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# Auricular transcutaneous vagus nerve stimulation improves memory persistence in naïve mice and in an intellectual disability mouse model

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ABSTRACT

*Background:* Vagus nerve stimulation (VNS) using non-invasive approaches have attracted great attention due to their anti-epileptic, anti-depressive and pro-cognitive effects. It has been proposed that auricular transcutaneous VNS (atVNS) could benefit intellectual disability disorders, but preclinical data supporting this idea is limited.
*Objective:* To develop an atVNS device for mice and to test its efficacy on memory performance in naïve mice and in a mouse model for intellectual disability.
*Methods:* Naïve outbreed CD-1 mice and a model for fragile X syndrome, the *Fmr1* knockout (*Fmr1*KO), were used to assess the effect of atVNS in the novel object-recognition memory performance.
*Results:* We found that atVNS significantly improves memory persistence in naïve mice. Notably, atVNS was efficacious in normalizing the object-recognition memory deficit in the *Fmr1*KO model.
*Conclusion:* Our data show that atVNS improves memory persistence in naïve mice and in a model of intellectual disability and support further studies taking advantage of preclinical mouse models of cognitive disorders.


## Introduction

The vagus nerve (cranial nerve X) contains mostly afferent fibers (80%) carrying sensorial inputs from visceral organs and superficial areas [1]. Vagus nerve electrostimulation (VNS) through invasive approaches has shown effectiveness in controlling refractory epilepsy, as a co-adjuvant in the treatment of major depression, and the improvement of cognitive performance [1,2]. The innervation of the external ear is supplied by a heterogeneous distribution of cranial branchial nerve and somatic cervical nerves [3]. The helix of the auricle is mainly supplied by the auriculotemporal nerve (91%) and to a minor extent by the great auricular nerve (9%) [3], while the *cymba conchae* is entirely (100%) supplied by the auricular branch of the vagus nerve (ABVN) [3], which makes it a readily accessible site for electrostimulation. In rats, the ABVN emerges from the superior ganglion [4] and terminates in the nucleus of the tractus solitarius (NTS) of the brainstem [5]. As the therapeutic effects of invasive VNS involve the activation of the NTS, the ABVN has gained interest as a target for non-invasive auricular transcutaneous vagus nerve stimulation (atVNS) [1].

Neurodevelopmental disorders resulting in intellectual disability may benefit from non-invasive VNS. Unfortunately, adequate VNS set-ups combined with relevant behavioral outcomes are not currently available for well-established animal models of cognitive disorders which hampered the possibility of producing a thorough preclinical assessment. Fragile X syndrome is the most common monogenic cause of inherited intellectual disability and autism produced by the silencing of the *FMR1* gene [6]. The constitutive knockout mouse for the *Fmr1* gene (*Fmr1*KO) [7] shows significant cognitive alterations [8,9] and has been long

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used as a well-accepted tool for experimental therapeutic assessment [10].

In this study, we designed, produced and tested the effect on memory persistence of a non-invasive auricular stimulator for mice. Our data further support the relevance of atVNS in cognitive modulation in naive mice and in a model of intellectual disability.

## Materials and methods

### *Animals*

Young-adult male CD-1 mice (10–12 weeks old) were purchased from Charles River Laboratories (France). Young-adult *Fmr1*KO mice (12–14 weeks old) and wildtype (WT) littermates in a C57BL/6 J congenic background (B6.129P2-*Fmr1*$^{tm1Cgr}$/J) [7] were bred at the Barcelona Biomedical Research Park (PRBB) Animal Facility. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 2010/63/EU). Mice were housed in a temperature-controlled (21 ± 1 °C) and humidity-controlled (55 ± 10%) environment. Lighting was maintained at 12 h cycles (on at 8 a.m. and off at 8 p.m.). Food and water were available *ad libitum*. Mice were handled for 1 week before starting the experiment. All behavioral experiments were performed by experimenters blind to the experimental conditions.

