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MNEMONIC AND BEHAVIOURAL EFFECTS OF BIPERIDEN, AN M1-SELECTIVE MUSCARINIC ANTAGONIST, IN THE RAT

PAMĚŤOVÉ A BEHAVIORÁLNÍ VLIVY BIPERIDENU, M1-SELEKTIVNÍHO ANTAGONISTY, U LABORATORNÍHO POTKANA

Diploma thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Statistiku vypracoval Mgr. et Ing. Štěpán Bahník, Ph. D. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstrakt:

Vzhledem k přetrvávajícímu nedostatku spolehlivých zvířecích modelů kognitivních poruch s dobrou translační validitou, soustředí se současný výzkum na vývoj nových způsobů a nástrojů pro imitaci příznaků lidských neurodegenerativních onemocnění u hlodavců. Biperiden, M1-selektivní antagonista muskarinových receptorů, byl nedávno navržen jako potenciální nástroj pro vytváření rychlých screeningových modelů paměťových poruch obdobných těm u pacientů s Alzheimerovou chorobou. Jelikož je vysoce selektivní pro M1 receptor, převládající typ muskarinových acetylcholinových receptorů v mozku, účastnící se kognitivních procesů, bylo spekulováno, že by mohl ovlivňovat pouze kognici, aniž by vyvolával vedlejší sensorimotorické účinky. Studie zabývající se využitelností tohoto farmaka ukázaly různé, často protichůdné výsledky. My jsme se rozhodli rozšířit množství experimentálních dat a zhodnotit validitu biperidenu v několika variantách Morrisova vodního bludiště.

Výsledky této studie neukázaly žádný signifikantní efekt biperidenu na kognitivní flexibilitu, testovanou v přeučení (*reversal*). V *delayed-matching-to-position* testu, hodnotícím pracovní paměť, byl nalezen rozdíl mezi skupinami; nelze však jednoznačně určit, zda šlo o narušení paměti. Žádný deficit nebyl pozorován v úloze s viditelným ostrůvkem, což potvrzuje, že patrně nedošlo k vyvolání sensorimotorických vedlejších účinků. V *counter-balanced acquisition* testu bylo ukázáno zvýšení času potřebného k nalezení ostrůvku, což ukazuje nedostatky v získávání paměťových stop. V testovacích plavbách (*probe trials*) bylo pozorováno signifikantní snížení času stráveného v cílovém kvadrantu, což naznačuje poruchy v uchování paměti. Vezmeme-li v potaz rozporuplné výsledky jiných studií, nezdá se biperiden jako dostatečně spolehlivý nástroj pro generování modelů kognitivních poruch a jeho další využití v tomto směru bychom tedy nedoporučili.

Klíčová slova:

biperiden, cholinergní systém, M1-receptor, Morrisovo vodní bludiště, chování, učení a paměť, animální modely, potkan

Abstract:

Due to the persisting lack of reliable animal models of cognitive impairment with good translational validity, researches strive to discover new ways and tools to replicate symptoms of human neurodegenerative diseases in rodents. Recently, biperiden, an M1-selective muscarinic antagonist, has been proposed as a potential tool for generating fast screening models of mnemonic deficits such as seen in patients with Alzheimer's disease. Being highly selective for the M1 receptor, a predominant type of muscarinic acetylcholine receptors in the brain involved in cognitive processes, it has been speculated to possibly only influence cognition without causing sensorimotor side effects. Studies assessing the usability of this drug reported conflicting results. We have decided to expand the experimental data and evaluate biperiden's validity in several variants of the Morris water maze.

The results of this study showed no significant effect of biperiden on cognitive flexibility, tested by reversal learning. In delayed-matching-to-position paradigm, which tests assesses working memory, we found a difference in performance between the two experimental groups; however, it cannot be unequivocally attributed to a memory impairment. No effects were observed in visible platform task, confirming a lack of sensorimotor side effects. We found an increase in escape latencies in the counter-balanced acquisition paradigm, pointing to a disruptive influence on memory acquisition. In probe trials, a significant decrease of time spent in the target quadrant was observed, suggesting a memory retention impairment. In conclusion, taking into account the conflicting results from other studies, biperiden does not seem reliable enough to serve as a tool for generating models of cognitive impairment, and as such we would not recommend its use in this field.

Key words:

biperiden, cholinergic system, M1-receptor, Morris water maze, behaviour, learning & memory, animal models, rat

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ABBREVIATIONS

AAPA .. active allothetic place avoidance

AC ... adenylyl cyclase

ACh ... acetylcholine

AChE … acetylcholine esterase

AChR … acetylcholine receptors

AD ... Alzheimer's disease

Asn … asparagine

BBB … blood-brain barrier

BIP ... biperiden

C … control (group)

CA ... counter-balanced acquisition

cAMP ... cyclic adenosine monophosphate

ChAT … choline acetyl transferase

CNS … central nervous system

DMP ... delayed matching to position

DMSO ... dimethyl-sulfoxide

ETM ... elevated T-maze

GABA … gama-amino-butyric acid

GLM … general linear model

GPCRs ... G-protein coupled receptors

i. p. … intraperitoneally

ITI ... inter-trial interval

mAChR … muscarinic acetylcholine receptors

MDMA … 3,4-methylendioxy-metamphtamine

mPFC ... posterior medial frontal cortex

MWM ... Moriss watermaze

nAChR … nicotinic acetylcholine receptors

NMDA … N-methyl-D-aspartate

NSP ... non-spatial pre-training

OF ... open field test

PAM … positive allosteric modulator

PLC ... phospholipase C

PPI … pre-pulse inhibition

QNB … quinuclidinyl benzilate

s. c. ... subcutaneously

SCOP ... scopolamine

VAChT … vesicular acetylcholine transporter

VP ... visible platform

1. INTRODUCTION

This study explores the effectiveness and validity of biperiden as a potential tool for animal modelling of cognitive and memory deficits such as observed in human patients with Alzheimer's disease (AD). This compound has been suggested as an alternative to scopolamine, a commonly used muscarinic receptor antagonist. Being non-selective for any of the subtypes (M1 - 5) of these receptors, this drug produces a range of non-cognitive effects (such as hyper-locomotion, etc.) which may alter the results of behavioural tests. Biperiden, on the other hand, is highly selective for the M1 subtype of the muscarinic cholinergic receptors, thus prompting a hypothesis that it might exert influence on cognitive abilities only. Various other studies have attempted to investigate the potential use of biperiden in this field, however the authors reported unclear and conflicting results. To determine the validity of this model, or to at least contribute to the pool of data, we have decided to test the properties of this drug in several variations of the Morris water maze task (MWM).

2. LITERATURE OVERVIEW

2.1. Acetylcholine System in the Brain

Acetylcholine (ACh) counts among one of the major neurotransmitters and modulators in the nervous system (Figure 1); its receptors are abundantly expressed in a wide variety of tissues, from neuromuscular junctions and parasympathetic system to cortical regions involved in cognitive functions such as learning and memory (VanPatten & Al-Abed, 2016). The cholinergic system has been shown to play an important role in processes such as circadian rhythmicity (Hut & Van der Zee, 2011), addiction (Leslie, Mojica, & Reynaga, 2013), motivation, pain, reward (VanPatten & Al-Abed, 2016), as well as cognitive flexibility (Prado, Janickova, Al-Onaizi, & Prado, 2016), perceptual memory (Robinson, Platt, & Riedel, 2011), spatial learning (Deiana, Platt, & Riedel, 2011), and many more. It comes as no surprise that any abnormalities in function of the cholinergic system and its components underlie a multitude of pathologies such as Parkinson's disease (Schliebs & Arendt, 2011), Alzheimer's disease (Jiang et al., 2014), schizophrenia, bipolar disorder (Carruthers, Gurvich, & Rossell, 2015; Pittaras et al., 2016), and depression (Witkin et al., 2014). For these reasons the cholinergic system has been extensively studied in the recent years, however many mechanisms of its workings remain unclear.

The main components of cholinergic signalling are: (1) *acetylcholine*, synthesized in the neural terminus by (2) *choline acetyltransferase* (ChAT) and subsequently transported into vesicles by (3) *vesicular acetylcholine transporter* (VAChT). When released into the synaptic cleft, the neurotransmitter binds to an (4) *acetylcholine receptor* (AChR) which may be located both presynaptically and postsynaptically. The signal is promptly terminated by (5) *acetylcholine esterase* (AChE) which cleaves acetylcholine into acetate residue and choline that are subsequently transported from the synaptic cleft back into the terminal button of the presynaptic neuron. All the components, i. e. the AChR and the enzymes involved in signalling via acetylcholine, have been a subject of study for both purely scientific research purposes, as well as potential therapeutic targets (Prado et al., 2016). We will further focus on the AChR.

There are two main types of acetylcholine receptors, named historically after their naturally occurring alkaloid agonists: (1) **nicotinic**[1], a family of ionotropic receptors which act as ligand-dependent cation channels, and (2) **muscarinic**[2], a metabotropic G-protein coupled receptor (GPCRs) family whose activation may trigger various responses depending on the specific subtype and context of the signal (Jiang et al., 2014).

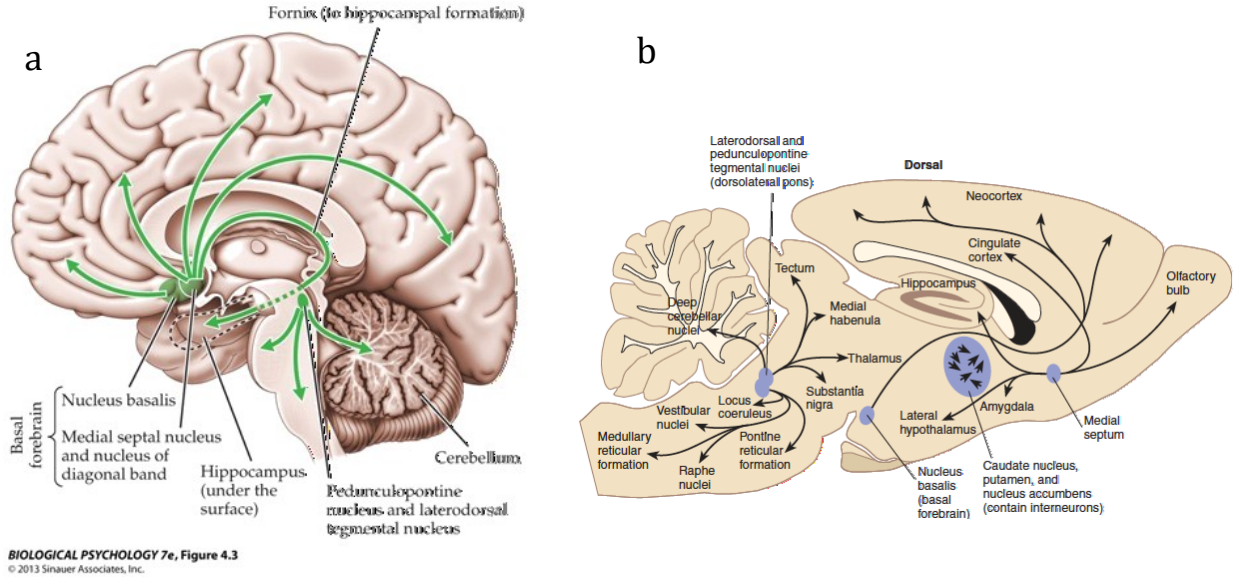

Figure 1: The pictures above show the cholinergic system in the (a) human, and (b) rat brain. There are two main groups of the cholinergic pathways: (1) the pedunculopontine complex, originating in the pedunculopontine nucleus and the laterodorsal nucleus with projection into the midbrain, the cerebellum and the medulla oblongata, and (2) the basal forebrain complex, projecting from the nucleus basalis Meynerti and medial septal nucleus into the cortex and the hippocampus (Carruthers et al., 2015). Taken from (Breedlove & Watson, 2013) and (Carlson, 2013).

