BACHELOR'S THESIS / BIOMEDICAL ENGINEERING 2021

Auricular transcutaneous vagus nerve stimulation device in the form of an ear pod for mice: A first step towards chronic non-invasive auricular vagus nerve stimulation in animal models

Sergi Navarro Guimerà

upf. Universitat Pompeu Fabra Barcelona

Auricular transcutaneous vagus nerve stimulation device in the form of an ear pod for mice: A first step towards chronic non-invasive auricular vagus nerve stimulation in animal models

Sergi Navarro Guimerà

Bachelor's Thesis UPF 2020/2021

Thesis Supervisors:

Dr. Andrés Ozaita, (Principal investigator in NeuroPhar and Full Professor at UPF)
Dr. Antoni Ivorra, (Head of BERG and Full Professor at UPF)

Acknowledgments

I would first like to express my sincere gratitude to my two bachelor's thesis supervisors Dr. Andrés Ozaita and Dr. Antoni Ivorra for their assistance at every stage of the project. Apart from being the architects of the original idea, they have actively provided me with insightful feedback and suggestions. Your plentiful expertise and patience have pushed me to work at my highest level.

I would also like to express my special thanks to Cecilia, who together with Dr. Andrés Ozaita have been my mentors in my first experimental contact with mice. From the very first moment you provided me with your invaluable time and unwavering support, both technical and emotional, which have encouraged me to believe in this project. I just have no words to express how thankful I am for this.

I would like to extend my sincere thanks to Dr. Laura Becerra-Fajardo and Ianire Gallego. Laura has attended my proposals and inquires, lending me a hand at many times and helping me choose the right direction, always with kind words. Ianire has also made key contributions in experimental processes.

I am also grateful to the Neuropharmacology and BERG laboratories, as well as to all their members. I have really been embraced by them.

Finally, I would like to acknowledge my reviewers in advance, Dr. Rubén Moreno and pre-doc Anna Vázquez-Oliver, for acceding to read my thesis. I strongly believe that the feedback will be essential to help my grow personally and professionally.

Getting to the point where we are now would not have been possible without all of you. You have shown me that communication, collaboration, effort and passion are the recipe for good work, and this, together with the amazing experience and the encouraging results is what I value the most.

Abstract

The pro-cognitive effects of Vagus Nerve Stimulation (VNS) observed in some small off-label studies have raised enthusiasm for its use to ameliorate cognitive performance. Relatedly, auricular transcutaneous VNS (atVNS) has gained a lot of attention as the most affordable, non-invasive VNS technique. Unfortunately, mechanisms underlying atVNS cognitive effect are still unclear. Further research in the mouse is required. So far it has been limited to acute non-invasive stimulation, or to chronic invasive stimulation. While the underlying effects of acute stimulation are probably far from the physiological and molecular pathways activated in chronic stimulation, invasivity is accompanied by surgical challenges and morbidity. There is a need for a simple, well-characterized, and reliable non-invasive chronic atVNS technique. In the present study, after intense trial and error, we design and produce the S-pod: a device in the form of an ear pod for atVNS in mice. To test proper functioning, we corroborate the already proven memory persistence enhancement triggered by acute atVNS in anesthetized naïve mice in a novel object-recognition test. The implementation of an innovative technique to test the stimulation sites, and the steadiness of the S-pod in the concha might make the results more reproducible than the ones obtained with the previous and only atVNS device for mice, also designed by our group. A further advantage of the S-pod with respect to the previous atVNS device is its compatibility with a proposed preliminary model of a chronic atVNS setup for electrostimulation in non-anesthetized mice. The latter includes an Elizabethan collar to avoid ear scratching, for which a habituation validation in CD-1 mice is provided. The present work supposes a first step to implementing non-invasive chronic atVNS in mice. Further research could conceivably open new doors to unearthing the molecular, neurological, and anatomical mechanisms underlying the cognitive effect of chronic atVNS.

Keywords

vagus nerve stimulation, auricular transcutaneous vagus nerve stimulation, cognitive enhancement, stimulation sites, elizabethan collar, single-axis lever arm, chronic atVNS, free-moving mice

Prologue

This is a joint project between BERG and NeuroPhar, both belonging to the UPF. The research of BERG laboratory is focused on exploring bioelectrical phenomena to develop new methods and devices for biomedical applications. NeuroPhar laboratory mainly centers on developing lines of research to identify new therapeutic targets at the level of the nervous system. Additionally, the group led by Prof. Andrés Ozaita is now putting big effort to find new pharmacological and non-pharmacological treatments to modulate cognition. As a matter of fact, in a previous collaboration between these two laboratories they yielded an atVNS device to perform acute auricular transcutaneous vagus nerve stimulation (atVNS) in mice. Testing showed enhanced memory performance in a novel object-recognition test. The encouraging results triggered the start of a further investigation of the cognitive effects of acute atVNS with the stimulation device. This is part of a PhD project of pre-doc Cecilia Brambilla, being the study also conducted by Prof. Andrés Ozaita.

The cognitive modulation capacity of acute atVNS derived from these studies asks for further investigation on this field. For so, there is a need for a chronic atVNS setup for the mouse. This is, although acute atVNS is already showing very encouraging cognitive effects, the underlying activated pathways are probably far from the ones in chronic atVNS.

In the present study we design, produce and test an atVNS device in the form of an ear pod for mice: the S-pod. This supposes an advance with respect to the previous and only atVNS device for mice in three main aspects: (1) S-pod allows reproducibility of the stimulation sites due to the perfect fitting in the concha, which was not possible in the previous design. This can be demonstrated thanks to our technique, the first to the best of our knowledge, to mark stimulation sites in atVNS using DC current (2) S-pod eases acute atVNS thanks to easy placement (3) S-pod is compatible with a proposed chronic atVNS setup, for which a preliminary habituation validation is provided.

The S-pod, the new technique to mark stimulation sites, and the proposed chronic atVNS setup are beneficial at the level of BERG and NeuroPhar laboratories to continue investigating on the field of atVNS and cognition, but they also suppose a global step further in atVNS research on animal models.

Further research could center on unearthing the possible inflammatory reduction or cognitive reserve increase effects of atVNS. This could be life-changing not only for people suffering from dementia - being it the main cause of cognitive impairment and tightly related to neuroinflammation -, but also for every single human, bearing in mind that the cognitive reserve strengthens resistance to cognitive decline typical from ageing.

Index

List of Figures

List of Tables

1 Introduction

Cognition refers to a broad range of higher cortical functions carried out by the human brain including memory, thinking, remembering, reasoning, learning, comprehension, orientation, analyzing, planning, language, judgement, and attention [1][2]. In broad strokes, cognition includes all process by which a person becomes aware of a situation, needs, goals, and required actions, and uses this information to implement problem solving strategies for optimal living [1]. When a person permanently looses or lacks this ability, up to a minor or major degree, we talk about cognitive impairment, being the two main causes intellectual disability and dementia.
Intellectual disability is a feature of numerous neurodevelopmental disorders characterized by significantly impaired intellectual and adaptive functioning that originates and manifests before the age of 18 [3]. The etiology is normally genetic (e.g. fragile X syndrome, Down syndrome, Prader-Willi syndrome), but it can also be triggered by certain environmental exposures (e.g. fetal alcohol spectrum disorder, viral or bacterial infections during pregnancy, exposure to toxins, malnutrition). The prevalence of intellectual disability in the Western population is estimated to range from 1% to 3% [3], presenting about 85% of the affected individuals a mild intellectual disability, corresponding to the lowest level of severity - mild, moderate, severe, and profound in order of severity -.
In contrast, dementia is a syndrome derived from diseases (e.g. Alzheimer's Disease, vascular dementia, Parkinson's Disease) or injury of the brain that affect cognition beyond what might be expected from normal ageing [2]. Hence, similarly to intellectual disability, the capacity of the individual to understand reality and interact with it is lessened, lacking them competence in social, conceptual and practical skills [3]. Nowadays, the estimated number of people living with dementia worldwide is 35.6 million. However, the prevalence is expected to globally rise, indicating projections that by 2050, 151.4 million people will suffer from dementia [4].

At the present time there is still no cure for cognitive impairment in intellectual disability or dementia. Treatments mostly aim at improving adaptive behavioral skills with supportive care or avoiding rapid progression in dementia, but they fail at enhancing cognition. Accordingly, there is a need for innovative treatments with the capacity for improving the performance of higher cortical functions in mentally disabled individuals. It is under such premise that Vagus Nerve Stimulation (VNS) has recently attracted a lot of attention as a possible non-pharmacological cognitive enhancing therapy. Relatedly, a non-invasive variant of VNS, auricular transcutaneous VNS (atVNS), has been defined as the most affordable non-invasive manipulation of the central nervous system [5]. Although some case studies already suggest atVNS to have pro-cognitive effects [6, 7], the underlying mechanisms are still unclear. Further research on the mouse is required, being it the preferred species to derive the bio-physiological basic mechanisms behind emerging therapies [8]. Research in mice so far been limited to acute atVNS [6], or to chronic invasive [8] or non-invasive VNS [9]. There is a need for a simple, well-characterized, and reliable non-invasive chronic atVNS technique for mice. The better understanding of the cognitive pathways activated by atVNS could lead to the grounding of therapies to enhance cognition, as well as to a more exact definition of the possible side effects. This would be life-changing for millions of people suffering from cognitive impairment around the world.

The objective of the present study is to do a first step towards the implementation of a non-invasive system to perform chronic atVNS arrays in mice. With such purpose, we provide with 3 main tools. In the first place, in Introduction and Additional Information sections we supply with contrasted state-of-the-art regarding the physiological pathway of atVNS, the cognitive effects observed so far, and apparently optimal stimulation parameters in mice. Secondly, we yield an atVNS device in the form of a pod for stimulation in mice, which is compatible with the third tool: a preliminary setup for chronic non-invasive atVNS in non-anesthetized mice. These three tools are complementary. While the chronic atVNS setup requires the pod for stimulation, the contrasted state-of-the-art supposes a guide to know which directions to take in research using the proposed setup to derive the activated cognitive pathways in chronic atVNS. All in all, this will give a chance to advance in the treatment of cognitive impairment, a major need that will escalate in the coming years.

1.1 Vagus Nerve

The vagus nerve (VN) - also known as tenth cranial nerve (CN X) - is a mixed Autonomous Nervous System (ANS) nerve composed of 20% efferent (motor) fibers and 80% afferent (sensory) fibers [10]. This means that the VN mainly conveys sensory information from the body surface, muscles, and viscera to the spinal cord [11], although presenting it also some fibers responsible for carrying impulses away from the central nervous system (CNS).

Vagal afferents can be classified in two main categories depending on their neuroanatomical functions: general visceral afferents (GVA) and general somatic afferents (GSA). The first type of afferent fibers carry information from the larynx, trachea, lungs, heart and gastrointestinal tract [12, 13] to four nuclei in the medulla: the spinal nucleus of the trigeminal nerve, the nucleus ambiguous, the dorsal motor nucleus of the VN, and the nucleus tractus solitarius (NTS) [14]. Alternatively, GSA relay sensory impulses from the lower pharynx, larynx, trachea, oesophagus, and posterior dura mater, and carry somatic sensory information of pain, touch and temperature [13] from the posterior meninges, tympanic membrane and external ear (e.g. conchae, skin on the back of the ear, external acoustic meatus, and part of the tympanic membrane) to the brainstem nuclei of the trigeminal nerve [7].

Vagal efferents originate in the nucleus ambiguous and the dorsal motor nucleus. These two medulla nuclei send widespread efferent visceromotor fibers to govern neurogenic, myogenic, and endocrine actions within end organs (i.e. pharynx, larynx, trachea, heart, aorta, lungs, and the entire gastrointestinal tract) [7].

The combination of CNS modulatory (afferent fibers) and systemic effects (efferent fibers) makes from VN an essential driver of the mutual connection between the brain and major body structures. Accordingly, the activity of VN is positively associated with health, well-being, relaxation, and emotions like empathy, and negatively associated with morbidity, mortality, and stress [7].

1.2 Vagus Nerve Stimulation (VNS)

The term "Vagus Nerve Stimulation" (VNS) refers to any technique that originates electric pulses in the CN X [10], being it popularly described as an adjunctive neuromodulation therapy [15].

1.2.1 History and Indications

The first U.S. Food and Drug Administration (FDA) approved indication of VNS was partial onset seizures in 1997. The approval led Cyberonics to release an invasive VN neck implantable stimulator [13] for adjunctive treatment refractory to antiepileptic medications in adults and adolescents aged at least 12 years old [14].

Since 1997, an evolution beyond invasive VNS has led to three other very common VNS techniques in humans: non-invasive VNS, auricular transcutaneous VNS, and auricular percutaneous VNS [16].

Invasive VNS is typically performed via cuff electrodes wrapped around the left cervical branch, and connected to an implantable pulse generator. This is compatible with fixed non-adaptive and on-demand adaptive stimulation, and it has been FDA approved to treat epilepsy and depression (PMA P970003/S207) [16].
Non-invasive VNS also activates the VN at the cervical level, but surface skin electrodes in the neck are used instead. Intermittent transcutaneous stimulation using this non-invasive approach has FDA approval as a preventive and/or acute treatment for migraine and episodic cluster headache (DEN150048) [17, 16].
Auricular transcutaneous VNS uses two skin electrodes to deliver intermittent bipolar pulses on the outer ear. The method has CE approval for epilepsy, depression, pain (CerboMed-Nemos), and migraine (GammaCore) [16].
Finally, auricular percutaneous VNS is a minimally invasive technique consisting in the insertion of miniature needle electrodes in the targeted outer ear sites. Intermittent activation of the VN using this technique has been authorized as a pain reliever for multiple disease conditions (e.g. chronic cervical pain, chronic low back pain, migraine, acute post-operative pain, pain due to peripheral arterial occlusive disease) [16].

Beyond the authorized indications of the four main VNS methods, off-label studies and case series reports have suggested VNS to have positive effects in the treatment of multiple psychiatric disorders (e.g. rapid cycling bipolar disorder, treatment-resistant anxiety disorders [18], autism spectrum disorders [5]), neurodegenerative disorders (e.g. Alzheimer's Disease [19]), cognitive impairment (enhanced divergent thinking [20], facilitation of visceral pain-related emotional affective memory, extinction of conditioned fear [5], face-name associations consolidation [21], intellectual disability [6], cerebral ischemia [22], recovery after traumatic brain injury [5]), and other disease conditions (e.g. obesity [18]). However, the FDA hasn't still approved none of these indications. This is mainly due to the lack of standardized methods of employment, which manifests the need for more preclinical studies.

1.2.2 Auricular transcutaneous Vagus Nerve Stimulation (atVNS)

Most GSA fibers terminating the central process in the NTS are driven by the auricular branch of the VN (ABVN) - also known as Alderman's nerve or Arnold's nerve -, accounting for the only peripheral branch of the VN [7]. On its way, ABVN innervates most of the area around the external auditory meatus and the auricular concha [23, 24, 25], which includes the cavity of the concha, the crus of helix and the cymba conchae (see Figure 1 for further information on outer ear innervations and parts). Due to the entire supply (100%) of the latter by fibers of ABVN, cymba conchae forms a cutaneous receptive field susceptible to external stimuli. Consequently, atVNS in cymba conchae offers the most affordable non-invasive manipulation of the CNS by acting upon NTS [5].

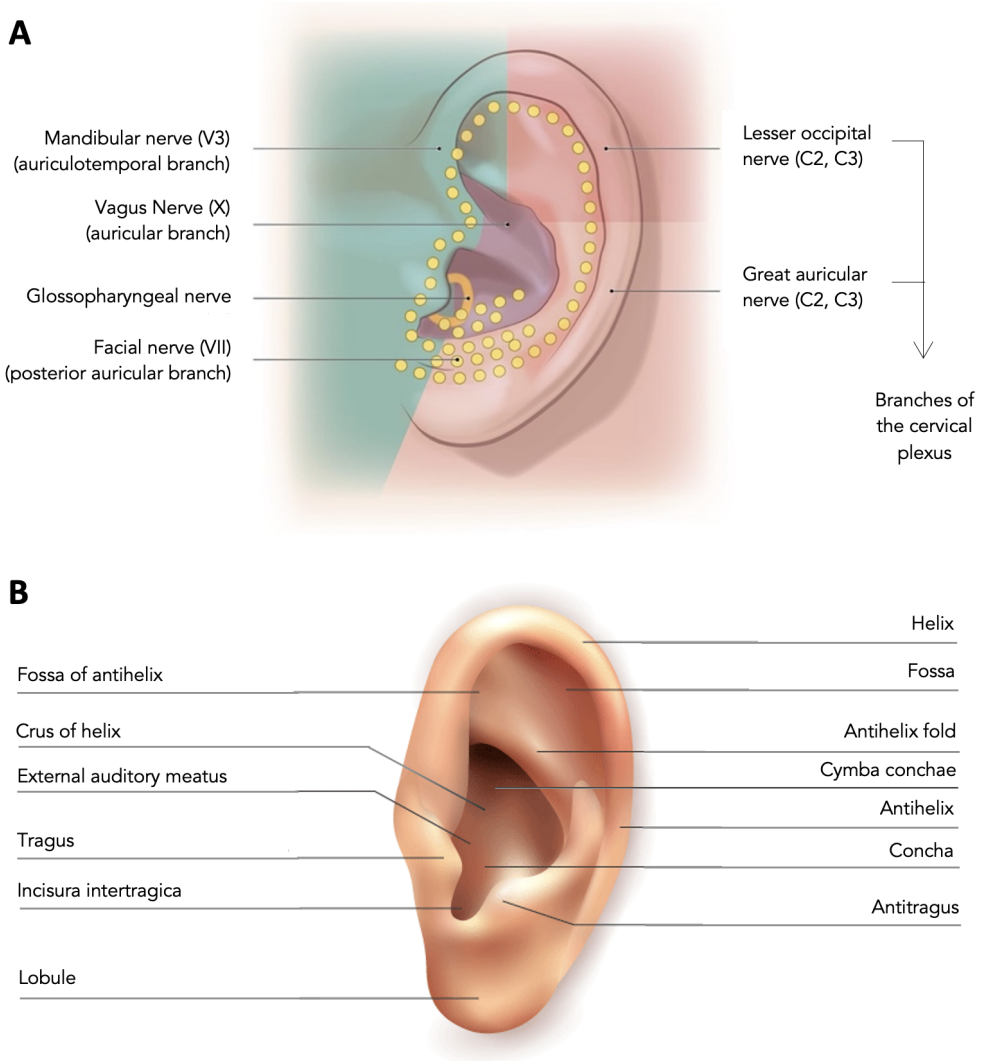

Figure 1: **Human external auricle.** (A) Display of the different parts of the human outer ear adapted from Byju's [26]; (B) Innervations of the external auricle adapted from AMBOSS [27]. Green represents for the innervations of the auriculotemporal nerve, blue of the ABVN, light red of the lesser occipital nerve, beige of the greater auricular nerve, yellow of the facial nerve, and gold of the glossopharyngeal nerve.

