FEDERAL UNIVERSITY OF MINAS GERAIS

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Ana Caroline Nogueira Souza

# EVALUATION OF THE EFFECTS OF PhKv TOXIN, ISOLATED FROM THE *Phoneutria nigriventer* VENOM, ON MEMORY, IN MICE DEFICIENT FOR THE VESICULAR ACETYLCHOLINE TRANSPORTER

Avaliação dos efeitos da toxina PhKv, isolada do veneno de *Phoneutria nigriventer*, na memória, em camundongos deficientes do transportador vesicular de acetilcolina

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**Final version**

Dissertation presented to the Postgraduate Program in Molecular Medicine, at the Federal University of Minas Gerais (UFMG) School of Medicine, as partial fulfillment of the requirements for a master's degree.

Advisor: Prof. Marcus Vinícius Gomez, PhD

Co-advisor: Hèlia Tenza Ferrer, PhD

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## FOLHA DE APROVAÇÃO

**Avaliação dos efeitos da toxina PhKv, isolada do veneno de *Phoneutria nigriventer*, na memória, em camundongos deficientes do transportador vesicular de acetilcolina**

**ANA CAROLINE NOGUEIRA SOUZA**

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## ABSTRACT

Acetylcholine (ACh) is an essential neurotransmitter for cognition. It modulates circuits related to attention, cognitive flexibility, memory, and social interaction, among other functions. Low ACh levels in cholinergic synaptic clefts result in several issues, including memory and sociability disorders. VAChT $KD^{HET}$ mice present reduced vesicular ACh transporter (VAChT) protein production, which could elicit low ACh quantal release and, consequently, memory and sociability impairments. It has been shown that the PhKv toxin, isolated from the *Phoneutria nigriventer* venom, inhibits acetylcholinesterase, an enzyme responsible for ACh hydrolysis. Consequently, the neurotransmitter could remain longer in the synaptic cleft, which may alleviate cognitive problems caused by low levels of ACh. Thus, here we aimed to explore VAChT $KD^{HET}$ mice cognitive deficits in two behavioral assays - the novel object recognition task (NORT) and the three-chamber social test (3-CST) - and then investigate the potential effect of the PhKv toxin improving memory in this mouse model. First, we observed that VAChT $KD^{HET}$ mice presented object recognition memory deficits and social novelty preference impairments but no sociability issues. Subsequently, in order to evaluate the effects of PhKv toxin in memory, we injected PhKv (100 pmol/site, i.c.v.) or galantamine (1 mg/kg, s.c.) in VAChT $KD^{HET}$ mice and compared their performance in the NORT. We observed that mice treated with PhKv showed similar performance in this behavioral assay compared to mice treated with galantamine or both vehicles. We also noted that mice treated with both vehicles presented memory improvement compared to non-treated (naive) mutant mice. Then, we suggest that the surgical procedure might have impacted our results. Overall, our study proposes that PhKv could have a neuroprotective effect, which may be further investigated in order to elucidate its mechanisms of action.



**Keywords:** *Phoneutria nigriventer*, Brazilian wandering spider, PhKv, spider toxin, acetylcholine, acetylcholinesterase, memory, vesicular acetylcholinesterase transporter.

## RESUMO

A acetilcolina (ACh) é um neurotransmissor importante para a cognição. Dentre diversas funções, ela atua como reguladora de circuitos relacionados à atenção, flexibilidade cognitiva, memória e interação social. Níveis reduzidos de ACh nas fendas sinápticas colinérgicas resultam em vários problemas, incluindo distúrbios de memória e de sociabilidade. Os camundongos VAChT $KD^{HET}$ apresentam produção reduzida da proteína transportadora vesicular de ACh (VAChT), o que pode acarretar em baixa liberação de ACh e, consequentemente, disfunções de memória e de sociabilidade. Foi demonstrado que a toxina PhKv, isolada do veneno de *Phoneutria nigriventer*, inibe a acetilcolinesterase, enzima responsável pela hidrólise da ACh. Ao inibir a enzima, o neurotransmissor pode permanecer mais tempo na fenda sináptica, o que pode aliviar os problemas cognitivos acarretados pelos baixos níveis de ACh. Portanto, no presente trabalho, exploraramos os déficits cognitivos de camundongos VAChT $KD^{HET}$ em dois ensaios comportamentais - a tarefa de reconhecimento de novo objeto (NORT) e o teste social de três câmaras (3-CST) - e, em seguida, propomos investigar os efeitos da toxina PhKv na melhoria da memória nesse modelo animal. Primeiro, observamos que os camundongos VAChT $KD^{HET}$ apresentaram déficits de memória de reconhecimento de objetos e de preferência por novidades sociais, sem exibir problemas de sociabilidade. Dentro desse contexto, a fim de avaliar os efeitos da toxina PhKv na memória, injetamos PhKv (100 pmol/sítio, icv) ou galantamina (1 mg/kg, s.c.) em camundongos mutantes e comparamos os efeitos destas na NORT. Observamos que camundongos tratados com PhKv demonstraram desempenho semelhante na tarefa, em comparação com camundongos tratados com galantamina ou ambos veículos. Notamos também que o grupo controle/*sham* apresentou uma melhoria de memória, em comparação com camundongos não-tratados (*naive*). Então, sugerimos que o procedimento cirúrgico pode ter interferido em nossos resultados. No geral, nosso estudo propõe que a PhKv pode ter um efeito neuroprotetor, que precisa ser mais investigado, principalmente em uma rota de administração menos invasiva, a fim de elucidar os mecanismos de ação da toxina.



**Palavras-chave:** *Phoneutria nigriventer*, aranha-armadeira, PhKv, toxina de aranha, acetilcolina, acetilcolinesterase, memória, transportador vesicular de acetilcolina.

## LIST OF FIGURES

## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **3-CST** | Three-chamber social test |
| **Aβ** | Amyloid beta |
| **Acetyl-CoA** | Acetyl coenzyme A |
| **ACh** | Acetylcholine |
| **AChE** | Acetylcholinesterase |
| **AD** | Alzheimer's disease |
| **ANOVA** | Analysis of variance |
| **AP** | Anteroposterior |
| **ATP** | Adenosine triphosphate |
| **CEUA** | Ethics Committee on the Use of Animals |
| **Ch** | Choline |
| **ChAT** | Choline transferase |
| **CHT1** | High-affinity choline transporter 1 |
| **CI** | Confidence interval |
| **cm** | Centimeter |
| **CNS** | Central nervous system |
| **Da** | Dalton |
| **DV** | Dorsoventral |
| **e.g.** | *Exempli gratia* (for example) |
| ***F*** | F-test |
| **Funed** | Ezequiel Dias Foundation |
| **GAL** | Galantamine |
| **h** | Hour |
| **HET** | Heterozygous |
| **HOM** | Homozygous |
| **i.c.v.** | Intracerebroventricular |
| **i.e.** | *Id est* (that is) |
| **i.p.** | Intraperitoneal |
| **ICB** | Institute of Biological Sciences |

| | |
|---|---|
| **IQR** | Interquartile range |
| **$K^+$** | Potassium |
| **KD** | Knockdown |
| **kg** | Kilogram |
| **L** | Liter |
| **mACh** | Muscarinic acetylcholine receptor |
| **MD** | Difference between means |
| **MedianD** | Difference between medians |
| **mg** | Milligram |
| **min** | Minute |
| **ML** | Mediolateral |
| **mL** | Milliliter |
| **mm** | Millimeter |
| **MS** | Ministry of Health of Brazil |
| **nAChR** | Nicotinic acetylcholine receptor |
| **NIA** | National Institute of Aging |
| **nL** | Nanoliter |
| **NMDA** | N-methyl-D-aspartate |
| **NORT** | Novel object recognition task |
| **ºC** | Celsius degree |
| ***p*** | p-value |
| **PBS** | Phosphate buffered saline |
| **pmol** | Picomol |
| **s** | Second |
| **SAL** | Saline |
| **s.c.** | Subcutaneous |
| **S.E.M.** | Standard error of the mean |
| **SLC18** | Solute carrier family 18 |
| **SNP** | Social novelty preference |
| **ST** | Sociability test |
| ***t*** | t-factor |

| | |
|---|---|
| **UFMG** | Federal University of Minas Gerais |
| **uL** | Microliter |
| **VAChT** | Vesicular acetylcholinesterase transporter |
| **VMAT1** | Vesicular monoamine transporter 1 |
| **VMAT2** | Vesicular monoamine transporter 2 |
| **WT** | Wild-type |

**SUMMARY**

# 1 INTRODUCTION

Acetylcholine (ACh) was the first neurotransmitter described in the literature. It was discovered in in 1913 by Sir Henry Dale and Arthur Ewins. Eight years later, in 1921, its function was described by Otto Loewi. These findings later culminated in a shared Nobel Prize in Physiology and Medicine “for their discoveries relating to chemical transmission of nerve impulses” (EWINS, 1914; LOEWI, 1921; TANSEY, 2006; BORGES & GARCIA, 2021; NOBELPRIZE.ORG, 2022).

More than a century after its discovery, the role of ACh has still been investigated. In the peripheral nervous system, ACh appears to act as a primary excitatory fast-acting neurotransmitter; however, in the central nervous system, ACh seems to operate as a neuromodulator. It presents a variety of functions in the brain, such as changing neuronal excitability, modifying the release of neurotransmitters, inducing synaptic plasticity, and coordinating the firing of groups of neurons (PICCIOTTO, HIGLEY & MINEUR, 2012). ACh is also linked to modulating circuits related to attention, cue detection, learning and memory, and social interaction (NEWMAN et al., 2012; BICKS et al., 2015).

ACh is synthesized by the choline acetyltransferase (ChAT) enzyme (**Figure 1**). It catalyzes the reaction between choline (Ch) and acetyl coenzyme A (acetyl-CoA) in the cytoplasm of nerve terminals, where an acetyl molecule from acetyl-CoA is transferred to Ch. Acetyl-CoA is derived from the mitochondrial metabolism, whereas Ch is uptaken in the synaptic cleft by the high-affinity choline transporter 1 (CHT1), a $Na^+$-dependent transporter located at the cell membrane. Then, ACh is loaded in synaptic vesicles by the vesicular acetylcholine transporter (VAChT). When a nerve impulse arrives, the vesicles containing ACh fuse to the cell membrane, releasing the neurotransmitter. In the synaptic cleft, ACh can bind into nicotinic (nAChR) or muscarinic (mAChR) receptors. In order to terminate signaling between cholinergic synapses, ACh is hydrolyzed by acetylcholinesterase (AChE) into acetate and choline, which is reuptaken by CHT1, and so the cycle continues (ARVIDSSON et al., 1997; PURVES et al., 2001; SOREQ & SEIDMAN, 2001; DEUTCH & ROTH, 2004; TRANG & KHANDHAR, 2021).

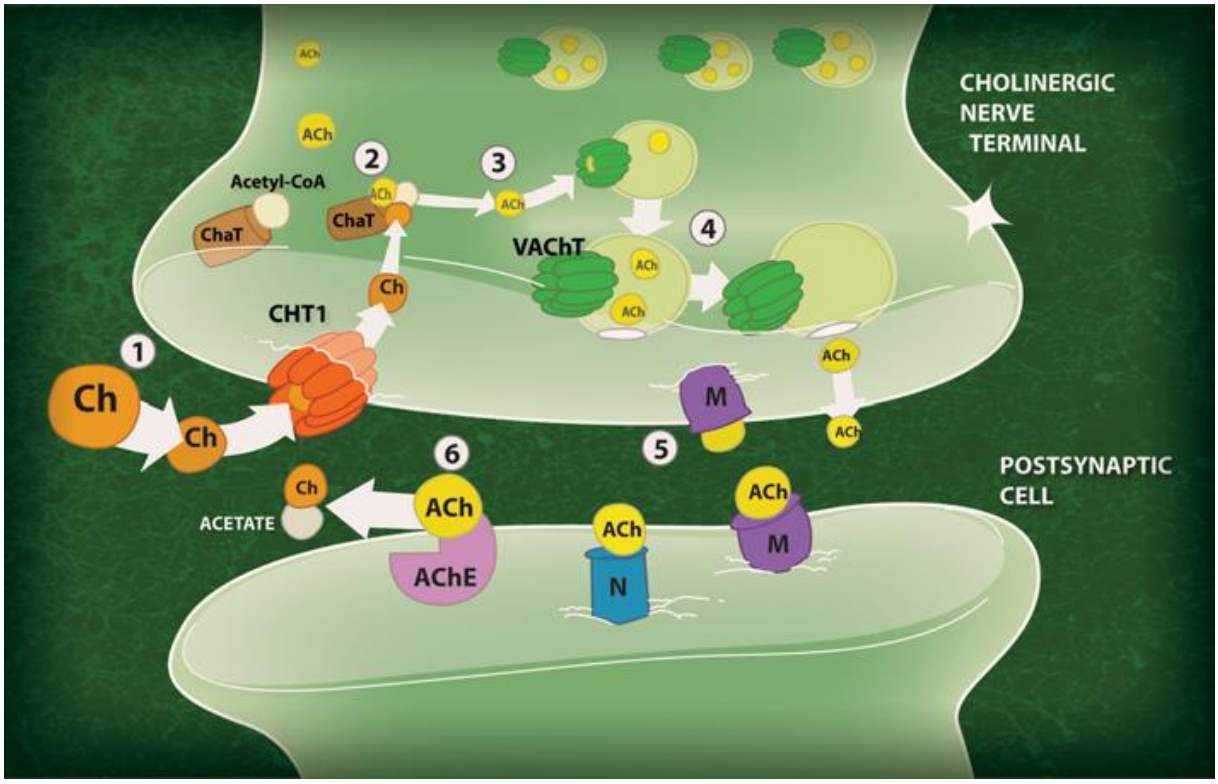


**Figure 1. Acetylcholine synthesis and metabolization.** (1) Choline (Ch), acetylcholine (ACh) precursor, is uptaken by high-affinity choline transporter 1 (CHT1). (2) Choline acetyltransferase (ChAT) synthesizes ACh from Ch and acetyl coenzyme A (acetyl-CoA). (3) ACh is loaded into synaptic vesicles by the vesicular ACh transporter (VAChT). (4) Vesicles full of ACh fuse to the cell membrane and release the neurotransmitter. (5) ACh can bind into nicotinic (N) or muscarinic (M) receptors. (6) ACh is degraded into acetate and Ch by the acetylcholinesterase (AChE). (PRADO et al., 2013).

As seen, VAChT (also known as SLC18A3) is a crucial protein for cholinergic neurotransmission. It is a transmembrane protein that belongs to the solute carrier family 18 (SLC18) of the major facilitator superfamily of transporters, together with the vesicular monoamine transporters 1 and 2 (VMAT1 and VMAT2 or SLC18A1 and SLC18A2, respectively). These active transporters utilize the electrochemical generated by a vacuolar-type ATPase to carry and accumulate neurotransmitters in vesicles. It has been suggested that VAChT is a slow-type vesicular transporter. Thus, VAChT protein expression and activity could influence the release of ACh directly, performing as a limiting factor in the recycling of cholinergic synaptic vesicles in order to maintain ACh release (VAROQUI & ERICKSON, 1996; NGUYEN, COX & PARSONS, 1998; PARSONS, 2000; VARDY et al., 2004; LAWAL & KRANTZ, 2013; PRADO et al., 2013).