2.1.1. Nicotinic Receptors

Despite being best known for their involvement in signal transduction at neuromuscular junctions, these receptors are also expressed throughout the nervous system. As mentioned above, nicotinic acetylcholine receptors are ionotropic, i. e. ligand-gated cation channels, whose activation by an agonist evokes an influx of K^+, Ca^{2+}, and Na^+

[1] As it is commonly known, it is named after nicotine, its prototypical agonist. Probably the most famous antagonist is D-tubocurarin, a compound found in the curare poisons (Malca Garcia et al., 2015; Role & Berg, 1996).
[2] Named after muscarine, a toxic alkaloid synthesized in *Amanita muscaria*. Possibly the best known antagonist is atropine, found in *Atropa belladonna* (Albuquerque et al., 2009).

ions (however not all subtypes of nAChR are permeable for Na^+), which in turn triggers mechanisms of Ca^{2+} signalling (VanPatten & Al-Abed, 2016). These receptors typically comprise of five subunits: either a homomeric combination of α subunits (for example α7), or a heteromeric combination of α(1 – 10), β (1 – 4), δ, and ε subunits. The specific combination of the subunits generates different pharmacological properties of the individual subtypes, such as ion selectivity and ligand affinity (Albuquerque, Pereira, Alkondon, & Rogers, 2009). The most common nAChR subtypes found in the brain are α7 and α4β2 receptors (although recently a new receptor type α7β2 has been found). Located both pre- and postsynaptically, they play a pivotal role various processes, such as learning and cognition (He, Johnston, Zeitlinger, City, & City, 2015), decision-making (Pittaras et al., 2016), regulation of postnatal development of visual cortex (Sadahiro, Sajo, & Morishita, 2016).

2.1.2. Muscarinic Receptors

Muscarinic receptors are abundantly expressed throughout the brain, however they are found in various other tissues in the body, such as the heart (De Sarno et al., 2003; Tomankova, Valuskova, Varejkova, & Rotkova, 2015), the bladder, pulmonary system (Dale et al., 2014), and the intestine (Muise, Gandotra, Tackett, Bamdad, & Cowles, 2016). As mentioned above, unlike the nicotinic receptors, the muscarinic receptors do not serve as cation channels, but instead are coupled with G-proteins (Figure 2), which transmit the signal into the cell by affecting the activity of certain enzymes (such as the adenylyl cyclase, phospholipase C, etc.) (Albuquerque et al., 2009; Picciotto, Higley, & Mineur, 2012).

Five subtypes, M1 - M5, of the muscarinic receptors have been described. They differ in their level of expression in different parts of the body and the signal cascades they trigger after binding an agonist. Located mostly postsynaptically, the M1, M3, and M5 receptors (sometimes referred to as "M1-like") activate phospholipase C (PLC) via $G_{q/11}$ protein, thus inducing calcium influx into the cell[3]. M2 and M4 on the other hand (the "M2-like" group), when activated work towards lowering the level of cyclic adenosine mono-phosphate (cAMP) in the cell by $G_{o/i}$ protein-mediated inhibition of adenylyl cyclase (AC).

[3] As an example of neuromodulation on molecular level through M1-like AChR might be mentioned that activation of these receptors results in depletion of phosphatidylinositol-bisphosphate (PIP_2) which in turn reduces the current through KCNQ potassium channels (Suh, Horowitz, Hirdes, Mackie, & Hille, 2004).

They are found both pre- and postsynaptically (Jiang et al., 2014; Picciotto et al., 2012; Zhang et al., 2002).

The outputs of signalling through specific cholinergic receptor subtypes may vary tremendously depending on the subtype of the receptors and their pre- or postsynaptic localization. The specific tissue and the type of the cell that expresses the receptors is also of major importance, as well as the metabolic state of the neuron at the precise time of receiving the signal, i. e. a cell with high intracellular levels of calcium may react differently to a signal than one with low intracellular concentration of calcium. To further complicate any predictions of outcomes of cholinergic signalling and behavioural analysis, many neurons co-release ACh and glutamate, or ACh and gama-amino-butyric acid (GABA) (Picciotto et al., 2012; Prado et al., 2016)

As an example of the complexity of pharmacological modulation of a process in the brain might pose the involvement of the central muscarinic system in startle reflex and pre-pulse inhibition (PPI) where administration of various muscarinic antagonists has been shown to lead to different results. Jones et al. (2000) reported that whereas the non-selective antagonist scopolamine decreased PPI but had no effect on startle response itself, dicyclomine and biperiden did not affect PPI, but decreased amplitude of the startle response (Jones & Shannon, 2000). Sipos et al (2001) on the other hand observed an increase in startle response amplitude and a decrease in PPI following administration of both scopolamine and biperiden (Sipos, Burchnell, & Galbicka, 2001).

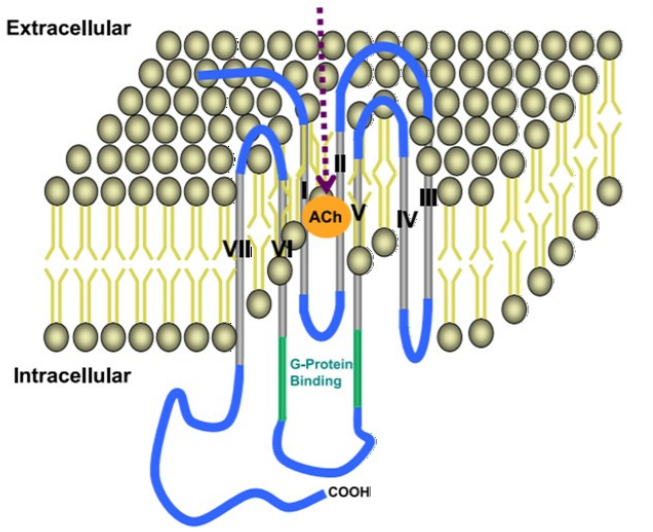

Figure 2: A schematic representation of the structural organization of muscarinic receptors in the cell membrane. The N-terminal of the protein is located extracellularly, whilst the -COOH-terminal is intracellular. The transmembrane domains are positioned in a way that creates

a central pore with a binding site for acetylcholine. The specific G-protein binds to the third intracellular loop. Picture taken from (Shah, Khurana, Cheng, & Raufman, 2009).

One of the major characteristics of the molecular structure of the muscarinic receptors is the evolutionarily highly conserved orthosteric acetylcholine binding site, with a key asparagine residue (Asn105). This means a great difficulty in developing direct agonists and antagonists selective for a specific receptor subtype[4]. Thus, the researchers have rather focused on developing compounds acting as allosteric ant/agonists and positive allosteric modulators (PAMs) (Digby, Shirey, & Conn, 2010; Jiang et al., 2014).

2.1.2.1. M1 Muscarinic Receptor

The M1 receptor is considered to be the most abundant subtype (50 – 60 % of all mAChR) of muscarinic receptors in the brain. It plays an essential role in many cognitive functions such as learning and memory and thus has become the target of research focusing on developing therapeutics for neurodegenerative diseases (Carruthers et al., 2015; Foster, Choi, Jeffrey Conn, & Rook, 2014; Jiang et al., 2014). For example, Ragozzino et al. (2011) reported an enhancing effect of CDD-0102A, a partial M1 agonist, on working memory and strategy changing in rats. The compound improved the rats' performance in a spontaneous alteration task (designed to test working memory) and, under changed circumstances, their ability to deem a previously useful strategy irrelevant and to find and retain a new one. This study shows the involvement of M1 receptors in these processes; furthermore the authors suggest the CDD-0102A, emphasizing its enhancing influence and the lack of observed adverse effects, as a potential therapeutic agent for disorders such as AD and schizophrenia (Ragozzino et al., 2012).

The M1 receptor is also expressed in other tissues than the brain; for example it has been shown to participate in regulation of non-quantal ACh release in neuromuscular junctions (Malomouzh, Mukhtarov, Nikolsky, & Vyskočil, 2007).

2.2. Antimuscarinic Drugs

Due to the diverse expression and functions of AChR in the brain, compounds affecting the cholinergic neurotransmission are employed in the treatment of a wide

[4] An example of such an non-selective agent might be scopolamine, an antiemetic drug, widely use in research of memory impairment (Pergolizzi et al., 2012; Sambeth et al., 2014), or 3-iodothyronamine (Laurino, Matucci, Vistoli, & Raimondi, 2016).

range of conditions and diseases. They are generally used for antiparkinsonian treatment, specifically targeting extrapyramidal symptoms such as rigidity, tremors, and bradykinesia. For example, it is generally accepted that an imbalance of cholinergic and dopaminergic transmission in the brain is one of the mechanisms underlying schizophrenia, namely causing negative symptoms and cognitive impairment. However, anticholinergic drugs are often prescribed along with antipsychotics, to alleviate their unwanted side effects. Their usage is often questioned as they themselves cause a range of side effects, such as cognitive impairment, tardive dyskinesia, blurred vision, dry mouth, problems with urinary retention, psychosis, addiction, and many more (Desmarais, Beauclair, & Margolese, 2012; Ogino, Miyamoto, Miyake, & Yamaguchi, 2014; Vinogradov et al., 2009). To give an example, Veselinović et al. (2015) have investigated the effect of administration of anticholinergics on cognition in untreated patients with schizophrenia and healthy control subjects. Their results show a marked impairment in both experimental groups, which was however more pronounced in the schizophrenia patients, thus again casting doubt on the suitability of these drugs in the treatment of schizophrenia (Veselinović et al., 2015).

Muscarinic antagonists are also used in treatment of organophosphate poisoning. Various studies tested many compounds to select those with good effectiveness and minimum side effects. Using the acoustic startle response test and pre-pulse inhibition (PPI), Sipos et al. (2001) investigated the effects of aprophen, atropine, azaprophen, benactyzine, biperiden, procyclidine, scopolamine, and trihexyphenidyl. Based on their findings, the authors recommend biperiden, procyclidine, triheyphenidyl, and benactyzine as delivering the best results at dosages low enough not to cause unwanted side effects (Sipos et al., 2001). A similar study was conducted by Myhrer et al. (2008), who tested benactyzine, biperiden, caramiphen, procyclidine, and trihexyphenidyl in a novelty test, ultimately recommending procyclidine (Myhrer, Enger, & Aas, 2008).

Interestingly, some antimuscarinic agents (namely scopolamine) also appear to possess antidepressant qualities, especially in treatment of those patients who are unresponsive to the standard therapy. Witkin et al. (2014) report these antidepressant effects might be mediated specifically by the blockage of the M1 and M2 receptors (Witkin et al., 2014).

The general consensus is that anticholinergics disrupt acquisition learning and long-term memory processing. As such, these compounds are often employed for

inducing memory and cognitive impairments in laboratory animals in order to simulate pathological states observed in human diseases such as schizophrenia, Alzheimer's disease and other dementias (Robinson et al., 2011). For example, atropine was shown to impair memory retention in in mice in a step-through inhibitory avoidance task (Boccia, Blake, Acosta, & Baratti, 2003).

2.2.1. Mechanism of Action

As mentioned above, the acetylcholine binding site is evolutionarily highly conserved across all five muscarinic receptor subtypes, which in turn complicates the search for selective ligands. However, there is an abundance of allosteric sites that facilitate receptor activity modulation, and are specific for each receptor subtype. These enable development of highly selective compounds. (Jiang et al., 2014).

