Universidade de Lisboa

Faculdade de Farmácia

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Catarina Francisco Alves Tavares

Dissertation supervised by Professor Aliz Judit Ernyey and co-supervised by Professor Cecília Maria Pereira Rodrigues

Mestrado em Ciências Biofarmacêuticas

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Resumo

A escopolamina, um composto anticolinérgico, é frequentemente aplicada como modelo farmacológico para debilitar funções cognitivas. É usado principalmente em dose única e em animais jovens. O objetivo deste estudo foi investigar os efeitos do tratamento crónico com escopolamina em várias funções cognitivas em ratos experientes. Também foi testado até que ponto o donepezil pode melhorar o deficite cognitivo.

Ratos da espécie Long-Evans de 8,5 meses de idade foram treinados regularmente em paradigmas como 5CSRTT (medindo a atenção), MWM (aprendizado espacial), Pot jumping (aprendizado da função motora), discriminação em tela sensível ao toque de caixa (TS) (aprendizagem visual) e uma tarefa de cooperação (aprendizagem social). Após as medições iniciais, os ratos foram distribuídos aleatoriamente em três grupos de tratamento: solução salina, escopolamina (0,3 mg/kg) e escopolamina+donepezil (3 mg/kg). Os grupos de salina e escopolamina receberam tratamento via ip por 20 dias, o grupo escopolamina+donepezil foi injetado com escopolamina por 10 dias e então escopolamina e donepezil durante os 10 dias seguintes.

A escopolamina prejudicou significativamente o desempenho em 5CSRTT, COOP e PJ: em 5CSRTT, os animais de controlo acertaram mais e produziram menos omissões do que os animais tratados. Os ratos tratados com escopolamina realizaram ensaios fracos em testes de cooperação, quando comparado com os de controlo. No entanto, o desempenho melhorou gradualmente durante o período de tratamento. Em PJ, o grupo de controlo pôde saltar distâncias significativamente maiores do que o grupo tratado com escopolamina, e a magnitude do efeito da escopolamina aumentou com tratamentos repetidos. A escopolamina exerceu um efeito fraco em TS e não mostrou efeitos significativos em MWM. Donepezil não melhorou o deficite de desempenho de aprendizagem em nenhum dos testes. Todos os grupos apresentaram desempenho semelhante aos níveis iniciais logo dois dias após a descontinuação dos tratamentos.

Com base nos nossos resultados, o tratamento repetido com escopolamina não induziu mudanças duradouras no funcionamento das redes neurais cognitivas, portanto, provavelmente não será um modelo adequado para testar medicamentos de tratamento para demência especialmente em animais jovens.

Palavras Chave: farmacologia comportamental translacional cognitiva, doença de Alzheimer, memória, modelos de distúrbios cognitivos humanos

Abstract

Scopolamine, an anticholinergic compound is frequently applied as a pharmacological model of cognitive impairment. It is mainly used in single dose and in naïve animals. The aim of this study was to investigate the effects of repeated scopolamine treatment on several cognitive functions in experienced rats. It was also assessed to what extent donepezil could improve the induced impairment.

8.5 months old Long-Evans rats had been trained regularly in 5-choice serial reaction time task (5CSRTT, measuring attention), Morris water maze (MWM, spatial learning), pot jumping test (PJ, motor learning), pairwise discrimination in touchscreen box (TS, visual learning) and a cooperation task (social learning). After baseline measurements rats were randomly assigned into three treatment groups: saline, scopolamine (0.3 mg/kg) and scopolamine+donepezil (3 mg/kg). Saline and scopolamine groups received ip. treatment for 20 days, the scopolamine+donepezil group was injected scopolamine for 10 days then scopolamine and donepezil during the following 10 days. Scopolamine significantly impaired performance in 5CSRTT, COOP and PJ: in 5CSRTT, control animals gave more correct answers and produced less omissions than treated animals. The scopolamine-treated rats yielded less successful trials in cooperation tests, than the control. However, these impairments gradually decreased during the treatment period. In PJ, the control group could jump significantly longer distances than the scopolamine-treated, and the magnitude of the scopolamine-effect increased by repeated treatments. Scopolamine exerted a weak effect in TS and did not show significant effects in MWM. Donepezil did not ameliorate the learning performance deficit in any of the tests. All groups showed similar performance to their baseline levels already two days after discontinuation of the treatments.

Based on our results, repeated scopolamine treatment could not induce lasting changes in the functioning of cognitive neural networks. Therefore, it may not be an appropriate model for testing potential antidementia drugs, especially in young animals.

Key words: cognitive translational behavioral pharmacology, Alzheimer's disease, memory, models of human cognitive disorders

List of abbreviations

Coop – Cooperation task

5-CSRTT – 5-Choice Serial Reaction Time Task

PJ – Pot Jumping Task

TS – Touchscreen task

MWM – Morris Water-maze

Scop – Scopolamine

Donep – Donepezil

1. INTRODUCTION

1.1. Problems in relation to the study of drugs for dementia and Alzheimer's disease: the translational gap

In the beginning of this third millennium, due to prolonged ageing, neurodegenerative disorders are growing in number, and a much deeper knowledge of the brain is necessary for scientific and technological research from molecular to behavioral levels. Research has been conducted in many different places of the world, but knowledge has not been developed in a deep level enough.

Dementia is a neurological disorder defined as a clinical syndrome characterized by progressive deterioration in multiple cognitive domains. It interferes with daily functioning (Xu *et al*., 2013) and cognitive functions including memory, comprehension, language, attention, reasoning, and judgment. Alzheimer's disease (AD) is the most common type of dementia, accounting for at least two-thirds of cases of dementia in people at age of 65 and older (Kumar *et al*., 2021). There have been a lot of promising new therapies progressing through preclinical development offering the potential for improved treatment options for neurodegenerative diseases characterized by cognitive impairments, like Alzheimer's or Parkinson's disease and for neurodevelopmental diseases like autism spectrum disorder (ASD). But despite the abundance of cognitive enhancer mechanisms identified in basic research, many studies resulted in ineffective clinical outcome because the suggested cognitive enhancer mechanisms were still doubtful. There is insufficient clinical efficacy of several tested drugs against defects in complex cognitive domains such as working -, semantic -, episodic - and visual memory, attention and information processing, social cognition, executive function and procedural memory (Millan *et al*, 2012). The few approved drugs for cognitive disorders have a limited and scanty efficacy (Gyertyán, 2017). Some enhancer drugs are not even approved by FDA due to the lack of clinical evidence (Gyertyán, 2017, Gupta and Samant, 2021).

There are many reasons for the lack of progress in cognitive disorders' research and development. Studying the brain directly is more complicated than studying other organs of the body. The brain is the most complex organ in the body, and it is well-accepted that mental illnesses involve overly complex interactions of genetic factors, environment and experience (Cuthbert and Insel, 2013). Farther, another reason why promising preclinical effects might not be translated to clinical research is the way as

different aspects of memory and other cognitive functions are tested in different phases of drug development. For example, spatial navigation testing in animals in the Morris water-maze (MWM) showed positive effects of some enhancer drugs while testing in AD Assessment Scale (ADAS) the same effects were not replicated (Hort *et al*., 2013).

The clinical trials for the treatment of Alzheimer's disease have been related to serial failures due to the low translational value of animal experimental models in predictions for human efficacy. Alzheimer's disease treatment approaches were based mainly on the amyloid cascade hypothesis and its key models were transgenic mouse lines carrying human mutant transgenes characteristic for the familial form of the disease (Gáspár *et al*., 2021, Barage and Sonawane, 2015). These strains are characterized by massive human β-amyloid overproduction, but this can be considered as a model of amyloid intoxication rather than the disease itself (Gyertyán, 2017). Then, non-transgenic models for Alzheimer's disease have become the focus of this disease research.

Yet, little is known about existing pharmacological treatments and their relation to clinical outcome. Also attempts to link distinct cognitive dysfunctions with genetic loci have been largely unsuccessful so far (Hvoslef-Eide *et al*., 2015).

This unfortunate situation raises questions about the appropriateness and specificity of the used animal models. Gyertyán affirms that there is a translational gap where preclinical validation of the potential cognitive enhancer drugs had been failed (Gyertyán, 2017). The research of drugs should be also based on previous testings underpinning a validation methodology. The methodology should be changed by approximating clinical studies regarding patient population, treatment length and outcome measures. The research results must be also on convergence of different and non-connected scientific fields for example pharmacy and biology research.

1.2. Strategy for testing the efficacy of cognitive enhancer drugs in animal models

Gyertyán suggests that the potential drugs should be tested on multiple types of cognitive functions, to increase the predictive power of positive findings (World Health

Organization, 2006, Gyertyán, 2017). Therefore, Gyertyán's research group aims to establish a cognitive test battery with rats that might be an appropriate tool for testing and developing effective cognitive enhancer drugs (Gyertyán, 2017). For example, Kozma *et al*. (2019) formulated an assay – which was integrated in the cognitive test battery - for studying cognitive domains such as cooperation, a social cognitive function that has a decisive importance in daily life and social integration. Another study by Gyertyán's research group was done for the purpose to translate the cognitive domain 'orientation', placing rats in a water-maze task to investigate whether they can determine which time of the day they are (Ernyey *et al*., 2019b). The study serves as one of the paths to construct a system investigating episodic-like memory which is deteriorated during Alzheimer's disease.

Therefore, a range of learning tasks in a test battery in rats has been applied for impairing cognitive functions such as attention, working memory, visual memory, social cognition and procedural learning to test the efficacy of cognitive enhancer drugs.