In order to explore the outcomes of reduced expression of VAChT on cholinergic neurotransmission *in vivo*, Prado and colleagues (2006) developed a VAChT KD (knockdown) mouse (PRADO et al., 2006). VAChT KD mice present a decreased gene and protein expression of VAChT rather than complete deletion, as VAChT deleted allele mice (knockout) die after birth (DE CASTRO et al., 2009a).

VAChT KD mice present different rates of VAChT protein expression diminution, according to their genotype: VAChT $KD^{HOM}$ (homozygous) mice have a 65% decrease in VAChT protein expression, whereas VAChT $KD^{HET}$ (heterozygous) have a 45% reduction in this protein expression. The reduction of VAChT protein production prejudiced cholinergic neurotransmission, which resulted in cognitive and motor losses in VAChT $KD^{HOM}$ mice, and exclusively cognitive deficits in VAChT $KD^{HET}$ mice (PRADO et al., 2006; DE CASTRO et al., 2009a; DE CASTRO et al., 2009b; CAPETTINI et al., 2011; DE JAEGER et al., 2013; MAGALHÃES-GOMES et al., 2018).

VAChT $KD^{HET}$ mice present progressive impairment of cognitive flexibility, attention span, and recognition memory (mainly social and object recognition memories), characteristics also described in neurodegenerative disorders, such as Alzheimer's disease (AD) (PRADO et al., 2006; KARANTZOULIS & GALVIN, 2011; TARAWNEH & HOLTZMAN, 2012). These aspects make VAChT $KD^{HET}$ mice appropriate to study how cholinergic agents and cholinesterase inhibitors aid in reversing cognitive deterioration observed in AD (KOLISNYK et al., 2013).

AD is a progressive, irreversible, and fatal neurodegenerative disorder, clinically characterized by the deterioration of many cognitive functions, such as attention, language, and memory. AD patients typically present changes in behavior and impairment in performing daily tasks. Progressive synaptic losses and neuronal death in regions responsible for cognitive functions (e.g., cortex and hippocampus) are some examples of histopathological evidence of AD (SCHELTENS et al., 2016).

There are a few hypotheses that explain the leading cause of senile dementia (e.g., "amyloid hypothesis", "cholinergic hypothesis", "tau hypothesis"). The "cholinergic hypothesis" was elaborated by Bartus et al. (1982). They suggested that a gradual deterioration of cholinergic neurons in the forebrain, followed by a progressive reduction of ACh levels in the cerebral cortex and other areas, contributes to the impairment of cognitive function, seen in many AD patients. Therefore, this cholinergic function loss could be the principal cause of AD, which could lead to the other described symptoms (BARTUS et al., 1982; FRANCIS et al., 1999). In this

context, in order to retard, for a limited period of time, this clinical condition and provide symptomatic relief to AD patients, the treatment of this disorder includes cholinesterase inhibitors, such as donepezil, galantamine, and rivastigmine, pharmacological agents currently approved for the symptomatic treatment of AD by Brazilian and international health agencies (MS, 2017; SABBAGH, HENDRIX & HARRISON, 2019; NIA, 2021).

As previously seen, an example of cholinesterase is AChE, the enzyme responsible for ACh hydrolysis. Carvajal and Inestrosa (2011) suggested that AChE could be involved in the histopathology of AD. They observed that the enzyme interacted directly with Aβ (amyloid beta) peptides, accelerating the deposition of the insoluble peptide on plaques (CARVAJAL & INESTROSA, 2011). Aβ aggregation in the brain is also suggested as a leading cause of the pathogenesis of AD ("amyloid hypothesis") (HAASS & SELKOE, 1993, CHEN et al., 2017). This vital role of AChE indicates that AChE inhibitors could act not only as mere palliatives in AD but also as modifying agents of this disease. However, nowadays commercialized cholinesterase inhibitors agents present numerous side effects (i.e., gastrointestinal anomalies-nausea, vomiting, diarrhea, anorexia, abdominal pain, headache, bradycardia, syncope, dizziness), which may be uncomfortable for most patients. In this context, new cholinesterase inhibitor agents may be excellent candidates for future therapy for AD, especially those presenting less side effects (REES & BRIMIJOIN, 2003; REES et al., 2003; GARCÍA-AYLLÓN et al., 2011; COLOVIC et al., 2013). PhKv, a toxin isolated from the *Phoneutria nigriventer* spider venom, has been recently investigated as a potential alternative cholinesterase inhibitor (ALMEIDA et al., 2011; RIGO et al., 2017).

*P. nigriventer* (Araneidae, Ctenidae), also known as “Brazilian wandering spider”, is a synanthropic, solitary, and aggressive species. They dwell in neotropical forests from Southern Central America throughout South America. *P. nigriventer* venom (PNV) effects have been studied since the 1920s and it is considered a "pharmacological treasure". PNV presents a diverse range of molecules, such as proteases and peptides. Some of these peptides can interact with neuromuscular chemical receptors, neuronal ion channels, or both, affecting neurotransmitter release and ion channels function (GOMEZ et al., 2002; PEIGNEUR, DE LIMA & TYGAT, 2018). Rezende-Júnior and colleagues (1991) described a method for isolating different fractions of PNV so that PNV could be separated into five fractions: a single non-toxic to mammals fraction, named PnM, and four different toxic fractions active on mammals, PnTx1 to 4 (**Figure 2**) (REZENDE-JÚNIOR et al., 1991). In each toxic

fraction, many toxins, including neurotoxins, have been identified and characterized for their function and structure (CORDEIRO et al., 1995; DE LIMA et al., 2015).

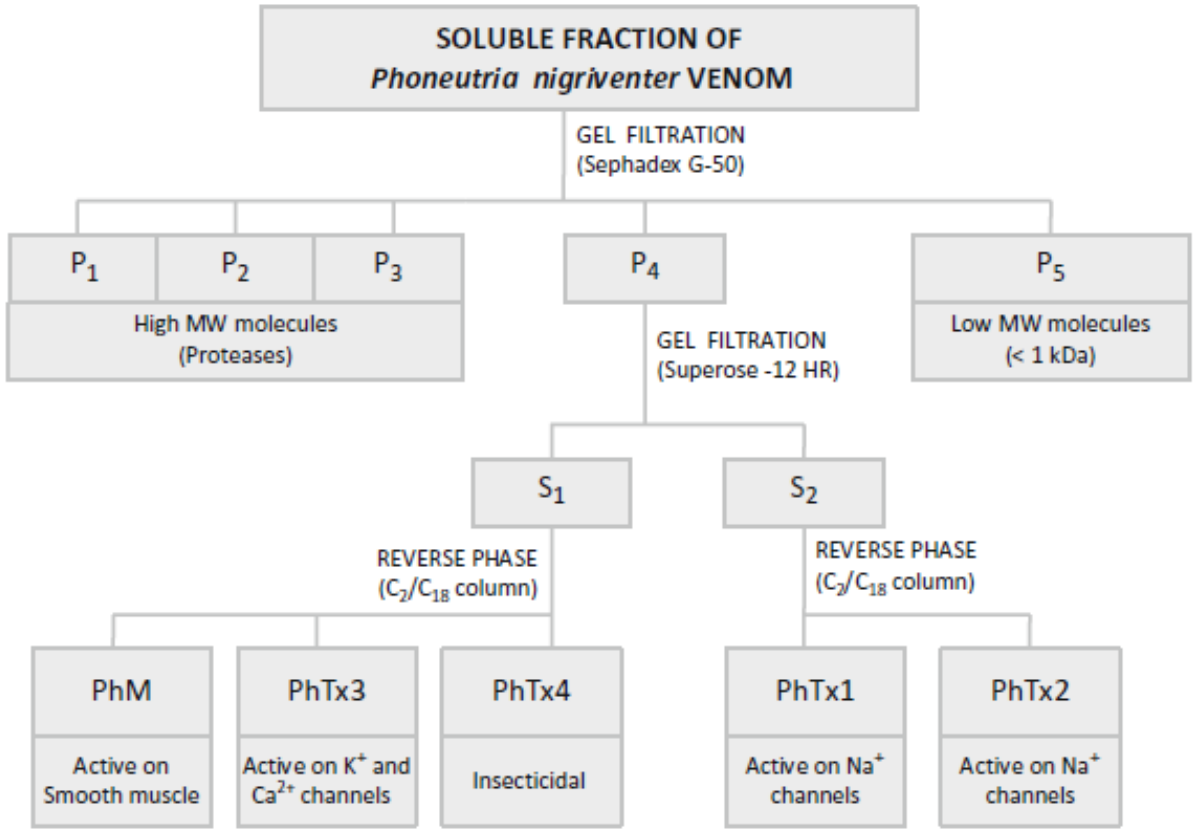


**Figure 2. The purification process of *Phoneutria nigriventer* venom.** The procedure results in five fractions: PhTx1 to -4 and PhM. PhKv, also known as Tx3-1, is isolated from the PhTX3 fraction. (de Lima et al., 2015).

The PhKv toxin (AECAAVYERCGKGYKRCCEERPCKCNIVMDNCTCKKFISEL molecular weight = 4582.93 Da), originally named Tx3-1, is an example of neurotoxin isolated from PNV (GOMEZ et al., 2002; PEIGNEUR, DE LIMA & TYGAT, 2018). It was first purified and described by Cordeiro et al. (1993), as it was purified from the PhTx3 fraction of PNV (CORDEIRO et al., 1993).

PhKv effects were first inquired by Kushmerick and collaborators (1999). They observed that PhKv inhibited a specific voltage-gated calcium-independent potassium channel type of current, the A-type $K^+$ current ($I_A$), through patch-clamp experiments in $GH_3$ neuroendocrine cell culture (KUSHMERICK et al., 1999).

Almeida and colleagues (2011) showed that PhKv could reduce the duration of cardiac arrhythmias in rats (ALMEIDA et al., 2011). Gomes and collaborators (2013) investigated the effects of PhKv in cognition. They noticed that PhKv improved memory

in $A\beta_{25-35}$-treated mice, without any adverse effects (GOMES et al., 2013). Rigo et al. (2017) explored the potential analgesic effect of PhKv toxin and observed that PhKv could reduce the capsaicin nociceptive process *ex vivo*. They also noted that PhKv was able to inhibit AChE in mice spinal cord *in vivo* (RIGO et al., 2017).

Together, these studies show the therapeutic effects of PhKv toxin and its possible clinical pharmacological use. Thus, here, we investigated the potential effects of the PhKv toxin, administered directly in the CNS, in improving memory deficits in VAChT $KD^{HET}$ mice.

# 2 OBJECTIVES

## 2.1 General objective

Investigate the effects of the PhKv toxin, administered through intracerebroventricular (i.c.v.) route, on memory in VAChT $KD^{HET}$ mice.

## 2.2 Specific objectives

- Select the best behavioral assay to evaluate cognitive deficits presented by VAChT $KD^{HET}$ mice.
- Assess the cognitive function alterations evoked by the treatment with the PhKv toxin (i.c.v.) in VAChT $KD^{HET}$ mice.
- Compare the effects of the PhKv toxin (i.c.v.) and galantamine, an acetylcholinesterase inhibitor approved for clinical use, in VAChT $KD^{HET}$ mice.

# 3 MATERIALS AND METHODS

## 3.1 Animals

Heterozygous VAChT KD (VAChT $KD^{HET}$) mice were donated by the Laboratório de Biologia de Neurotransmissão (ICB-UFMG) and were backcrossed with C57BL/6 animals for at least three generations. The offspring were genotyped at postnatal weeks. Animals were housed in clear polyethylene cages, with pinewood shaving bedding and enrichment, in a temperature-controlled room (22 ± 1°C), with 12h:12h light-dark cycles. Food and water were provided *ad libitum*. Female and male mice were selected for the behavior testing aged 17 to 22 weeks. All experimental procedures were approved by the Ethics Committee on the Use of Animals at the Federal University of Minas Gerais (CEUA-UFMG), under protocol number 345/2019.

## 3.2 Behavioral testing

### *3.2.1 Novel object recognition task*

The apparatus used for assessing the object recognition memory of the subjects was an open MDF (medium-density fiberboard) square box (40 x 40 x 40 cm), with white opaque walls and floor. Objects used were made of ABS (acrylonitrile butadiene styrene) or PLA (polylactic acid), two distinct types of plastic with different shapes, textures, colors, and sizes. A lampshade placed 60 cm next to the apparatus provided constant illumination of about 15 lux, and a speaker provided background sound isolation. Animals were acclimated to the room for at least 30 min before the beginning of each trial.

The novel object recognition task (NORT) was performed according to de Jaeger and colleagues (2013), with some alterations. The task consisted of three stages: habituation (HAB), familiarization (FAM), and tests (**Figure 3**). In the HAB phase, mice were individually placed on the empty apparatus for 10 min, free to explore. 24h after HAB, mice were introduced to two identical objects (A1 and A2), which were placed in a symmetrical position from the walls of the apparatus, for a single 10 min session. This phase was named familiarization (FAM). The last stage consisted of the tests phase, divided into a short-term memory (STM) test and a long-

term memory (LTM) test. In the STM test, animals were reintroduced to the apparatus 90 min after FAM and submitted a new set of objects, a familiar object (A) and a novel object (B), placed at the exact locations as during the FAM stage, for 10 min. In the LTM test, mice were reset on the apparatus 24h after FAM and exposed to two objects, a familiar object (A) and a novel object (C), for 10 min (DE JAEGER et al., 2013).

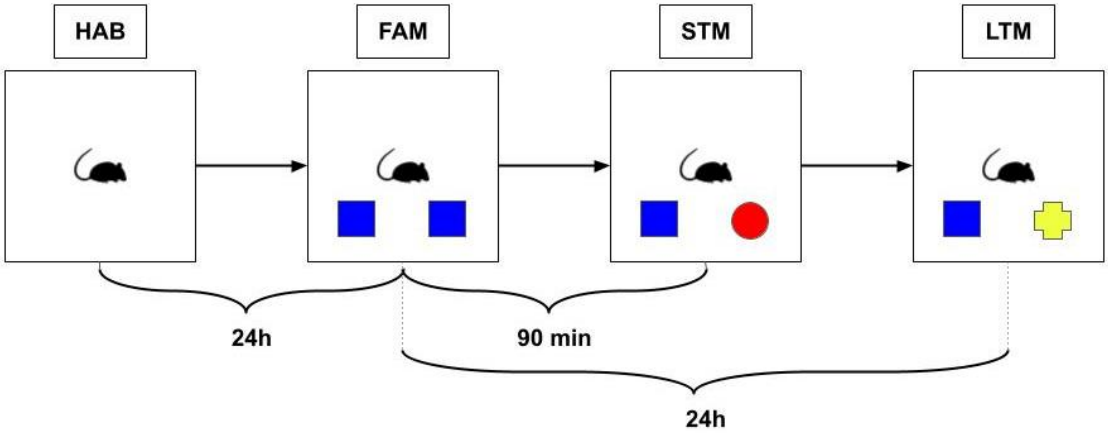


**Figure 3. Novel object recognition task stages.** NORT presents three stages: habituation (HAB), familiarization (FAM), and tests (STM and LTM). Mice are first accustomed to the apparatus and tested for motor impairments (HAB). 24h later, they are introduced to two identical objects (FAM). 90 min after this stage, mice are presented to a familiar object and an unfamiliar object (STM). 24h after FAM, they are presented to a familiar object and a novel object (LTM).