Orthosteric subtype-selective agents are scarce, however some may be found; for example a recent study reported a novel compound PCS1055 which exhibits high selectivity for M4 receptor (Croy et al., 2016). Also, some ligands have been shown to bind at the orthosteric site as well as one of the allosteric sites, thus achieving relatively high selectivity for a specific mAChR subtype. An example may be provided in the work of Jakubík et al. (2014) where the mechanism of action of M2-selective antagonist methoctramine was put under scrutiny. The authors report that methoctramine binds with high affinity to the orthosteric site and at the same time interacts with lower affinity with an allosteric site at the second and third extracellular loops. Interestingly, in the presence of another orthosteric-binding ligand (such as N-methyl-scopolamine), methoctramine may still bind to the allosteric site, thus preventing the other ligand from dissociating from the receptor. This antagonist occasionally binds M3 receptor as well, but with much lower affinity due to the lack of the allosteric site found on M2 (Jakubík et al., 2014). Also, that the time antagonists take to bind to the receptor has been shown to be of crucial importance for the efficacy of receptor blockage. For example, due to its relatively slow binding, tiotropium seems less effective at blocking the M3AChR (Deng, Wang, Su, & Fang, 2012).

As to the effects of antimuscarinic drugs on the organism, these naturally depend on the means and place of administration (which determines where the agent exerts its influence, such as the brain following an intraventricular injection or the heart after a systemic application of a drug unable to cross the blood-brain barrier). Thus, as the M1

and M4 receptors are abundantly expressed in parts of the brain affected in neurodegenerative diseases such as AD, it seems probable - and it has been repeatedly proven – that stimulating cholinergic transmission via these receptors would enhance cognitive abilities, learning and memory, whereas blocking it would result in cognitive impairment (Digby et al., 2010). However, the detrimental effect of antimuscarinic drugs on cognition may also be caused or supplemented via other, less direct means. For example, the results of the studies investigating antimuscarinic agents in the acoustic startle reaction test and PPI seem to suggest that one of the possible mechanisms of the scopolamine's disruptive effect on cognition might be its inhibiting pre-pulse inhibition, i. e. enhancing startle reactions (Jones & Shannon, 2000; Robinson et al., 2011).

2.3. Clinical Potential

In spite of the risk of various undesirable side effects such as cognitive impairment, dry mouth, or even psychosis and addiction, etc., if dosed with care, antimuscarinic drugs provide therapeutic effects in a number of conditions. For illustration, aclidinium and tiotropium are often prescribed in treatment of chronic pulmonary disease, as well as asthma, overactive bladder, and irritable bowel syndrome (Busse, Dahl, Jenkins, & Cruz, 2016; Callegari et al., 2011; Peretto, Petrillo, & Imbimbo, 2009; Zhong, Roth, J., & M., 2014)

Quite recently, scopolamine, a non-selective antagonist capable of crossing the blood-brain barrier, has been found to exhibit antidepressant properties (mediated probably by its binding to M1 and M2 receptors), even in patients unresponsive to standard therapy (Witkin et al., 2014). It has proven beneficial not only to patients with major depressive disorder, but to also to those suffering from bipolar disorder (Jeon, Dean, Scarr, & Gibbons, 2015). Other than that, scopolamine is also used as an antiemetic, for example in treating post-operational nausea (Pergolizzi, Philip, Leslie, Taylor, & Raffa, 2012).

As mentioned previously, muscarinic receptor antagonists (e. g. biperiden, trihexyphenidyl) are also employed as prophylaxis and/or treatment of side effects of antipsychotics prescribed in diseases such as schizophrenia. However, this method is currently on the decline due to the multitude of unwanted side effects of the anticholinergic treatment (Desmarais et al., 2012; Veselinović et al., 2015).

Biperiden, amongst other antimuscarinics, also acts as an antiparkinsonian agent and is thus sometimes prescribed to patients with Parkinson's disease, as well as other

diseases manifesting with parkinsonian symptoms. However, even here the risk of addiction and detrimental side effects still remains (Brocks, 1999; Espi Martinez, Espi Forcen, Shapov, & Martinez Moya, 2012).

Quite surprisingly, given the amount of criticism regarding the cognitive side effects of muscarinic antagonists, a recent study investigating the properties of a new potential treatment for Alzheimer's disease reported an M1-antagonism of these agents. The tested drug candidate has been developed in light of a newly proposed approach to treating multifactorial diseases such as AD; which aims to hit multiple therapeutic targets with a single drug. This comprises of a series of compounds, in this case combining 7-methoxytacrine and memantine. As the results of other tests (such as successful prevention of β-amyloid fibrillization, AChE inhibition, etc.) look rather promising, the authors recommend the novel compound as a potential treatment, claiming the observed M1-antagonism did not seem to exhibit noticeable effect (Gazova et al., 2016).

2.4. Biperiden as a Prototype Drug

Biperiden hydrochloride (or lactate) is a proven M1-receptor selective antagonist (Figure 3). Approved for human usage and sold under the brand name of Akineton, it is prescribed for Parkinsonism (to improve motor abilities such as gait and tremor) and occasionally to suppress the side effects of neuroleptics. It is administered orally, in a dose of 2 – 16 mg a day (for adults). The commonly observed side effects of Akineton include blurred vision, dry mouth, constipation, drowsiness and dizziness, mental confusion and agitation (AHFS DI Essentials, 2017).

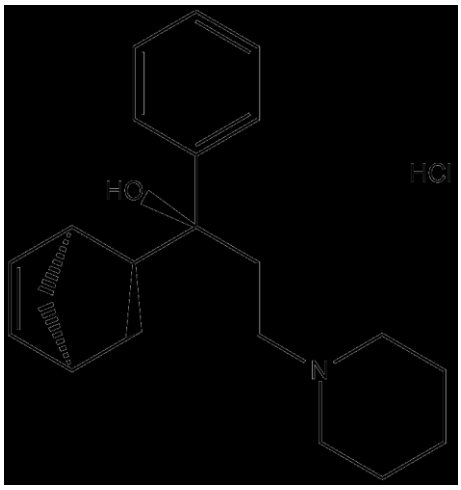

Figure 3.: The chemical formula of biperiden hydrochloride (1-(5-bicyclo[2.2.1]hept-2-enyl)-1-phenyl-3-piperidin-1-ylpropan-1-ol;hydrochloride). Copied from (APExBIO, 2017).

Apart from clinical practice, biperiden is also used in research as a cognitive impairer (Asth, Lobão-Soares, André, Soares, & Gavioli, 2012; Gieling et al., 2013). Biperiden hydrochloride for laboratory practice is sold in the form of white powder. The information about solubility and other properties differ depending on the manufacturer. For example, Sigma-Aldrich states that it is insoluble in water, but readily soluble in DMSO (> 20 mg/ml) instead and that LD_{50} for rats is 750 mg/kg (Sigma-Aldrich Co. LLC, 2017). APExBIO on the other hand mentions only a limited solubility and recommends heating and the use of an ultrasonic bath. They also suggest intraperitoneal (i. p.) injections or oral administration of the drug solution (APExBIO, 2017).

Biperiden has been shown to cross the blood-brain barrier (BBB) without difficulties, thus enabling a simple administration of the drug, such as intraperitoneal or subcutaneous injections (s. c.). The tissue distribution (V_d) for biperiden has been reported to be relatively high: with brain to plasma ratio reaching up to 7 – 12 (Brocks, 1999). The uptake of the drug by the tissues is quite rapid; possibly also due to its substantial transport into lysosomes (Ishizaki, Yokogawa, Nakashima, Ohkuma, & Ichimura, 1998).

2.5. Place Navigation

To increase their chances of survival, successful foraging for food and other resources, as well as finding their nest or burrow, animals employ a variety of spatial navigation strategies. These are mostly a combination of **idiothetic** (also called egocentric) or **allothetic** navigation. In the first case, an individual finds its way based on the information from motor regions of the brain, vestibular receptors, and muscle proprioceptors, whereas in the second case, the spatial representation is established upon external cues (Bures, Fenton, Kaminsky, & Zinyuk, 1997). Three strategies may be used to reach a goal:

(1) a *praxis* strategy, when an animal follows a set of learned movements that lead to a known goal,

(2) a *taxis* strategy, when the goal is clearly visible from a distance or marked by other cues,

(3) spatial strategy or mapping, when long-distance external cues become the spatial reference points, as the goal cannot be located otherwise (by sight or smell) (D'Hooge & De Deyn, 2001; Morris, 1981; Sutherland, Whishaw, & Regehr, 1982).

To illustrate, a man waking up at night and finding his way to the bathroom in the dark employs a *praxis* strategy; he knows it takes approximately four steps to the door of the room and then he has to turn right in the hallway and walk five more steps. A *taxis* strategy is used for example by a man approaching a bank – a large conspicuous building bearing an easy-to-see sign "Bank". Whereas the mapping strategy focuses on finding the correct configuration of distal external cues, such as a man searching for a buried treasure (after his unsuccessful errand in the bank): he has to stand at a place with the big pine tree to his left, the strangely-shaped mountain on the horizon behind him, and the lake a short distance in front of him.

Spatial navigation is based on the so-called *place coding* (Kitanishi et al., 2016). The key structure of the brain involved in these processes is generally thought to be the hippocampus (more specifically the ventral part), however other parts of the brain play important roles as well. The neuronal substrate consists of (1) **place cells**, large hippocampal pyramidal neurons with characteristic complex spikes that fire only in a specific part (or parts) of a given environment (the so-called *firing fields* or *place fields*). Interestingly, their structural organisation in the brain is not topological, i. e. it does not reflect the outside world. Groups of these cells constitute *ensembles*, which serve as representations of the environment. Apart from these, there are (2) **grid cells**, located in the entorhinal cortex. The spatial pattern of their firing fields resembles a hexagonal grid. And the final type is represented by (3) **head direction cells**, found in the Papez's circuit, and whose activity is dependent on the inclination or direction of an individual's head (Bures et al., 1997; Burgess, 2006; Kitanishi et al., 2016; Yan, Wang, Qu, & Chen, 2016). The specific roles and mechanisms of function of these cells are not yet fully understood. A recent study has proposed a model for spatial navigation based on cooperation between place cells and grid cells, in which place cells are responsible mainly for locating a goal, whereas grid cells are in charge of directing an individual towards the goal (Yan et al., 2016).

Another important aspect of effective spatial navigation are sets of spatial stimuli that yield so-called **frames of reference**. An individual often needs to be able to distinguish and correctly assess conflicting information from several of these frames to solve a task. An example of a behavioural test specifically assessing this ability is the Active Allothetic Place Avoidance (AAPA; see section 1.5.3.). Hippocampus has been shown to be the structure responsible for organising this spatial information into

representations correctly corresponding the outside world (Stuchlik, Rezacova, Vales, Bubenikova, & Kubik, 2004)[5].

Behavioural tests based on spatial navigation are largely used by researchers in studying certain types of memory. It has been shown that in rodents, the most similar equivalent to episodic memory, generally considered to be unique to humans, are processes employed in spatial navigation. These follow the same neural circuits, even the left vs. right hippocampus functional asymmetry is analogous, as well as the wave oscillation patterns that orchestrate the brain function (Kitanishi et al., 2016).

2.5.1. Morris Water Maze

First conducted and described by Richard Morris in 1981, the behavioural test now commonly known as the Morris water maze (MWM) was the first test enabling researchers to confirm the existence of spatial mapping and assess its features, as the animals have no visual, olfactory or any other way of detecting the goal other than distant external cues. Unlike many other behavioural tests, it is relatively simple to set up and perform, but still enables quite detailed discerning of various behavioural mechanisms (D'Hooge & De Deyn, 2001; Morris, 1981).

