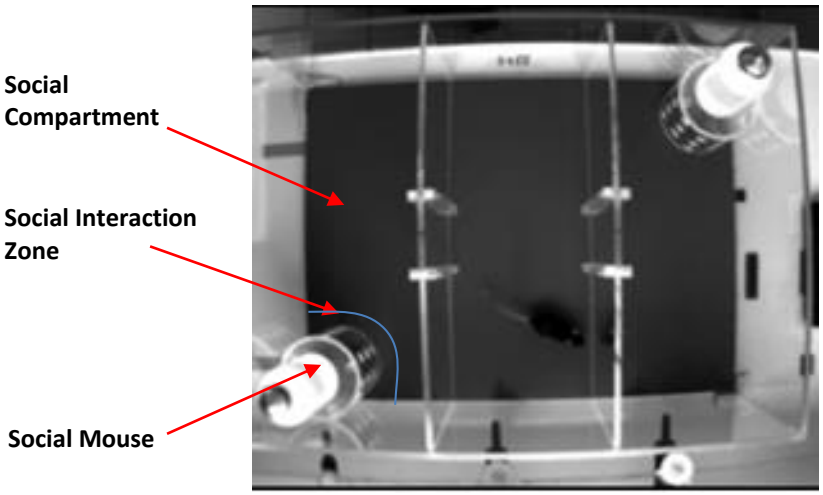
Mice are considered highly social species that participate in great degrees of social approach, social exploration, reciprocal social interactions, communal nesting, sexual behaviors, parenting and territorial aggressive behaviors [44]. Simple evaluation of social approach was achieved in a Plexiglas three-chambered apparatus with a novel object in one

side compartment and a stranger or unfamiliar mouse in the other side compartment as shown in Fig. 4.9. This test measures social motivation as more time spent in the chamber with the mouse than in the chamber with the non-social novel object. It should be noted that the stranger mouse was enclosed in a wire cup that allowed visual, olfactory, auditory and some tactile contact, while the object was placed in an inverted empty wire cup which provoked a high level of exploratory behaviors in mice, and the center chamber was totally empty forming a control for general locomotor activity. Ethovision (Noldus, Netherlands) was used to track the movements of the subject mouse from one chamber to another and determine the amount of time that the tested mouse occupies in each of the three chambers including the social chamber and social contact zone, as well as the distance covered by this mouse throughout the test duration.

The session started by placing the subject mouse in the center chamber for a 10-minute habituation period, through which the center area becomes a familiar “home base.” Sliding doors within the chamber divider panels are then elevated, permitting the subject mouse to discover and become familiarized to all three empty chambers for another 10-minute period. After that, the tested mouse is enclosed in the center chamber whereas an empty inverted wire pencil cup containing the novel object is located in one side chamber. At the same time, a new stranger mouse which had no prior contact with the subject is located in an inverted wire pencil cup cage in the other side chamber. Weights are positioned over both cups to prevent the tested mouse from ascending on top of the cups. Retractable doors between the chambers are elevated to start the 10-minute sociability test. Since the wire cup permits olfactory, visual, auditory and some tactile contact, the tested mouse can perceive social signs produced by the stranger mouse and thus respond to it by initiating several components of social interaction. The stranger mice used were usually of same gender and age, but had no previous physical contact with the tested mouse. Every stranger mouse was already habituated to the wire cage to become inactive throughout the test session. Placing the stranger mouse inside the wire cage guarantees that all social approaches are originated only by the tested mouse and prohibits aggressive fighting.



(a)



(b)

Figure 4.9: Three-chambered apparatus for quantitating social approach behaviors in mice (a) Front view (b) Top view.

- Free Social Interaction Test (Mice Profiler for Social Interaction and Ultrasonic Vocalization for Communication)

Another simple approach for testing all types of social interactions is the free social interaction test shown in Fig. 4.10. The test started by placing the tested mouse in a Plexiglas transparent cage for a 30-minute habituation period to allow the subject to explore freely and become familiar with the environment. At the end of the 30-minute session, a second mouse

of the same strain, sex and age was added to the cage for another 8-minute testing period through which the behavior of the two mice was monitored. Thus, one mouse was the resident of the cage (tested mouse) and the other an intruder. A video camera with MediaRecorder software (Noldus, Netherlands) was used to video record the behavior throughout the whole session. A microphone was utilized with UltraVox XT (Noldus, Netherlands) to record and analyze the ultrasonic vocalizations.

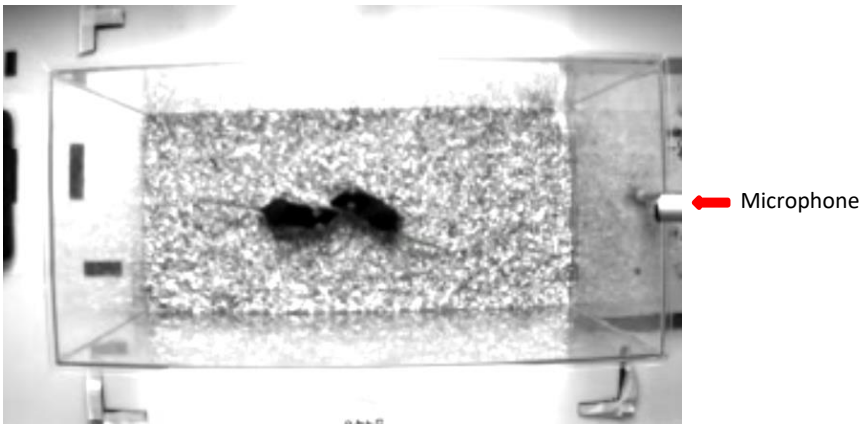


Figure 4.10: Illustration of free social interaction test.

The social interaction/behavior was analyzed for the first 4 minutes using the mice profiler tracker (Fig. 4.11) within ICY software [128]. 12 social events were discriminated with their number and duration. Some of the social events detected involved normal contact (minimum distance between mice <1 cm), oral-oral contact, oral-genital contact, following behaviors, escapes, one mouse reach the other and the other escapes, etc.

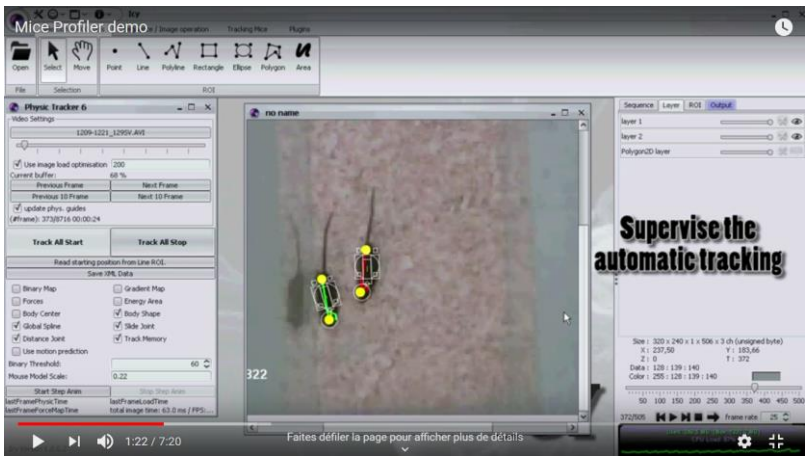


Figure 4.11: Mice profiler tracker demonstration.

Belayed language and poor communication skills are core features of ASD. Although mice do not use language and do not have the same qualitative level of communication as human speech, they show social communication mechanisms through emitting auditory signals involving ultrasonic vocalizations, and olfactory social signals including deposition of pheromones in the environment [41]. Conspecifics understand and reply to these auditory and olfactory emissions. Ultrasonic vocalizations are uttered during free interactions involving male-male interaction, female-female interaction and mating [129]. Quantitative measures of ultrasonic vocalizations were used to identify deficits in communicative interactions in mice during free social interaction test. Ultrasonic vocalizations were analyzed for the first 2 minutes of free interaction. It is supposed that autistic-like mice will show fewer ultrasonic vocalization calls compared to healthy mice, and might fail to reply to the ultrasonic vocalizations of the conspecifics. Fig. 4.10 shows a detector microphone that recorded the ultrasonic vocalizations during free social interaction test. Fig. 4.12 shows an example of ultrasonic vocalizations detected by the microphone and analyzed with SASLab Pro (Avisoft Bioacoustic, Berlin, Germany).

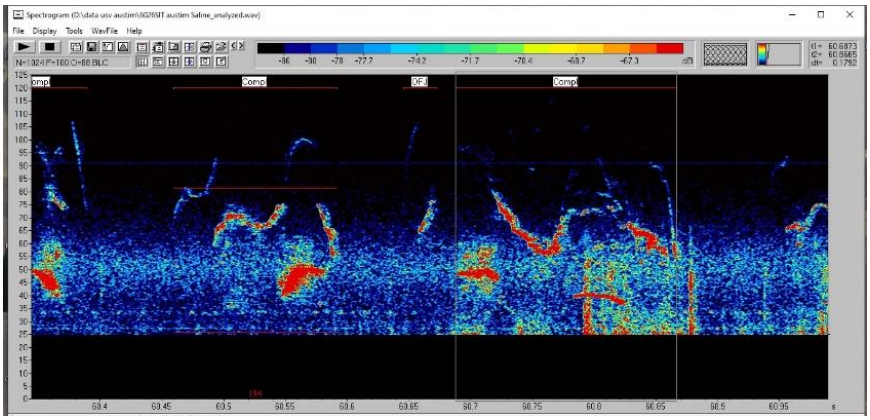
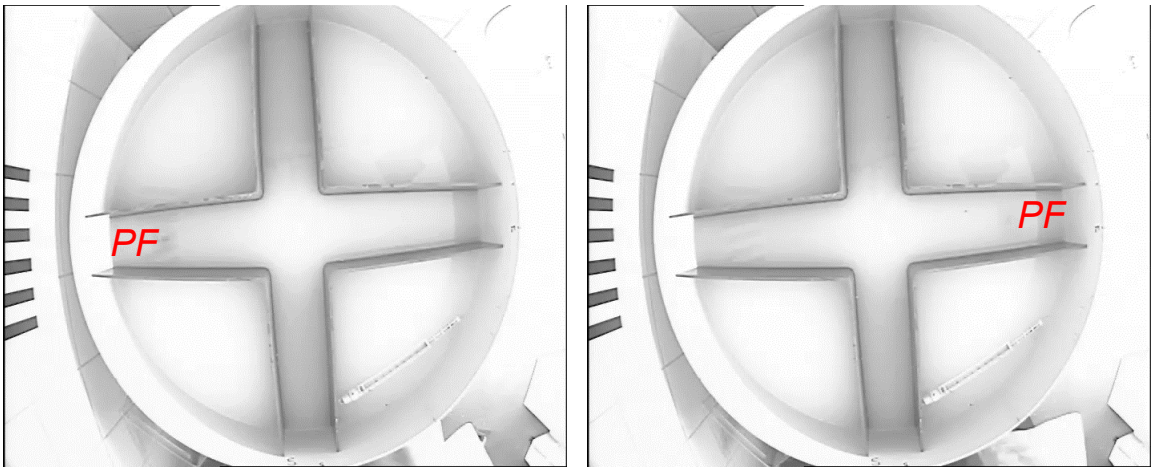


Figure 4.12: Example of ultrasonic vocalization calls emitted by a mouse.

- Cognitive Rigidity and Resistance to Change in Routine
  - Reversal Learning in Water Maze

Autistic individuals usually preserve rigid habits, and display a robust persistence on sameness and anxious to change in routine [41]. It is supposed that mice could be trained to develop a habit and then requested to make a change in the established routine. An approach used to establish a habit in mice is to train the mouse on an instinctive task with a spatially

liable supporter. Water plus-maze learning includes placing a hidden escape platform in one quadrant location of a circular swimming pool of water. Reversal learning asks the mouse to quench the position of the hidden platform in one quadrant and learn the new location of the hidden platform in another quadrant. Reversal learning necessitates different skills than the original acquisition learning. Ability, resistance and responses to the change in routine would be evaluated. Ability of the subjects to shift rapidly to the new position is quantified by the number of re-training trials needed to constantly indicate the different escape platform site in the pool. The number of errors done by the mouse to reach a certain criteria of correct trails shows the level of flexibility to change, however, failure to switch to the new position habit might correspond to the inflexibility in routine which is a symptom of autism. Fig. 4.13 represents the circular swimming pool used for water plus-maze reversal learning with the platform (PF) positioning change during learning, retrieval and reversal learning phases. In our project, the tested mouse was subjected to 6 blocks of learning phase, each consisting of 5 trials, thus forming a total of 30 trials for the learning stage in one day. After that, the subject was exposed in the second day to a retrieval phase consisting of 12 trials through which the test is stopped directly after the mouse successfully achieved 5 consecutive correct trials. Following and in the same day, a reversal learning stage composing of a total of 25 trial tests was performed during which the test is terminated directly after detecting 5 successive correct attempts.

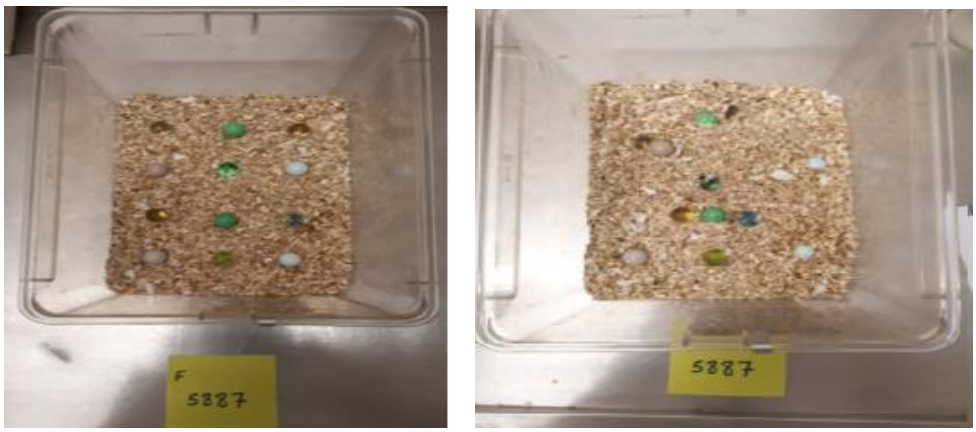


(a) **(b)**

Figure 4.13: Reversal learning test (a) Platform location during learning and retrieval phases (b) Platform location during reversal learning phase.

- Anxiety and Impaired Interest Toward New Objects
  - Marble Burying Test

Another simple test for measuring anxiety and interest toward new objects is the marble burying test. In this test, 12 different colored marbles were organized and positioned in a Plexiglas cage as displayed in Fig. 4.14. Then, the mouse was placed into a corner of the cage containing marbles while making sure to place the mouse on bedding as far from marbles as possible and allow it undisturbed for 30-minute test duration. After test completion, the mouse was removed and returned it to its home cage while being careful in not moving or displacing the marbles. The number of marbles buried was counted and used for evaluation of anxiety and interest in new objects. A marble was scored as buried when two-thirds of its surface area was covered by the bedding material.



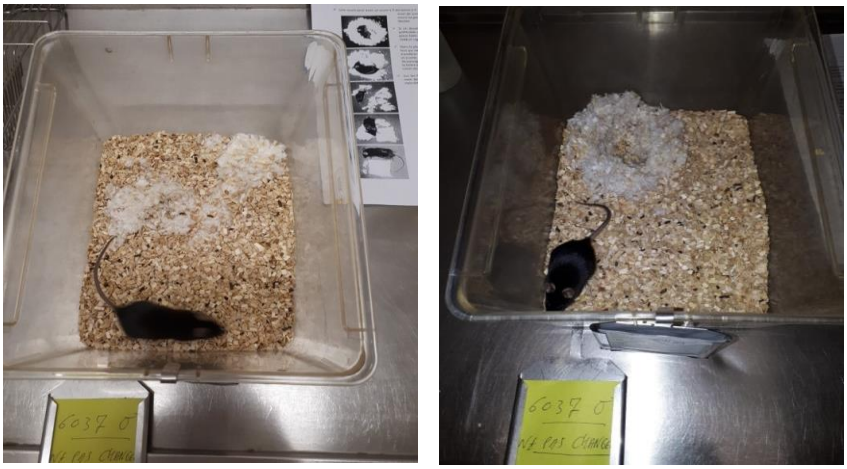
(a) **(b)**

Figure 4.14: Marble burying test (a) Positioning of the marbles in the cage before the test (b) Example of the positioning of marbles after the test.

- Nesting Test

Nesting building test is other useful test for measuring interest toward new or non-familiar objects. Few grams of cotton nesting material ranging from 2.4 to 3.2 grams were added to the Plexiglas cage where the subject mouse is settled. Nestlet weight was monitored at 5 hours and 24 hours after the new material is provided as shown in Fig. 4.15 for nest scoring (calculating the rating scale). The nest scoring protocol used depended on the manipulation of the material by the mouse. The score ranged from 0 to 5 [130]. If the nesting

material was not touched by the mouse, then the whole nest received a score of 0. If the nesting material was spread throughout the cage without being gathered to a central site, the nest received a score of 1. However, if the nest site was presented, the nest was considered as a square and each of the 4 sides of the square was given a separate score from 2 to 5. The 4 scores were then averaged for the cage nest score, at which a score of 2 was given when the nest was flat with no shallow walls, a score of 3 was provided when the nest had a slightly cupped shape where the wall of the nest is less than half the height of a dome that would cover a mouse, a score of 4 was agreed when the wall of the nest was half the height of a dome, and 5 given when the walls were taller than half the height of a dome that may or may not fully enclose the nest.



(a) **(b)**

Figure 4.15: Nest building test (a) Nestlet at 5 hours from the beginning of the test with nest scoring of 3 (b) Nestlet at 24 hours from the beginning of the test with nest scoring of 5.

4.6 CONCLUSION

An experimental design and methodology for examining tFUS on VPA animal model of ASD is described in this chapter. Additionally, a detailed procedure and protocol for motor cortex ultrasonic neurostimulation in mice is explained. Moreover, the process of stimulating the ACC is given. Furthermore, the practice of developing the VPA model and testing tFUS on it is covered along with the set of behavioral measures performed for evaluating the impact of stimulation.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 ULTRASONIC NEUROSTIMULATION IMPLEMENTATION AND SAFETY

5.1.1 Success Rate of Motor Cortex Stimulation

Motor responses characterized by muscular movements of the left upper limb during tFUS stimulation were visually detected and video recorded on the computer for post-treatment. After validating the real time visual detection of muscular responses with the video records, the success rate of stimulation was studied. The success rate was defined as the ratio of the detected number of muscular responses divided by the total number of applied stimulation trials made (10 trials per each pressure) and expressed in percentage. The muscular responses counted included any muscular movements at the left upper limb directly following tFUS stimulation regardless of its strength. The success rate of motor responses at different acoustic pressures was calculated for each mouse (refer to Fig. 5.1) and then averaged for all animals (refer to Fig. 5.2).

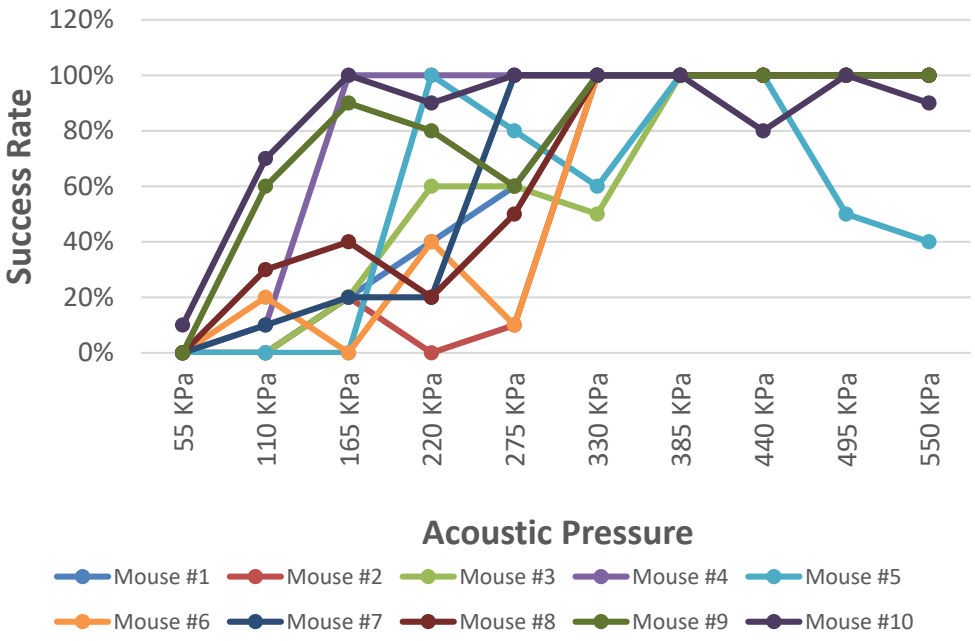


Figure 5.1: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials detected visually at different acoustic pressures for each mouse.

The success rate of motor responses increased with increasing the acoustic pressure until it reached its peak at 385 kPa and then it weakly reduced with continuous pressure increase, as shown in Fig. 5.2. At the lowest acoustic pressure (55 kPa), a total failure of motor responses (1% success rate) was observed. A 50% success rate appeared at 198 kPa. High-success rates (>91%) were achieved from 330 kPa (91±0.18%) to 550 kPa (93±0.18%) while having the highest possible success rate of 100% at 385 kPa. The experimental data was fit into a sigmoidal curve till 385 kPa, following the intensity-response relationship [131]. However, a decline of the success rate was observed at pressures higher than 385 kPa, which might be ascribed to neuron saturation. Normally, the neuron response increases with acoustic pressure until the response reaches saturation [132] [133] [134]. This nerve saturation could be related to the acoustic pressure or stimulation duration [135]. Repeated measures analysis was done using ANOVA. The result was considered significant when p-value, the statistical measure used, was less than 0.05. Evaluated parameters included degree of freedom = 9 and p = 0.

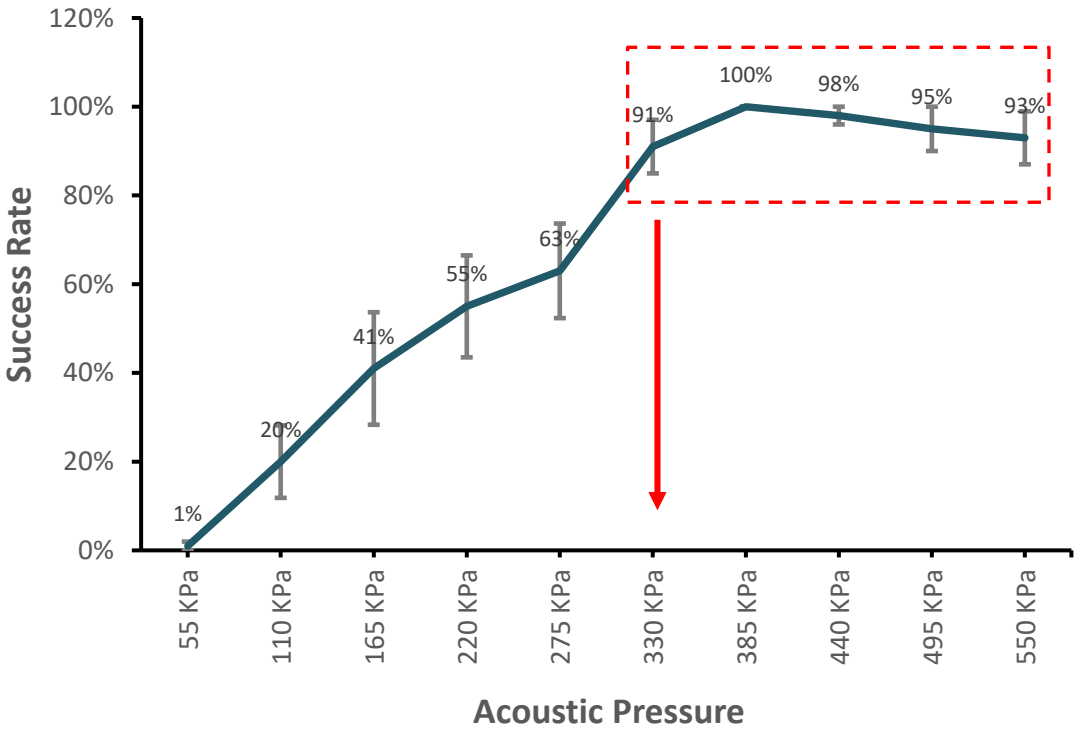


Figure 5.2: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials detected visually at different acoustic pressures for 10 tested mice. Results represent mean ± SEM.