To avoid displacing the objects throughout the experiment, they were fixed with tape on the bottom, 12 cm away from the walls. Objects were used in a counterbalanced manner to prevent preference by the subjects. The apparatus and objects were narrowly cleaned with 70% ethanol and ventilated between animals and across sessions. After each stage, animals were returned to their home cages. The time spent exploring the apparatus and the objects was recorded. Total distance traveled, time spent in the periphery, exploration time of each of the objects, and total exploration time (sum of both times of exploration of both objects) were scored using the ANY-maze software (Stoelting Co., Illinois, IL, USA), version 7.0. Exploration time of the object was counted when mice were in direct contact with the object or when they stretched off their necks in an area 5 cm around the container, with their nose pointed towards the object. The discrimination index (DI) was used as a memory parameter. DI was calculated according to the following formula: $(TN - TF)/(TN + TF)$, where $TN$ is the total exploration time of the novel object, and $TF$ is the total exploration time of the familiar object.

*3.2.2 Three-chamber social test*

The apparatus used for evaluating the social memory of the subjects was a rectangular box (120 x 40 x 30 cm), with transparent plexiglass-made walls and an opaque dark floor. The arena is divided into three chambers (40 x 40 x 30 cm), divided by two transparent walls, with an entry middle section, allowing mice free access to the chambers. A cylinder wire cup-like container (7 x 7 x 15 cm) with removable lids was placed in each lateral chamber (left or right). A lampshade set 100 cm above the apparatus provided direct illumination of about 600 lux, and a speaker provided background sound isolation. In addition to the subjects, another animal category was required: the intruder (or stranger) mouse. Two intruder mice were selected for each subject, with similar genetic background, sex, age, and weight but no previous contact (non-littermates). Animals were acclimated to the room for at least 60 min before the beginning of each trial.

The three-chamber social test (3-CST) was performed according to Kaidanovich-Beilin and collaborators (2011), with a few modifications. The test comprised three stages: habituation (HAB), sociability test (ST), and social novelty preference (SNP) test (**Figure 4**). In the HAB phase, mice were individually placed on the apparatus with the two containers placed on each lateral chamber for 10 min, free to explore. In the ST, an intruder mouse (stranger 1) was positioned in one of the containers (left or right), and the subject mouse was left free to explore the three chambers for 10 min. In the last stage, the SNP test, another unfamiliar mouse (stranger 2) was set on the remaining container, and the subject mouse was left free to explore the apparatus and the containers for 10 min (KAIDANOVICH-BEILIN et al., 2011).

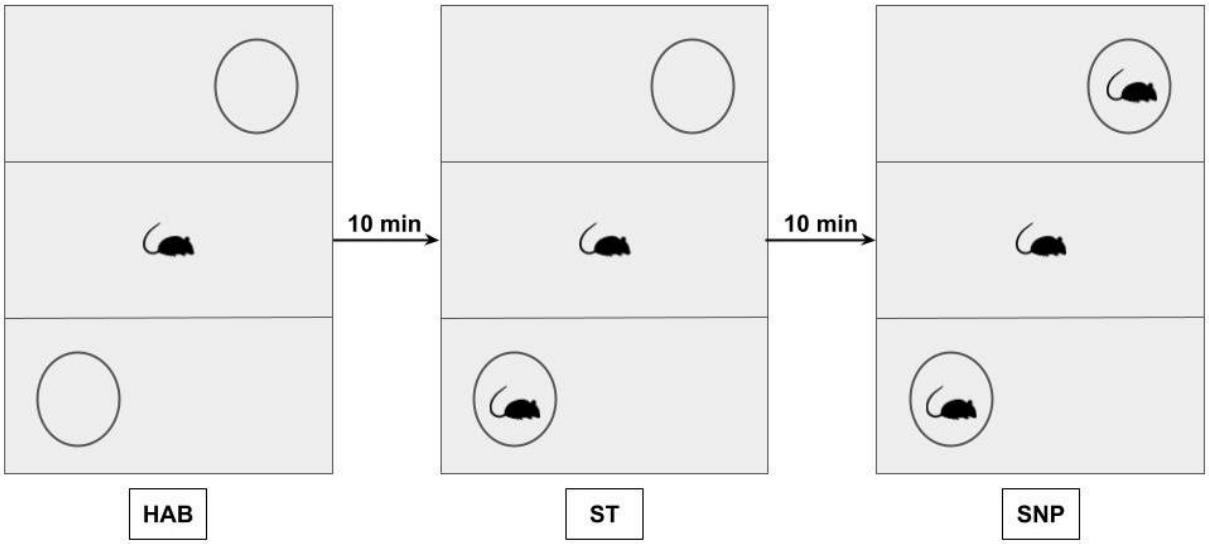


**Figure 4. Three-chamber social test stages.** 3-CST presents three stages: habituation (HAB), sociability test (ST), and social novelty preference test (SNP). Mice are first accustomed to the apparatus and tested for chamber preference (HAB). 10 min after HAB, they are introduced to an intruder conspecific in one of the chambers (ST). 10 min after ST, mice are presented to a second intruder (SNP).

The apparatus and containers were minutely cleaned with 70% ethanol and ventilated between subjects and across sessions. At the end of the test, animals were returned to their home cages. Time spent exploring the apparatus and the containers was recorded. Mice were considered inside a chamber when their head was pointed towards the entry, and 70% of their bodies entered the chamber. Total distance traveled, exploration time of each of the chambers, exploration time of each of the intruders, total exploration time of chambers (sum of both time of exploration of both chambers), and total exploration time intruders (sum of both time of exploration of both intruders) were scored using ANY-maze software. Exploration time of the intruder was accounted for when the subject animal was in direct contact with the stranger or when it stretched off its neck in an area 5 cm around the container, with its nose pointed in the direction of the intruder.

### 3.3 Drugs and treatments

Galantamine was purchased from Tocris Bioscience (Minneapolis, MN, USA). PhKv toxin was donated by the Serviço de Proteômica e Aracnídeos, at Ezequiel Dias Foundation (Funed). Galantamine and PhKv stock solutions were prepared one day

prior to the treatments and stored at -20ºC. Galantamine (1 mg/kg) or vehicle (saline 0.9%) was administered by subcutaneous (s.c.) route (PRADO et al., 2006). PhKv (100 pmol/site) or vehicle (PBS 1x) was injected via intracerebroventricular (i.c.v.) route (GOMES et al., 2013; RIGO et al., 2017).

The surgical procedure was performed according to Magno and collaborators (2019), with some modifications. Briefly, for the i.c.v. injection, mice were fully anesthetized with a mix of ketamine/xylazine (80 mg/kg per 8 mg/kg), through intraperitoneal (i.p.) injection. Animals were maintained on oxygen support (1 L/min) and under deep anesthesia (isoflurane 1%) during the surgical procedure. Mice were placed into a stereotaxic frame with a body temperature control. To access the lateral ventricle, a craniotomy was performed by a dental drill with a 0.75 mm burr according to the following coordinates from bregma: anteroposterior (AP) -0.20 mm, mediolateral (ML) -1.00 mm, and dorsoventral (DV) -2.20 mm. PhKv or vehicle was injected through a pulled borosilicate glass micropipette. The volume injected was 3000 nL (3 uL), at an automated rate of 150 nL/min, and posterior 5 min to guarantee the diffusion of the liquid. After surgery, animals received proper post-surgical care. They were injected with a mix of ketoprofen (5 mg/kg) and Ringer's lactate solution, via s.c., and were placed in a pre-warmed (37 ºC) clean cage. Mice received wet food pellets placed in a small Petri dish to facilitate feeding. They were closely supervised by the experimenter until completely awake (MAGNO et al., 2019).

### 3.4 Experimental design

To select the best behavioral testing to assess cognitive deficits of VAChT KD$^{HET}$/WT mice, 18 animals (F = 9, M = 9) performed NORT and 3-CST, according to the protocols described in section 3.2. Mice were divided in two groups, according to genotype: VAChT KD$^{HET}$ (n = 10), VAChT WT (n = 8).

To evaluate the effects of the PhKv toxin on cognition, 36 VAChT KD$^{HET}$ mice (F = 18, M = 18) were divided in three groups: PhKv (n = 12), galantamine (n = 12), control/sham (n = 12). The PhKv group received vehicle (saline 0.9%) s.c. and PhKv toxin (100 pmol/site) i.c.v., the galantamine group received galantamine (1 mg/kg) s.c. and vehicle (PBS 1x) i.c.v., and the control/sham group received both vehicles s.c. and i.c.v. Prior to the treatments, animals performed the first and second stages of NORT (HAB and FAM, respectively) (**Figure 5**). Right after FAM, mice underwent treatment

with galantamine or vehicle (sterile 0.9% saline) s.c., followed by PhKv or vehicle (sterile PBS 1x) i.c.v., to prevent any effects of injection during the FAM stage (GOMES et al., 2013). 24h after FAM, animals performed the LTM stage.

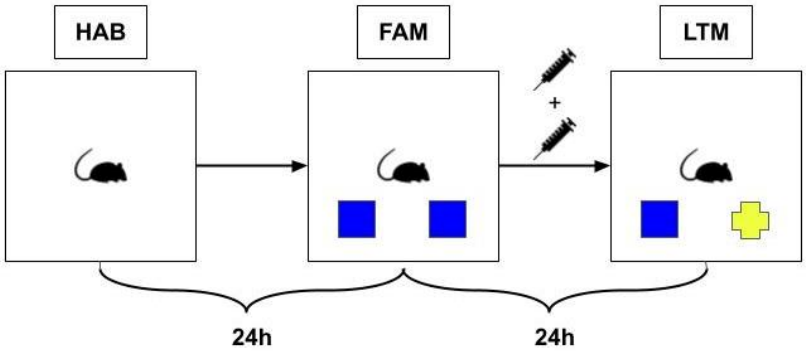


**Figure 5. Treatments experimental design.** HAB and FAM were performed before treatments. Right after FAM, mice were treated with galantamine or vehicle (s.c.) and PhKv toxin or vehicle (i.c.v.). 24h after FAM, they performed the LTM stage.

### 3.5 Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA), version 8. Results were expressed as mean ± S.E.M. (standard error of the mean) for all measures. Grubb's test (alpha = 0.05) was performed to establish significant outliers, which were excluded from the analysis, followed by the Shapiro–Wilk normality test, used to determine normal distribution of data. Unpaired Student's t-test or Mann-Whitney test, ordinary one-way analysis of variance (ANOVA) or Kruskal-Wallis test, and ordinary two-way ANOVA were performed, depending on the experiment. Values of $p < 0.05$ were considered significant.

# 4 RESULTS

## 4.1 VAChT KD$^{HET}$ mice present impaired object recognition memory

One of the peculiar aspects of VAChT KD mice is an impairment of object recognition memory. In order to evaluate such cognitive impairment, we submitted VAChT KD$^{HET}$/WT mice to the novel object recognition task (NORT). NORT is divided into three stages: habituation (HAB), familiarization (FAM), and test (PRADO et al., 2006; DE CASTRO et al., 2009a; DE JAEGER et al., 2013).

Mice were allowed to explore the empty apparatus to assess motor function and anxiety-like behavior (HAB stage). We observed no significant difference in mobility aspects [WTxKD$^{HET}$, HAB: Student's t-test: MD 1.678, 95% CI -2.861 to 6.217, $t$ = 0.7837, $p$ = 0.4447] (**Figure 6A**) nor in anxiety-like behavior parameters [WTxKD$^{HET}$, HAB: Student's t-test: MD -40.62, 95% CI -100.7 to 19.43, $t$ = 1.434, $p$ = 0.1718; WTxKD$^{HET}$, HAB: Student's t-test: MD 38.89, 95% CI -19.93 to 97.70, $t$ = 1.402, $p$ = 0.1801] (**Figures 6B-6C**). Then, mice were introduced to two identical objects (FAM stage). The total exploration time of two objects was similar for VAChT KD$^{HET}$ and VAChT WT mice, demonstrating that both genotypes explored the objects likewise [WTxKD$^{HET}$, FAM: Mann-Whitney test: MedianD -31.20, $p$ = 0.0545] (**Figure 6D**). We also observed that mice had no preference for one of the objects since the discrimination index (DI) was similar to both genotypes and closer to zero [WTxKD$^{HET}$, FAM: Student's t-test: MD -0.09125, 95% CI -0.4027 to 0.2202, $t$ = 0.6212, $p$ = 0.5432] (**Figure 6E**).

After a latency of 90 min, mice were submitted to the first test to assess their short-term memory (STM), in which they were introduced to a familiar object and a novel object. We observed that VAChT WT mice spent more time exploring the objects than VAChT KD$^{HET}$ mice [WTxKD$^{HET}$, STM: Mann-Whitney test: MedianD -43.30, $p$ = 0.0014] (**Figure 6F**). DI was significantly different between genotypes. VAChT WT did not show preference for the novel object (DI value closer to zero), whereas VAChT KD$^{HET}$ mice preferred examining the familiar object (DI value closer to -1) [WTxKD$^{HET}$, STM: Student's t-test: MD -0.3282, 95% CI -0.6509 to -0.005427, $t$ = 2.156, $p$ = 0.0467] (**Figure 6G**).

24h after FAM, animals were submitted to the second test to evaluate their LTM, in which they explored a familiar object and an unfamiliar object. Total exploration time was significantly distinct between VAChT $KD^{HET}$ and VAChT WT mice, as seen on STM [$WTxKD^{HET}$, LTM: Student's t-test: MD -50.53, 95% CI -87.52 to -13.53, $t = 2.895$, $p = 0.0105$] (**Figure 6H**). Nonetheless, mutant mice were not able to differentiate the objects (DI value close to zero), whereas WT mice could distinguish them (DI value closer to +1) [$WTxKD^{HET}$, LTM: Student's t-test: MD -0.3233, 95% CI -0.4985 to -0.1481, $t = 3.911$, $p = 0.0012$] (**Figure 6I**).