Although different laboratories may alter the set-up slightly, it generally consists of a large pool (approximately 1.5 – 2 m in diameter) filled with water rendered opaque by addition of milk or non-toxic paint, and an escape platform submerged approximately 1 cm under the water surface. (Some authors report using clear water in combination with black pool and black or transparent platform.) The surrounding environment should offer a rich amount of cues employable for navigation. The animal's performance is usually recorded by an overhead camera and a tracking program (Figure 4), that enable various parameters (such as the time taken to reach the platform – the so-called escape latency, distance, thigmotaxis, and floating) to be analysed. The rats (or mice) are placed in the pool (facing a wall) and allowed to swim for a given amount of time (usually 60 – 90 s) or until they find and climb onto the hidden platform, where they are allowed a short time to become acquainted with the position of the platform in relation to the surroundings.

[5] A similar task testing the ability to mentally coordinate conflicting information exists for humans as well: in a Stroop test, a subject is presented with a sheet with words for colours, which, however, are printed in ink of a different colour than the one described by the word (i. e. the word 'yellow' is printed in blue ink, etc.). The respondent is asked to say the colours of the ink; he has to avoid reading the actual words. It has been shown that patients suffering from schizophrenia are incapable of completing this task (Laurenson et al., 2015).

Different numbers of testing days, swims (otherwise called trials), varying lengths of intervals between the trials, as well as diverse locations of the hidden-platform and their un/changing are used in the many variations of this test (D'Hooge & De Deyn, 2001; Morris, 1981; Terry, 2009). A quite detailed information on conducting a Morris water maze experiment may be found here (Terry, 2009). For an all-embracing comprehensive review, see (D'Hooge & De Deyn, 2001).

The most basic form of a MWM experiment is simple *acquisition*, in which the animals are trained to find the hidden platform whose location does not change. The number of trials and testing days may vary due to differences in learning abilities of the given rat (or mouse) strain and in protocols used by specific laboratories (Entlerova et al., 2013; Morris, 1981). Another example of a design of a MWM experiment is *reversal learning*. In this task, the animals are trained for five consecutive days (eight trials per session). For the first three days, the platform remains at a constant position (for example the north-east part of the pool), but is changed to the polar opposite (i. e. in this example to the south-west part) for the remaining two days. This tests the rats' ability to relearn the new location of the platform, in other words cognitive flexibility. Probe trials, in which the platform is removed from the pool, and the animals are allowed to swim freely for a given amount of time, may be included. These in turn investigate how much time the rats spend in the quadrant of the pool, where the platform used to be, thus assessing memory retention (Devan, Tobin, Dunn, & Magalis, 2016). Other variants of the design include *delayed-matching-to-position* (DMP) which enables testing of working memory: the location of the hidden platform changes every session, thus creating the need to learn it anew every time (O'Carroll, Martin, Sandin, Frenguelli, & Morris, 2006; von Linstow Roloff, Harbaran, Micheau, Platt, & Riedel, 2007). The animals may of course undertake this testing after having been subjected to pharmacological, surgical or other manipulation, in order to test for the effects on memory and cognition of the given treatment. Especially in cases like these, the hidden-platform testing is often supplemented by *visible platform*, in which the escape platform emerges above water surface and may also be marked by a ring or a hanging cue for the animals to see clearly. A poor performance in this paradigm indicates visual, motor, etc. impairment, whereas if learning deficits are observed in the hidden-platform but not here, the impairment is probably of cognitive nature (D'Hooge & De Deyn, 2001; Entlerova et al., 2013; Laczó et al., 2016). An interesting variation of the task is the '*on-demand-platform'*, in which the

platform is deep underwater and only emerges after the rat has spent a designated amount of time swimming over the area where the platform is located (Bures et al., 1997).

Several variants fit for use in humans of the MWM have been developed, such as the blue velvet arena (Laczó et al., 2016), and the virtual maze environment (Schoenfeld, Schiffelholz, Beyer, Leplow, & Foreman, 2017). Studies comparing the performance of human subjects in these tests with that of rodents in the MWM showed no major differences, thus confirming the validity of the MWM experimental design (Laczó et al., 2016; Schoenfeld et al., 2017). Quite interestingly, an analogous test to the MWM has been described also for frogs (Bilbo, Day, & Wilczynski, 2000).

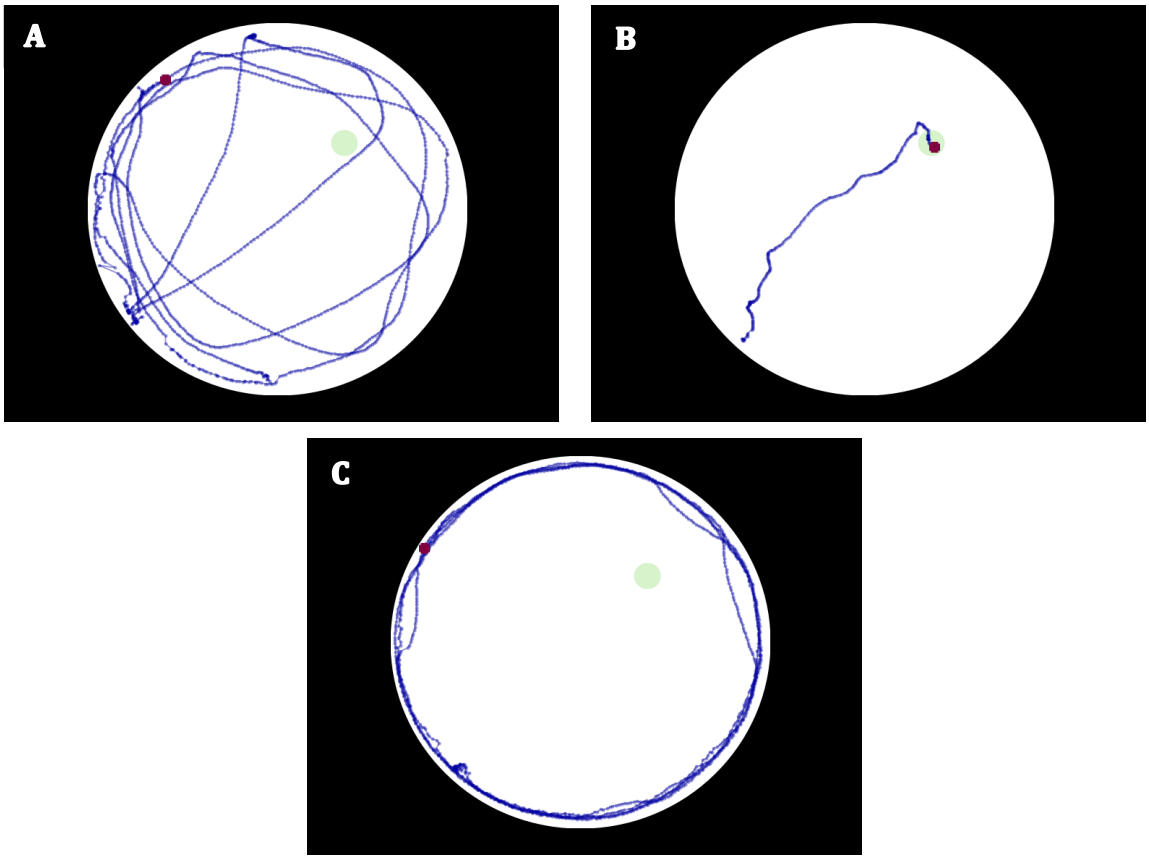

Figure 4: The diagrams above show several examples of a rat's trajectory in the MWM recorded by a tracking program. In picture (a), the rat was placed in the pool for the first time and was therefore unfamiliar with the task. First, it swam around the pool, keeping close to the sides, then it started exploring the middle part as well, however was unable to find the hidden platform. After 60 s, the trial was stopped and the animal was gently guided to the platform. Picture (b) shows a path of a rat well acquainted with the task and who had already learned to locate the hidden platform almost immediately. In (c), the animal displayed a very prominent thigmotaxis,

not searching for the platform at all. This behaviour might reflect a heightened level of anxiety. Pictures are part of the data for this study.

2.5.2. Effects of Muscarinic Antagonists in the Morris Water Maze

2.5.2.1. Scopolamine

Antagonists of muscarinic receptors have been repeatedly shown in various behavioural testing paradigms to impair cognitive performance, the MWM being no exception. Possibly one of the most frequently used antimuscarinic agents in this particular test is scopolamine, which has been reported to disrupt different types of memory. In spite of becoming something of a 'golden standard' in research of cognitive impairment, its validity as a model has often been questioned on the grounds of its considerable side effects. As it lacks selectivity for any of the subtypes of muscarinic receptors, apart from memory and cognition, it also affects sensorimotor functions of the treated subjects, thus sometimes compromising the results of the behavioural tests (Klinkenberg & Blokland, 2011). However, Robinson et al. (2004) reported an impaired performance in the MWM in both rats and mice following scopolamine administration at a dose that exhibited no effect on visual acuity. This was studied in a variant of the MWM task specially adjusted to test for compromised visual perception, in which the animals were required to discriminate between two marginally differing cards in order to successfully find the hidden platform (Robinson, Harbaran, & Riedel, 2004). A lack of effect on performance in a mainly vision-reliant task (the visible platform variant of the MWM) was also reported by Entlerova et al. (2013) in their study focusing on comparison of two commonly used rat strains (Wistar and Long-Evans) and their performance and sensitivity to anticholinergic blockade in the MWM and the AAPA. Following scopolamine treatment, they found no marked differences in the MWM between the two strains, unlike the AAPA, where the Wistar rats exhibited significantly worse performance than the Long-Evans group, which suggests a higher sensitivity of the former group (Entlerova et al., 2013).

Furthermore, Von Linstow Roloff et al. (2007) set out to investigate whether the poor performance of scopolamine-treated rats in the MWM is in any part due to an effect on memory processes, or whether it is just the result of the compromised sensorimotor abilities. In a series of experiments consisting of acquisition tasks combined with both spatial and non-spatial pre-training, as well as delayed-matching-to position (DMP), and

a variant of the DMP with an on-cue platform (also called the Atlantis platform), they were able to show that although scopolamine undoubtedly causes side effects leading to altered swimming speed and higher levels of thigmotaxis, these can be eliminated by extensive spatial pre-training. In such a case however, scopolamine-treated animals still perform more poorly than controls, thus confirming that scopolamine does indeed affect spatial memory. In the Atlantis platform paradigm, the researchers were able to discriminate between the effects on procedural and spatial memory: scopolamine was found to impair the latter (von Linstow Roloff et al., 2007).

Scopolamine-induced cognitive impairment was also shown to possess good validity as a translational model in research: Laczó et al. (2016) compared the effects of scopolamine administration (as well as its co-administration with donepezil, an AChE inhibitor) in rats and humans in the MWM and the Hidden Goal Task, an analogue of the water maze fit for use in humans. The authors reported successful validation of the tasks and scopolamine, as no significant differences were found between the human volunteers and the animals. Donepezil was shown to exhibit some ameliorative effect, however this was not clearly marked in all cases (Laczó et al., 2016).

2.5.2.2. Quinuclidinyl Benzilate

The use of MWM also occurred in a report assessing the properties of 3-quinuclidinyl benzilate (QNB), a non-selective muscarinic antagonist, which has also been proposed as a potential agent for modelling cognitive deficit in rats. The study showed a significant detrimental effect of QNB on acquisition in the MWM, whereas no impairment was found in memory consolidation and retrieval. Apart from hyperlocomotion, leading to higher swimming speed the authors observed no adverse side effects of QNB on vision and sensorimotor functions (Misik, Vanek, Musilek, & Kassa, 2014).