One of those learning paradigms is the Morris Water-maze (MWM), used to assess the skill of spatial memory and working memory on a spatial navigation assay (Morris, 1984. MWM is a task for rodents that relies on distal cues to navigate from start locations around the perimeter of an open swimming arena to locate a submerged escape platform (Vorhees and Williams, 2006). The primary performance parameter is the escape latency to find the hidden platform from the time of placing rodents into the maze. This assay has several advantages including absence of motivational factors such as food and water deprivation, electrical stimulations or buzzer sounds (Vogel *et al*, 1997)

Another learning paradigm that has been used for measuring effects of enhancer cognitive drugs on attentional performance is the so called 5-choice serial reaction time task (5-CSRTT). Animals are trained to nose-poke into a randomly chosen hole marked by turning on the stimulus light. Correct responses are rewarded. The basic task essentially tests the ability of the rat to sustain spatial attention divided among a number of locations over a large number of trials (Robbins, 2002). Robbins claims it can measure several distinct types of performance that include aspects of attention

and impulse control. It might be considered that increases in premature responses, as well as impairments in accuracy, all reflect changes in stimulus control.

Regarding the visual memory, one of the most used tests to assess it is the touchscreen task. During this task the animals are trained to discriminate between two images (one is correct and the other is incorrect) presented randomly in the left and right window of the touchscreen. Nose-poking the correct image is rewarded with a pellet (Hvoslef-Eide *et al*., 2015). This task reflects learning of a new association by doing observation trial by trial. Batteries of rodent touchscreen behavioral tasks that closely parallel human touchscreen tests have been developed considering visual memory as one of the studied cognitive functions (Hvoslef-Eide *et al*. 2015, Bussey *et al*. 2012, Horner et al, 2013).

Social cognition has been part of the process used to monitor and interpret social signals from others, to decipher their state of mind, emotional status and intentions and select appropriate social behavior (Gyertyán, 2017), which is studied by Kozma *et al*., 2019 in cooperation assay in rats. In this task two rats are placed in the same Skinner box. Both parts of the chamber are equipped with one nose-poke module and one magazine. If two animals perform simultaneous nose-pokes after a stimulus light is turned on in both modules, they obtain food reward. This assay served to study the learning to cooperate and social skills and cognition which may be deteriorated in autistic conditions and/or might be improved by potential anti-autism agents.

Furthermore, procedural memory as well as its age-associated decline or deficits caused by lesions of the motor system can be studied by Rotarod learning task (Rustay *et al*., 2003) or ‘pot jumping’ task (Ernyey *et al*., 2019a). In the latter study Long-Evans rats were allowed to move freely on 12 pots placed in circle form with gradually increasing distances between the centers of the adjacent pots. Their behavior was observed measuring their procedural memory capabilities, i.e. the longest inter-pot distance jumped over was recorded. The method seems to be sensitive to detect the beginning and progression of aging process in rats.

Further, in Gyertyán’s opinion, potential cognitive enhancer molecules should be tested not simply in models of human cognitive functions but instead in models of defective human cognitive functions. Therefore, the validity of any animal models

essentially and critically depends on the construct of cognitive deficiency or in other words, how impaired performance is brought about (Gyertyán, 2017).

There are, essentially, two ways against this issue: disease models and symptom models. For the first models the pathomechanism of the disease should be known or, at least, they should be based on a solid theory, e.g. in AD, genetic models of joint beta-amyloid and tau pathology (Gyertyán, 2020). Regarding the symptom models there is no requirement to replicate the pathomechanism. Instead, they are constructed to generate cognitive deficits in healthy animals via different ways of interventions, e.g. pharmacological treatment, brain lesion, modulation of gene expression, stress, aging and increasing task difficulty (Gyertyán, 2017).

There is yet another aspect not well studied 'translation-wise', that is the memory characteristics of the animals on which testing is carried out. Gyertyán claims: if we make predictions for the human patient population the use of naïve animals should be avoided (Gyertyán, 2020) because it goes in contrast to the human population where the cognitive deterioration affects subjects with substantial accumulated knowledge, experience, and history. Most of the tasks above mentioned were used for the novel translational approach for clinical prediction using a complex model system in rats by Gyertyán.

1.3. Modelling Alzheimer's disease using scopolamine as a cognitive impairing agent

Normally, to study a battery of cognitive tests one impairing drug depending on the studied symptom is used. Administration of scopolamine, an acetylcholine antagonist, is a frequently used way to impair cognitive functions in rats (Flood and Cherkin, 1986, Lindner *et al*, 2006) Scopolamine is a muscarinic receptor antagonist, it acts by blocking cholinergic signaling (Figure 1), thereby inducing the concomitant appearance of transient cognitive amnesia and electrophysiological changes, which resemble those observed in Alzheimer's Disease (Reis *et al*, 2013, Muhammad *et al*, 2019). In rats, scopolamine can cause symptoms such as motor or vision impairment, attention deficits, drowsiness, dizziness and memory loss (Luyten *et al*., 2017), and a low dose can cause a detectable cognitive decline in executive function and working

memory (Laczó et al., 2017). In animals, numerous behavioral studies with scopolamine have employed place navigation tasks, with convincing results about the impairment of hippocampus-based learning and memory (Lobellova *et al*. 2013, Andalib *et al*, 2022). Therefore, in the current study scopolamine was used as the compound to induce memory loss as in Alzheimer's disease or schizophrenia and is commonly used in behavioral studies (Snyder *et al*. 2014).

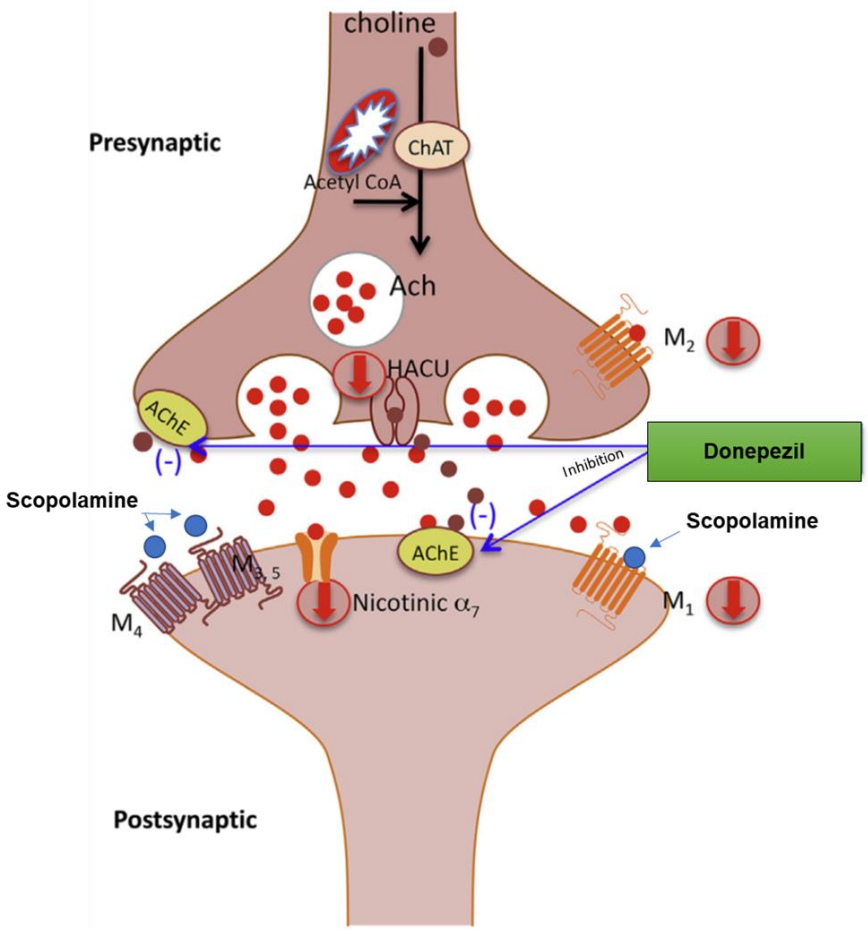

***Figure 1**-Mode of action of scopolamine and donepezil in the cholinergic synapse, Figure modified after Lombardero et al, 2020*

The deterioration of cholinergic neurons in the brain and the loss of neurotransmission are major causes of the decline in cognitive function in Alzheimer's disease. Therefore, cholinesterase is a significant therapeutic target and cholinesterase inhibitors are used as improving agents in animal models of cognitive disorders, too (Marucci *et al*., 2021) As an example of a cognitive enhancer drug donepezil is commonly used to improve mental and cognitive functions (Lindner *et al*, 2006). Donepezil acts by binding to and

reversibly inactivating the cholinesterase enzyme, thus inhibiting hydrolysis of acetylcholine presented in cerebral cortex and other areas of the brain. Donepezil has a long duration of action, with a half-life of approximately 70h, which allows once-daily administration (Seltzer, 2005). Studies showed that patients with Alzheimer's symptoms receiving donepezil showed significant improvements in a range of cognitive and neuropsychiatric symptoms over a 6 months-period (Birks *et al*., 2018, Cummings *et al*., 2016, Wallin *et al*., 2007). Lindner *et al*. (2006) used Sprague–Dawley rats with scopolamine-induced deficits in a battery of cognitive/behavioral tests to assess the effects of donepezil. The magnitude of the effects of donepezil were calculated between the scopolamine-treated group and the donepezil + scopolamine-treated group in each of the tests, using the optimal dose of donepezil for the comparison (Lindner *et al*., 2006). Goverdhan *et al*. (2012) used a range of other tests to study the neuroprotective effect of Meloxicam and Selegiline in scopolamine induced cognitive impairment in rats based on the translation approach (Goverdhan *et al*., 2012).