### *Electrode system*

The electrode prototype was based in a description of a setup for atVNS in rat [5]. Briefly, silver wires with a diameter of 0.5 mm were mounted on a newly designed transparent methacrylate surface and fixed with epoxy resin (Fig. 1A).

### *Stimulation parameters*

Rectangular bipolar pulses were delivered with a Beurer EM49 stimulator (Beurer, Germany). The stimulation parameters were: 1 mA, 20 pulses/second, 30 s ON and 5 min OFF, total length of 30 min, with 330 μs pulse width [5,11].

Current delivery was monitored using a Hantek DSO8060 oscilloscope (Qingdao Hantek Electronic, China), measuring the voltage drop across an external reference resistance $R_{ref}$ in series with the electrode system $Z_{load}$. Then, the current was calculated as $I = V/R_{ref}$, where $I$ is the delivered current and $V$ the voltage drop calculated across the external reference resistance $R_{ref}$.

### *Electrostimulation procedure*

Mice were anesthetized with isoflurane (2% induction; 1.5% maintenance) in 0.8 L/min $O_2$ during 30 min. Normothermic conditions were maintained during anesthesia with a heating pad. For atVNS condition, electrodes were placed in the *concha* of the left ear (Fig. 1B) to avoid cardiac complications, as the right branch of the vagus nerve innervates the sinoatrial node and can have undesirable effects on heart rate [12]. For sham condition, electrodes were placed on the *helix* of the left auricle, outside of the ABVN innervated area. For the "no stimulation" condition mice were anesthetized, but no electrical stimulation was delivered. True atVNS, sham stimulation and no stimulation procedures were performed immediately after the familiarization phase of the novel object-recognition test.

### *Novel object-recognition memory test*

Object-recognition memory was assayed as described previously [13] (Fig. 1C). Briefly, on the first day, mice were habituated to an empty V-shaped maze (V-maze) for 9 min (habituation phase). Then, on the second day, mice were introduced into the V-maze where two identical objects were presented for 9 min (familiarization phase). Immediately after the familiarization phase, mice were exposed to one of the following conditions: (atVNS condition, sham condition or no stimulation) as described above. Then, on the third day, the memory persistence test was performed. To this end, one familiar and one novel object were presented to the mice for 9 min in the V-maze. In naïve CD-1 mice, object-recognition