2.5.2.3. Atropine

Although mostly of an older date, studies examining the effects of other antimuscarinic agents may also be found. One such report focused on atropine. In an older study by Sutherland et al. (1982), atropine sulfate-treated rats were found to lack the ability to employ spatial mapping as means of learning the location of the hidden platform, thus turning to a combination of taxis and praxis strategies (i. e. not remembering the position of the platform but instead rather a way of finding it). No such deficit was

observed in control animals and a group treated with atropine methylnitrate (a substance acting solely in periphery as it is unable to cross the blood-brain barrier), hence confirming the hypothesis that central cholinergic system underlies spatial mapping strategies (Sutherland et al., 1982).

2.5.2.4. Pirenzepine

Another such example: the study of Hagan et al. (1987) investigated properties of pirenzepine, an M1-selective antagonist. Although less potent than scopolamine, it was nevertheless shown to impair spatial navigation in the MWM. However, one of the major drawbacks of this drug is its incapability to cross the blood-brain barrier, consequently requiring an intraventricular administration (Hagan, Jansen, & Broekkamp, 1987).

2.5.2.5. M2 Receptor Antagonists – Ameliorative Exceptions

An exception to the 'rule' of muscarinic antagonist having a detrimental effect on learning and memory are compounds selective for receptors expressed pre-synaptically (such as M2), which by blocking the pre-synaptically mediated inhibition of ACh release actually help to increase the levels of ACh in the synapse, and thus also cholinergic transmission (Greenlee et al., 2001; Rowe et al., 2003). For example, BIBN-99, a selective M2 antagonist, has been shown to improve performance of aged rats in the MWM (Rowe et al., 2003).

2.5.3. Other Behavioural Tests

2.5.3.1. Spontaneous Alteration Tasks

The tasks in this category are all based on the natural tendency of rodents to explore unknown environment, i. e. enter those arms of a maze that they have not visited previously. If an animal's (working, in most cases) memory capabilities are compromised, it will not be able to recall which places are new and thus keep randomly returning to the parts it has already visited. The tests used for the assessment of this behaviour include the so-called T-maze, Y-maze, and (four-way) cross maze (Myhrer, 2003). To give an example, Ragozzino et al. (2012) used a cross maze task to show and confirm the enhancing effects of CDD-0102A on working memory, a partial M1 agonist (Ragozzino et al., 2012).

Some of the arms in the mazes may also be closed and others open and the apparatus may be elevated – in this case, apart from memory, the tasks become tools for measuring levels of anxiety in laboratory animals. For example, Asth et al. (2012) used the elevated T-maze to study effects of biperiden and diazepam administration in mice; the results suggesting a memory acquisition impairment following the drug treatment (Asth et al., 2012). Elevated plus maze was employed, for example, by Gupta et al. (2012) who investigated the potential beneficial effects of resveratrol on scopolamine-induced cognitive impairment in mice, however no differences were found between the control group and the group pre-treated with resveratrol (Gupta, Gupta, Mediratta, & Bhattacharya, 2012).

2.5.3.2. Passive and Active Avoidance Tasks

In passive avoidance tasks, a rodent is required to avoid a natural behavioural response, such as moving down from an elevated platform (a *'step-down' test*) or escape from a brightly lit compartment into a dark one (a *'step-through' test*); this behaviour is punished by a mild electric foot-shock (Myhrer, 2003).

As an example, a *step-through* passive avoidance task was used by Misik et al. (2014) to investigate the influence of QNB on various memory stages; the authors reported a detrimental effect on acquisition, but not consolidation and retrieval of memory (Misik et al., 2014). The *step-down* paradigm was utilised for example in study aforementioned study by Gupta et al. (2012) assessing whether resveratrol might reverse cognitive impairment of scopolamine, yet again no alleviating effects were observed (Gupta et al., 2012).

In contrast, in active avoidance paradigms, the animals are required to actively escape to a different part of the testing apparatus, lest they receive an aversive stimulus (a mild electric shock). An example of such a procedure is the *two-way active avoidance*. The apparatus typically consists of a shuttle box with gridded floor and light or sound mechanism for presenting a conditioned stimulus: the animal learns to move to the other compartment of the shuttle box upon the occurring of a brief light or sound signal, otherwise it receives a foot-shock. This task was used, for example, by Carballo-Márquez et al. (2011) in assessing the effects of cholinergic blockade by scopolamine in basolateral amygdala on this aversion learning. Quite interestingly, no deficit in learning was

observed in the scopolamine-treated animals, there was even a tendency towards better performance (Carballo-Márquez et al., 2011).

2.5.3.3. Active Allothetic Place Avoidance

Active allothetic place avoidance (AAPA) is a behavioural test specifically focusing on a rat's ability to coordinate two conflicting frames of reference. An animal is placed into a slowly rotating arena where he needs to learn to avoid a 'forbidden sector', upon stepping into which it receives a foot-shock. The position of this sector does not change relative to the room frame, i. e. the animal has to actively move to another place in the arena so as not to be carried into the forbidden sector. The arena's surroundings ought to contain distinct extra-maze cues for the rats to navigate by (Stuchlik et al., 2004).

This task was used for example in a study by Entlerova et al. (2013) which compared performance of two rat strains (Long-Evans and Wistar) in the MWM and the AAPA, following scopolamine treatment. Whereas in the MWM, the disruption in learning and memory was similar, in the AAPA the Wistar rats exhibited higher sensitivity to scopolamine than the Long-Evans group (Entlerova et al., 2013).

2.5.3.4. Radial Arm Maze

The Radial arm maze presents another task used to test spatial cognition, namely working and reference memory, but the procedure may also be adjusted to assess acquisition and memory retrieval (Myhrer, 2003; Pilcher, Sessions, & McBride, 1997). The apparatus consists of several corridors – 'arms' (mostly six or eight but other variants are also possible) which may via a system of pulleys be closed by the experimenter. A food reward is placed at the end of each arm. The animals have a free choice of which arm to visit; they are consequently tested upon their ability to recall where they have already been, represented by the number of 'wrong' entries, i. e. entering a previously visited arm (Rosengarten & Quartermain, 2002).

This task was used for example in the study of Kay et al. (2010), which showed that scopolamine elicits stronger effect on working memory, whilst 3,4-methylendioxy-metamphtamine (MDMA) administration affects reference memory more prominently (Kay, Harper, & Hunt, 2010). Similar results regarding scopolamine administration had also been reported by Pilcher et al. (1997), who compared the effects of scopolamine on

working memory, acquisition and memory retrieval, concluding stronger impairment in working memory relative to the other types (Pilcher et al., 1997).

This task may also be used for investigating the differences in consequences of acute vs. chronic drug administration, as shown for example by Ortega-Alvaro et al. (2006). In their study, the authors found a significant impairment in rats's performance in the radial arm maze following an acute injection of atypical antipsychotics (olanzapine and clozapine, used in treatment of schizophrenia) and scopolamine, marked among others by a lower speed of movement. However, when following a chronic drug treatment, the observed deficits were absent, hence hinting at the ability to build a tolerance. The authors also concluded that chronic muscarinic antagonism may exert little or no influence over working memory (Ortega-Alvaro, Gibert-Rahola, & Micó, 2006).

2.5.3.4.1. Radial Arm Water Maze

A combination of water maze and the radial arm maze, this task requires the animals to swim towards hidden platforms located at the ends of the maze corridors, whilst avoiding the previously visited arms and arms not containing an escape platform. It has been suggested to be more advantageous over the classic radial arm maze as the setup eliminates the possibility of using odours for navigation as well as the need for food deprivation (Penley, Gaudet, & Threlkeld, 2013).

2.5.3.5. Barnes Maze

In the so-called Barnes-maze, a rat is placed in the centre of a circular platform with holes at the edges. An escape cylinder is placed under one of these holes; the animals are trained to locate the position of this cylinder based one distal external cues. The use of odour trails is eliminated by rotating the platform in between trials (Barnes, 1979).

This paradigm was employed for example by Seeger et al. (2004) for investigating the changes in cognition and behaviour in M2 knock-out mice, reporting a severe impairment in learning with both short-term and long-term potentiation significantly decreased (Seeger et al., 2004). Another example of usage of this test is the study by Gawel et al. (2016) in which the authors examined the potential of cholinesterase inhibitors (donepezil and rivastigmine) to alleviate ethanol-induced cognitive impairment. The results showed an improvement in both memory retention and cognitive flexibility, the latter being more pronounced in rivastigmine. Similarly to the study of Seeger et al.

(2004), the authors as well recommend their findings as note-worthy in relation to potential advances in clinical therapy (Gawel et al., 2016).

2.5.3.6. Cone-field Test

The cone-field task represents another experimental paradigm for testing spatial learning and memory. It consists of dodecagonal field with a number of cones topped with un/baited food cups in the middle and four starting boxes on the borders, from which the animal is released into the field. The ability of the rat to learn and remember the position of the baited cones is assessed. A suggested advantage of this test over tasks like the MWM is that it is based on positive reward learning (whereas the MWM relies on aversive learning). This task was used for example by Van der Staay et al. (2005) to investigate the effects of AChE inhibitors (donepezil and metrifonate) on scopolamine-induced learning deficit in rats. The results showed that metrifonate, but not donepezil, was able to alleviate the working memory disruption produced by scopolamine (Van Der Staay & Bouger, 2005). Another example of usage of the cone-field test is the study of Szcodry et al. (2014) evaluating biperiden as a potential neurodegeneration modelling tool (see discussion) (Szczodry, van der Staay, & Arndt, 2014).

2.5.3.7. Hole-board Task

In the hole-board task, an animal is placed in a rectangular box with a number of holes in the floor. Some of these are baited with food reward, i. e. the tested animal's ability to learn and remember the position of the baited holes as well as the holes it has already visited, is evaluated. Different variations and adaptions of this task have been used. For example, Post et al. (2011) published a paper on a hole-board paradigm specially designed for mice (the so-called COGITAT) and presented its validation as a tool for testing spatial learning and memory via a scopolamine-induced performance deficit and its alleviation by metrifonate (Post et al., 2011).

2.5.3.8. Starmaze

The starmaze has been developed quite recently as a combination of the MWM and the T-maze. It consists of circular pool with a system of corridors creating the outline of a five-pointed star – five alleys lead from the sides of the pool into the centre where they interconnect in a pentagon-shaped ring. Similar to the MWM, the pool is filled with water

render opaque and an escape platform is placed in one of the ends of the alleys. The goal of the task is to find the hidden platform; this may be achieved by using an egocentric or allocentric navigational strategy or the combination of both. The overall design of the apparatus and the chosen behavioural paradigm allows to discern the employed strategies and/or prompt the animals to favour one of them. As such, it was used for example by Rondi-Reig et al. (2006) in their study investigating the hypothesis that hippocampal N-methyl-D-aspartate (NMDA) receptors located in the CA1 region may play a role in spatiotemporal memory consisting of a sequence of actions. The results from the NMDA-knock-out mice suggest that this might be the case, as these animals were unable to use either egocentric or allocentric strategy to sole the task (Rondi-Reig et al., 2006).

3. AIMS OF THE DIPLOMA THESIS

3.1. Proposed Paradoxical Usage of Biperiden as a Cognitive Impairer

In light of the persisting need for reliable animal models of neurodegenerative diseases with stronger validity, suggestions of new potential candidates keep arising. One of such proposed possibilities is biperiden, antiparkinsonian drug selectively antagonising M1 muscarinic receptor, thus making it a potential tool for generating a fast screening model of memory impairment. Despite being prescribed for treatment of Parkinson's disease and to ease side effects of antipsychotics, it has also been reported to exhibit cognition-impairing properties. As it is highly selective for the M1 receptor, it has been suggested as a potentially superior alternative to scopolamine, as it should elicit little or no side effects (Klinkenberg & Blokland, 2011; Sambeth, Riedel, Klinkenberg, Kähkönen, & Blokland, 2014). However, the up-to-date studies using this agent report conflicting results: whilst some authors observed clear disruption of learning and memory following biperiden treatment (Klinkenberg & Blokland, 2011), others did not or only after an extremely high dose (Szczodry et al., 2014).

