UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE MEDICINA
Programa de Pós-Graduação em Medicina Molecular

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INVESTIGAÇÃO MOLECULAR E COMPORTAMENTAL DA ESTIMULAÇÃO TRANSCRANIANA POR CORRENTE ALTERNADA EM CAMUNDONGOS

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Molecular da Faculdade de Medicina da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Mestre em Medicina Molecular.

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INVESTIGAÇÃO MOLECULAR E COMPORTAMENTAL DA ESTIMULAÇÃO TRANSCRANIANA POR CORRENTE ALTERNADA EM CAMUNDONGOS

FERNANDA DONIZETE REZENDE

Dissertação de Mestrado defendida e aprovada, no dia dezesseis de agosto de dois mil vinte e um, pela Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação Medicina Molecular da Universidade Federal de Minas Gerais constituída pelos seguintes professores doutores:

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RESUMO

Nas últimas duas décadas, o uso de técnicas de neuromodulação tornou-se presente no tratamento de transtornos neuropsiquiátricos, como depressão maior, ansiedade, transtornos de humor e personalidade, abuso de substâncias, doença de Alzheimer e transtorno do déficit de atenção e hiperatividade (TDAH). Dentre as estimulações cerebrais não invasivas, destacam -se as estimulações transcranianas elétricas, sendo amplamente utilizadas em humanos com finalidade terapêutica ou em indivíduos saudáveis. A Estimulação Transcraniana por Corrente Alternada (ETCA) chama a atenção devido sua característica corrente elétrica com alternância entre os polos, promovendo um padrão de onda sinusoidal que se assemelha ao padrão de disparos neuronais, possibilitando a mimetização das oscilações cerebrais endógenas. Apesar disso, ainda não há informações suficientes para elucidar seus mecanismos de ação. Por isso, faz-se necessário mais estudos, preferencialmente em modelos animais. Portanto, nosso objetivo foi avaliar o efeito da Estimulação Transcraniana por Corrente Alternada no comportamento e no cérebro de camundongos C57Bl/J6 estimulados sob sedação e em diferentes protocolos. Nossos resultados não demonstraram ação positiva ou negativa da ETCA. Considerando nossas limitações e perspectivas futuras para a utilização da estimulação transcraniana por corrente alternada, sugerimos mais estudo, sobretudo com modelos animais, leituras de encefalografia e protocolos de ETCA em animais acordados durante realização de tarefas.

Palavras-chave: Estimulação Transcraniana por Corrente Alternada. ETCA. Expressão Gênica. Labirinto de Barnes. Modelo Animal.

ABSTRACT

In the last two decades, the use of neuromodulation techniques has become present in the treatment of neuropsychiatric disorders, such as major depression, anxiety, mood and personality disorders, substance abuse, Alzheimer's disease, and attention deficit hyperactivity disorder (ADHD). Among the non-invasive brain stimulations, transcranial electrical stimulations stand out, being widely used in humans for therapeutic purposes or in healthy individuals. Transcranial Alternating Current Stimulation (tACS) draws attention due to its characteristic electrical current with alternation between the poles, promoting a sinusoidal wave pattern that resembles the one of neuronal firings, enabling the mimicry of endogenous brain oscillations. Despite this, there is still not enough information to elucidate its action mechanisms. Therefore, further studies are needed, preferably in animal models. Our goal was to evaluate the effect of Transcranial Alternating Current Stimulation on the behavior and brain of C57Bl/J6 mice stimulated under sedation and in different protocols. Our results did not demonstrate positive or negative action of tACS. Considering our limitations and future perspectives for the use of transcranial alternating current stimulation, we suggest further study, especially with animal models, encephalography readings, and tACS protocols in animals awake while performing tasks.

Key-words: Transcranial Alternating Current Stimulation. tACS. Gene Expression. Barnes Maze. Animal Model.

LISTA DE ABREVIATURAS E SIGLAS

°C	grau Celsius / degree Celsius
ADHD	attention deficit hyperactivity disorder
ANOVA	análise de variancia / analysis of variance
AP	anteroposterior
ARC	activity-regulated cytoskeleton-associated protein
CAMKAIIa	calcium/calmodulin dependent protein kinase II alpha
CDK5	cyclin dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CEUA	comissão de ética na utilização de animais
cFOS	fos proto-oncogene
DNA	deoxyribonucleic acid
DV	dorsoventral
ECG	electrocardiogram
ECT	eletroconvulsoterapia / electroconvulsive therapy
EEG	eletroencefalograma
ETCA	estimulação transcraniana por corrente alternada
ETCC	estimulação transcraniana por corrente contínua
ETFU	estimulação transcraniana por foco de ultrassom
ETM	estimulações transcraniana magnética
ETRA	estimulação transcraniana por ruído aleatório
GAD67	glutamate decarboxylase 1
GFAP	glial fibrillary acidic protein
GRIA	glutamate ionotropic receptor AMPA type subunit 1
Hz	Hertz
IDT	Integrated DNA Technologies
L	litro
mA	mili Amper
MD	mean difference
MedD	median difference
min	minuto
mL	mililitro
mm	milímetro

NaCl	cloreto de sódio
PLA	poly (lactic acid)
PSD95	discs large MAGUK scaffold protein 4
RNA	ribonucleic acid
RPL13A	ribosomal protein l13a
RT-PCR	reverse transcription polymerase chain reaction
S.E.M.	standart error of mean
SYN	synapsin I
tACS	transcranial alternating current stimulation
TDAH	transtorno do déficit de atenção com hiperatividade
tDCS	transcranial direct current stimulation
tES	transcranial electric stimulation
tMS	transcranial magnectic stimulation
tRNS	transcranial random noise stimulation

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SUMÁRIO

1. INTRODUÇÃO

Nas últimas duas décadas, o uso de técnicas de neuromodulação tornou-se presente no tratamento de transtornos neuropsiquiátricos. Sua capacidade de modular a fisiologia, morfologia e circuitarias cerebrais aumentaram as possibilidades terapêuticas para além dos tratamentos convencionais. Atualmente, as principais ferramentas de neuromodulação utilizam campo magnético, elétrico ou ondas de ultrassom (POLANÍA, NITSCHE, RUFF, 2018).

Entre as estimulações magnéticas, destaca-se a Estimulação Transcraniana Magnética (ETM). Ela utiliza bobinas que geram um campo eletromagnético capaz de modular a atividade neuronal. Por ser a técnica de estimulação não-invasiva mais antiga, a ETM possui uma gama de protocolos e variações da técnica possibilitando uma maior adaptação às necessidades de cada usuário (HALLETT, 2007)

Em contrapartida, as técnicas mais recentes são as derivadas de ultrassom. Esta ferramenta é amplamente conhecida em outras áreas da saúde, como na medicina diagnóstica e tratamentos fisioterapêuticos. Considerando os aspectos desta técnica, os estudos envolvendo estimulações transcranianas por foco de ultrassom são respaldados na capacidade de atravessar tecidos biológicos, promovendo uma ação mais profunda (BIASE, FALATO, LAZZARO, 2019).

Por fim, as técnicas de estimulação elétrica se destacam pelas variáveis aplicações, desde eletroconvulsoterapia (ECT) a diversas técnicas de estimulação elétrica de baixa intensidade (MAIXNER et al., 2021)

As estimulações transcranianas por corrente contínua (ETCC), corrente alternada (ETCA) e por ruído aleatório (ETRA) são as mais estudadas atualmente. Elas diferem entre si, essencialmente, por seus formatos de onda (**Figura A**) (VOSSKUHL et al., 2018).

Na ETCC, a aplicação da corrente se dá por um fluxo contínuo, com cátodo e ânodo bem estabelecidos. Esta característica facilita a observação de efeitos excitatórios ou inibitórios da técnica. Em oposição, a ETRA apresenta uma entrega da corrente que alterna entre os eletrodos, com frequência e amplitude de onda pré-definida (VANNESTE, FREGNI, DE RIDDER, 2013).

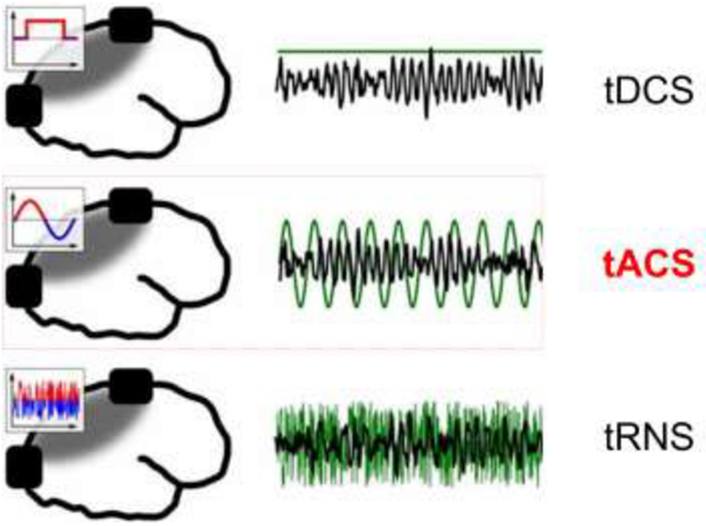

Figura A. Comparação entre estimulações elétricas de baixa intensidade. *tDCS (transcranial direct current stimulation* - estimulação trancraniana por corrente contínua); *tACS (transcranial alternating current stimulation* - estimulação trancraniana por corrente alternada); *tRNS (transcranial random noise stimulation* - estimulação transcraniana por ruído aleatório). Adaptado de Vosskuhl et al., 2018. (doi.org/10.3389/fnhum.2018.00211)

A ETCA foi inicialmente relatada como a aplicação de uma corrente elétrica com alternância entre os polos, promovendo um padrão de onda sinusoidal. Esta característica da ETCA se assemelha ao padrão de disparos neuronais, possibilitando a mimetização das oscilações cerebrais endógenas (**Figura B**) (TAVAKOLI, YUN, 2017).

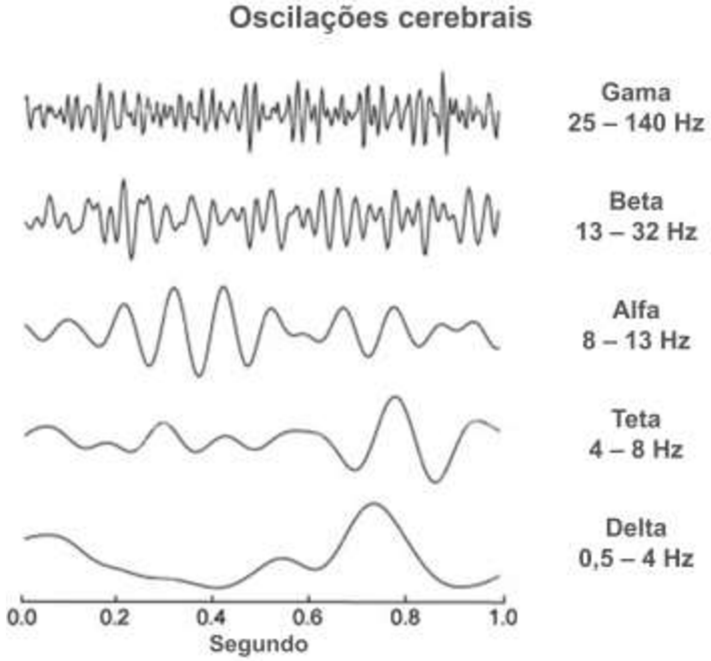

Figura B. Faixas de oscilações cerebrais lidas por eletroencefalograma. Adaptado de Kolb e Mohajerani, 2020. (doi.org/10.1016/j.pneurobio.2020.101878)

Entretanto, pouco ainda se sabe sobre esta técnica. Com isso, estudos utilizando ferramentas para captura de informações cerebrais, como o eletroencefalograma (EEG), instrumentos de neuronavegação e neuroimagem estão sendo incluídos junto à aplicação da ETCA para melhor compreensão da técnica. Com

isso, novas hipóteses de como a ETCA atua ou novas possibilidades de aplicação foram surgindo.