The strength and reliability of stimulation at different acoustic pressures (55 kPa to 550 kPa) were assessed using EMG recordings in the right upper limb during tFUS stimulation. EMG recordings obtained at the right forelimb validated the experimental data obtained visually at the left forelimb. The success rate of detecting EMG signals in response to tFUS stimulation of the M1 was evaluated and displayed in Fig. 5.3. An EMG signal was noted to confirm the muscular contractions detected at the right upper limb regardless of its strength.

EMG recordings detected at the right forelimb confirmed the experimental results of success rates obtained visually which increased with increasing the acoustic pressure till 385 kPa followed by a slight decline with continuous pressure increase, as shown in Fig. 5.3. Also, high-success rates (>92%) were obtained from 330 kPa to 550 kPa with the highest possible success rate of 100% at 385 kPa. However, 49% success rate of motor responses appeared at 165 kPa and 64% at 220 kPa, in contrary to the visual observation that showed 41% success rate at 165 kPa and 55% at 220 kPa. This can be due to muscular contractions of small amplitude that were recorded on the EMG while could not be visually detected and distinguished. Moreover, a decline of the success rate was observed at pressures higher than 385 kPa, which might be ascribed to neuron saturation.

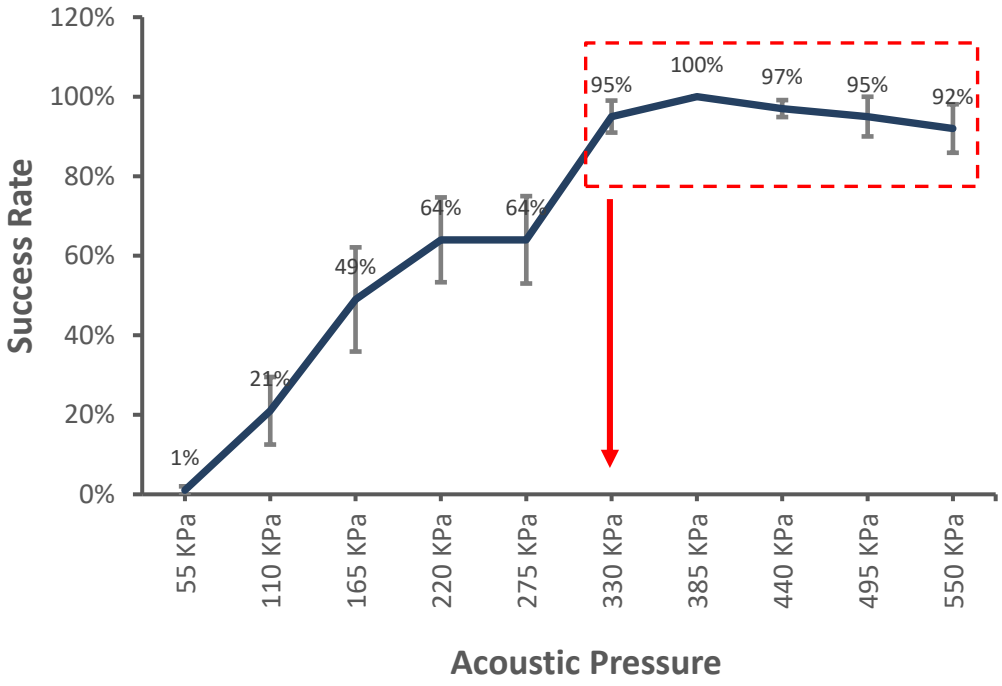


Figure 5.3: Chart representing the success rate of tFUS stimulation at the primary motor cortex over 10 trials obtained using EMG at different acoustic pressures for 10 tested mice. Results represent mean ± SEM.

Elicited EMG signals were post-treated in LabChart 8. A band pass digital filter, between 300 Hz and 1 kHz, was applied to the EMG signal [38]. Then the absolute value with a gain of 1000 was extracted [38]. Smoothing was performed using the triangular (Bartlett) window with 301 samples [38]. A single-event motor evoked potential (MEP) was monitored up to a 590-msec bin for late polysynaptic waves. Averaged MEP amplitudes were then obtained for each individual pressure regardless of motor success. The mean of these averaged amplitudes was then evaluated along with the standard error and plotted as shown in Fig. 5.4. The strength of the elicited EMG signals which was represented by its amplitude differed at diverse acoustic pressures. Measures analyzed using ANOVA and included degree of freedom = 9 and p = 0.001. The result was considered significant when p was less than 0.05.

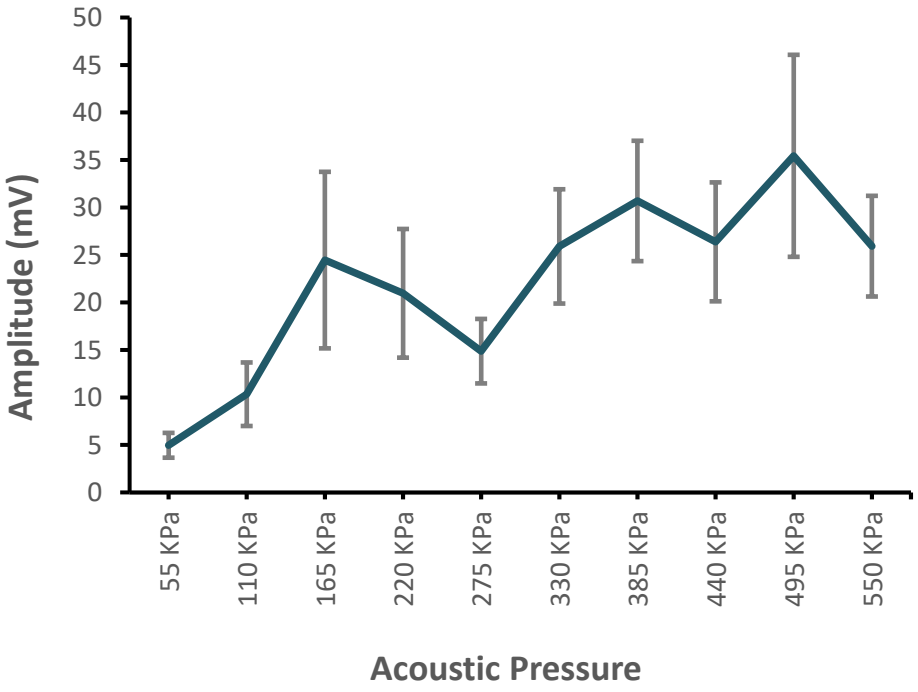


Figure 5.4: Amplitude of EMG signals detected versus acoustic pressure. Results represent mean ± SEM.

Elicited EMG signals varied in shape, amplitude and duration during the same stimulus pressure and even for the same mouse, as illustrated in Fig. 5.5. The following figure shows an example of EMG signals obtained from 6 randomly selected mice at 500 kPa (10 pulses each). The mouse number is written next to the left of each electromyogram. You can also refer to Fig. 5.6 for additional examples. EMG signals also diverged in shape, amplitude and

duration during distinct stimulus pressures. Fig. 5.6 represents examples of electromyograms attained at various acoustic pressures from randomly selected mice. Taking these examples into consideration, we realize that the shape, amplitude and duration of the signals varied with the stimulus pressure.

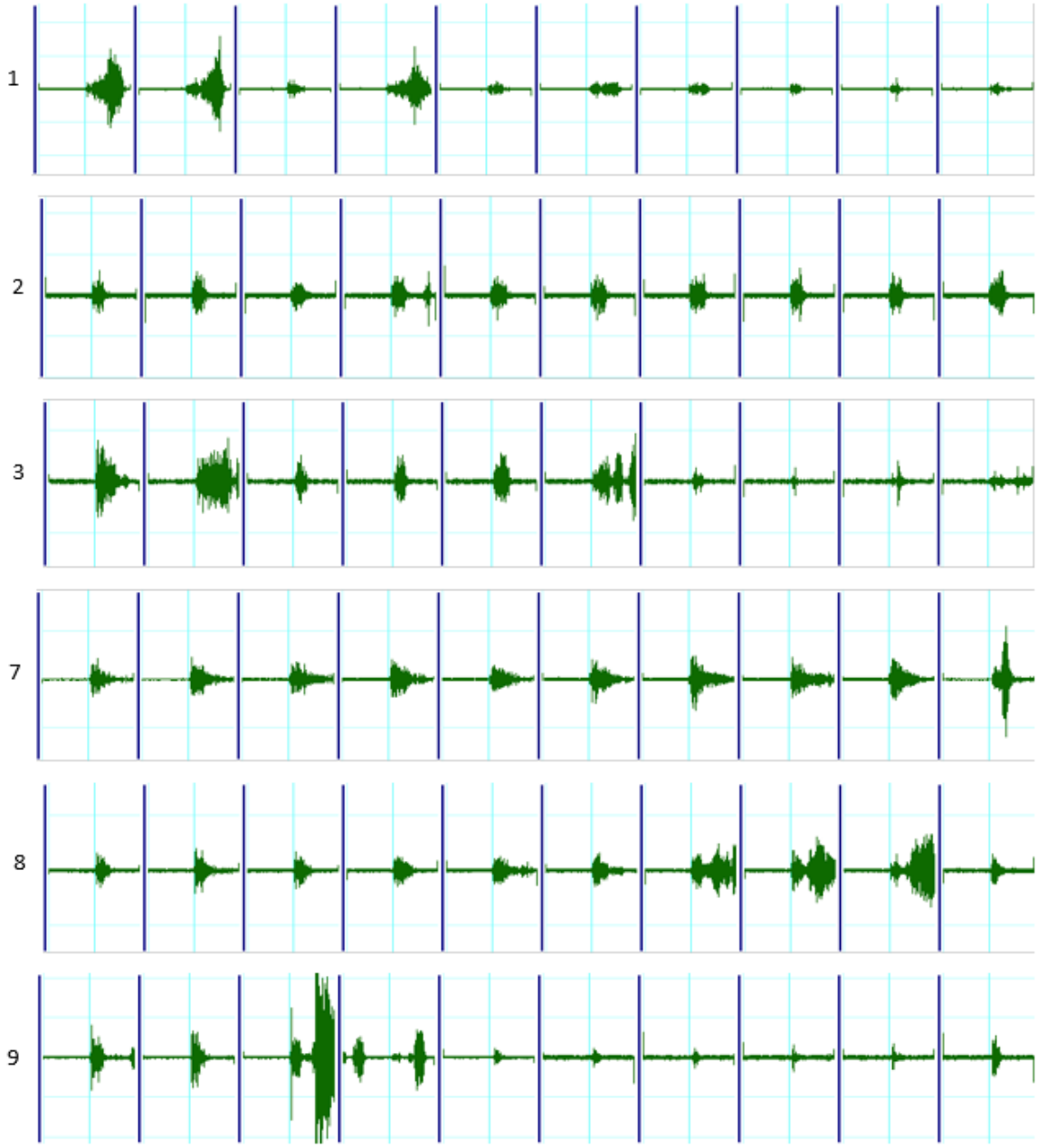


Figure 5.5: Electromyogram of 6 randomly selected mice showing the diversity in shape, amplitude and duration of EMG signals obtained at 500 kPa.

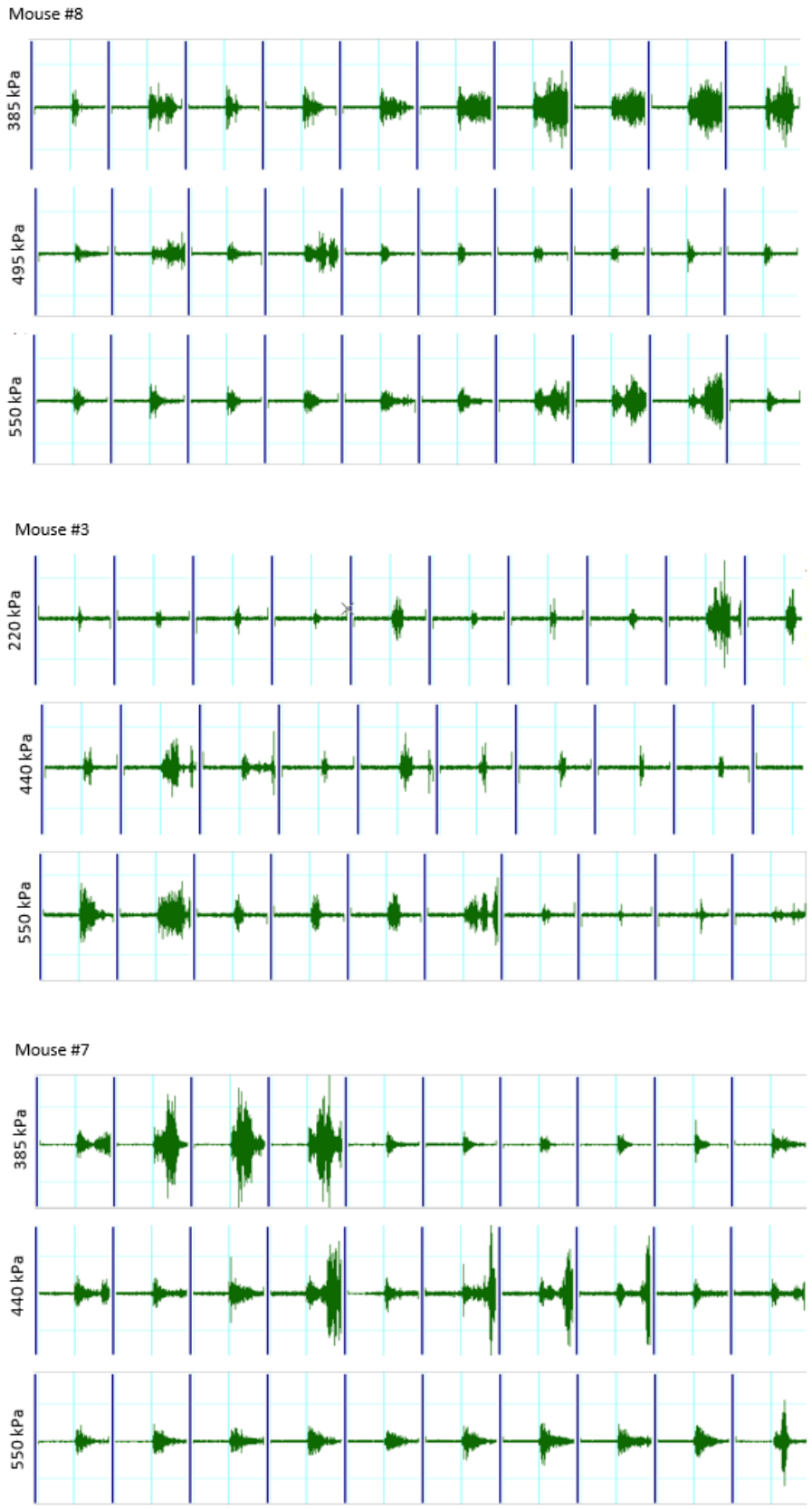


Figure 5.6: Electromyograms obtained at various acoustic pressures from randomly selected mice showing the diversity in shape, amplitude and duration of elicited EMG signals.

Visual observations and EMG recordings showed increased stimulus success and reliability at the pressures ranging from 330 kPa to 550 kPa. However, the motor response detected appeared to be an all-or nothing phenomenon, i.e. stronger stimulus pressure increased the probability of a motor response without affecting the duration or amplitude of the response. This was confirmed by King's work [131].

Based upon the overall data obtained from M1 stimulation, the optimal US parameters for tFUS were determined at 330 kPa (160 msec pulse length), displaying criteria of motor success (91±0.18% to 95±0.12%) compared to lower or higher-pressure steps.

5.1.2 Effect of Anesthesia on Motor Responses

The effect of anesthesia level on the motor responses was evaluated through tFUS stimulation of the primary motor cortex. Fig. 5.7 (a) shows the raw EMG signals detected from the right forelimb following tFUS for 3 different mice (numbered in ascending order from top to bottom) at 220, 330 and 440 kPa, and at 0.5 and 0% anesthesia levels. The red spikes present the sync pulses of stimulation and the green traces represent the EMG signals. At 0.5% isoflurane, out of 9 stimulations, mouse 1 showed no response at all at the 3 pressures used, mouse 2 displayed 2 responses only at 440 kPa, and mouse 3 exhibited 6 responses at 330 and 440 kPa. However, at 0% isoflurane and out of 9 stimulations, mouse 1 demonstrated 6 responses starting at 330 kPa, and mice 2 and 3 showed 9 responses starting from 220 kPa. The results demonstrate that more motor responses were achieved at lower acoustic pressures at 0% anesthesia. Our results confirm others reports who showed that the success of motor cortex stimulation decreases with increasing anesthesia levels [131] [136] [137] [138] [139].

The averaged amplitude of the elicited EMG signals was assessed at different acoustic pressures (220, 330 and 440 kPa) and anesthesia levels (0.5 and 0% isoflurane) for the 3 tested mice as displayed in Fig. 5.7 (b). At 0.5% isoflurane, the amplitude of the EMG signals detected increased with increasing the acoustic pressure with 5.3 mV at 220 kPa, 25 mV at 330 kPa, and 38 mV at 440 kPa. However, at 0% isoflurane, the amplitude of the elicited EMG signals were higher that than obtained at 0.5% isoflurane, with 57 mV at 220 kPa, 93.4 mV at 330 kPa, and 83 mV at 440 kPa. Also at 0% isoflurane, the amplitude of the EMG signals increased

with increasing the acoustic pressure from 220 to 330 kPa, but it then slightly declined with continuous pressure increase at 440 kPa which might be related to neuron saturation.

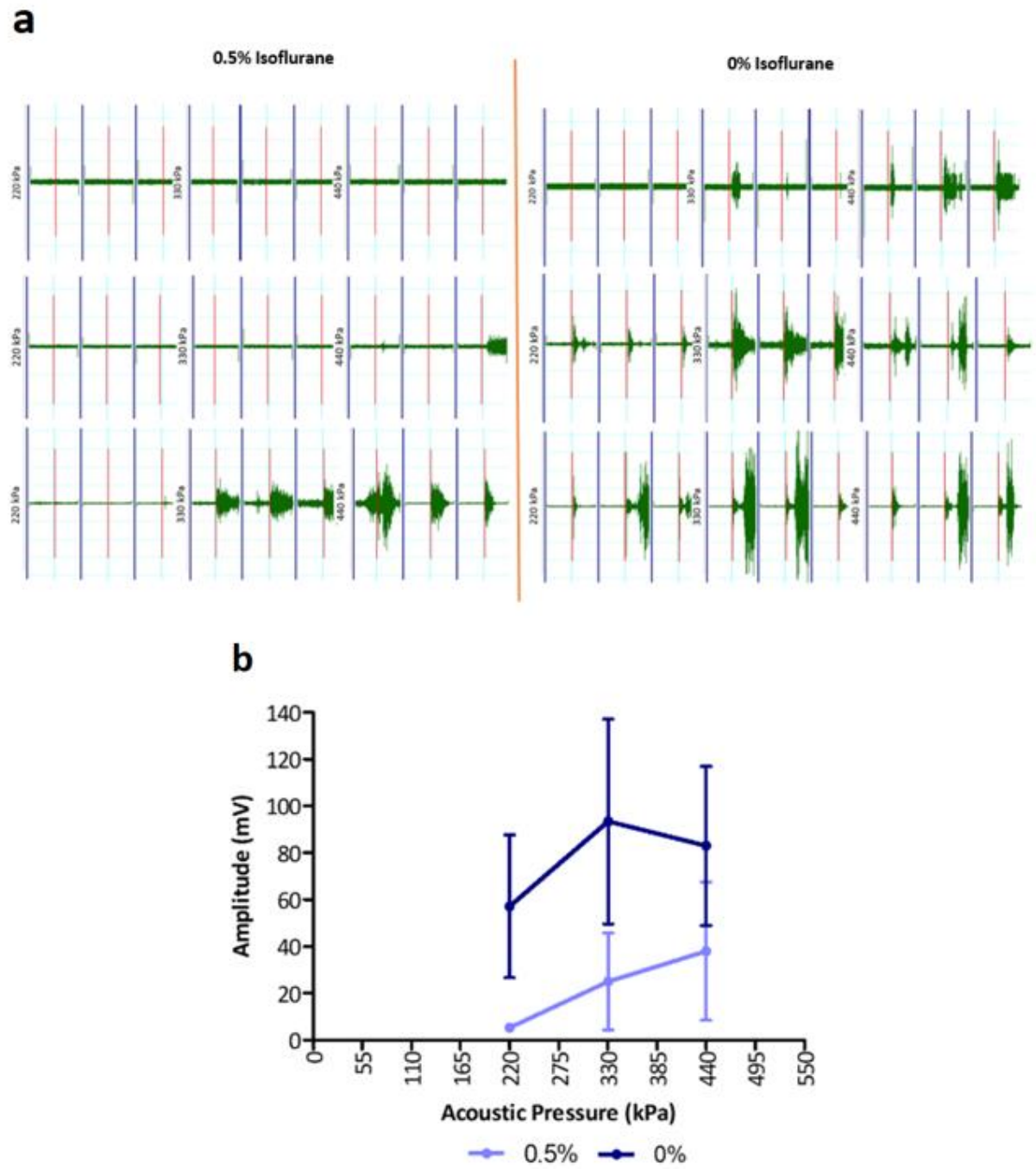


Figure 5.7: tFUS stimulation targeting primary motor cortex induced motor response depending on anesthesia level. Motor responses following tFUS stimulation of the left primary motor cortex were estimated by electromyography through (a) Visual detection of a signal and (b) Evaluation of the averaged amplitude according to acoustic pressure and anesthesia level. Results in (b) represent mean ± SEM (n = 3 mice).

5.1.3 Safety and Effect of ACC Stimulation

Open field test provides qualitative and quantitative measurements of locomotion and exploration. It gives information about locomotor activity levels, willingness to explore, as well as information about anxiety and stereotypic behaviors. Thus, it presents information about the general behavior and condition of the animal. Open field tests performed demonstrated that tFUS has no side effects on locomotor activity as it did not show a significant locomotion effect. Statistical analysis was performed using ANOVA. The result was considered significant when p-value was less than 0.05. As displayed in Fig. 5.8, the distance moved by mice of control group was nearly the same as the distance moved by mice that received one tFUS stimulation at three different tests sessions. However, a significant difference (degree of freedom = 4 and p = 0.015) was temporary detected in the first test session between the above mentioned mice and the mice that received five tFUS stimulations noting that this effect could be linked to the small sample size of mice used for five stimulations i.e. 4 mice. Also, this significant difference could also be related to the possibility that mice might be tired or showing anxiety after 5 consecutive days of undergoing experimentation involving being prepared for stimulation, undergoing anesthesia, and receiving brain stimulation.