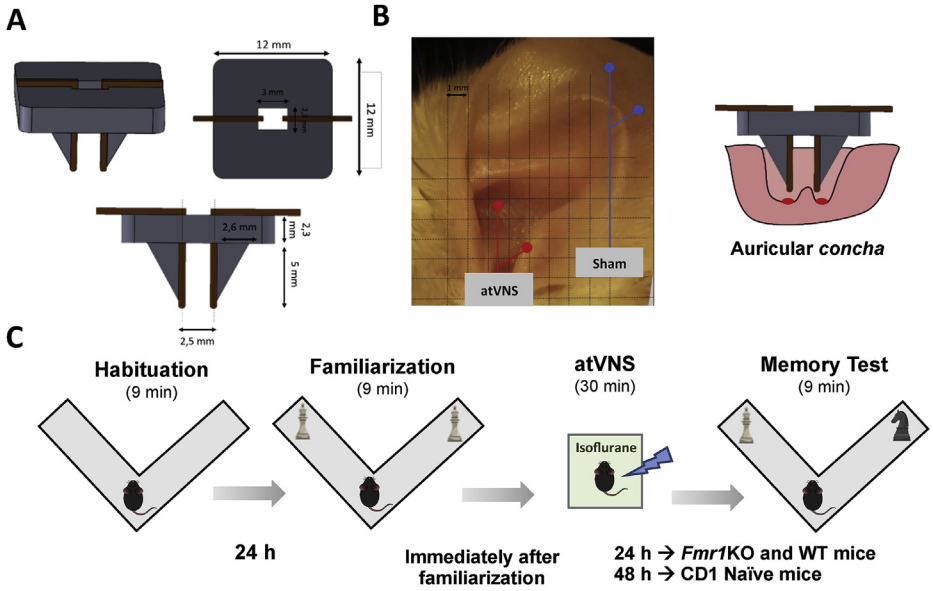
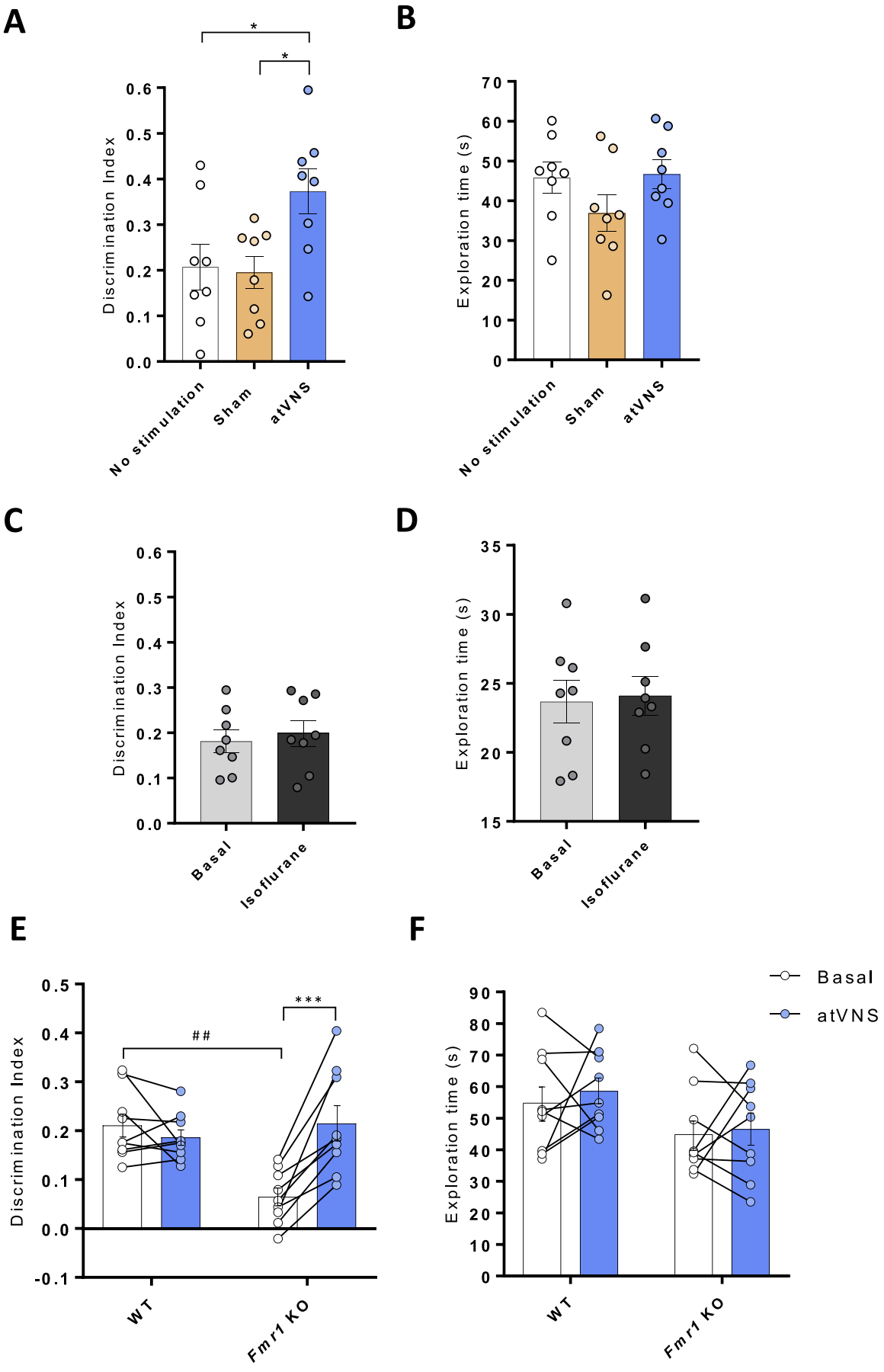



**Fig. 1. Experimental design. (A)** atVNS prototype design and dimensions. **(B)** Sites of electrostimulation in the auricle for true atVNS and sham condition (top panel) and detail on the position of electrodes on the true atVNS condition (bottom panel). **(C)** Different phases of the novel object-recognition memory test performed in a V-maze (top view). Electrostimulation was performed immediately after the familiarization phase, and memory performance was assessed 48 h later to measure improvements in object-recognition memory persistence in naïve mice, or 24 h later in the Fmr1KO model of fragile X syndrome.