As estimulações cerebrais não invasivas estão sendo amplamente utilizadas em humanos com finalidade terapêutica, como em casos de depressão maior, ansiedade, transtornos de humor e personalidade, abuso de substâncias, doença de Alzheimer e transtorno do déficit de atenção e hiperatividade (TDAH), ou em indivíduos saudáveis. Entretanto, apenas a ETM possui liberação internacional para uso clínico sem a necessidade de vínculo com pesquisa científica (POLANÍA, NITSCHE, RUFF, 2018).

Os dados científicos provenientes da aplicação destas técnicas são majoritariamente frutos de ensaios clínicos sem respaldo de pesquisas em modelos não-humanos. Com isso, lacunas na literatura científica são evidentes sobre este assunto, principalmente na caracterização de mecanismos de ação molecular destas técnicas. (PELLETIER et al., 2015).

Apesar das evidências e hipóteses relativas ao funcionamento da ETCA serem potenciais explicações para os efeitos evocados por estímulos elétricos, ainda não há informações suficientes para elucidar seus mecanismos e alterações significantes, principalmente por ainda ser comparada com outras estimulações elétricas, como a ETCC. Esta falta de evidências traz a necessidade de mais estudos, preferencialmente em modelos não-humanos, como estudos computacionais e modelos em roedores.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar o efeito da Estimulação Transcraniana por Corrente Alternada no comportamento e no cérebro de camundongos C57Bl/J6 estimulados sob sedação

2.2. Objetivos Específicos

Investigar a expressão de genes relacionados a atividade neuronal e glial, atividade e plasticidade sináptica, estruturação e lesão neuronal em camundongos submetidos a ETCA sob sedação em diferentes frequências.

Investigar aspectos cognitivos de camundongos submetidos a ETCA sob sedação por meio de avaliação comportamental.

3. ARTIGO

TRANSCRANIAL ALTERNATING CURRENT STIMULATION EFFECT ON GENE EXPRESSION AND COGNITIVE BEHAVIOR IN MICE

3.1. Abstract

In the last two decades, the use of neuromodulation techniques has become present in the treatment of neuropsychiatric disorders, such as major depression, anxiety, mood and personality disorders, substance abuse, Alzheimer's disease, and attention deficit hyperactivity disorder (ADHD). Among the non-invasive brain stimulations, transcranial electrical stimulations stand out, being widely used in humans for therapeutic purposes or in healthy individuals. Transcranial Alternating Current Stimulation (tACS) draws attention due to its characteristic electrical current with alternation between the poles, promoting a sinusoidal wave pattern that resembles the one of neuronal firings, enabling the mimicry of endogenous brain oscillations. Despite this, there is still not enough information to elucidate its action mechanisms. Therefore, further studies are needed, preferably in animal models. Our goal was to evaluate the effect of Transcranial Alternating Current Stimulation on the behavior and brain of C57Bl/J6 mice stimulated under sedation and in different protocols. Our results did not demonstrate positive or negative action of tACS. Considering our limitations and future perspectives for the use of transcranial alternating current stimulation, we suggest further study, especially with animal models, encephalography readings, and tACS protocols in animals awake while performing tasks.

Key-words: Transcranial Alternating Current Stimulation. tACS. Gene Expression. Barnes Maze. Animal Model.

3.2. Introduction

In the last two decades, neuromodulation has become present in the treatment of neuropsychiatric disorders. Its ability to modulate brain physiology, morphology, and

circuitry has increased therapeutic possibilities beyond conventional treatments. (POLANÍA, NITSCHE, RUFF, 2018).

Among the non-invasive brain stimulations, transcranial electrical stimulations stand out. They are being widely used in humans for therapeutic purposes, such as in cases of major depression, anxiety, mood and personality disorders, substance abuse, Alzheimer's disease, and attention deficit hyperactivity disorder (ADHD), or in healthy individuals. However, only transcranial magnetic stimulation (tMS) has international approval for clinical use without the need for a link with scientific research (POLANÍA, NITSCHE, RUFF, 2018).

Transcranial alternating current stimulation (tACS) was initially reported as the application of an electrical current alternating between the poles, promoting a sinusoidal waveform. This feature of tACS is similar to the pattern of neuronal firings, enabling the mimicry of endogenous brain oscillations (TAVAKOLI, YUN, 2017).

The scientific data from the application of this technique are mostly the result of clinical trials without research support in non-human models. Thus, gaps in the scientific literature are evident on this subject, especially in the characterization of molecular mechanisms of action of these techniques. (PELLETIER et al., 2015).

However, little is known about this technique. Thus, studies using tools to capture brain information, such as the electroencephalogram (EEG), neuronavigation, and neuroimaging instruments are being included with the application of the tACS for a better understanding of the technique.

Still, there is a wide range of protocols being published and few studies repeating parameters to enrich the literature.

Despite the evidence and hypotheses related to the functioning of tACS being potential explanations for the effects evoked by electrical stimuli, there is still not enough information to elucidate its mechanisms and significant alterations. This lack of evidence brings the need for further studies, preferably on non-human models, such as computational studies and rodent models.

Therefore, our goal was to evaluate the effect of Transcranial Alternating Current Stimulation on the behavior and brain of C57Bl/J6 mice stimulated under sedation. We used the expression of genes related to neuronal activity in animals submitted to different tACS protocols and behavioral assessment for this investigation.

3.3. Materials and Methods

3.3.1. Animals

Male C57BL/6J mice (Central Animal Facility of UFMG) were used in this study. The animals were 8-9 weeks old and had a minimal weight of 20 g. They were provided with food and water *ad libitum.* All procedures were approved by the Animal Use Ethics Committee (CEUA) - UFMG under protocol 321/2019.

3.3.2. Stereotaxic surgery and post-surgery care

The stereotaxic surgery protocol was performed according to NICOLAU et al., 2018. Mice received were anesthetized with ketamine (Dopalen®, 80 mg/kg) and xylazine (Anasedan®, 8 mg/kg) intraperitoneally. Then, trichotomy (Philips Multigroom) and stereotaxic (KOPF®) fixation procedures were performed on the animal, which remained throughout the surgery on a heated platform (Physitemp TCAT-2LV Controller, 37ºC). In addition, a mask for continuous oxygen and isoflurane (Isoforine®, 1%/L) support was attached. The incision site was disinfected with proper asepsis procedures and cut to expose the skull.

A customized implant (poly (lactic acid) (PLA); silver; tinned copper), was glued with cyanoacrylate superglue (Loctite Super Bonder Original) in the animal's skull at the following coordinates: +1 mm AP (anteroposterior); 0 mm ML (mediolateral); 0 mm DV (dorsoventral) (**Figure 1**). We applied three layers of dental acrylic resin (Duralay Reliance) for more fixation. Chemical suture was performed with surgical glue (Vetbond™).

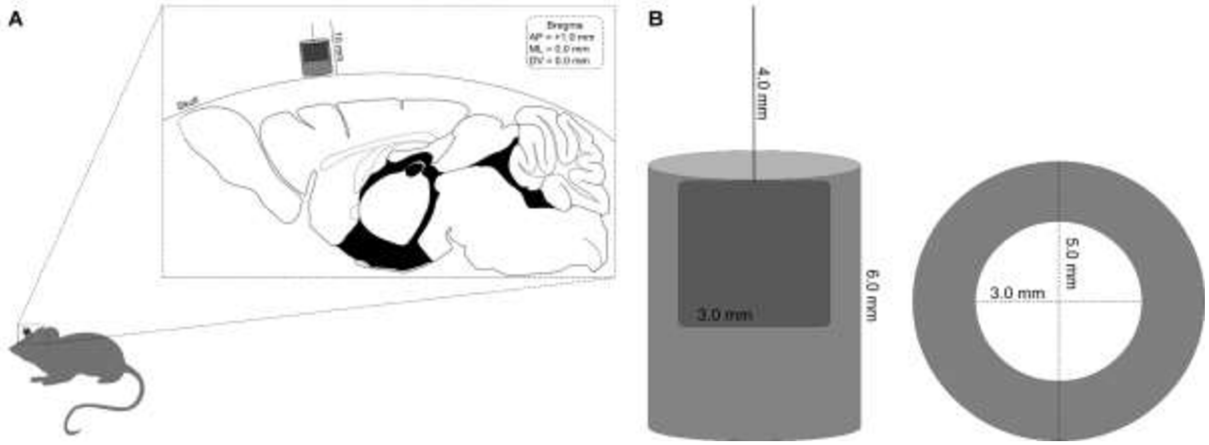

Figure 1. Schematic design of head electrode. (A) Electrode coordinate. AP: anteroposterior; ML: mediolateral; DV: dorsoventral. **(B)** Electrode dimensions.

We applied lidocaine hydrochloride solution (Xylestesin, 2%) on the incision site and adjacent tissues for local anesthesia. Subcutaneous injection in the animal's dorsum containing 50 µL of the anti-inflammatory and analgesic ketoprofen (Profenid®, 5 mg/kg) diluted in 450 µL of a sterile Ringer's Lactate solution (Eurofarma®) was applied to control pain and rehydrate the animal. Then, the mice were relocated to a new cage, previously heated to 25ºC, for monitoring the animal until it woke up. For three days following surgery, animals received a daily subcutaneous injection containing 50 µL of ketoprofen (Profenid®, 5 mg/kg) and 450 µL of a sterile solution Ringer's Lactate (Eurofarma®). Animals were monitored and fully recovered for 7 days.

3.3.3. Transcranial Alternating Current Stimulation

The stimulation protocols were conducted by Animal Transcranial Electrical Stimulator equipment (Soterix Medical Inc.). This device had power, start and abort buttons; waveform, duration (min), frequency (Hz), and current intensity (mA) controllers; on/off SHAM mode, polarity (bipolar/unipolar), and current relaxation switches. Its display shows the time remaining, contact quality score (1 to 10), frequency, and true current (**Figure 2**).