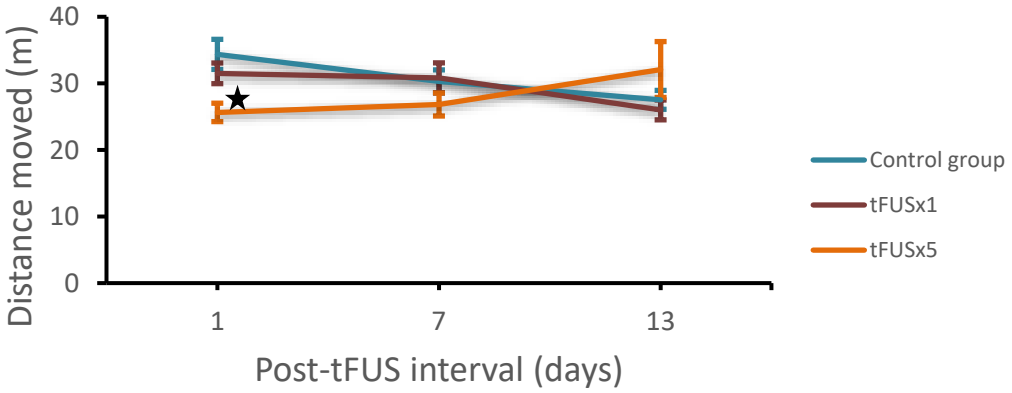
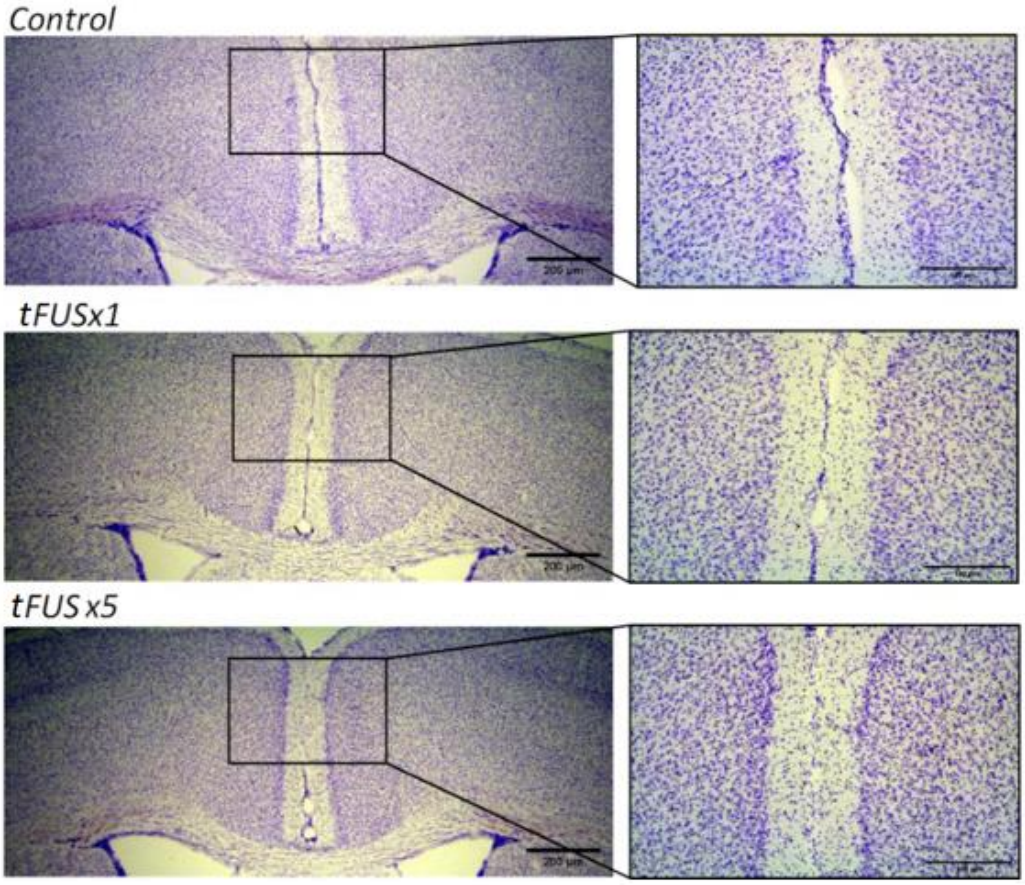
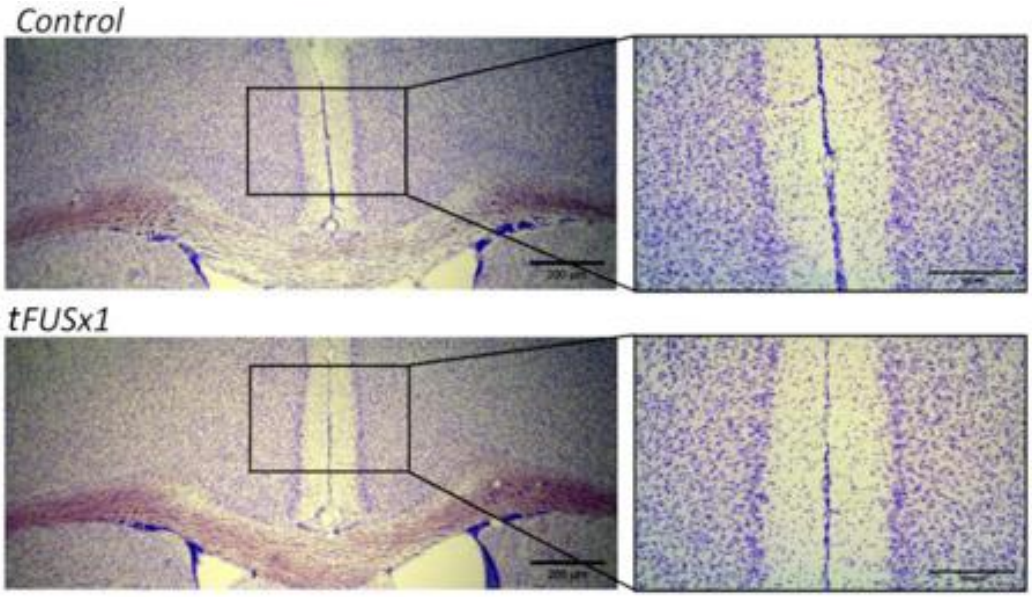


Figure 5.8: Distance moved by mice in the open-field test at three different intervals from tFUS. Results represent mean ± SEM.

Images of Nissl staining of the cortical tissue corresponding to the anterior cingulate cortex at 90-minutes and 14 days post-tFUS interval are shown in Figs. 5.9a and 5.9b respectively. No lesions or injuries were detected in these images.



(a)



(b)

Figure 5.9: tFUS targeting the anterior cingulate cortex showed no significant effect on tissue integrity over a 2-week post-tFUS period. (a) Representative images of Nissl staining of the cortical tissue (area corresponding to anterior cingulate cortex) at 90-minutes post-tFUS interval at x25 and x100 magnification. (b) Representative images of Nissl staining of the cortical tissue (area corresponding to anterior cingulate cortex) at 14-days post-tFUS interval at x25 and x100 magnification.

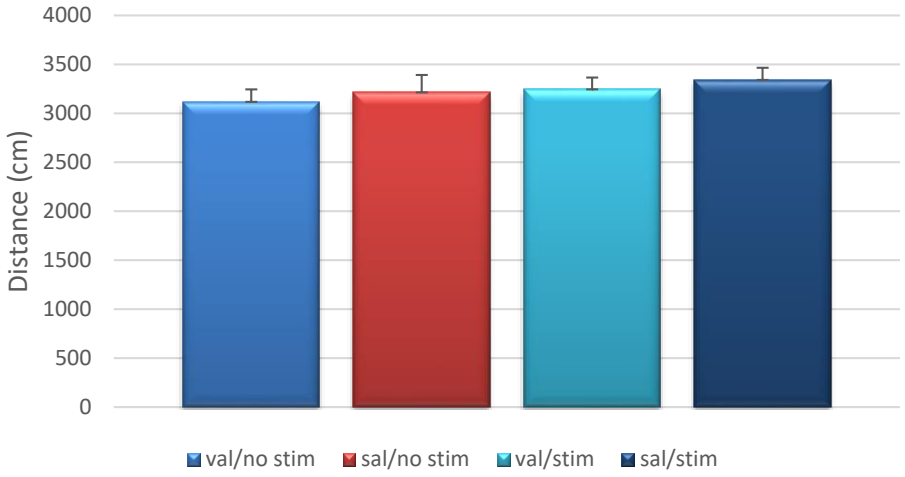
An effective and safe protocol for ultrasonic neuromodulation using the 500 kHz single element transducer was showed in preclinical research. A detailed and reliable methodology for successful tFUS in mice was presented and could be directed particularly to young students and researchers who are starting in the brain stimulation field and encountering practical challenges. Existing tFUS studies do not show the method for locating the brain region to be stimulated non-invasively, which is a very challenging phase prior to stimulation. In our project, a simple and reliable non-invasive method for locating and targeting brain regions in mice was offered. Additionally, we validated that a successful stimulation could be achieved using 0 anesthesia level through which we confirmed other's reports who showed that the success of motor cortex stimulation decreases with increasing anesthesia levels [136] [131] [137] [138]. We also found that the success rate of motor responses increases with increasing the acoustic pressure until it reaches its peak and then it weakly reduces with continuous pressure increase. Our observation of this decline of the success rate which might be ascribed to neuron saturation that in turn could be related to the acoustic pressure or stimulation duration [135], supports other's work who showed that normally the neuron response increases with acoustic pressure until the response reaches saturation [132] [133] [134]. Moreover, we found that high success of tFUS is achieved at pressures ranging from 330 to 550 kPa. Our results also confirmed the all-or-nothing motor response phenomenon reported by some researches [131].

5.2 VPA MODEL AND EFFECT OF STIMULATION

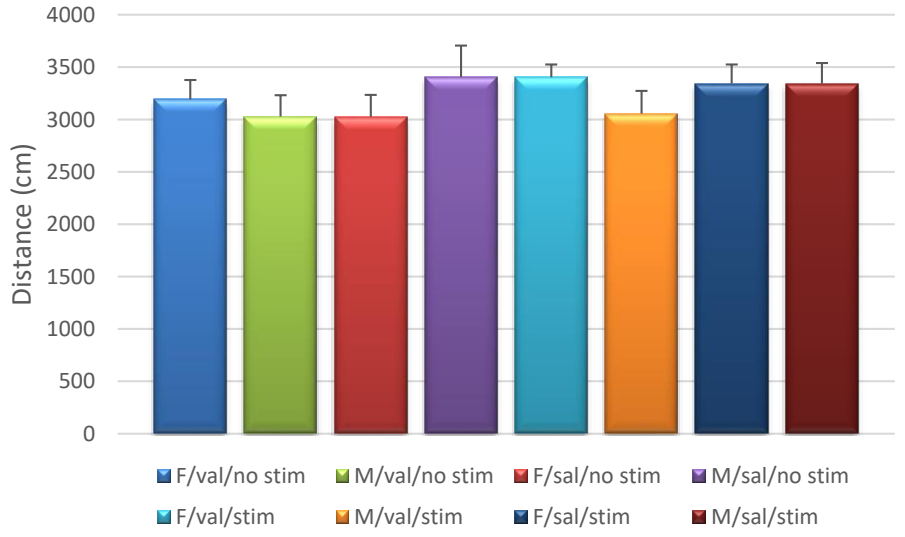
5.2.1 3-Chamber Interaction Test

The mean distance covered by the mouse throughout the 10-minutes sociability test duration was evaluated for all tested mice. Fig. 5.10 (a) shows the distance moved in cm versus mice groups that are categorized into valproic or control, and sham or stimulated groups. "val/ no stim" refers to VPA mice that received no stimulation (sham stimulation), "sal/ no stim" refers to saline (control) mice that received sham stimulation, "val/ stim" refers to VPA mice that received stimulation, and "sal/ stim" refers to control mice that received stimulation. No significant difference in the distance moved was detected between these four mice groups using Kruskall-Wallis analysis (all $p>0.05$, non-significant) achieved through

Statview 4.57 software (StatView® SAS Institute Inc.). With an interest to determine the existence of a gender effect, the mean distance was reanalyzed using the same method and software, and plotted in Fig. 5.10 (b), but with females separated from males' groups (groups used as described previously in details in section 4.4.2). In this chart, F refers to female, M: male, val: VPA, sal: saline or control, stim: stimulated, no stim: sham or no stimulation. Similarly, no variation in the distance moved between saline and VPA groups as well as between stimulated and non-stimulated groups for males and females was observed through Kruskall-Wallis analysis (all $p>0.05$, non-significant).



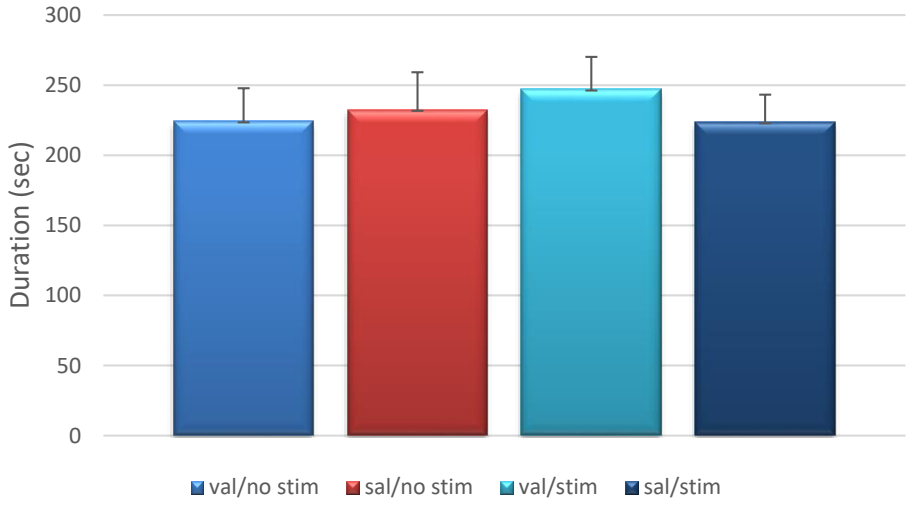
(a)



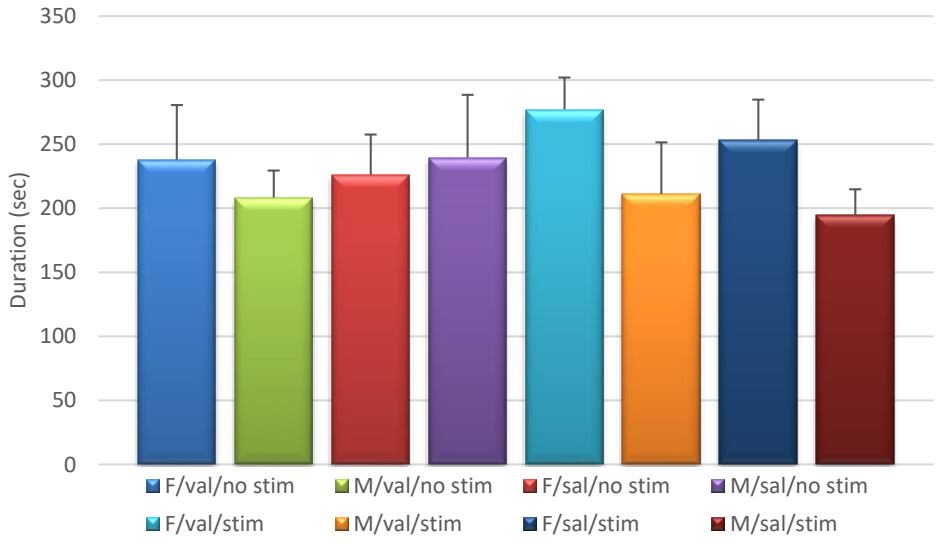
(b)

Figure 5.10: Distance covered by mice in 10-minutes sociability test period with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM.

The time duration spent by the tested mouse in the social chamber was also determined for the same mice groups used previously i.e. grouping both genders in Fig. 5.11 (a) and differentiating females from males in Fig. 5.11 (b). Kruskall-Wallis statistical analysis did not show significant difference in the time duration spent in the social chamber between saline and VPA groups as well as between stimulated and non-stimulated groups for males and females (all $p>0.05$, non-significant).



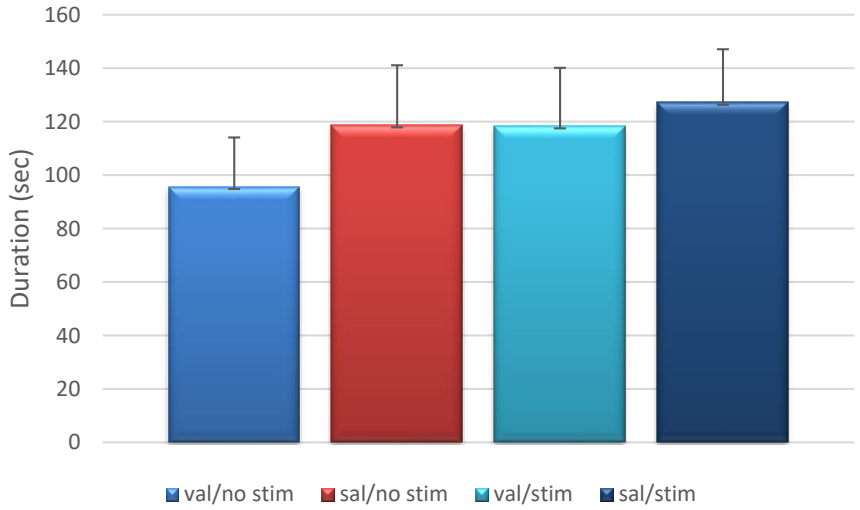
(a)



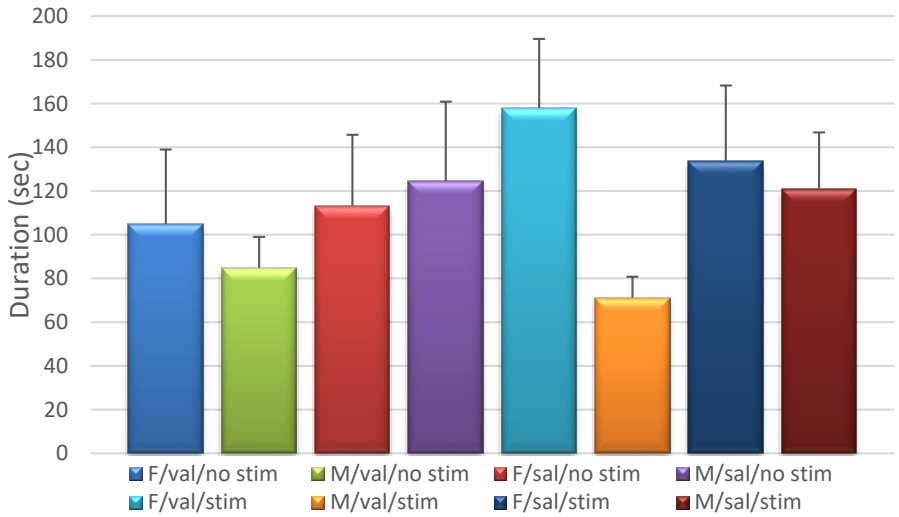
(b)

Figure 5.11: Duration of time spent by mice in the social chamber with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM.

Additionally, the time duration spent by the mouse in the social contact zone was assessed for the same mice groups used before. Fig. 5.12 (a) shows the time duration spent in the social contact zone by mice groups of both genders and Fig. 5.12 (b) represents the time duration spent in the social contact zone by mice while differentiating females from males. Similarly, here, no variation in the time duration spent in the social contact zone between saline and VPA groups, and stimulated and non-stimulated groups for males and females was realized through Kruskall-Wallis analysis (all $p>0.05$, non-significant).



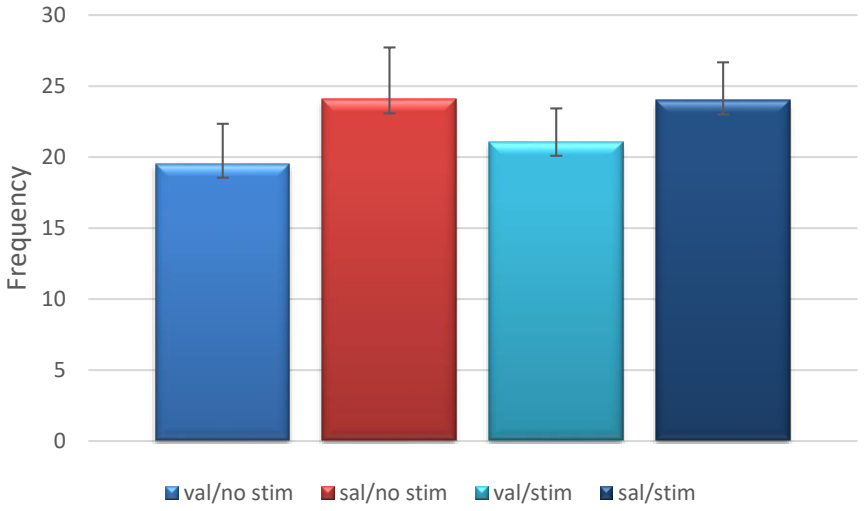
(a)



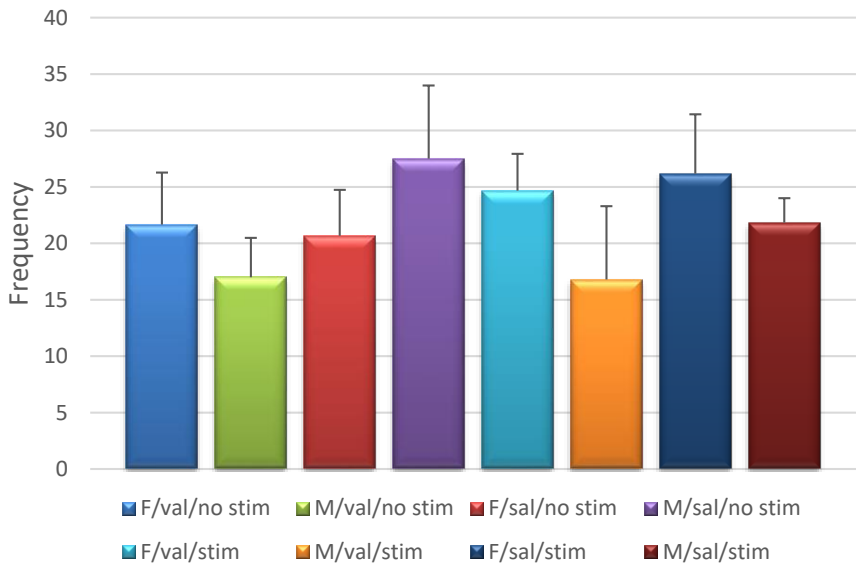
(b)

Figure 5.12: Duration of time spent by mice in the social contact zone with (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM.

Moreover, the frequency of entries in the social contact zone was measured for the same mice groups utilized previously while gathering both genders in Fig. 5.13 (a) and separating females and males' groups in Fig. 5.13 (b). Likewise, no variation in the frequency of entries in the social contact zone between saline and VPA groups as well as between stimulated and non-stimulated groups for males and females was noted through Kruskall-Wallis statistical analysis (all $p>0.05$, non-significant).



(a)



(b)

Figure 5.13: Frequency of entries in the social contact zone for (a) Males and females together and (b) Males and females separated. Results represent mean ± SEM.

5.2.2 Free Social Interaction Test

a) Mice Profiler Data Analysis for Social Interaction

The behavior of each tested mouse was analyzed for the first 4 minutes of interaction, where 12 social events were discriminated with number and duration. The events noticed included: minimum distance between the tested mouse and the visitor is < 1 cm, distance between the centers of both mice heads is < 2 cm, distance between the center of the oral's head of the visitor mouse and the start of the genital's tail of the tested mouse is < 1.5 cm, tested mouse goes to visitor starting from no contact finishing with contact, visitor mouse goes to the tested mouse and visitor escapes, visitor mouse stops, visitor mouse speed > tested animal speed and visitor get to tested mouse, visitor mouse is going to tested mouse and finishing with contact while starting from no contact, tested mouse escapes from the visitor starting from contact finishing without contact, tested mouse gets to visitor and visitor escapes, visitor mouse gets to tested mouse and tested mouse escapes, and tested mouse follows the visitor mouse. All events were studied during the first 2 minutes of interaction then from 2 to 4 minutes to evaluate possible evolution across session, and overall from 0 to 4 minutes.