A
B
C
D
E
F
Discrimination Index
Exploration time (s)
No stimulation
Sham
atVNS
Basal
Isoflurane
WT
Fmr1 KO
*
*
##
***
Basal
atVNS

memory was tested 48 h after the familiarization phase, a time at which memory persistence enhancements can be measured. In the case of the fragile X syndrome model, memory persistence was tested 24 h after the familiarization phase, at a time when Fmr1KO mice show a significant memory impairment [14]. Time exploring both novel and familiar objects was considered as the time mice spent within 2 cm from the object and with their nose facing it. The exploration time was used to calculate the discrimination index (DI): the difference between the exploration time for the novel and the familiar object related to the time exploring both objects. Higher discrimination indexes were considered to reflect greater object-recognition memory persistence.

### *Statistical analysis*

Data were analyzed with Statistica Software using one-way analysis of variance (ANOVA) or repeated-measures two-way ANOVA for multiple group comparison. Subsequent *post-hoc* analysis (Newman-Keuls) was used when required (significant interaction between factors). Comparisons were considered statistically significant when $p < 0.05$. Data are represented as mean ± standard error of the mean (s.e.m.).

## Results

### *atVNS improves novel object-recognition memory performance in naïve mice*

We first assessed the effect of atVNS in cognitive function in naïve CD-1 mice. atVNS condition showed a significantly improvement in object-recognition memory performance at 48 h as revealed by the discrimination index values for the different experimental groups (atVNS = 0.37 ± 0.05; Sham = 0.19 ± 0.03, p = 0.01; No stimulation = 0.21 ± 0.05, p = 0.03) (Fig. 2A), while sham condition reproduced the data on discrimination indexes obtained in mice that did not receive any electrostimulation (Sham = 0.19 ± 0.03; No stimulation = 0.21 ± 0.05, p = 0.84) (Fig. 2A). No differences were observed in overall exploration during the memory test phase (Fig. 2B).

To discard possible confounding effects of anesthesia in the consolidation of novel object-recognition memory delivered after the familiarization session, we assessed object-recognition performance in anesthetized and non-anesthetized naïve mice. Discrimination indexes corresponding to object-recognition memory tested 48 h showed no difference between experimental groups (Basal = 0.18 ± 0.02; Isoflurane = 0.20 ± 0.03, p = 0.65) (Fig. 2C). As expected, there were no differences in exploratory behavior during the memory test phase (Fig. 2D).

### *atVNS improves novel object-recognition memory performance in Fmr1KO mice*

We used the *Fmr1*KO as a model of intellectual disability. In this case, a cohort of *Fmr1*KO and WT littermates was first investigated for their novel object-recognition memory performance 24 h after the familiarization phase in basal conditions (WT Basal = 0.21 ± 0.02; *Fmr1*KO Basal = 0.065 ± 0.02, p = 0.00016) (Fig. 2E), a time known to show clear genotype differences in discrimination indexes [14]. Two weeks later, all mice were submitted to atVNS to assess the effect of electrostimulation. Notably, atVNS had a significant effect in novel object-recognition memory in *Fmr1*KO mice compared to previous basal levels (*Fmr1*KO Basal = 0.065 ± 0.02; *Fmr1*KO atVNS = 0.21 ± 0.03, p = 0.0003), while it did not modify the performance of WT littermates (WT Basal = 0.21 ± 0.02; WT atVNS = 0.19 ± 0.02, p = 0.34) (Fig. 2E). The improvement was observed in almost every *Fmr1*KO animal analyzed (Fig. 2E). No changes were observed in the overall exploration of experimental groups at the time of the memory test (Fig. 2F).