Donepezil was administered in the current study as a positive control against scopolamine induced impairment.

Based on the strategy proposed by Gyertyán (2020) in order to improve the 'translation-wise' cognitive system, an experiment was performed with five conjugated cognitive tasks representing different cognitive domains. The same cohort of Long-Evans rats was used in all tasks to create a population with wide-spread-knowledge. This strain was chosen because of its good learning capability, which is an essential requirement in a system imposing heavy cognitive load on the subjects. The effect of scopolamine impairment on the various cognitive functions could then be simultaneously measured in this trained population. These impaired states served then the target of potential cognitive enhancer treatments such as donepezil in a "clinical trial-like" study. The treatment started with the application of scopolamine for 10 days to get an impaired "patient population". In the literature, single dose of scopolamine is mainly used, but we chose a 10 days long continuous treatment in order to simulate a maintained "disease" state by a long lasting cholinergic blockade. While continuing scopolamine injections - modelling that the underlying "disease" is sustained - donepezil was started to be administered for 9 days. Again, repeated administration was chosen to translate the clinical situation. The tasks applied in the

system were 5-choice serial reaction time task (5CSRTT), Morris water-maze task (MWM), cooperation task (Coop), touchscreen task (TS) and pot Jumping task (PJ) and are described in *Material and Methods.* They were chosen to cover the main cognitive domains such as procedural memory (PJ), spatial memory (MWM), attention (5CSRTT), social cognition (Coop) and visual memory (Touchscreen) (Ernyey *et al*., 2019a; Morris, 1984; Robbins, 2002; Kozma *et al*., 2019; Hvoslef-Eide *et al.,* 2015).

2. AIMS

- The general aim of this study was to apply a paradigm system that is translationally interpreted and could be effective in testing potential cognitive enhancer drugs; and
- the particular aim was to induce impairment by scopolamine in Long-Evans rats and using donepezil as positive cognitive enhancer drug to verify that the system can predict clinical efficacy

3. MATERIAL AND METHODS

3.1. Subjects

The experimental subjects were 35 9 months old male Long-Evans rats (Janvier, France), housed in groups of three in 50x38x22 cm cages with elevated grid top in a temperature and light controlled animal care unit (22 ±2 C, relative humidity 70 ± 10%, 12:12h light on at 5pm). In case of frequent and major fighting episodes in the home cages, aggressive animals were placed in a separate cage, but were kept in olfactory, visual, and hearing vicinity of their previous cage mates. The average body mass of the animals was 352 g (range: 278-402g) at the beginning and 363g (range: 298-412g) at the end of the study.

Aspen bricks and cardboard tubes were placed in the cages. Rats were fed at the end of an experiment with commercial pellet rat feed R/ M-Z+H produced by SSniff Spezialdiäten GmbH. Daily food intake was limited to 40-45g per cage, proportionally reduced for two or one rat in a cage. Access to tap water was ad libitum. Housing of the animals and testing procedures conformed to the rules and principles of the 2010/63 EU Directive and the Animal Experiments Government Ordinance 40/2013. The experiments were conducted under the project license of the Pest County Government Office (PE/EA/785-5/2019).

3.2. Learning Paradigms

3.2.1. 5-Choice Serial Reaction Time Task

The equipment for 5-Choice Serial Reaction Time Task (5-CSRTT) was an operant chamber (TSE, Germany) and was equipped with five nose-poke modules (Kassai *et al*, 2022; Figure 2). Animals were trained to nose-poke into a randomly chosen hole marked for 1 sec. Turning on the stimulus light served as a signal to nose-poke. Correct responses were rewarded with a pellet (45 mg purified dustless precision pellets, Bio-Serv) delivered into the magazine. Nose-poke into the magazine initiated the next trial. The animal made an incorrect response if nose-poked into one of the non-signalled holes, a premature response, if nose poked into any of the holes during the 5s long inter-trial interval, and an omission if it did not respond to the stimulus during its duration and a 5s long post-stimulus

hold period. Incorrect and premature responses as well as omissions were punished with a 5s time-out period when the house light was turned off. Duration of a daily test session was 20 min. Rats were started to be trained for the 5-CSRTT at their 2 months of age in stages with gradually increasing difficulty.

Completion of the training took 2 months and afterwards rats participated in regular maintenance training involving 1-2 sessions a week until the start of this study. During the measurements the computer recorded response accuracy, premature responding, percent correct responding, percent omissions. The primary outcome parameter was the percentage correct response ratio, i.e. mean of ((correct responses/ total trials)x100).

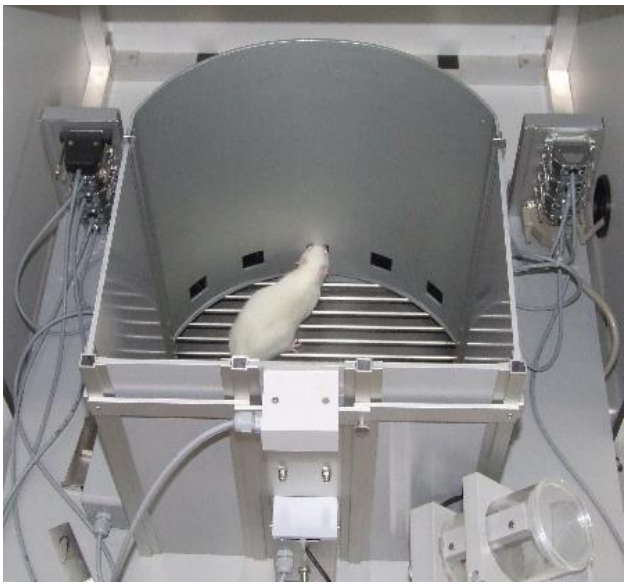

Figure 2 *– 5-Choice Serial Reaction Time Task apparatus*

3.2.2. Morris water-maze

The task of the animals was to find a hidden 10cm diameter platform in a 190 cm diameter, 60 cm deep circular tank filled with 39 cm water (23± 1°C). The platform was 1cm under the water surface, at about 40 cm distance from the side wall of the pool. The location of the platform in the initial training was in the SE quadrant while animals were trained for four days to escape onto the hidden platform. On the wall of the experimental room extra maze cues were placed to facilitate the orientation during swimming. At the start of a trial the rat was placed into the pool

at one of the four possible start points ((North, East, West or South). After finding the platform the rats were allowed to remain on the platform for 30 s, afterwards were taken out, dried by a cloth and returned to their cage. When the animal did not find the platform, it was gently guided to the platform and allowed to climb onto it. Movement of animals was recorded with Smart v3.0 video tracking system software. During the measurements, the computer recorded the latency time until the animal reached the platform and in a maximum of 3 minutes. Rats completed 3 daily trials with an intertrial interval of 30 min. For the maintenance trainings (once a month, 3 daily trials, 30 min intertrial interval) the place of the platform varied from session to session among the four quadrants of the maze (south- east [SE], south-west [SW], north-east [NE], north-west NW], Figure 3). During the treatment period rats performed the task once a week, with 3 daily trials and 24 min intertrial interval. The primary performance parameter was the time to find the target (escape latency); daily average of the 3 trials was used as individual value in the statistical calculation.

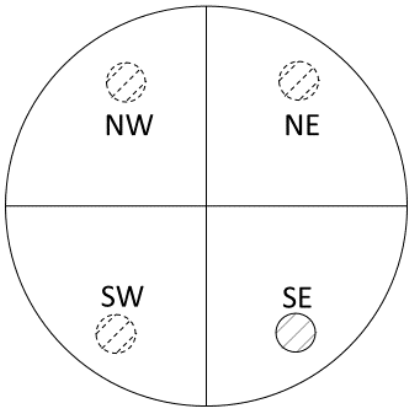

Figure 3 *– Morris Water-maze apparatus. NW: North-west; NE: North-east; SW: South-west; SE: South-east; The platform is shown as a circle, with diagonal lines*

3.2.3. Cooperation task in skinner box

Conditioning took place in a 30x24x21 cm Skinner box system (Med Associates, Fairfax, Vermont). The opposite walls of the chamber were equipped with one nose-poke module, one lever press module and one magazine for each (figure 4). During the task, the animals worked in pairs but were separated from each other by a separating fence. A trial started with lighting up both nose-poke modules for 15 seconds. One of the animals had to nose poke into its nose poke module for 3

sec, then it activated the lever for 15 sec at the opposite side. The other animal had to push the lever, as a result of which they received a reward pellet and started a new trial. The task was unsuccessful if one of the steps was missing. Rats were trained for the task in increasing difficulty stages. In early stages, the animals were kept alone in the box, and they had to learn how to use the nose poke module. The rats were learning through stages with gradually increasing the time they needed to hold their noses in the nose poke module to receive a reward pellet. After this, they learnt how to use the lever press module. If they successfully used these modules separately, in the next stage they learnt how to use them in sequence: a 3 sec long nose poke – instead of earning one pellet – activated the lever which had to be pressed in order to get the reward. After the rats learnt successfully this combined response, they were put together in pairs and had to work together to obtain the reward pellets. Whichever animal made a nose-poke, it activated the other rat's lever, which had to be pressed in order to get a pellet. Animals could step to the next training stage when they collected at least 30 pellets during a training session. An omission response was recorded when the rats did not make any nose-poke or lever-press during the time these modules were activated. Out of sequence and incorrectly timed responses were punished with 5 sec timeout. Length of a daily test session was 20 min. During the measurements the computer recorded the number of omissions (when the rat missed the trial), the number of times the animals pressed lever /nose-poke and the number of times the animals got rewards. Mean values of the recorded parameters were calculated for the entire session. The daily performance of the animals was characterized by the ratio of successful trials (number of rewarded trials/total number of trialsx100).