Using Statview analysis, only few limited differences in the events between VPA and control (saline) mice were detected, as shown in Fig. 5.14, including: i) the number of events of having the minimum distance between the tested mouse and the visitor is < 1 cm from 2 to 4 minutes in (a) (Mann-Whitney: $z = -3.556$, $p = 0.0004$), ii) the duration of the events of having the visitor mouse goes to the tested mouse and visitor escapes from 0 to 4 minutes (Mann-Whitney: $z = -2.339$, $p = 0.0193$) in (b), iii) the number of events of having the tested mouse goes to visitor starting from no contact finishing with contact from 2 to 4 minutes (Mann-Whitney: $z = -1.969$, $p = 0.0489$) in (c), as well as the duration of this event from 2 to 4 minutes (Mann-Whitney: $z = -2.400$, $p = 0.0164$) and from 0 to 4 minutes (Mann-Whitney: $z = -2.154$, $p = 0.0312$) in (d). Therefore, little difference is noticed between VPA and control mice.

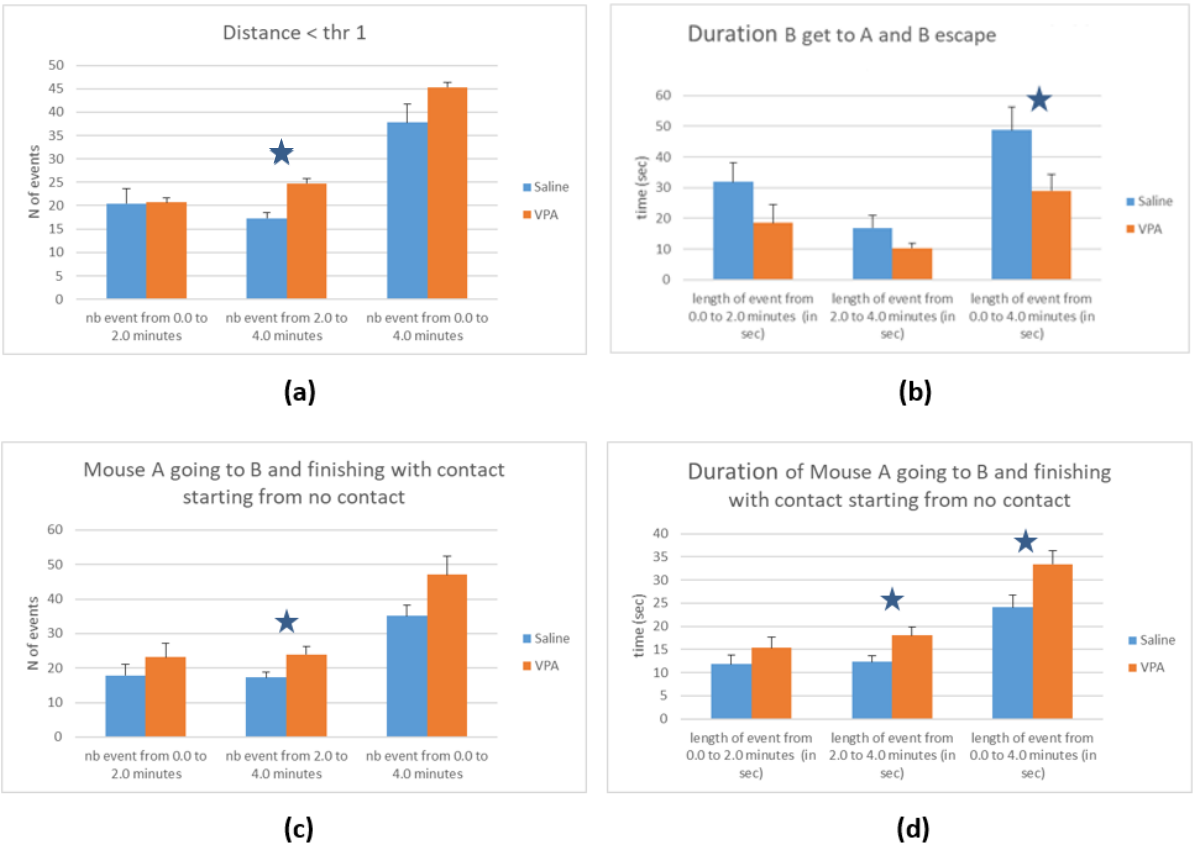
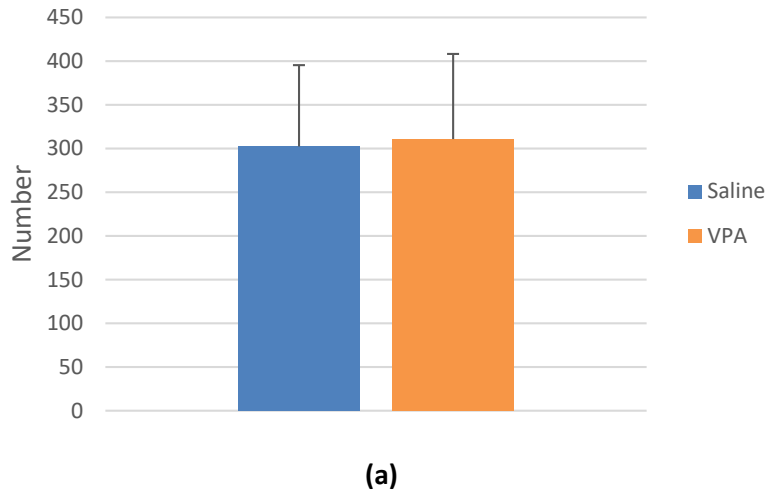


Figure 5.14: Different events evaluated during free social interaction test. A refers to the tested mouse and B indicates the visitor mouse. Results represent mean ± SEM.

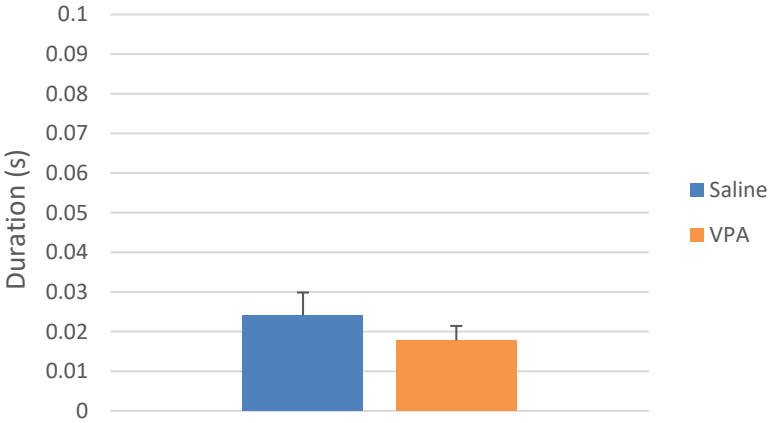
b) Ultrasonic Vocalization for Communication

The emitted ultrasound vocalizations, between 20 and 100 kHz, in mice were recorded during free interactions involving male-male and female-female interactions. Fig. 5.15 shows the mean number of ultrasonic vocalizations emitted during the first 2 minutes of interaction and their mean duration. No significant difference was detected in the number and duration of the recorded ultrasonic vocalizations between control and VPA mice according to the statistical analysis performed using Statview (all $p>0.05$, non-significant).

Different ultrasonic vocalizations subtypes exist [140]. The percentage of the different vocalizations subtypes emitted by control and VPA mice are shown in Fig. 5.16 with USVs emitted by control mice to the left and by VPA mice to the right. No significant difference was observed in the emitted vocalizations subtypes between control and VPA mice according to the statistical analysis achieved using Shapiro-Wilk test (all $p>0.05$, non-significant).



(a)



(b)

Figure 5.15: Charts representing ultrasonic vocalizations emitted by VPA and control mice during the first 2 minutes of interaction with (a) Number of USVs emitted and (b) Their duration. Results represent mean ± SEM.

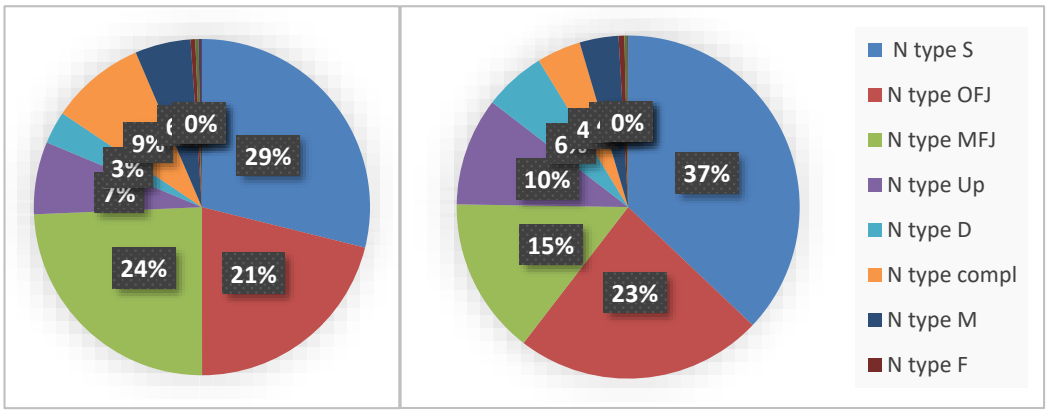
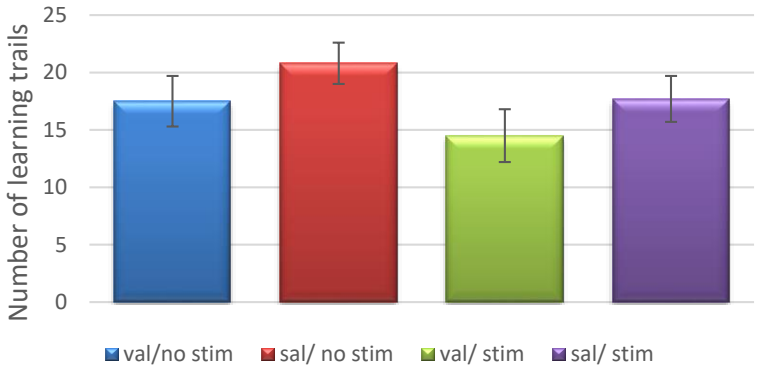


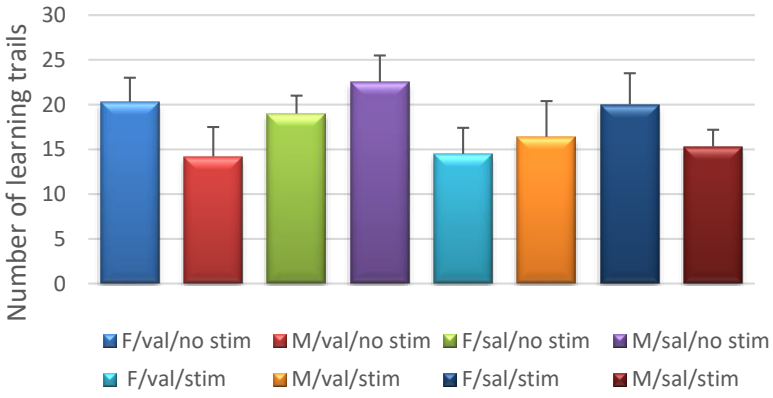
Figure 5.16: Percentage of ultrasonic vocalizations subtypes emitted by control mice to the left and VPA mice to the right.

5.2.3 Reversal Learning in Water Maze

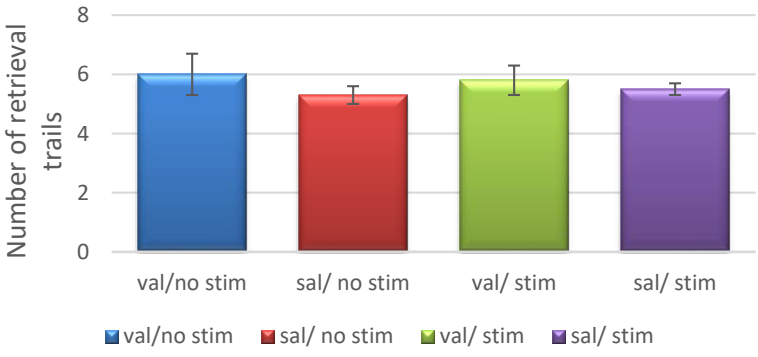
The number of trails detected during the learning phase, retrieval phase, and reversal learning phase for the 4 and 8 mice groups used previously in the 3-chamber test is displayed in Fig. 5.17. Statistical analysis performed through Statistica showed no variation between all the mice groups utilized in all three phases of the test, demonstrating that no gender effect, no VPA effect, and no stimulation effect exist (all $p>0.05$, non-significant).



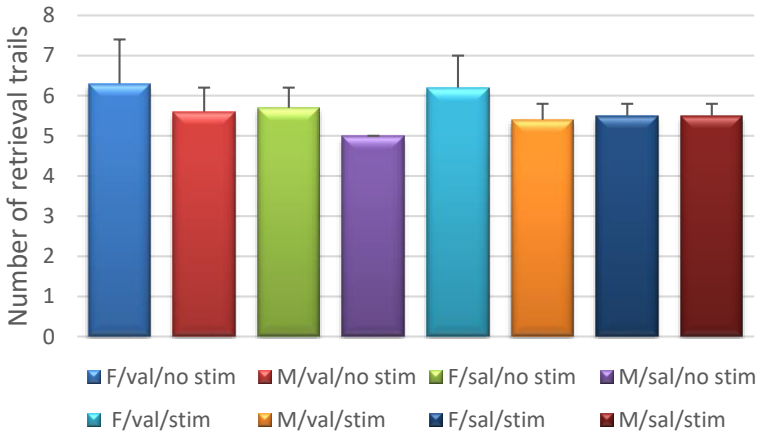
(a)



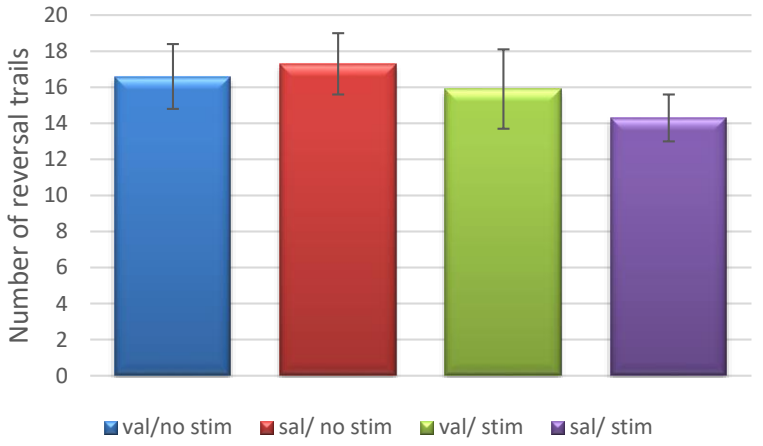
(b)



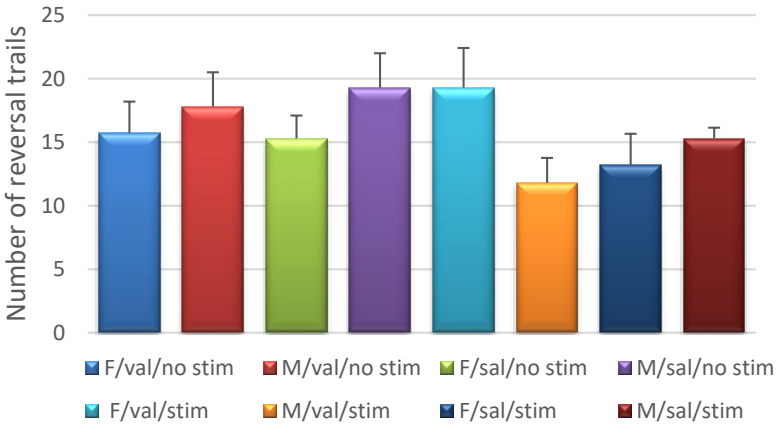
(c)



(d)



(e)

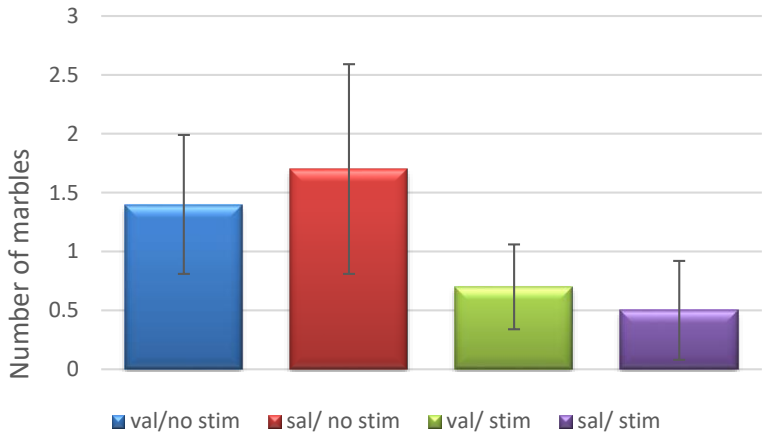


(f)

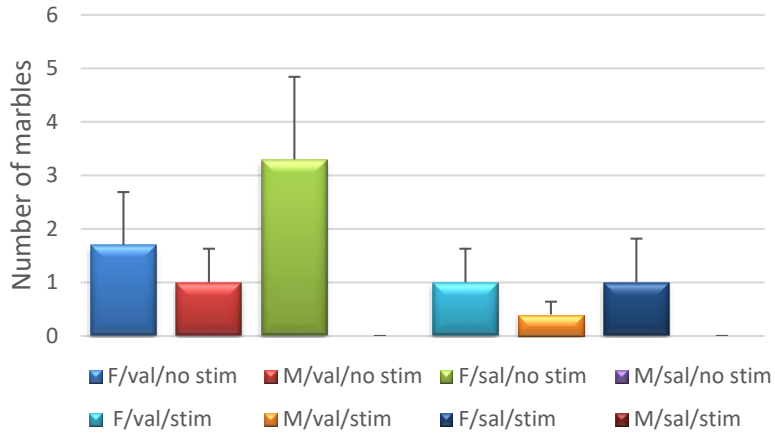
Figure 5.17: The number of trails detected during (a) Learning phase for 4 mice groups, (b) Learning phase for 8 mice groups, (c) Retrieval phase for 4 mice groups, (d) Retrieval phase for 8 mice groups, (e) Reversal learning phase for 4 mice groups, (f) Reversal learning phase for 8 mice groups. Results represent mean ± SEM.

5.2.4 Marble Burying Test

The number of marbles buried during the 30-minutes test duration was measured for the four mice groups involving gathering males and females (refer to Fig. 5.18 (a)), and then for the 8 mice groups with females separated from males (refer to Fig. 5.18 (b)). Statistica analysis through ANOVA showed a significant gender effect ($p = 0.019$). However, no variation was detected between VPA and control mice, and between stimulation and sham stimulation groups (all $p>0.05$, non-significant), implying that no VPA effect and no stimulation effect existed. Furthermore, the gender effect detected appeared to be linked to stimulation and not related to VPA.



(a)

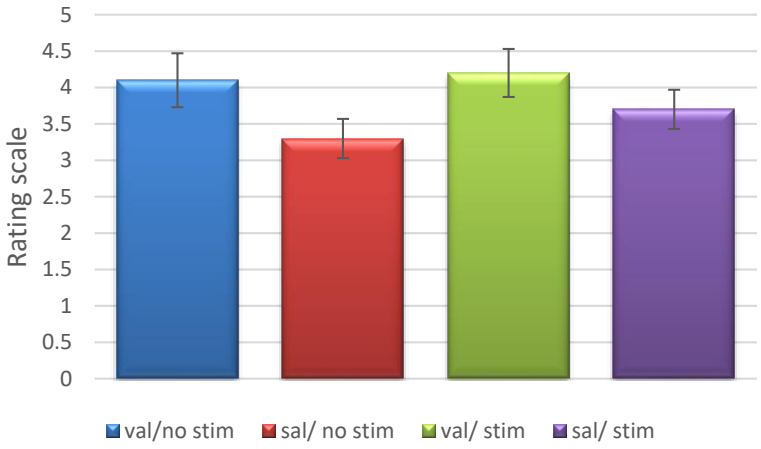


(b)

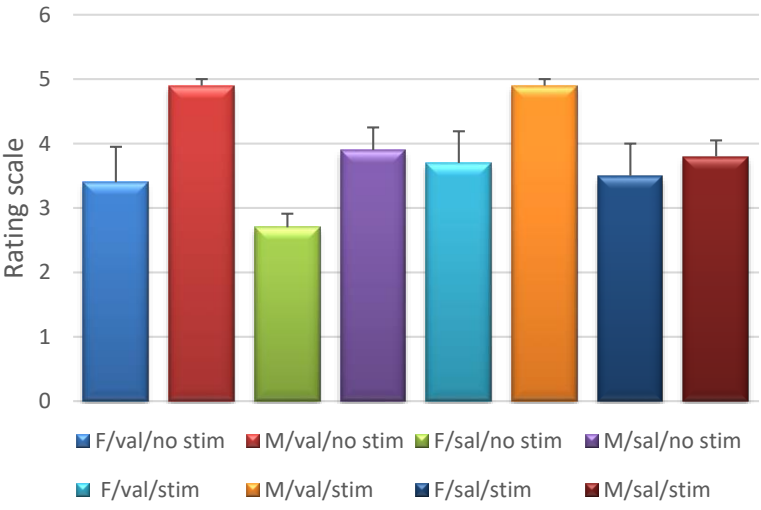
Figure 5.18: The number of marbles buried during the 30-minutes test duration versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM.

5.2.5 Nesting Test

Nestlet weight was monitored at 5 hours and 24 hours after the new material is provided for calculating the rating scale. The mean rating scale calculated at 5 hours was plotted for the 4 mice groups and 8 mice groups used before in Fig. 5.19. Statistica analysis demonstrated significant difference between VPA and control mice ($p = 0.0088$) at 5 hours rating. However, no variation was noticed between males and females, and stimulated and non-stimulated groups (all $p>0.05$, non-significant).



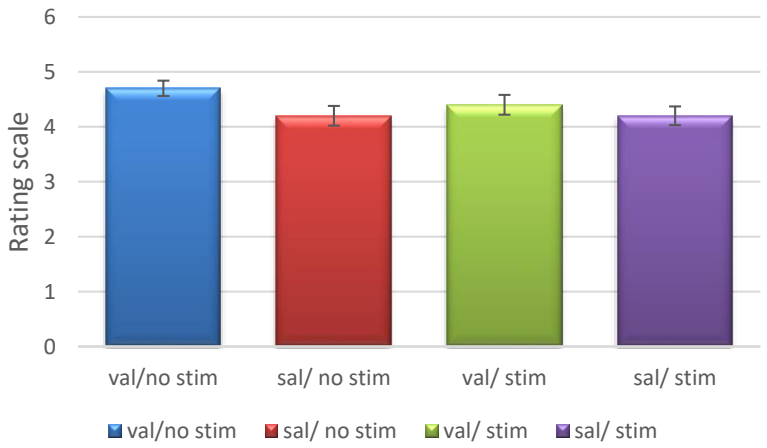
(a)



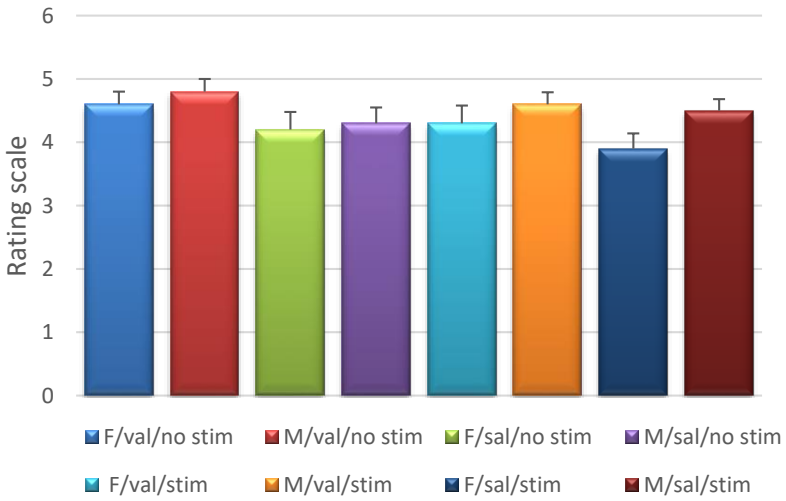
(b)

Figure 5.19: The rating scale calculated at 5 hours versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM.