## Discussion

This study describes a novel non-invasive transcutaneous vagus nerve stimulation method for mice with a direct impact on memory performance.

Vagus nerve stimulation has emerged as a therapy for the treatment of drug-resistant epilepsy and refractory major depression, since vagal afferents onto the brainstem convey relevant inputs to numerous brain areas deregulated in both pathological states. Among those, brain regions such as the amygdala, the prefrontal cortex and the hippocampus, are also relevant for attention and memory [15]. In agreement, previous studies have revealed the modulation of memory function using invasive and non-invasive approaches of VNS in animal models and in humans [16–18], but, to the best of our knowledge non-invasive transcutaneous approaches had not been assessed in mouse models.

We run atVNS under normothermic conditions and using a low dose of isoflurane to prevent alterations in hippocampal signaling pathways relevant for cognition [19,20]. Our anesthesia conditions revealed no alteration of memory performance compared to non-anesthetized mice, ruling out this step as a potential bias in our behavioral results.

Notably, atVNS in the *concha* of the left external ear in naïve CD-1 mice improved memory retention compared to no stimulation or to sham stimulation conditions. These results are reminiscent of previous reports in other species using invasive techniques for the enhancement of memory retention [21] and point to a potential role of endogenous modulators such as noradrenaline, which extracellular concentrations are enhanced by VNS in rodents [22,23]. We then tested the potential of our atVNS protocol in a well-characterized mouse model of fragile X syndrome, the Fmr1KO model [7], which shows a poor object-recognition memory performance 24 h after familiarization. This marked phenotype of Fmr1KO mice, which can be improved through pharmacological interventions [24,25], was also normalized through atVNS. Discrimination index of WT littermates was not affected after atVNS, since the novel object-recognition test presents a ceiling effect. Therefore, it is not possible to reveal an improvement in the performance of the task in models that do not display a deficit in the test phase 24 h after the familiarization phase.

Together, these results further confirm the potential of atVNS in modulating memory retention in naïve mice, and as a therapeutic tool worth exploring in the context of neurodevelopmental disorders, as previously proposed for invasive forms of VNS [26]. Future studies should focus on the cellular and molecular outcomes of atVNS to elucidate the mechanisms involved in the pro-cognitive effects of this non-invasive technique.

**Fig. 2. atVNS improves object-recognition memory persistence in naïve and *Fmr1*KO mice.** Discrimination index **(A)** and total exploration time **(B)** in novel object-recognition test (NORT) for atVNS, sham and not stimulated naïve mice (atVNS condition, $n = 8$; Sham condition, $n = 8$; No stimulation condition, $n = 8$). Discrimination index **(C)** and total exploration time **(D)** in NORT for naïve mice in basal and anesthetized conditions (Basal, $n = 8$; Isoflurane, $n = 8$). Discrimination index **(E)** and total exploration time **(F)** in NORT for WT and *Fmr1*KO mice under basal conditions (basal) and after the same mice received atVNS (atVNS) (WT, $n = 9$; *Fmr1*KO, $n = 9$). $^{*}p < 0.05$, $^{***}p < 0.001$ (electrostimulation effect); $\#\#p < 0.01$ (genotype effect) by one-way ANOVA (A, B, C, D) and repeated measures two-way ANOVA (E, F).

## Author contribution section

A.V.-O. participated in experimental design, conducted and analyzed behavioral experiments and wrote the manuscript.

C.B.-P. participated in experimental design, conducted and analyzed behavioral experiments and wrote the manuscript.

M.D.-G. designed and generated auricular transcutaneous vagal nerve stimulator and wrote the manuscript.

R.M. participated in the supervision and experimental design, funded the project and revised the manuscript.

A.I. participated in the supervision and stimulator design and generation, funded the project and revised the manuscript.

A.O. conceptualized, participated in experimental design, supervised, funded the project and wrote the manuscript.