The animal was sedated using isoflurane (Isoforine®, 3%/L) and oxygen in a chamber (VetEquip Inc.) for two minutes. After, it was fixed to the stereotaxic apparatus through the incisor teeth, on a heated platform (37°C). The sedation was maintained

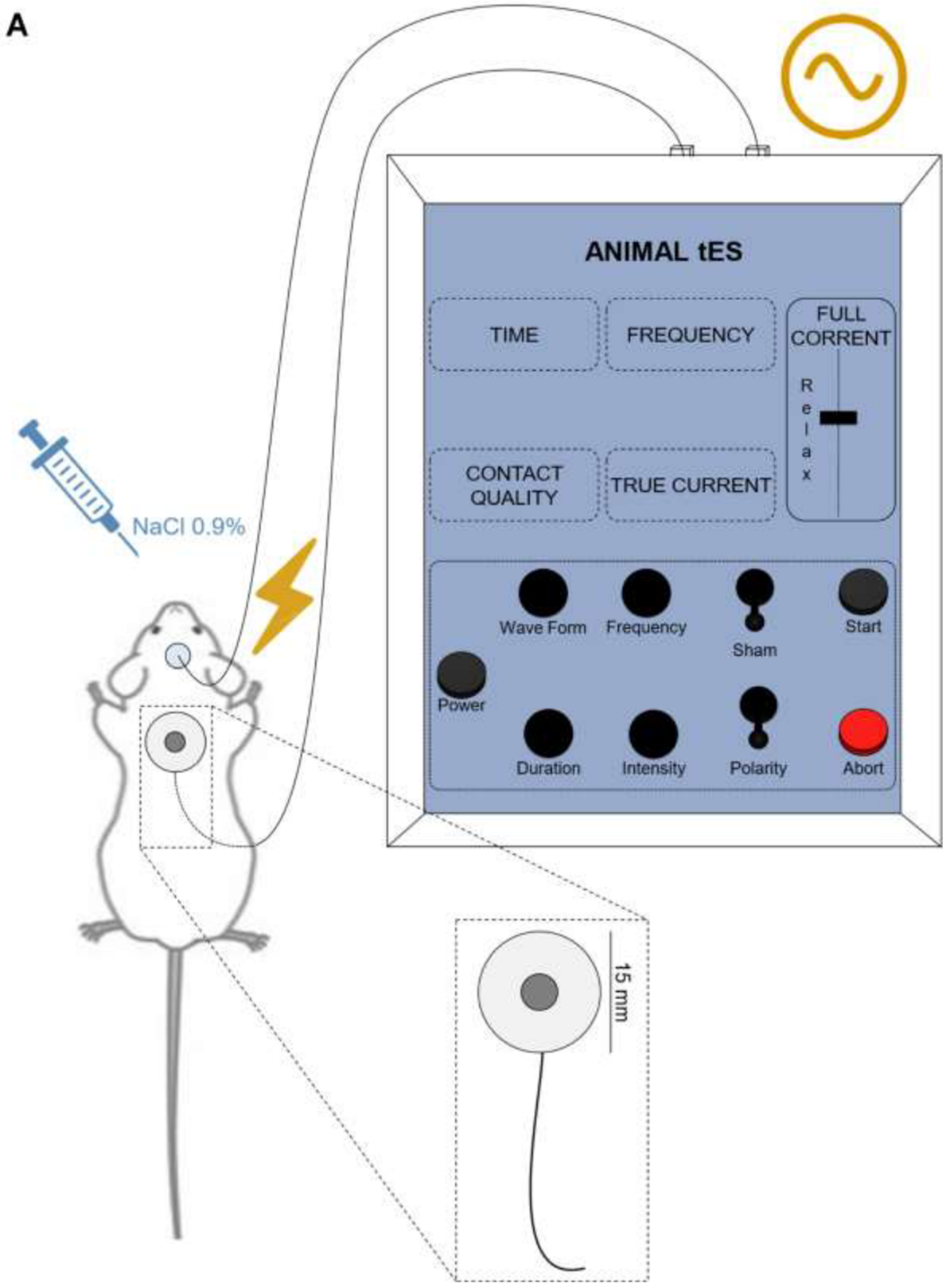

Figure 2. Animal tES and stimulation setup. (A) Device's scheme and stimulation setup with body electrode dimensions.

during the procedure (Isoforine®, 1%/L) with an oxygen continuous flow of 1L/min. The head implant was filled with saline solution 0.9% using a syringe for electrical conduction. The body electrode (electrocardiogram (ECG) electrode, nickel-plated

brass) was placed under the animal, in the thoracic region, and filled with saline solution 0.9%. The stimulation started after checking all parameters and certifying that the animal did not present reflexes and the contact quality was stable between 7 and 10.

The device set was: bipolar sinusoidal waveform; the current intensity of 35 mA; for 10 minutes. For the tACS groups, the SHAM mode was off and the frequencies applied were 8 Hz, 30 Hz, and 80 Hz, in the first experiment, and 80 Hz in the second experiment. For SHAM groups, the frequency set was chosen randomly and the SHAM mode was on. The stimulation has an ascent ramp of 30 seconds at the beginning and 30 seconds of a descent ramp at the end of the time. After, the animal was carefully transferred back to its home cage, heated at 37°C, and was monitored until the animal recovered from the sedation.

3.3.4. Barnes Maze

The Barnes Maze is a complex behavioral assessment of visuospatial learning and memory in rodents (BARNES, 1979). The apparatus was a circular platform with 90 cm of diameter and 100 cm elevated from the floor, with 19 false holes and one true hole - escape chamber (Stoelting Co.). The animals were habituated at the testing room for, at least, 30 minutes. The apparatus was cleaned with 70% alcohol before all trials. All stages were recorded on video for further analysis. Speakers were placed in the testing room for white noise. Visual cues were placed on the four cardinal points to assist in spatial location (**Figure 3**).

To avoid positioning bias, we used an opaque hollow rectangular prism made of acrylic to place the animals in the apparatus. This way they were randomly positioned before the test started.

This test has three different stages: habituation, training, and test. The habituation stage is one trial on the first day to introduce the apparatus to the animals. The mice were placed in the middle of the platform (inside the prism), and after 10 seconds, the prism was lifted, freeing the animal to explore for 120 seconds. To help it learn the task, we used a glass cylinder to help the animal locate it by itself. If after 60 seconds the animal still didn’t enter the escape chamber, we gently placed it inside the

chamber through the hole. Mice stayed in the chamber for 60 seconds and then returned to their home cage.

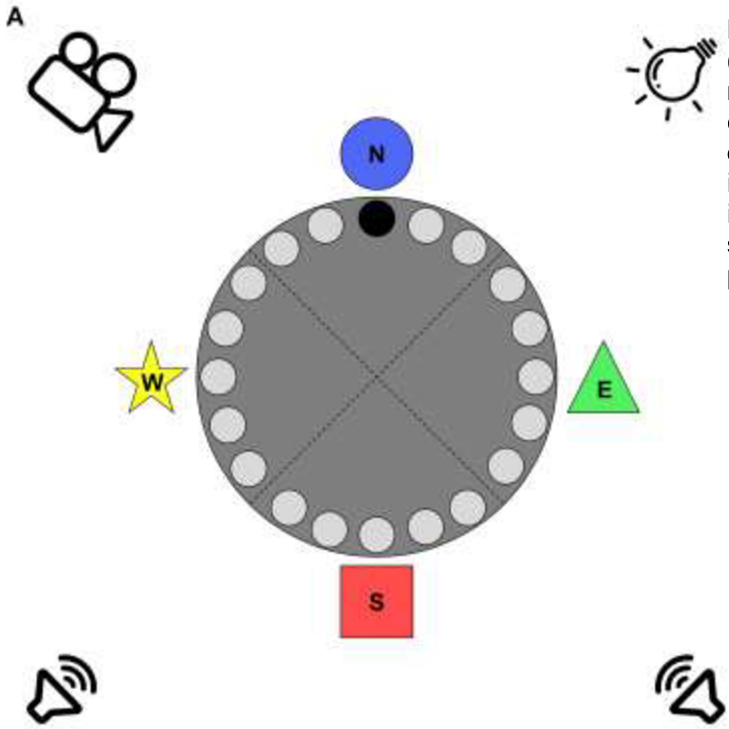

Figure 3. Barnes Maze room setup. (A) Banes Maze apparatus in the middle with cues around in the cardinal points. N: north; S: south; E: east; W: west. Camera image to illustrate video record; lap image to illustrade a lighting room and speakears to illustrde white noise playing.

The training stage is conducted for three days, with three trials each day, to learn the task. The mice were placed in the middle of the platform (inside the prism), and after 10 seconds, the prism was lifted, freeing the animal to explore for 180 seconds. We used a glass cylinder to help the animal locate it and enter the escape chamber by itself. If after 30 seconds the animal still didn't enter the escape chamber, we gently placed it inside the chamber through the hole. If the animal enters the chamber before the time ends, we go to the next step. Mice stayed in the chamber for 60 seconds and then returned to their home cage. Then, after three minutes, the animal returns for the next trial.

The fifth and last day of Barnes Maze was the test stage, to understand if the mice had retained the information on the location of the correct hole zone. Therefore, it's only one trial where the chamber was replaced for a false hole. The mice were placed in the middle of the platform (inside the prism), and after 10 seconds, the prism was lifted, freeing the animal to explore for 90 seconds, then, the animal returned to its home cage.

Parameters such as mean speed, max speed, distance traveled, primary latency, primary errors were analyzed by using automated video-tracking software (ANY-maze version 6.3, Stoelting). Adopted strategies were manually analyzed and

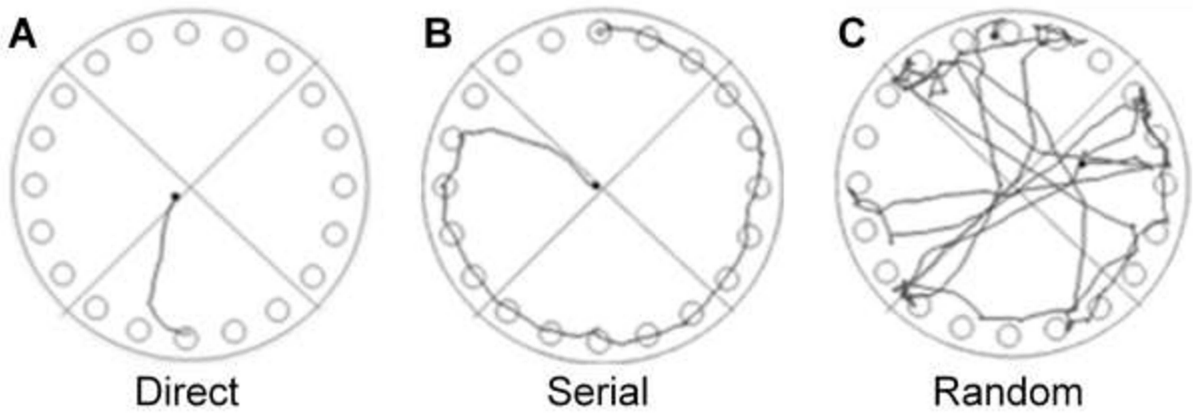

Figure 4. Strategies' types. Schematic view of Barnes Maze strategies analyzed: Direct **(A)**, Serial **(B)**, Random **(C).**

divided into three categories: random, serial, and direct. The first is a type of exploration without spatial location (randomly). In the serial strategy, the mice explore the hole in sequence but do not use visuospatial clues to find the correct direction. The direct strategy is a visuospatial response where the animal goes to the correct place and made until two primary errors (**Figure 4**)

3.3.5. Gene expression (RT-PCR)

The stimulated cortex was obtained by dissection of fresh unfixed tissue from animals euthanized by cervical dislocation 24 hours after the last tACS session. The tissues were homogenized and total RNA was extracted through the TRIzol™ (ThermoFisher) method. Then, the RNA was submitted to cDNA synthesis by High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). The gene expression was assessed through the CFX96 (Bio-Rad) equipment, using SsoAdvanced Universal

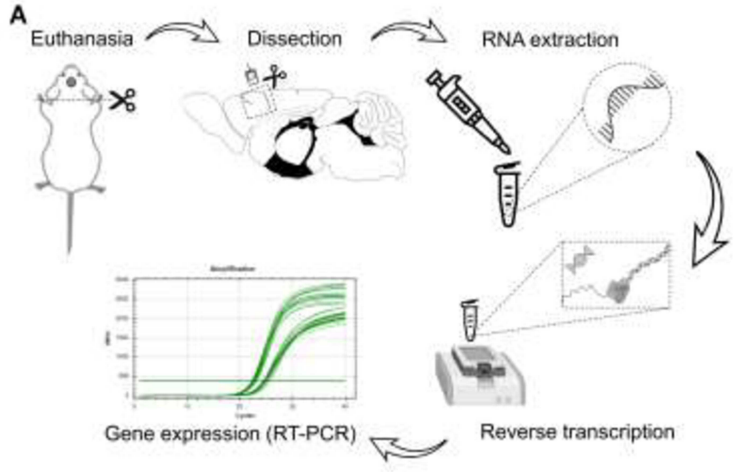

Figure 5. Gene Expression. (A) Step-by-step from euthanasia method to RT-PCR data collect.