Auricular transcutaneous VNS is emerging in a context where bioelectronic medicine is perceived as a non-pharmaceutical treatment option for various diseases. A similar path is being followed by invasive, non-invasive, and auricular percutaneous VNS. How-

ever, atVNS is outlying over them given its non-invasivity and low recruitment of efferent fibers associated with adverse effects.

Current atVNS is being performed through surface skin electrodes. The number ranges from 2 to 3, and the most common stimulation sites are the cymba conchae, antihelix, tragus, and cavity of the concha. However, the exact location of electrodes in each of these areas is still unclear. Apparently, there seems to be a dependence on the desired effects, as well as on the fibers that are intended to be recruited [28]. Despite the entire suppliance (100%) of the cymba conchae by ABVN, the antihelix (73%), the tragus (45%), and the cavity of the concha (45%) also present non-vagal fibers that can be recruited under atVNS given the diffuse stimulation fields most devices result in [16, 7].

When it comes to safety and tolerability of atVNS, it has been demonstrated by several groups including central and peripheral nervous system effects, and behavioral effects in neuropsychiatric populations [29, 30]. Furthermore, side-effects are minimal, with skin irritation or redness being the most common ones [16].

Auricular transcutaneous VNS has CE approval for various indications in humans (i.e. epilepsy, depression, pain, and migraine), but there is extensive literature based on human models proposing atVNS as a promising therapeutic tool for neurological disorders, inflammation reduction [7], disorders of consciousness [31], and to enhance cognitive and social functioning [21]. An extended and more detailed summary of previous atVNS clinical trials and studies is provided by Y. Y. Yap et al. (2020) [32].

1.2.3 Physiological mechanism of action of atVNS

Nucleus tractus solitarius serves as a major relay center of vagal afferent projections, being it also targeted by some efferents from the spinal nucleus of the trigeminal nerve [12]. Namely, electrical shocks to the ABVN activate action potentials in vagal primary afferent neurons, which are conducted centrally to trigger action potentials in second-order neurons within the NTS [33]. These second-order neurons project axons to different sites across the CNS, in a way that the activation of ABVN has the potential to modulate the activity of subcortical and cortical circuitry [34, 35].

NTS, apart from directly or indirectly activating the CNS by innervating brain regions, governs systemic parameters of cardiovascular, respiratory, and visceral functions to stay within their homeostatic limits [7].

As a matter of fact, the activation of the VN not only can modulate brain functions by activating the CNS, but also have systemic effects [25]. This is, the VN is involved in closed-loop reflex pathways along efferent and afferent pathways, being it a master key in thermo-regulation, immune-regulation, and blood pressure control of the body. A summary of all the pathways derived from atVNS can be seen in Figure 2. This includes 5 cortical pathways (i.e. lower brainsteam pathway, upper brainsteam pathway, serotonin pathway, cholinergic pathway, and norepinephrine pathway), and a simplification of closed-loop reflex pathways.

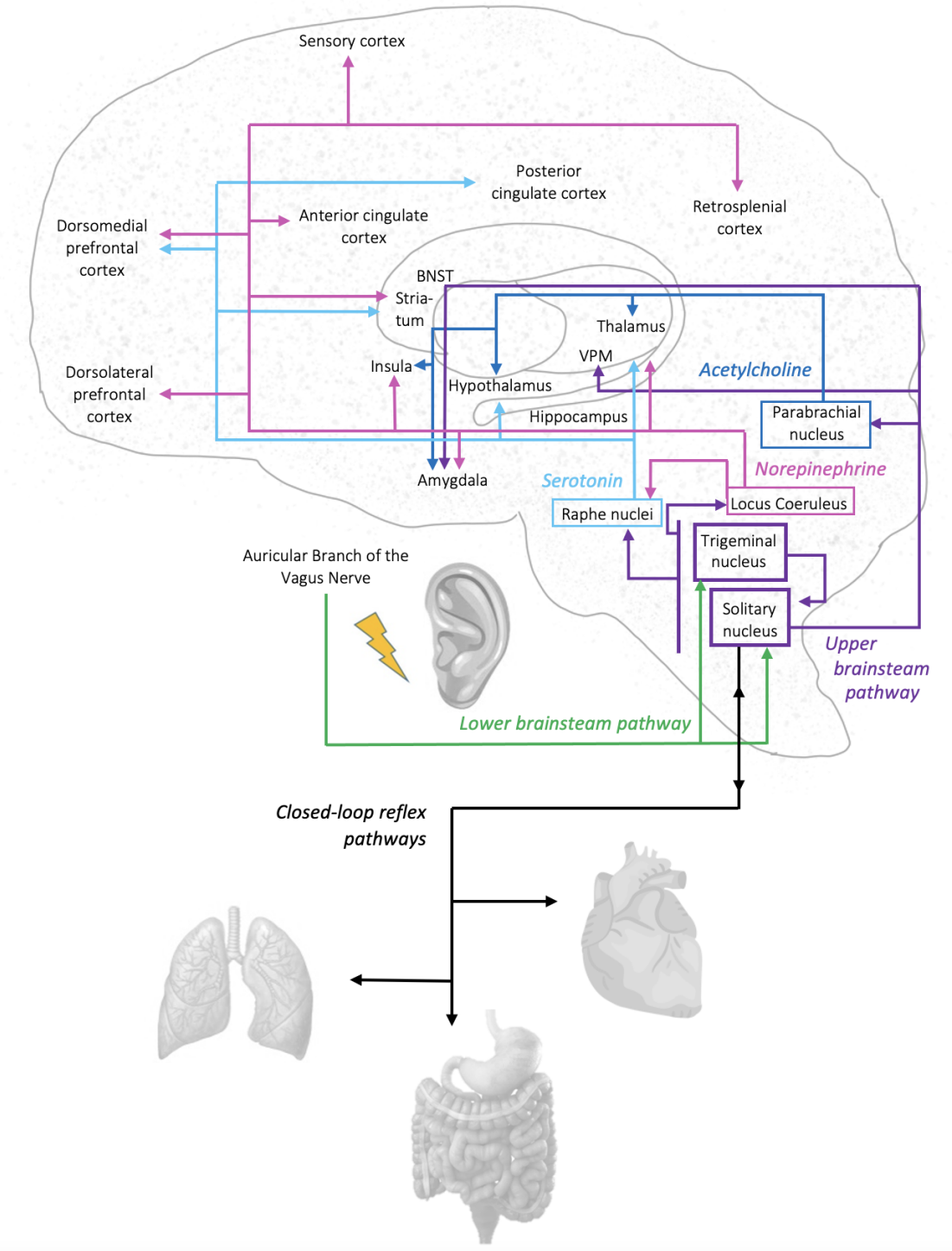

Figure 2: **Vagal cortical pathways model based on Briand et al. [12] and Kaniusas et al. [16]**. It consists in 5 main pathways: (1) lower brainsteam pathway; activation of the trigeminal nucleus and the nucleus tractus solitarius located in the lower brainstem (purple arrows) (2) upper brainsteam pathway; further activation of the locus coeruleus, raphe nuclei, and parabrachial complex, all of them localized in the upper brainstem (green arrows) (3 and 4) norepinephrine and cholinergic pathways; the production of norepinephrine and acetylcholine by the locus coeruleus and the parabrachial nucleus, respectively, modulate global brain activity (pink and dark blue arrows, respectively). (5) serotonin pathway: the delayed production of serotonin of raphe nuclei targets some structures of the limbic system and the frontal cortex (light blue arrows). Additionally, a simplification of closed-loop reflex pathways is also present (black arrows)

1.3 VNS in mice

Although cognitive impairment has been proposed to be positively treated by VNS both in humans [36] and rodents [6, 37, 38], the underlying mechanisms of VNS in cognitive enhancement are still unclear [39].

There is a need for research on animal models, and more importantly, mouse models. Animal models are essential drivers to derive the basic mechanisms behind disease conditions and emerging therapies. This can be explained by the tightly controlled settings in experimental animal studies [7]. Furthermore, the mouse is currently the species of choice in the study of disease pathophysiology [8] given the broad spectrum of disorder models it offers.

1.3.1 VNS parameters for cognitive impairment treatment in mice

Concern arises regarding VNS parameters for cognitive enhancement in mice. Namely, despite the fact that a controlled optimization can be performed by varying the pulse amplitude (intensity in mA), frequency of the pulses (in Hz), pulse width (duration of each pulse), and on/off cycle, there is still no defined optimal parameters. Here, we provide with the parameters defined in most relevant works up to date, bearing in mind that identifying the optimal stimulation parameters and "dose" may represent the crucial next step for atVNS research.

Current amplitude. There are plenty of studies in rodent models [40, 41, 42] that demonstrate effects may bear an inverted-U profile [43]. In other words, moderate stimulation (0.4 - 0.8 mA) enhances memory and plasticity at a larger level [43]. Similarly, in human models, markers of noradrenergic activity are known to be more elevated after moderate atVNS (25 Hz, 1.3 mA [44] or 30 Hz, 1.5-1.75 mA [45]).

Frequency. VNS delivered at 30 Hz in rats has been proven to drive map expansion in the auditory cortex more effectively than other stimulation frequencies [46].

Pulse width. An investigation by Elizabeth P. Buell et al. suggests that 0.5s duration VNS positive pulses enhance plasticity over 0.125 s and 2 s trains [46]. This result correlates with the one from the Laboratory of Neuropharmacology at UPF [6], where a 330 μs pulse width was used [6]. Furthermore, a similar number of pulses per second in both studies was employed, being it 32 and 20, respectively.

ON/OFF cycles. The majority of VNS studies [43, 44, 45] coincide in the fact that the ON training state must have an approximate duration of 30 s. This is preferably complemented by OFF periods of 5 min [6] and 48 s [45] in rodent and human models, respectively. However, it has been suggested that an OFF period of less than 5 min could result in positiver effects in mice [47].

Duration and periodicity of the treatment. These are difficult to set parameters. The success in terms of safety and inflammation reduction of the first long-term invasive VNS implant in mice brings to think that chronic stimulation would probably be optimal [48], referring chronic in this case to the permanent application of successive ON/OFF cycles. However, for example, atVNS in humans is intermittently activated (e.g. 3–4

stimulation sessions per day for in total 4–5 h) [16]. Further research is needed to set standard values of duration and periodicity for cognitive enhancement in mice.

Last but not least, it is important to keep in mind that VNS gated to the exhalation phase of respiration is suggested to be more efficient in the activation of NTS due to VN activation tunning with respiration [5, 49]. This could condition future experiments in the search for efficacious stimulation parameters.

1.3.2 VN Stimulators

Up to date there are 3 main types of VN stimulators for rodents: chronic invasive [8], acute auricular transcutaneous [6], and chronic non-invasive [9].

Starting with the chronic invasive VN stimulator, Mughrabi, Hickman, et al. [8] designed a surgical technique for long-term invasive VNS in mice (persistence of responses for at least 4 weeks). The surgery features the implantation of a bipolar platinum-iridium micro-cuff electrode on the cervical VN, and ECG electrodes to measure heart rate. The ECG and cuff leads are connected to a multi-channel nanoconnector and tunneled to a headcap secured with dental cement to the skull (see image A from Figure 3). Reported results were the first demonstration of chronic VNS in the mouse, providing longitudinal evidence of stimulus-elicited physiological responses [8]. Such responses include a successful reduction of serum tumour necrosis factor levels, a key regulator of the inflammatory response, without interfering with physiological VN-mediated reflexes (i.e. baro-, lung stretch- and feeding reflex). Other observations were that animals receiving VNS did not show any signs of distress, and that VNS at large frequencies and/or intensities causes reduction in heart rate through activation of efferent cardio-inhibitory fibers. To avoid compensatory reflexes triggered by VNS, they proposed using a current intensity lower than the heart rate threshold. This is the minimum current intensity required to elicit a 5-15% reduction in heart rate given the selected stimulation parameters. In the end, this study represented the first proof in mice that long-term VNS can modulate inflammation, being it a potential treatment of chronic diseases.

For atVNS, the group led by Prof. Ozaita developed a device to be used in anesthetized mice. The prototype consisted in two silver wires with 0.5 mm diameter mounted on a transparent methacrylate surface and fixed with epoxy resin (see image C of Figure 3) [6]. In this study, authors used acute atVNS in the concha of the left external ear of anesthetized mice. In their words, electrodes were placed in the concha of the left ear to avoid cardiac complications, as the right branch of the VN innervates the sinoatrial node and can have undesirable effects on heart rate [6]. They tested two mouse models: (1) CD-1 naïve mice (2) Fmr1 knock-out mice, which mimic the genetic alteration in fragile X syndrome subjects, a disorder characterized by intellectual disability. In both models atVNS improved memory performance as measured by an object recognition memory test.

L. Oshinsky et al. (2014) explored the mechanism of action of non-invasive VNS for the treatment of trigeminal allodynia in rats. Namely, they placed an Elizabethan collar with 2 silver-coated electrodes (0.8cm diameter) for transcutaneous stimulation to conscious naïve and allodynic rats (see image B from Figure 3). Results suggested non-invasive VNS to be useful to treat trigeminal allodynia [9].

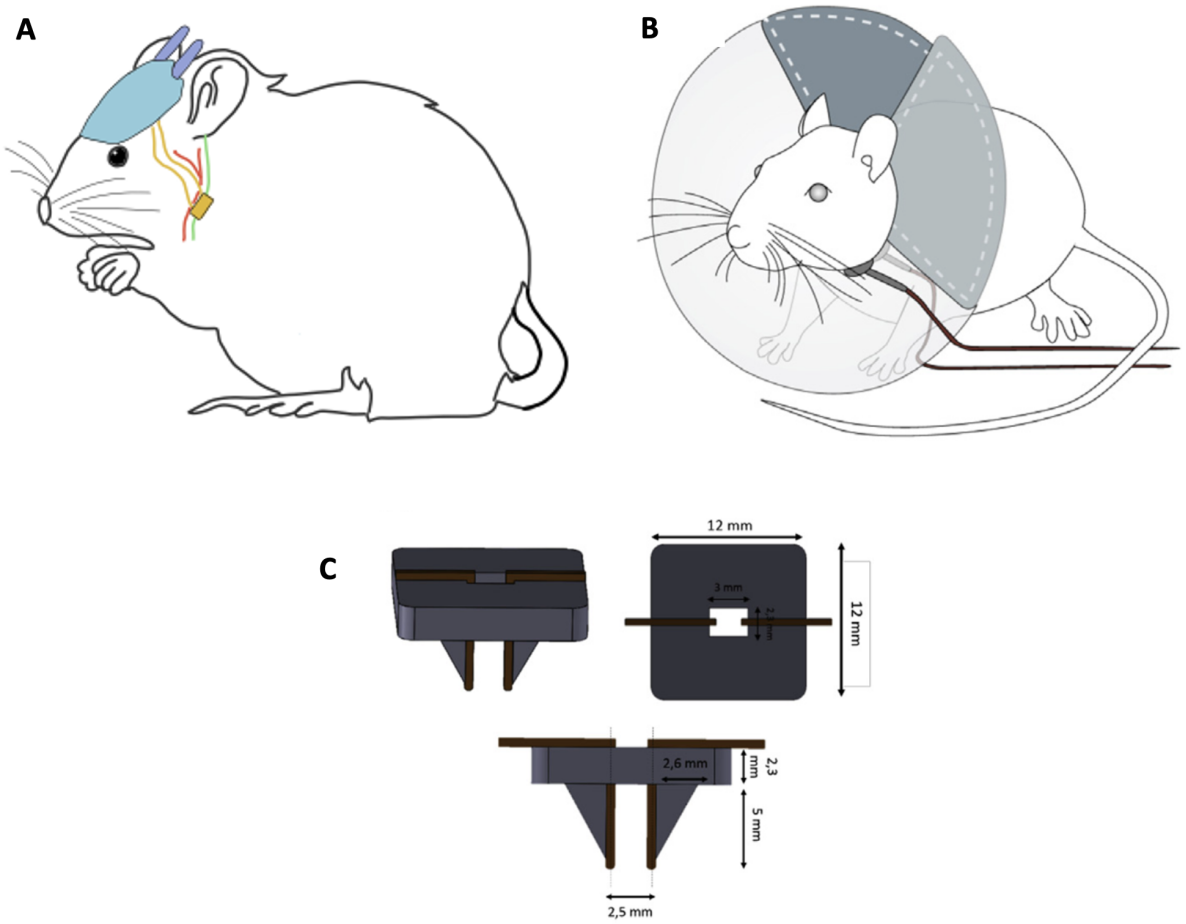

Figure 3: **(A) Mughrabi, Hickman, et al. VNS invasive implant.** Cervical long-term VNS with a 4-weeks response persistence. Light blue represents for the dental cement headcap, dark blue for the electrode pins, green for the VN, and yellow for the cuff electrode **(B) Vázquez-Oliver et al. atVNS device.** atVNS device developed by Vázquez-Oliver et al. to deliver current in the concha of the left ear of mice **(C) L. Oshinsky et al. (2014) non-invasive VNS technique including an Elizabethan collar and 2 silver-coated electrodes.**

Despite the relevance of VNS research executed so far, it has been limited to acute atVNS [6], chronic invasive VNS [8], and chronic non-invasive VNS [9]. While the underlying effects of acute stimulation are probably far from the physiological and molecular pathways activated in chronic stimulation, invasivity is accompanied with surgical and technical challenges with the microscopic anatomy of the mouse [8], and non-invasive stimulation can trigger motor efferent fibers and visceral afferents with undesired side effects [16]. There is a need for a simple, well-characterized, and reliable non-invasive chronic atVNS technique. In the present study, after intense trial and error, we design, produce, and test a device in the form of a pod for atVNS in mice: the S-pod. Its functioning is tested in terms of memory persistence enhancement in a novel object-recognition test in anesthetized mice, where the implementation of an innovative technique to test the stimulation sites, and the better adaptation of the pod in the concha of the mouse might make the results more reproducible than the ones obtained with the atVNS device from Vázquez-Oliver et al. Additionally, the S-pod is also compatible with a proposed preliminary model of a chronic atVNS setup. Such setup consists of the pod, an Elizabethan collar to avoid ear scratching, and a single-axis lever arm for free movement.