Similarly, the mean rating scale was assessed at 24 hours for the same mice groups used before and displayed in Fig. 5.20. Statistica analysis showed significant difference between VPA and control mice (p = 0.042), while no gender effect and no stimulation effect was observed.



(a)



(b)

Figure 5.20: The rating scale calculated at 24 hours versus (a) 4 mice groups gathering females and males together (b) 8 mice groups with females and males separated. Results represent mean ± SEM.

Several behavioral tests have been applied to evaluate the VPA model used and the effect of stimulation performed. These tests involved 3-chamber interaction test, free social interaction test, reversal learning in water maze test, marble burying test and nesting test.

Statistical analysis of the behavioral tests done which was achieved through ANOVA, Statistica and Statview did not show significant differences between VPA and control mice groups throughout the listed behavioral tests except for only one test. Variation was only observed between these two mice groups through the nesting test. So, lack of VPA model effect was noticed, contrary to what was expected, suggesting that the VPA model used did not work. One reason for having VPA mice with no autistic phenotypes could be linked to error in the gestation age at which the VPA was injected. The pregnant female mice have been purchased with an identified gestation day (the mating was not performed in our lab). ip injection of VPA (600 mg/kg) was planned to be done at the gestation day 12.5, however, we might have injected them at the wrong gestation day due to error in the information we have received about the pregnant females. On the other hand, the absence of distinction between stimulated and non-stimulated mice groups obtained throughout almost of the behavioral tests achieved, confirmed the safety of the tFUS protocol and methodology applied.

CHAPTER 6

CONCLUSION AND FUTURE WORK

The ultimate goal of this project was to examine and assess ultrasound neurostimulation technique as a therapeutic tool in a VPA animal model of ASD. To do so, a first part of the study was dedicated to setup a detailed and precise procedure for brain stimulation of the motor cortex. This step is essential to determine the required ultrasound parameters for therapy.

Once the required parameters get identified, another main objective was to study the effect and safety of stimulation on the anterior cingulate cortex, a deep major brain structure implicated in various psychiatric diseases such as autism spectrum disorder and depression.

The implementation and optimization of tFUS stimulation required targeting the primary motor cortex at various acoustic pressures and assessing the motor evoked responses at the two forelimbs, for determining the minimum stimulation thresholds from which the activation of the neuronal population of the target area is effective. This has been achieved by real time visual observation of muscular responses on one upper limb and EMG elicited from the second upper limb. Evaluating the impact and safety of tFUS required stimulating healthy mice at the ACC followed by behavioral open field testing and histological analysis.

The use of animal model was an essential step in the implementation and characterization of new therapeutic strategies. Since ASD is a complex disorder that is strictly linked to human, so it was not possible to summarize all the dimensions of ASD in rodents or "autistic-like" mice. VPA animal model was chosen for our stimulation experiments as it is one of the mostly used models in recent research. Examining the phenomenological validation of the VPA animal model used and the effects of tFUS was performed using wide variety of behavioral tests. Set of tests used to measure behavioral deficits presenting conceptual analogies with several symptoms of ASD at the cognitive and social levels. However, statistical analysis of the tests done did not show significant differences between VPA and control mice as variation was observed between these two groups through only the nesting test. So, lack of VPA model

effect was noticed, contrary to what was expected, proposing that the VPA model used did not work. On the other hand, the absence of distinction between stimulated and non-stimulated mice obtained through the same statistical analysis, confirmed the safety of the tFUS protocol and methodology applied.

An effective and safe protocol for ultrasonic neuromodulation using the 500 kHz single element transducer was shown in preclinical research. A detailed and reliable methodology for successful ultrasonic neurostimulation in mice was presented and could be directed particularly to young students and researchers who are starting in the brain stimulation field and encountering practical challenges. Existing ultrasonic neurostimulation studies do not explain the method for locating the brain region to be stimulated non-invasively, which is a very challenging phase prior to stimulation. In our project, a simple and reliable non-invasive method for locating and targeting brain regions in mice was offered. This was achieved and confirmed using healthy mice that underwent surgical opening of the head as well as through the high success rates of stimulation reached. Additionally, we validated that a successful stimulation could be achieved using 0 anesthesia level. We also found that the success rate of motor responses increases with increasing the acoustic pressure until it the response saturated. Moreover, we demonstrated that high success of ultrasonic neurostimulation is achieved at pressures ranging from 330 to 550 kPa. Furthermore, we confirmed the all-or-nothing motor response phenomenon reported by some researches. Evaluation of the impact and safety of tFUS was mainly confirmed by an analysis of the motor and exploratory behavior of healthy mice stimulated at the ACC, as well as histological analysis.

Conducting this project provided us with an opportunity to acquire new knowledge on the applicability of this technology and examine its therapeutic potential in ASD. Our thesis is characterized by its originality as it is the first study that examines the application of tFUS in the case of autism spectrum disorder. It is a novel project through which an in-vivo tFUS system is optimized and adapted to “autistic-like” mice, and a major deep brain region implicated in ASD, the anterior cingulate cortex, is stimulated. We have also experimented developing a preclinical model of autism i.e. the VPA model.

We believe that this project will represent the launch of a long-term research project and will therefore serve as an initial reference for several following studies that would extend

and validate this approach in the same model as well as in other models of ASD and then ultimately in humans. Finally, this project thus presents a unique opportunity to initiate a new research activity with significant translational potential at the medical level: offering new prospects for autism therapy.

In future work, the focus will be on re-experimenting and re-developing VPA model for providing a validated model to be able to re test the stimulation and its effects. Additional studies for examining this ultrasonic approach on different models of ASD, which could be of genetic etiological factor such as BTBR, would be effective. Moreover, ultrasonic stimulation can be tested on different rodent's species such as rats. Furthermore, the functional and neurobiological characterizations underlying the effects induced by the model and tFUS could be carried out along with the behavioral tests. This can be achieved through recording the activity of neuronal populations in in-vivo calcium imaging. Additionally, immunohistochemistry can be used to assess the integrity of markers involved in the anomalies of ASD and test whether tFUS has been able to affect these systems in inducing possible effects. Noting that we have planned and started performing this step, however we could not obtain the results due to the COVID-19 pandemic.

REFERENCES

[1] J. Crawley, "Translational Animal Models of Autism and Neurodevelopmental Disorders," *Dialogues in Clinical Neuroscience,* vol. 14, no. 3, p. 293–305, 2012.

[2] R. Zhang, J. Zhou, J. Ren, S. Sun, Y. Di, H. Wang, X. An, K. Zhang, J. Zhang, Z. Qian, M. Shi, Y. Qiao, W. Ren and Y. Tian, "Transcriptional and Splicing Dysregulation in the Prefrontal Cortex in Valproic Acid Rat Model of Autism," *Reproductive Toxicology,* vol. 77, pp. 53-61, 2018.

[3] K. Iwata, H. Matsuzaki, N. Takei, T. Manabe and N. Mori, "Animal Models of Autism: An Epigenetic and Environmental Viewpoint," *Journal of Central Nervous System Disease,* vol. 2, p. 37–44, 2010.

[4] S. LeClerc and D. Easley, "Pharmacological Therapies for Autism Spectrum Disorder: A Review," *Pharmacy and Therapeutics,* vol. 40, no. 6, p. 389–397, 2015.

[5] T. Kim, J. E. Ryu and G. H. Bahn, "Brain Stimulation and Modulation for Autism Spectrum Disorder," *Hanyang Medical Reviews,* vol. 36, pp. 65-71, 2016.

[6] D. Papadopoulos, "Mothers' Experiences and Challenges Raising a Child with Autism Spectrum Disorder: A Qualitative Study," *Brain Sci,* vol. 11, no. 3, p. 309, 2021 Mar.

[7] A. Ludlow, C. Skelly and P. Rohleder, "Challenges Faced by Parents of Children Diagnosed with Autism Spectrum Disorder," *Journal of Health Psychology,* vol. 17, no. 5, pp. 702-711, 2011.

[8] E. Rezayat and I. Toostani, "A Review on Brain Stimulation Using Low Intensity Focused Ultrasound," *Basic and Clinical Neuroscience,* vol. 7, no. 3, p. 187–194, 2016.

[9] P. Cristancho, K. Akkineni, J. Constantino, A. Carter and J. O'Reardon, "Transcranial Magnetic Stimulation (TMS) in a 15 Year Old Patient with Autism and Comorbid Depression," *Journal of ECT,* vol. 30, no. 4, 2014.

[10] H. Schneider and J. Hopp, "The Use of the Bilingual Aphasia Test for Assessment and Transcranial Direct Current Stimulation to Modulate Language Acquisition in Minimally Verbal Children with Autism," *Clinical Linguistics and Phonetics,* vol. 25, no. 6-7, pp. 640-654, 2011.

[11] M. Casanova, J. Baruth, A. El-Baz, A. Tasman, L. Sears and E. Sokhadze, "Repetitive Transcranial Magnetic Stimulation (rTMS) Modulates Event-Related Potential (ERP) Indices of Attention in Autism," *Transl Neurosci,* vol. 3, no. 2, p. 170–180, 2012.

[12] T. Warwick, J. Griffith, B. Reyes, B. Legesse and M. Evans, "Effects of Vagus Nerve Stimulation in a Patient with Temporal Lobe Epilepsy and Asperger Syndrome: Case Report and Review of the Literature," *Epilepsy & Behavior,* vol. 10, no. 2, pp. 344-347, 2007.

[13] E. Sokhadze, A. El-Baz, L. Sears, I. Opris and M. Casanova, "rTMS Neuromodulation Improves Electrocortical Functional Measures of Information Processing and Behavioral Responses in Autism," *Frontiers in Systems Neuroscience,* vol. 8, no. 134, 2014.

[14] V. Sturm, O. Fricke, C. Bührle, D. Lenartz, M. Maarouf, H. Treuer, J. Mai and G. Lehmkuhl, "DBS in the Basolateral Amygdala Improves Symptoms of Autism and Related Self-Injurious Behavior: a Case Report and Hypothesis on the Pathogenesis of the Disorder," *Frontiers in Human Neuroscience,* vol. 6, no. 341, 2013.

[15] G. D'Urso, R. Ferrucci, D. Bruzzese, A. Pascotto, A. Priori, C. A. Altamura, S. Galderisi and C. Bravaccio, "Transcranial Direct Current Stimulation for Autistic Disorder," *Biological psychiatry,* vol. 76, no. 5, 2013.

[16] S. G. Sajith and S. F. Liew, "Response to Electroconvulsive Therapy in Patients With Autism Spectrum Disorder and Intractable Challenging Behaviors Associated With Symptoms of Catatonia," *The Journal of ECT,* vol. 33, no. 1, pp. 63-67, 2017.

[17] J. Bronstein, M. Tagliati, R. Alterman, A. Lozano, J. Volkmann, A. Stefani, F. Horak, M. Okun, K. Foote, P. Krack, R. Pahwa, J. Henderson, M. Hariz, R. Bakay, A. Rezai and W. Marks, "Deep Brain Stimulation for Parkinson Disease: An Expert Consensus and Review of Key Issues," *Archives of Neurology,* vol. 68, no. 2, p. 165–171, 2011.

[18] A. Bystritsky, A. Korb, P. Douglas, M. Cohen, W. Melega, A. Mulgaonkar, A. DeSalles, B.-K. Min and S.-S. Yoo, "A Review of Low-Intensity Focused Ultrasound Pulsation," *Brain Stimulation,* vol. 4, no. 3, pp. 125-36, 2011.

[19] A. Thielscher, A. Opitz and M. Windhoff, "Impact of the Gyral Geometry on the Electric Field Induced by Transcranial Magnetic Stimulation," *NeuroImage,* vol. 54, no. 1, pp. 234-243, 2011.

[20] J. Baudewig, W. Paulus and J. Frahm, "Artifacts Caused by Transcranial Magnetic Stimulation Coils and EEG Electrodes in T2∗-Weighted Echo-Planar Imaging," *Magnetic Resonance Imaging,* vol. 18, no. 4, pp. 479-484, 2000.

[21] G. Hartwigsen, T. Kassuba and H. R. Siebner, "Combining Transcranial Magnetic Stimulation with (f)MRI," in *fMRI*, Berlin, Springer Heidelberg, 2013, pp. 283-297.

[22] S. Rauch, D. Dougherty, D. Malone, A. Rezai, G. Friehs, A. Fischman, N. Alpert, S. Haber, P. Stypulkowski and M. Brown, "A Functional Neuroimaging

Investigation of Deep Brain Stimulation in Patients with Obsessive-Compulsive Disorder," *J Neurosurg,* vol. 104, no. 4, pp. 558-565, 2006.

[23] D. Dormont, D. Seidenwurm, D. Galanaud, P. Cornu, J. Yelnik and E. Bardinet, "Neuroimaging and Deep Brain Stimulation," *AJNR Am J Neuroradiol,* vol. 31, no. 1, pp. 15-23, 2010.

[24] A. Rezai, M. Phillips, K. Baker, A. Sharan, J. Nyenhuis, J. Tkach, J. Henderson and F. Shellock, "Neurostimulation System Used for Deep Brain Stimulation (DBS): MR Safety Issues and Implications of Failing to Follow Safety Recommendations," *Invest Radiol.,* vol. 39, no. 5, pp. 300-303, 2004.

[25] J. Henderson, J. Tkach, M. Phillips, K. Baker, F. Shellock and A. Rezai, "Permanent Neurological Deficit Related to Magnetic Resonance Imaging in a Patient with Implanted Deep Brain Stimulation Electrodes for Parkinson's Disease: Case Report," *Neurosurgery,* vol. 57, no. 5, p. E1063, 2005.

[26] H. Baek, K. J. Pahk and H. Kim, "A Review of Low-Intensity Focused Ultrasound for Neuromodulation," *Biomedical Engineering Letters,* vol. 7, no. 2, p. 135–142, 2017.

[27] Y. Tufail, A. Matyushov, N. Baldwin, M. Tauchmann, J. Georges, A. Yoshihiro, S. Helms Tillery and W. Tyler, "Transcranial Pulsed Ultrasound Stimulates Intact Brain Circuits," *Neuron,* vol. 66, no. 5, pp. 681-694, 2010.

[28] S.-S. Yoo, H. Kim, E. Filandrianos, S. Taghados and S. Park, "Non-Invasive Brain-to-Brain Interface (BBI): Establishing Functional Links between Two Brains," *PloS One,* vol. 8, no. 4, 2013.

[29] S.-S. Yoo, A. Bystritsky, J.-H. Lee, Y. Zhang, K. Fischer, B.-K. Min, N. McDannold, A. Pascual-Leone and F. Jolesz, "Focused Ultrasound Modulates Region-Specific Brain Activity," *Neuroimage,* vol. 56, no. 3, pp. 1267-1275, 2011.

[30] H. Kim, M. Park, S. Lee, W. Lee, A. Chiu and S.-S. Yoo, "Suppression of EEG Visual-Evoked Potentials in Rats via Neuromodulatory Focused Ultrasound," *Neuroreport,* vol. 26, no. 4, p. 211–215, 2015.

[31] T. Deffieux, Y. Younan, N. Wattiez, M. Tanter, P. Pouget and J.-F. Aubry, "Low-Intensity Focused Ultrasound Modulates Monkey Visuomotor Behavior," *Current Biology,* vol. 23, no. 23, p. 2430–2433, 2013.

[32] W.-T. Lin, R.-C. Chen, W.-W. Lu, S.-H. Liu and F.-Y. Yang, "Protective Effects of Low-Intensity Pulsed Ultrasound on Aluminum-Induced Cerebral Damage in Alzheimer's Disease Rat Model," *Sci Rep,* vol. 5, no. 9671, 2015.

[33] B.-K. Min, A. Bystritsky, K.-I. Jung, K. Fischer, Y. Zhang, L.-S. Maeng, S. In Park, Y.-A. Chung, F. Jolesz and S.-S. Yoo, "Focused Ultrasound-Mediated Suppression of Chemically-Induced Acute Epileptic EEG Activity," *BMC Neurosci,* vol. 12, no. 1, p. 23, 2011.

[34] K. Yoon, W. Lee, J. E. Lee, L. Xu, P. Croce, L. Foley and S.-S. Yoo, "Effects of Sonication Parameters on Transcranial Focused Ultrasound Brain Stimulation in an Ovine Model," *PLoS One,* vol. 14, no. 10, 2019.

[35] W. Legon, T. Sato, A. Opitz, J. Mueller, A. Barbour, A. Williams and W. Tyler, "Transcranial Focused Ultrasound Modulates the Activity of Primary Somatosensory Cortex in Humans," *Nat Neurosci,* vol. 17, no. 2, pp. 322-329, 2014.

[36] S. Hameroff, M. Trakas, C. Duffield, E. Annabia, M. Geraced, P. Boylea, A. Lucasa, Q. Amosa, A. Buaduc and J. Badal, "Transcranial Ultrasound (TUS) Effects on Mental States: A Pilot Study," *Brain Stimul,* vol. 6, no. 3, pp. 409-415, 2013.

[37] W. Legon, P. Bansal, R. Tyshynsky, L. Ai and J. Mueller, "Transcranial Focused Ultrasound Neuromodulation of The Human Primary Motor Cortex," *Scientific Reports,* vol. 8, no. 1, 2018.

[38] M. Legrand, L. Galineau, A. Novell, B. Planchez, B. Brizard, S. Leman, C. Tauber, J.-M. Escoffre, A. Lefèvre, P. Gosset, W. El-Hage, P. Emond, C. Belzung and A. Bouakaz, "Efficacy of Chronic Ultrasound Neurostimulation on Behaviors and Distributed Brain Metabolism in Depressive-Like Mice," *bioRxiv,* 2019.

[39] R. Ruhela, A. Prakash and B. Medhi, "An Urgent Need for Experimental Animal Model of Autism in Drug Development," *Ann Neurosci,* vol. 22, no. 1, p. 44–49, 2015.

[40] D. Mabunga, E. Gonzales, J.-w. Kim, K. C. Kim and C. Y. Shin, "Exploring the Validity of Valproic Acid Animal Model of Autism," *Experimental Neurobiology,* vol. 24, no. 4, p. 285–300, 2015.

[41] J. Crawley, "Mouse Behavioral Assays Relevant to the Symptoms of Autism," *Brain Pathology,* vol. 17, no. 4, pp. 448-459, 2007.

[42] P. Patterson, "Modeling Autistic Features in Animals," *Pediatric Research,* vol. 69, no. 5, p. 34–40, 2011.

[43] E. Bryda, "The Mighty Mouse: The Impact of Rodents on Advances in Biomedical Research," *Mo Med.,* vol. 110, no. 3, p. 207–211, 2013.

[44] M. Kas, J. Glennon, J. Buitelaar, E. Ey, B. Biemans, J. Crawley, R. Ring, C. Lajonchere, F. Esclassan, J. Talpos, L. Noldus, P. Burbach and T. Steckler, "Assessing Behavioural and Cognitive Domains of Autism Spectrum Disorders in Rodents: Current Status and Future Perspectives," *Psychopharmacology,* vol. 231, no. 6, pp. 1125-1146, 2014.

[45] S. Klauck and A. Poustka, "Animal Models of Autism," *Drug Discovery Today: Disease Models,* vol. 3, no. 4, pp. 313-318, 2006.

[46] S. McBride, C. Choi, Y. Wang, D. Liebelt, E. Braunstein, D. Ferreiro, A. Sehgal, K. Siwicki, T. Dockendorff, H. Nguyen, T. McDonald and T. Jongens, "Pharmacological Rescue of Synaptic Plasticity, Courtship Behavior, and

Mushroom Body Defects in A Drosophila Model of Fragile X Syndrome," *Neuron,* vol. 45, no. 5, pp. 753-764, 2005.

[47] V. Tropepe and H. Sive, "Can Zebrafish be Used As A Model to Study The Neurodevelopmental Causes of Autism?," *Genes Brain Behav,* vol. 2, no. (5, pp. 268-281, 2003.

[48] J. Brandão, C. Souza, P. Fernandes, A. M. Soares, M. Costa and R. Romcy-Pereira, "Epigenetic Inheritance of Behavior and Organization of Cortical Circuits in The VPA Animal Model of Autism," in *9th World Congress International Brain Research Organization*, Rio de Janeiro, 2015.

[49] N. Sosa-Diaz, M. E. Bringas, M. Atzori and G. Flores, "Prefrontal Cortex, Hippocampus, and Basolateral Amygdala Plasticity in a Rat Model of Autism Spectrum," *Synapse,* vol. 68, p. 468–473, 2014.

[50] A. Banerjee, F. García-Oscos, S. Roychowdhury, L. Galindo, S. Hall, M. Kilgard and M. Atzori, "Impairment of Cortical GABAergic Synaptic Transmission in An Environmental Rat Model of Autism," *International Journal of Neuropsychopharmacology,* vol. 16, no. 6, p. 1309–1318, 2013.

[51] E. Lauber, F. Filice and B. Schwaller, "Prenatal Valproate Exposure Differentially Affects Parvalbumin-Expressing Neurons and Related Circuits in the Cortex and Striatum of Mice," *Front Mol Neurosci.,* vol. 9, no. 150, 2016.

[52] W. SnowKelly, H. Hartle and T. Ivanco, "Altered Morphology of Motor Cortex Neurons in the VPA Rat Model of Autism," *Developmental Psychobiology,* vol. 50, no. 7, pp. 633-639, 2008.

[53] T. Rinaldi, C. Perrodin and H. Markram, "Hyper-Connectivity and Hyper-Plasticity in the Medial Prefrontal Cortex in the Valproic Acid Animal Model of Autism," *Front Neural Circuits,* vol. 2, no. 4, 2008.

[54] G. Wagner, K. Reuhl, M. Cheh, P. McRae and A. Halladay, "A New Neurobehavioral Model of Autism in Mice: Pre- and Postnatal Exposure to

Sodium Valproate," *Journal of Autism and Developmental Disorders,* vol. 36, p. 779–793, 2006.

[55] S. Kataoka, K. Takuma, Y. Hara, Y. Maeda, Y. Ago and T. Matsuda, "Autism-Like Behaviours with Transient Histone Hyperacetylation in Mice Treated Prenatally with Valproic Acid," *Int J Neuropsychopharmacol.,* vol. 16, no. 1, pp. 91-103, 2013.

[56] T. Al Sagheer, O. Haida, A. Balbous, M. Francheteau, E. Matas, P.-O. Fernagut and M. Jaber, "Motor Impairments Correlate with Social Deficits and Restricted Neuronal Loss in an Environmental Model of Autism," *Int J Neuropsychopharmacol,* vol. 21, no. 9, p. 871–882, 2018.

[57] M. Campolongo, N. Kazlauskas, G. Falasco, L. Urrutia, N. Salgueiro, C. Höcht and A. M. Depino, "Sociability Deficits after Prenatal Exposure to Valproic Acid are Rescued by Early Social Enrichment," *Molecular Autism,* p. 9:36, 2018.