3.2. Experimental Questions

- Does biperiden cause impairment in spatial learning and memory in the MWM?
    - Does it affect working memory, memory acquisition and/or retrieval? Does it influence cognitive flexibility?
- May biperiden be recommended as a useful tool for modelling neurodegenerative diseases in rodents?

4. METHODS

4.1. Animals

The total of eighty male Wistar rats (2.5 months old, 270 - 450 g at the beginning of the experiments) obtained from the breeding colony of the Institute of Physiology of the Czech Academy of Sciences were used in this study. The animals were housed in transparent plastic cages (25 x 25 x 40 cm) with water and feed available ad libitum. They were kept in an air-conditioned room with a constant temperature (21 °C), humidity (40 %), and light-dark cycle 12/12. Separate groups of animals were used for different tasks employed in this study (i. e. reversal, DMP, and CA). The behavioural training took place between 8 am and 5 pm (during the light part of the 12/12 cycle). The animals were handled in compliance with the Animal Protection Code of the Czech Republic and the corresponding directives of the European Community Council (2010/63/EC).

4.2. Drugs

The M1-selective muscarinic acetylcholine receptor antagonist biperiden hydrochloride (BIP; obtained from APExBIO) was first dissolved in dimethyl-sulfoxide (DMSO; 100 µl DMSO per 1 mg BIP) and then sterile saline (NaCl 0.9%) was added to reach the final concentration of 3 mg/ml. The solution was prepared a day before the drug treatment. Thirty minutes prior to testing, the rats were subcutaneously (s. c.) injected with either biperiden at a dose of 3mg/kg, or a control solution consisting of DMSO in saline (300 µl DMSO per 1 ml saline).

4.3. Apparatus and Behavioural Procedures

The rats were trained in several versions of the Morris Water Maze Task (MWM). The apparatus consisted of a pale blue pool (180 cm in diameter) filled with water (temperature approximately 22 °C) which was rendered opaque by addition of non-toxic black paint (Swingcolor, black). A transparent plastic escape platform was placed in the pool (submerged underwater), its position depending on the specific design of a given test. The surroundings of the pool provided an abundance of extra-maze cues usable for spatial learning and navigation. The rats' performances were recorded by an overhead camera connected to a tracking program (Tracker, Biosignal Group, USA). The aim was for the rat to learn to find the hidden platform when released into the pool from different

locations. If the animal failed to do so within 60 s from the start of each swim, it was gently guided to the platform. The rats were allowed approximately 15 – 30 s on the platform in order to memorise its position. Rats were returned back to the cage for 10 – 15 mins before next trial (with the exception of the DMP, where the rats were returned either after having finished all four swims, or for 30 mins between the first and second trials).

4.3.1. Reversal

The so-called reversal tests cognitive flexibility, i. e. the ability to relearn a previously acquired task when the circumstances have slightly changed (Deiana et al., 2011; Prado et al., 2016). The animals underwent five days of training with 8 trials per day (Figure 5). For the first 3 days (acquisition phase), the hidden platform was placed in the center of the north-east quadrant of the pool. For the remaining 2 days (reversal phase), it was repositioned in the south-west quadrant, and the rats received drug treatment. A probe trial was added at the end of the third, fourth and fifth day to test memory retention; the platform was taken out of the pool and the rats were allowed to swim freely for a minute.

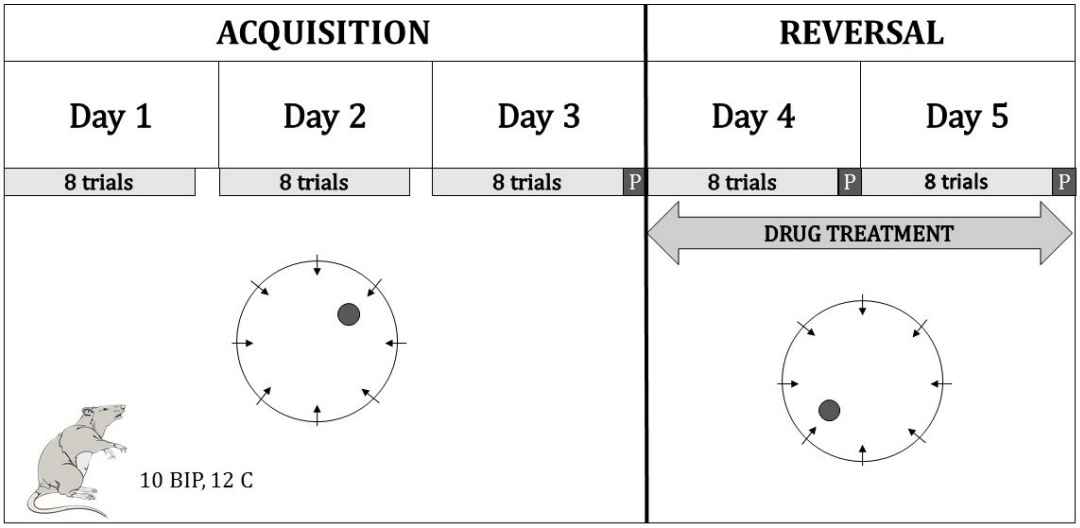

Figure 5: The diagram above represents the experimental design of the reversal task. The upper part shows a time line (day 1 - 5) with the corresponding number of trials for each day (a dark box marked P stands for a probe trial). The double arrow denotes the days when the animals were subjected to drug treatment. The circles represent the pool, the position of the platform for the given set of days is marked by a filled circle, and the arrows signify the different starting positions. The rat in the bottom left corner stands for the total number of animals used in this task (i. e. 10 rats treated with biperiden, 12 rats treated with vehicle). (The picture of the rat was obtained and modified from (Clker-Free-Vector-Images, 2016).)

4.3.2. Delayed Matching to Position

This variant of the MWM tests working memory and memory trace persistence (von Linstow Roloff et al., 2007). Before the experiment itself, the animals underwent a one-day non-spatial pre-training (NSP): any external cues were hidden by a black curtain and the rats were subjected to four swims, to become aware of the existence of the hidden platform and to get acquainted with the new settings. The DMP was then conducted over eight consecutive days with 4 trials per session, the position of the platform changing every day (Figure 6). The rats were under drug treatment for the whole experiment (except for the non-spatial pretraining) and the inter-trial interval (ITI) between the first and second swim pseudo-randomly changed between 15 s and 30 minutes each day for each animal. The DMP was followed by a visible platform test (VP), i. e. one session with 4 trials in which the platform protruded 1-2 cm above the water surface and was clearly marked with a ring and a hanging cue (a cross made out of two compact discs hanging on a string) for the rats to see.

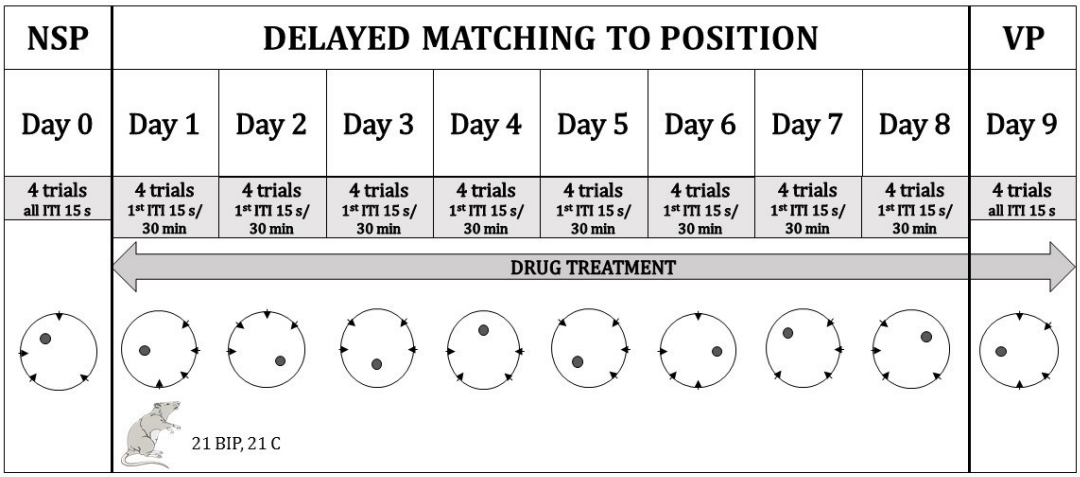

Figure 6: This diagram illustrates the DMP design used in this study. For an explanation of the symbols used see the commentary to Fig. 5. (The picture of the rat was obtained and modified from (Clker-Free-Vector-Images, 2016).)

The NSP part of the design ensures the rats are familiar with the settings and the existence of the hidden platform from the beginning of the DMP task itself. Due to the everyday changing of the platform position, the DMP presents a good tool for testing working memory. The subsequent one-day four-trial visible-platform task serves as a

control of whether the results of the animals' performance might not have been influenced by, or due to a visual or motor impairment.

4.3.3. Counter-balanced Acquisition

This design consisted of four consecutive testing days with eight trials per session and a probe trial at the end of the second and fourth day (Figure 7). The position of the platform (NE) remained constant during the whole experiment. The rats were divided into two groups. The first group (B1) received biperiden treatment for the first two days, whereas the other group (B2) was treated with vehicle. For the remaining two days the drug treatment was switched between the groups, i. e. B1 were injected with saline + DMSO, and B2 with biperiden.

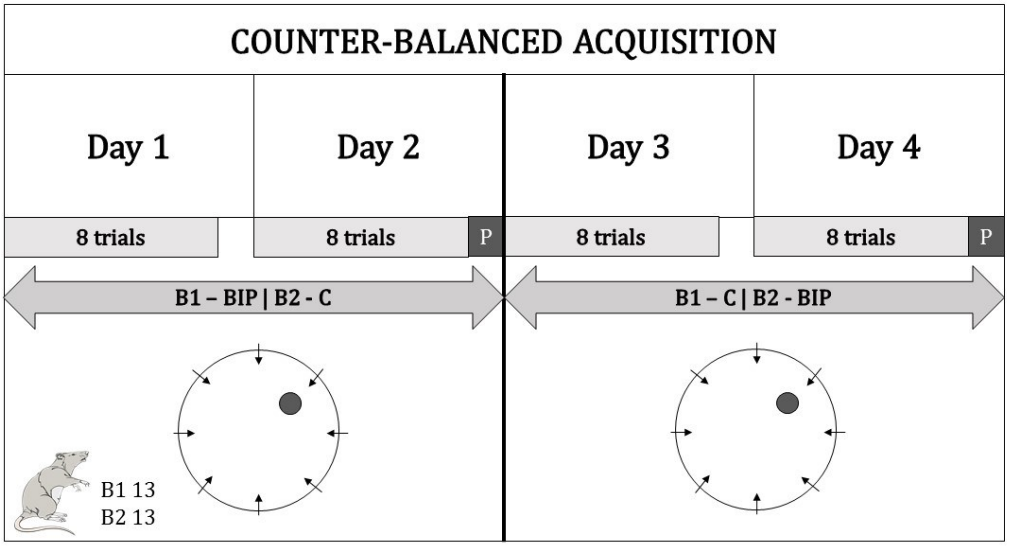

Figure 7: This diagram shows the design of the counter-balanced acquisition test. For an explanation of the symbols used see the commentary to Fig. 4. The double arrows denote what treatment each group of animals received for the given time span (BIP stands for biperiden, C for control, i. e. vehicle). (The picture of the rat was obtained and modified from (Clker-Free-Vector-Images, 2016).)