All in all, the present work supposes a first step towards the implementation of non-invasive chronic atVNS in mice. This is, apart from presenting an improved atVNS device, and a preliminary technique to chronically stimulate awake mice in a non-invasive way, it also provides with contrasted information on the supposedly optimal stimulation parameters to enhance cognition, and the possible physiological and molecular cognitive effects of atVNS. Further work using the provided tools could conceivably open new doors to validating the proposed molecular, neurological, and anatomical mechanisms underlying the cognitive effect of chronic atVNS.

2 Materials and Methods

2.1 Animals

CD-1 and C57BL/6J young adult (10-12 weeks) male mice from the Barcelona Biomedical Research Park Animal Facility participated in the study. Animal welfare in all procedures was ensured by complying with the standard ethical guidelines for the use of animals in research (Directive 2010/63/EU of the European Parliament and of the Council). Mice were housed in a temperature-controlled (21 $\pm$ 1$^{\text{o}}$C) and humidity-controlled (55 $\pm$ 10%) environment. Lighting was maintained on a regular 12h–12h light–dark cycle (on at 8 a.m.; off at 8 p.m.). Food and water were available ad libitum [6].

2.2 Electrode System

The electrode prototype accounts for the S-pod, a left ear pod made of ultraviolet (UV) resin (i.e. *Bostik Fix Flash 5G*[1]). The UV resin structure contains two silver wires in the stimulation sites, accounting for the electrodes and residing in the concha and the cymba conchae, respectively, and a magnet inside, in the top right part. The latter aims at holding and maintaining the pod steady when placing another identical magnet in the same place, but in the behind part of the ear. Figure 7 shows the S-pod captured from a digital microscope.

For production, the left ear of CD-1 euthanized mice was used as a mold. The process consisted of the following steps: (1) drilling of the stimulation sites with a 0.6 mm diameter needle (2) 90$^{\text{o}}$ bending of the last 3 mm of two silver wires with a diameter of 0.5 mm, and a length of 1.5 cm (3) introduction of the two bent parts of the silver wires into the drilled sites (4) placement of a magnet into the top right part of the concha, held with another identical magnet in the behind part of the ear (5) pouring of UV resin into the concha (6) application of UV light into the external ear for curation (7) extraction of the pod from the ear of euthanized mice (8) filing of the pod and the tip of silver wires to better adapt them to the morphology of the concha of the mouse.

With a view to facilitate the production of the S-pod for atVNS in mice, we created a 3D printable version with *AutoDesk Fusion 360*[2]. The morphology of the S-pod was depicted by making manual measures of the devices obtained with the traditional method. This printable version contains two holes in the stimulation sites to introduce the silver wires, and a cavity to fit the magnet in the top right part.

[1]Bostik FIX FLASH: https://diy.bostik.com/en-UK/products/repair-assembly/fix-flash
[2]AutoDesk Fusion 360 2.0.10148: https://www.autodesk.com/products/fusion-360/free-trial

2.3 Marking of stimulation sites

Two parameters where measured in euthanized CD-1 mice to test the correct functioning of the S-pod: the contact of the electrodes with the skin of the concha, and the correct position of the stimulation sites (i.e. cavity of the concha and cymba conchae). Both of them were derived from the consequences of the delivery of Direct Current (DC) through the electrodes of the S-pod. This is the first time DC is used to guarantee contact and correct stimulation sites, as far as we know.

For DC delivery, an electronic board was used to build a circuit containing a 9 V battery, a potentiometer acting as a variable resistor - $0 - 4.7\ k\Omega$ range -, a 38XR-A TRMS Digital Multimeter (Amprobe, USA), and the S-pod, all connected in series.

The test proceeded as follows: (1) we placed a small amount of *Aquasonic 100 Ultrasound Transmission Gel*[3] at the tips of the two electrodes and slightly spread it over the contact surface of the S-pod (2) we fitted the S-pod into the left concha and pinned it down with a magnet placed in the behind part of the ear (3) out of the two electrode connections, the anode was attached to the electrode in the cymba conchae, and the cathode to the electrode in the cavity of the concha (4) DC was delivered through the electrodes, adjusting the impedance of the potentiometer to make the current be 1 mA in the beginning (5) after 5 min of stimulation, the anode and the cathode were exchanged (6) after 5 more min, the current was turned off.

If the electrodes were making correct contact with the skin of the concha, a black stain resulting from skin damage appeared in the stimulation sites. Hence, we argued there was contact from skin damage. To verify the stimulation sites, we checked whether the marks were in the cymba conchae and the cavity of the concha. In consequence, were there two marks in the concha and cymba conchae, the S-pod was classified as valid.

2.4 Novel object-recognition test

The novel object-recognition test is a relatively fast and efficient means to test non-emotional memory in mice [50]. It can be completed over 3 sessions: habituation, familiarization, and testing. In the present study, given the will to reproduce the previous results of our group [6], we used the same parameters and procedures as in the so-mentioned paper.

During the first session, animals were habituated to an empty V-shaped maze for 9min. The aim was to reduce stress and to avoid a potential neophobic response [51]. 24 h after, in the familiarization phase, mice were let to freely explore two identical objects for 9 min, which was promoted by the habituation phase. Right after familiarization, they were anesthetized with isoflurane and exposed to atVNS or no stimulation, as specified in 2.5. In the last session 48 h after the familiarization phase, a 9-min memory persistence test was carried out. We recorded rodents in the V-shaped apparatus, where one of the identical objects was replaced by a novel, unfamiliar object [50].

[3]Aquasonic 100 Ultrasound Transmission Gel Single Use: https://aquasonicgel.com/aquasonic-100-single-use-packette-100-per-box

In the end, we collected and analyzed data from the recordings regarding the exploratory behavior of rodents towards the objects. We chose to score object exploration as follows: 'directing the nose' toward the object 'at a distance less than or equal to 2 cm' [52]. The exploration time was used to calculate the discrimination index (DI), accounting for the difference between the exploration time of the old (T_o) and the familiar object (T_f), divided by the total exploration time (T_e) ($DI = (T_o + T_f)/T_e$) [6]. Based on the innate preference of mice to explore the novel object rather than the familiar one [50], higher DI was considered to reflect greater object-recognition memory persistence [6].

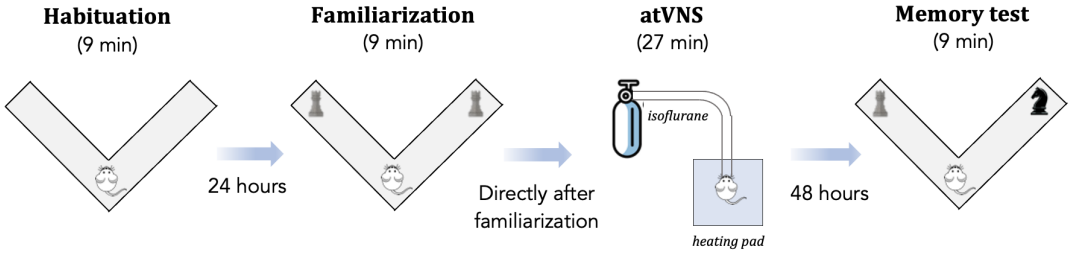

Figure 4: **Novel object-recognition test based on Vázquez-Oliver et al. (2019).** The novel object-recognition test is completed in 3 sessions: habituation to the V-shaped maze, familiarization with two novel identical objects 24 h after, and testing of memory persistence by changing one of the objects for a novel one 48 h after familiarization.

2.5 Electrostimulation procedure

In accordance with the statement of reproducibility in 2.4, the electrostimulation procedure basis was also taken from Vázquez-Oliver et al. [6]. Right after the familiarization phase of the novel object-recognition test, mice were anesthetized with isoflurane (1.5% induction and maintenance) in 0.8 L/min O_2 [6]. A heating pad was used during anesthesia to maintain normothermic conditions, and we put *Xilin Night*[4] on the eyes of mice to prevent corneal abrasion triggered by eye dryness [53]. Two different S-pods were then placed into the concha. For so, we put a small amount of Aquasonic Gel at the tips of the electrodes and slightly spread it over the contact surface of the S-pod, and then the latter into the left concha and pinned it down with a magnet placed in the behind part of the ear. Electrical stimulation was only performed in atVNS condition (8 out of 16 CD-1 mice). Rectangular bipolar pulses were delivered with a Beurer EM49 stimulator (Beurer, Germany), and we monitored current delivery using a Hantek DSO1202S oscilloscope (Qingdao Hantek Electronic, China). To do so, we measured the voltage (V) drop across an external reference resistance $R_{ref} = 15\ k\Omega$ in series with the electrode system Z_{load}. Then, we calculated the current as $I = V/R_{ref}$ [6]. As further specified in 2.6, intensity was set to approximately 1 mA by adjusting V to $\sim 15\ V$, considering the impedance of the skin with gel to be negligible. The procedure, whether in atVNS or no stimulation condition, had a duration of 27 min.

2.6 Stimulation parameters

Rectangular bipolar pulses were delivered with a Beurer EM49 stimulator. The stimulation parameters can be seen in Table 1:

[4]Xilin Night 5g: https://www.visufarma.es/product/xilin-night/

Table 1: **Stimulation Parameters in anesthetized mice.** Stimulation parameters used in atVNS condition after the familiarization phase of the novel object-recognition test. These were obtained from Vázquez-Oliver et al. (2019) [6].

Parameter	Value
Current amplitude	1 mA
Pulse width	330 μs
Frequency	20 Hz
ON/OFF Cycle	30 s/5 min
Duration	27 min

2.7 Elizabethan collar

We conducted a behavioral test in CD-1 and C57BL/6J mice to assess adaptation and habituation of these two mouse strains to wearing an Elizabethan collar, accounting for the most commonly used restraint collar for research animals [54]. This was done to determine whether this medical device could be use to avoid the mouse from scratching the left ear while wearing the S-pod.

First, We bent Elizabethan collars 90^{o} in the inferior part using a heat gun, and cut the area of the bent surface laying below the mouth (see Figure 5). The aim was to ease walking and exploration, and permit grooming while preventing mice from reaching the ear.

For the placement of the collar, mice were briefly anesthetized with isoflurane (1.5% induction) in 0.8 L/min O_2. After on, animals were placed in separate spare beakers and recorded in pairs. A white sheet was placed between the two glass recipients to avoid external stimuli to alter the results. We did recordings of 1 h of mice in the beakers in 3 consecutive days in a week. After each test, mice were removed the collar and put back to their cages.

In the posterior visualization of the recordings, we analyzed qualitative and quantitative parameters to derive the behavior of mice while wearing the Elizabethan collar. Quantitative parameters included the number of defecation, stand ups with and without the wall, scratching, jumps, grooming, and the number of times mice managed to reach their ear; locomotion was the only qualitative parameter. In consequence, we measured anxiety-associated parameters (i.e. locomotion, defecation, and grooming), exploration parameters (i.e. stand-ups with and without the wall, and jumps) and collar discomfort parameters (i.e. ear touches, and scratching).

To prevent the effect of anesthesia from altering the results, videos were analyzed from minute 5 to 29. Furthermore, the quantification of parameters was divided in 3 periods (5-13 min, 13-21 min and 21-29 min) to not only spot interday, but also intraday behavioral changes.

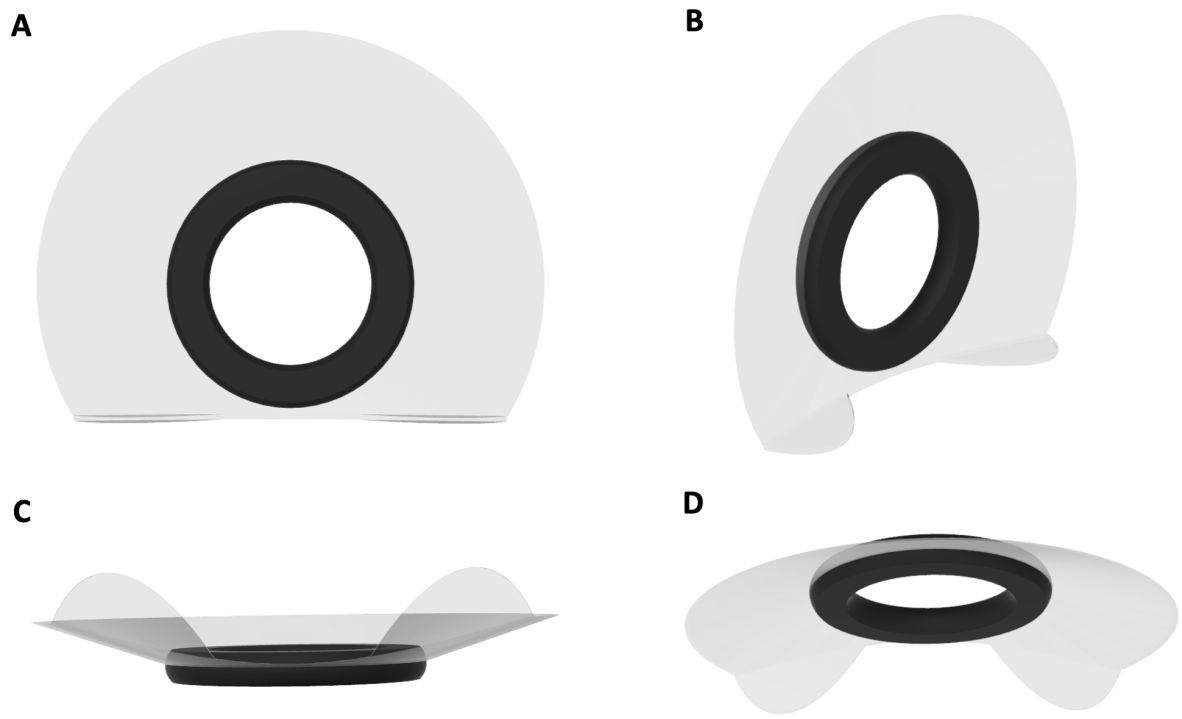

Figure 5: **3D representation of the Elizabethan Collar.** They are 90º bent in the inferior part and lack the area of the bent surface laying below the mouth. This is to improve comfort of the animal. (A) frontal view of the Elizabethan Collar (B) left diagonal view of the Elizabethan Collar (C) lower view of the Elizabethan Collar (D) upper view of the Elizabethan Collar

2.8 Chronic atVNS setup

The proposed preliminary chronic atVNS setup is composed of 3 main elements: the S-pod, the Elizabethan collar, and a single-axis lever arm. In addition, the structure can be divided in two main parts joined by a Molex (Molex, USA; Connector KK 254 Series 2 Way). Figure 12 in 3.5 shows a 3D representation of the whole setup, whilst in Figure 13 each part is displayed independently.

The first part consists of the S-pod and the Elizabethan collar, being the latter attached to the wire-to-board header of the Molex (Molex, USA; series: KK 254; RS code: 483-8461). Namely, the lower limbs of this header are used to drill the Elizabethan collar and fixed with *Loctite*[5] in a manner that they reside in the frontal part, laying over the head, while the rest of the structure resides in the back part. This permits to drive two coated copper wires (Multicomp Pro; manufacturer reference: MPRRW-A-105) from the electrodes of the S-pod right to the lower limbs of the wire-to-board header, making the connection between the S-pod and the Elizabethan collar feasible. These coated copper wires are connected to the electrodes with epoxy resin (Chemtronics; manufacturer reference: CW2400), and welded to the limbs of the header.