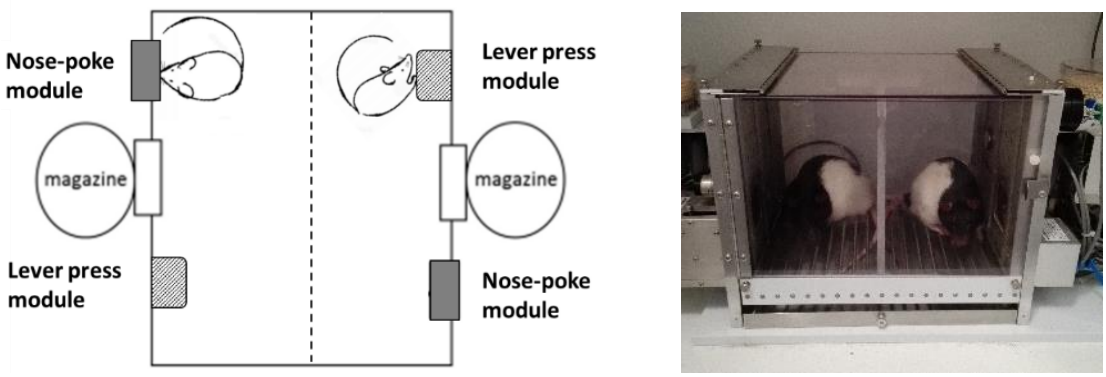

Figure 4 *– Cooperation task in Skinner box system.*

3.2.4. Pot jumping task

The equipment was a 190-cm-diameter circular open arena with 60-cm-high walls where 12 flowerpots (16 cm high, 10 and 17.5 cm wide at base and top, respectively) were placed upside down in a circle form with increasing distances between the centers of two adjacent pots from 18–46 cm in anticlockwise direction (Figure 5). A horizontally placed paper tube (20 cm long, 8-cm diameter) was suspended above pot 12 on the wall of the arena, so that the animal could climb and hide inside, where one piece of peanut reward could be obtained (Figure 5). The test served to measure procedural learning capabilities and was designed according to Ernyey *et al*, 2019a. The tank was filled with 6 cm deep water to restrain rats climbing off the pots. During a session, animals were placed onto the start pot, which was within the shortest distance from the next pot (18 cm). They could freely move on the pots for 3 min and their behavior was observed and recorded with a video camera system. Animals had a training period of 4 months practicing the task once a week. During the measurements, the experimenter analyzing the assay registered the latency time that the animal jumped in each pot, the number of jumps and the longest distance the animal could jump during a three-minutes session. The longest interpot distance jumped over was the primary performance parameter.

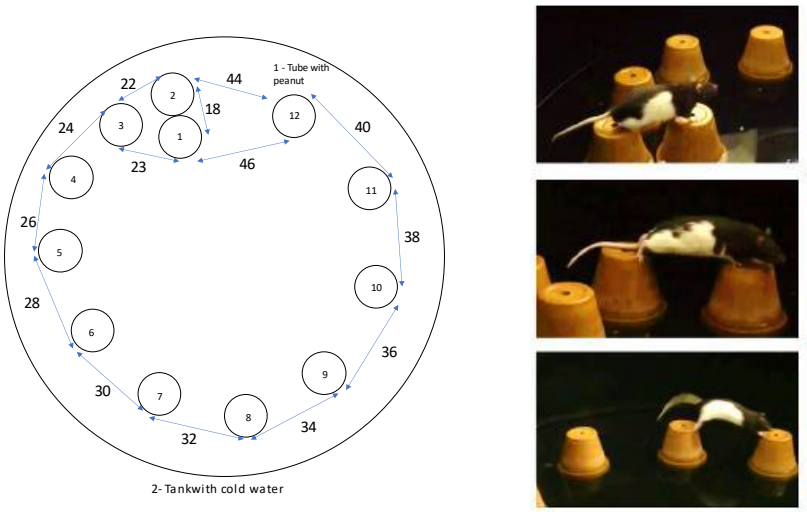

Figure 5 *– Pot Jumping scheme. In the figure, from pot 1 to 12, the length between the pots is written in cm. 1- a tube with a peanut is hanged above the last pot. 2- The tank was filled with cold water up to 6 cm. Photo: movement patterns of rats in the task (from top to bottom): walking, overarching and jumping between two pots in the test arena.*

3.2.5. Touchscreen

The rat touchscreen apparatus was a trapezoid shape chamber with a touch screen at the front and a magazine at the back wall (Figure 6). A sheet of Perspex mask covers the screen with two response windows through which the rat could make a nose-poke toward the screen. The trial begun with the presentation of two images on the screen; one is programmed as correct and one as incorrect. Whether the correct response was on the right or left is determined pseudorandomly. The rat must nose poke the correct stimulus to elicit the reward tray light and food delivery response. If the rat nose-poked the incorrect image, no reward was delivered, and a time-out of 5 sec followed before the rat was given the opportunity to complete a correction trial (the two images in the same position as in the previous trial). Animals were pre-trained for this task with increasing difficulty levels consisting of 5 stages. A stage was completed when the rat gained 20 pellets. The task was considered learnt when the rat achieved 75% correct responses 3 times in the last stage. The length of a daily session was 20 min. Number of completed trials, correct and incorrect responses were registered by ABET II Software v2.15 software. The image projection and nose poke detections were controlled by Whisker Server v4.0.0 control System (Cambridge University Technical Services Ltd., Cambridge, UK). The number of rewards was recorded and correct percentage was calculated and registered.

Figure 6 *– Touchscreen Apparatus*

3.3. Study design

One week before factual drug treatment rats participated in the routine maintenance training in 5CSRTT, touchscreen, pot jumping and cooperation assays. These results served as data for the baseline. The baseline tests in Morris water-maze were performed three weeks before the treatment The flow of the study is shown in Figure 7.

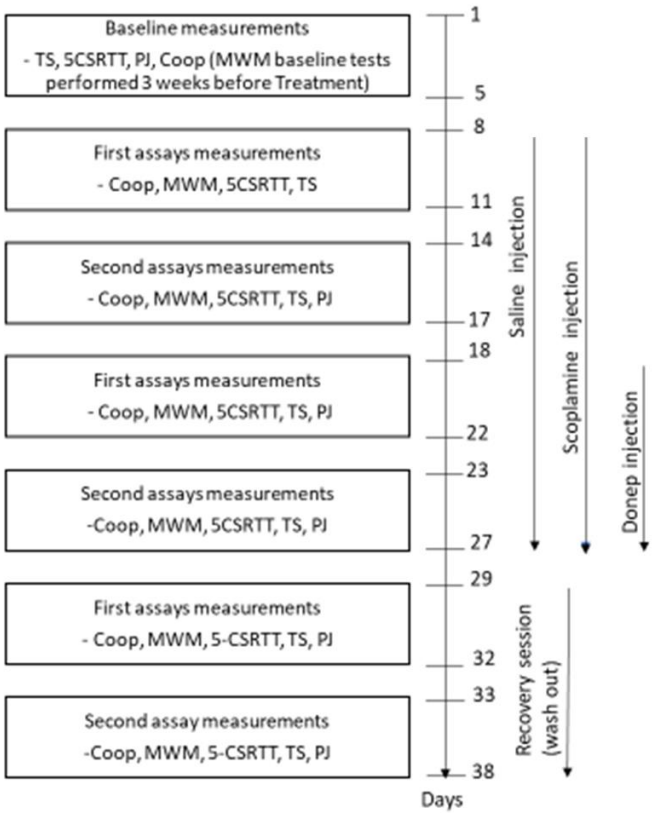

Figure 7 *– Flow of the study (Coop – Cooperation task; MWM – Morris Water Maze; 5CSRTT– 5-Choice serial real time task; TS – Touchscreen; PJ – Pot jumping task)*

Each of the different tasks were performed two times during scopolamine/saline treatment, two times during scopolamine/donepezil-treatment and two follow-up measurements were done after finishing the treatments. Pot jumping task was done only once during the scopolamine/saline treatment. Logistically, Morris water-maze assay could be performed with only one-third of the animals a day, so each measurement was run in three parts with one day shift. Accordingly, 5CSRTT and touchscreen tests were performed also in three parts.

3.4. Drug treatment

During the drug treatment period, 11 of 35 animals were administered saline (2 mL/kg) once a day for 20 days, 30 minutes before the actual learning tasks. Scopolamine (0.3 mg/kg, Sigma-Aldrich) was administered intraperitoneally to 24 animals for 20 days. Donepezil (3 mg/kg, Tokyo Chemical Industries) was injected also intraperitoneally to 12 out of the 24 animals that received scopolamine for the second 10 days period of the scopolamine treatment. Scopolamine was dissolved in saline (0.9% NaCl) while donepezil was co-dissolved with scopolamine in saline (2 mL/kg injection volume). Separate persons performed the learning assays and none of them were aware of which treatment the animals received.

3.5. Statistical evaluation

Group means and standard error of the means were calculated for each measured variable. Data were analyzed with repeated measures ANOVA with treatment as the between group factor and measurement days as the repeated measures factor. Duncan-test was applied for post-hoc comparisons. Statistica 13.5.0.17 software package (TIBCO 323 Software Inc.) was used. The Evaluation Parameters for the statistical analysis are shown in Table 1.