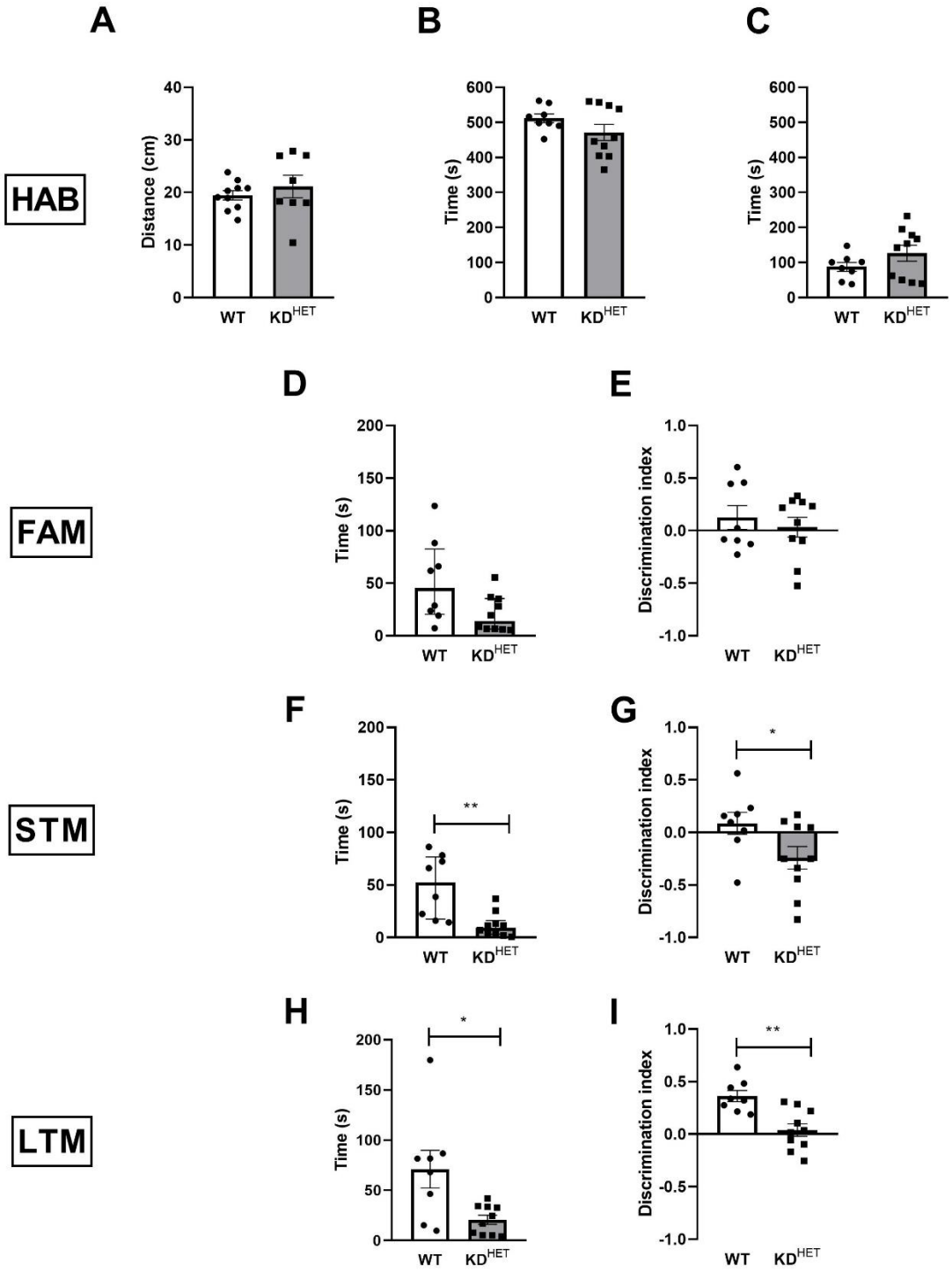


**Figure 6. VAChT KD$^{HET}$ mice present impaired object recognition memory. (A, B, C)** Evaluation of HAB parameters: total distance traveled **(A)**, time spent in the periphery **(B)**, and time spent in the center **(C)**. **(D, E)** Assessment of FAM parameters: total exploration time **(D)** and discrimination index **(E)**. **(F, G)** Evaluation of STM parameters: total exploration time **(F)** and discrimination index **(G)**. **(H, I)** Assessment of LTM parameters: total exploration time **(H)** and discrimination index **(I)**. Data represented as mean ± S.E.M in A, B, C, E, G, H, I. Data represented as median and IQR in D, F. (n = 8-10/group). White bars indicate VAChT WT mice. Gray bars indicate VAChT KD$^{HET}$ mice. (*) indicates statistically different performance when compared to VAChT WT mice. * $p < 0.05$, ** $p < 0.01$.

Since a previous study showed sexual dimorphism in VAChT KD$^{HET}$ mice, we segregated the groups according to sex (CAPETTINI et al., 2011). First, comparing each sex according to the genotypes, we observed that, in STM (**Figure 7A**), female VAChT KD$^{HET}$ mice showed statistically significant impaired recognition memory compared to female VAChT WT mice. Female mutant mice also preferred to explore the familiar object than the novel object (DI value closer to -1) [FWTxFKD$^{HET}$, STM: Student's t-test: MD -0.6011, 95% CI -1.143 to -0.05889, $t$ = 2.621, $p$ = 0.0343]. In LTM (**Figure 7B**), female mutant mice also displayed statistically significant impaired recognition memory compared to female WT mice, although they did not show a preference for either of the objects (DI value closer to zero) [FWTxFKD$^{HET}$, LTM: Student's t-test: MD -0.2874, 95% CI -0.5724 to -0.002539, $t$ = 2.386, $p$ = 0.0485]. In terms of male mice, we noticed that VAChT KD$^{HET}$ male mice presented memory deficits in both stages; however, mutant mice performance in STM stage was not statistically significant compared to male WT mice, whereas it was statistically significant in LTM stage [MWTxMKD$^{HET}$, STM: Student's t-test: MD -0.05526, 95% CI -0.4643 to 0.3538, $t$ = 0.3194, $p$ = 0.7587; MWTxMKD$^{HET}$, LTM: Student's t-test: MD -0.3591, 95% CI -0.5401 to -0.1782, $t$ = 4.694, $p$ = 0.0022] (**Figures 7A-B**).

Lastly, comparing both sexes and genotypes, we noted that, in STM, female and male VAChT KD$^{HET}$ mice and also male VAChT WT mice presented a statistically significant poor performance compared to female WT mice [MWTxFWT, STM: Student's t-test: MD -0.3900, 95% CI -0.7798 to -8.321e-005, $t$ = 2.447, $p$ = 0.0500; MKD$^{HET}$xFKD$^{HET}$, STM: Student's t-test: MD 0.1559, 95% CI -0.3543 to 0.6660, $t$ = 0.7046, $p$ = 0.5011; FWTxMKD$^{HET}$, STM: Student's t-test: MD -0.4452, 95% CI -0.8134 to -0.07708, $t$ = 2.860, $p$ = 0.0243; MWTxFKD$^{HET}$, STM: Student's t-test: MD 0.2111, 95% CI -0.3597 to 0.7819, $t$ = 0.8746, $p$ = 0.4108; FWTxFKD$^{HET}$xMWTxMKD$^{HET}$, STM: two-way ANOVA: effect of interaction: $F$(1,14) = 3.611, $p$ = 0.0782; effect of sex $F$(1,14) = 0.6643, $p$ = 0.4286; effect of genotype: $F$(1,14) = 5.221, $p$ = 0.0384] (**Figure 7A**). In LTM female and male mutant mice presented statistically significant impaired memory in comparison to female WT mice; furthermore, male VAChT WT mice perform similarly to female VAChT KD$^{HET}$ [MWTxFWT, LTM: Student's t-test: MD -0.1506, 95% CI -0.3886 to 0.08726, $t$ = 1.549, $p$ = 0.1723; MKD$^{HET}$xFKD$^{HET}$, LTM: Student's t-test: MD -0.2223, 95% CI -0.4549 to 0.01025, $t$ = 2.204, $p$ = 0.0586; FWTxMKD$^{HET}$, LTM: Student's t-test: MD -0.5098, 95% CI -0.7688 to -0.2507, $t$ = 4.653, $p$ = 0.0023; MWTxFKD$^{HET}$, LTM: Student's t-test: MD 0.1368, 95% CI -0.07954 to 0.3531, $t$ = 1.495,

$p$ = 0.1785; FWTxFKD$^{HET}$xMWTxMKD$^{HET}$, LTM: two-way ANOVA: effect of interaction: $F(1,14)$ = 0.2523, $p$ = 0.6233; effect of sex: $F(1,14)$ = 6.829, $p$ = 0.0204; effect of genotype: $F(1,14)$ = 20.52, $p$ = 0.0005] (**Figure 7B**).

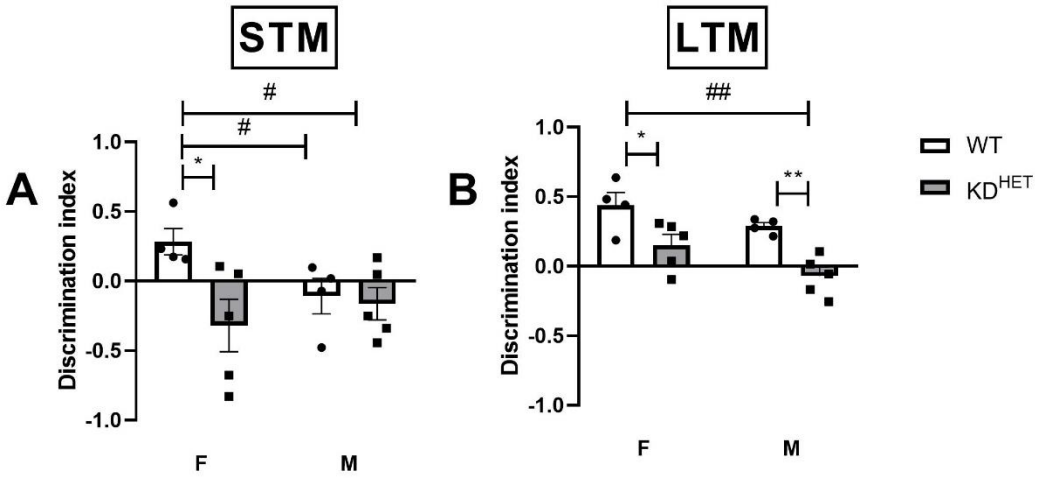


**Figure 7. Male mice present impaired object recognition in comparison to female mice. (A-B)** Discrimination index according to sex in STM **(A)** and LTM **(B)**. Data represented as mean ± S.E.M. (n = 4-5/group). White bars indicate VAChT WT mice. Gray bars indicate VAChT KD$^{HET}$ mice. (*) indicates statistically different performance when compared to WT mice. (#) indicates statistically different performance when compared to female VAChT WT mice. * p < 0.05, ** p < 0.01. # p < 0.05, ## p < 0.01.

These results suggest that mutant mice appear to have a cognitive deficit that affects their performance in NORT in both stages: STM and LTM. Also, they propose that there might have a sexual dimorphism related to memory in mice, regardless of genotype in STM, but genotype- and sex-related in LTM.

### 4.2 Mutant mice present impaired performance in the three-chamber social test

Another typical characteristic of VAChT KD mice is social memory deficits. Nevertheless, this aspect was not explored in this mouse model as the object recognition memory. Therefore, we submitted VAChT KD$^{HET}$/WT mice to the three-chamber social test (3-CST) to assess social memory impairment. The 3-CST protocol includes three stages: habituation (HAB), sociability test (ST), and social novelty preference test (SNP) (KAIDANOVICH-BEILIN et al., 2011).

In the first stage (HAB), mice were exposed to the arena without intruders, in order to avoid any previous preference for a particular chamber. Total exploration time [WTxKD$^{HET}$, HAB: Student's t-test: MD -15.50, 95% CI -76.98 to 45.98, $t$ = 0.5344, $p$ =

0.6004] (**Figure 8A**) and exploration time of each empty chamber [WT, HAB: E1xE2: Student's t-test: MD 25.29, 95% CI -27.79 to 78.37, $t$ = 1.022, $p$ = 0.3242; $KD^{HET}$, HAB: E1xE2: Student's t-test: MD 8.820, 95% CI -40.10 to 57.74, $t$ = 0.3788, $p$ = 0.7093; WTE1xWTE2x$KD^{HET}$E1x$KD^{HET}$E2, HAB: two-way ANOVA: effect of interaction: $F$(1,32) = 0.2321, $p$ = 0.6333; effect of objects $F$(1,32) = 0.9956, $p$ = 0.3259; effect of genotype: $F$(1,32) = 0.2055, $p$ = 0.6533] (**Figure 8B**) was similar in VAChT $KD^{HET}$ and VAChT WT mice. In the ST stage, 10 min after HAB, mice were presented to the first intruder (stranger 1). Both genotypes spent statistically significantly more time exploring the stranger 1 than the empty cage [WT, ST: S1xE: Student's t-test: MD 88.94, 95% CI 45.97 to 131.9, $t$ = 4.440, $p$ = 0.0006; $KD^{HET}$, ST: S1xE: MD 80.47, 95% CI 44.88 to 116.1, $t$ = 4.751, $p$ = 0.0002; WTS1xWTEx$KD^{HET}$S1x$KD^{HET}$E, ST: two-way ANOVA: effect of interaction: F(1,32) = 0.1056, p = 0.7474; effect of objects F(1,32) = 42.26, p < 0.0001; effect of genotype: F(1,32) = 0.6843, p = 0.4142] (**Figure 8D**), in spite of spending similar time exploring them [WTx$KD^{HET}$, ST: Student's t-test: MD -21.56, 95% CI -65.10 to 21.99, $t$ = 1.049, $p$ = 0.3096] (**Figure 8C**). 10 min after ST, mice were introduced to the second intruder (stranger 2). We observed that, notwithstanding the fact that both genotypes presented similar total exploration time [WTx$KD^{HET}$, SNP: Student's t-test: MD 23.54, 95% CI -21.24 to 68.31, $t$ = 1.114, $p$ = 0.2816] (**Figure 8E**), WT mice significantly spent more time exploring stranger 2, whereas mutant mice did not show preference for the novel intruder [WT, SNP: S1xS2: Student's t-test: MD 81.78, 95% CI 39.39 to 124.2, $t$ = 4.138, $p$ = 0.0010; $KD^{HET}$, SNP: S1xS2: MD 29.22, 95% CI 12.43 to 70.87, $t$ = 1.474, $p$ = 0.1578; WTS1xWTS2x$KD^{HET}$S1x$KD^{HET}$S2, SNP: two-way ANOVA: effect of interaction: $F$(1,32) = 3.432, $p$ = 0.0732; effect of objects $F$(1,32) = 15.30, $p$ = 0.0004; effect of genotype: $F$(1,32) = 0.6860, $p$ = 0.4130] (**Figure 8F**).

These results suggest that both genotypes prefer interacting with the intruder than with the empty cage (i.e., have intact sociability patterns). However, we observed that VAChT $KD^{HET}$ did not present social novelty preference, proposing that mutant mice present social memory deficits.