The second part is only formed by the single-axis lever arm. The cables in the single-axis lever arm are protected all along with a spring to permit free movement of the mouse

[5]Locitite Super Glue-3 Líquido Precisión: https://www.loctitesuperglue-3.com/es/productos/super-glue-3-liquido/super_glue_3_liquidoprecision.html

in the cage and avoid tension. These cables are attached to crimp terminals and inserted into the crimp housing part of the Molex (Molex, USA; series: KK 254; RS code: 188-8796). Nonetheless, the single-axis lever arm includes a connector that permits connection to an stimulator to deliver rectangular bipolar pulses.

2.9 Statistical analysis

Data were analyzed with *Prism*[6] Software using T-test, except for an Ordinary one-way ANOVA analysis for locomotion in CD-1 mice. Comparisons were considered statistically significant when $p < 0.05$. Data are represented as mean ± standard error of the mean.

3 Results

3.1 Design and production of a novel atVNS device: S-pod

The final design was derived from the knowledge acquired during the R and D process (see 6.3 from Additional Information for further insight). The most relevant prototypes prior to the S-pod can be seen in Figure 6. All of them already incorporated the concept of pod. They are described as support methods if they are attached to the pod, forming a single structure, and external supporting elements if they are independent from the pod but intend to maintain it steady in the concha.

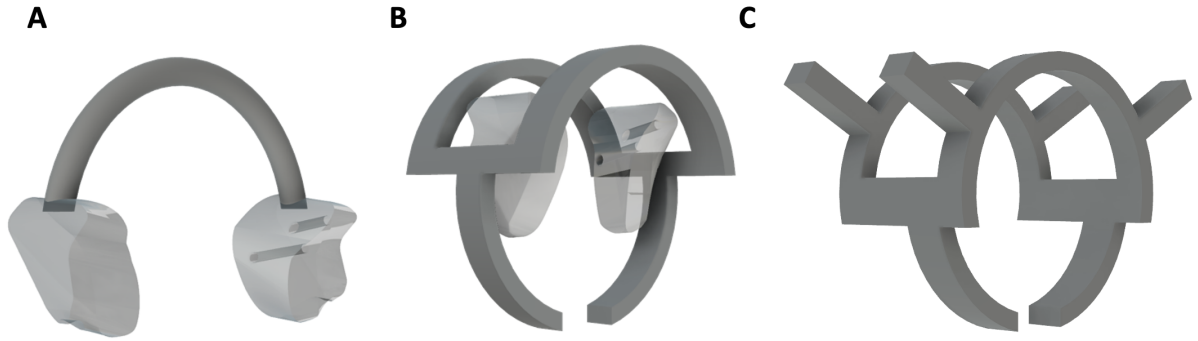

Figure 6: **Preliminary prototypes prior to the design of the S-pod.** These include support methods and external supporting elements. (A) Headphones support method; it was discarded due to slipping up (B) Helmet support method; it was discarded due to difficult placement (C) Helmet external supporting element; it was discarded given extreme behaviors, being it too loose, or too tight. The four sticks in the top bands aim at easen opening of the helmet.

The first support method that we tested was a pair of headphones (image A from Figure 6). Namely, we built a pod for each ear, and stuck them to the tips of a 180º arch 3D-printed with *Creality Ender 3*[7], being infill and perfil parameters 20 and 0.2, respectively. We also designed an extended arch (320º) with two bands instead of one in the upper half that we called "helmet" (infill = 40; perfil = 0.2), which was tested both as a support method and as an external supporting element (images B and C from Figure 6).

[6]Prism 9.0.0: https://www.graphpad.com/scientific-software/prism/

[7]Creality Ender-3: https://www.creality3dshop.eu/collections/3d-printers/products/creality3d-ender-3-pro-high-precision-3d-printer

The final atVNS device, namely S-pod, consists in a pod for the left ear of CD-1 mice containing a magnet for fixation. Figure 7 shows the physical pod captured from a digital microscope, whilst Figure 8 shows the 3D printable version.

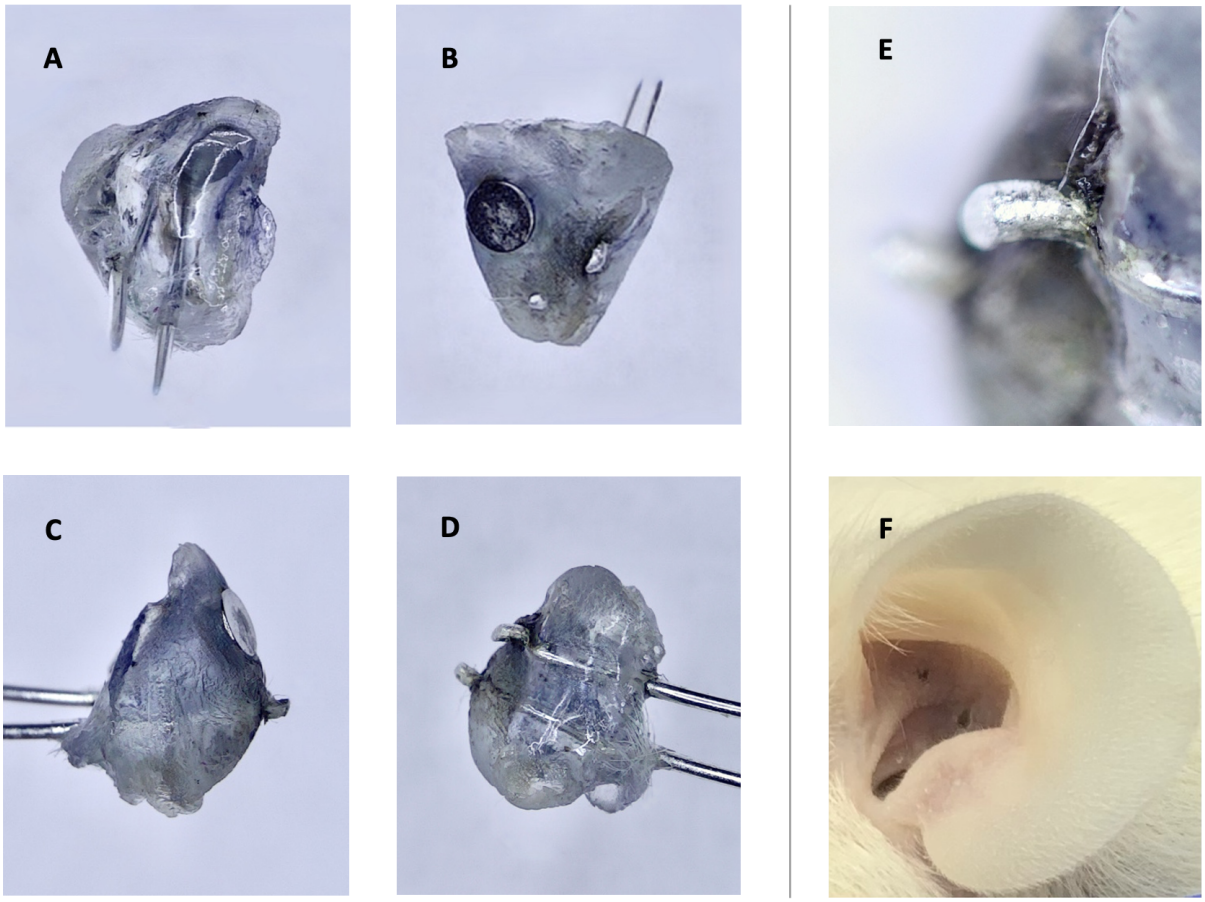

Figure 7: **Physical S-pod.** Images of the S-pod taken with a digital microscope. The two silver wires account for the electrodes, whilst the magnet is the supporting element; (A) front side (B) back side (C) right side (D) left side. **Electrode and marking of stimulation sites.** (E) rough surface of an electrodes of the S-pod (F) marking of stimulation sites using DC current in an euthanized mouse. The marks assesses contact, and that stimulation is being performed in the cavity of the concha (lower mark) and cymba conchae (upper mark).

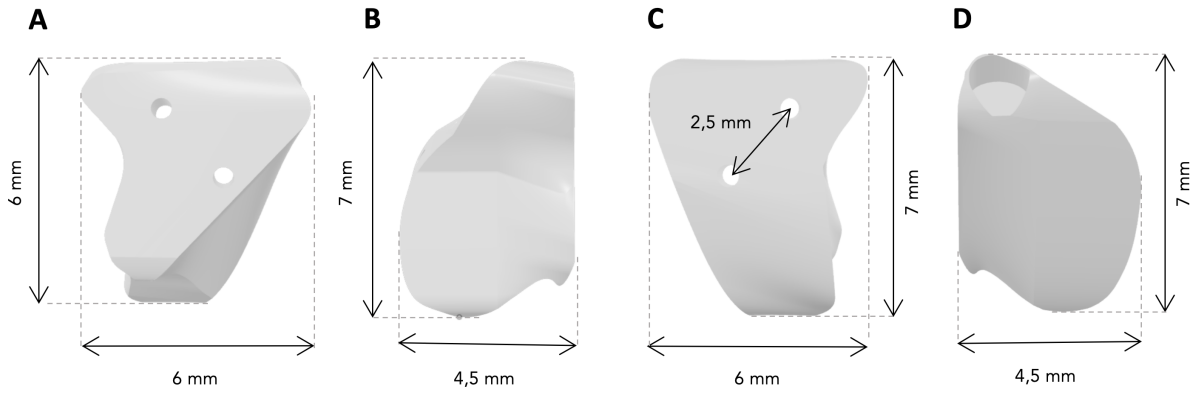

Figure 8: **3D printable version of the S-pod.** Fusion model of the S-pod: (A) front side of the pod (B) left side of the pod (C) back side of the pod (D) right side of the pod.

3.2 Marking of stimulation sites

To ensure correct stimulation sites of the S-pod, we used it to deliver DC current in the left ear of euthanized mice. The subsequent damage in the skin consisted in black stains on the site of stimulation of the electrode connected to the cathode. Also the one connected to the anode showed skin damage, but in the form of a small crust, sometimes indiscernible. Consequently, polarity of electrodes was exchanged to ease the marking of the two sites. Results obtained using one of the S-pods can be seen in image F from Figure 7. In addition, image E shows the surface of the electrodes, which were filed before use to increase roughness, and subsequently, the contact surface.

3.3 S-pod electrostimulation enhances memory persistence in anesthetized mice

We conducted a novel object-recognition test to assess the correct functioning of the S-pod. Out of 16 CD-1 naïve mice, 8 of them were stimulated, and the others were considered for sham conditions. The DI values 48 h after stimulation show enhanced cognitive function compared to no stimulation (atVNS = 0.2498; no stimulation=0.0324; difference=0.2175 ± 0.08167; p=0.0186). The total exploration time, accounting for the sum of the exploration time dedicated to the new and the old objects, shows no significant difference between atVNS and sham conditions (atVNS=27.03; no stimulation=23.04; difference=-3.99 ± 4.05; p=0.34).

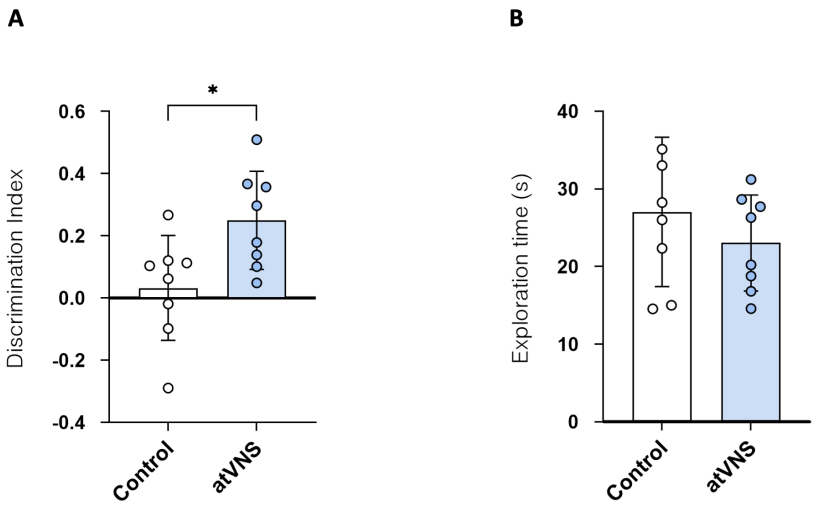

Figure 9: **(A) Discrimination index and exploration time in the novel object-recognition test.** Results obtained in the test session of the NORT; atVNS condition (DI=0.2498) shows enhanced memory persistence with respect to no stimulation (DI=0.0324; p=0.0186) **(B) Total exploration time.** There are no significant differences (atVNS=27.03; no stimulation=23.04; difference=-3.99 ± 4.05; p=0.34).

Out of the 8 mice in atVNS condition, 4 of them were stimulated with one S-pod, and the 4 remaining were stimulated with another S-pod. Considering that the stimulation positions of the electrodes were slightly different, Figure 10 shows the DI obtained with each pod independently. No significant differences are observed neither in DI (pod 1=0.2187; pod 2=0.2809; difference=-0.0622 ± 0.1180; p=0.6171) between the pods, nor in the exploration time (pod 1=22.80; pod 2=23.28; difference=0.49 ± 4.71; p=0.92).

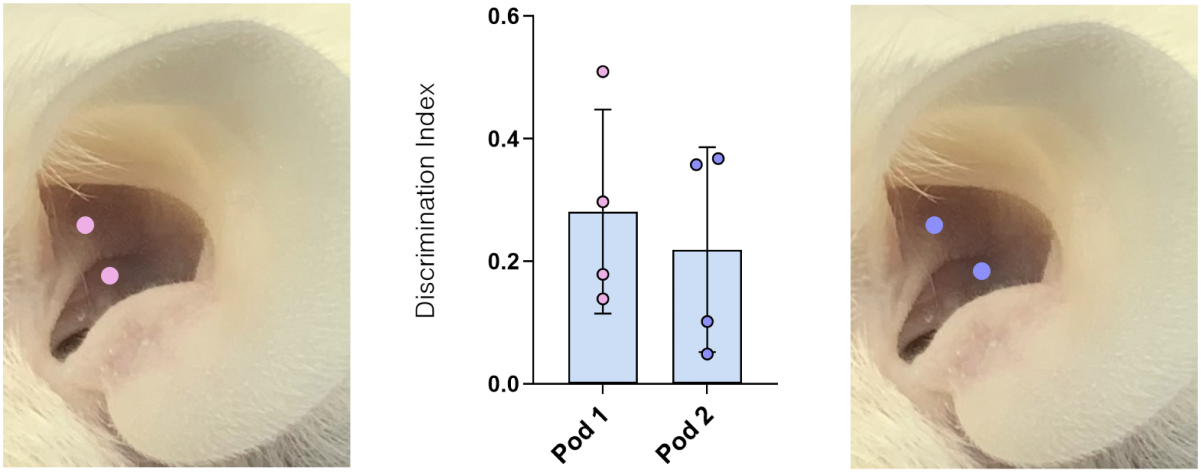

Figure 10: **Discrimination index obtained in atVNS condition with each pod independently.** Comparison of DI of stimulated mice, bearing in mind that two different S-pods were used in 4 animals each; results show no significant differences (pod 1=0.2187; pod 2=0.2809; difference=-0.0622 ± 0.1180; p=0.6171)

3.4 Assessment of the behavioral response to the Elizabethan collar

We assessed the effect in behavior of placing an Elizabethan collar in naïve CD-1 and C57BL/6J mice, as these are the most used naïve strains in biomedical research. The quantitative measures obtained for each individual and parameter are displayed in Table 2 (CD-1 mice) and 3 (C57BL/6J). Out of the 8 analyzed parameters, significance with ordinary One-way Anova was only found in locomotion from CD-1 mice, indicating a progressive habituation to wearing the collar and suitability for use as part of a chronic atVNS setup. Figure 11 shows the so-mentioned adaptation of CD-1 mice (CD-1=3.00; C57BL/6J=2.08; difference=-0.92 ± 0.25; p=0.0047).

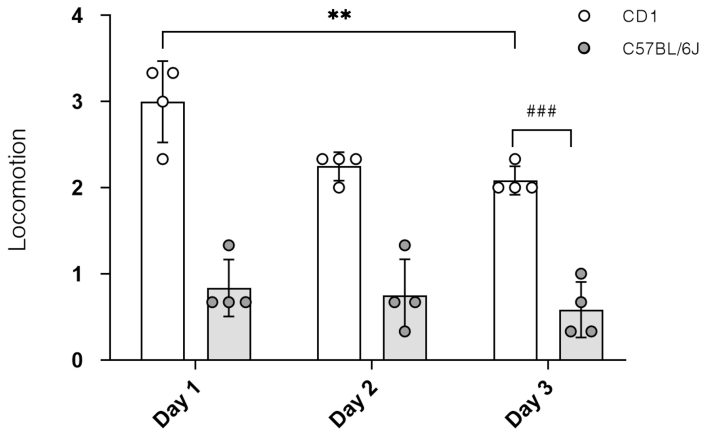

Figure 11: **CD-1 and C57BL/6J Locomotion.** Measure of the locomotion of CD-1 and C57BL/6J mice. Regarding the quantification, 0 means no movement, 1 is assigned to mice performing 1 or 2 long walks, 2 to movement with long pauses, 3 to mice moving with pauses, and 4 to movement with practically no pauses. It is perceptible how there is a significant reduction of locomotion in CD-1 mice from day 1 to 3 ($p = 0.0047$ using ordinary one-way Anova). Also, CD-1 mice show a significantly higher movement pattern than C57BL/6J mice in day 3 ($p = 0.0002$ using t-test)

3.5 Chronic atVNS setup

A simple first preliminary setup for chronic atVNS in mice is presented in Figure 12. This is the first setup, as far as we know, that permits to perform atVNS in awake mice.