Table 1 *– Evaluation Parameters used for statistical analysis*

Paradigm	Evaluation Parameters
5-Choice Serial Reaction Time Task	Correct Percentage (%)
Morris water maze	Latency Time (s)
Cooperation Assay	Correct Percentage (%)
Pot Jumping	Longest Distance (cm)
Touchscreen	Correct Percentage (%)

4. RESULTS

4.1. 5CSRTT

In the 5CSRTT task, the repeated measures ANOVA revealed significant difference between the treatment groups (F (2,32)=11,256, p=0,0002); significant interaction between the treatment and days (F(12,192)=3,212, p=0,00032), and a significant time effect (F(6,192)=4,022), p<0,0001) (Figure 8). Significant effect between treatment groups were found in sessions “scop1”, “scop2”, “donep1” and “donep2”. Scopolamine caused substantial decrease in performance, however, its efficacy – though remaining significant – gradually diminished by repeated injections; it was significantly weaker at the end of treatment than at the beginning. Donepezil could not block the action of scopolamine.

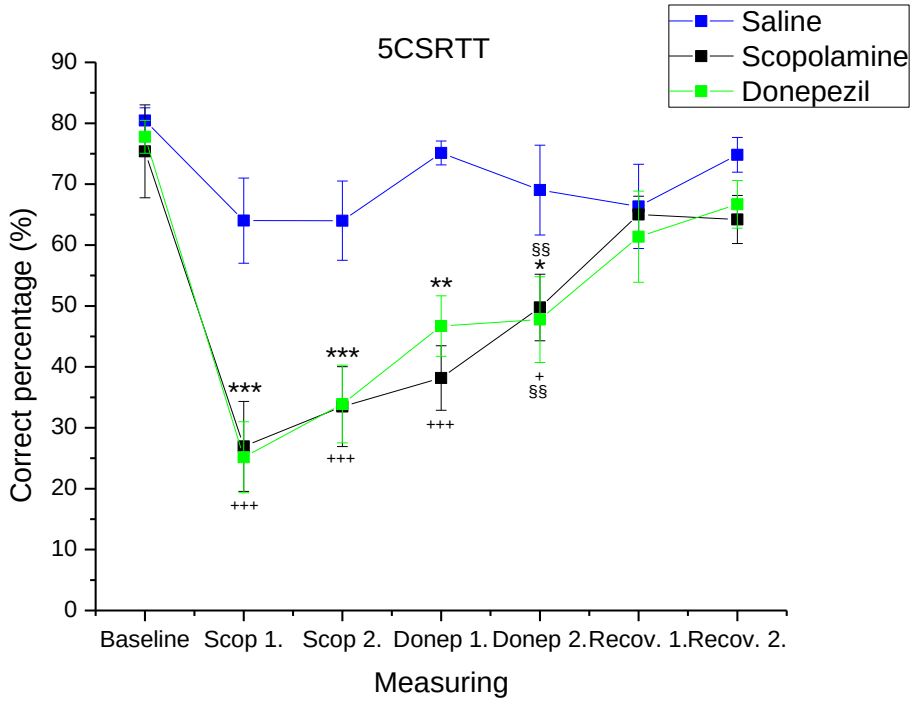

Figure 8 *– Performance of the rats in 5-choice serial reaction time task. Shown are means of correct percentage during the treatment. Blue curve: saline treated rats, black: scopolamine treated rats, green: scopolamine plus donepezil treated rats. ***: p<0,001 vs saline treated-group; **/++: p<0,01 vs saline-treated group;*: p<0,05 vs the same group; , §§: p<0,01 vs “scop 1” measurement (post-hoc Duncan test).*

4.2. Morris water-maze

No significant difference was found between the three treatment groups ($F(2,32)=0,969$, $p=0,39$) during the treatment period (Figure 9). The ANOVA also did not reveal significant interaction between treatment and days ($F(12,192)=0,687$, $p=0,76$), but it did show significant time effect ($F(6,192)=9,865$, $p<0,001$).

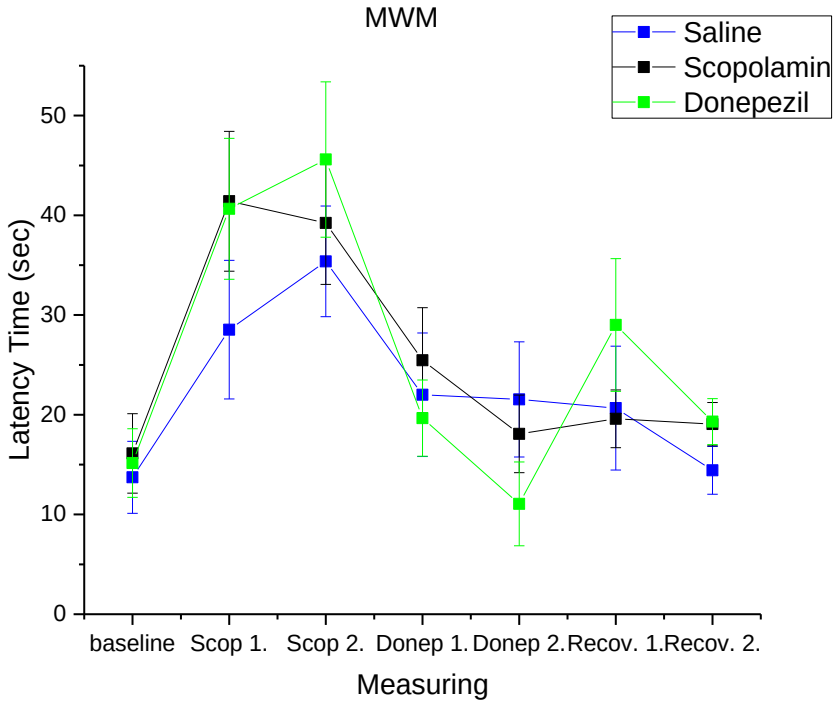

Figure 9 *– Performance of the rats in Morris Water-maze. Blue curve indicates the performance of saline treated rats, black curve indicates scopolamine treated rats, green curve indicates scopolamine plus donepezil treated rats. Shown are means of escape latency time (s) during the period of the study. No significant difference was found between the treatment groups.*

4.3. Cooperation Task

The ANOVA revealed significant difference between the treatment groups ($F(2,27)=4,19$, $p=0,026$), significant interaction between the treatment and days ($F(12,162)=12,997$, $p<0,001$) and significant time effect ($F(6,162)=43,367$, $p<0,001$). Difference in performances between saline treated and the other two groups were significant in sessions “scop1” “scop2”, “donep1” and “donep2” (Figure 10). Scopolamine caused substantial decrease in performance, however, its efficacy – though remaining significant – gradually diminished by repeated injections; it was significantly weaker at the end of treatment than at the beginning. Donepezil could not block the action of scopolamine.

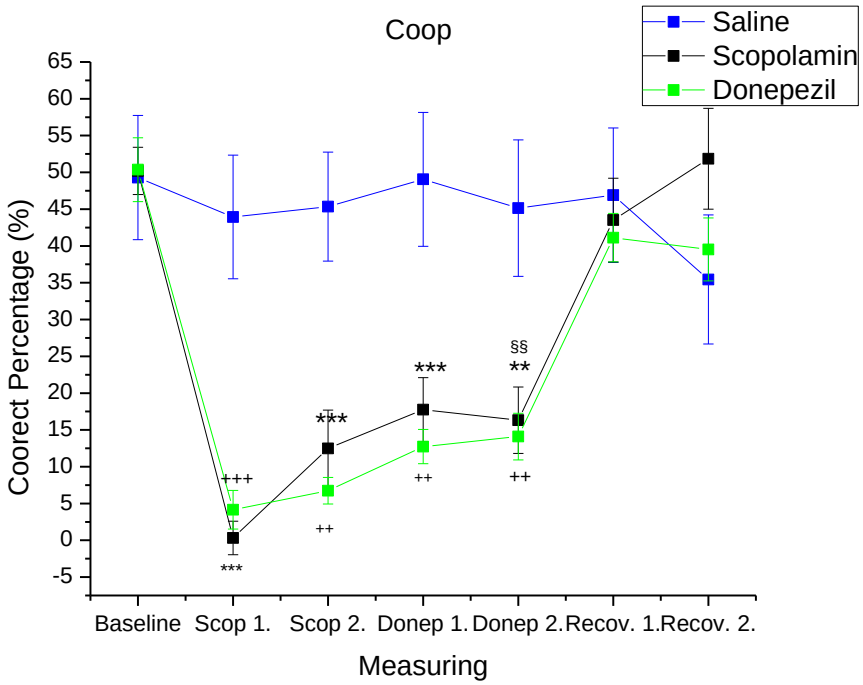

Figure 10 – *Performance of the rats in Cooperation task. Shown are means of correct percentage during the treatment. Blue curve: saline treated rats, black: scopolamine treated rats, green: scopolamine plus donepezil treated rats. ***/+++: p<0,001 vs saline-treated group; **/++: p<0,01 vs saline-treated group, §§: p<0,01 vs “scop 1” measurement (post-hoc Duncan test).*

4.4. Pot Jumping Task

The ANOVA did not reveal significant difference between the treatment groups (F(2,32)=2,265, p=0,12), but showed significant interaction between the treatment and days (F(10,160)=2,035, p=0,033) and significant time effect (F(5,160)=13,085, p<0,001) (Figure 11). In the session when donepezil was started to be injected the mean of the longest distance in the donepezil treated group significantly differed from the performance of saline treated group. The same significant effect was detected on the second session of the donepezil treatment (”donep2”) but this time the mean of the scopolamine-treated group also significantly differed from that of the controls. Donepezil treatment did not exert any change in the effect of scopolamine.