SYBR Green Supermix (Bio-Rad) (**Figure 5**). Nine genes were selected and analyzed. Primers were synthesized by Integrated DNA Technologies (IDT) for the sequences below (**Table 1**). The relative gene expression was determined using the Livak method ($2^{-\Delta\Delta CT}$) with *RPL13A* housekeeping gene normalization.

Gene	Primers (forward - reverse)
ARC	5' TTGGTAAGTGCCGAGCTGAG 3' - 3' CGGTAGAAGACCTCCCTCCA 5'
CAMKIIa	5' AGCCCTAGTTCCCAGCCTAA 3' - 3' CCCCACCAGTAACCAGATCG 5'
CDK5	5' GGGACCTGTTGCAGAACCTAT 3' - 3' ACTGGGGTTCAGAGAGCCTA 5'
cFOS	5' TCTGTCCGTCTCTAGTGCCA 3' - 3' GATCTGTCTCCGCTTGGAGT 5'
GAD67	5' TACTCCTGTGACAGAGCCGA 3' - 3' TCATACGTTGTAGGGCGCAG 5'
GFAP	5' GGCGAAGAAAACCGCATCAC 3' - 3' ACACCTCACATCACCACGTC 5'
Gria1	5' AGTCTGCAGAACCGTCTGTG 3' - 3' GCTCAGAGCACTGGTCTTGT 5'
PSD95	5' AGCCCCAGGATATGTGAACG 3' - 3' ATGGAACCCGCCTCTTTGAG 5'
SYN1	5' CAGAAACCCAGCCAGGATGT 3' - 3' GGAGGGGCTGGCTTTGAG 5'
RPL13A	5' GAGGGGCAGGTTCTGGTATTG 3' - 3' GGGGTTGGTATTCATCCGCT 5'

Table 1. Used gene primers sequences

3.3.6. Statistics

Dataset normality was tested using the D'Agostino & Pearson normality test ($\alpha > 0.05$) or Shapiro–Wilk normality test ($\alpha < 0.05$) for small *n* values. All tests were two-tailed and had an $\alpha = 0.05$. For two-sample comparisons of a single variable, we used Unpaired Student's *t* test or Mann-Whitney test. For comparisons of three or more groups with a single variable, we used one-way ANOVA followed by Tukey's multiple-comparisons tests or Kruskal-Wallis's test followed by Dunn's multiple-comparisons tests. Two-way repeated measures ANOVA followed by Sidak's multiple-comparisons tests were applied for multiple comparisons between two groups. For multiple comparisons between three or more groups, we used Two-way ANOVA followed by Tukey's multiple-comparisons tests (for repeated measures or unpaired datasets).

Values were expressed with mean and standard error of mean (mean ± SEM). Asterisks (*) in the figures indicates the *P* values for the two-sample simple tests or multiple comparisons tests (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). Hash (#) was used for *P* values ANOVA tests (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$; #### $P < 0.0001$). All statistical analysis and graphs were performed using Prism version 7 (GraphPad Software).

3.4. Results

3.4.1. tACS did not alter gene expressions under sedation protocols

First, we designed a protocol to start the investigation with different frequencies ranges (**Figure 6A**). To confirm the current supply, we analyze the contact quality score provided from the tES device. All groups had a satisfying score and had no differences between groups (Interaction effect: $F_{(12, 52)}$=0,2294, p=0,9960; Days effect: $F_{(4, 52)}$=1,621, p=0,1829; Treatment effect: $F_{(3, 13)}$=1,7, p=0,2160; Subjects (matching) effects: $F_{(13, 52)}$=1,057, p=0,4156; two-way repeated-measures ANOVA. **Figure 6B**).

The next question was if tACS was capable of modifying molecular parameters. Then, we choose nine genes linked to cortex neuron functions. We saw a downregulation for *cFOS* gene under 8 Hz stimulation (*cFOS* (Fos Proto-Oncogene) - SHAM vs. 8 Hz, MD=0,5575±0,1163, 95%CI=0,273 to 0,842, $t_{(6)}$=4,796, p=0,0030; SHAM vs. 30 Hz, MD=0,1175±0,1607, 95%CI=-0,2756 to 0,5106, $t_{(6)}$=0,7313, p=0,4921; SHAM vs. 80 Hz, MD=0,115±0,2755, 95%CI=-0,5365 to 0,7665, $t_{(7)}$=0,4174, p=0,6889; unpaired Student's t test. Treatment effect, $F_{(3, 13)}$=2,232, p=0,1331; ordinary one-way ANOVA; **Figure 6F**)

We did not see differences in the others: *ARC* (Activity Regulated Cytoskeleton Associated Protein) (SHAM vs. 8 Hz, MD=0,335±0,1374, 95%CI=-0,01829 to 0,6883, $t_{(5)}$=2,438, p=0,5029; SHAM vs. 30 Hz, MD=-0,04±0,1202, 95%CI=-0,3489 to 0,2689, $t_{(5)}$=0,3328, p=0,1969; SHAM vs. 80Hz, MD=-0,06 ± 0,2267, 95%CI=-0,6147 to 0,4947, $t_{(6)}$=0,2647, p=0,8001; unpaired Student's t test. Treatment effect: $F_{(3, 12)}$= 2,54, p=0,1056; ordinary one-way ANOVA; **Figure 6C**), *CAMKIIa* (Calcium/Calmodulin Dependent Protein Kinase II Alpha) (SHAM vs. 8 Hz, MD=0,185±0,2003, 95%CI=-

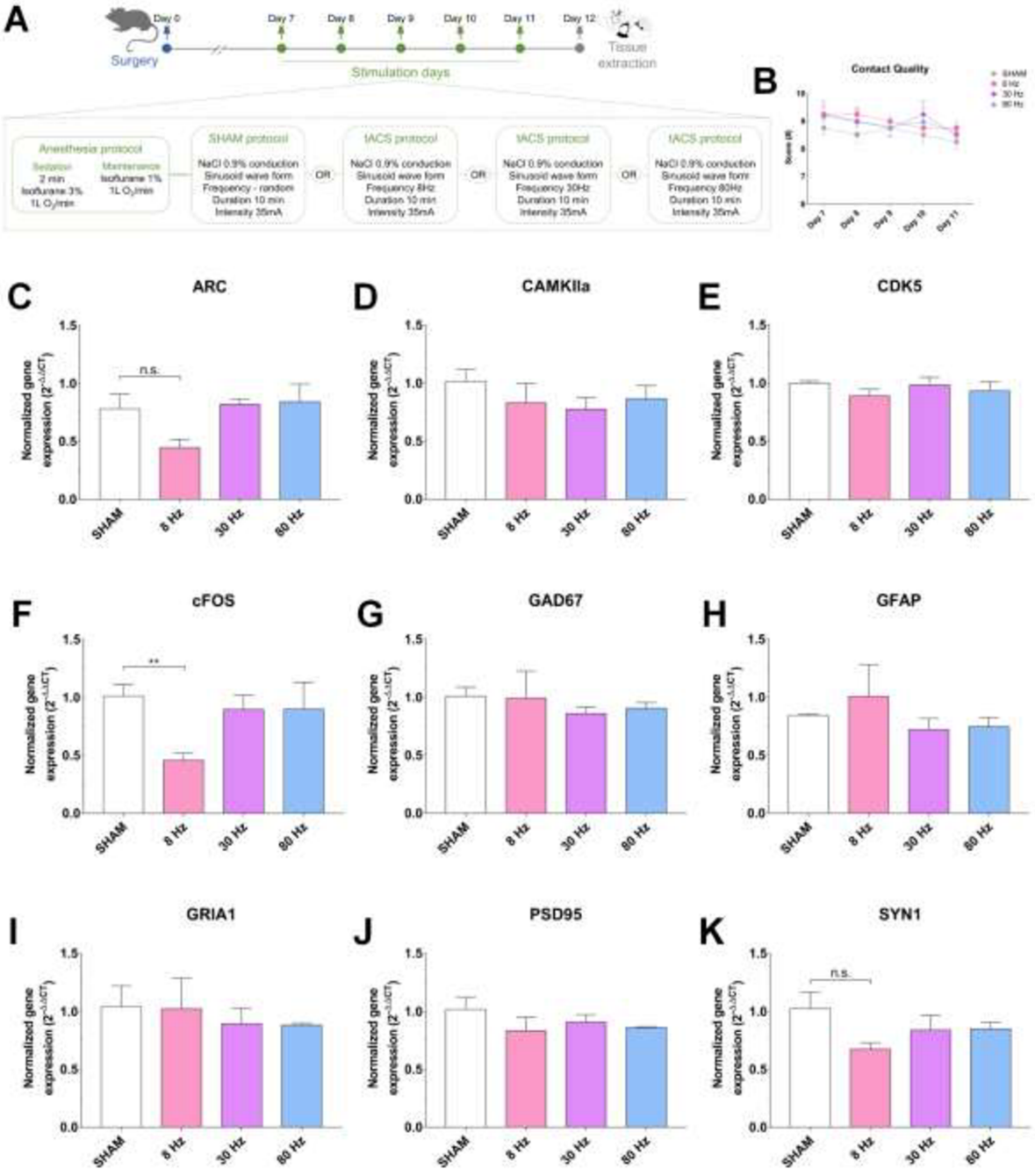

Figure 6. tACS did not alter gene expressions under sedation protocols. (A) Experimental design. **(B)** Contact quality. **(C, D, E, F, G, H, I, J and K)** Gene expression results. Data represented as mean ± S.E.M. (n = 4 SHAM, 4 8Hz; 4 30 Hz; 5 80 Hz). Simple comparison (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001). ANOVA (# P < 0.05; ## P < 0.01; ### P < 0.001; #### P < 0.0001).