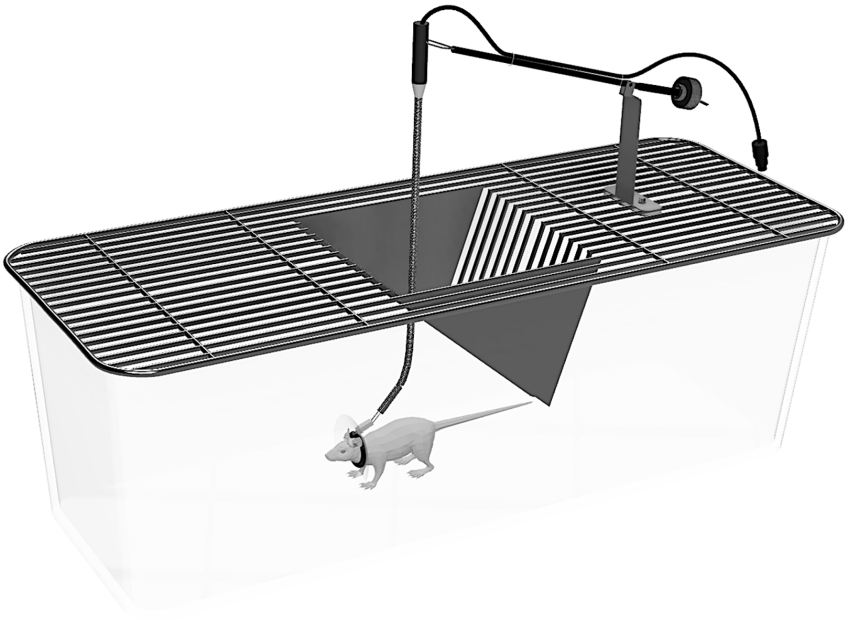

Figure 12: **chronic atVNS setup.** First structure to perform atVNS in awake mice. Therefore, the main remarks of this new setup are the non-invasivity, the compatibility with atVNS in non-anesthetized mice, and subsequently, the fact that it permits to perform chronic stimulation.

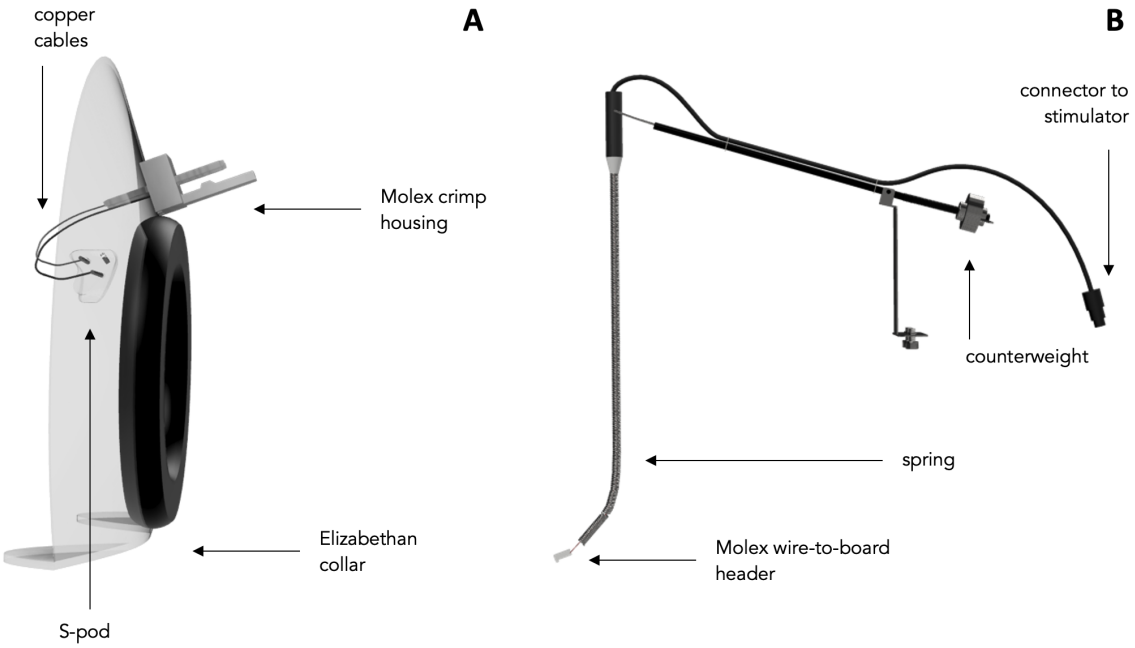

Figure 13: **Parts chronic atVNS setup.** The proposed chronic atVNS setup can be divided in two parts united by a Molex. This is of high relevance to ease the placement of the structure on the mouse. (A) the main elements of the first part are the Elizabethan collar and the S-pod; while the first avoids ear scratching, the second allows non-invasive auricular electrostimulation. (B) The second part is only formed by the single-axis lever arm, which allows connection to a stimulator.

The structure can be divided in two main parts united by a Molex, as shown in Figure 13. The first part mainly consists of the Elizabethan collar and the S-pod, and the second part is the single axis-lever arm by itself, which allows connection to a stimulator.

The proposed setup is described as feasible, resistant to breaking, and compatible with the normal movement of the mouse, but further investigation is needed for validation.

4 Discussion

The lack of contrasted information on the cognitive effect of atVNS raises the need for more preclinical studies to help derive the underlying pathways. In the present study we do a step forward towards the better understanding of this phenomenon by providing with an improved atVNS device for stimulation in anesthetized mice, a new technique to mark stimulation sites in euthanized mice, and a preliminary chronic atVNS setup to stimulate awake mice.

4.1 S-pod

To the best of our knowledge, our group was the first to use non-invasive transcutaneous approaches in mouse models [6]. The employed atVNS device (see image C from Figure 3) was thought for auricular transcutaneous stimulation in anesthetized mice. Spotting the need for an atVNS device compatible with chronic stimulation in a free movement mouse model, we designed, produced and tested a new electrode system fulfilling these requirements that we called S-pod.

The final design was preceded by intense trial and error, which was of high use to better define the anatomical characteristics of the mouse, and how we could take advantage of them for an optimized and more comfortable device.
The first observation was that the two stimulation sites, residing in the cymba conchae and the cavity of the concha, lay against the cranium. Namely, they do not belong to the prominent part of the ear. This anatomical feature makes it difficult to use a device in the form of a clip, typical from atVNS in humans [55], also bearing in mind the membrane-like helix of the mouse. In this way, we considered the concha to be the only part that could be relied on in terms of support.
The choosing of the concha as support brought us directly to the concept of "pod", accounting for a piece that perfectly fits the concha. Having in mind that we wanted to use the left ear of euthanized mice as a mold, different materials that curate/solidify under different conditions were tried for the pod body, including Loctite, *Dentalon*[8] and UV resin. On the one hand, Loctite and Dentalon, which solidify at room temperature, were discarded because of stickiness and slow solidification, and variability of viscosity, respectively. On the other hand, UV resin, which curates under UV light, was chosen as the material to use given the fast curation, reproducible viscosity and easy removal of the ear of the mouse after production.

Given the susceptibility of the UV resin pod to popping out under movement observed in first tests, we decided to incorporate an extra support to press the pod. For so, two

[8]Resina Dentalon Plus Polvo: https://www.dentaltix.com/es/kulzer/resina-dentalon-plus-polvo-100gr

options were considered, both including the pod: the so-called support methods, and the external supporting methods. Support methods account for prototypes were the supporting piece is fixed to two pods, one for each ear, to enhance stability. Contrarily, external supporting elements are pieces that aim at maintaining the left pod steady in the concha, but they are independent from it. It was through the testing of support methods and external supporting elements that we derived the second anatomical observation: the head of the mouse is highly compressible. While the headphones support method (see image A from Figure 6) resulted in slipping up or down, the helmet - including the support method concept and the external supporting element concept in images B and C from Figure 6 - was difficult to place, and normally resulted in too tight or too loose fitting. Thereby, we discarded the option of using support methods and external supporting elements to press the pod against the ear.

Finally, we changed the pressing concept, which had been considered to be a mild-stone, for just holding. Namely, we added a magnet in the atVNS device, which can be hold with another magnet in the behind part of the ear. This has shown to be a very effective way of holding the pod steady in the concha, even in a free-exploration setting. In addition, the contact with the stimulation sites occurs properly and reproducibly, which is essential for the stimulation to take place.

The S-pod, perceptible in Figure 7 supposes a very important step forward for atVNS in anesthetized mice, presenting it some very relevant advantages with respect to the previous model. In the first place, the perfect fitting of the pod provides with constant stimulation sites along time, and also between individuals stimulated with the same pod. This is especially remarkable in a context where the previous and only atVNS device up to date fails at precisely stimulating the desired sites. As a matter of fact, marking with DC current using the previous design of the atVNS device assessed a large variability in the stimulation sites between sessions; in some cases, we even obtained the mark of both electrodes in the cavity of the concha. Therefore, the use of the S-pod might increase the reproducibility of results and make the findings more consistent.
Secondly, stimulation procedure becomes easier thanks to the easy placement and perfect fitting of the S-pod in the ear of mice. Moreover, whilst the previous device required the application of pressure to ensure contact of the electrodes with the skin, the new design works without the need of external pressure and so, is much less sensitive to small movements of the animal while anesthetized.
Finally, the S-pod is compatible with a chronic stimulation setup (read 4.5 for further information). Therefore, it could be key to allow chronic non-invasive atVNS in mice in the near future.

When it comes to limitations of the pod, the main one is the production procedure (see 2.2). The need for euthanized mice, the small anatomy of the ear of the mouse, and the manual placing of electrodes in the stimulation sites can be limiting and/or time-consuming. Additionally, the resulting pods can be variable in shape, and even in terms of the stimulation sites.
To compensate for this limitation, we have built a 3D printable model of the S-pod, perceptible in Figure 8. The main body is complemented by two 0.6 mm diameter holes in the stimulation sites to place the silver wires - electrodes -, and a cavity to stick the magnet. In this manner, we do away with the tedious and imprecise production

procedure, get reproducible stimulation sites among devices, and reduce the number of animals involved in the study. We also give a tool to test different stimulation sites, which can be easily done by changing the position of the holes in the provided stl file. This is of high interest considering the lack of standardized stimulation sites in atVNS.
Also importantly, the 3D printable S-pod is compatible with percutaneous atVNS, opening new doors for the investigation of this alternative minimally-invasive auricular VNS technique in mice.

4.2 Marking of Stimulation sites

After design and production of the S-pod, we encountered a remarkable problem: there was no way to know the exact position of the electrodes in the concha. Up to date, blind stimulation with respect to the stimulation sites was being performed. This is, the morphology of the previous atVNS device impeded the visual definition of the exact position of the electrodes. This was also a limitation of the S-pod, and to compensate for this, we devised a technique, the first to the best of our knowledge, that allows to mark stimulation sites in euthanized mice using DC current. A visual representation of the results can be seen in image F from Figure 7.

This new technique can only be used to ensure correct stimulation sites with the S-pod, as these are always the same in all stimulation sessions, and in all animals. Namely, a definition of the position of the electrodes in one mouse ensures their reproducibility in other individuals, which can not be extrapolated to the previous atVNS device.

The setting of this new technique to mark stimulation sites in atVNS using DC current supposes a very significant advance to ensure electrostimulation efficiency and reproducibility. Additionally, the precise definition of the position of the electrodes raises the possibility to look for different effects according to the stimulation sites.

4.3 Memory persistence enhancement in anesthetized mice

To prove correct functioning of the S-pod, we tested memory persistence in mice 48 h after atVNS in a novel object-recognition test (see 2.4 for further information). The idea was to recreate the results obtained with the previous device of our group, where a significant positive difference in DI of stimulated naïve mice with respect to no stimulation and sham conditions was observed. With the aim of reproducing the same environmental conditions, we ran atVNS under normothermia and using a low dose of isoflurane already proven not to alter memory performance compared to no anesthesia conditions [6].

The results of the novel object-recognition test can be seen in Figure 9. As expected, atVNS using the S-pod after the familiarization phase resulted in increased memory retention (DI=0.025) compared to no stimulation condition (DI=0.03). Namely, mice that received atVNS spent more time exploring the novel object than the familiar one. This is translated in larger DI values in Figure 9 based on the innate preference of mice to explore novel objects rather than already-known/familiar ones [50].

As the stimulation sites of the two S-pods used in the experiment were not exactly the same, we decided to analyze DI for each pod independently (see Figure 10).

The results show no significant differences. However, further research should center on determining which are the optimal stimulation sites to enhance cognition the most, whilst reducing adverse effects to the minimum. Additionally, to better understand atVNS activated pathways involved in cognition, chronic stimulation should be performed. The brain plasticity follows different time scales: changes can take place in seconds, which are the ones we rely on in acute stimulation, or within weeks, or up to a year [7]. Proof of so are the expected delayed effects of atVNS on the synthesis and transmission of serotonin, and on the amygdala and hippocampus plasticity [35]. Therefore, a chronic atVNS setup is needed to unearth the mechanisms underlying cognitive enhancement through transcutaneous stimulation of the auricle.

4.4 Elizabethan collar

As aforementioned, having the need for a chronic atVNS setup for mice in mind, we designed the S-pod with a view to using it in a free-movement mouse model. However, bearing in mind the easy access of mice to the outer ear with the posterior limbs, the removal of the S-pod or damage to the cables driven to the stimulator becomes a very feasible scenario when used in awake mice. To prevent them from scratching the left ear, we thought of the Elizabethan collar. This is the most commonly used restraint collar for research animals [54], having it already been used for non-invasive cervical VNS in rats.

To assess the responses of of CD-1 and C57BL/6J mice to wearing the Elizabethan collar, we performed a behavioral test (see details in 2).

The analysis of measurements displayed in Table 2 and 3 shows that over the three days, neither a significant increase, nor a significant decrease in any parameter but locomotion in CD-1 mice occurred. Thus, although some parameters denoting anxiety like grooming and defecation remained more or less the same over the three days in CD-1 mice, a significant ($p = 0.0047$) decrease in the quantified movement from day 1 to 3 (see Figure 11) can be interpreted as a sign of habituation and adaptation. In contrast, C57BL/6J mice stayed hypoactive all the time ($p = 0.0002$ with respect to CD-1 mice locomotion in the third day). This is a marked freezing response, accounting for an absence of movement except for breathing, which suggests fear.

In consequence, by overlooking locomotion, no mouse breed adapted to wearing the collar, but they also didn't get sensitive to it in both intraday and interday periods. However, when considering locomotion, CD-1 mice seem to go from hyperactivity to a normal movement pattern over days in Figure 11. Thus, although C57BL/6J mice are sensitive to wearing the collar from the first moment, results assess that this protective medical device could be used in CD-1 mice to avoid ear touches without comprising normal behavior. Such statement can be derived from the non-sensitization of this mouse strain to wearing the collar, the locomotion pattern normalization denoting habituation, and the difficult ear access the Elizabethan collar provokes (see ear touches in Table 2 and 3). However, results suggest that an adaptation period of minimum 3 days might be necessary.

A study with a larger number of mice and with longer session times could help define the maximum amount of time animals can wear the collar per day without comprising

normal behavior and/or being damaged (e.g. chafing, chocking). In addition, it would give place to a more reliable validation of adaptation to wearing the collar.

4.5 Chronic atVNS setup

The trigger for the setting up of this project was the lack of a simple, well-characterized, and reliable non-invasive chronic atVNS technique in mice. While the use of atVNS in humans is growing, being this variant of VNS already CE authorized to treat epilepsy, depression, pain, and migraine [16], the understanding of its functioning is still unclear [36]. Further research on the activation patterns underlying atVNS would not only allow a better understanding of the benefits and risks for treatment of the already approved disease conditions, but also open new doors to using it for other indications. A clear example is cognitive enhancement. Although multiple clinical trials have already proved the pro-cognitive effect of the four main methods to stimulate the VN [36], the lack of information on the bases of the therapy in terms of functioning is limiting its use. Here, to get out of this dead end, we propose a preliminary atVNS chronic setup for the mouse, the preferred species to unearth basic mechanisms behind disease conditions and emerging therapies [8].

The proposed model consists in a 2-parts structure; the first one accounts for the S-pod and the Elizabethan collar, whilst the second is a single-axis lever arm containing a connector for a stimulator (see Chronic atVNS setup for further information). Both parts are united with a Molex.
The independence of the Elizabethan collar and the pod of the single-axis lever arm is of high importance in a context were the single-axis lever arm is though to permit the movement inside the cage, being it set in the top part of it. This is, the placement of the collar when attached to the single-axis lever arm becomes challenging. Consequently, the division of the setup in two parts aims at the easing of the placement of the structure on awake animals.

This chronic atVNS setup, although being a very preliminary version, might succeed in chronically stimulating mice during various hours per day. Small trials in few individuals have shown the structure to be robust and compatible with normal movement of the animal. However, considering the fact that the mouse is not only wearing the Elizabethan collar, but also connected to the single-axis lever arm and wearing the pod, the adaptation time might be larger than the 3 days proposed for just the Elizabethan collar.

The proposed combined approach - chronicity and auricular non-invasivity - representative for the setup can have numerous advantages with respect to so-far VNS techniques in mice (i.e. acute atVNS, and chronic invasive and non-invasive VNS), which are listed in continuation.