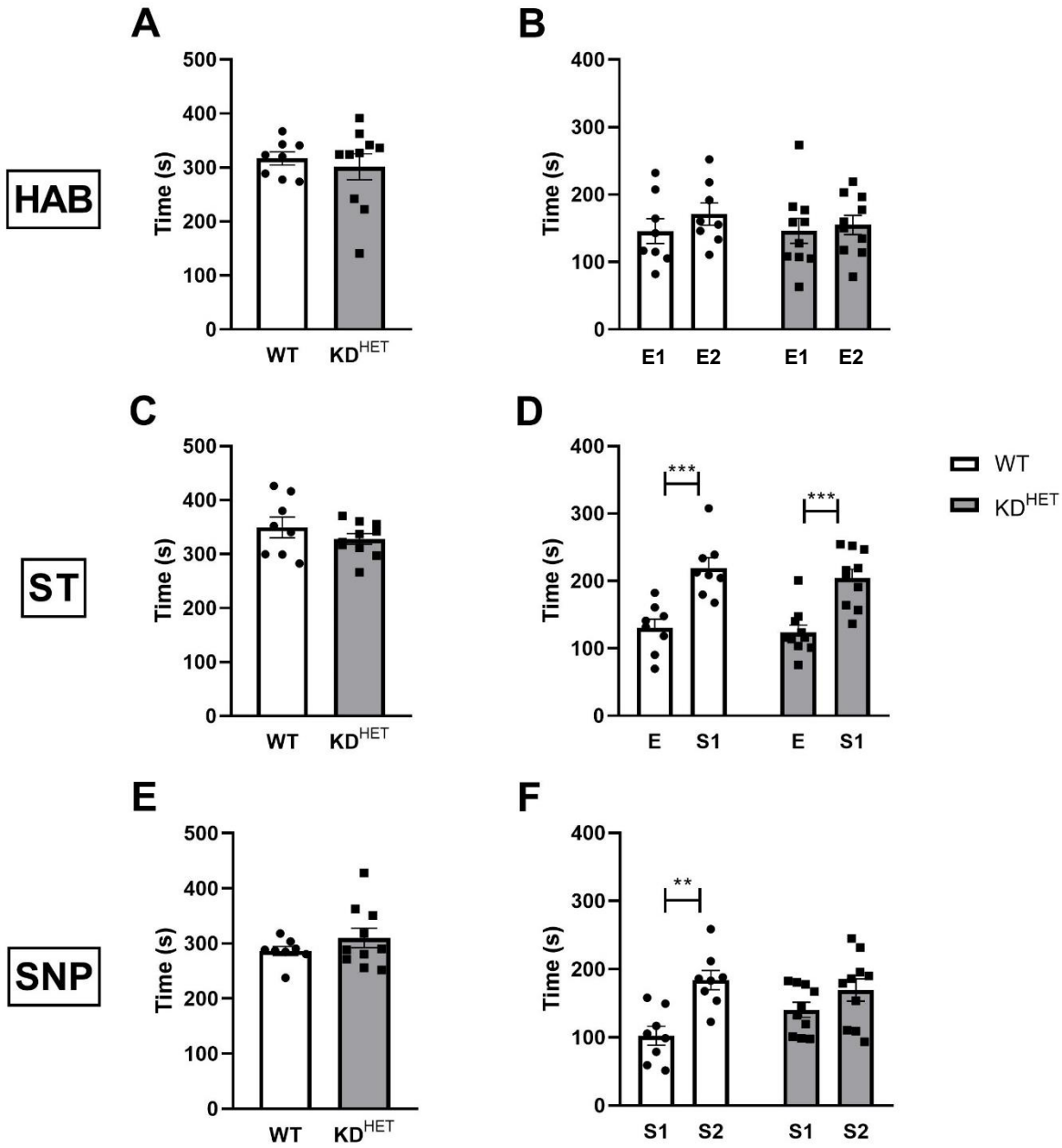


**Figure 8. VAChT KD$^{HET}$ mice present impaired performance in the three-chamber social test. (A, B)** Evaluation of HAB parameters: total exploration time **(A)** and exploration time of each empty chamber (E1 and E2) **(B)**. **(C, D)** Assessment of ST parameters: total exploration time **(C)** and exploration time of the remaining empty chamber (E) and the first intruder (S1) **(D)**. **(E, F)** Evaluation of SNP parameters: total exploration time **(E)** and exploration time of S1 and the novel conspecific (S2) **(F)**. Data represented as mean ± S.E.M. (n = 8-10/group). White bars indicate VAChT WT mice. Gray bars indicate VAChT KD$^{HET}$ mice. (*) indicates statistically different performance when compared to the exploration of S1 (in ST) or S2 (in SNP). ** $p < 0.01$, *** $p < 0.001$.

### 4.3 VAChT KD$^{HET}$ mice treated with PhKv via intracerebroventricular showed similar performance in novel object recognition task in comparison to mice treated with galantamine or both vehicles

Gomes and colleagues (2013) showed that PhKv improved STM and LTM of A$\beta_{25-35}$-treated mice (GOMES et al., 2013). Likewise, Rigo and collaborators (2017) investigated the antinociceptive effect of PhKv and suggested this phenomenon was linked to the inhibition of AChE (RIGO et al., 2017). As VAChT KD mice could present a cholinergic quantal release deficit, and PhKv inhibits AChE, which increases ACh levels in the synaptic cleft, we inquired the effects of PhKv on VAChT KD$^{HET}$ object recognition memory. Thus, we selected NORT as behavioral testing for this experiment since it is a standard test used in pharmacological behavior assays and suited our treatment protocol better (ANTUNES & BIALA, 2012).

VAChT KD$^{HET}$ mice did not demonstrate motor impairment [(SAL+PBS)x(GAL+PBS), HAB: Mann-Whitney test: MedianD -3.384, $p$ = 0.0545; (SAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: MedianD -4.801, $p$ = 0.1135; (GAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: Median D -1.417, $p$ = 0.7987; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), HAB: Kruskal-Wallis test: Kruskal-Wallis statistic 5.734, $p$ = 0.0569] (**Figure 9A**) nor anxiety-like behavior [(SAL+PBS)x(GAL+PBS), HAB: Mann-Whitney test: MedianD 2.750, $p$ = 0.4865; (SAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: MedianD -1.100, $p$ = 0.7399; (GAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: Median D -3.850, $p$ = 0.2476; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), HAB: Kruskal-Wallis test: Kruskal-Wallis statistic 1.427, $p$ = 0.4899; (SAL+PBS)x(GAL+PBS), HAB: Mann-Whitney test: MedianD -2.750, $p$ = 0.6075; (SAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: MedianD 1.100, $p$ = 0.7859; (GAL+PBS)x(SAL+PhKv), HAB: Mann-Whitney test: Median D 3.850, $p$ = 0.4428; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), HAB: Kruskal-Wallis test: Kruskal-Wallis statistic 0.7202, $p$ = 6976] (**Figures 9B-C**), as shown in HAB. 24h after HAB, mice were introduced to two similar objects (FAM). They presented similar total exploration time [(SAL+PBS)x(GAL+PBS), FAM: Student's t-test: MD -2.675, 95% CI -20.19 to 14.84, $t$ = 0.3167, $p$ = 0.7544; (SAL+PBS)x(SAL+PhKv), FAM: Student's t-test: MD 12.12, 95% CI -7.107 to 31.34, $t$ = 1.307, $p$ = 0.2046; (GAL+PBS)x(SAL+PhKv), FAM: Student's t-test: MD 14.79, 95% CI -6.080 to 35.66, $t$ = 1.470, $p$ = 0.1558; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), FAM:

one-way ANOVA: $F(2,33) = 1.442$, $p = 0.2510$] (**Figure 9D**) and did not show a preference for one of the objects [(SAL+PBS)x(GAL+PBS), FAM: Student's t-test: MD 0.04818, 95% CI -0.1128 to 0.2092, $t = 0.6242$, $p = 0.5396$; (SAL+PBS)x(SAL+PhKv), FAM: Student's t-test: MD 0.1077, 95% CI -0.04433 to 0.2598, $t = 1.4473$, $p = 0.1555$; (GAL+PBS)x(SAL+PhKv), FAM: Student's t-test: MD 0.05955, 95% CI -0.1206 to 0.2397, $t = 0.6873$, $p = 0.4994$; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), FAM: one-way ANOVA: $F(2,31) = 0.9434$, $p = 0.4002$] (**Figures 9E**). Right after FAM, mice were injected with galantamine (1 mg/kg, s.c.) or vehicle and PhKv (100 pmol/site, i.c.v.) or vehicle. To allow an appropriate recovery after the surgical procedure, mice were not submitted to the first test (STM). 24h after FAM, mice were presented to the familiar object and the novel object. We noted that all three groups, including control/sham, showed similar total exploring time [(SAL+PBS)x(GAL+PBS), LTM: Mann-Whitney test: MedianD -0.6500, $p = 0.7125$; (SAL+PBS)x(SAL+PhKv), LTM: Mann-Whitney test: MedianD 7.850, $p = 0.4428$; (GAL+PBS)x(SAL+PhKv), LTM: Mann-Whitney test: MedianD 8.500, $p = 0.1645$; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), LTM: Kruskal-Wallis test: Kruskal-Wallis statistic 1.895, $p = 0.3878$] (**Figure 9F**) and exhibited higher DI index in this stage than in FAM, indicating that mice spent more time exploring the novel object than the familiar object. We also noticed that none of the groups displayed a statistically significant memory improvement [(SAL+PBS)x(GAL+PBS), LTM: Student's t-test: MD -0.06417, 95% CI -0.3568 to 0.2285, $t = 0.4547$, $p = 0.6538$; (SAL+PBS)x(SAL+PhKv), LTM: Student's t-test: MD -0.09583, 95% CI -0.3649 to 0.1732, $t = 0.7387$, $p = 0.4679$; (GAL+PBS)x(SAL+PhKv), LTM: Student's t-test: MD -0.03167, 95% CI -0.3607 to 0.2974, $t = 0.1996$, $p = 0.8436$; (SAL+PBS)x(GAL+PBS)x(SAL+PhKv), LTM: one-way ANOVA: $F(2,33) = 0.2310$, $p = 0.7950$] (**Figure 9G**).

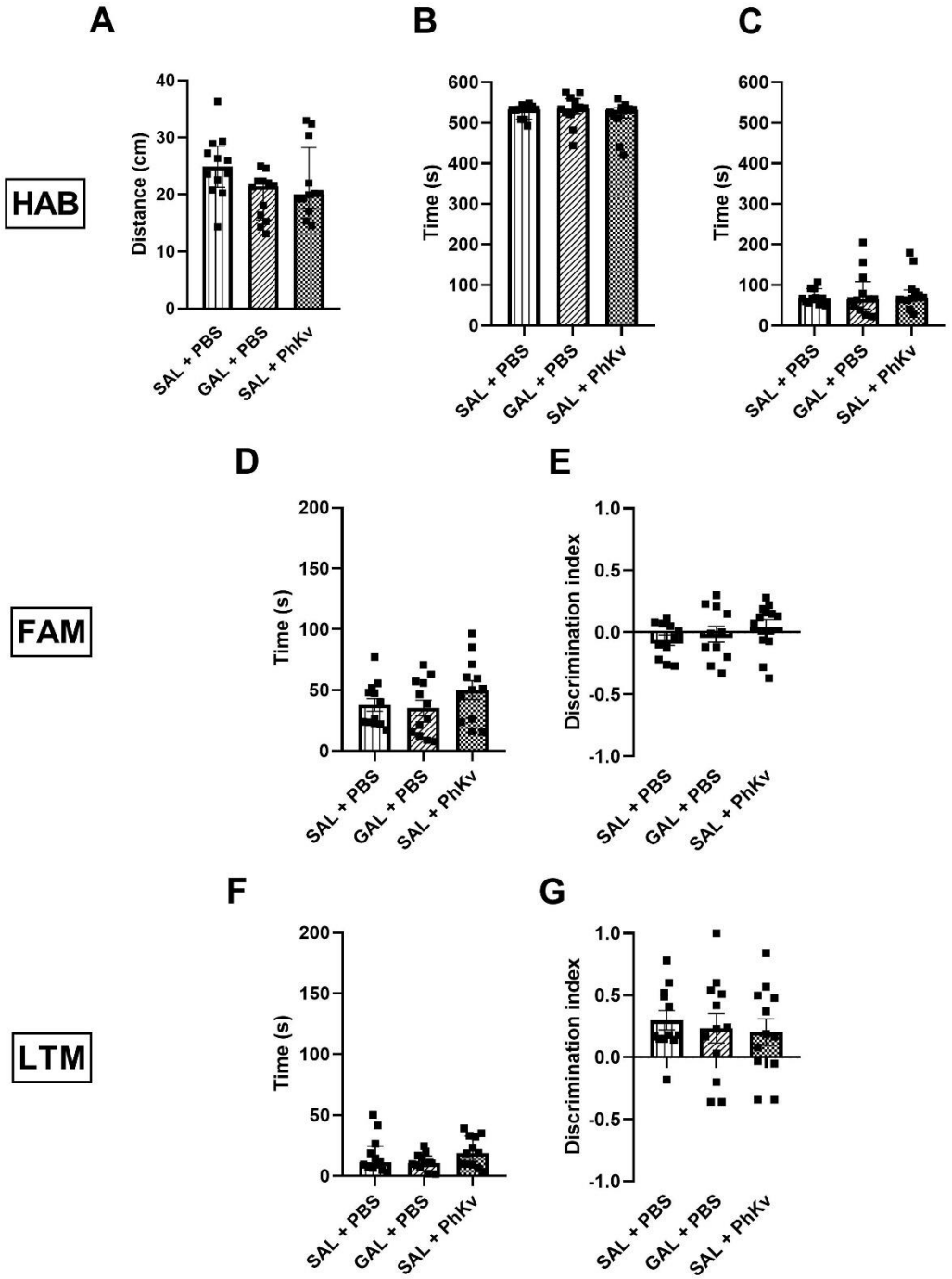


**Figure 9. Mutant mice treated with PhKv via intracerebroventricular showed similar performance in novel object recognition tasks compared to mice treated with galantamine or both vehicles. (A, B, C)** Evaluation of HAB parameters: total distance traveled **(A)**, time spent in the periphery **(B)**, and time spent in the center **(C)**. **(D, E)** Assessment of FAM parameters: total exploration time **(D)** and discrimination index **(E)**. **(F, G)** Evaluation of LTM parameters: total exploration time **(F)** and discrimination index **(G)**. Data represented as mean ± S.E.M in D, E. Data represented as median and IQR in A, B, C, F, G. (n = 12/group). Patterned bars indicate the type of treatment VAChT $KD^{HET}$ mice received: vehicle + vehicle (vertical lines), galantamine + vehicle (diagonal hatch), and vehicle + PhKv toxin (checkered).

Therefore, in order to further explore these outcomes, we compared the results of this experiment to the results of the previous NORT experiment, in which animals did not undergo surgery (naive-VAChT KD$^{HET}$ mice and naive-VAChT WT mice). We observed a statistically significant difference between the control/sham group and naive-VAChT KD$^{HET}$ mice [(SAL+PBS)xKD$^{HET}$naive, LTM: Student's t-test: MD -0.2596, 95% CI -0.4682 to -0.05106, $t$ = 2.597, $p$ = 0.0173; (SAL+PBS)xWTnaivexKD$^{HET}$naive, LTM: one-way ANOVA: $F$(2, 27) = 6.065, $p$ = 0.0067]. However, we did not note a statistically significant difference between galantamine or PhKv-treated mice and naive-VAChT KD$^{HET}$ mice [(GAL+PBS)xKD$^{HET}$naive, LTM: Student's t-test: MD -0.1955, 95% CI -0.4912 to 0.1002, $t$ = 1.379, $p$ = 0.1831; (GAL+PBS)xWTnaivexKD$^{HET}$naive, LTM: one way ANOVA: $F$(2, 27) = 4.019, $p$ = 0.0297; (SAL+PhKv)xKD$^{HET}$naive, LTM: Student's t-test: MD -0.1638, 95% CI -0.4306 to 0.1030, $t$ = 1.281, $p$ = 0.2150; (SAL+PhKv)xWTnaivexKD$^{HET}$naive, LTM: one way ANOVA: $F$(2, 27) = 3.247, $p$ = 0.0545] (**Figure 10**).