0,3051 to 0,6751, $t_{(6)}$=0,9236, p=0,3913; SHAM vs. 30 Hz, MD=0,015±0,06946, 95%CI=-0,155 to 0,185, $t_{(6)}$=0,2159, p=0,8362; SHAM vs. 80 Hz, MD=0,066±0,08729, 95%CI=-0,1404 to 0,2724, $t_{(7)}$=0,7561, p=0,4742; unpaired Student's t test. Treatment effect: $F_{(3, 13)}$=0,6388, p=0,6033; ordinary one-way ANOVA; **Figure 6D**), *CDK5* (Cyclin Dependent Kinase 5) (SHAM vs. 8 Hz, Actual MedD=0,15, U=3,5, p=0,2571; SHAM

vs. 30 Hz, MD=0,015±0,06946, 95%CI=-0,155 to 0,185, $t_{(6)}$=0,2159, p=0,8362; SHAM vs. 80 Hz, MD=0,066±0,08729, 95%CI=-0,1404 to 0,2724, $t_{(7)}$=0,7561, p=0,4742; unpaired Student's t test or Mann-Whitney test. Treatment, *KW*=2,934, p=0,4270; Kruskal-Wallis test; **Figure 6E**), *GAD67* (Glutamate Decarboxylase 1), (SHAM vs. 8 Hz, Actual MedD=0,085, *U*=6,5, p=0,7429; SHAM vs. 30 Hz, Actual MedD=0,045, *U*=4,5, p=0,4000; SHAM vs. 80 Hz, Actual MedD=-0,005, *U*=8,5, p=0,7778; Mann-Whitney test. Treatment, *KW*=1,118, p=0,7987; Kruskal-Wallis test; **Figure 6G**), *GFAP* (Glial Fibrillary Acidic Protein) (SHAM vs. 8 Hz, Actual MedD=-0,075, *U*=6, p>0,9999; SHAM vs. 30 Hz, Actual MedD=0,085, *U*=6, p>0,9999; SHAM vs. 80 Hz, Actual MedD=0,03, *U*=5, p=0,5357; Mann-Whitney test. Treatment, *KW*=0,4919, p=0,9328; Kruskal-Wallis test; **Figure 6H**), *GRIA1* (Glutamate Ionotropic Receptor AMPA Type Subunit 1) (SHAM vs. 8 Hz, Actual MedD=0,165, *U*=6, p=0,6857; SHAM vs. 30 Hz, MD=0,1475±0,22, 95%CI=-0,3909 to 0,6859, $t_{(6)}$=0,6704, p=0,5276; SHAM vs. 80 Hz, MD=0,16±0,1775, 95%CI=-0,2744 to 0,5944, $t_{(6)}$=0,9013, p=0,4021; unpaired Student's t test or Mann-Whitney test. Treatment, *KW*=1,665, p=0,6784; Kruskal-Wallis test; **Figure 6I**), *PSD95* (Discs Large MAGUK Scaffold Protein 4) (SHAM vs. 8 Hz, Actual MedD=0,24, *U*=3, p=0,2000; SHAM vs. 30 Hz, Actual MedD=0, *U*=6, p=0,6286; SHAM vs. 80 Hz, MD=0,155±0,1067, 95%CI=-0,106 to 0,416, $t_{(6)}$=1,453, p=0,1964; unpaired Student's t test or Mann-Whitney test. Treatment, *KW*=3,207, p=0,3835; Kruskal-Wallis test; **Figure 6J**), *SYN1* (Synapsin I) (SHAM vs. 8 Hz, MD=0,3517±0,17, 95%CI=-0,08538 to 0,7887, $t_{(5)}$=2,068, p=0,0934; SHAM vs. 30 Hz, MD=0,185±0,1887, 95%CI=-0,2768 to 0,6468, $t_{(6)}$=0,9802, p=0,3649; SHAM vs. 80 Hz, MD=0,175±0,1367, 95%CI=-0,1483 to 0,4983, $t_{(7)}$=1,28, p=0,2413; unpaired Student's t test. Treatment effect: $F_{(3, 12)}$=1,699, p=0,2201; ordinary one-way ANOVA; **Figure 6K**).

3.4.2. tACS did not interfere in cognitive behavior

To understand if tACS was capable of modulating cognitive function, we chose the Barnes Maze test as a behavioral tool for this investigation. This test is a complex behavior test where we can evaluate mice's learning and memory skills (BARNES, 1979).

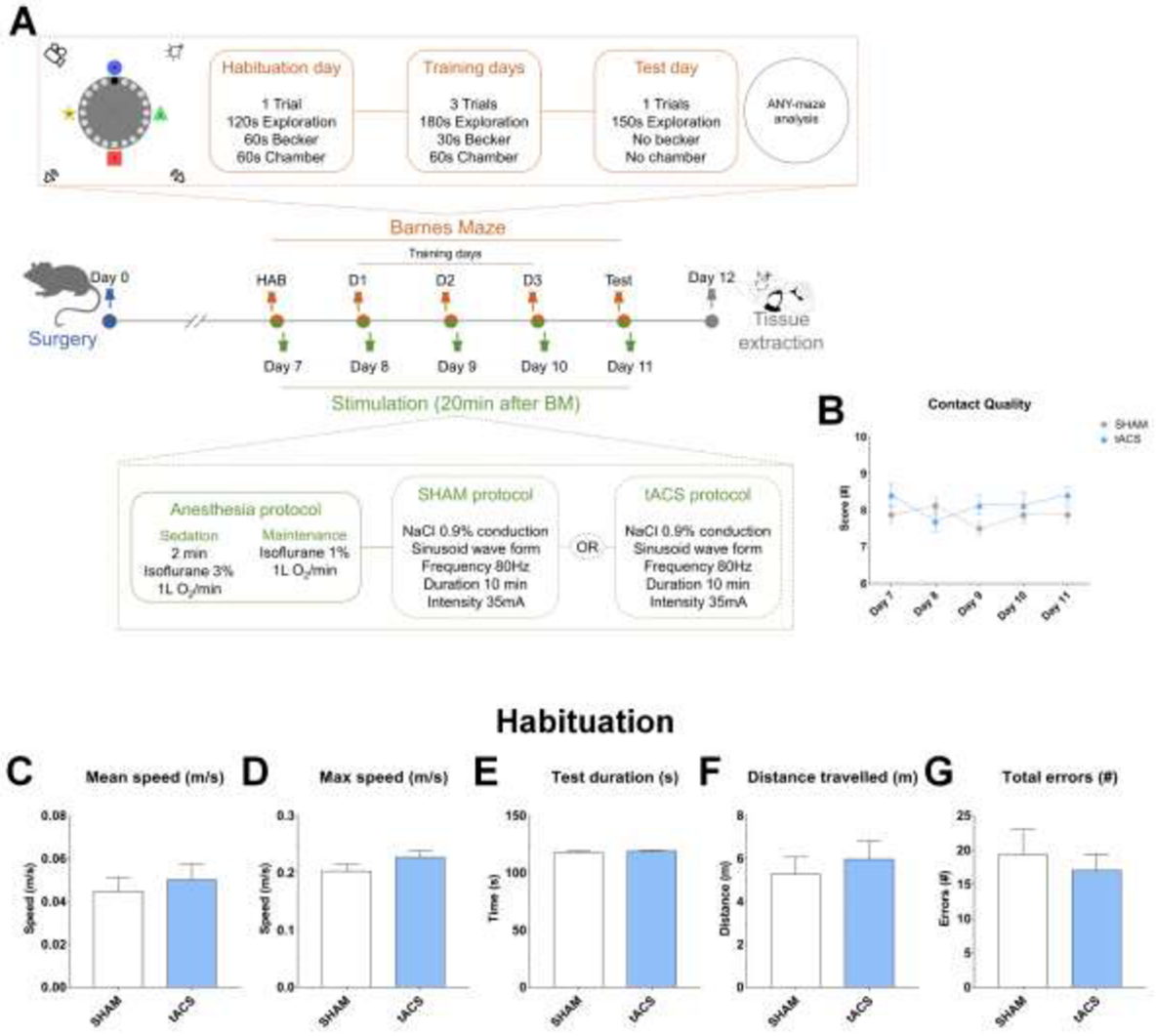

Figure 7. tACS did not interfere in cognitive behavior (part one). (A) Experimental design. **(B)** Contact quality. **(C, D, E, F and G)** Barnes Maze Test – Habituation results; mean speed **(C)**, max speed **(D)**, test duration **(E)**, distance travelled **(F)**, total errors **(G)**. Data represented as mean ± S.E.M. (n = 8 SHAM, 7 tACS). Simple comparison (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001). ANOVA (# P < 0.05; ## P < 0.01; ### P < 0.001; #### P < 0.0001).

For this, we organized an experimental design with the test's protocol and stimulation sections occuring in the same days (**Figura 7A**). We availed the contact quality, as we did in the first experiment, and it showed no differences (Interaction effect: $F_{(4,\ 52)}$=1,655, p=0,1747; Days effect: $F_{(4,\ 52)}$=0,7406, p=0,5686; Treatment effect: $F_{(1,\ 13)}$=3,106, p=0,1015; Subjects (matching) effect: $F_{(13,\ 52)}$=1,468, p=0,1618; two-way repeated-measures ANOVA; **Figure 7B**).

First, we characterized the habituation trial to show that the groups were in the same conditions. For this, parameters as mean speed (SHAM vs. tACS, MD=-0,005518±0,0098, 95%CI=-0,02669 to 0,01565, $t_{(13)}$=0,5631, p=0,5830; unpaired Student's t test; **Figure 7C**), max speed (SHAM vs. tACS, MD=-0,02425±0,0167, 95%CI=-0,06032 to 0,01182, $t_{(13)}$=1,453, p=0,1701; unpaired Student's t test; **Figure**

7D), test duration (SHAM vs. tACS, Actual MedD=0, *U*=25, *p*=0,8564; Mann-Whitney test; **Figure 7E**), distance travelled (SHAM vs. tACS, MD=-0,6923±1,181, 95%CI=-3,244 to 1,859, $t_{(13)}$=0,5861, *p*=0,5678; unpaired Student's t test; **Figure 7F**) and total errors (SHAM vs. tACS, MD=2,375±4,493, 95%CI=-7,332 to 12,08, $t_{(13)}$=0,5286, *p*=0,6060; unpaired Student's t test; **Figure 7G**) were analyzed. No differences were seen.

After, we analyzed training days for learning context. We didn't observe differences between groups in any parameters. The results show the learning process. Mean speed, max speed and primary errors (Interaction effect: $F_{(2, 26)}$=1,119, *p*=0,3419; Days effect: $F_{(2, 26)}$=2,545, *p*=0,0978; Treatment effect: $F_{(1, 13)}$=4,256e-005, *p*=0,9949; Subjects (matching): $F_{(13, 26)}$=2,225, *p*=0,0400; two-way repeated-measures ANOVA; **Figure 8A**), (Interaction effect: $F_{(2, 26)}$=0,9702, *p*=0,3923; Days effect: $F_{(2, 26)}$=0,7581, *p*=0,4786; Treatment effect: $F_{(1, 13)}$=0,0759, *p*=0,7873; Subjects (matching): $F_{(13, 26)}$=1,61, *p*=0,1460; two-way repeated-measures ANOVA; **Figure 8B**), (Interaction effect: $F_{(2, 26)}$=3,137, p=0,0602; Days effect: $F_{(2, 26)}$=2,411, *p*=0,1095, Treatment effect: $F_{(1, 13)}$=0,8023, *p*=0,3867; Subjects (matching): $F_{(13, 26)}$=2,155, *p*=0,0464; two-way repeated-measures ANOVA; **Figure 8E**) had no alteration.