All authors reviewed and approved the final version of the manuscript

## Declaration of competing interest

Authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2019.12.024.

## References

[1] Johnson RL, Wilson CG. A review of vagus nerve stimulation as a therapeutic intervention. J Inflamm Res 2018;16(11):203–13. https://doi.org/10.2147/JIR.S163248. Available from.

[2] Jacobs HIL, Riphagen JM, Razat CM, Wiese S, Sack AT. Transcutaneous vagus nerve stimulation boost associative memory in older individuals. Neurobiol Aging 2015;36(5):1860–7. https://doi.org/10.1016/j.neurobiolaging.2015.02.023. Available from.

[3] Peuker ET, Filler TJ. The nerve supply of the human auricle. Clin Anat 2002;15(1):35–7. https://doi.org/10.1002/ca.1089. Available from.

[4] Tekdemir I, Aslan A, Elhan A. A clinico-anatomic study of the auricular branch of the vagus nerve and Arnold’s ear-cough reflex. Surg Radiol Anat 1998;20(4):253–7. https://doi.org/10.1007/BF01628484. Available from.

[5] He W, Jing XH, Zhu B, Zhu XL, Li L, Bai WZ, Ben H. The auriculo-vagal afferent pathway and its role in seizure suppression in rats. BMC Neurosci 2013;14(1):85. https://doi.org/10.1186/1471-2202-14-85. Available from.

[6] Penagarikano O, Mulle JG, Warren ST. The pathophysiology of fragile x syndrome. Annu Rev Genom Hum Genet 2007;8(1):109–29. https://doi.org/10.1146/annurev.genom.8.080706.092249. Available from.

[7] The Dutch-Belgian Fragile X Consortium. Fmr1 knockout mice: a model to study fragile X mental retardation. 15 Cell 1994;78(1):23–33. https://doi.org/10.1016/0092-8674(94)90569-X. Available from.

[8] Levenga J, de Vrij FM, Buijsen RA, Li T, Nieuwenhuizen IM, Pop A, Oostra BA, Willemsen R. Subregion-specific dendritic spine abnormalities in the hippocampus of Fmr1 KO mice. Neurobiol Learn Mem 2011;95(4):467–72. https://doi.org/10.1016/j.nlm.2011.02.009. Available from.

[9] Eadie BD, Zhang WN, Boehme F, Gil-Mohapel J, Kainer L, Simpson JM, Christie BR. Fmr1 knockout mice show reduced anxiety and alterations in neurogenesis that are specific to the ventral dentate gyrus. Neurobiol Dis 2009;36(2):361–73. https://doi.org/10.1016/j.nbd.2009.08.001. Available from.

[10] Kazdoba TM, Leach PT, Silverman JL, Crawley JN. Modeling fragile X syndrome in the Fmr1 knockout mouse. Intractab Rare Dis Res 2014;3(4):118–33. https://doi.org/10.5582/irdr.2014.01024. Available from.

[11] Huffman WJ, Subramaniyan S, Rodriguiz R, Wetsel WC, Grill WM, Terrando N. Modulation of neuroinflammation and memory dysfunction using percutaneous vagus nerve stimulation in mice. Brain Stimulation 2019;12(1):19–29. https://doi.org/10.1016/j.brs.2018.10.005. Available from.

[12] Kaniusas E, Kampusch S, Tittgemeyer M, Panetsos F, Gines RF, Papa M, Kiss A, Podesser B, Cassare Am, Tanghe E, Samoudi Am, Tarnaud T, Joseph W, Marozas V, Lukosevicius A, Istuk N, Sarolic A, Lechner S, Klonowski W, Varoneckas G, Széles JC. Current directions in the auricular vagus nerve stimulation I - a physiological perspective. Front Neurosci 2019;13:854. https://doi.org/10.3389/fnins.2019.00854. Available from.

[13] Puighermanal E, Marsicano G, Busquets-Garcia A, Lutz B, Maldonado R, Ozaita A. Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. Nat Neurosci 2009;12(9):1152–8. https://doi.org/10.1038/nn.2369. Available from.