[58] S. Roux and J. L. Bossu, "Valproic Acid and Autism Spectrum Disorder: from Clinical Observations to Animal Studies," *Neurology,* vol. 11, 2017.

[59] J.-L. Bossu and S. Roux, "The Valproate Model of Autism," *Med Sci.,* vol. 35, p. 236–243, 2019.

[60] H. Atladóttir, P. Thorsen, L. Østergaard, D. Schendel, S. Lemcke, M. Abdallah and E. Parner, "Maternal Infection Requiring Hospitalization during Pregnancy and Autism Spectrum Disorders," *J Autism Dev Disord.,* vol. 40, no. 12, pp. 1423-1430, 2010.

[61] S. K. Kar and S. Sarkar, "Neuro-stimulation Techniques for the Management of Anxiety Disorders: An Update," *Clinical Psychopharmacology and Neuroscience,* vol. 14, no. 4, p. 330–337, 2016.

[62] S. Ansari, K. Chaudhri and K. Al Moutaery, "Vagus Nerve Stimulation: Indications and Limitations," *Acta Neurochirurgica Supplement,* vol. 97, pp. 281-286, 2007.

[63] Y. Jin and J. Kong, "Transcutaneous Vagus Nerve Stimulation: A Promising Method for Treatment of Autism Spectrum Disorders," *Frontiers in Neuroscience,* vol. 10, 2017.

[64] "Bizzie Dow- Epilepsy and Vagus Nerve Stimulation".*Neurobiology of Disease: New Treatment Strategies.*

[65] A. Tekriwal and G. Baltuch, "Deep Brain Stimulation: Expanding Applications," *Neurol Med Chir (Tokyo),* vol. 55, no. 12, p. 861–877, 2015.

[66] J. Pilitsis, O. Khazen and S. Patel, "Deep Brain Stimulation".*American Association of Neurological Surgeons.*

[67] V. Chalmers, "Controversial Electric Shock Therapy Approved on The NHS to Treat Depression Is Not Safe and Should Be Stopped, Leading Researcher Argues," *MailOnline,* 30 January 2019.

[68] G. Eryılmaz, G. Sayar, B. Ünsalver, I. Gül, E. Özten and E. Sağlam, "Adverse Effects of Transcranial Direct Current Stimulation (TDCS) in a Group of Psychiatric Patients," *Scholars Journal of Applied Medical Sciences,* pp. 294-297, 2014.

[69] H. Matsumoto and Y. Ugawa, "Adverse Events of tDCS and tACS: A Review," *Clinical Neurophysiology Practice,* vol. 2, p. 19–25, 2017.

[70] T. Sirin, S. Aksu, A. Kurt, S. Karamürsel and B. Baykan, "Efficacy and Mechanisms of Transcranial Electrical Stimulation in Headache Disorders," *Neurological Sciences and Neurophysiology,* vol. 36, no. 2, pp. 57-68, 2019.

[71] J. Rozisky, L. Antunes, A. Brietzke, A. C. de Sousa and W. Caumo, "Transcranial Direct Current Stimulation and Neuroplasticity," in *Transcranial Direct Current Stimulation (tDCS): Emerging Uses, Safety And Neurobiological Effects*, Brazil, Nova Science Publishers, 2015.

[72] D. Spronk, M. Arns and P. Fitzgerald, "Chapter 10 - Repetitive Transcranial Magnetic Stimulation in Depression: Protocols, Mechanisms, and New Developments," in *Neurofeedback and Neuromodulation Techniques and Applications*, Academic Press, 2011, pp. 257-291.

[73] S. Cooley, "Transcranial Ultrasound: Future of Brain Stimulation," 2018.

[74] G. Li, W. Qiu, Z. Zhang, Q. Jiang, M. Su, R. Cai, Y. Li, F. Cai, Z. Deng, D. Xu, H. Zhang and H. Zheng, "Noninvasive Ultrasonic Neuromodulation in Freely Moving Mice," *IEEE Transactions on Biomedical Engineering,* vol. 66, no. 1, pp. 217-224, 2019.

[75] H. Kim, S. Taghados, K. Fischer, L.-S. Maeng, S. Park and S.-S. Yoo, "Noninvasive Transcranial Stimulation of Rat Abducens Nerve by Focused Ultrasound," *Ultrasound in Medicine & Biology,* vol. 38, no. 9, pp. 1568-1575, 2012.

[76] D. Daniels, S. Sharabi, D. Last, D. Guez, S. Salomon, Z. Zivli, D. Castel, A. Volovick, J. Grinfeld, I. Rachmilevich, T. Amar, S. Liraz-Zaltsman, N. Sargsyan, Y. Mardor and S. Harnof, "Focused Ultrasound-Induced Suppression of Auditory Evoked Potentials in Vivo," *Ultrasound in Medicine & Biology,* vol. 44, no. 5, pp. 1022-1030, 2018.

[77] T. Sato, M. Shapiro and D. Tsao, "Ultrasonic Neuromodulation Causes Widespread Cortical Activation via an Indirect Auditory Mechanism," *Neuron,* vol. 98, no. 5, pp. 1031-1041, 2018.

[78] P. Xie, S. Zhou, X. Wang, Y. Wang and Y. Yuan, "Effect of Pulsed Transcranial Ultrasound Stimulation at Different Number of Tone-Burst on Cortico-Muscular Coupling," *BMC Neuroscience,* vol. 19, no. 60, 2018.

[79] S.-S. Yoo, H. Kim, B.-K. Min and S. P. E. Franck, "Transcranial Focused Ultrasound to the Thalamus Alters Anesthesia Time in Rats," *Neuroreport,* vol. 22, no. 15, p. 783–787, 2011.

[80] S.-S. Yoo, K. Yoon, P. Croce, A. Cammalleri, R. Margolin and W. Lee, "Focused Ultrasound Brain Stimulation to Anesthetized Rats Induces Long-Term Changes in Somatosensory Evoked Potentials," *Int J Imaging Syst Technol,* vol. 28, no. 2, p. 106–112, 2018.

[81] H. Kim, M.-A. Park, S. Wang, A. Chiu, K. Fischer and S.-S. Yoo, "PET⁄CT Imaging Evidence of FUS-mediated (18)F-FDG Uptake Changes in Rat Brain," *Medical Physics,* vol. 40, no. 3, 2013.

[82] S. Sharabi, D. Daniels, D. Last, D. Guez, Z. Zivli, D. Castel, Y. Levy, A. Volovick, J. Grinfeld, I. Rachmilevich, T. Amar, Y. Mardor and S. Harnof, "Non-thermal Focused Ultrasound Induced Reversible Reduction of Essential Tremor in a Rat Model," *Brain Stimulation,* vol. 12, no. 1, pp. 1-8, 2019.

[83] H. Kim, A. Chiu, S. Lee, K. Fischer and S.-S. Yoo, "Focused Ultrasound-Mediated Non-Invasive Brain Stimulation: Examination of Sonication Parameters," *Brain Stimulation,* vol. 7, no. 5, pp. 748-756, 2014.

[84] H. Guo, M. Hamilton, S. Offutt, C. Gloeckner, T. Li, Y. Kim, W. Legon, J. Alford and H. Lim, "Ultrasound Produces Extensive Brain Activation via a Cochlear Pathway," *Neuron,* vol. 98, no. 5, pp. 1020-1030, 2018.

[85] N. Wattiez, C. Constans, T. Deffieux, P. Daye, M. Tanter, J.-F. Aubry and P. Pouget, "Transcranial Ultrasonic Stimulation Modulates Single-Neuron Discharge in Macaques Performing an Antisaccade Task," *Brain Stimulation,* vol. 10, no. 6, pp. 1024-1031, 2017.

[86] P.-F. Yang, A. Phipps, A. Newton, V. Chaplin, J. Gore, C. Caskey and L. M. Chen, "Neuromodulation of Sensory Networks in Monkey Brain by Focused Ultrasound with MRI Guidance and Detection," *Scientific Reports,* vol. 8, no. 7993, 2018.

[87] R. Mihran, F. Barnes and H. Wachtel, "Temporally-Specific Modification of Myelinated Axon Excitability in Vitro Following a Single Ultrasound Pulse," *Ultrasound in Medicine & Biology,* vol. 16, no. 3, pp. 297-309, 1990.

[88] B. Min, P. S. Yang, M. Bohlke, S. Park, D. Vago, T. Maher and S. Yoo, "Focused Ultrasound Modulates the level of cortical neurotransmitters: Potential as a new functional brain mapping technique," *International Journal of Imaging Systems and Technology,* vol. 21, no. 2, pp. 232-240, 2011.

[89] D. Zhang, H. Li, J. Sun, W. Hu, W. Jin, S. Li and S. Tong, "Antidepressant-Like Effect of Low-Intensity Transcranial Ultrasound Stimulation," *IEEE Transactions on Biomedical Engineering,* vol. 66, no. 2, pp. 411-420, 2019.

[90] T. Guo, H. Li, Y. Lv, H. Lu, J. Niu, J. Sun, G.-Y. Yang, C. Ren and S. Tong, "Pulsed Transcranial Ultrasound Stimulation Immediately After The Ischemic Brain Injury Is Neuroprotective," *IEEE Transactions on Biomedical Engineering,* vol. 62, no. 10, pp. 2352-2357, 2015.

[91] K. Yu, A. Sohrabpour and B. He, "Electrophysiological Source Imaging of Brain Networks Perturbed by Low-Intensity Transcranial Focused Ultrasound," *IEEE Transactions on Biomedical Engineering,* vol. 63, no. 9, pp. 1787-1794, 2016.

[92] W. Tyler, Y. Tufail, M. Finsterwald, M. Tauchmann, E. Olson and C. Majestic, "Remote Excitation of Neuronal Circuits Using Low-Intensity, Low-Frequency Ultrasound," *PLoS One,* vol. 3, no. 10, 2008.

[93] W. Lee, S. Lee, M. Park, L. Foley, E. Purcell-Estabrook, H. Kim, K. Fischer, L.-S. Maeng and S.-S. Yoo, "Image-Guided Focused Ultrasound-Mediated Regional Brain Stimulation in Sheep," *Ultrasound in Medicine & Biology,* vol. 42, no. 2, pp. 459-470, 2016.

[94] W. Lee, H. Kim, Y. Jung, I.-U. Song, Y. A. Chung and S.-S. Yoo, "Image-Guided Transcranial Focused Ultrasound Stimulates Human Primary Somatosensory Cortex," *Scientific Reports,* vol. 5, 2015.

[95] W. Lee, H.-C. Kim, Y. Jung, Y. A. Chung, I.-U. Song, J.-H. Lee and S.-S. Yoo, "Transcranial Focused Ultrasound Stimulation of Human Primary Visual Cortex," *Scientific Reports,* vol. 6, 2016.

[96] L. Ai, J. Mueller, A. Grant, Y. Eryaman and W. Legon, "Transcranial Focused Ultrasound for BOLD fMRI Signal Modulation in Humans," in *38th Annual International Conference of the IEEE Engineering in Medicine and Biology Society*, USA, 2016.

[97] L. Ai, P. Bansal, J. Mueller and W. Legon, "Effects of Transcranial Focused Ultrasound on Human Primary Motor Cortex Using 7T fMRI: A Pilot Study," *BMC Neuroscience,* vol. 19, no. 1, p. 56, 2018.

[98] W. Legon, L. Ai, P. Bansal and J. Mueller, "Neuromodulation With Single-Element Transcranial Focused Ultrasound in Human Thalamus," *Human Brain Mapping,* vol. 39, no. 5, 2018.

[99] B. Gibson, J. Sanguinetti, B. Badran, A. Yu, E. Klein, C. Abbott, J. Hansberger and V. Clark, "Increased Excitability Induced in the Primary Motor Cortex by Transcranial Ultrasound Stimulation," *Frontiers in Neurology,* vol. 9, no. 1007, 2018.

[100] W. Lee, Y. A. Chung, Y. Jung, I.-U. Song and S.-S. Yoo, "Simultaneous Acoustic Stimulation of Human Primary and Secondary Somatosensory Cortices Using Transcranial Focused Ultrasound," *BMC Neuroscience,* vol. 17, no. 1, p. 68, 2016.

[101] M. Monti, C. Schnakers, A. Korb, A. Bystritsky and P. Vespa, "Non-Invasive Ultrasonic Thalamic Stimulation in Disorders of Consciousness after

Severe Brain Injury: A First-in-Man Report," *Brain Stimulation,* vol. 9, no. 6, pp. 940-941, 2016.

[102] J. Blackmore, S. Shrivastava, J. Sallet, C. Butler and R. Cleveland, "Ultrasound Neuromodulation: A Review of Results, Mechanisms and Safety," *Ultrasound in Medicine & Biology,* vol. 45, no. 7, pp. 1509-1536, 2019.

[103] M. Prieto, Ö. Oralkan, B. Khuri-Yakub and M. Maduke, "Dynamic Response of Model Lipid Membranes to Ultrasonic Radiation Force," *Plos One,* vol. 8, no. 10, 2013.

[104] W. Tyler, Y. Tufail and S. Pati, "Pain: Noninvasive Functional Neurosurgery Using Ultrasound," *Nature Reviews. Neurology,* vol. 6, no. 1, pp. 13-14, 2010.

[105] D. Dalecki, "Mechanical Bioeffects of Ultrasound," *Annual Review of Biomedical Engineering,* vol. 6, pp. 229-248, 2004.

[106] W. O'Brien, "Ultrasound-Biophysics Mechanisms," *Progress in Biophysics and Molecular Biology,* vol. 93, no. 1-3, pp. 212-255, 2007.

[107] O. Altland, D. Dalecki, V. Suchkova and C. Francis, "Low-Intensity Ultrasound Increases Endothelial Cell Nitric Oxide Synthase Activity and Nitric Oxide Synthesis," *Journal of Thrombosis and Haemostasis,* vol. 2, no. 4, pp. 637-643, 2004.

[108] K. Iida, H. Luo, K. Hagisawa, T. Akima, P. Shah, T. Naqvi and R. Siegel, "Noninvasive Low-Frequency Ultrasound Energy Causes Vasodilation in Humans," *Journal of the American College of Cardiology,* vol. 48, no. 3, pp. 532-537, 2006.

[109] Y. Sugita, S. Mizuno, N. Nakayama, T. Iwaki, E. Murakami, Z. Wang, R. Endoh and H. Furuhata, "Nitric Oxide Generation Directly Responds to Ultrasound Exposure," *Ultrasound in Medicine & Biology,* vol. 34, no. 3, pp. 487-493, 2008.

[110] A. El Hady and B. Machta, "Mechanical Surface Waves Accompany Action Potential Propagation," *Nature Communications,* vol. 6, no. 6697, 2015.

[111] S.-S. Yoo, J.-H. Lee, Y. Zhang, W. Lee, K. Fischer, A. Golby, N. McDannold and F. Jolesz, "FUS-Mediated Reversible Modulation of Region Specific Brain Function," *Washington DC: Proceedings of MRgFUS,* p. 10, 2008.

[112] S.-S. Yoo, B.-K. Min, Y. Zhang, N. McDannold, A. Bystritsky and F. Jolesz, "Non-Invasive Suppression of Animal-Model Chronic Epilepsy Using Image-Guided Focused Ultrasound," in *Proceeding of SMRM-ESMRMB Joint Annual Meeting in Stockholm*, Sweden, 2010.

[113] W. Tyler, "Noninvasive Neuromodulation with Ultrasound? A Continuum Mechanics Hypothesis," *Neuroscientist,* 2010.

[114] M. Legrand, R. Troubat, B. Brizard, A.-M. Le Guisquet, C. Belzung and W. El-Hage, "Prefrontal Cortex rTMS Reverses Behavioral Impairments and Differentially Activates c-Fos in A Mouse Model of Post-Traumatic Stress Disorder," *Brain Stimul,* vol. 12, no. 1, pp. 87-95, 2019.

[115] K. Tennant, D. Adkins, N. Donlan, A. Asay, N. Thomas, J. Kleim, Jones and Theresa, "The Organization of the Forelimb Representation of the C57BL/6 Mouse Motor Cortex as Defined by Intracortical Microstimulation and Cytoarchitecture," *Cereb Cortex,* vol. 21, no. 4, p. 865–876, 2011.

[116] M. Simms, T. Kemper, C. Timbie, M. Bauman and G. Blatt, "The Anterior Cingulate Cortex in Autism: Heterogeneity of Qualitative and Quantitative Cytoarchitectonic Features Suggests Possible Subgroups," *Acta Neuropathol,* vol. 118, no. 5, pp. 673-684, 2009.

[117] X. Hua, P. Thompson, A. Leow, S. Madsen, R. Caplan, J. Alger, J. O'Neill, K. Joshi, S. Smalley, A. Toga and J. Levitt, "Brain Growth Rate Abnormalities Visualized in Adolescents with Autism," *Hum Brain Mapp,* vol. 34, no. 2, pp. 425-436, 2013.

[118] M. Gomot, F. Bernard, M. Davis, M. Belmonte, C. Ashwin, E. Bullmore and S. Baron-Cohen, "Change Detection in Children with Autism: An Auditory Event-Related fMRI Study," *Neuroimage,* vol. 29, no. 2, pp. 475-484, 2006.

[119] D. Kennedy, E. Redcay and E. Courchesne, "Failing to Deactivate: Resting Functional Abnormalities in Autism," *Proc Natl Acad Sci U S A,* vol. 103, no. 21, pp. 8275-8280, 2006.

[120] D. Vargas, C. Nascimbene, C. Krishnan, A. Zimmerman and C. Pardo, "Neuroglial Activation and Neuroinflammation in the Brain of Patients with Autism," *Ann Neurol,* vol. 57, no. 1, pp. 67-81, 2005.

[121] R. Kana, T. Keller, N. Minshew and M. Just, "Inhibitory Control in High-Functioning Autism: Decreased Activation and Underconnectivity in Inhibition Networks," *Biol Psychiatry,* vol. 62, no. 3, pp. 198-206, 2007.

[122] N. Schmitz, K. Rubia, T. v. Amelsvoort, E. Daly, A. Smith and D. Murphy, "Neural Correlates of Reward in Autism," *Br J Psychiatry,* vol. 192, no. 1, pp. 19-24, 2008.

[123] K. Fraklin and G. Paxinos, The Mouse Brain in Stereotaxic Coordinates, Academic Press, 2007.

[124] M. Aggarwal, J. Zhang, M. Miller, R. Sidman and S. Mori, "Magnetic Resonance Imaging and Micro-Computed Tomography Combined Atlas of Developing and Adult Mouse Brains for Stereotaxic Surgery," *Neuroscience,* vol. 162, no. 4, pp. 1339-1350, 2009.

[125] J. Bancroft and A. Stevens, Theory and Practice of Histological Techniques, 6th ed., New York: Churchill Livingstone, 2008, pp. 365-381.

[126] C. Nicolini and M. Fahnestock, "The Valproic Acid-Induced Rodent Model of Autism," *Experimental Neurology,* vol. 299, pp. 217-227, 2018.

[127] M. Gandal, J. Edgar, R. Ehrlichman, M. Mehta, T. Roberts and S. Siegel, "Validating Gamma Oscillations and Delayed Auditory Responses as Translational Biomarkers of Autism," *Biological Psychiatry,* vol. 68, no. 12, pp. 1100-1106, 2010.

[128] F. de Chaumont, S. Dallongeville, N. Chenouard, N. Hervé, S. Pop, T. Provoost, V. Meas-Yedid, P. Pankajakshan, T. Lecomte, Y. Le Montagner, T. Lagache, A. Dufour and J.-C. Olivo-Marin, "ICY: An Open Bioimage Informatics Platform for Extended Reproducible Research," *Nat Methods,* vol. 9, no. 7, pp. 690-696, 2012.

[129] M. Premoli, S. A. Bonini, A. Mastinu, G. Maccarinelli, F. Aria, G. Paiardi and M. Memo, "Specific Profile of Ultrasonic Communication in A Mouse Model of Neurodevelopmental Disorders," *Scientific Reports,* vol. 9, 2019.

[130] C. Neely, K. Pedemonte, K. Boggs and J. Flinn, "Nest Building Behavior as an Early Indicator of Behavioral Deficits in Mice," *J Vis Exp.,* vol. 19, no. 152, 2019.

[131] R. King, J. Brown, W. Newsome and K. B. Pauly, "Effective Parameters for Ultrasound-Induced In Vivo Neurostimulation," *Ultrasound in Medicine & Biology,* vol. 39, no. 2, pp. 312-331, 2013.

[132] M. Menz, Ö. Oralkan, P. Khuri-Yakub and S. Baccus, "Precise Neural Stimulation in the Retina Using Focused Ultrasound," *J Neurosci,* vol. 33, no. 10, p. 4550–4560, 2013.

[133] P.-A. Lo, K. Huang, Q. Zhou, M. Humayun and L. Yue, "Ultrasonic Retinal Neuromodulation and Acoustic Retinal Prosthesis," *Micromachines (Basel),* vol. 11, no. 10, p. 929, 2020.

[134] M. Menz, P. Ye, K. Firouzi, A. Nikoozadeh, K. B. Pauly, P. Khuri-Yakub and S. Baccus, "Radiation Force as a Physical Mechanism for Ultrasonic

Neurostimulation of the Ex Vivo Retina," *J Neurosci,* vol. 39, no. 32, p. 6251–6264, 2019.

[135] J. Vion, "Study of the Mechanisms Underlying Neurostimulation Induced by Low- Energy Pulsed Ultrasound : Towards Approaches for the Management of Cancer-Related Chronic Pain," Lyon, 2019.

[136] W. Lee, P. Croce, R. Margolin, A. Cammalleri, K. Yoon and S.-S. Yoo, "Transcranial Focused Ultrasound Stimulation of Motor Cortical Areas in Freely-Moving Awake Rats," *BMC Neurosci,* vol. 19, no. 57, 2018.

[137] Y. Younan, T. Deffieux, B. Larrat, M. Fink, M. Tanter and J.-F. Aubry, "Influence of The Pressure Field Distribution in Transcranial Ultrasonic Neurostimulation," *Med Phys,* vol. 40, no. 8, 2013.

[138] Y. Tufail, A. Yoshihiro, S. Pati, M. Li and W. Tyler, "Ultrasonic Neuromodulation by Brain Stimulation with Transcranial Ultrasound," *Nat Protoc,* vol. 6, no. 9, pp. 1453-1470, 2011.

[139] Y. Yuan, X. Wang, J. Yan and X. Li, "The Effect of Anesthetic Dose on The Motor Response Induced by Low-Intensity Pulsed Ultrasound Stimulation," *BMC Neurosci,* vol. 19, no. 78, 2018.

[140] J. Chabout, P. Serreau, E. Ey, L. Bellier, T. Aubin, T. Bourgeron and S. Granon, "Adult Male Mice Emit Context-Specific Ultrasonic Vocalizations That Are Modulated by Prior Isolation or Group Rearing Environment," *PLoS One,* vol. 7, no. 1, 2012.

[141] N. Barnea-Goraly, H. Kwon, V. Menon, S. Eliez, L. Lotspeich and A. Reiss, "White Matter Structure in Autism: Preliminary Evidence from Diffusion Tensor Imaging," *Biological Psychiatry,* vol. 55, no. 3, pp. 323-326, 2004.

APPENDIX A

Neurostimulation for Autism Spectrum Disorder: An Overview of Preclinical Studies

Rasha Noureddine[a,b], Yassine Mofid[a,e], Mohamad Nassereddine[c], Alexander Surget[a], Jamal Charara[c], Ayache Bouakaz[a*], Frédérique Bonnet Brilhault[a,d,e]

[a]UMR 1253, iBrain, Université de Tours, Inserm, Tours, France.

[b]Lebanese University, Doctoral School of Science & Technology, Hadath, Lebanon.

[c]Lebanese University, Faculty of Sciences I - Department of Physics – Electronics, Hadath, Lebanon.