Primary latency, primary distance, test duration, distance travelled, total errors, time in correct zone and escape zone visits had no differences (Interaction effect: $F_{(2, 26)}$=1,883, *p*=0,1723; Days effect: $F_{(2, 26)}$=12,66 , *p*=0,0001; Treatment effect: $F_{(1, 13)}$=0,1026, *p*=0,7538; Subjects (matching): $F_{(13, 26)}$=1,738, *p*=0,1116; two-way repeated-measures ANOVA; **Figure 8C**), (Interaction effect: $F_{(2, 26)}$=1,334, *p*=0,2809; Days effect: $F_{(2, 26)}$=8,404 , *p*=0,0015; Treatment effect: $F_{(1, 13)}$=0,1219, *p*=0,7325; Subjects (matching): $F_{(13, 26)}$=1,851, *p*=0,0880; two-way repeated-measures ANOVA; **Figure 8D**), (Interaction effect: $F_{(2, 26)}$=3,197, *p*=0,0574; Days effect: $F_{(2, 26)}$=57,63, *p*<0,0001; Treatment effect: $F_{(1, 13)}$=2,221, *p*=0,1600; Subjects (matching): $F_{(13, 26)}$=5,252, *p*=0,0002; two-way repeated-measures ANOVA; **Figure 8F**), (Interaction effect: $F_{(2, 26)}$=1,713, *p*=0,2001; Days effect: $F_{(2, 26)}$=35,15, *p*<0,0001; Treatment effect: $F_{(1, 13)}$=2,568, *p*=0,1331; Subjects (matching): $F_{(13, 26)}$=3,03, *p*=0,0078; two-way repeated-measures ANOVA; **Figure 8G**), (Interaction effect: $F_{(2, 26)}$=2,826, *p*=0,0776; Days effect: $F_{(2, 26)}$=15,4, *p*<0,0001; Treatment effect: $F_{(1, 13)}$=4,556, *p*=0,0524; Subjects (matching): $F_{(13, 26)}$=2,482, *p*=0,0235; two-way repeated-measures ANOVA; **Figure 8H**), (Interaction effect: $F_{(2, 26)}$=3,205, *p*=0,0570; Days effect: $F_{(2, 26)}$=18,85, *p*<0,0001; Treatment effect: $F_{(1, 13)}$=1,986, *p*=0,1822; Subjects (matching): $F_{(13,}$

$_{26)}$=4,696, p=0,0004; two-way repeated-measures ANOVA; **Figure 8I**), (Interaction effect: $F_{(2, 26)}$=1,281, p=0,2948; Days effect: $F_{(2, 26)}$=4,903, p=0,0156; Treatment effect: $F_{(1, 13)}$=2,13, p=0,1682; Subjects (matching): $F_{(13, 26)}$=3,831, p=0,0017; two-way

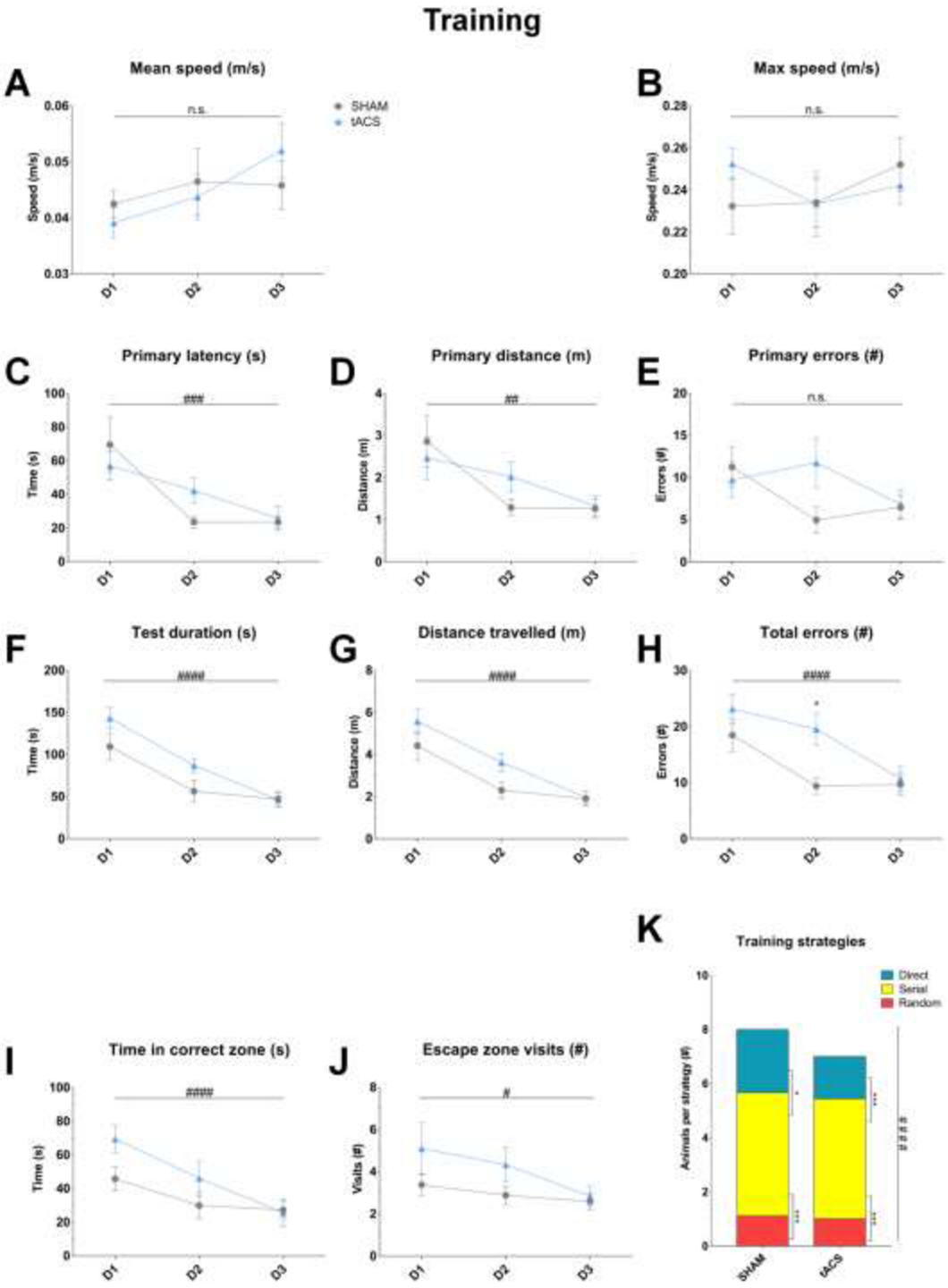

Figure 8. tACS did not interfere in cognitive behavior (part two). (A, B, C, D, E, F, G, H, I and J) Barnes Maze Test – Training results; mean speed **(A)**, max speed **(B)**, primary latency **(C)**, primary distance **(D)**, primary errors **(E)** test duration **(F)**, distance travelled **(G)**, total errors **(H)**, time in correct zone **(I)** and escape zone visits **(J). (K)** Strategies used in training days. Data represented as mean ± S.E.M. (n = 8 SHAM, 7 tACS). Simple comparison (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001). ANOVA (# P < 0.05; ## P < 0.01; ### P < 0.001; #### P < 0.0001).

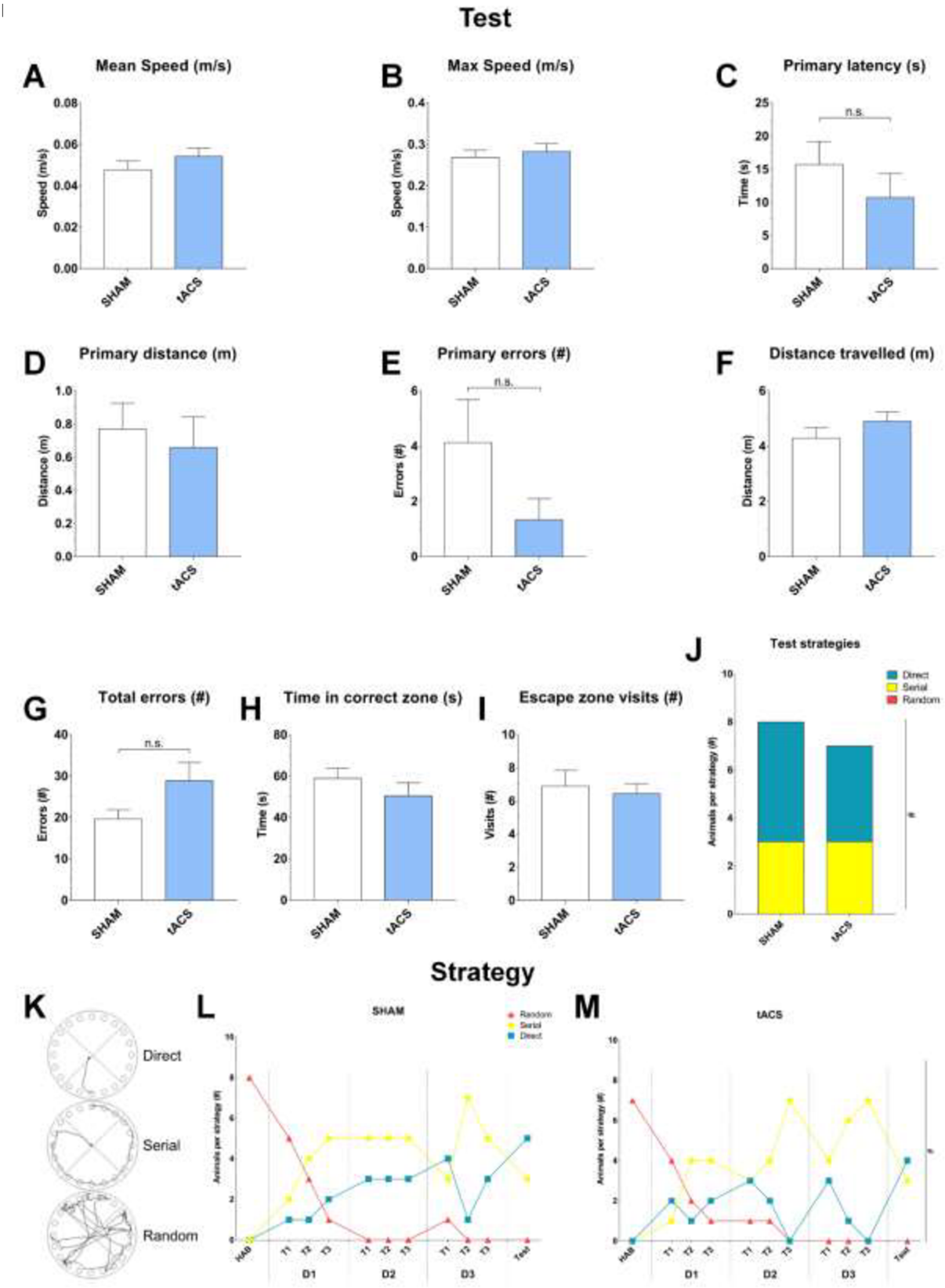

Figure 9. tACS did not interfere in cognitive behavior (part three). (A, B, C, D, E, F, G, H and I) Barnes Maze Test – Test results; mean speed **(A)**, max speed **(B)**, primary latency **(C)**, primary distance **(D)**, primary errors **(E)**, distance travelled **(F)**, total errors **(G)**, time in correct zone **(H)** and escape zone visits **(I). (J)** Strategies used in test day. **(K, L and M)** Strategy results; Types of strategies **(K)**, SHAM strategies results per trial **(L)**, tACS strategies results per trial **(L).** Data represented as mean ± S.E.M. (n = 8 SHAM, 7 tACS). Simple comparison (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001). ANOVA (# P < 0.05; ## P < 0.01; ### P < 0.001; #### P < 0.0001).

with no differences between groups.