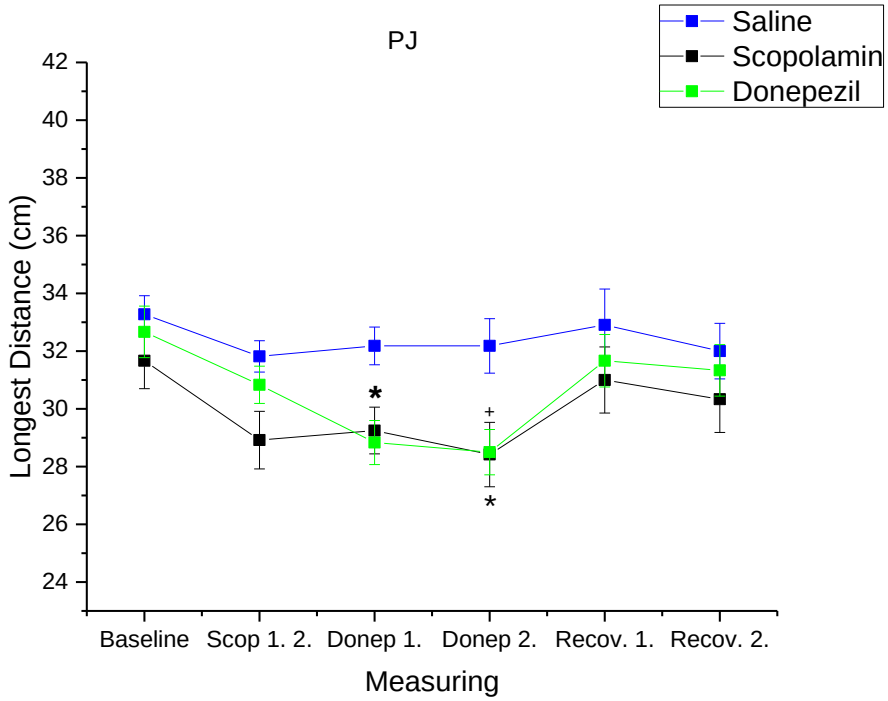

Figure 11 *– Performance of the rats in Pot Jumping task. Shown are means of longest distance spanned (cm) during the treatment. Blue curve: saline treated rats, black: scopolamine treated rats, green: scopolamine plus donepezil treated rats. *: p<0,05 (post-hoc Duncan test for the difference between saline treated rats and scopolamine plus donepezil treated rats); +: p<0.05 between saline treated rats and scopolamine treated rats).*

4.5. Touchscreen

In the touchscreen task, the ANOVA did not reveal significant difference between the treatment groups (F (2,32)=0,749, p=0,48);), but showed significant interaction between the treatment and days (F (12,192)=2,098, p=0,0187), and a significant time effect (F(6,192)=8,186, p<0,001). In “donep2” session performance of saline treated group significantly differed from scopolamine treated and donepezil treated groups (*: p<0,05) (Figure 12).

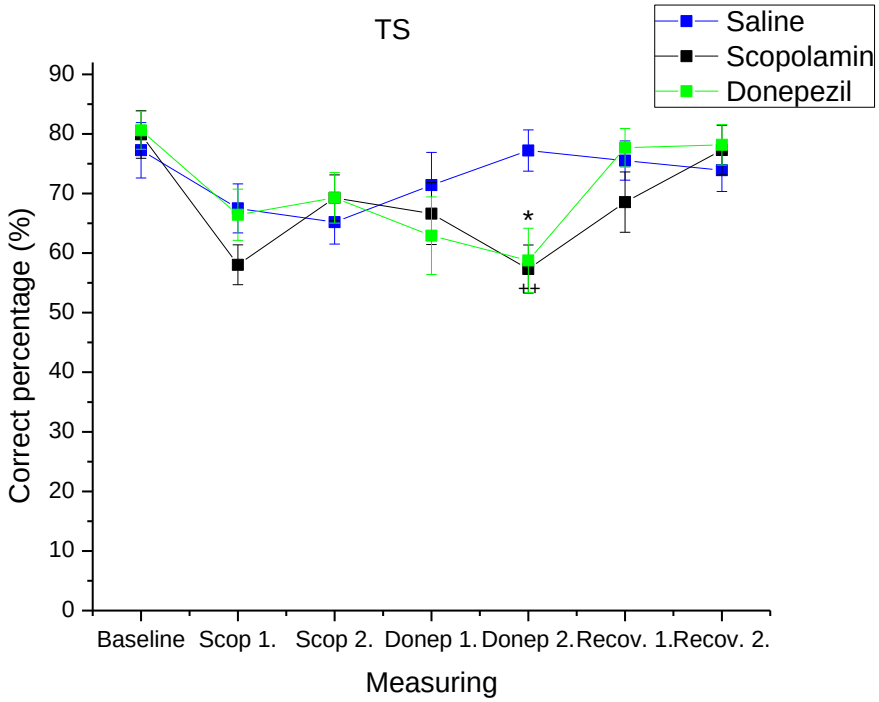

Figure 12 *– Performance of the rats in Touchscreen Task. Shown are means of correct percentage during the treatment. Blue curve: saline treated rats, black: scopolamine treated rats, green: scopolamine plus donepezil treated rats. *: p<0,05 vs saline-treated group; ++: p<0,01 vs saline-treated group (post-hoc Duncan test).*

5. DISCUSSION

The aim of this study was to establish an effective paradigm system that is translationally interpreted in different cognitive domains. All the animals were able to learn all tasks except for 3 animals in the cooperation assay. There were essential impairment effects by scopolamine in 5-choice serial reaction time task, cooperation task and pot jumping task but the same did not happen in Morris water maze task and Touchscreen task. Regarding scopolamine effects, different results were seen for each paradigm.

In 5-choice serial reaction time task, considerable effects on percentage of correct responses are shown in figure 8. Treated animals showed worse performance than the control animals from baseline mean values to first scopolamine injection. During the remaining scopolamine injections correct answers gradually increased, demonstrating diminishing effect of scopolamine. This could happen due to tolerance to scopolamine when the availability of its receptors is less or there are changes in the efficiency of cholinergic receptors, which results in a reduced effectiveness of scopolamine (Overstreet and Yamamura, 1979). Kirkby *et al*. (1996) used scopolamine to impair attention in the 5CSRTT (Kirkby *et al*, 1996). Scopolamine significantly increased omissions and premature responses in this study. Similar results were obtained by Hodges *et al*. (2009). But in another previous study the systematic application of 0.03–0.1 mg/kg of scopolamine did not much impair choice accuracy in 5CSRTT in young rats except in a condition employing interpolated bursts of white noise in the intertrial interval (Jones and Higgins, 1995) suggesting that it mainly impaired selective attention.

Regarding Morris Water maze performance, there was no significant difference between the treated animal groups and the control group. There was a slight increase in latency time for the control group at the first and second session, compared to baseline, possibly due to the stress caused by the i.p. injections. The small effect of scopolamine at the first two sessions disappeared by the third session representing a sign of tolerance to scopolamine. Stress may have affected control group performance (Du Preez *et al*., 2020). Previous studies where MWM task was conducted showed big impairing effects of scopolamine on spatial memory in rats (*Chen et al.,* 2002, see Klinkenberg and Blokland, 2010 for review). Other studies showed that the effect of scopolamine in Morris water

maze was absent in pretrained rats (Saucier *et al*, 1996; Hodges *et al*,, 2009) – similar to our results obtained in well-trained animals.

In cooperation task, the rats' performance tremendously decreased at the initiation of treatment with correct percentage approximated to null values. Second, third and fourth scopolamine injection still decreased the performance in treated groups compared to controls but the magnitude diminished gradually. The effect of scopolamine after the 4th dose was significantly lower than that after the 1st injection (figure 10 - §§: $p<0,01$ vs first measurement) what indicates a tolerance to the scopolamine doses. In the literature there are only a few methods that impair social cognition with scopolamine, probably due to the difficulty to assess this function. Sensitivity to the partner and coordination are among the studied factors on social cognition (Schuster and Perelberg, 2004, Kozma *et al*., 2019).

In pot jumping, the rats' performance seemed to be affected by scopolamine representing an impairment on motor function. Figure 9 shows the magnitude of the effect increased on all rats' performance by repeated treatments that did not happen in the other paradigms. Few studies used scopolamine as an imparing agent for motor functions and only as single dose (Thouvarecq *et al*., 2001, Goverdhan *et al*, 2012, Parasuraman *et al.,* 2019; Shabani and Mirshekar, 2018)

In touchscreen task there was no effect except after the 4th injection of scopolamine. It may reflect a delayed scopolamine effect or be mere chance. Previous studies demonstrated that visual memory methods with rats does not appear sensitive to the muscarinic antagonists like scopolamine (Talpos *et al*, 2009). Then, it has been demonstrated that systemic injections of scopolamine are problematic for use in rats when tested in touchscreen equipped operant boxes (Talpos *et al*., 2012). Visual accuracy may be less sensitive to scopolamine effect (Leaton and Kreindler, 1972, Andrews *et al*, 1992,).

Summing up, the results of the scopolamine injections (from "scop1" to "donep2") suggest that this impairing agent could affect the cognitive functions depending on the paradigms. Noticeable decrease in control groups's performance in 5CSRTT, MWM and touchscreen was registered after baseline measurements which suggests that the injections created stressful period for the rats. In three of

the assays tolerance developed to the scopolamine effect during the chronic treatment, i.e. the effect of scopolamine is not maintained during the treatment. So, the disease “cured itself”. Furthermore, the performance soon returned to control level after cessation of treatment showing that scopolamine could not cause a long lasting impairment in the performance. These findings strongly suggest that subchronic scopolamine treatment may not be an appropriate model for testing potential antidementia drugs. (A single dose of scopolamine would not be sufficient either for this purpose).