[d]Centre Universitaire de Pédopsychiatrie, CHRU de Tours, Tours, France.

[e]EXcellence Center in Autism and Neurodevelopmental Disorders - Tours ExAC-T, Tours, France.

[*]To whom correspondence should be addressed. Dr. Ayache Bouakaz: UMR 1253, iBrain, Université de Tours, Inserm, 10 boulevard Tonnellé, 37032 Tours, France. E-mail: ayache.bouakaz@univ-tours.fr

Abstract

Background: Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder with uncertain etiology, varying deficits, and no effective therapies making it a major public health problem.

Objective: The objective of the survey is to review the preclinical research using neurostimulation methods applied to ASD and their therapeutic potential. There is a huge need to develop new therapeutic and targeted approaches.

Methods: We surveyed human studies to document the Human Brain Regions hypothesized to be involved in ASD. We then described studies conducted with the various neurostimulation techniques in preclinical scenarios for autism that were based on findings from human studies and we sought to investigate the biological mechanisms involved in brain stimulation.

Results: The review shows that the used neurostimulation techniques face various limitations, either in terms of invasiveness, targeting and selectivity, or in terms of accessibility to deep brain zones. We also introduce a novel neurostimulation technique using ultrasound waves. This emerging technology offers real therapeutic opportunity as it might overcome the limitations of currently available neurostimulation approaches.

Conclusions: We believe that in the future low intensity focused ultrasound stimulation could be a promising therapeutic strategy as it overcomes the limitations in terms of resolution and spatial targeting.

Keywords: Autism Spectrum Disorder; Brain regions; Neurostimulation; Preclinical research, Ultrasound.

Introduction

Autism spectrum disorder (ASD) is a common and heterogeneous lifelong developmental disorder characterized by (i) social interaction and communication deficits, and (ii) restricted, repetitive patterns of behavior, interests, or activities as per the diagnostic and statistical manual of mental disorders (DSM 5).[1] Autism Spectrum is related to a large spectrum of clinical expression from high functioning people to ASD children or adults with multiple comorbidities involving self injurious behaviors, epilepsy and obsessive compulsive behaviors. The precise etiology of autism remains unknown but it is know well admitted that it involves interaction of environmental and genetic factors as well as epigenetic factors.[2,3] The prevalence of autism, is approximated 1% in the general population, with a gender ratio of about 4 males for every female.[3] Huge progress has been made in early intervention and rehabilitation and educative programs for ASD, such as the exchange and development therapy and applied behavior analysis. However, some patients and their family are still facing clinical challenges. Only two drugs, risperidone and aripiprazole, were approved by the food and drug administration (FDA) only for the treatment of irritability in ASD patients.[4] Thus, for the time being, the pharmacological treatments that can treat or improve core symptoms of ASD have not been discovered.[5] Therefore, there is a big need for developing new complimentary interventional therapy in ASD.

Transcranial stimulation is based on a neurostimulation of a specific region in the brain, which directly or indirectly affects the electrical activity of the brain that successively prompts localized protracted neurochemical changes and is aiming at inducing beneficial therapeutic effects.[5] For example, repetitive transcranial magnetic stimulation could result in antidepressant effects when applied to depressed patients.[6] Also, Transcranial direct current

stimulation is undergoing clinical trials to evaluate whether this approach can improve certain mental abilities in patients with autism.

In this paper, we present an overview of the human brain regions implicated in ASD. In addition, we provide the first review that encompasses all neurostimulation studies examined in the field of ASD in preclinical research, and explain the underlying mechanisms of each. Since the preclinical studies were based on findings issued from human research, we have surveyed human studies to report on the Human Brain Regions Implicated in ASD and then described the preclinical studies in ASD. Finally, we propose the use of low intensity focused ultrasound stimulation (LIFUS) as a new application in ASD.

Human Brain Regions Implicated in ASD

A network of different brain regions (listed and explained below) that are functionally connected, is implicated in this disorder.[7] Table 1 displays several studies on the human brain regions most critically implicated in ASD.

Prefrontal Cortex

The prefrontal cortex has been extensively implicated in autism as it is linked to higher order cognitive, language, social and normal emotion processing.[8] Gomot et al.[9] reported dysfunctioning in the left prefrontal cortex in autistic children, while, Koshino et al.[10] and Price[11] reported more right lateralized activation and hypo-functionality in the prefrontal region in ASD subjects respectively.

Taking into consideration that the **medial prefrontal cortex (mPFC)** is of special importance for human social cognition and behavior, it was one of the most zones examined in autism. Courchesne et al.[12] showed an abnormal excess number of neurons within the

mPFC in the case of autism. Kennedy[13] demonstrated lack of activity in the mPFC in ASD patients. However, Castelli et al.[14] and Pierce et al.[15] detected hypo-activity of this zone in autistic patients, and Bertero et al.[16] showed reduced global connectivity in the mPFC.

The **dorsolateral prefrontal cortex (DLPFC)**, a brain region responsible for processing components of working memory, decision making process, and regulating the ability to focus attention, is another area of interest for researchers in ASD. Courchesne et al.[12] reported an abnormal excess number of neurons in the DLPFC in autistic patients, while Koshino et al.[10] showed less activity in the DLPFC in autistic participants.

Anterior Cingulate Cortex

Anterior cingulate cortex (ACC) is a region linked to attention and cognitive control. ACC combines inputs from sensory, motor, cognitive, and emotional information to modulate value attribution, personality patterns, and social functioning. Neuroanatomical abnormalities related to ASD have been found in ACC. Simms et al.[17] showed decrease in cell size and cell packing density of the ACC. However, Hua et al.[18] demonstrated abnormal overgrowth of this region in autistic individuals. In addition, abnormal white matter diffusion patterns in the ACC region of autistic patients was demonstrated in a study achieved by Barnea Goraly et al.[19] Considering ASD related functional changes in ACC, Daniel Kennedy et al.[13] and Gomot et al.[20] reported lack of activity and reduced activation of the ACC in autistic patients respectively. Based on post-mortem study Vargas et al.[21] showed neuroglial activation and neuroinflammation.

Thalamus

Thalamus, which consists of different nuclei each with specific function involving relaying sensory and motor signals, and regulation of alertness, pain, consciousness, mood and motivation, is also implicated in ASD. Morphological alterations in the thalamus were reported in individuals with ASD by Schuetze et al.[22] However, Woodward et al. observed thalamocortical dysconnectivity in autistic participants.[33]

Cerebellum

The cerebellum is recognized to be involved in many brain functions, including cognitive functions such as language and attention, in emotional control, voluntary movements such as posture, balance, coordination, and speech. Some neuroanatomical evidences have linked the cerebellum to ASD. Reduction of cerebellar Purkinje cells (PCs) was observed in individuals with autism in the study of Bailey et al.[24]. Fatemi et al.[25] also found decreased size of PCs in this region in ASD subjects. While, Vargas et al.[21] highlighted significant neuroglial activation and neuroinflammation in the cerebellum of patients with autism.

Temporal Lobe

The temporal lobe is responsible for processing auditory information from the ears and involved in higher order cognitive functions such as declarative memory encoding, spatial navigation, language processing and complex visual processing. It has been also implicated in ASD. Castelli et al.[14] demonstrated hypo-functionality of the basal temporal area, while, Koshino et al.[10] showed hyper-functionality of the temporal lobe in autistic participants.

Moreover, Bertero et al.[16] showed reduced connectivity in the superior and medial temporal gyrus in autistic subjects.

Parietal Lobe

The parietal lobe is another brain zone connected to autism. It involves somatosensory treatment and perception, and is concerned with integrating sensory input, especially with the visual system. Koshino et al.[10] observed a hypo-functioning of the left inferior parietal lobe and hyper-functioning of the right inferior parietal lobe in subjects with ASD. Furthermore, Bertero et al.[16] showed recently a reduced connectivity in the parietal areas of autistic individuals.

Temporoparietal Junction

One of the most reported brain regions implicated in ASD is the temporoparietal junction. Castelli et al.[14] demonstrated a hypo-functionality of the bilateral superior temporal sulcus, which is essential in many aspects of social cognition, in autistic individuals. However, Just et al.[26] noticed a hyper-functioning of the Wernicke's area, which is a part of the temporoparietal junction responsible for the comprehension of speech, in participants with autism. Moreover, Bertero et al.[16] showed a reduced connectivity in the temporal parietal junctions of autistic participants.

Amygdala

Amygdala, which is responsible for emotions, survival instincts, and emotional memory, was also related to ASD. Bauman and Kemper[27] reported increased packing density and reduced cell size in certain amygdalar subregions in autistic man compared to normal control. While, Schumann et al.[28] detected amygdalar enlargement in children with ASD.

However, Baron-Cohen et al.[29] reported lack of activity and Pierce et al.[15] detected hypo-functionality of the amygdala in ASD participants respectively.

Hippocampus

Abnormalities in the hippocampus, which plays important roles in declarative memory and merging information from short term memory to long term memory, are also implicated in ASD. Schumann et al.[28] showed enlargement in the hippocampus in children with ASD. However, Lawrence et al.[30] observed an alteration of hippocampal shape and alterations in interneuron density in the hippocampus in autism cases respectively. Recently, Cooper et al.[31] showed reduced hippocampal connectivity in individuals with ASD compared to controls.

Other Regions

Broca's area, occipital lobe, occipitotemporal region, and **precentral sulcus** are other brain regions linked to ASD. Over and above, more brain regions were recently associated with ASD involving **angular gyrus**, **inferior frontal gyrus**, **superior parietal lobe**, **postcentral gyrus**, **precentral gyrus**, **superior temporal sulcus**, **lateral occipital cortex**, and **supplementary motor area/paracentral gyrus**.[32]

All the previously mentioned brain regions are functionally connected and form a network "the Social brain" that is linked to ASD. Thus, there is no individual brain zone underlying either the ASD syndrome or single endophenotypes of autism. Rather, we observe neuroanatomical and functional alterations of a network of connected regions that may underlie as a whole for this disorder. Atypical functional connectivity has been implicated with mixed and conflicting reports of hypo- and hyper-connectivity.[33] For instance, as per a recent

study, the default mode network, which includes several of the brain areas described above, has been related to social dysfunction and implicated in ASD.[34]

Neurostimulation Techniques in ASD: Preclinical Studies

The development of validated animal models imitating ASD causes and symptoms would be of high value for studying autism, deciphering its neurobiological underpinning and probing new therapeutic strategies. However, ASD is a complex neurodevelopmental disorder with unrevealed reasons and varied symptoms, and firmly restricted to human, making difficult to develop models capable of recapitulating this disorder in its entirety. However, researchers have developed strategies focusing on specific environmental or genetic risk factors, or on specific endophenotypes allowing to assess some hypothesis about etiology and pathophysiology of ASD. Attempts to develop such models have been made in species like mice and rats principally, but also in guinea pigs, Microtus, zebrafish, and fly.[35-36] It exists therefore two main different types of ASD animal models: the genetic models and the environmental risk factors models.[37] Genetic models involve chromosome engineering or loss of function mutations.[38, 39] Various forms of genetic modifications can enhance the risk of developing autistic features including monogenic syndromes, Mendelian mutations, and de novo chromosomal deletions or duplications.[40] On the other hand, models based on environmental risk factors have involved prenatal and neonatal environmental challenges including maternal valproic acid (VPA) exposure, maternal bacterial and viral infections, neonatal isolation, and stress experiences during gestation.[3, 41, 42]

Neurostimulation studies in the case of ASD in preclinical research is limited to few recent studies described below.

Deep Brain Stimulation

DBS requires the implantation of stimulated generator and electrodes in specific areas of the brain. It consists in sending the electrical stimulus through the implanted electrodes to prompt an electrical field that alters the activity in the neuronal circuits. DBS has been recently examined on valproate (VPA)-induced rat ASD model by Wu et al.[43] in 2018. The authors administered DBS and 5-HT_{1A} receptor agonist treatment with 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) to VPA offspring. DBS was applied via implanted electrodes in the right medial prefrontal cortex (2.2 mm anteroposterior, 0.5 mm mediolateral) consisting of a multi-array stainless steel wire electrode. DBS of 160 Hz frequency, a 60-μs pulse width and 150-μA current, was applied for 7 days, 30 min per day. Rats were then evaluated 24 hours after the last stimulation by social interaction testing and molecular approaches. Social interaction tests included three-chamber social interaction test, elevated plus maze (EPM), open field test, and molecular approaches involved Nissl staining (fluorescence microscope), and western blotting analysis. The authors demonstrated that employing DBS and 5-HT1A receptor agonist treatment with 8-OH-DPAT improved autistic symptoms consisting of sociability deficits, anxiety and hyperactivity in the rats. They explained the mechanism of DBS effect on autistic behaviors through the 5-HT system in the infra-limbic prefrontal cortex. They showed that both 8-OH-DPAT and DBS treatment improved autistic behaviors by lowering the expressions of NR2B subunit of N-Methyl-D-aspartate receptors and the β3 subunit of γ-aminobutyric acid type A receptors ($GABA_AR$) in the prefrontal cortex region.

Another study was newly performed to discover the effect of DBS in rats. Lin et al.[44] tested the potential of DBS on VPA rat offspring model of ASD in 2019. DBS was applied to

the central thalamic nucleus (CTN) for 3 days (30 minutes per day) using magnetic resonance compatible neural probe that provided microstimulations during fMRI. The authors used fMRI, three-chamber social interaction test, and Western blotting analysis to assess the impact of their stimulation. They showed that DBS was capable of enhancing the functional connectivity in corticostriatal and corticolimbic circuits, and social interaction, while diminishing the social avoidance. Additionally, they observed reduction of dopamine D2 receptor, proposing modifications in synaptic plasticity and brain circuits. They suggested that brain circuit connectivity improvement might have modified the expression of dopaminergic receptors that modulate striatal synaptic plasticity to control downstream signaling cascades for improved social behavior related brain function. Also, they added that CTN-DBS in the ASD rats caused increase in the functional connectivities in the corticolimbic and the corticostriatal circuits, associated with modifications in the social interaction in ASD.

Electroconvulsive therapy

ECT is a noninvasive technique that entails general anesthesia. It provides an electric stimulus to the surface of the head in which widespread seizure is induced with neurotransmitters liberation. The effect of ECT on ASD was tested in 2015 by Hagen et al.[45] ECT was administered to BTBR mice models of autism on 7 days while applying a series of 13 electroconvulsive shocks (ECS) twice a day. ECS of 0.1 ms pulse duration, 1 s ECS duration, 100 pulses, and 25 mA was delivered to the mouse brain via auricular clip electrodes positioned on the mouse's ears. Behavioral testing relying on the 3-chamber test for examining sociability, social novelty, self-directed repetitive behavior was performed 24 hours after the last ECS. ECT was capable of enhancing the autism-like behavioral impairments consisting of impaired sociability, social novelty, and repetitive behavior in BTBR mice up to 2

weeks after ECT. The authors did not explain the mechanism of the therapeutic effect of ECT in improving autistic-like symptoms but they suggested that it may involve plastic changes in the brain and may be mediated by central oxytocin receptors in a complicated behavior-dependent style. Furthermore, they proposed that the basis of the ECT effect can compromise set of targets rather than one molecule or one pathway. Moreover, they considered that two effects involving restoring a specific dysfunction and overcompensation of otherwise unaffected pathways.

The impact of ECT followed by DBS was studied lately on mice models of ASD by Chang et al.[46] in 2016. The authors started by administering one electroconvulsive seizure (ECS) via bilateral earclip electrodes followed by DBS to two genetically mice models of ASD displaying excessive self-grooming, particularly, Viaat-Mecp2$^{-/y}$ and Shank3B$^{-/-}$ mice. High-frequency stimulation for DBS was applied at the subthalamic nucleus (STN-HFS) through bilaterally implanted monopolar stimulation electrodes. Constant-current square waves (130 Hz, 60 μs, 30 μA) were administered at each electrode with a maximum radius of tissue activation surrounding the stimulation electrodes of approximately 0.093–0.332 mm. HFS was applied on three consecutive days for 3 hours per day. The effect of the stimulation was evaluated by behavioral testing, self-grooming and locomotion activity, and social interaction tests. The authors demonstrated the ability of ECS to suppress the excessive self-grooming in Viaat-Mecp2$^{-/y}$ mice as well as the potency of high frequency stimulation at the subthalamic nucleus to suppress excessive self-grooming in both genetic models intensely during stimulation and for several days after stimulation. They realized the selective and specific effect of STN-HFS and that suppression in excessive self-grooming was not due to hypo-activity. They proposed

that ECT affects GABAergic function and it was not clear to explain the mechanisms underlying the function or impact of ECT on GABAergic tone to suppress excessive self-grooming.

Repetitive Transcranial Magnetic Stimulation

The repetitive transcranial magnetic stimulation is based on the application of magnetic fields to the brain. These fields, generated via a stimulating coil, pass through skull and induce an electrical current in the brain to modulate its activity. Low frequency rTMS (LF rTMS, 1 Hz) has been experienced by Tan et al.[47] in 2018 on rats displaying ASD features using neonatal isolation rat model. Most regions of the brain of the rat were stimulated by placing the coil center over the intersection of the midline and interocular line. The stimulation covered an area between 6 mm anterior and 13.5 mm posterior to Bregma, so almost all the brain. Stimulation was applied on consecutive 14 days. Each rTMS session was composed of 20 burst trains, each train containing 30 pulses at 1 Hz with 2-s inter-train intervals (600 stimuli in total). Intensity was set at 100% of the average resting motor threshold and remained constant during the whole experiments. Magnetic field intensities were 2.60, 1.80 and 1.32 Tesla at 0, 5 and 10 mm below the surface of the coil center respectively. Impact of the used low frequency stimulation was assessed after rTMS by behavioral tests consisting sequentially of social interaction (three-chamber test), self-grooming test, EPM test and forced swimming test, with 24 hours pause between tests. Additionally, electrophysiology and western blotting were utilized. The authors have found that LF rTMS significantly relieved symptom of ASD involving enhancement of social interaction and reduction of self-grooming, anxiety and depression. Moreover, the authors showed that this rescue of autistic-like symptoms was accompanied by a restoration of the balance between excitability and inhibitory (E/I) activity, especially at the level of synaptic transmission and receptors in

synaptosomes. They supposed that LF rTMS improved autistic-like behavior through regulating the synaptic GABA transmission.

Discussion and Conclusions

Only few recent preclinical neurostimulation studies in animal model of ASD have been performed to date and were limited to rodent's research particularly in mice and rats. Although these available investigations could reach positive outcomes in treating autism-like symptoms such as enhancing social interaction, and reducing anxiety, repetitive behaviors, hyperactivity and self-grooming, they were implemented on either environmental factor based or genetically modified animal models of ASD. Actually, despite of the various existing animal models of autism, there is no ideal accepted model developed yet. [48, 49] The major reason behind that is that a perfect animal model should fit construct, face and predictive validity.[48, 50] However, it is not the case for available autism models as models generated by genetic modifications can cause autistic-like features without ensuring its relevance regarding the human disorders.[51] Moreover, some of them can create other serious conditions such as tumors and physical malformations.[40] Moreover, the animal models did not create the male bias existing in autism.[40] Furthermore, different little stimulation techniques specifically DBS, ECT, and rTMS with various stimulation parameters, variety of sessions, distinct targeted brain regions, and different evaluation techniques have been only tested in animal excluding other important modulation methods which might be beneficial in autism such as tDCS.

Despite of the fact that these previously mentioned well known neurostimulation techniques have been widely used in neuroscience and in the treatment of mental and motor disorders, they have specific limitations for ASD.[52-55] These neurostimulation techniques are

not yet approved by FDA for autism and the majority of them offer low spatial resolution or necessitate invasive procedure.[56, 57] Although DBS provides high spatial resolution, it is considered as an invasive technique that requires complex neurosurgery.[58] Additionally, rTMS provides low spatial resolution while targeting the region of interest (ROI) which is a superficial zone. rTMS generates electrical fields with length scales on the order of several centimeters that can cross and affect anatomically and functionally various neighboring brain circuits.[59, 60]

Therefore, there is a need for developing a complimentary neurostimulation technique that can beat the restrictions of the available techniques and target deep brain regions implicated in ASD such as anterior cingulate cortex, amygdala and hippocampus that can cause new behavioral impacts and improve the outcome. As per recent findings, low intensity focused ultrasound stimulation is proposed to be the moderator that can overcome all the deficiencies of these neurostimulation techniques due to its noninvasiveness, ability to penetrate into deep brain tissues with high resolution, and safety.[61, 62] Table 2 recaps the advantages and disadvantages of the commonly used neurostimulation techniques along with LIFUS.

LIFUS is a noninvasive stimulation technique that directs low intensity ultrasound waves through specialized transducer into a small focus on the head. LIFUS has reversible effect on neurons and can cause neuronal excitation and inhibition.[58, 62] It is thought that LIFUS modulates the neuronal functions in the brain through nonthermal mechanical effect on cellular membranes and ion channels.[61, 63] LIFUS modulates the brain activity in the ROI without causing tissue damage and can target deep regions in the brain without affecting surrounding tissues.[63] Although the mechanisms underlying how LIFUS modulates neuronal

functions are poorly understood, recent reviews have indicated the ability of LIFUS to mechanically alter the state of mechanosensitive ion channels existing within the cellular membranes. Compression, tension and shear forces generated by ultrasound can enhance the possibility of opening/activating voltage gated ion channels including sodium, calcium, and potassium channels, and thus triggering action potentials and synaptic transmission.[61, 62]

LIFUS has been studied in brain pathways as reported elsewhere.[61] LIFUS activated the motor cortex and produced muscular contractions when applied to rodents.[64, 65] It also produced eyeball movement while stimulating the abducens nerve in rat, inhibited the primary visual cortex in rabbit, suppressed EEG visual evoked potentials in rats, and modulated the latencies in anti saccade task when it targeted the left frontal eye field in monkey. [66, 67] Moreover, LIFUS showed neuroprotective effects against cerebral damages and caused memory retention in Alzheimer disease in rats, as well as reduction in the occurrence of epileptic EEG bursts in rat model of epilepsy. [68, 69] Lately, it has been tested and safely performed in sheep.[70] Furthermore, LIFUS has been examined in human by stimulating the primary somatosensory cortex and posterior frontal cortex; resulting in enhancing the performance on sensory discrimination tasks and improving mood/global affect respectively. [63, 71] Additionally, it modulated the excitability of the human motor cortex.[72]

These finding, along with the enormous potential of LIFUS, offer interesting opportunities for the future application of LIFUS in the treatment of neuropsychiatric disorders (autism, depression, post-traumatic stress disorder) and other neurodegenerative diseases (alzheimer, parkinson).[58, 61] Preclinical neurostimulation research in ASD is recent and so limited compared to the clinical studies available. Future research in autism should focus on more animal studies.

Acknowledgements: R.N. acknowledges the financial support of Walid Joumblatt Foundation for University Studies.

Disclosure: none.

References

1. Crawley J. Translational Animal Models of Autism and Neurodevelopmental Disorders. *Dialogues Clin Neurosci*. 2012;14(3):293–305.

2. Zhang R, Zhou J, Ren J, et al. Transcriptional and Splicing Dysregulation in the Prefrontal Cortex in Valproic Acid Rat Model of Autism. *Reprod Toxicol*. 2018;77:53-61.

3. Iwata K, Matsuzaki H, Takei N, Manabe T, Mori N. Animal Models of Autism: An Epigenetic and Environmental Viewpoint. *J Cent Nerv Syst Dis*. 2010;2:37–44.

4. LeClerc S, Easley D. Pharmacological Therapies for Autism Spectrum Disorder: A Review. *P T*. 2015;40(6):389–397.

5. Kim T, Ryu JE, Bahn GH. Brain Stimulation and Modulation for Autism Spectrum Disorder. *Hanyang Med Rev*. 2016;36:65-71.