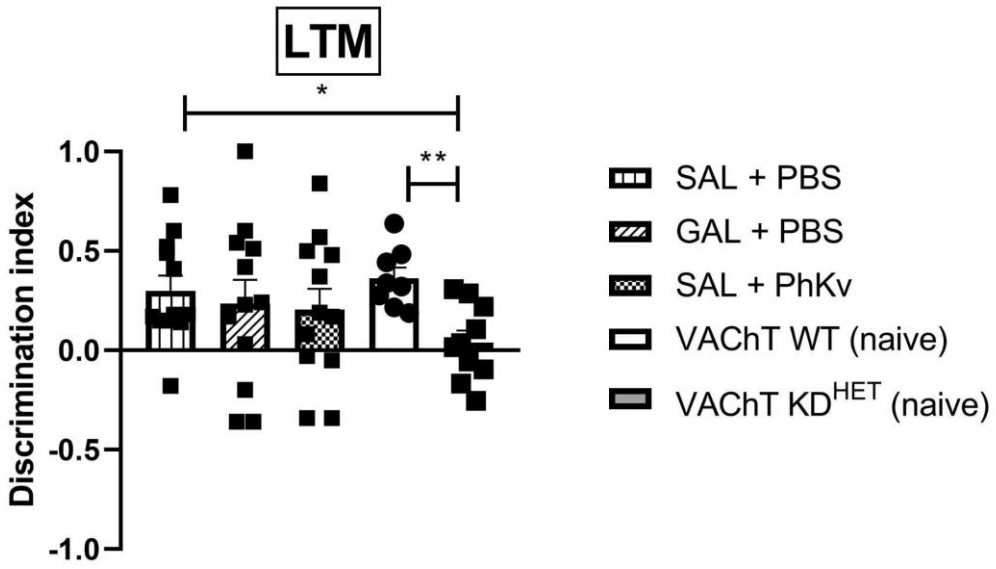


**Figure 10. VAChT KD$^{HET}$ mice treated with both vehicles perform significantly better in object recognition test compared to non-treated mutant mice.** Discrimination index of treated and non-treated VAChT KD$^{HET}$ mice. Data represented as mean ± S.E.M. (n = 8-12/group). Patterned bars indicate the type of treatment VAChT KD$^{HET}$ mice received: vehicle + vehicle (vertical lines), galantamine + vehicle (diagonal hatch), and vehicle + PhKv toxin (checkered). White bars indicate naive-VAChT WT mice. Gray bars indicate VAChT KD$^{HET}$ mice. (*) indicates statistically different performance when compared to naive-VAChT KD$^{HET}$ mice. * p <0.05, ** p < 0.01.

These results imply that PhKv toxin could not wholly reverse object recognition memory impairment in VAChT $KD^{HET}$ mice, as mutant mice treated with PhKv did not show a better performance in NORT compared to VAChT $KD^{HET}$ mice that did not undergo surgery. Mutant mice treated with galantamine also did not exhibit a statistically significant improvement of performance in NORT than VAChT $KD^{HET}$ mice that did not undergo surgery, which was unexpected. Thus, as only mice that received both vehicles, s.c. and i.c.v., presented a memory improvement compared to naive VAChT $KD^{HET}$ mice, we suggest that the surgical process might have somehow impacted their performances (e.g., anesthetics used, drugs interaction).

## 5 DISCUSSION

The VAChT KD mouse model is known for its decreased expression of VAChT protein, which could promptly reduce ACh release in the synaptic cleft. Hence, VAChT KD mice present motor or cognitive deficits, or both. Studies showed that VAChT $KD^{HET}$ mice exhibit impaired performance in social and object recognition tests, but no motor prejudice (PRADO et al., 2006; DE CASTRO et al., 2009b; CAPETTINI et al., 2011; DE JAEGER et al., 2013). In order to assess cognitive deficits in VAChT $KD^{HET}$ mice, we evaluated their performance in two behavioral assays: one for object recognition memory (NORT) and another for sociability (3-CST).

NORT was first described by Ennaceur and Delacour (1988) and is broadly used to evaluate pharmacological and neurological alterations on the memory in rodents (ENNACEUR & DELACOUR, 1988). This assay is based on two components of recognition memory: familiarity and recollection (SQUIRE et al., 2007). Access to novelty can evoke approach behaviors in rodents; therefore, mice are more likely to explore an unfamiliar object than a familiar one (LEGER et al., 2013). In NORT, mice were presented to two familiar objects and, after a delay of 90 min (STM test) or 24h (LTM test), they were introduced to a novel object and a familiar one (LUEPTOW, 2017).

Here, we showed that VAChT $KD^{HET}$ mice present STM and LTM impairment, according to previous studies (PRADO et al., 2006; DE CASTRO et al., 2009b; DE JAEGER et al., 2013). These studies did not specify the sex of mice used or only utilized male mice, a common practice in animal research (BEERY, 2018). Capettini and colleagues (2011) examined the role of sexual dimorphism on object recognition memory in VAChT $KD^{HET}$ mice. They demonstrated that female mutant mice presented intact STM and impaired LTM, whereas male VAChT $KD^{HET}$ mice exhibited both STM and LTM deficits. Nevertheless, female mutant mice that underwent ovariectomy displayed similar performance to male mutant mice. Thus, they suggested that ovarian hormones allowed STM maintenance by restoring the cholinergic network activity (CAPETTINI et al., 2011).

In our study, we did not observe that since female VAChT $KD^{HET}$ mice showed impaired recognition memory in both stages. However, we noted that female WT mice presented intact recognition memory in STM and LTM stages, whereas male WT mice

showed impaired performance in STM. The poorer male WT performance in STM might have reflected a DI closer to zero. Even though the low number of subjects in each group (n = 4-5/group) might have influenced our results, it has been shown that estrogen plays an essential role as a positive memory regulator, which could also explain our outcome (FRICK, FERNANDEZ & BULINSKI, 2002; YAN et al., 2017; RENCZES et al., 2020; TAO et al., 2020; LUO et al., 2021). Therefore, these results show the importance of including female subjects in experiments, as they could exhibit differences in behavioral performances.

Besides NORT, we also examined the performance of VAChT $KD^{HET}$ mice in a social context. Mice are inherently social animals; therefore, they tend to approach and investigate novel conspecifics (YANG, SILVERMAN & CRAWLEY, 2011). However, some mice genotypes do not present social behavior. In order to evaluate sociability and SNP aspects of VAChT $KD^{HET}$ mice, we utilized an alternative version of 3-CST (MOY et al., 2004; KAIDANOVICH-BEILIN et al., 2011). In 3-CST, mice were presented to an apparatus divided into three chambers, where an intruder restrained in a cage was placed in one of the lateral chambers, and an empty cage was set in the remaining lateral chamber (ST). After 10 min latency, mice were allowed to interact with another intruder placed in the previous empty cage (SNP).

In the present study, we observed that VAChT $KD^{HET}$ mice were as social as VAChT WT mice. We also noted that mutant mice preferred the social stimulus (stranger 1) similarly to VAChT WT mice in ST. Prado and collaborators (2006) also showed that VAChT $KD^{HET}$ mice exhibited sociability behavior similarly to VAChT WT mice, as we observed. However, in their study, mutant mice also spent as much time exploring the non-social stimulus as exploring the social stimulus (PRADO et al., 2006). Contrariwise, we did not observe this behavior pattern in our study since both genotypes explored less the non-social stimulus. As emphasized previously, we used female and male mice, which may have contributed to the results we found once estrogen receptors have been linked to modulating social memory in mice (TANG et al., 2005; SÁNCHEZ-ANDRADE & KENDRICK, 2011).

Regarding the SNP test, we noticed that mutant mice did not prefer social novelty (stranger 2), suggesting a social memory deficit. No other studies showed that SNP in a 3-CST context in VAChT $KD^{HET}$mice. Although our study suggests that VAChT $KD^{HET}$ mice present intact sociability and impaired SNP pattern, additional

investigation is needed in order to explore this subject, as social memory is a complex neurobiological feature, which hampers its investigation (LUNARDI et al., 2021).

VAChT is a crucial protein for cholinergic neurotransmission. It is responsible for loading ACh into synaptic vesicles. Likewise, VAChT limits ACh release, as it is a slow-type vesicular transporter. Thus, VAChT protein activity and its precedent expression may influence ACh release directly (PRADO et al., 2013). As ACh plays a crucial role in learning and memory function, treatments that improve ACh levels in the brain could result in increased release of ACh and consequently revert learning and memory issues (VAROQUI & ERICKSON, 1996; MICHEAU & MARIGHETTO, 2011).

An important ACh-related pharmacological target is AChE. AChE is the enzyme responsible for ACh hydrolysis, which implies the end of neurotransmission at cholinergic synapses. AChE inhibition increases ACh levels in the synaptic cleft and, as a result, enhances the duration of cholinergic neurotransmission. AChE inhibitors are frequently utilized to treat neurodegenerative disorders, particularly in AD patients, since they improve cognitive dysfunction (DVIR et al., 2010; COLOVIC et al., 2013). Rigo and colleagues (2017) showed PhKv toxin inhibitory effect over AChE in mice spinal cord, which could have reduced capsaicin nociceptive process (RIGO et al., 2017). Gomes and collaborators (2013) demonstrated that PhKv was able to ameliorate cognitive deficits in $A\beta_{25-35}$-treated mice (GOMES et al., 2013).

Therefore, we studied the potential effect of the PhKv toxin in improving memory deficits in VAChT $KD^{HET}$ mice. In NORT, we observed that PhKv-treated mutant mice did not have a statistically significant improvement of performance in comparison with galantamine (an AChE inhibitor approved for clinical use) and sham-treated animals. So, as all three groups underwent surgical processes (i.c.v. treatment), we decided to compare the performances of animals that underwent surgery and naive mice. We noted that mice treated with PhKv presented a better performance in NORT than naive-VAChT $KD^{HET}$; however, the difference between both groups was not statistically significant. Unexpectedly, only the control/sham group presented a statistically significant improvement of performance when compared to naive-VAChT $KD^{HET}$ mice.

In the surgical procedure, we utilized a mix of ketamine/xylazine and isoflurane in order to anesthetize the animals and maintain them under deep anesthesia, respectively. Although the anesthetic protocols we used are safe for mice (ADAMS & PACHARINSAK, 2015), anesthetic drugs could affect cognition. Thus, we decided to investigate the effects of each of them on memory further. Ketamine is a non-

competitive N-methyl-D-aspartate (NMDA) receptor antagonist, which has four crucial clinical therapeutic properties: anti-inflammatory, antidepressant, analgesic, and dissociative anesthetic activities (ZANOS et al., 2018). Concerning its antidepressant effects, Yang et al. (2018) showed that ketamine (5 mg/kg, i.p.) ameliorated memory dysfunction in a depression mouse model (YANG et al., 2018). Contrariwise, several studies have identified adverse effects of subanesthetic administration of ketamine on memory and learning (PITSIKAS, 2018).

In terms of object recognition in rodents, as NMDA receptors are directly involved in the formation of object recognition memory, especially in the LTM stage, ketamine may affect the performance of mice in the NORT behavioral assay (WARBURTON, BARKER, BROWN, 2013; IWAMURA, YAMADA, ICHITANI, 2016). Most authors suggested that acute or chronic treatment with a subanesthetic (lower than 80 mg/kg) or anesthetic (equal or higher than 80 mg/kg) dose of ketamine, i.p., in rodents, implied object recognition memory deficits (CHAN et al., 2008; PITSIKAS et al., 2008; PITSIKAS & BOULTADAKIS, 2009; GOULART et al., 2010; BOULTADAKIS & PITSIKAS, 2011). Fan and collaborators (2021) found that a single subanesthetic ketamine dose (10 mg/kg, i.p.) administered immediately after the second FAM stage (reactivation stage) in NORT enhanced object recognition memory in mice (FAN et al., 2021). Shi and colleagues (2021) showed a similar result in rats that performed the Morris water maze test, a behavioral assay utilized to assess spatial memory (SHI et al., 2021).

Ketamine could also facilitate ACh liberation in the hippocampus, mainly because of dopamine increase, which might help memory consolidation in rodents; however, in clinical efficient concentrations, it may as well inhibit ACh release mediated by the NMDA receptor, as demonstrated *in vitro* by Furuya and collaborators (1999). Lastly, despite presenting a weaker affinity for ACh receptors than NMDA receptor binding site, ketamine presents a direct inhibiting effect on both nicotinic and muscarinic receptors, which may impact memory (ARONSTAM, NARAYANAN & WENGER, 1982; KOHRS & DURIEUX, 1998; FURUYA et al., 1999; MION & VILLEVIEILLE, 2013).

So, the absence of consensus in the literature suggests that more pre-clinical studies should be performed in order to clarify the role of ketamine in memory. As to the other anesthetics used in our study, there was no evidence found in the literature

that the acute use of xylazine or isoflurane, or both, influences the performance of rodents in cognitive behavioral assays.

Interestingly, VAChT KD$^{HET}$ mice treated with galantamine (1 mg/kg, s.c.) did not present an improvement in LTM stage performance, contrary to what has been suggested in previous studies (PRADO et al., 2006; DE JAEGER et al., 2013). Galantamine is an AChE reversible inhibitor and an allosteric modulator of neuronal nAChR. This dual pharmacological mechanism increases cholinergic transmission in the CNS and, consequently, ameliorates cognition (RAZAY & WILCOCK, 2008). Moriguchi and colleagues (2004) showed that galantamine potentiates the actions of the NMDA receptor, which could also be partially responsible for the cognitive improvements seen in AD patients (MORIGUCHI et al., 2004). As previously seen, ketamine is a non-competitive NMDA receptor antagonist (i.e., it binds to the receptor blocking the NMDA receptor channel activity) (ZHANG et al., 2021). Therefore, both drugs could interact, reducing the effects promoted by galantamine. Nikiforuk et al. (2016) showed that a single galantamine injection (1 and 3 mg/kg, i.p.) was able to improve object recognition memory in ketamine-induced (20 mg, i.p.) schizophrenia-like rat model (NIKIFORUK et al., 2016). Nevertheless, there is no evidence in the literature about galantamine (1 mg/kg, s.c.) and ketamine (80 mg/kg, i.p.) concomitant treatment and its effects in rodents. Therefore, this plausible interaction – as well as the possible interaction between ketamine and the PhKv toxin – might be further studied.