Improvements over acute atVNS from Vázquez-Oliver et. al (2019) are mainly two: (1) no anesthesia is needed, being it a very common source of variation by decreasing vagal tone [56] and suppressing the immune response [8] (2) chronic stimulation allows to well-characterize the underlying pathways of atVNS.

The proposed stimulation method might also be more attractive than the invasive one proposed by Mughrabi, Hickman, et al. as (1) surgical and technical challenges and high

costs accompany a stable long-term setup with the microscopic anatomy of the mouse VN [8] (2) implantation of the electrode is irreversible (3) invasive devices are less likeable for human treatment, reducing reproducibility for future therapy in persons (4) infection-associated morbidity can be high [16] (5) there is an unwanted stimulation of motor VN fibers and visceral afferents.
The associated morbidity is apparent in the so-mentioned invasive experiment, where only 64% (38 out of 59) of mice were responsive to surgery and recovery; 22% (13 out of 59) were non-responsive, 12% died (7 out of 59), and one of them had a headcap failure, representing for the 2%. Although these are good results, there is danger, up to a major or minor degree, in performing invasive VNS. In contrast, the use of our chronic atVNS setup is expected to also properly activate the CN X through the S-pod, and to not significantly change the behavior of animals or generate damage in any case.
The recruitment of visceral afferents and motor VN fibers leads to unfavorable multiple side effects such as cough, voice alteration (hoarseness), swallowing difficulties, or bradycardia, present in up to 30% of human patients [16].

The possible outperformance of our chronic atVNS setup for mice over the existing chronic non-invasive one from L. Oshinsky et al. (2014) could come from two main disadvantages of the latter: (1) need for strong currents to circumvent the skin barrier (2) recruitment of efferent fibers with associated adverse effects.
While the application of strong currents can pickle the stimulation sites, the activation of efferent fibers can result in headache, nasopharyngitis, and oropharyngeal pain [16].

Apart from the mentioned advantages of our model with respect to other already existing VNS techniques in mice, there is another feature to take in mind: the proposed atVNS setup is also compatible with chronic percutaneous auricular VNS, which has never been conducted in mice. Namely, the changing of the electrodes of the pod for miniature needles permits to use the setup to perform this latter VNS technique.

Given the resemblance of percutaneous auricular VNS with atVNS, it doesn't come as a surprise that they both coincide at succeeding in not innervating efferent fibers associated to adverse effects, and at showing few side effects (e.g. pain and skin irritation in the stimulation sites, headache and dizziness). When it comes to the differences, they mainly differ in how diffuse their stimulation fields are: whilst atVNS yields to diffuse stimulation fields - backed up by the non-significant differences in DI in mice stimulated with pods with different stimulation sites (see Figure 10) -, auricular percutaneous VNS allows spatially focused ones [16]. This is where the trade-off between both stimulation techniques comes: atVNS is non-invasive, which is desired, but percutaneous auricular VNS permits to more precisely stimulate nerves. In consequence, there is no best technique, but it mainly depends on the purpose.
In this case we decided to opt for atVNS given the non-invasivity of the treatment, and considering its major link with cognitive modulation in terms of indications (e.g. depression, epilepsy). Having been percutaneous auricular VNS mostly related to pain relieve, atVNS seems to better suit the requirements for cognitive enhancement. This is curious, as they stimulate the same fibers, permitting percutaneous auricular VNS to make it more precisely. A possible explanation for this could be that the diffuse fields of atVNS activate other fibers apart from the VN that might be implicated in the cognitive effect of atVNS [7].

Despite the numerous advantages of the proposed preliminary chronic atVNS setup, there are still some important limitations. In the first place, a behavioral experiment to test adaptation of the mouse to the 2-parts structure has not been conducted. In consequence, although the proving of the adaptation of CD-1 mice to the Elizabethan collar, and the observed normal movement pattern of animals when wearing the whole structure, there is no guarantee that the setup can be used for chronic atVNS. The second limitation is the time-constrain of the proposed chronic atVNS model. The reported neck injuries, and increased thickness of the epidermal, keratin layers, and inflammatory cell counts of mice wearing the Elizabethan collar [57] suggest the impossibility to use it for long periods without damage.

Further work could center on testing the time the setup can be used without comprising animal integrity and behavior. This could also be accompanied with an improving of the proposed model to allow a permanent stimulation during various consecutive days.

All in all, the chronic atVNS setup presented in this work supposes a first step towards chronic atVNS in mice. This is of high interest in a context where the mechanisms underlying the pro-cognitive effect of atVNS are unclear. Therefore, the better understanding of the functioning of atVNS through the proposed atVNS setup together with its encouraging cognitive effects could be key to developing the first therapy to enhance cognition and change the life of millions of people suffering from cognitive impairment.

5 Conclusion

Although some case studies suggest atVNS to have pro-cognitive effects, the mechanisms underlying this stimulation technique are still unclear. Further research in the mouse is required, being it the preferred species to derive the bio-physiological basic mechanisms behind emerging therapies. For so, there is a need for a simple, well-characterized, and reliable non-invasive chronic atVNS technique for mice.

In the present study we do a first step towards the implementation of non-invasive chronic atVNS in mice. With such purpose, we provide with 3 main tools:

In the first place, we supply a contrasted state-of-the-art regarding the physiological pathway of atVNS, the cognitive effects observed so far, and the optimal stimulation parameters to enhance cognition.

Secondly, we propose a brand new atVNS device to be used in mice: the S-pod. The device consists in a left ear pod containing two electrodes for stimulation in the cavity of the concha and cymba conchae, and a magnet in the top right part to ensure proper holding. Additionally, we provide with the first technique, to the best of our knowledge, to mark stimulation sites in atVNS delivering DC current in euthanized mice. The perfect fitting in the ear combined with the marking of the stimulation sites supposes an advance with respect to the previous and only atVNS model for anesthetized mice, easing the stimulation procedure and enhancing the reproducibility of the electrode positions.
To test the correct functioning of the S-pod, we run a novel object-recognition test in anesthetized naïve CD-1 mice, where atVNS condition shows increased memory persis-

tence with respect to no stimulation. This supposes a significant advance in atVNS in anesthetized mice, being the S-pod more comfortable for use than the previous atVNS device, and also enhancing reproducibility of stimulation sites thanks to the perfect fitting in the concha.

Finally, we propose a preliminary model for a chronic atVNS setup for mice, accounting for the third tool. This is a 2-parts structure united by a Molex; the first part consists of the S-pod and an Elizabethan Collar, and the second is simply a single-axis lever arm. To prove the feasibility of the proposed model, we drive a behavioral test in CD-1 and C57BL/6J mice when wearing the Elizabethan collar. Results show an adaptation of CD-1 mice after 3 training days. Additionally, small trials in few individuals have shown the setup to be robust and compatible with normal movement of the animal, being further research needed for validation.

These three tools are complementary. Whilst the chronic atVNS setup requires the S-pod for stimulation, the contrasted state-of-the-art supposes a guide to know which directions to take in research performed with the setup to derive the activated cognitive pathways in atVNS. All in all, the possibility to chronically and non-invasively stimulate the auricle of mice could open new doors to better understanding the cognitive effect of atVNS, subsequently permitting to advance in the grounding of new therapies to enhance cognition in intellectually disabled individuals.

Bibliography

[1] Soo Borson. "Cognition, Aging and Disabilities: Conceptual Issues". In: *Physical medicine and rehabilitation clinics of North America* 21.2 (2010), pp. 375–382.

[2] Christine A. F. von Arnim et al. "Diagnosis and treatment of cognitive impairment". In: *Gerantology and Geriatrics* 52 (2019), pp. 309–315. DOI: https://doi.org/10.1007/s00391-019-01560-0.

[3] Keun Lee, Marco Cascella, and Raman Marwaha. "Intellectual Disability". In: *StatPearls* (2020).

[4] Angelique Mavrodaris, John Powell, and Margaret Thorogood. "Prevalences of dementia and cognitive impairment among older people in sub-Saharan Africa: a systematic review". In: *StatPearls* 91 (2013), pp. 773–783. DOI: http://dx.doi.org/10.2471/BLT.13.118422.

[5] Eugenijus Kaniusas et al. "Current Directions in the Auricular Vagus Nerve Stimulation I – A Physiological Perspective". In: *frontiers in Neuroscience* 13 (2019), p. 854. DOI: https://doi.org/10.3389/fnins.2019.00854.

[6] Anna Vázquez-Oliver et al. "Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model". In: *Brain Stimulation* 13.2 (2020), pp. 494–498. DOI: https://doi.org/10.1016/j.brs.2019.12.024.

[7] Eugenijus Kaniusas et al. "Current Directions in the Auricular Vagus Nerve Stimulation I – A Physiological Perspective". In: *Frontiers in Neuroscience* 13 (2019). DOI: 10.3389/fnins.2019.00854.

[8] Ibrahim T. Mughrabi et al. "Development and characterization of a chronic implant mouse model for vagus nerve stimulation". In: *eLife* 10 (2021). DOI: 10.7554/eLife.61270.

[9] Michael L. Oshinsky et al. "Noninvasive vagus nerve stimulation as treatment for trigeminal allodynia". In: *Pain* 155.5 (2014), pp. 1037–1042. DOI: 10.1016/j.pain.2014.02.009.

[10] Robert H. Howland. "Vagus Nerve Stimulation". In: *Current Behavioral Neuroscience Reports* 1.2 (2014), pp. 64–73. DOI: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4017164/.

[11] Paul E. Summers, Jonathan C.W. Brooks, and Julien Cohen-Adad. "Spinal Cord fMRI". In: *Quantitative MRI of the Spinal Cord*. Ed. by Julien Cohen-Adad and Claudia Wheeler-Kingshott. 1st. ScienceDirect, 2014, pp. 221–239. DOI: https://doi.org/10.1016/C2011-0-07594-4.

[12] Marie-Michele Briand et al. "Transcutaneous Auricular Vagal Nerve Stimulation and Disorders of Consciousness: A Hypothesis for Mechanisms of Action". In: *Frontiers in Neuroscience* 11 (2020). DOI: 10.3389/fneur.2020.00933.

[13] R S McLachlan. "Vagus nerve stimulation for intractable epilepsy: a review". In: *Journal of Clinical Neurophysiology* 14.5 (1997), pp. 358–368. DOI: 10.1097/00004691-199709000-00002.

[14] Riccardo Ruffoli et al. "The chemical neuroanatomy of vagus nerve stimulation". In: *Journal of Chemical Neuroanatomy* 42.4 (2011), pp. 288–296. DOI: https://doi.org/10.1016/j.jchemneu.2010.12.002.

[15] Jeyul Yang and Ji Hoon Phi. "The Present and Future of Vagus Nerve Stimulation". In: *Journal of Korean Neurosurgical Society* 62.3 (2019), pp. 344–352. DOI: 10.3340/jkns.2019.0037.

[16] Eugenijus Eugenijus Kaniusas et al. "Current Directions in the Auricular Vagus Nerve Stimulation II – An Engineering Perspective". In: *Frontiers in Neuroscience* 14.5 (2019), pp. 358–368. DOI: 10.3389/fnins.2019.00772.

[17] Piero Barbanti et al. "Non-invasive vagus nerve stimulation for acute treatment of high-frequency and chronic migraine: an open-label study". In: *The Journal of Headache and Pain* 16 (2015). DOI: 10.1186/s10194-015-0542-4.

[18] Howland RH et al. "The emerging use of technology for the treatment of depression and other neuropsychiatric disorders". In: *Annals of Clinical Psychiatry* 23.1 (2011), pp. 48–62.

[19] J.P. Beekwilder and T Beems. "Overview of the clinical applications of vagus nerve stimulation". In: *Journal of Clinical Neurophysiology* 27.2 (2010), pp. 130–138. DOI: 10.1097/WNP.0b013e3181d64d8a.

[20] S. S. Colzato M. Ritter and L. Steenbergen. "Transcutaneous vagus nerve stimulation (tVNS) enhances divergent thinking". In: *Neuropsycologia* 111 (2018), pp. 72–76. DOI: 10.1016/j.neuropsychologia.2018.01.003.

[21] Jacobs H. I. et al. "Transcutaneous vagus nerve stimulation boosts associative memory in older individuals." In: *Neurobiology of Aging* 36 (2015), pp. 1860–1187. DOI: 10.1016/j.neurobiolaging.2015.02.023.

[22] Ilknur Ay, Vitaly Napadow, and Hakan Ay. "Electrical stimulation of the vagus nerve dermatome in the external ear is protective in rat cerebral ischemia". In: *Brain Stimulation* 8.1 (2015), pp. 7–12. DOI: 10.1016/j.brs.2014.09.009.

[23] P. Bermejo et al. "Innervation of the Human Cavum Conchae and Auditory Canal: Anatomical Basis for Transcutaneous Auricular Nerve Stimulation". In: *BioMed Research International* (2017), pp. 1–10. DOI: 10.1155/2017/7830919.

[24] Wei He et al. "The auriculo-vagal afferent pathway and its role in seizure suppression in rats". In: *BMC Neuroscience* (2013). DOI: 10.1186/1471-2202-14-85.

[25] Elmar T. Peuker and Timm J. Filler. "The nerve supply of the human auricle". In: *Clinical Anatomy* 15.1 (2002), pp. 35–37. DOI: https://doi.org/10.1002/ca.1089.

[26] *Byjus Human Ear*. https://byjus.com/biology/human-ear/. Accessed: 2021-05-27.

[27] *AMBOSS The ear*. https://www.amboss.com/us/knowledge/The_ear. Accessed: 2021-05-27.

[28] Yusuf Ozgur Cakmak. "Concerning Auricular Vagal Nerve Stimulation: Occult Neural Networks". In: *Frontiers in Human Neuroscience* 13 (2010). DOI: 10.3389/fnhum.2019.00421.

[29] Henry Stefan et al. "Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: A proof of concept trial". In: *Epilepsia* 53.7 (2012), pp. 115–118. DOI: 10.1111/j.1528-1167.2012.03492.x.

[30] Jennifer A. Clancy et al. “Non-invasive Vagus Nerve Stimulation in Healthy Humans Reduces Sympathetic Nerve Activity”. In: *Brain Stimulation* 7.6 (2014). DOI: 10.1016/j.brs.2014.07.031.

[31] Ming Song et al. “Prognostication of chronic disorders of consciousness using brain functional networks and clinical characteristics”. In: *eLife* 7 (2018). DOI: 10.7554/eLife.36173.

[32] Jonathan Y. Y. Yap et al. “Critical Review of Transcutaneous Vagus Nerve Stimulation: Challenges for Translation to Clinical Practice”. In: *Frontiers in Neuroscience* 14 (2020), p. 284. DOI: https://doi.org/10.3389/fnins.2020.00284.

[33] Ezure K and Tanaka I. “Contralateral projections of barosensitive neurons of the nucleus tractus solitarii”. In: *Neuroscience Letters* 219.1 (1996), pp. 37–40. DOI: https://doi.org/10.1016/S0304-3940(96)13169-4.

[34] Eric Beaumont et al. “Cervical vagus nerve stimulation augments spontaneous discharge in second- and higher-order sensory neurons in the rat nucleus of the solitary tract”. In: *American Journal of Physiology-Heart and Circulatory Physiology* 313.2 (2017), H354–H367. DOI: 10.1152/ajpheart.00070.2017.

[35] Laureen D. Hachem, Simeon M. Wong, and George M. Ibrahim. “The vagus afferent network: emerging role in translational connectomics”. In: *Journal of Neurosurgery* 45.3 (2018). DOI: https://doi.org/10.3171/2018.6.FOCUS18216.

[36] Adam Broncel, Katarzyna Bliźniewska, and Monika Talarowska. “How Does Vagus Nerve Stimulation (VNS) Affect Cognition? A Review”. In: *Medical Science and Technology* 58.5 (2017), pp. 67–72. DOI: 10.12659/MST.904180.

[37] William J Huffman et al. “Modulation of neuroinflammation and memory dysfunction using percutaneous vagus nerve stimulation in mice”. In: *Brain Stimulation* 12.1 (2018), pp. 19–29. DOI: 10.1016/j.brs.2018.10.005.

[38] Ai-fen Liu et al. “Effects of vagus nerve stimulation on cognitive functioning in rats with cerebral ischemia reperfusion”. In: *Journal of translational medicine* 14.101 (2016). DOI: https://doi.org/10.1186/s12967-016-0858-0.

[39] Teresa H. Sanders et al. “Cognition-Enhancing Vagus Nerve Stimulation Alters the Epigenetic Landscape”. In: *The Journal of Neuroscience* 39.18 (2019), pp. 3454–3469. DOI: https://doi.org/10.1523/JNEUROSCI.2407-18.2019.

[40] Clark KB et al. “Post-training unilateral vagal stimulation enhances retention performance in the rat.” In: *Neurobiology of Learning and Memory* 63.3 (1995), pp. 213–216. DOI: 10.1006/nlme.1995.1024.

[41] Clark KB et al. “Enhanced recognition memory following vagus nerve stimulation in human subjects”. In: *Nature Neuroscience* 2.1 (1999), pp. 94–98. DOI: 10.1038/4600.

[42] Borland MS et al. “Cortical Map Plasticity as a Function of Vagus Nerve Stimulation Intensity.” In: *Brain Stimulation* 9.1 (2016), pp. 117–123. DOI: 10.1016/j.brs.2015.08.018.