6. Machado S, Arias-Carrión O, Paes F, et al. Repetitive Transcranial Magnetic Stimulation for Clinical Applications in Neurological and Psychiatric Disorders: An Overview. *Eurasian J Med*. 2013 Oct;45(3):191–206.

7. Bonnet-Brilhault F, Tuller L, Prévost P, et al. A Strategic Plan to Identify Key Neurophysiological Mechanisms and Brain Circuits in Autism. *J Chem Neuroanat*. 2018;89:69-72.

8. Rinaldi T, Perrodin C, Markram H. Hyper-Connectivity and Hyper-Plasticity in the Medial Prefrontal Cortex in the Valproic Acid Animal Model of Autism. *Front Neural Circuits*. 2008;2(4).

9. Gomot M, Giard MH, Adrien JL, Barthelemy C, Bruneau N. Hypersensitivity to Acoustic Change in Children With Autism: Electrophysiological Evidence of Left Frontal Cortex Dysfunctioning. *Psychophysiology*. 2002;39(5):577-584.

10. Koshino H, Carpenter P, Minshew N, Cherkassky V, Keller T, Just M. Functional Connectivity in an fMRI Working Memory Task in High-Functioning Autism. *Neuroimage*. 2005;24(3):810-821.

11. Price J. Prefrontal Cortex. *Understanding Autism: From Basic Neuroscience to Treatment*: CRC Press; 2006.

12. Courchesne E, Mouton P, Calhoun M, et al. Neuron Number and Size in Prefrontal Cortex of Children with Autism. *JAMA*. 2011;306(18):2001-2010.

13. Kennedy D, Redcay E, Courchesne E. Failing to Deactivate: Resting Functional Abnormalities in Autism. *Proc Natl Acad Sci U S A*. 2006;103(21):8275-8280.

14. Castelli F, Frith C, Happé F, Frith U. Autism, Asperger Syndrome and Brain Mechanisms for the Attribution of Mental States to Animated Shapes. *Brain*. 2002;125(8):1839-1849.

15. Pierce K, Haist F, Sedaghat F, Courchesne E. The Brain Response to Personally Familiar Faces in Autism: Findings of Fusiform Activity and Beyond. *Brain*. 2004;127(12):2703-2716.

16. Bertero A, Liska A, Pagani M, et al. Autism-Associated 16p11.2 Microdeletion Impairs Prefrontal Functional Connectivity in Mouse and Human. *Brain*. 2018;141(7):2055–2065.

17. Simms M, Kemper T, Timbie C, Bauman M, Blatt G. The Anterior Cingulate Cortex in Autism: Heterogeneity of Qualitative and Quantitative Cytoarchitectonic Features Suggests Possible Subgroups. *Acta Neuropathol*. 2009;118(5):673-684.

18. Hua X, Thompson P, Leow A, et al. Brain Growth Rate Abnormalities Visualized in Adolescents with Autism. *Hum Brain Mapp*. 2013;34(2):425-436.

19. Barnea-Goraly N, Kwon H, Menon V, Eliez S, Lotspeich L, Reiss A. White Matter Structure in Autism: Preliminary Evidence from Diffusion Tensor Imaging. *Biological Psychiatry*. 2004;55(3):323-326.

20. Gomot M, Bernard F, Davis M, et al. Change Detection in Children with Autism: An Auditory Event-Related fMRI Study. *Neuroimage*. 2006;29(2):475-484.

21. Vargas D, Nascimbene C, Krishnan C, Zimmerman A, Pardo C. Neuroglial Activation and Neuroinflammation in the Brain of Patients with Autism. *Ann Neurol*. 2005;57(1):67-81.

22. Schuetze M, Park MT, Cho IY, MacMaster F, Chakravarty M, Bray S. Morphological Alterations in the Thalamus, Striatum, and Pallidum in Autism Spectrum Disorder. *Neuropsychopharmacology*. Oct 2016;41(11):2627–2637.

23. Woodward N, Giraldo-Chica M, Rogers B, Cascio C. Thalamocortical Dysconnectivity in Autism Spectrum Disorder: An Analysis of the Autism Brain Imaging Data Exchange. *Biol Psychiatry Cogn Neurosci Neuroimaging*. Jan 2017;2(1):76–84.

24. Bailey A, Luthert P, Dean A, et al. A clinicopathological study of autism. *Brain*. 1998;121(5):889-905.

25. Fatemi S, Halt A, Realmuto G, et al. Purkinje Cell Size is Reduced in Cerebellum of Patients with Autism. *Cell Mol Neurobiol*. 2002;22(2):171-175.

26. Just M, Cherkassky V, Keller T, Minshew N. Cortical Activation and Synchronization During Sentence Comprehension in High-Functioning Autism: Evidence of Underconnectivity. *Brain*. 2004;127(8):1811-1821.

27. Bauman M, Kemper T. Histoanatomic Observations of the Brain in Early Infantile Autism. *Neurology*. 1985 ;35(6):866-874.

28. Schumann C, Hamstra J, Goodlin-Jones B, et al. The Amygdala is Enlarged in Children But Not Ndolescents with Autism; the Hippocampus is Enlarged at All Ages. *J Neurosci*. 2004;24(28):6392-6401.

29. Baron-Cohen S, Ring H, Wheelwright S, et al. Social Intelligence in the Normal and Autistic Brain: an fMRI Study. *Eur J Neurosci*. 1999;11(6):1891-1898.

30. Lawrence Y, Kemper T, Bauman M, Blatt G. Parvalbumin-, Calbindin-, and Calretinin-Immunoreactive Hippocampal Interneuron Density in Autism. *Acta Neurol Scand*. 2010;121(2):99-108.

31. Cooper R, Richter F, Bays P, Plaisted-Grant K, Baron-Cohen S, Simons J. Reduced Hippocampal Functional Connectivity During Episodic Memory Retrieval in Autism. *Cereb Cortex*. 2017;27(2):888–902.

32. Huang Y, Zhang B, Cao J, et al. Potential Locations for Noninvasive Brain Stimulation in Treating Autism Spectrum Disorders—A Functional Connectivity Study. *Front Psychiatry*. 2020;11(388).

33. Mash L, Reiter M, Linke A, Townsend J, Müller RA. Multimodal Approaches to Functional Connectivity in Autism Spectrum Disorders: An Integrative Perspective. *Dev Neurobiol*. 2018;78(5):456-473.

34. Padmanabhan A, Lynch C, Schaer M, Menon V. The Default Mode Network in Autism. *Biol Psychiatry Cogn Neurosci Neuroimaging*. 2017;2(6):476–486.

35. Klauck S, Poustka A. Animal Models of Autism. *Drug Discovery Today: Disease Models*. 2006;3(4):313-318.

36. McBride S, Choi C, Wang Y, et al. Pharmacological Rescue of Synaptic Plasticity, Courtship Behavior, and Mushroom Body Defects in A Drosophila Model of Fragile X Syndrome. *Neuron*. March 2005;45(5):753-764.

37. Hrabovska S, Salyha, Yu. Animal Models of Autism Spectrum Disorders and Behavioral Techniques of their Examination. *Neurophysiology*. October 2016;48(5):380–388.

38. Nakatani J, Tamada K, Hatanaka F, Ise S, Ohta H, Inoue K. Abnormal Behavior in A Chromosome-Engineered Mouse Model for Human 15q11-13 Duplication Seen in Autism. *Cell*. June 2009;137(7):1235-1246.

39. Jamain S, Radyushkin K, Hammerschmidt K, Granon S, Boretius S, Varoqueaux F. Reduced Social Interaction and Ultrasonic Communication in A Mouse Model of Monogenic Heritable Autism. *Proc Natl Acad Sci U S A*. Feb 2008;105(5):1710-1715.

40. Patterson P. Modeling Autistic Features in Animals. *Pediatric Research*. 2011;69(5):34–40.

41. Nicolini C, Fahnestock M. The Valproic Acid-Induced Rodent Model of Autism. *Exp Neurol*. 2018;299(Pt A):217-227.

42. Tartaglione AM, Schiavi S, Calamandrei G, Trezza V. Prenatal Valproate in Rodents As A Tool to Understand The Neural Underpinnings of Social Dysfunctions in Autism Spectrum Disorder. *Neuropharmacology*. 2019;159(107477).

43. Wu HF, Chen YJ, Chu MC, et al. Deep Brain Stimulation Modified Autism-Like Deficits via the Serotonin System in a Valproic Acid-Induced Rat Model. *International Journal of Molecular Sciences*. 2018;19(9):2840.

44. Lin TC, Lo YC, Lin HC, et al. MR Imaging Central Thalamic Deep Brain Stimulation Restored Autistic-Like Social Deficits in The Rat. *Brain Stimulation*. 2019;12(6):1410-1420.

45. Hagen E, Shprung D, Minakova E, et al. Autism-Like Behavior in BTBR Mice is Improved by Electroconvulsive Therapy. *Neurotherapeutics*. 2015;12(3):657-666.

46. Chang A, Berges V, Chung S, Fridman G, Baraban J, Reti I. High-Frequency Stimulation at the Subthalamic Nucleus Suppresses Excessive Self-Grooming in Autism-Like Mouse Models. *Neuropsychopharmacology*. 2016;41(7):1813-1821.

47. Tan T, Wang W, Xu H, Huang Z, Wang YT, Dong Z. Low-Frequency rTMS Ameliorates Autistic-Like Behaviors in Rats Induced by Neonatal Isolation Through Regulating the Synaptic GABA Transmission. *Frontiers in Cellular Neuroscience. PubMed. Feb 28;*. 2018;12(46).

48. Ruhela R, Prakash A, Medhi B. An Urgent Need for Experimental Animal Model of Autism in Drug. *Ann Neurosci*. 2015;22(1):44–49.

49. Tania M, Asaduzzaman Khan M, Xia K. Recent Advances in Animal Model Experimentation in Autism Research. *Acta Neuropsychiatr*. October 2014;26(5):264-271.

50. Crawley J. Designing Mouse Behavioral Tasks Relevant to Autistic-Like Behaviors. *Ment Retard Dev Disabil Res Rev*. 2004;10(4):248-258.

51. Hulbert S, Jiang Yh. Monogenic Mouse Models of Autism Spectrum Disorders: Common Mechanisms and Missing Links. *Neuroscience*. May 2016;321:3–23.

52. Dowling J. Deep Brain Stimulation: Current and Emerging Indications. *Mo Med*. 2008;105(5):424-428.

53. Larson P. Deep Brain Stimulation for Movement Disorders. *Neurotherapeutics*. July 2014;11(3):465-474.

54. Burt T, Lisanby S, Sackeim H. Neuropsychiatric Applications of Transcranial Magnetic Stimulation: A Meta Analysis. *Int J Neuropsychopharmacol*. March 2002;5(1):73-103.

55. Kerner N, Prudic J. Current Electroconvulsive Therapy Practice and Research in The Geriatric Population. *Neuropsychiatry (London)*. 2014;4(1):33–54.

56. Bronstein J, Tagliati M, Alterman R, et al. Deep Brain Stimulation for Parkinson Disease: An Expert Consensus and Review of Key Issues. *Arch Neurol*. February 2011;68(2):165.

57. Parasuraman R, Christensen J, Grafton S. Neuroergonomics: The Brain in Action and at Work. *Neuroimage*. January 2012;59(1):1-3.

58. Bystritsky A, Korb A, Douglas P, et al. A Review of Low-Intensity Focused Ultrasound Pulsation. *Brain Stimul*. 2011;4(3):125-36.

59. Thielscher A, Opitz A, Windhoff M. Impact of the Gyral Geometry on the Electric Field Induced by Transcranial Magnetic Stimulation. *NeuroImage*. 2011;54(1):234-243.

60. Opitz A, Windhoff M, Heidemann RTR, Thielscher A. How the Brain Tissue Shapes the Electric Field Induced by Transcranial Magnetic Stimulation. *Neuroimage*. 2011;58:849–859.

61. Rezayat E, Toostani I. A Review on Brain Stimulation Using Low Intensity Focused Ultrasound. *Basic Clin Neurosci*. 2016;7(3):187–194.

62. Baek H, Pahk KJ, Kim H. A Review of Low-Intensity Focused Ultrasound for Neuromodulation. *Biomed Eng Lett*. 2017;7(2):135–142.

63. Legon W, Sato T, Opitz A, et al. Transcranial Focused Ultrasound Modulates the Activity of Primary Somatosensory Cortex in Humans. *Nat Neurosci*. 2014;17(2):322-329.

64. Tufail Y, Matyushov A, Baldwin N, et al. Transcranial Pulsed Ultrasound Stimulates Intact Brain Circuits. *Neuron*. 2010;66(5):681-694.

65. Yoo SS, Kim H, Filandrianos E, Taghados S, Park S. Non-Invasive Brain-to-Brain Interface (BBI): Establishing Functional Links between Two Brains. *PloS One*. 2013;8(4).

66. Yoo SS, Bystritsky A, Lee JH, et al. Focused Ultrasound Modulates Region-Specific Brain Activity. *Neuroimage*. 2011;56(3):1267-1275.

67. Kim H, Park M, Lee S, Lee W, Chiu A, Yoo SS. Suppression of EEG Visual-Evoked Potentials in Rats via Neuromodulatory Focused Ultrasound. *Neuroreport*. 2015;26(4):211–215.

68. Lin WT, Chen RC, Lu WW, Liu SH, Yang FY. Protective Effects of Low-Intensity Pulsed Ultrasound on Aluminum-Induced Cerebral Damage in Alzheimer's Disease Rat Model. *Sci Rep*. 2015;5(9671).

69. Min BK, Bystritsky A, Jung KI, et al. Focused Ultrasound-Mediated Suppression of Chemically-Induced Acute Epileptic EEG Activity. *BMC Neurosci*. 2011;12(1):23.

70. Yoon K, Lee W, Lee JE, et al. Effects of Sonication Parameters on Transcranial Focused Ultrasound Brain Stimulation in an Ovine Model. *PLoS One*. 2019;14(10).

71. Hameroff S, Trakas M, Duffield C, et al. Transcranial Ultrasound (TUS) Effects on Mental States: A Pilot Study. *Brain Stimul*. 2013;6(3):409-415.

72. Legon W, Bansal P, Tyshynsky R, Ai L, Mueller J. Transcranial Focused Ultrasound Neuromodulation of The Human Primary Motor Cortex. *Scientific Reports*. 2018;8(1).

73. Deffieux T, Younan Y, Wattiez N, Tanter M, Pouget P, Aubry JF. Low-Intensity Focused Ultrasound Modulates Monkey Visuomotor Behavior. *Curr Biol*. 2013;23(23):2430–2433.

74. Kim H, Taghados S, Fischer K, Maeng LS, Park S, Yoo SS. Noninvasive Transcranial Stimulation of Rat Abducens Nerve by Focused Ultrasound. *Ultrasound Med Biol*. 2012;38(9):1568-1575.

75. Tropepe V, Sive H. Can zebrafish be used as a model to study the neurodevelopmental causes of autism? *Genes Brain Behav*. Oct 2003;2(5):268-281.

Tables

Table 1: Human brain regions implicated in ASD.

Brain Region	Subject	Result	Evaluation Technique	Reference/Year
Prefrontal cortex	15 children with autism (12 boys and 3 girls with age range of 5 to 9 years) and 15 healthy children (12 boys and 3 girls with age range of 5 to 9 years)	Dysfunctioning	EEG and Electro oculograms (EOG)	9 - 2002
	14 high functioning participants with autism (13 males and 1 female, mean age of 25.7 years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)	Hyperactivity	fMRI	10 - 2005

Medial prefrontal cortex (mPFC)	7 autistic patients and 6 control male children aged 2 to 16 years	Excess number of neurons	Stereological methods	12 - 2011
	15 patients with ASD (10 with high functioning autism, 3 with Asperger's syndrome, and 2 with pervasive developmental disorder) and 14 healthy controls	No activity	Behavioral Data and fMRI	13 - 2006
	10 adults (mean age of 33 years) with autism or Asperger syndrome, and 10 normal individuals (mean age of 25 years)	Hypo-activity	Behavioural data along with PET and MRI	14 - 2002
	7 autistic and 9 normal control adults	Hypo-activity	fMRI	15 - 2004
	19 children with autism associated 16p11.2 deletion and 28 control subjects	Reduced global connectivity	fMRI	16 - 2018
Dorsolateral prefrontal cortex (DLPFC)	7 autistic patients and 6 control male children aged 2 to 16 years	Excess number of neurons	Stereological methods	12 - 2011

	14 high functioning participants with autism (13 males and 1 female, mean age of 25.7 years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)	Hypo-activity	fMRI	10 - 2005
Anterior cingulate cortex (ACC)	9 autistic individuals and healthy controls	Decrease in cell size and cell packing density	Stereological techniques and microscopic examination	17 - 2009
	13 autistic and 7 healthy children and adolescents aged 6 to 17 years	Abnormal overgrowth	MRI	18 - 2013
	7 male children and adolescents with autism and 9 control subjects	Disruption of white matter tracts between regions implicated in social functioning	Diffusion tensor imaging	19 - 2004
	15 patients with ASD (10 with high functioning autism, 3 with Asperger's syndrome, and 2 with pervasive developmental	No activity	Behavioral Data and fMRI	13 - 2006

	disorder) and 14 healthy controls			
	12 children with autism (age range of 10 to 15 years) and 12 age and sex matched healthy controls	Hypo-activity	fMRI	20 - 2006
	11 patients with autism	Neuroglial activation and neuroinflammation	Immunocytochemistry, cytokine protein arrays, and enzyme linked immunosorbent assays	21 - 2005
Thalamus	373 male participants with ASD (age range of 7–35 years) and 384 typically developing	Morphological alterations	T1-weighted anatomical images	22 - 2016
	228 individuals with ASD and 228 healthy individuals	Prefrontal cortex, temporal lobe, and sensorimotor cortex exhibited hyper-connectivity with the thalamus	Autism Brain Imaging Data Exchange	23 - 2017
Cerebellum	6 individuals with autism of ages 4, 20, 23, 24, and 27 years	Reduced number of PCS	Morphometry	24 - 1998
	5 autistics and 5 normal individuals	Decreased size of PCs	Computer assisted image analysis	25 - 2002

	11 patients with autism	Neuroglial activation and neuroinflammation	Immunocytochemistry, cytokine protein arrays, and enzyme linked immunosorbent assays	21 - 2005
Temporal lobe	10 adults (mean age of 33 years) with autism or Asperger syndrome, and 10 normal individuals (mean age of 25 years)	Hypo-activity	Behavioural data along with PET and MRI	14 - 2002
	14 high functioning participants with autism (13 males and 1 female, mean age of 25.7 years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)	Hyperactivity	fMRI	10 - 2005
	19 children with autism associated 16p11.2 deletion and 28 control subjects	Reduced connectivity	fMRI	16 - 2018
Parietal lobe	14 high functioning participants with	Hypo-activity of the left inferior parietal lobe and	fMRI	10 - 2005

	autism (13 males and 1 female, mean age of 25.7 years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)	hyperactivity of the right inferior parietal lobe		
	19 children with autism associated 16p11.2 deletion and 28 control subjects	Reduced connectivity	fMRI	16 - 2018
Temporoparietal junction	10 adults (mean age of 33 years) with autism or Asperger syndrome, and 10 normal individuals (mean age of 25 years)	Hypo-activity	Behavioural data along with PET and MRI	14 - 2002
	17 high functioning autistic participants and 17 healthy individuals	Hyperactivity	fMRI	26 - 2004
	19 children with autism associated 16p11.2 deletion and 28 control subjects	Reduced connectivity	fMRI	16 - 2018

Amygdala	29 years old autistic man and 29 years old healthy man	Reduced cell size	Zeiss comparison microscope	27 - 1985
	male children of an age range of 7.5 to 18.5 years were participated in four diagnostic groups: autism with mental retardation, autism without mental retardation, Asperger syndrome, and age matched typically developing controls	Larger volume	MRI	28 - 2004
	Patients with high functioning autism or Asperger syndrome (AS) and normal participants	Lack of activity	fMRI	29 - 1999
	7 autistic and 9 normal control adults	Hypo-activity	fMRI	15 - 2004
Hippocampus	7.5 to 18.5 years were participated in four diagnostic	Larger right and left hippocampal volume	MRI	28 - 2004

	groups: autism with mental retardation, autism without mental retardation, Asperger syndrome, and age matched typically developing controls			
	5 autistic and 5 postmortem control cases	Alterations in interneuron density	Stereological techniques	30 - 2010
	48 subjects (age range of 18 to 45 years) including 24 adults with ASD (14 males) and 24 healthy participants (13 males)	Reduced connectivity	fMRI	31 - 2017
Broca's Area	17 high functioning autistic participants and 17 healthy individuals	Hypo-activity	fMRI	26 - 2004
Occipital lobe	14 high functioning participants with autism (13 males and 1 female, mean age of 25.7	Hyperactivity	fMRI	10 - 2005

	years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)			
Precentral sulcus	14 high functioning participants with autism (13 males and 1 female, mean age of 25.7 years) and 14 healthy normal control participants (13 males and 1 female, mean age of 29.8 years)	Hypo-activity	fMRI	10 - 2005

Table 2: Comparison between some neurostimulation methods and LIFUS.[61]

Specification	DBS	rTMS	LIFUS
Invasiveness	Invasive	Noninvasive	Noninvasive
Spatial Resolution	~1 mm	~3 to 5 cm	1 to 5 mm
Stimulation Depth	Unlimited	~1 to 1.5 cm unless H coil is used	10 to 15 cm or more

Rasha NOUREDDINE

Impact of Focused Ultrasound Stimulation on Brain Activity in The Framework of Autism – In-Vivo Study in ‘‘Autistic-Like’’ Behavior Animal Model

Résumé

L'objectif de ce projet est d'examiner la stimulation par ultrason focalisé transcrânien (tFUS) comme outil thérapeutique, dans un modèle animal de valproate (VPA), des troubles du spectre autistique (TSA). La mise en œuvre et l'optimisation de la stimulation tFUS nécessitaient de cibler le cortex moteur à diverses pressions acoustiques et d'évaluer les réponses motrices évoquées au niveau des membres antérieurs. Un autre objectif est d'étudier l'effet et l'innocuité de la stimulation sur le cortex cingulaire antérieur (ACC) qui nécessitait la stimulation de souris saines à l'ACC suivie d'essais en plein champ et d'une analyse histologique. L'examen de la validation du modèle animal VPA utilisé et des effets du tFUS a été effectué à l'aide de divers tests comportementaux. Ces tests n'ont pas montré de différences significatives entre les souris VPA et les souris témoins, ce qui suggère que le modèle VPA utilisé n’était pas convenable. Cependant, l'absence de différences a également été remarquée entre les souris stimulées et non stimulées confirmant l'innocuité de la stimulation appliquée. De plus, une méthodologie et un protocole fiables pour une neuromodulation ultrasonore efficace et sûre chez la souris ont été réalisés. En outre, une méthode non invasive simple et cohérente pour localiser et cibler les régions du cerveau chez la souris a été réalisée.

Résumé en anglais

The objective of this project was to examine transcranial focused ultrasound stimulation (tFUS) as a therapeutic tool in a valproate (VPA) animal model of autism spectrum disorder (ASD). The implementation and optimization of tFUS stimulation required targeting the motor cortex at various acoustic pressures and assessing the motor evoked responses at the forelimbs. Another aim was to investigate the effect and safety of stimulation on the anterior cingulate cortex (ACC) which required stimulating healthy mice at the ACC followed by open field testing and histological analysis. Examining the validation of the VPA animal model used and the effects of tFUS was performed using variety of behavioral tests. Behavioral tests did not show significant differences between VPA and control mice, suggesting that the VPA model used did not work. However, lack of differences was also noticed between stimulated and non-stimulated mice confirming the safety of the stimulation applied. Moreover, a reliable methodology and protocol for effective and safe ultrasonic neuromodulation in mice was accomplished. Furthermore, a simple and consistent non-invasive method for locating and targeting brain regions in mice was achieved.