4.4. Measured Parameters and Statistical Analysis

4.4.1. Reversal

The analysis was conducted using mixed-effect regression. Escape latency served as a dependent variable and group (biperiden- or vehicle-treated), day, trial, and phase

(acquisition or reversal) as well as their interactions served as predictors. Linear and quadratic contrasts were used for the effect of the trial. The data analysis was conducted with an exclusion of the data from the first day which was regarded as required for learning the task. The remaining days were coded as 0.5 for the third day of acquisition and second day of reversal phase and -0.5 for the second day of acquisition and first day of reversal. The subjects were nested within a run to take into account a possible dependence of data for subjects belonging to the same run. All analyses were conducted using R (R Core Team, 2016).

Probe trials were analyzed with mixed-effect regression as well. The time spent in the target quadrant (i.e., the quadrant where the platform had been placed previously) served as a dependent variable and group (biperiden- or vehicle-treated) and day as well as their interaction served as predictors. Deviation coding was used for days. The subjects were nested within a run to take into account a possible dependence of data for subjects belonging to the same run.

4.4.2. Delayed Matching to Position

The analysis was conducted using mixed-effect regression. Escape latency served as a dependent variable and group (biperiden- or vehicle-treated), day, trial, and ITI served as predictors. Apart from the main effect of group, we also included its interaction with day, trial, and ITI in the model. Linear and quadratic contrasts were used for the effect of the day. For the effect of the trial, we used forward difference coding to test the changes between successive trials, and linear and quadratic contrasts to test the trend of changes between the trials. The subjects were nested within a run to take into account a possible dependence of data for subjects belonging to the same run. The data analysis was conducted with an exclusion of the data from the first two days which were regarded as required for learning the task.

4.4.3. Visible Platform

The analysis of performance in this task was conducted similarly to the DMP, excluding the ITI and day predictors. We used polynomial contrasts for the trial effect.

4.4.4. Counter-balanced Acquisition

We used mixed-effect regression for analysis of the latency to reach the platform. As predictors, we included the effect of group (biperiden administered the first two days vs. biperiden administered the last two days), the effect of biperiden, linear and quadratic contrasts for effects of trial and day, the interactions of group effects, trial effects, and group, and the interaction between the effect of biperiden and trial effects. The model was selected by removing predictors from the full model based on Akaike information criterion. We also nested the random effect for a subject under the effect of run.

For the probe trial results, the proportion of time spent in the target quadrant was analyzed using mixed-effect regression with the administration of biperiden and day as well as their interaction as predictors.

5. RESULTS

5.1. Reversal

Escape latencies were lower in the last days of a phase, $t(653.1) = -6.60$, $p < .001$, $b = -8.21$, 95% CI = [-10.65, -5.78], but they did not differ between the two phases, $t(653.1) = -0.12$, $p = .91$, $b = -0.15$, 95% CI = [-2.58, 2.29]. Escape latencies were shorter in later trials as suggested by the linear effect of a trial, $t(653.2) = -11.75$, $p < .001$, $b = -20.72$, 95% CI = [-24.18, -17.26], but the improvement was lower in later trials, $t(653.2) = 2.64$, $p = .008$, $b = 4.66$, 95% CI = [1.20, 8.12]. Most importantly, there was no effect of biperiden administration, $t(19.9) = -0.49$, $p = .63$, $b = -1.37$, 95% CI = [-6.79, 4.05], as well as no interaction of biperiden administration with the effect of a day, $t(653.1) = 0.39$, $p = .70$, b = 0.98, 95% CI = [-3.90, 5.85], phase, $t(653.1) = 0.26$, $p = .80$, $b = 0.64$, 95% CI = [-4.24, 5.52], or linear effect of a trial, $t(653.2) = -0.99$, $p = .32$, $b = -3.49$, 95% CI = [-10.40, 3.43]. The interaction between group and quadratic effect of trial was significant, $t(653.2) = -2.11$, $p = .04$, $b = -7.43$, 95% CI = [-14.35, -0.51], suggesting that biperiden-treated animals did not improve as much as animals in the control group with subsequent trials, but this effect was not specific just to the reversal phase where biperiden was administered. The linear effect of a trial was weaker in the last day of a phase, $t(653.1) = 3.45$, $p < .001$, $b = 12.15$, 95% CI = [5.24, 19.07] and this interaction was weaker in the reversal phase as suggested by the significant interaction of phase, day, and linear effect of a day, $t(653.1) = -2.13$, $p = .03$, $b = -15.03$, 95% CI = [-28.86, -1.21]. No other effect was significant. See Figure 8 for the results.

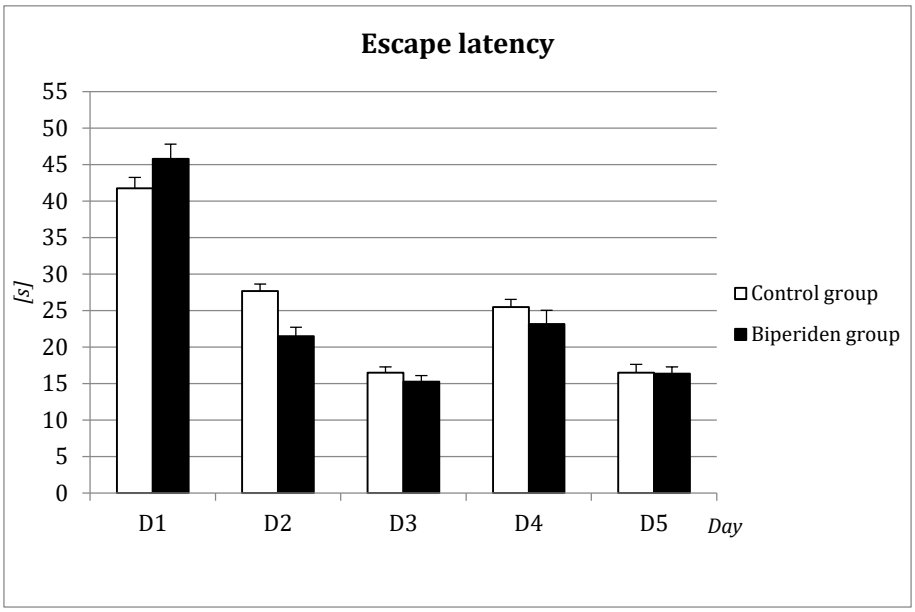

Figure 8: The graph above shows the escape latency (i. e. the time taken by the rats to find the hidden platform). No significant difference is present between the two experimental groups; both successfully relearned the task.

5.1.1. Probe Trials - Reversal

Time spent in the target quadrant did not differ between the two groups, $t(54.7) = 0.57$, $p = .57$, $b = 1.99$, 95% CI = [-4.82, 8.80], and it was not lower in the first day of reversal, $t(39.1) = -1.21$, $p = .24$, $b = -2.65$, 95% CI = [-6.96, 1.66], or the second day of reversal, $t(39.5) = -0.29$, $p = .78$, $b = -0.64$, 95% CI = [-5.00, 3.72], than in the last day of the acquisition phase. The difference between the last day of acquisition and first day of reversal phase did not differ between the two groups, $t(39.1) = -1.44$, $p = .16$, $b = -6.35$, 95% CI = [-14.97, 2.27], but it differed between the last day of acquisition and second day of reversal, $t(39.5) = -2.47$, $p = .02$, $b = -10.97$, 95% CI = [-19.70, -2.25], showing that biperiden-treated animals stayed in the target quadrant for a shorter duration than the control animals in the second day of the reversal phase, $t(19) = -2.97$, $p = .008$, $d = -1.27$, 95% CI = [-2.19, -0.33], $M_{\text{biperiden}} = 22.05$ s, $M_{\text{control}} = 30.87$ s. See Figure 9 for the results.

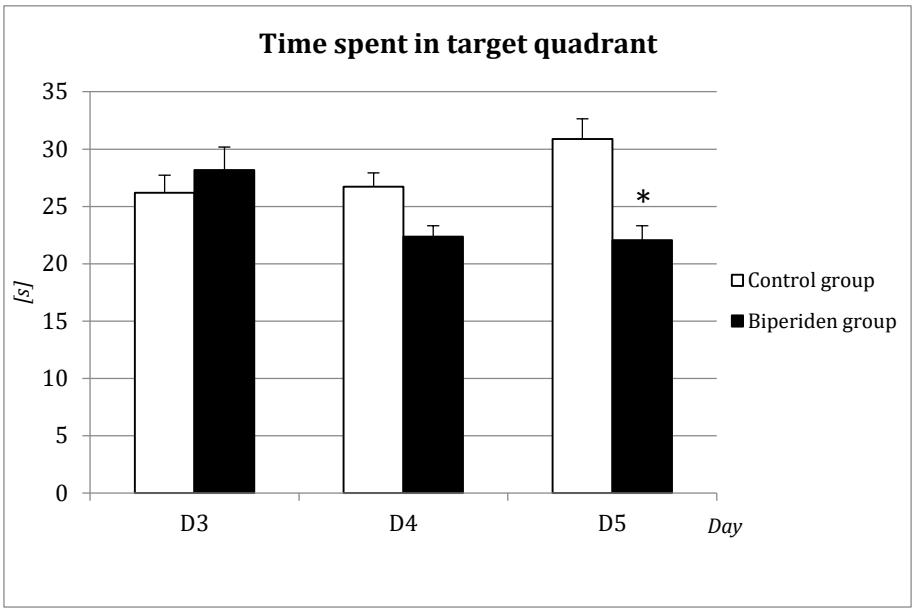

Figure 9: The results of the probe trials on the last day of acquisition phase and the subsequent reversal phase (during which drugs were administered prior to testing; the time spent in the target quadrant on the fifth day was found to be significantly shorter in the biperiden group.

5.2. Delayed Matching to Position

There was no main effect of administration of biperiden on escape latency, $t(38.6) = 1.07$, $p = .29$, $b = 1.94$, 95% CI = [-1.62, 5.49]. Escape latency decreased linearly with subsequent days, $t(950.2) = -3.24$, $p = .001$, $b = -4.47$, 95% CI = [-7.16, -1.77], but there was no quadratic effect of the day, $t(950.2) = 0.43$, $p = .67$, $b = 0.59$, 95% CI = [-2.11, 3.30]. The improvement between days did not seem to level out within the 8 days of the experiment. The effect of the day also did not differ between the two groups either for the linear, $t(950.2) = 0.26$, $p = .80$, $b = 0.70$, 95% CI = [-4.69, 6.10], or for the quadratic effect, $t(950.2) = -0.22$, $p = .83$, $b = -0.60$, 95% CI = [-6.01, 4.81]. There was no effect of ITI on escape latency, $t(953.9) = 0.10$, $p = .92$, $b = 0.11$, 95% CI = [-2.11, 2.33], and no interaction of ITI with group was found as well, $t(953.8) = -0.16$, $p = .87$, $b = -0.36$, 95% CI = [-4.79, 4.07]. Escape latencies decreased between the first two trials, $t(950.3) = -5.38$, $p < .001$, $b = -8.57$, 95% CI = [-11.69, -5.45], and between the second and third trials, $t(950.2) = -4.04$, $p < .001$, $b = -6.42$, 95% CI = [-9.54, -3.30], but there was no further change between the last two trials, $t(950.2) = -1.14$, $p = .25$, $b = -1.82$, 95% CI = [-4.93, 1.29]. The two groups of rats did not differ significantly in the change of escape latencies between the first two trials, $t(950.3) = 0.99$, $p = .32$, $b = 3.17$, 95% CI = [-3.07, 9.41], between the second and

third trial, $t(950.2) = 1.26$, $p = .21$, $b = 4.01$, 95% CI = [-2.23, 10.24], and between the last two trials, $t(950.2) = 1.45$, $p = .15$, $b = 4.62$, 95% CI = [-1.61, 10.85].