[43] Lindsey J. Noble, Rimenez R. Souza, and Christa K. McIntyre. “Vagus nerve stimulation as a tool for enhancing extinction in exposure-based therapies”. In: *Psychopharmacology (Berl)* 236.1 (2019), pp. 355–367. DOI: 10.1007/s00213-018-4994-5.

[44] Niels Hansen. "Memory Reinforcement and Attenuation by Activating the Human Locus Coeruleus via Transcutaneous Vagus Nerve Stimulation". In: *Frontiers in Neuroscience* 12.955 (2018). DOI: https://doi.org/10.3389/fnins.2018.00955.

[45] Lihua Lihua Sun et al. "Vagus nerve stimulation improves working memory performance". In: *Journal of Clinical and Experimental Neuropsychology* 39.10 (2017). DOI: 10.1080/13803395.2017.1285869.

[46] Elizabeth P. Buell et al. "Vagus nerve stimulation rate and duration determine whether sensory pairing produces neural plasticity". In: *Neuroscience* 406 (2019), pp. 290–299. DOI: 10.1016/j.neuroscience.2019.03.019.

[47] Crystal M. Noller et al. "Vagus Nerve Stimulation in Rodent Models: An Overview of Technical Considerations". In: *Frontiers in Neuroscience* 13 (2019). DOI: https://doi.org/10.3389/fnins.2019.00911.

[48] Ibrahim T. Mughrabi et al. "An implant for long-term cervical vagus nerve stimulation in mice". In: *bioRxiv* (2020). DOI: https://doi.org/10.1101/2020.06.20.160473.

[49] Roberta Sclocco et al. "The influence of respiration on brainstem and cardiovagal response to auricular vagus nerve stimulation: A multimodal ultrahigh-field (7T) fMRI study". In: *Brain Stimulation* 12.4 (2019), pp. 911–921. DOI: 10.1016/j.brs.2019.02.003.

[50] Marianne Leger et al. "Object recognition test in mice". In: *Nature Protocols* 8 (2013), pp. 2531–2537. DOI: https://doi.org/10.1038/nprot.2013.155.

[51] C. Messier. "Object recognition in mice: improvement of memory by glucose". In: *Neurobiology of Learning and Memory* 67.2 (1997), pp. 172–175. DOI: https://doi.org/10.1038/nprot.2013.155.

[52] A. Ennaceur and J. Delacour. "A new one-trial test for neurobiological studies of memory in rats". In: *Brain Research* 31.1 (1988), pp. 47–59. DOI: 10.1016/0166-4328(88)90157-x.

[53] E. White. "Care of the eye during anaesthesia". In: *Anaesthesia Intensive Care Medicine* 5.9 (2004), pp. 302–303. DOI: 10.1383/anes.5.9.302.49894.

[54] Emert White. "Care of the eye during anaesthesia". In: *Anaesthesia Intensive Care Medicine* 5.9 (2004), pp. 302–303. DOI: 10.1383/anes.5.9.302.49894.

[55] Pei-Jing Rong et al. "Transcutaneous vagus nerve stimulation for the treatment of depression: A study protocol for a double blinded randomized clinical trial". In: *MC Complementary and Alternative Medicine* 12.1 (2012). DOI: 10.1186/1472-6882-12-255.

[56] G. Marano et al. "Development and characterization of a chronic implant mouse model for vagus nerve stimulation". In: *Journal of Cardiovascular Pharmacology* 28.4 (1996). DOI: 10.1097/00005344-199610000-00006.

[57] Yura Jang et al. "The vest-collar as a rodent collar to prevent licking and scratching during experiments". In: *The Journal of Headache and Pain* 50.4 (2015), pp. 296–304. DOI: https://doi.org/10.1177/0023677215610971.

[58] M.A. Lebow and A. Chen. "Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders". In: *Molecular Psychiatry* 21 (2016), pp. 450–463. DOI: https://doi.org/10.1038/mp.2016.1.

[59] W. J. H. Nauta. "Fibre degeneration following lesions of the amygdaloid complex in the monkey". In: *Journal of Anatomy* 95 (1961), pp. 515–531.

[60] Clifford B. Saper. "The Central Autonomic Nervous System: Conscious Visceral Perception and Autonomic Pattern Generation". In: *Annual Review of Neuroscience* 25 (2002), pp. 433–469. DOI: 10.1146/annurev.neuro.25.032502.111311.

[61] Valerio Zerbi et al. "Rapid Reconfiguration of the Functional Connectome after Chemogenetic Locus Coeruleus Activation". In: *Neuron* 103.4 (2020), pp. 702–718. DOI: https://doi.org/10.1016/j.neuron.2019.05.034.

[62] Erno J Hermans et al. "Dynamic adaptation of large-scale brain networks in response to acute stressors". In: *Trends in Neurosciences* 37.6 (2014), pp. 304–314. DOI: 10.1016/j.tins.2014.03.006.

[63] Andrea Giorgi et al. "Brain-wide Mapping of Endogenous Serotonergic Transmission via Chemogenetic fMRI". In: *Cell Reports* 21.4 (2017), pp. 910–918. DOI: 10.1016/j.celrep.2017.09.087.

[64] Dorr AE and Debonnel G. "Norepinephrine: A Neuromodulator That Boosts the Function of Multiple Cell Types to Optimize CNS Performance". In: *Neurochemical Research* 37.11 (2012), pp. 2496–2512. DOI: 10.1007/s11064-012-0818-x.

[65] Cunningham JT et al. "Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation". In: *Neuropsychopharmacology* 33 (2008), pp. 1884–1895. DOI: 10.1038/sj.npp.1301570.

[66] Dorr AE and Debonnel G. "Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission". In: *Journal of Pharmacology and Experimental Therapeutics* 318 (2006), pp. 890–898. DOI: 10.1124/jpet.106.104166.

[67] Yves Charnay. "Brain serotonergic circuitries". PhD thesis. Hôpitaux Universitaires de Genève, Département de Psychiatrie, 2008, pp. 471–487. DOI: 10.31887/DCNS.2010.12.4/ycharnay.

[68] Michael Caton, Enrique L. M. Ochoa, and Francisco J. Barrantes. "The role of nicotinic cholinergic neurotransmission in delusional thinking". In: *NPJ Schizophrenia - Nature* 6.16 (2020). DOI: https://doi.org/10.1038/s41537-020-0105-9.

[69] Sombat Muengtaweepongsa. "General Principles of Somatosensory Evoked Potentials". In: *Medscape* (2019).

[70] Christine Ibrahim et al. "The Insula: A Brain Stimulation Target for the Treatment of Addiction". In: *Frontiers in Pharmacology* 10 (2019), p. 720. DOI: https://doi.org/10.3389/fphar.2019.00720.

[71] Follesa P et al. "Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain." In: *Brain Research* 1179 (2007), pp. 28–34. DOI: 10.1016/j.brainres.2007.08.045.

[72] Biggio F et al. "Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus". In: *The International Journal of Neuropsychopharmacology* 12 (2009), pp. 1209–1221. DOI: 10.1017/S1461145709000200.

[73] Cao J et al. "Vagal nerve stimulation triggers widespread responses and alters large-scale functional connectivity in the rat brain". In: *PLoS ONE* (2017). DOI: 10.1371/journal.pone.0189518.

[74] Célia Fourrier, Gaurav Singhal, and Bernhard T. Baune. "Neuroinflammation and cognition across psychiatric conditions". In: *CNS Spectrums* 24.1 (2019), pp. 4–15. DOI: 10.1017/S1092852918001499.

[75] Michael A. Galic, Kiarash Riazi, and Quentin J. Pittman. "Cytokines and brain excitability". In: *Frontiers in Neuroendocrinology* 33.1 (2011), pp. 116–125. DOI: 10.1016/j.yfrne.2011.12.002.

[76] Sigrid Breit et al. "Vagus Nerve as Modulator of the Brain–Gut Axis in Psychiatric and Inflammatory Disorders". In: *Frontiers in Psychiatry* 9 (2018). DOI: https://doi.org/10.3389/fpsyt.2018.00044.

[77] Andrea C. Sartori et al. "The Impact of Inflammation on Cognitive Function in Older Adults: Implications for Health Care Practice and Research". In: *Journal of Neuroscience Nursing* 44.4 (2012), pp. 206–217. DOI: 10.1097/JNN.0b013e3182527690.

[78] Fabiola N. Velazquez, Beatriz L. Caputto, and François D. Boussin. "c-Fos importance for brain development". In: *Frontiers in Pharmacology* 7.12 (2015), pp. 1028–1029. DOI: 10.18632/aging.100862.

[79] Cunningham JT et al. "Induction of c-Fos and DeltaFosB immunoreactivity in rat brain by vagal nerve stimulation". In: *Neuropsychopharmacology* 33 (2008), pp. 1884–1895. DOI: 10.1038/sj.npp.1301570.

6 Additional Information

6.1 Extended description of physiological mechanisms of action of atVNS

The NTS serves as a major relay center of vagal afferent projections, being it also targeted by some efferents from the spinal nucleus of the trigeminal nerve [12]. Namely, electrical shocks to the ABVN activate action potentials in vagal primary afferent neurons, which are conducted centrally to trigger action potentials in second-order neurons within the NTS [33]. These second-order neurons project axons to different sites across the CNS, in a way that the activation of ABVN has the potential to modulate the activity of subcortical and cortical circuitry [34, 35].

Most pathways ascending from the interoceptive portion of the NTS reach the forebrain, and in particular, the bed nucleus of the stria terminalis. From this center of integration for limbic information [58], projections to the amygdala come into beginning, most of which end in its central and medial nuclei [59]. Nonetheless, vagal sensory information from the thoracic and abdominal viscera is also transferred through the NTS to the parabrachial complex (PBN) and the ventral posteromedial nucleus of the thalamus (VPM) [60]. The activation of the former alters acetylcholine transmission to its diffuse outputs, including the insular cortex, amygdala, hypothalamus and thalamus [26], the latter of which connects to the anterior cingulate (ACC) and insular cortices [35].

The specific modulation of other brain structures by NTS have also been observed, including the dorsal raphe nuclei (DRN) and the locus coeruleus (LC) [35]. On the one hand, increased activity in LC results in elevation of norepinephrine (NE) concentrations within multiple brain areas (i.e. the sub-thalamic nucleus, zona incerta, thalamus, claustrum, globus pallidus, hippocampus, nucleus accumbens, amygdala, ventromedial striatum, prefrontal cortex, insular area, anterior cingulate cortex (ACC) and retrosplenial cortex [61, 62]). On the other hand, indirect projections by way of LC to the DRN have a regulatory effect over serotonergic pathways [35] affecting the thalamus, hippocampus, hypothalamus, nucleus accumbens, cerebellum, dorsomedial prefrontal cortex, and anterior and posterior cingulate cortices [12, 63].

NTS, apart from directly or indirectly activating the CNS by innervating brain regions, governs systemic parameters of cardiovascular, respiratory, and visceral functions to stay within their homeostatic limits [7]. For instance, the caudal part of the NTS is an important center for the regulation of blood pressure and vagal parasympathetic premotor neurons controlling heart rate. NTS is also very relevant in the mediation of the visceral sensation, and the control of motoneurons of the nucleus ambiguous innervating striate muscles involved in swallowing [12, 14]. Last but not least, it regulates the expiratory center, situated in the reticular formation of the brain medulla, by relaying inputs coming from the vagal general visceral afferent fibers [14].

As a matter of fact, the activation of the VN not only can modulate brain functions by activating the CNS, but also have systemic effects [25]. This is, the VN is involved in closed-loop reflex pathways along efferent and afferent pathways, being it a master key in thermo-regulation, immune-regulation, and blood pressure control of the body.

6.2 Implication of atVNS in cognition

As mentioned in 1.2.3, the innervation of NTS by the VN triggers the activation of multiple brainstem nuclei, forebrain limbic structures and cortical regions. Such stimuli alter neurotransmitter systems and neural circuit mediators in the pathway of multiple diseases. Assuming atVNS to have the same cortical effects as VNS, given that these are triggered by the stimulation of VN afferents and the consequent activation of NTS in both stimulation techniques [7], the most common molecular and physiological cognitive consequences of atVNS are listed in continuation.

LC activation. A greater activity in the LC results in major norepinephrine (NE) concentrations within some brain regions [35]. Such phenomenon facilitates both long-term-potentiation (LTP) and long-term-depression (LTD) of synapses, triggers broad changes in network excitability via effects on microglia and synaptic scaling, and syncs changes in neural activity to changes in metabolic rate. Driving these changes optimal functional responses on immediate and long-term timescales, LC activation provides a substrate for the transformation of behaviorally relevant events into permanent changes in brain function and behavior. In consequence, major NE release mediated by LC can have effects on network gating, plasticity and memory consolidation with a huge breadth of effects on cognition [64].
Additionally, LC projections may promote activity in many structures of the Salience Network (SN) like the fronto-insular cortex and the dorsal Anterior Cingulate Cortex (ACC). This limbic-paralimbic network drives attention and working memory. Namely, it is involved in cognitive and affective tasks by improving negative connectivity between the Default Mode Network (DMN), a self-awareness network associated to inner speech, day-dreaming or mind-wandering, and the External fronto-parietal Network (ExN), responsible for the relationship with the environment driving action selection [12].
Finally, NE boost seems to lead to enhanced gamma coherence, increasing awareness and alertness [12].

Regulatory effect on serotonergic pathways. Delayed regulatory effect on the synthesis and transmission of serotonin through dorsal raphe nuclei (DRN) modulation [35] explains why although acute VNS fails at changing DRN neuronal activity [65], chronic treatment maintains long-term enhancement of baseline activity [66]. This raises enthusiasm to using chronic VNS to treat psychiatric and neurodegenerative disorders, given the potential implication of serotonergic dysfunction in their progression and in the deregulation of the DMN [67, 12].

Regulation of acetylcholine transmission. Knowing that cholinergic dysfunction is tightly related to cognitive impairment, psychosis, sleep abnormalities and autonomic dysfunction, the hope to use VNS for their treatment is growing [68].

Increase of the latency of somatosensory evoked potentials (SSEP). Thalamocortical SSEP are a series of waves that reflect sequential activation of neural structures along the somatosensory pathways. The growth of their latency strengthens connections to the anterior cingulate cortex [35, 69], the impairment of which is bound to feelings of anxiety and alterations in cognition, mood, threat recognition, and conscious urges [70].

Alteration of amygdala and hippocampus plasticity. Upregulation of important drivers of excitatory synapse formation such as neurexin-1a, cadherin 13, and a2d1 proteins in the amygdala and piriform cortex have been documented following 1 week of stimulation [35]. VNS has also shown to boost hippocampal gene expression of brain-derived neurotrophic factor (BDNF) and fibroblast growth factor (FGF) [71]. Finally, it induces neurogenesis within the hippocampus and long-term potentiation (LTP) of dentate gyrus synapses [44]. These are crucial in declarative memory, resulting the latter in a lateral inhibition of structures such as the hippocampus, parahippocampus, anterior cingulated cortex, frontal cortex and amygdala in declarative memory [72]. All these effects have been postulated to be attuned by noradrenergic and serotonergic inputs [35]. Accordingly, the odds are that the formation of new synapses and rewiring of dysregulated circuitry in the amygdala and hippocampus comes from the stimulation of LC, which gives place to a major NE release [72].

Limbic Circuitry Reorganization. Findings pose possible connectivity changes between areas promoting positive effects on memory mediation, and cognition [35, 73].

CNS inflammation reduction. Raised inflammatory processes could disrupt neurobiological mechanisms regulating cognition such as Hebbian and homeostatic plasticity, neurogenesis, neurotrophic factor synthesis, the hypothalamic–pituitary–adrenal (HPA) axis interaction, and the kynurenine pathway [74]. Therefore, the reported antiinflamatory effect of VNS via inhibition of cytokine production and activation of the adrenocorticotropic hormone–glucocorticoid pathway [35, 75, 76] could make from it an encouraging treatment to beat cognitive impairment and dementia in older age [77], or even in psychiatric conditions (e.g. major depressive disorder, bipolar disorder, schizophrenia, and posttraumatic stress disorder) [74].

Boost of c-Fos expression. Up-regulation of this marker of neuronal activity, which is a master switch that transduces short-term stimuli into long-term responses [78], has been observed within the NTS, paraventricular nucleus of the hypothalamus, parabrachial nucleus, ventral bed nucleus of the stria terminalis, and locus coeruleus [34, 79].

6.3 Trial and error process to design the S-pod

The final design of the S-pod was preceded by a long trial and error process, where we designed, produced and tested multiple materials, support methods, and external supporting elements. This was essential to derive the possibilities offered by the anatomical characteristics of the mouse, always having in mind the comfort of the animal, and the ensuring of a correct functioning of the atVNS device. Here, we present the most relevant trials, all of them performed in euthanized mice, and explain why they were discarded, and what conclusions we extracted from them.

6.3.1 S-pod material

With the aim of using the left ear of euthanized mice as a mold, our approach was to use a liquid material that could solidify under a certain condition. We selected 3 of them: Loctite, Dentalon and UV Resin. Loctite and Dentalon solidify at room temperature, and UV resin curates under UV light.

Loctite was classified as ineffective to generate the pod for two main reasons: (1) large solidification time (2) extreme stickiness. The waiting time was a limitation, as the mouse had to be kept in the same position to avoid pouring out of the ear. Also extreme stickiness was uncomfortable, as the pod was difficult to remove.
Dentalon showed good results. However, it was discarded given the variability of the viscosity before solidification. Dentalon is sold in the form of dust, and then it has to be mixed with water to result in a solid after some time. The main drawback was the setting of the level of viscosity. While too low viscosity dramatically increased the solidification time, a large viscosity hindered the easy pouring of Dentalon in the concha.
UV resin was selected as the material to be used. The fact that it is delivered inside a tube with a small tip, and the ideal and reproducible viscosity among use sessions make the utilization more comfortable than Dentalon. Additionally, the cured pod is very easy to remove from the concha, leaving intact skin. A picture of a final result can be seen in Figure 7.