Hereafter the measurements about donepezil and following treatments sessions are discussed.

Donepezil served as a positive control during the experiment. The application of donepezil occurred in the 11th day of treatment and the tests were carried out in two sessions: “donep1” and “donep2”. The expected improving effect of donepezil could not be observed in any of the tests, showing no attenuation of scopolamine-induced effects. In contrast to our results, previous studies have reported that repeated donepezil treatment reduces scopolamine-induced deficits in the MWM and 5CSRTT (Hupparage *et al.,* 2020, Pattanashetti *et al.,* 2017). However, the results of studies are variable. One study reported that donepezil produced a small effect and only partially attenuated scopolamine-induced deficits (Ogura *et al*. 2000) and another study reported that the effect was not complete, amounting to only about 50% of task accuracy in the control group (Buccafusco, 2009). Lindner *et al*. (2006) showed lack of efficacy of donepezil against scopolamine in the MWM.

There are possible explanations for the donepezil ineffectiveness in this study. Donepezil ineffectiveness could be explained by the injection timing that differs from previous studies that found donepezil to be effective against repeated scopolamine treatment. Parasuraman *et al*. (2019), Goverdhan *et al*. (2012) and Andalib *et al*. (2022) applied a concomitant donepezil – scopolamine treatment regime for 21, 9 and 14 days, respectively, with donepezil preceding scopolamine injections each day. The peak effect of scopolamine is reached faster than the peak effect of donepezil, hence it could be more beneficial to administer donepezil earlier than scopolamine. In the literature the standard pre-treatment time for scopolamine injection is 30 min. Donepezil is never given afterwards,

sometimes given simultaneously (Saleh *et al*, 2021,) but more often before (Biradar *et al*, 2022, Hupparage *et al.*, 2020). Thus, the increased level of endogenous acetylcholine (evoked by donepezil) may effectively compete with scopolamine on the cholinergic receptor. In our study, however, the preceding chronic scopolamine treatment may have caused a permanent massive blockade of the receptors that could have impeded binding of endogenous acetylcholine. Moreover, repeated scopolamine treatment was shown to increase the activity of acetylcholinesterase (Goverdhan *et al*., 2012; Lian *et al*. 2017); this effect may have also contributed to the ineffectiveness of donepezil. One may also argue that a higher dose of donepezil could have been effective, however, a variety of studies with donepezil in cognitive tests have used this dose and, at higher doses of donepezil, disruptive effects on behavior emerge (Dawson and Iversen, 1993; Kirkby *et al.,* 1996)

1. conclusion on the model

Based on our results and in comparisons to other studies we can conclude that the chronic scopolamine administration before chronic donepezil administration while the scopolamine administration is continued could result in ineffectiveness of donepezil. We can also conclude that chronic scopolamine treatment may not be translationally appropriate for predicting clinical efficacy in testing cognitive enhancer drugs for the following reasons. The effect of scopolamine is not maintained during the treatment, tolerance was seen in 3 assays. Another reason is no lasting change, performance returned soon to control levels after cessation of treatment. For all tasks there were a noticeable increase on the performance during the recovery session after cession of treatment in 2 or 3 days which means the scopolamine/ donepezil were well eliminated from animal's body. And the last reason is the ineffectiveness of donepezil itself that shows no signs of improvement of scopolamine-induced cognitive functions. In conclusion, the chosen timing and dose of scopolamine and donepezil treatment - that has not been tested yet in the literature - did not verify our expectations: the described model is not appropriate for testing cognitive enhancer effect under the chosen circumstances.

2. limitations of the study

One of the limitations of the study is that only single dose of donepezil was used leaving open the possibility that higher doses would have been effective. Another

one is the duration of donepezil treatment that may have been too short. Thus, donepezil administration may be extended and/or donepezil dose could be raised in order to increase its effectiveness. The lack of habituating saline i.p. injection during the baseline measurements in the protocol may be also a limitation in this study.

3. how to improve in the future

The model could be improved if there were more assays modelling cognitive domains, like working memory (e.g. Y-maze spontaneous alteration), egocentric navigation (labyrinth test), episodic memory (NOR), fear extinction (fear conditioning). Increasing the dose of donepezil relative to scopolamine dose in order to increase cognitive improving effects is also an option. A dose-response curve experiment may be a better approach of treatment results. In addition, prolonging the length of donepezil treatment, and increasing the pre-treatment time of donepezil may also give chance to detect cognitive improvement. The timing for donepezil plus scopolamine administration may be in the correspondent order: first donepezil than scopolamine for better drug absorption as Parasuraman *et al*, 2019 and Malik *et al*, 2013 showed a significant increase in the cognitive performances.

6. REFERENCES

Andalib S, Anoush M, Pourmansouri Z, Javadi R, Ghorbanpour B, Sharafi A, Mohamadpour H, Anarkooli IJ. (2022). Clavulanic Acid: A Novel Potential Agent in Prevention and Treatment of Scopolamine-Induced Alzheimer's Disease. *ACS Omega 2022*, 7, 16, 13861-13869

Andrews JS, Grützner M, Stephens DN, (1992). Effects of cholinergic and noncholinergic drugs on visual discrimination and delayed visual discrimination performance in rats. *Psychopharmacology* 106, 523–530.

Barage, SH, and Sonawane KD (2015). Amyloid Cascade Hypothesis: Pathogenesis and Therapeutic Strategies in Alzheimer's Disease. *Neuropeptides*, 52, 1-18.

Biradar P, Patil V, Joshi H. *et al*. (2022). Experimental validation and network pharmacology evaluation to decipher the mechanism of action of *Erythrina 33ariegate L.* bark against scopolamine-induced memory impairment in rats. *ADV TRADIT MED (ADTM),* 22, 193–

Birks JS, Harvey RJ. Donepezil for dementia due to Alzheimer's disease. *Cochrane Database of Systematic Reviews* 2018, Issue 6.

Buccafusco JJ. (2009). Methods of Behavior Analysis in Neuroscience. *Boca Raton (FL): CRC Press/Taylor & Francis*

Bussey TJ, Holmes A, Lyon L *et al*. (2012). New translational assays for preclinical modelling of cognition in schizophrenia: the touchscreen testing method for mice and rats. *Neuropharmacology*, 62, 1191–1203

Chen Z, Xu AJ, Li R, Wei EQ. (2002). Reversal of scopolamine induced spatial memory deficits in rats by TAK-1471. *Acta Pharmacol Sin* 23:355–360

Cummings J, Morstorf T, Lee G. (2016). Alzheimer's drug-development pipeline: 2016. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, 2(4), 222–232.

Cuthbert BN, Insel TR. (2013). Toward the future of psychiatric diagnosis: the seven pillars of RDoC. *BMC Medicine*, 11, 126

Dawson GR, Iversen SD. (1993). The effects of novel cholinesterase inhibitors and selective muscarinic receptor agonists in tests of reference and working memory. *Behav Brain Res*, 30,57(2),143-53

Du Preez A, Law T, Onorato D. *et al*. (2020). The type of stress matters: repeated injection and permanent social isolation stress in male mice have a differential effect on anxiety- and depressive-like behaviours and associated biological alterations. *Transl Psychiatry 10*, 325

[(a)]Ernyey AJ, Grohmann Pereira T, Kozma K, Kouhnavardi S, Kassai F, Gyertyán I. (2019a). Following of aging process in a new motor skill learning model, "pot jumping" in rats. *Geroscience*. 2019a, 41,309-19

[(b)]Ernyey AJ, Bögi E, Kassai F, Plangár I, Gyertyán I. (2019). Translational Difficulties in Querying Rats on "Orientation". *BioMed Research International*, Vol 2019

Flood JF, Cherkin A. (1986). Scopolamine effects on memory retention in mice: a model of dementia? *Behav Neural Biol*, 45(2), 169-84

Gáspár A, Hutka B, Ernyey AJ, Tajti BT, Varga BT, Zádori ZS and Gyertyán I (2021) Intracerebroventricularly Injected Streptozotocin Exerts Subtle Effects on the Cognitive Performance of Long-Evans Rats. *Front. Pharmacol*. 12:662173.

Goverdhan P, Sravanthi A, Mamatha T. (2012) Neuroprotective effects of meloxicam and selegiline in scopolamine-induced cognitive impairment and oxidative stress. *Int J Alzheimer's Dis*, 974013

Gupta GL, Samant NP. (2021) Current druggable targets for therapeutic control of Alzheimer's disease. Elsevier. *Contemporary Clinical Trials*

Gyertyan I, Kassai F, Kozma K, Kitka T, Ernyey AJ. (2020). Procognitive profiling of a serotonin 5-HT6 receptor antagonist in a complex model system in rats: A novel translational approach for clinical prediction. *Brain Research Bulletin*, 165, 238-245

Gyertyán I. (2017). Cognitive 'Omics': Pattern-Based Validation of Potential Drug Targets. Elsevier. Trends in Pharmacological Sciences, Vol 38, 113-126

Gyertyán I. (2020). How can preclinical cognitive research further neuropsychiatric drug discovery? Chances and challenges. *Expert Opin Drug Deliv,* 2020, 15(6), 659-70

Hodges DB, Lindner MD, Hogan JB, Jones KM, Markus EJ. (2009). Scopolamine induced deficits in a battery of rat cognitive tests: comparisons of sensitivity and specificity. *Behavioural Pharmacology*, 20(3), 237–251

Horner AE, Heath CJ, Hvoslef-Eide M *et al*. (2013). The touchscreen operant platform for testing learning and memory in rats and mice*. Nat Protoc* 8, 1961–1984

Hort JR, Andel I, Mokrisova I, Gazova J, Amlerova, Valis M, Coulson EJ, Harrison J, Windisch M, Laczó J. (2013). Effect of Donepezil in Alzheimer Disease Can Be Measured by a Computerized Human Analog of the Morris Water Maze. *Neurodegener Dis 2014*, 13:192–196

Hupparage B, Rasal P, Vishal P, Patil G., Mulange P, Malgi A, Karade P. (2020). Ameliorative effect of *Amaranthus tricolor L.* leaves on scopolamine-induced cognitive dysfunction and oxidative stress in rats. *Journal of Applied Pharmaceutical Science*

Hvoslef-Eide M, Nilsson SRO, Saksida LM and Bussey TJ (2015). Cognitive Translation Using the Rodent Touchscreen Testing Approach. *Curr Topics Behav Neurosci* 2016, 28, 423–447

Jones DNC, Higgins, GA. (1995). Effect of scopolamine on visual attention in rats. *Psychopharmacology*, 120(2), 142–149

Kassai F, *et al*. (2022): Lack of general learning ability factor in a rat test battery measuring a wide spectrum of cognitive domains." *Journal of Integrative Neuroscience* 21, 1, 12.