[14] Busquets-Garcia A, Gomis-González, Guegan T, Agustín-Pavón C, Pastor A, Mato S, Pérez-Samartín A, Matute C, de la Torre R, Dierssen M, Maldonado R, Ozaita A. Targeting the endocannabinoid system in the treatment of fragile X syndrome. Nat Med 2013;19(5):603–7. https://doi.org/10.1038/nm.3127. Available from.

[15] Grimonprez A, Raedt R, Baeken C, Boon P, Vonck K. The antidepressant mechanism of action of vagus nerve stimulation: evidence from preclinical studies. Neurosci Biobehav Rev 2015;56:26–34. https://doi.org/10.1016/j.neubiorev.2015.06.019. Available from.

[16] Clark KB, Smith DC, Hassert DL, Browning RA, Naritoku DK, Jensen RA. Post-training electrical stimulation of vagal afferents with concomitant vagal efferent inactivation enhances memory storage processes in the rat. Neurobiol Learn Mem 1998;70(3):364–73. https://doi.org/10.1006/nlme.1998.3863. Available from.

[17] Clark KB, Naritoku DK, Smith DC, Browning RA, Jensen RA. Enhanced recognition memory following vagus nerve stimulation in human subjects. Nat Neurosci 1999;2(1):94–8. https://doi.org/10.1038/4600. Available from.

[18] Vonck K, Raedt R, Naulaerts J, De Vogelaere F, Thiery E, Van Roost D, Aldenkamp B, Miatton M, Boon P. Vagus nerve stimulation…25 years later! what do we know about the effects on cognition? Neurosci Biobehav Rev 2014;45:63–71. https://doi.org/10.1016/j.neubiorev.2014.05.005. Available from.

[19] Cohen SJ, Stackman Jr RW. Assessing rodent hippocampal involvement in the novel object recognition task. A review. Behav Brain Res 2015;15(285):105–17. https://doi.org/10.1016/j.bbr.2014.08.002. Available from.

[20] Hao H, Wang S. Hypothermia induced by anesthesia regulates various signals expressions in the hippocampus of animals. Biomed Pharmacother 2017;95:1321–30. https://doi.org/10.1016/j.biopha.2017.09.046. Available from.

[21] Ghacibeh GA, Shenker JI, Shenal B, Uthman BM, Heilman KM. The influence of vagus nerve stimulation on memory. Cogn Behav Neurol 2006;19(3):119–22. https://doi.org/10.1097/01.wnn.0000213908.34278.7d. Available from.

[22] Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. 1119 Brain Res 2006;13(1):124–32. https://doi.org/10.1016/j.brainres.2006.08.048. Available from:.

[23] Raedt R, Clinckers R, Mollet L, Vonck K, El Tahry R, Wyckhuys T, De Herdt V, Carrette E, Wadman W, Michotte Y, Smolders I, Boon P, Meurs A. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. J Neurochem 2011;117(3):461–9. https://doi.org/10.1111/j.1471-4159.2011.07214.x. Available from.

[24] Busquets-Garcia A, Maldonado R, Ozaita A. New insights into the molecular pathophysiology of fragile X syndrome and therapeutic perspectives from the animal model. Int J Biochem Cell Biol 2014;53:121–6. https://doi.org/10.1016/j.biocel.2014.05.004. Available from.

[25] Gomis-González M, Busquets-Garcia A, Matute C, Maldonado R, Mato S, Ozaita A. Possible therapeutic doses of cannabinoid type 1 receptor antagonist reverses key alterations in fragile X syndrome mouse model. Genes 2016;7(9):56. https://doi.org/10.3390/genes7090056. Available from.

[26] Engineer CT, Hays SA, Kilgard MP. Vagus nerve stimulation as potential adjuvant to behavioral therapy for autism and other neurodevelopmental disorders. J Neurodev Disord 2017;9(20):1–8. https://doi.org/10.1186/s11689-017-9203-z. Available from.