6.3.2 Support methods

The decision to use a pod brought to the next step: the need for a method to hold it in the ear. Bearing in mind that in the previous and only atVNS device for mice there was a need for pressure against the ear to make proper contact, we assumed that this was a mildstone. The first support method that we tried was a pair of headphones. Namely, we built a pod for each ear, and stuck them to the tips of a 3D-printed 180^{o} arch using UV resin. Arches with different diameters (13 mm, 14 mm, 16 mm) and widths (1.5 mm, 2 mm) were printed using Creality Ender 3. The infill and perfil parameters were set to 20 and 0.2, respectively. A 3D representation of the 16 mm arch together with the pods can be seen in Figure 14.

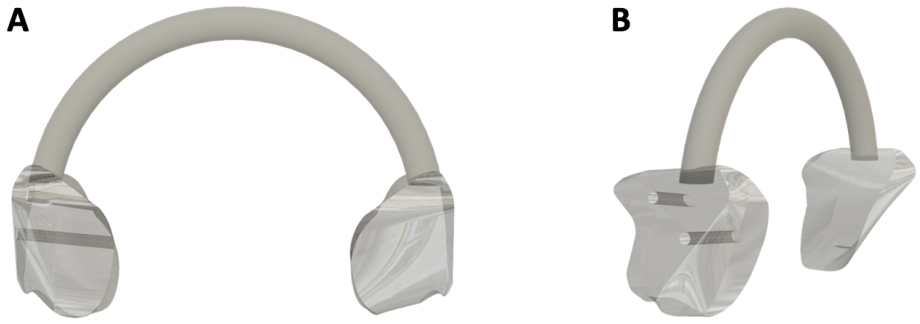

Figure 14: **Concept of headphones.** Structure including two pods built as depicted in 2.2, and a plastic arch as a uniting piece; (A) frontal view (B) diagonal view

This support method failed at maintaining the pod in the concha. Due to the laying of the ear of mice in the upper part of the head, a slipping up occurred. Additionally, the placement of the two pods in each respective ear was uncomfortable and challenging.

To avoid slipping up, there was a need for a more stable uniting piece. We designed an extended arch (320^{o}) with two bands instead of one in the upper half that we called "helmet". The goal was to enhance fitting to the head to prevent the pod from getting off the concha.

Pieces with a width of 1.5 mm and different diameters (15.5 mm, 16 mm, 17 mm) were printed using Creality Ender 3. The infill and perfil parameters were set to 40 and 0.2, respectively. A representation of this support method can be seen in Figure 15.

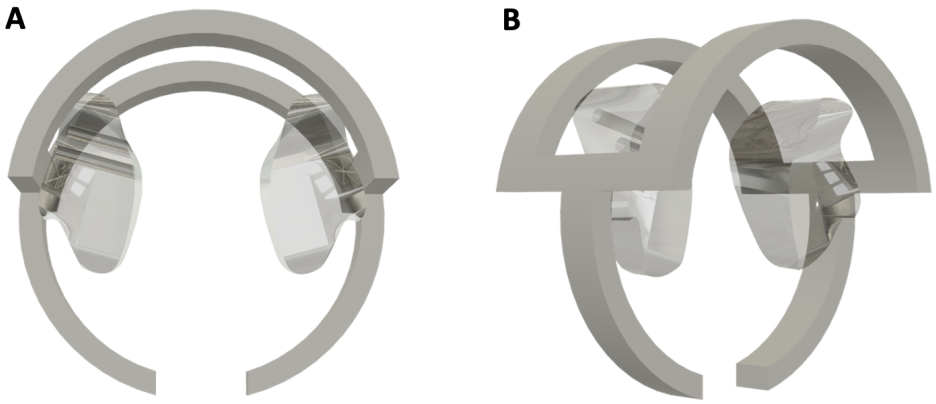

Figure 15: **Helmet concept.** 320º arch with two bars in the upper half thought to be placed in the head of mice to pressure the pod of the left ear and avoid slipping up.

The new support method eliminated slipping up, but also resulted in a new problem: the challenge to correctly place the helmet fitting the pods in the ears. Under such premise, we decided to separate the helmet from the pod.

6.3.3 External supporting elements

This new approach for the helmet was not a uniting piece between ear pods, but an external supporting element instead. Namely, the placing of the arch took place once the left pod was already in the concha. This brought 2 big advantages with: (1) need for just the left pod (2) easing of the placement of the pod. The idea was to maintain it still in the concha with the lateral bar separating the upper and lower part of the arch. Following with the idea of the helmet, we made some variants from this concept taking three main directions: (1) try of different heights of the lateral bar (2) redesign of the lateral bar to enhance holding of the pod in the concha (3) addition of a structure compatible with the electrodes in the lateral band to disregard the pod (4) changing of the lower half of the arch to enhance fitting to the head. A recompilation of all designed variants can be seen in Figure 16.

In the first place, we observed that the proposed height in Figure 15 was the optimal. Secondly, the redesign of the lateral bar showed no better performance than the basic horizontal bar. In the third place, the 3D printed pieces that disregarded the pod were not feasible due to the lack of resolution of Creality Ender 3. Finally, the addition of a bar in the lower half of the arch demonstrated not to enhance adaptation to the head, and to reduce comfort. Apart from the failure of all the variants, we also observed that the placement of the helmet by itself, even without the pods, was still complicated. Therefore, we decided to add some vertical sticks in the upper bands to easen the opening of the arch with a single hand, as perceptible in Figure 17.

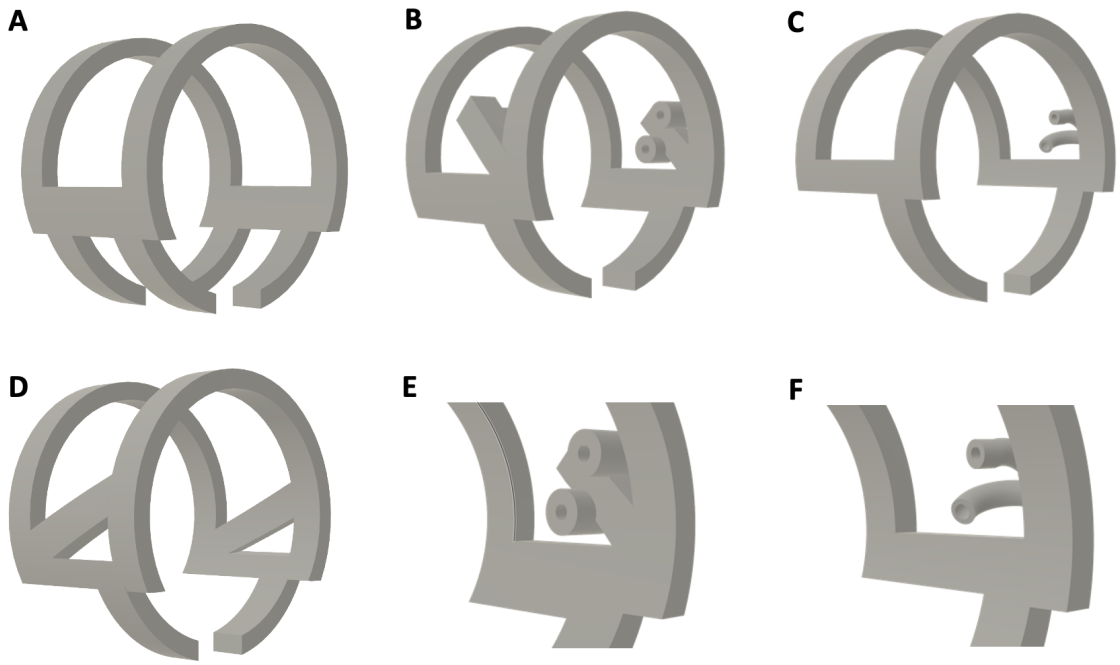

Figure 16: **Helmet variants.** (A) helmet with two below bars for enhanced support (B, C, E and F) helmet including cable incorporators in the lateral band to disregard the pod (D) helmet with the lateral band at a lower level and presenting an extra diagonal bar for better maintenance of the pod in the concha.

Figure 17: **Easy-open Helmet.** 320^{o} arch with two bars in the upper half including each of them two vertical bars to easen the opening of the piece.

Despite the improved functioning of this new approach, the fitting of the helmet around the head was still a very challenging task. We observed the high compressibility of the head of the mouse to be the main hindering feature. 17 mm diameter structures were easy to place, but were not holding good enough in the head. Conversely, 15.5 mm diameter arches were very difficult to place, but once placed, the provided pressure was optimal.

The impossibility to find a diameter permitting easy placement of the helmet, providing enough pressure, and ensuring comfort, we decided to discard it as an external supporting method. As a last try, we thought of a combined approach between the headphones concept, and the helmet approach. Namely, we designed an extended 320^{o} arch with a single band including the two vertical sticks for easy opening. A representation of

this combined approach can be seen in Figure 18.

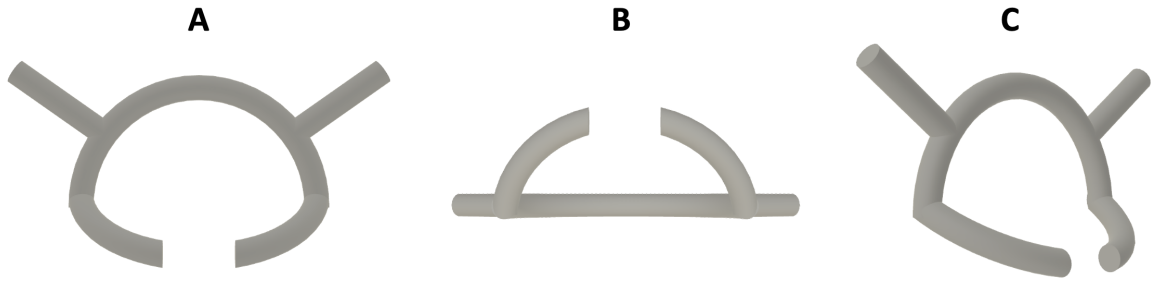

Figure 18: **Combined approach.** 320º bent arch with a single bar including two vertical sticks to easen the opening of the piece.

In parallel to the design of the 3D-printed arches, we though of a head cap to hold the pod in the ear and make pressure. For the cap, we cut a part of a finger of a latex glove. Again, the extreme compressibility of the head of the animal triggered the fail of this technique. Whilst a low cap diameter resulted in difficult placement, a larger one did not hold the pod.

With the aim of avoiding piercing at all costs, which would make the stimulation method minimally invasive, we tried to use a pair of magnets to hold the pod in the left concha. This approach presented 3 main advantages: (1) need for a single pod, accounting for the one in the left ear (2) avoiding of slipping up (3) no need to deal with head compressibility. In contrast, it presented one big disadvantage, which was the reason why we discarded the option in the first place: it only holds the pod, but it makes no pressure against the ear.
Against all odds, by incorporating the pod as depicted in Electrode System, the electrodes showed to make proper contact. In consequence, what we had considered to be a mildstone, accounting for the pressure, was not necessary for the correct functioning of the atVNS device.

This is why, in the end we selected the UV resin body containing the two silver wires and the magnet as our final atVNS device, which we called S-pod.

6.4 Trial and error process to design the chronic atVNS setup

As depicted in 2.8, the setup consists in a 2-parts structure. The first part includes the pod and the Elizabethan collar; the second part only consists of the single-axis lever arm. Additionally, these two parts are joint together with a Molex.

In the trial and error process we were hindered by two main problems. The first one accounted for the selection of the union piece between the single-axis lever arm and the Elizabethan collar. As stated above, we finally decided to use a Molex. Secondly, we also faced challenges in the selection of the type of cable to connect the electrodes of the S-pod to the Molex, and in the methodology to do so.

6.4.1 Single-axis lever arm connection to Elizabethan collar

Having in mind the idea to separate the structure in two parts to easen the placement on the animal, a connector was mandatory.
The first connector that we tried was a jack connector (Lumberg; female connector manufacturer reference: KLB 13; male connector manufacturer reference: KLS 13). This was tested in a single animal, but the discomfort was patent. The movement was conditioned by the elevated weight of the connector, in a manner that the well-being and locomotion of the mouse was hindered. Accordingly, we decided to discard this option.

Looking for a lighter and simpler connector, we decided to use a Molex. The much lower weight, and also the smaller dimensions resulted in success. Namely, we decided to use the Molex as the connector between the single-axis lever arm and the Elizabethan collar. With such purpose, we welded the cables of the single-axis lever were attached to crimp terminals and inserted into the crimp housing part of the Molex.

6.4.2 Elizabethan collar connection to pod

Apart from the connection between the single-axis lever arm and the crimp housing part of the Molex, a further connection between the electrodes and the lower limbs of the wire-to-board header of the Molex was necessary to permit stimulation.

In the first place, we tried a quite rigid multiwired cable. In the process of welding the cable to the electrodes, we observed that the body of the S-pod was modified due to the heat. This was undesired, as the position of the electrodes was altered. Alternatively, we decided to use epoxy resin (Chemtronics) for the connection between the electrodes of the pod and the cable. When it comes to the connection of the cable to the lower limbs of the wire-to-board header of the Molex, we observed welding to be optimal.
To test the functioning of the cable, we placed this first part of the chronic atVNS setup - Elizabethan collar + cable + S-pod - to an euthanized mouse. Although the collar was very easy to place, the rigidity of the cable made the placement of the S-pod very challenging.

Seeking for a more flexible cable, we decided to use a coated copper cable (Multicomp Pro). Epoxy resin and welding were used for connection to the pod, and Elizabethan collar, respectively. The testing of this new approach in an euthanized mouse resulted in success. However, the cable shows susceptibility to breaking, which might be problematic in long term.

Table 2: **CD-1 Behavioral Test Parameters.** Statement of the number of times each parameter action was carried out by 4 different mice (a1, a2, a3, and a4) along three consecutive days divided in 3 periods of time.

		Day 1				**Day 2**				**Day 3**			
		a1	**a2**	**a3**	**a4**	**a1**	**a2**	**a3**	**a4**	**a1**	**a2**	**a3**	**a4**
Defecation	13 min	0	3	0	1	0	0	0	0	1	2	0	0
	21 min	0	2	2	1	0	2	0	4	0	2	0	4
	29 min	1	3	0	0	0	1	1	0	0	0	0	0
Locomotion	13 min	4	3	3	2	3	2	2	2	3	2	2	2
	21 min	3	3	3	3	2	3	2	3	2	2	2	2
	29 min	3	4	3	2	2	2	2	2	2	2	2	2
Jumps	13 min	0	0	0	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	0	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	0	0
Wall stand-ups	13 min	10	6	6	11	26	8	11	6	17	8	4	8
	21 min	5	9	1	34	6	18	1	13	3	8	2	4
	29 min	5	18	3	2	11	13	11	7	11	10	1	11
Stand-ups	13 min	0	1	0	0	9	1	0	0	7	0	0	1
	21 min	0	1	0	0	10	1	0	0	2	2	0	1
	29 min	1	3	0	0	4	1	0	0	1	2	1	0
Scratching	13 min	1	1	27	0	0	0	7	8	0	0	39	13
	21 min	14	11	43	2	4	0	49	0	5	24	61	3
	29 min	0	18	44	13	6	2	21	15	5	15	75	31
Grooming	13 min	3	7	8	4	12	4	7	6	2	4	6	17
	21 min	7	14	14	4	9	9	13	0	4	13	11	1
	29 min	4	13	15	16	10	22	8	14	7	16	18	32
Ear-touches	13 min	0	0	0	0	0	0	0	0	0	0	1	0
	21 min	0	0	0	0	0	0	0	0	0	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	0	0

Table 3: **C57BL/6J Behavioral Test Parameters.** Statement of the number of times each parameter action was carried out by 4 different mice (b1, b2, b3, b4) along three consecutive days divided in 3 periods of time.

		Day 1				Day 2				Day 3			
		b1	**b2**	**b3**	**b4**	**b1**	**b2**	**b3**	**b4**	**b1**	**b2**	**b3**	**b4**
Defecation	13 min	0	0	0	0	0	0	1	1	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	0	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	0	0
Locomotion	13 min	0	0	1	1	1	1	0	1	0	0	1	0
	21 min	1	1	0	2	1	1	1	1	1	0	1	1
	29 min	1	1	1	1	2	0	0	0	0	2	1	0
Jumps	13 min	0	0	0	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	0	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	0	0
Wall stand-ups	13 min	0	0	0	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	0	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	1	0
Stand-ups	13 min	0	0	3	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	1	0	0	2	0	0	0
	29 min	0	0	0	0	0	1	0	0	0	1	0	0
Scratching	13 min	0	0	0	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	13	0	0	0
	29 min	0	0	0	0	1	0	0	0	0	0	0	0
Grooming	13 min	0	0	0	0	5	3	0	0	3	0	0	0
	21 min	4	0	0	3	6	5	0	0	11	0	0	0
	29 min	0	0	0	0	0	4	0	0	0	11	0	0
Ear-touches	13 min	0	0	0	0	0	0	0	0	0	0	0	0
	21 min	0	0	0	0	0	0	0	0	11	0	0	0
	29 min	0	0	0	0	0	0	0	0	0	0	0	0