Kirkby DL, Jones DN, Barnes JC, Higgins GA. (1996) Effects of anticholinesterase drugs tacrine and E2020, the 5-HT(3) antagonist ondansetron, and the H(3) antagonist thioperamide, in models of cognition and cholinergic function. *Behavioural Pharmacology*,7(6), 513-525

Klinkenberg I, Blokland A. (2010). The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies. *Neurosci Biobehav Rev*., 34(8), 1307-50.

Kozma K, Kassai F, Ernyey AJ, Gyertyán I. (2019). Establishment of a rodent cooperation assay as a model of social cognition. *Journal of Pharmacological and Toxicological Methods*, 97, 44-51

Kumar A, Sidhu J, Goyal A, Tsao JW, Svercauski J. (2021) Alzheimer Disease (Nursing). *StatPearls Publishing, Treasure Island (FL)*

Laczó J, Markova H, Lobellova V, Gazova I. (2017). Scopolamine disrupts place navigation in rats and humans: a translational validation of the Hidden Goal Task in the Morris water maze and a real maze for humans. *Psychopharmacology*, 234, 535–547

Leaton RN, Kreindler M. (1972). Effects of physostigmine and scopolamine on operant brightness discrimination in the rat. *Physiology & Behavior*, 9(1), 121–123.

Lian W, Fang J, Xu L, Zhou W, De Kang, Xiong W, Jia H, Liu AL, Du GH. (2017). DL0410 Ameliorates Memory and Cognitive Impairments Induced by Scopolamine via Increasing Cholinergic Neurotransmission in Mice. *Molecules 22*, 3, 410.

Lindner MD, Hogan JB, Hodges DB, Orie AF, Chen P. (2006). Donepezil primarily attenuates scopolamine-induced deficits in psychomotor function, with moderate effects on simple conditioning and attention, and small effects on working memory and spatial mapping. *Psychopharmacology*, 188, 629–640

Lobellova V, Entlerova M, Svojanovska B, Hatalova H, Prokopova I, Petrasek T, Vales K, Kubik S, Fajnerova I, Stuchlik A. (2013). Two learning tasks provide evidence for disrupted behavioural flexibility in an animal model of schizophrenia-like behaviour induced by acute MK-801: a dose-response study. *Behav Brain Res*, 246,55-62.

Lombardero L, *et al*. (2020). Neurotransmitter receptors in Alzheimer's disease: from glutamatergic to cholinergic receptors. Genetics, *Neurology, Behavior, and Diet in Dementia. Academic Press*, 441-456.

Luyten L, Nuyts S, Beckers T. (2017). Low-dose systemic scopolamine disrupts context conditioning in rats. *J Psychopharmacol.* 31(6), 667–673

Malik J, Kumar M, Deshmukh R, Kumar P. (2013). Ameliorating effect of lyophilized extract of Butea frondosa leaves on scopolamine-induced amnesia in rats, *Pharmaceutical Biology*, 51, 2, 233-239,

Marucci G, Buccioni M, Dal Bem D, Lambertucci C, Volpini R, Amenta F. (2021). Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease, *Neuropharmacology*, Vol 190, 108352

Millan, M. J., Agid, Y., Brüne, M., Bullmore, E. T., Carter, C. S., Clayton, N. S., Young, L. J.(2012). Cognitive dysfunction in psychiatric disorders: characteristics, causes and the quest for improved therapy. *Nature Reviews Drug Discovery*, 11(2), 141–168

Morris R. (1984). Developments of a water-maze procedure for studying spatial learning in the rat. *J. Neurosci. Methods*, 11:47-60

Ogura H, Kosasa T, Kuriya Y, Yamanishi Y. (2000). Donepezil, a centrally acting acetylcholinesterase inhibitor, alleviates learning deficits in hypocholinergic models in rats. *Methods Find Exp Clin Pharmacol*, 22(2), 89-95.

Overstreet DH, Yamamura, HI. (1979). Receptor alterations and drug tolerance. *Life Sciences*, 25(22), 1865–1877

Parasuraman S, Hoong SS, Christapher PV, Zou LN, De Wei DL, Loshini S, *et al*. (2019). Effect of ethanolic extract of leaves of Solanum trilobatum on scopolamine-induced memory impairment in Sprague Dawley rats. *J Pharm Negative Results*, 10, 41-6.

Pattanashetti LA, Taranalli AD, Parvatrao V, Malabade RH, Kumar D. (2017). Evaluation of neuroprotective effect of quercetin with donepezil in scopolamine-induced amnesia in rats. *Indian J Pharmacol*, 49, 60-4

Reis RPM, Eckhardt H, Denise P, Bodem F. (2013). Localization of Scopolamine Induced Electrocortical Brain Activity Changes in Healthy Humans at *Rest. Pharmacodynamics*, 53, 619-625

Robbins TW, 2002, The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. *Psychopharmacology (Berl),* 163, 362-80.

Robert M. (2010) Attachment in Psychotherapy, by D. J. Wallin, *Journal of Trauma & Dissociation*, 12:1, 102-104

Rustay NR, Wahlsten D, Crabbe JC. (2003). Influence of task parameters on rotarod performance and sensitivity to ethanol in mice. *Behav Brain Res, 15*, 141(2), 237-49

Saleh SR, Masry, AM, Ghareeb DA *et al*. (2021). Trichoderma reesei fungal degradation boosted the potentiality of date pit extract in fighting scopolamine-induced neurotoxicity in male rats*. Sci Rep* 11, 14872

Saucier D, Hargreaves EL, Boon F, Vanderwolf CH, Cain DP. (1996). Detailed behavioral analysis of water maze acquisition under systemic NMDA or muscarinic antagonism: Nonspatial pretraining eliminates spatial learning deficits. *Behavioral Neuroscience*, 110(1), 103–116

Schuster R, Perelberg A. (2004). Why cooperate?: An economic perspective is not enough. *Behavioural Processes*, Vol 66, Issue 3

Seltzer B. (2005). Donepezil: A review. *Expert Opinion on Drug Metabolism and Toxicology*, 1, 527-536.

Shabani S, Mirshekar MA. (2018). Diosmin is neuroprotective in a rat model of scopolamine-induced cognitive impairment. *Biomedicine & Pharmacotherapy* 108, 1376–1383

Snyder PJ, Wrobleski KK, Brannan S, Miller DS, Schindler RJ, DeSanti S, Ryan JM, Morrison G, Grundman M, Chandler J, Caselli RJ, Isaac M, Bain L, Carrillo MC. (2014). Assessing cognition and function in Alzheimer's disease clinical trials: Do we have the right tools?. *Alzheimer's & Dementia*, 10, 6, Pages 853-860

Talpos JC, Fletcher AC, Circelli C, Tricklebank MD, Dix SL. (2012). The pharmacological sensitivity of a touchscreen-based visual discrimination task in the rat using simple and perceptually challenging stimuli. *Psychopharmacology (Berl)*, 221, 437–49.

Talpos JC, Winters BD, Dias R, Saksida LM, Bussey TJ. (2009). A novel touchscreen-automated paired-associate learning (PAL) task sensitive to pharmacological manipulation of the hippocampus: a translational rodent model of cognitive impairments in neurodegenerative disease. *Psychopharmacology (Berl),* 205(1):157-68

Thouvarecq R, Protais P, Jouen F, Caston J. (2001). Influence of cholinergic system on motor learning during aging in mice*. Behav Brain Res.*, 29, 118(2), 209-18.

Vogel HG, Vogel, WH (1997). Drug effects on learning and memory. *In: Vogel HG, Vogel WH Drug Discovery and Evaluation.* Springer, Berlin, Heidelberg

Vorhees CV, & Williams MT. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature protocols,* 1(2), 848–858.

Wallin AK, Andreasen N, Eriksson S, Båtsman S, Näsman B, Ekdahl A, Minthon L. (2007). Donepezil in Alzheimer's Disease: What to Expect after 3 Years of Treatment in a Routine Clinical Setting. *Dementia and Geriatric Cognitive Disorders*, 23(3), 150–160

World Health Organization. (2006). Neurological Disorders: Public Health Challenges. *World Health Organization*, 2006

Xu W, Marseglia A, Ferrari C, Wang H. (2013). Alzheimer's Disease: A Clinical Perspective. *Neurodegenerative Diseases*, Chapter 1, 3-33