We also analyzed the strategies used to understand how the mice learned the task. Results showed an increased use of serial and direct strategies in both groups over the random strategy. No differences were observed between groups (Interaction effect: $F_{(2, 48)}$=0,3032, p=0,7399; Treatment effect: $F_{(1, 48)}$=0,6821, p=0,4129; Strategy effect: $F_{(2, 48)}$=26,17, p<0,0001; ordinary two-way ANOVA; **Figure 8K**).

Finally, we analyzed the test trial. On this day of the protocol, we evaluate memory acquisition. We didn't see differences between groups on parameters such as mean speed, max speed, primary latency, primary distance, primary errors, distance travelled, total errors, time incorrect zone and escape zone visits (SHAM vs. tACS, MD=-0,006661±0,00578, 95%CI=-0,01915 to 0,005826, $t_{(13)}$=1,152, p=0,2699; unpaired Student's t test; **Figure 9A**), (SHAM vs. tACS, MD=-0,0142±0,02599, 95%CI=-0,07035 to 0,04196, $t_{(13)}$=0,5462, p=0,5942; unpaired Student's t test; **Figure 9B**), (SHAM vs. tACS, Actual MedD=2,9, U=19, p=0,3357; Mann-Whitney test; **Figure 9C**), (SHAM vs. tACS, Actual MedD=0,16, U=25, p=0,7789; Mann-Whitney test; **Figure 9D**), (SHAM vs. tACS, Actual MedD=1,5, U=14,5, p=0,2324; Mann-Whitney test, **Figure 9E**), (SHAM vs. tACS, MD=-0,6021±0,5181, 95%CI=-1,721 to 0,5171, $t_{(13)}$=1,162, p=0,2661; unpaired Student's t test; **Figure 9F**), (SHAM vs. tACS, MD=-9,232±4,745, 95%CI=-19,48 to 1,019, $t_{(13)}$=1,946, p=0,0736; unpaired Student's t test; **Figure 9G**), (SHAM vs. tACS, MD=8,47±7,881, 95%CI=-8,556 to 25,5, $t_{(13)}$=1,075, p=0,3021; unpaired Student's t test; **Figure 9H**), (SHAM vs. tACS, Actual MedD=-1, U=24, p=0,6684; Mann-Whitney test; **Figure 9I**). For strategies used on test trial, we saw more animals using direct strategy over serial and no mice using random strategy in both groups (Treatment effect: $F_{(1, 2)}$=1, p=0,4226; Strategy effect: $F_{(2, 2)}$=63, p=0,0156; ordinary two-way ANOVA; **Figure 9J**).

We also analyzed the strategies within the groups for a better comprehension of how the mice used different strategies (**Figure 9K**) in all apparatus' visits. In both groups, there was a decreasing trend of the random strategy until none of the animals were using it in the last trials. For direct and serial strategies, there was an increase of the number of animals per strategy. This showed that the mice were capable of learning the task and improved its performance, independent of the treatment. SHAM (Trial effect: $F_{(10, 20)}$=0, p>0,9999; Strategy effect: $F_{(2, 20)}$=2,498, p=0,1076; ordinary two-way ANOVA; **Figure 9L**), tACS (Trial effect: $F_{(10, 20)}$=0, p>0,9999; Strategy effect: $F_{(2, 20)}$=3,542, p=0,0482; ordinary two-way ANOVA; **Figure 9M**).

3.4.3. tACS didn't interfere with gene expression in mice after the Barnes Maze task

Our last question was if the stimulation maintained with no alterations on gene expression even after a behavioral trial. Then, the results showed no differences in gene expression, even after five days of behavior test (**Figure 10A)**. *ARC* (SHAM vs. tACS, MD=-0,045±0,2203, 95%CI=-0,5839 to 0,4939, $t_{(6)}$=0,2043, p=0,8449; unpaired Student's t test), *CAMKAIIa* (SHAM vs. tACS, MD=-0,1±0,2472, 95%CI=-0,705 to 0,505, $t_{(6)}$=0,4045, p=0,6999; unpaired Student's t test), *CDK5* (SHAM vs. tACS, MD=-0,035±0,131, 95%CI=-0,3556 to 0,2856, $t_{(6)}$=0,2672, p=0,7983; unpaired Student's t test), *cFOS* (SHAM vs. tACS, MD=-0,01±0,5879, 95%CI=-1,521 to 1,501, $t_{(5)}$=0,01701, p=0,9871; unpaired Student's t test), *GAD67* (SHAM vs. tACS, Actual MedD=-0,125, U=4, p=0,3143; Mann-Whitney test), *GFAP* (SHAM vs. tACS, MD=-0,2275±0,3122, 95%CI=-0,9914 to 0,5364, $t_{(6)}$=0,7287, p=0,4936; unpaired Student's t test), *GRIA* (SHAM vs. tACS, MD=-0,2775±0,2768, 95%CI=-0,9549 to 0,3999, $t_{(6)}$=1,002, p=0,3548; unpaired Student's t test), *PSD95* (SHAM vs. tACS, MD=-0,0825±0,113, 95%CI=-0,359 to 0,194, $t_{(6)}$=0,7302, p=0,4928; unpaired Student's t test), *SYN* (SHAM vs. tACS, MD=-0,14±0,2514, 95%CI=-0,7552 to 0,4752, $t_{(6)}$=0,5568, p=0,5978; unpaired Student's t test)

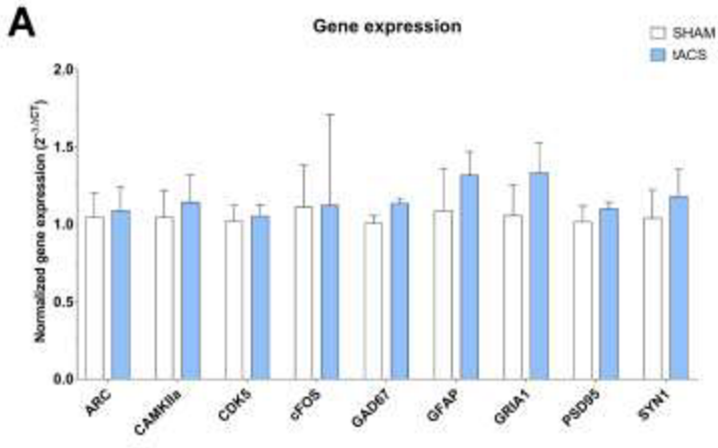

Figure 10. tACS didn't interfere with gene expression in mice after the Barnes Maze task. (A) Gene expression results. Data represented as mean ± S.E.M. (n = 4 SHAM, 4 8Hz; 4 30 Hz; 5 80 Hz). Simple comparison (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001). ANOVA (# P < 0.05; ## P < 0.01; ### P < 0.001; #### P < 0.0001).

Transcranial alternating current stimulation didn't interfere with gene expression, learning, and memory parameters analyzed in this paper. Although, the protocol applied had some limitations, such as the offline characteristic in the task paired experiment and the sedation step on both.

3.5. Discussion

Transcranial alternating current stimulation is a valid tool to aid in the treatment of psychiatric disorders. However, more studies are needed to elucidate their mechanisms of action and standardize their applications. Furthermore, studies in animal models are even more necessary, as some investigation tools and techniques can only be applied in animals and there are hardly any animal studies in this field. (HUANG et al., 2021), (ALI, SELLERS, FRÖHLICH, 2013), (SCHMIDT et al., 2014).

With this, our work comes to add information regarding protocols for application in rodents. Initially, we propose the use of three different stimulation protocols in sedated mice. After application, we performed the expression of genes that could be altered by stimulation. We observed that only the *cFOS* gene presented a downregulation in the 8 Hz group compared to the SHAM group. For the *cFOS* results, we suggest that the low frequency in tACS has an inhibitory effect, such as low frequency on tMS, (FERNANDEZ et al., 2018), (VOINESKOS et al., 2019), but none of the other genes show differences (AZEVEDO et al., 2020), (SHIN et al., 2017), (LI et al., 2017)

From this, we chose the 80 Hz protocol for a behavioral investigation, due to associations between high frequencies and cognitive processes (KAHANA, 2006), (HERRMANN, RACH, NEULING, STRÜBER, 2013), (KUCEWICZ et al., 2014), (JONES et al., 2017), (YU et al., 2018). The behavioral test chosen was the Barnes maze test, as it has a high cognitive complexity in addition to enabling motor assessment of the animals tested (O'LEARY, BROWN, 2012), (ILLOUZ et al., 2016), (GAWEL et al., 2019).

To show that both groups came from the same cognitive and motor background, we analyzed the data obtained in the habituation phase of the behavioral test. We then proceeded with the analysis of training days, where we observed differences only between days and not between groups. This result indicates that regardless of the treatment applied to the animals, in both groups, the task was understood and carried out. Even though the Barnes maze test is a highly complex task, only individuals with cognitive impairment cannot perform the task correctly (GAWEL et al., 2019). These results suggest that, although the stimulation did not improve their cognitive function and enhanced their performance, it did not cause any harm or negative effect.

In addition to the quantitative parameters, we qualitatively assess the strategy used by the animals to complete the task. Thus, we categorize three strategies: random, serial, and direct. In random analysis, the animal randomly visits false escape outlets, that is, it does not present a visuospatial strategy to locate and seek the escape outlet. This strategy is common in the first trials of the test, as the animals are still adapting to the task (O'LEARY, BROWN, 2012), (ILLOUZ et al., 2016), (PITTS, 2018), (HERREWEGEN et al., 2019).

The serial strategy is the animal choosing a direction followed by visits of false escape exits in sequence until finding the true exit. This strategy starts to appear as soon as the animals realize that there is a faster way to get back to their home cage, but they haven't acquired enough cognitive tools to carry out the direct strategy (PITTS, 2018).

The last strategy analyzed was direct. It consists of a complex visuospatial understanding of the apparatus and the use of visual cues arranged at the four cardinal points so that the animal can move towards the correct quadrant, making at most two primary errors (PITTS, 2018). Due to the high complexity of the direct strategy, it is not expected that all animals will be able to perform it over the days, however, as we can show, the last trials had mostly animals performing the direct and serial strategies and discarding the random strategy (PATIL et al., 2009), (ROSENFELD, FERGUSON, 2014), (ILLOUZ et al., 2016).

Finally, our last data was the evaluation of the gene expression of the stimulated cortex of the animals that went through the Barnes maze. We analyzed the expression for the same nine genes from the first experiment and we also did not observe changes in gene expression in these animals.

Considering the characteristics of our protocols, our data corroborate associations seen in the literature where offline tACS does not present neuromodulation traces, especially in short protocols, unlike other techniques, such as tDCS or tMS (HUANG et al., 2021), (ALI, SELLERS, FRÖHLICH, 2013), (SCHMIDT et al., 2014).