As we displayed, only a few studies showed object recognition and social memory deficits in VAChT KD$^{HET}$ mice (PRADO et al., 2006; DE CASTRO et al., 2009b; CAPETTINI et al., 2011; DE JAEGER et al., 2013). None of them explored the mechanisms behind these impairments. Lima et al. (2010) suggested that quantal ACh content and size were reduced, which implicated in decreasing ACh release in VAChT KD mice; however, they solely used VAChT KD$^{HOM}$ mice in their studies (LIMA et al., 2010). Thus, in order to understand the results achieved in our study, we suggest an additional inquiry into the molecular mechanisms underlying the observed deficits in VAChT KD$^{HET}$ mice.

## 6 CONCLUSION

The present study showed that VAChT $KD^{HET}$ mice presented impaired object recognition memory and SNP; however, they exhibited intact sociability. We also demonstrated that mutant mice treated with PhKv showed similar performance in this behavioral assay compared to VAChT $KD^{HET}$ mice treated with galantamine or both vehicles. Furthermore, we presented that the sham treatment could improve LTM, as the control/sham group displayed a statistically significant improvement in DI compared to non-treated (naive) mutant mice. Nevertheless, PhKv treatment did not evoke a memory improvement as seen in control/sham animals. Hence, we suggest that the surgical procedure might have impacted our results. We also propose that the mechanisms behind VAChT $KD^{HET}$ mice cognitive impairments might be more explored. Lastly, in order to elucidate the potential neuroprotective effect of PhKv toxin in cognitive deficits mouse models, we suggest that less invasive routes of administration would be considered in future studies, such as intravenous.

## REFERENCES


Adams, S., & Pacharinsak, C. (2015). Mouse anesthesia and analgesia. *Current Protocols in Mouse Biology*, *5*(1), 51–63. https://doi.org/10.1002/9780470942390.MO140179

Almeida, A. P., Andrade, A. B., Ferreira, A. J., Pires, A. C. G., Damasceno, D. D., Alves, M. N. M., Gomes, E. R. M., Kushmerick, C., Lima, R. F., Prado, M. A. M., Prado, V. F., Richardson, M., Cordeiro, M. N., Guatimosim, S., & Gomez, M. V. (2011). Antiarrhythmogenic effects of a neurotoxin from the spider *Phoneutria nigriventer*. *Toxicon: Official Journal of the International Society on Toxinology*, *57*(2), 217–224. https://doi.org/10.1016/J.TOXICON.2010.11.013

Antunes, M., & Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive Processing*, *13*(2), 93. https://doi.org/10.1007/S10339-011-0430-Z

Aronstam, R. S., Narayanan, L., & Wenger, D. A. (1982). Ketamine inhibition of ligand binding to cholinergic receptors and ion channels. *European Journal of Pharmacology*, *78*(3), 367–370. https://doi.org/10.1016/0014-2999(82)90040-1

Arvidsson, U., Riedl, M., Elde, R., & Bjorn M. (1997). Vesicular Acetylcholine Transporter (VAChT) Protein: A Novel and Unique Marker for Cholinergic Neurons in the Central and Peripheral Nervous Systems. *The Journal of Comparative Neurology*, *378*, 454–467. https://doi.org/10.1002/(SICI)1096-9861(19970224)378:4<454::AID-CNE2>3.0.CO;2-1

Bartus, R. T., Dean, R. L., Beer, B., & Lippa, A. S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science (New York, N.Y.)*, *217*(4558), 408–417. https://doi.org/10.1126/SCIENCE.7046051

Beery, A. K. (2018). Inclusion of females does not increase variability in rodent research studies. *Current Opinion in Behavioral Sciences*, *23*, 143. https://doi.org/10.1016/J.COBEHA.2018.06.016

Bicks, L. K., Koike, H., Akbarian, S., & Morishita, H. (2015). Prefrontal Cortex and Social Cognition in Mouse and Man. *Frontiers in Psychology*, *6*(NOV). https://doi.org/10.3389/FPSYG.2015.01805

Borges, R., & García, A. G. (2021). One hundred years from Otto Loewi experiment, a dream that revolutionized our view of neurotransmission. *Pflügers Archiv - European Journal of Physiology 2021 473:6*, *473*(6), 977–981. https://doi.org/10.1007/S00424-021-02580-9

Boultadakis, A., & Pitsikas, N. (2011). Anesthetic Ketamine Impairs Rats' Recall of Previous InformationThe Nitric Oxide Synthase Inhibitor N-nitro-l-arginine Methylester Antagonizes This Ketamine-induced Recognition Memory Deficit. *Anesthesiology*, *114*(6), 1345–1353. https://doi.org/10.1097/ALN.0B013E318219524E

Capettini, S. B., Moraes, M. F. D., Prado, V. F., Prado, M. A. M., & Pereira, G. S. (2011). Vesicular acetylcholine transporter knockdown mice show sexual dimorphism on memory. *Brain Research Bulletin*, *85*(1–2), 54–57. https://doi.org/10.1016/J.BRAINRESBULL.2011.02.005

Carvajal, F. J., & Inestrosa, N. C. (2011). Interactions of AChE with Aβ Aggregates in Alzheimer's Brain: Therapeutic Relevance of IDN 5706. *Frontiers in Molecular Neuroscience*, *4*. https://doi.org/10.3389/FNMOL.2011.00019

Chan, M. H., Chiu, P. H., Sou, J. H., & Chen, H. H. (2008). Attenuation of ketamine-evoked behavioral responses by mGluR5 positive modulators in mice. *Psychopharmacology 2008 198:1*, *198*(1), 141–148. https://doi.org/10.1007/S00213-008-1103-1

Chen, G. F., Xu, T. H., Yan, Y., Zhou, Y. R., Jiang, Y., Melcher, K., & Xu, H. E. (2017). Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacologica Sinica 2017 38:9*, *38*(9), 1205–1235. https://doi.org/10.1038/aps.2017.28

Colovic, M. B., Krstic, D. Z., Lazarevic-Pasti, T. D., Bondzic, A. M., & Vasic, V. M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology*, *11*(3), 315. https://doi.org/10.2174/1570159X11311030006

Cordeiro, M. do N., de Figueiredo, S. G., Valentim, A. do C., Diniz, C. R., von Eickstedt, V. R. D., Gilroy, J., & Richardson, M. (1993). Purification and amino acid sequences of six Tx3 type neurotoxins from the venom of the Brazilian "armed" spider *Phoneutria nigriventer* (Keys). *Toxicon: Official Journal of the International Society on Toxinology*, *31*(1), 35–42. https://doi.org/10.1016/0041-0101(93)90354-L

Cordeiro, M. D. N., Richardson, M., Gilroy, J., de Figueiredo, S. G., Beirão, P. S. L., & Diniz, C. R. (1995). Properties of the Venom from the South American ''Armed'' Spider *Phoneutria nigriventer* (Keyserling, 1891). *Journal of Toxicology-toxin Review*, *14*(3), 309–326. https://doi.org/10.3109/15569549509019466

de Castro, B. M., de Jaeger, X., Martins-Silva, C., Lima, R. D. F., Amaral, E., Menezes, C., Lima, P., Neves, C. M. L., Pires, R. G., Gould, T. W., Welch, I., Kushmerick, C., Guatimosim, C., Izquierdo, I., Cammarota, M., Rylett, R. J., Gomez, M. v., Caron, M. G., Oppenheim, R. W., … Prado, V. F. (2009a). The vesicular acetylcholine transporter is required for neuromuscular development and function. *Molecular and Cellular Biology*, *29*(19), 5238–5250. https://doi.org/10.1128/MCB.00245-09

de Castro, B. M., Pereira, G. S., Magalhães, V., Rossato, J. I., de Jaeger, X., Martins-Silva, C., Leles, B., Lima, P., Gomez, M. v., Gainetdinov, R. R., Caron, M. G., Izquierdo, I., Cammarota, M., Prado, V. F., & Prado, M. A. M. (2009b). Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. *Genes, Brain, and Behavior*, *8*(1), 23–35. https://doi.org/10.1111/J.1601-183X.2008.00439.X

de Lima, M. E., Figueiredo, S. G., Matavel, A., Nunes, K. P., da Silva, C. N., de Marco Almeida, F., Diniz, M. R. V., Cordeiro, M. do N., Stankiewicz, M., & Beirão, P. S. L. (2015). *Phoneutria nigriventer* Venom and Toxins: A Review. *Spider Venoms*, 71–99. https://doi.org/10.1007/978-94-007-6389-0_6

de Jaeger, X., Cammarota, M., Prado, M. A. M., Izquierdo, I., Prado, V. F., & Pereira, G. S. (2013). Decreased acetylcholine release delays the consolidation of object recognition memory. *Behavioural Brain Research*, *238*(1), 62–68. https://doi.org/10.1016/J.BBR.2012.10.016

Deutch, A. Y., & Roth, R. H. (2004). Pharmacology and Biochemistry of Synaptic Transmission: Classic Transmitters. *From Molecules to Networks*, 245–278. https://doi.org/10.1016/B978-012148660-0/50010-X

Dvir, H., Silman, I., Harel, M., Rosenberry, T. L., & Sussman, J. L. (2010). Acetylcholinesterase: From 3D Structure to Function. *Chemico-Biological Interactions*, *187*(1–3), 10. https://doi.org/10.1016/J.CBI.2010.01.042

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. *Behavioural Brain Research*, *31*(1), 47–59. https://doi.org/10.1016/0166-4328(88)90157-X

Ewins, A. J. (1914). Acetylcholine, a New Active Principle of Ergot. *Biochemical Journal*, *8*(1), 44–49. https://doi.org/10.1042/BJ0080044

Fan, J. F., Tang, Z. H., Wang, S. Y., Lei, S., Zhang, B., & Tian, S. W. (2021). Ketamine enhances novel object recognition memory reconsolidation via the BDNF/TrkB pathway in mice. *Physiology & Behavior*, *242*. https://doi.org/10.1016/J.PHYSBEH.2021.113626

Francis, P. T., Palmer, A. M., Snape, M., & Wilcock, G. K. (1999). The cholinergic hypothesis of Alzheimer's disease: a review of progress. *Journal of Neurology, Neurosurgery, and Psychiatry*, *66*(2), 137. https://doi.org/10.1136/JNNP.66.2.137

Frick, K. M., Fernandez, S. M., & Bulinski, S. C. (2002). Estrogen replacement improves spatial reference memory and increases hippocampal synaptophysin in aged female mice. *Neuroscience*, *115*(2), 547–558. https://doi.org/10.1016/S0306-4522(02)00377-9

Furuya, R., Oka, K., Watanabe, I., Kamiya, Y., Itoh, H., & Andoh, T. (1999). The Effects of Ketamine and Propofol on Neuronal Nicotinic Acetylcholine Receptors and P2x Purinoceptors in PC12 Cells. *Anesthesia & Analgesia*, *88*(1), 174–180. https://doi.org/10.1213/00000539-199901000-00033

García-Ayllón, M. S., Small, D. H., Avila, J., & Sáez-Valero, J. (2011). Revisiting the role of acetylcholinesterase in Alzheimer's disease: Cross-talk with β-tau and p-amyloid. *Frontiers in Molecular Neuroscience*, *4*(SEP), 22. https://doi.org/10.3389/FNMOL.2011.00022/BIBTEX

Gomes, G. M., Dalmolin, G. D., Cordeiro, M. do N., Gomez, M. v., Ferreira, J., & Rubin, M. A. (2013). The selective A-type $K^+$ current blocker Tx3-1 isolated from the *Phoneutria nigriventer* venom enhances memory of naïve and $A\beta_{(25-35)}$-treated mice. *Toxicon: Official Journal of the International Society on Toxinology*, *76*, 23–27. https://doi.org/10.1016/J.TOXICON.2013.08.059

Gomez, M. V., Kalapothakis, E., Guatimosim, C., & Prado, M. A. M. (2002). *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cellular and Molecular Neurobiology*, *22*(5–6), 579–588. https://doi.org/10.1023/A:1021836403433

Goulart, B. K., de Lima, M. N. M., de Farias, C. B., Reolon, G. K., Almeida, V. R., Quevedo, J., Kapczinski, F., Schröder, N., & Roesler, R. (2010). Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. *Neuroscience*, *167*(4), 969–973. https://doi.org/10.1016/J.NEUROSCIENCE.2010.03.032

Haass, C., & Selkoe, D. J. (1993). Cellular processing of β-amyloid precursor protein and the genesis of amyloid β-peptide. *Cell*, *75*(6), 1039–1042. https://doi.org/10.1016/0092-8674(93)90312-E

Iwamura, E., Yamada, K., & Ichitani, Y. (2016). Involvement of hippocampal NMDA receptors in retrieval of spontaneous object recognition memory in rats. *Behavioural Brain Research*, *307*, 92–99. https://doi.org/10.1016/J.BBR.2016.03.048

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., & Woodgett, J. R. (2011). Assessment of Social Interaction Behaviors. *Journal of Visualized Experiments: JoVE*, *48*, 2473. https://doi.org/10.3791/2473

Karantzoulis, S., & Galvin, J. E. (2011). Distinguishing Alzheimer's disease from other major forms of dementia. *Expert Review of Neurotherapeutics*, *11*(11), 1579. https://doi.org/10.1586/ERN.11.155

Kohrs, R., & Durieux, M. E. (1998). Ketamine. *Anesthesia & Analgesia*, *87*(5), 1186–1193. https://doi.org/10.1213/00000539-199811000-00039

Kolisnyk, B., Al-Onaizi, M. A., Hirata, P. H. F., Guzman, M. S., Nikolova, S., Barbash, S., Soreq, H., Bartha, R., Prado, M. A. M., & Prado, V. F. (2013). Forebrain deletion of the vesicular acetylcholine transporter results in deficits in executive function, metabolic, and RNA splicing abnormalities in the prefrontal cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(37), 14908–14920. https://doi.org/10.1523/JNEUROSCI.1933-13.2013

Kushmerick, C., Kalapothakis, E., Beirão, P. S. L., Penaforte, C. L., Prado, V. F., Cruz, J. S., Diniz, C. R., Cordeiro, M. N., Gomez, M. v., Romano-Silva, M. A., & Prado, M. A. M. (1999). *Phoneutria nigriventer* toxin Tx3-1 blocks A-type $K^+$ currents controlling $Ca^{2+}$ oscillation frequency in GH3 cells. *Journal of Neurochemistry*, *72*(4), 1472–1481. https://doi.org/10.1046/J.1471-4159.1999.721472.X

Lawal, H. O., & Krantz, D. E. (2013). SLC18: Vesicular neurotransmitter transporters for monoamines and acetylcholine. *Molecular Aspects of Medicine*, *34*(0), 360. https://doi.org/10.1016/J.MAM.2012.07.005

Leger, M., Quiedeville, A., Bouet, V., Haelewyn, B., Boulouard, M., Schumann-Bard, P., & Freret, T. (2013). Object recognition test in mice. *Nature Protocols*, *8*(12), 2531–2537. https://doi.org/10.1038/NPROT.2013.155

Lima, R. D. F. de, Prado, V. F., Prado, M. A. M., & Kushmerick, C. (2010). Quantal release of acetylcholine in mice with reduced levels of the vesicular acetylcholine transporter. *Journal of Neurochemistry*, *113*(4), 943. https://doi.org/10.1111/J.1471-4159.2010.06657.X