In addition, our experiments were carried out in animals under the effect of the sedative isoflurane and several studies show that sedatives can alter the expression of genes, especially of immediate early genes like most of the ones we tested (HAMAYA et al., 2000), (KÁDÁR et al., 2011), (LIU et al., 2014), (ZHONG et al., 2015),

(SMITH et al., 2016), (TANUSREE SEN, NILKANTHA SEN, 2016), (FRIESE et al., 2018).

Together, our results indicate that tACS is a safe technique, as it did not cause any negative effect in any protocol tested. However, we understand the limitations of our work and suggest, above all, more preclinical studies.

3.6. Conclusion

In this work, we seek to investigate the role of transcranial alternating current stimulation in the brain and the behavior of mice. We used a broader approach initially, seeking to evaluate more options for stimulation protocols. In addition, we use molecular and behavioral assessments.

Our results did not demonstrate positive or negative influence of tACS. However, considering that this technique is still very recent and little evaluated in preclinical studies, we can say that our data added to the literature.

Considering our limitations and future perspectives for the use of transcranial stimulation by alternating current, we suggest further study, especially with animal models. Furthermore, studies involving encephalography readings concomitant with tACS protocols in animals awake and during tasks performance will be essential to add to the literature, especially in cases of translational research.

REFERÊNCIAS

AZEVEDO, H.; FERREIRA, M.; MASCARELLO, A.; OSTEN, P. *et al.* Brain-wide mapping of c-fos expression in the single prolonged stress model and the effects of pretreatment with ACH-000029 or prazosin. **Neurobiology of Stress**, 13, p. 100226, 2020/11/01/ 2020.

BARNES, C. A. Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. **J Comp Physiol Psychol**, 93, n. 1, p. 74-104, Feb 1979.

DE SOUZA NICOLAU, E.; DE ALVARENGA, K. A. F.; TENZA-FERRER, H.; NOGUEIRA, M. C. A. *et al.* Transcranial Direct Current Stimulation (tDCS) in Mice. **J Vis Exp**, n. 139, Sep 23 2018.

DI BIASE, L.; FALATO, E.; DI LAZZARO, V. Transcranial Focused Ultrasound (tFUS) and Transcranial Unfocused Ultrasound (tUS) Neuromodulation: From Theoretical Principles to Stimulation Practices. **Frontiers in Neurology**, 10, p. 549, 2019. 10.3389/fneur.2019.00549.

ELYAMANY, O.; LEICHT, G.; HERRMANN, C. S.; MULERT, C. Transcranial alternating current stimulation (tACS): from basic mechanisms towards first applications in psychiatry. **European Archives of Psychiatry and Clinical Neuroscience**, 271, n. 1, p. 135-156, 2021/02/01 2021.

FERNANDEZ, L.; MAJOR, B. P.; TEO, W.-P.; BYRNE, L. K. *et al.* Assessing cerebellar brain inhibition (CBI) via transcranial magnetic stimulation (TMS): A systematic review. **Neuroscience & Biobehavioral Reviews**, 86, p. 176-206, 2018/03/01/ 2018.

FRIESE, M. B.; NATHAN, M.; CULLEY, D. J.; CROSBY, G. Isoflurane anesthesia impairs the expression of immune neuromodulators in the hippocampus of aged mice. **PLOS ONE**, 13, n. 12, p. e0209283, 2018.

GAWEL, K.; GIBULA, E.; MARSZALEK-GRABSKA, M.; FILAROWSKA, J. *et al.* Assessment of spatial learning and memory in the Barnes maze task in rodents—methodological consideration. **Naunyn-Schmiedeberg's Archives of Pharmacology**, 392, n. 1, p. 1-18, 2019/01/01 2019.

HALLETT, M. Transcranial Magnetic Stimulation: A Primer. **Neuron**, 55, n. 2, p. 187-199, 2007/07/19/ 2007.

HAMAYA, Y.; TAKEDA, T.; DOHI, S.; NAKASHIMA, S. *et al.* The Effects of Pentobarbital, Isoflurane, and Propofol on Immediate-Early Gene Expression in the Vital Organs of the Rat. **Anesthesia & Analgesia**, 90, n. 5, 2000.

HERRMANN, C.; RACH, S.; NEULING, T.; STRÜBER, D. Transcranial alternating current stimulation: a review of the underlying mechanisms and modulation of cognitive processes. **Frontiers in Human Neuroscience**, 7, p. 279, 2013. 10.3389/fnhum.2013.00279.

HUANG, W. A.; STITT, I. M.; NEGAHBANI, E.; PASSEY, D. J. *et al.* Transcranial alternating current stimulation entrains alpha oscillations by preferential phase synchronization of fast-spiking cortical neurons to stimulation waveform. **Nature Communications**, 12, n. 1, p. 3151, 2021/05/25 2021.

ILLOUZ, T.; MADAR, R.; CLAGUE, C.; GRIFFIOEN, K. J. *et al.* Unbiased classification of spatial strategies in the Barnes maze. **Bioinformatics**, 32, n. 21, p. 3314-3320, 2016.

JAFARI, Z.; KOLB, B. E.; MOHAJERANI, M. H. Neural oscillations and brain stimulation in Alzheimer's disease. **Progress in Neurobiology**, 194, p. 101878, 2020/11/01/ 2020.

JONES, N. C.; HUDSON, M.; FOREMAN, J.; RIND, G. *et al.* Brain-derived neurotrophic factor haploinsufficiency impairs high-frequency cortical oscillations in mice. **Eur J Neurosci**, 48, n. 8, p. 2816-2825, Oct 2018.

KAHANA, M. J. The cognitive correlates of human brain oscillations. **J Neurosci**, 26, n. 6, p. 1669-1672, Feb 8 2006.

KUCEWICZ, M. T.; CIMBALNIK, J.; MATSUMOTO, J. Y.; BRINKMANN, B. H. *et al.* High frequency oscillations are associated with cognitive processing in human recognition memory. **Brain**, 137, n. 8, p. 2231-2244, 2014.

LI, B.; JIA, S.; YUE, T.; YANG, L. *et al.* Biphasic Regulation of Caveolin-1 Gene Expression by Fluoxetine in Astrocytes: Opposite Effects of PI3K/AKT and MAPK/ERK Signaling Pathways on c-fos. **Frontiers in Cellular Neuroscience**, 11, n. 335, 2017-October-31 2017. Original Research.

LIU, J.; WANG, P.; ZHANG, X.; ZHANG, W. *et al.* Effects of different concentration and duration time of isoflurane on acute and long-term neurocognitive function of young adult C57BL/6 mouse. **International journal of clinical and experimental pathology**, 7, n. 9, p. 5828-5836, 2014.

MAIXNER, D. F.; WEINER, R.; RETI, I. M.; HERMIDA, A. P. *et al.* Electroconvulsive Therapy Is an Essential Procedure. **Am J Psychiatry**, 178, n. 5, p. 381-382, May 1 2021.

O'LEARY, T. P.; BROWN, R. E. The effects of apparatus design and test procedure on learning and memory performance of C57BL/6J mice on the Barnes maze. **Journal of Neuroscience Methods**, 203, n. 2, p. 315-324, 2012/01/30/ 2012.

PATIL, S. S.; SUNYER, B.; HÖGER, H.; LUBEC, G. Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze. **Behavioural Brain Research**, 198, n. 1, p. 58-68, 2009/03/02/ 2009.

PELLETIER, S. J.; CICCHETTI, F. Cellular and Molecular Mechanisms of Action of Transcranial Direct Current Stimulation: Evidence from In Vitro and In Vivo Models. **International Journal of Neuropsychopharmacology**, 18, n. 2, 2015.

PITTS, M. W. Barnes Maze Procedure for Spatial Learning and Memory in Mice. **Bio-protocol**, 8, n. 5, p. e2744, 2018.

POLANÍA, R.; NITSCHE, M. A.; RUFF, C. C. Studying and modifying brain function with non-invasive brain stimulation. **Nat Neurosci**, 21, n. 2, p. 174-187, Feb 2018.

ROSENFELD, C. S.; FERGUSON, S. A. Barnes Maze Testing Strategies with Small and Large Rodent Models. **JoVE**, n. 84, p. e51194, 2014/02/26/ 2014.

SCHMIDT, S. L.; IYENGAR, A. K.; FOULSER, A. A.; BOYLE, M. R. *et al.* Endogenous Cortical Oscillations Constrain Neuromodulation by Weak Electric Fields. **Brain Stimulation**, 7, n. 6, p. 878-889, 2014/11/01/ 2014.

SEN, T.; SEN, N. Isoflurane-induced inactivation of CREB through histone deacetylase 4 is responsible for cognitive impairment in developing brain. **Neurobiology of Disease**, 96, p. 12-21, 2016/12/01/ 2016.

SMITH, M. L.; LI, J.; COTE, D. M.; RYABININ, A. E. Effects of isoflurane and ethanol administration on c-Fos immunoreactivity in mice. **Neuroscience**, 316, p. 337-343, 2016/03/01/ 2016.

SU, Y.; SHIN, J.; ZHONG, C.; WANG, S. *et al.* Neuronal activity modifies the chromatin accessibility landscape in the adult brain. **Nature Neuroscience**, 20, n. 3, p. 476-483, 2017/03/01 2017.

TAVAKOLI, A. V.; YUN, K. Transcranial Alternating Current Stimulation (tACS) Mechanisms and Protocols. **Front Cell Neurosci**, 11, p. 214, 2017.

VAN DEN HERREWEGEN, Y.; DENEWET, L.; BUCKINX, A.; ALBERTINI, G. *et al.* The Barnes Maze Task Reveals Specific Impairment of Spatial Learning Strategy in the Intrahippocampal Kainic Acid Model for Temporal Lobe Epilepsy. **Neurochemical Research**, 44, n. 3, p. 600-608, 2019/03/01 2019.

VANNESTE, S.; FREGNI, F.; DE RIDDER, D. Head-to-Head Comparison of Transcranial Random Noise Stimulation, Transcranial AC Stimulation, and Transcranial DC Stimulation for Tinnitus. **Frontiers in Psychiatry**, 4, p. 158, 2013. 10.3389/fpsyt.2013.00158.

VOINESKOS, D.; BLUMBERGER, D. M.; ZOMORRODI, R.; ROGASCH, N. C. *et al.* Altered Transcranial Magnetic Stimulation–Electroencephalographic Markers of Inhibition and Excitation in the Dorsolateral Prefrontal Cortex in Major Depressive Disorder. **Biological Psychiatry**, 85, n. 6, p. 477-486, 2019/03/15/ 2019.

VOSSKUHL, J.; STRÜBER, D.; HERRMANN, C. S. Non-invasive Brain Stimulation: A Paradigm Shift in Understanding Brain Oscillations. **Frontiers in Human Neuroscience**, 12, p. 211, 2018. 10.3389/fnhum.2018.00211.

YU, C.-C.; WANG, Y.; SHEN, F.; KONG, L.-H. *et al.* High-frequency (50 Hz) electroacupuncture ameliorates cognitive impairment in rats with amyloid beta 1-42-

induced Alzheimer's disease. **Neural regeneration research**, 13, n. 10, p. 1833-1841, 2018.

ZHONG, T.; GUO, Q.; ZOU, W.; ZHU, X. *et al.* Neonatal Isoflurane Exposure Induces Neurocognitive Impairment and Abnormal Hippocampal Histone Acetylation in Mice. **PLOS ONE**, 10, n. 4, p. e0125815, 2015.