Loewi, O. (1921). Über humorale übertragbarkeit der Herznervenwirkung. *Pflüger's Archiv Für Die Gesamte Physiologie des Menschen und der Tiere 1921 189:1*, *189*(1), 239–242. https://doi.org/10.1007/BF01738910

Lueptow, L. M. (2017). Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. *Journal of Visualized Experiments: JoVE*, *2017*(126), 55718. https://doi.org/10.3791/55718

Lunardi, P., Mansk, L. M. Z., Jaimes, L. F., & Pereira, G. S. (2021). On the novel mechanisms for social memory and the emerging role of neurogenesis. *Brain Research Bulletin*, *171*, 56–66. https://doi.org/10.1016/J.BRAINRESBULL.2021.03.006

Luo, M., Zeng, Q., Jiang, K., Zhao, Y., Long, Z., Du, Y., Wang, K., & He, G. (2021). Estrogen deficiency exacerbates learning and memory deficits associated with glucose metabolism disorder in APP/PS1 double transgenic female mice. *Genes & Diseases*. https://doi.org/10.1016/J.GENDIS.2021.01.007

Magalhães-Gomes, M. P. S., Motta-Santos, D., Schetino, L. P. L., Andrade, J. N., Bastos, C. P., Guimarães, D. A. S., Vaughan, S. K., Martinelli, P. M., Guatimosim, S., Pereira, G. S., Coimbra, C. C., Prado, V. F., Prado, M. A. M., Valdez, G., & Guatimosim, C. (2018). Fast and slow-twitching muscles are differentially affected by reduced cholinergic transmission in mice deficient for VAChT: A mouse model for congenital myasthenia. *Neurochemistry International*, *120*, 1–12. https://doi.org/10.1016/J.NEUINT.2018.07.002

Magno, L. A. V., Tenza-Ferrer, H., Collodetti, M., Aguiar, M. F. G., Rodrigues, A. P. C., da Silva, R. S., do Prado Silva, J., Nicolau, N. F., Rosa, D. V. F., Birbrair, A., Miranda, D. M., & Romano-Silva, M. A. (2019). Optogenetic Stimulation of the M2 Cortex Reverts Motor Dysfunction in a Mouse Model of Parkinson's Disease. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *39*(17), 3234–3248. https://doi.org/10.1523/JNEUROSCI.2277-18.2019

Micheau, J., & Marighetto, A. (2011). Acetylcholine and memory: a long, complex and chaotic but still living relationship. *Behavioural Brain Research*, *221*(2), 424–429. https://doi.org/10.1016/J.BBR.2010.11.052

Ministério da Saúde. Secretaria de Atenção à Saúde. *Portaria Conjunta Nº 13, de 28 de novembro de 2017*. Retrieved Jan. 17, 2022, from http://conitec.gov.br/images/Relatorios/Portaria/2020/Portaria-Conjunta-13-PCDT-Alzheimer-atualizada-em-20-05-2020.pdf

Mion, G., & Villevieille, T. (2013). Ketamine Pharmacology: An Update (Pharmacodynamics and Molecular Aspects, Recent Findings). *CNS Neuroscience & Therapeutics*, *19*(6), 370. https://doi.org/10.1111/CNS.12099

Moriguchi, S., Marszalec, W., Zhao, X., Yeh, J. Z., & Narahashi, T. (2004). Mechanism of action of galantamine on N-methyl-D-aspartate receptors in rat cortical neurons. *The Journal of Pharmacology and Experimental Therapeutics*, *310*(3), 933–942. https://doi.org/10.1124/JPET.104.067603

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., Piven, J., & Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes, Brain, and Behavior*, *3*(5), 287–302. https://doi.org/10.1111/J.1601-1848.2004.00076.X

Nikiforuk, A., Kos, T., Hołuj, M., Potasiewicz, A., & Popik, P. (2016). Positive allosteric modulators of alpha 7 nicotinic acetylcholine receptors reverse ketamine-induced schizophrenia-like deficits in rats. *Neuropharmacology*, *101*, 389–400. https://doi.org/10.1016/J.NEUROPHARM.2015.07.034

National Institute on Aging. *How Is Alzheimer's Disease Treated?* (2021). Retrieved January 17, 2022, from https://www.nia.nih.gov/health/how-alzheimers-disease-treated

Newman, E. L., Gupta, K., Climer, J. R., Monaghan, C. K., & Hasselmo, M. E. (2012). Cholinergic modulation of cognitive processing: Insights drawn from computational models. *Frontiers in Behavioral Neuroscience*, *0*(JUNE), 24. https://doi.org/10.3389/FNBEH.2012.00024/BIBTEX

Nguyen, M. L., Cox, G. D., & Parsons, S. M. (1998). Kinetic parameters for the vesicular acetylcholine transporter: Two protons are exchanged for one acetylcholine. *Biochemistry*, *37*(38), 13400–13410. https://doi.org/10.1021/BI9802263/SUPPL_FILE/BI13400A.PDF

NobelPrize.org. *The Nobel Prize in Physiology or Medicine 1936*. Retrieved Feb.18, 2022, from https://www.nobelprize.org/prizes/medicine/1936/summary/

Parsons, S. M. (2000). Transport mechanisms in acetylcholine and monoamine storage. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *14*(15), 2423–2434. https://doi.org/10.1096/FJ.00-0203REV

Peigneur, S., de Lima, M. E., & Tytgat, J. (2018). *Phoneutria nigriventer* venom: A pharmacological treasure. *Toxicon*, *151*, 96–110. https://doi.org/10.1016/J.TOXICON.2018.07.008

Picciotto, M. R., Higley, M. J., & Mineur, Y. S. (2012). Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. *Neuron*, *76*(1), 116. https://doi.org/10.1016/J.NEURON.2012.08.036

Pitsikas, N., Boultadakis, A., & Sakellaridis, N. (2008). Effects of sub-anesthetic doses of ketamine on rats' spatial and non-spatial recognition memory. *Neuroscience*, *154*(2), 454–460. https://doi.org/10.1016/J.NEUROSCIENCE.2008.04.001

Pitsikas, N., & Boultadakis, A. (2009). Pre-training administration of anesthetic ketamine differentially affects rats' spatial and non-spatial recognition memory. *Neuropharmacology*, *57*(1), 1–7. https://doi.org/10.1016/J.NEUROPHARM.2009.03.015

Pitsikas, N. (2018). The Role of Ketamine in Object Recognition Memory in Rodents. *Handbook of Behavioral Neuroscience*, *27*, 553–560. https://doi.org/10.1016/B978-0-12-812012-5.00036-7

Prado, V. F., Martins-Silva, C., de Castro, B. M., Lima, R. F., Barros, D. M., Amaral, E., Ramsey, A. J., Sotnikova, T. D., Ramirez, M. R., Kim, H. G., Rossato, J. I., Koenen, J., Quan, H., Cota, V. R., Moraes, M. F. D., Gomez, M. v., Guatimosim, C., Wetsel, W. C., Kushmerick, C., …, Prado, M. A. M. (2006). Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. *Neuron*, *51*(5), 601–612. https://doi.org/10.1016/J.NEURON.2006.08.005

Prado, V. F., Roy, A., Kolisnyk, B., Gros, R., & Prado, M. A. M. (2013). Regulation of cholinergic activity by the vesicular acetylcholine transporter. *The Biochemical Journal*, *450*(2), 265–274. https://doi.org/10.1042/BJ20121662

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A.-S., McNamara, J. O., & Williams, S. M. (2001). *Acetylcholine*. https://www.ncbi.nlm.nih.gov/books/NBK11143/

Razay, G., & Wilcock, G. K. (2008). Galantamine in Alzheimer's disease. *Expert Review of Neurotherapeutics*, *8*(1), 9–17. https://doi.org/10.1586/14737175.8.1.9

Renczes, E., Borbelyova, V., Steinhardt, M., Hopfner, T., Stehle, T., Ostatníková, D., & Celec, P. (2020). The Role of Estrogen in Anxiety-Like Behavior and Memory of Middle-Aged Female Rats. *Frontiers in Endocrinology*, *11*, 734. https://doi.org/10.3389/FENDO.2020.570560/BIBTEX

Rees, T. M., & Brimijoin, S. (2003). The role of acetylcholinesterase in the pathogenesis of Alzheimer's disease. *Drugs of Today (Barcelona, Spain: 1998)*, *39*(1), 75–83. https://doi.org/10.1358/DOT.2003.39.1.740206

Rees, T., Hammond, P. I., Soreq, H., Younkin, S., & Brimijoin, S. (2003). Acetylcholinesterase promotes beta-amyloid plaques in cerebral cortex. *Neurobiology of Aging*, *24*(6), 777–787. https://doi.org/10.1016/S0197-4580(02)00230-0

Rezende-Júnior L., Cordeiro M.N., Oliveira E.B., Diniz C.R. Isolation of neurotoxic peptides from the venom of the 'armed' spider *Phoneutria nigriventer*. (1991) *Toxicon, 29*(10), 1225-33. https://doi: 10.1016/0041-0101(91)90195-w. PMID: 1801316.

Rigo, F. K., Rossato, M. F., Trevisan, G., de Prá, S. D. T., Ineu, R. P., Duarte, M. B., de Castro Junior, C. J., Ferreira, J., & Gomez, M. V. (2017). PhKv a toxin isolated from the spider venom induces antinociception by inhibition of cholinesterase activating cholinergic system. *Scandinavian Journal of Pain*, *17*, 203–210. https://doi.org/10.1016/J.SJPAIN.2017.09.019

Sabbagh, M. N., Hendrix, S., & Harrison, J. E. (2019). FDA position statement "Early Alzheimer's disease: Developing drugs for treatment, Guidance for Industry." *Alzheimer's & Dementia (New York, N. Y.)*, *5*, 13–19. https://doi.org/10.1016/J.TRCI.2018.11.004

Sánchez-Andrade, G., & Kendrick, K. M. (2011). Roles of α- and β-estrogen receptors in mouse social recognition memory: effects of gender and the estrous cycle. *Hormones and Behavior*, *59*(1), 114–122. https://doi.org/10.1016/J.YHBEH.2010.10.016

Scheltens, P., Blennow, K., Breteler, M. M. B., de Strooper, B., Frisoni, G. B., Salloway, S., & van der Flier, W. M. (2016). Alzheimer's disease. *Lancet (London, England)*, *388*(10043), 505–517. https://doi.org/10.1016/S0140-6736(15)01124-1

Shi, M., Ding, J., Li, L., Bai, H., Li, X., Lan, L., Fan, H., & Gao, L. (2021). Effects of Ketamine on Learning and Memory in the Hippocampus of Rats through ERK, CREB, and Arc. *Brain Sciences*, *11*(1), 1–11. https://doi.org/10.3390/BRAINSCI11010027

Squire, L. R., Wixted, J. T., & Clark, R. E. (2007). Recognition memory and the medial temporal lobe: a new perspective. *Nature Reviews. Neuroscience*, *8*(11), 872. https://doi.org/10.1038/NRN2154

Soreq, H., & Seidman, S. (2001). Acetylcholinesterase--new roles for an old actor. *Nature Reviews. Neuroscience*, *2*(4), 294–302. https://doi.org/10.1038/35067589

Tang, A. C., Nakazawa, M., Romeo, R. D., Reeb, B. C., Sisti, H., & McEwen, B. S. (2005). Effects of long-term estrogen replacement on social investigation and social memory in ovariectomized C57BL/6 mice. *Hormones and Behavior*, *47*(3), 350–357. https://doi.org/10.1016/J.YHBEH.2004.10.010

Tao, X., Yan, M., Wang, L., Zhou, Y., Wang, Z., Xia, T., Liu, X., Pan, R., & Chang, Q. (2020). Effects of estrogen deprivation on memory and expression of related proteins in ovariectomized mice. *Annals of Translational Medicine*, *8*(6), 356–356. https://doi.org/10.21037/ATM.2020.02.57

Tarawneh, R., & Holtzman, D. M. (2012). The Clinical Problem of Symptomatic Alzheimer Disease and Mild Cognitive Impairment. *Cold Spring Harbor Perspectives in Medicine*, *2*(5). https://doi.org/10.1101/CSHPERSPECT.A006148

Tansey, E. M. (2006). Henry Dale and the discovery of acetylcholine. *Comptes Rendus Biologies*, *329*(5–6), 419–425. https://doi.org/10.1016/J.CRVI.2006.03.012

Trang, A., & Khandhar, P. B. (2021). Physiology, Acetylcholinesterase. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK539735/

Vardy, E., Arkin, I. T., Gottschalk, K. E., Kaback, H. R., & Schuldiner, S. (2004). Structural conservation in the major facilitator superfamily as revealed by comparative modeling. *Protein Science: A Publication of the Protein Society*, *13*(7), 1832–1840. https://doi.org/10.1110/PS.04657704

Varoqui, H., & Erickson, J. D. (1996). Active Transport of Acetylcholine by the Human Vesicular Acetylcholine Transporter. *Journal of Biological Chemistry*, *271*(44), 27229–27232. https://doi.org/10.1074/JBC.271.44.27229

Warburton, E. C., Barker, G. R. I., & Brown, M. W. (2013). Investigations into the involvement of NMDA mechanisms in recognition memory. *Neuropharmacology*, *74*, 41–47. https://doi.org/10.1016/J.NEUROPHARM.2013.04.013

Yan, Y., Cheng, L., Chen, X., Wang, Q., Duan, M., Ma, J., Zhao, L., Jiang, X., Ai, J., Yan, Y., Cheng, L., Chen, X., Wang, Q., Duan, M., Ma, J., Zhao, L., Jiang, X., & Ai, J. (2017). Estrogen deficiency is associated with hippocampal morphological remodeling of early postmenopausal mice. *Oncotarget*, *8*(13), 21892–21902. https://doi.org/10.18632/ONCOTARGET.15702

Yang, M., Silverman, J. L., & Crawley, J. N. (2011). Automated Three-Chambered Social Approach Task for Mice. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N. Crawley ... [et Al.]*, *CHAPTER 8*(SUPPL. 56), Unit. https://doi.org/10.1002/0471142301.NS0826S56

Yang, Y., Ju, W., Zhang, H., & Sun, L. (2018). Effect of ketamine on LTP and NMDAR EPSC in hippocampus of the chronic social defeat stress mice model of depression. *Frontiers in Behavioral Neuroscience*, *12*, 229. https://doi.org/10.3389/FNBEH.2018.00229/BIBTEX

Zanos, P., Moaddel, R., Morris, P. J., Riggs, L. M., Highland, J. N., Georgiou, P., Pereira, E. F. R., Albuquerque, E. X., Thomas, C. J., Zarate, C. A., & Gould, T. D. (2018). Ketamine and Ketamine Metabolite Pharmacology: Insights into Therapeutic Mechanisms. *Pharmacological Reviews*, *70*(3), 621. https://doi.org/10.1124/PR.117.015198

Zhang, Y., Ye, F., Zhang, T., Lv, S., Zhou, L., Du, D., Lin, H., Guo, F., Luo, C., & Zhu, S. (2021). Structural basis of ketamine action on human NMDA receptors. *Nature*, *596:7871, 596*(7871), 301–305. https://doi.org/10.1038/s41586-021-03769-9