All the changes in escape latencies between trials were less marked for the biperiden-treated group. This can be seen when the analysis is done using polynomial contrasts for the trial effect instead of difference contrasts. Both linear, $t(952.2) = -3.25$, $p = .001$, $b = -4.46$, 95% CI = [-7.16, -1.77], and quadratic, $t(952.2) = 0.43$, $p = .67$, $b = 0.59$, 95% CI = [-2.11, 3.30], contrasts for trials were significant. More importantly, the linear effect of the trial differed between the two groups, $t(952.2) = 3.92$, $p < .001$, $b = 8.81$, 95% CI = [4.41, 13.21], with the rats administered biperiden showing generally smaller decrease of escape latency within a session. The quadratic effect did not differ between the two groups, $t(952.2) = 0.32$, $p = .75$, $b = 0.73$, 95% CI = [-3.68, 5.13]. The significant interaction of group with the linear effect of the trial suggests that biperiden-treated animals did not improve as fast as the control animals. When the analysis was done for each trial separately, biperiden treated animals had somewhat lower escape latencies – even if not significantly – than control animals in the first trial, $t(38.0) = -1.23$, $p = .22$, $b = -3.47$, 95% CI = [-8.99, 2.04], and second trial, $t(38.4) = -0.23$, $p = .82$, $b = -0.64$, 95% CI = [-6.20, 4.92], but they had higher escape latencies in the third trial, $t(40.0) = 1.31$, $p = .20$, $b = 3.61$, 95% CI = [-1.81, 9.03], and significantly higher escape latencies in the fourth trial, $t(40.2) = 3.87$, $p < .001$, $b = 8.35$, 95% CI = [4.12, 12.58]. See Figure 10 for the results.

Finally, we tested a specific prediction that biperiden would influence only long-term memory, which we tested by using only the change of escape latency between the first two trials for sessions with ITI of 30 minutes. The interaction between the effect of trial and group was not significant, $t(208.3) = 1.33$, $p = .19$, $b = 6.26$, 95% CI = [-3.00, 15.51], suggesting that the rats treated with biperiden do not improve less in the trials with long ITIs.

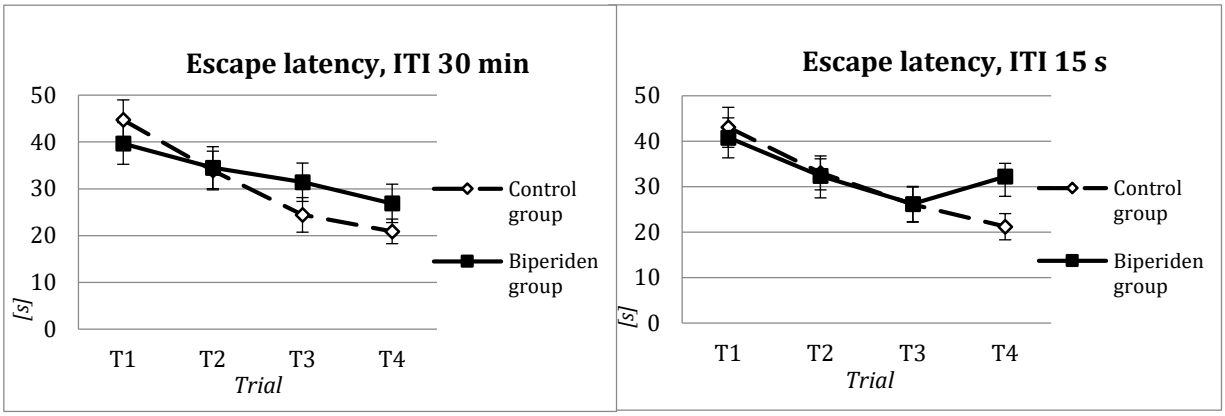

Figure 10: The graphs show the time the rats took to reach the hidden platform (escape latency) in the DMP task. The length of the first ITI clearly did not exert any influence on the rats' performance in the task. There seems to be a trend in the biperiden group towards a smaller progress in learning, which is especially pronounced in the third and fourth trials. The difference might indicate memory impairment; however, it may also be caused by an a priori worse performance of the control group during the first two trials.

5.2.1. Visible Platform

The results showed a significant effect of both a linear, $t(92.0) = -6.23$, $p < .001$, $b = -7.30$, 95% CI = [-9.60, -5.00], and quadratic, $t(92.0) = 2.43$, $p = .02$, $b = 2.84$, 95% CI = [0.55, 5.14], contrasts for trial. Most importantly, the two groups did not differ in their performance, $t(30.0) = -0.04$, $p = .97$, $b = -0.13$, 95% CI = [-6.21, 5.95], and unlike in the delayed matching to place task, they did not differ in their improvement within a session as well; $t(92.0) = 0.85$, $p = .40$, $b = 1.99$, 95% CI = [-2.60, 6.59], for interaction with the linear contrast; and, $t(92.0) = 0.11$, $p = .91$, $b = 0.25$, 95% CI = [-4.34, 4.85], for interaction with the quadratic contrast. See Figure 11 for the results.

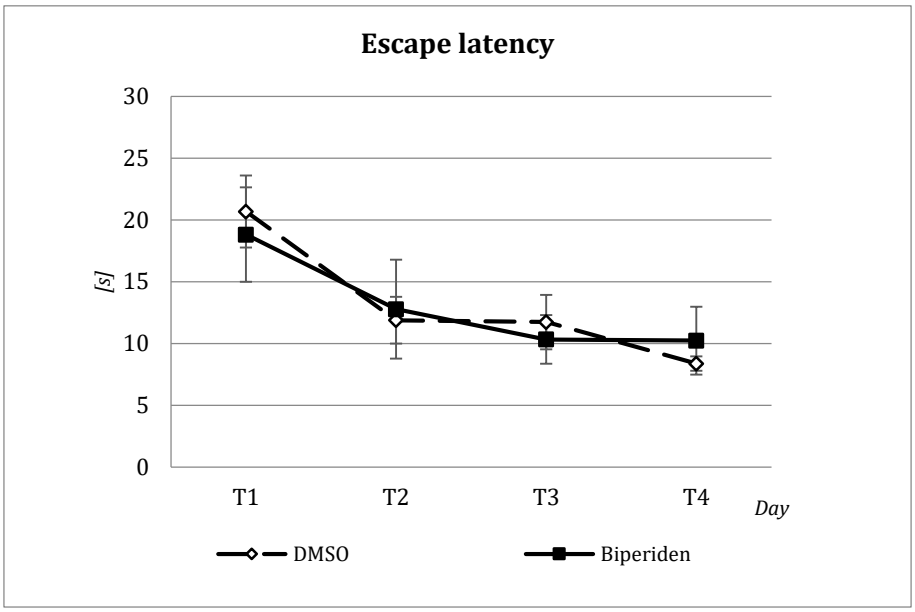

Figure 11: The graph above shows the results of the visible platform task: no differences were found between the two groups, which indicates a lack of sensorimotor deficit.

5.3. Counter-balanced Acquisition

The analysis of the data showed that latency times decreased with subsequent days, $t(782.1) = -18.76$, $p < .001$, $b = -21.10$, 95% CI = [-23.30, -18.90], and trials, $t(782.1) = -11.70$, $p < .001$, $b = -18.61$, 95% CI = [-21.73, -15.49]. The quadratic contrast was significant for both days, $t(782.1) = 4.26$, $p < .001$, $b = 4.79$, 95% CI = [2.58, 6.99], and trials, $t(782.1) = 4.50$, $p < .001$, $b = 7.15$, 95% CI = [4.04, 10.27], suggesting that the improvement in escape latencies was stronger in initial days and trials than in later days and trials. The interaction of the linear effect of day and trial, $t(782.2) = 1.93$, $p = .05$, $b = 6.15$, 95% CI = [-0.10, 12.40], suggests that the improvement within a day decreased for later days.

Administration of biperiden did not influence escape latency times, $t(782.1) = -0.51$, $p = .61$, $b = -1.27$, 95% CI = [-6.18, 3.65]. The order of administration of biperiden and saline did not have a significant effect on escape latency, $t(23.0) = 1.61$, $p = .12$, $b = 3.36$, 95% CI = [-0.74, 7.47]. However, the interaction of group and the linear effect of a day was significant, $t(782.1) = -2.04$, $p = .04$, $b = -10.23$, 95% CI = [-20.07, -0.39], which shows that the group that was administered biperiden in the last two days improved less with subsequent days than the group that was administered biperiden the first two days. Given that the effect of day is confounded with the effect of biperiden administration, this suggests that biperiden administration had smaller effect in the group that was

administered biperiden the last two days. This can be seen when the first two days and last two days are analyzed separately. Whereas for the first two days biperiden-treated animals had significantly worse results than the control animals, $t(22.8) = 2.29$, $p = .03$, b = 6.63, 95% CI = [0.96, 12.31], there was no difference in the last two days, t(24.1) = 0.03, p = .98, $b = 0.07$, 95% CI = [-5.00, 5.14]. See Figure 12 for the results.

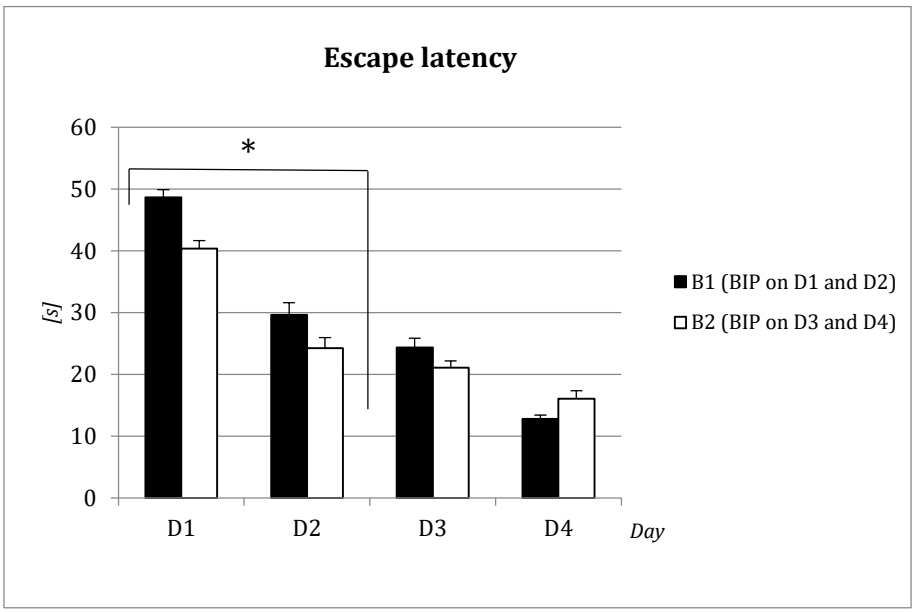

Figure 12: The graph above shows the escape latency results in the CA task. The rats treated with biperiden displayed increased escape latencies; the difference was significant during the first two days.

5.3.1. Probe Trials – Counter-balanced Acquisition

The proportion of time spent in the target sector in probe trials was analyzed using mixed-effect regression with the administration of biperiden and day as predictors. The time spent in the target sector did not differ significantly between the two days with probe trials, $t(24.0) = 1.16$, $p = .26$, $b = 0.03$, 95% CI = [-0.02, 0.08]. Administration of biperiden decreased the proportion of time spent in the target sector, $t(24.0) = -3.22$, $p = .004$, $b = -0.08$, 95% CI = [-0.14, -0.03]. The interaction between the day and administration of biperiden was not significant, $t(23.0) = -0.28$, $p = .78$, b = -0.02, 95% CI = [-0.17, 0.13]; that is, the effect of biperiden did not differ between the two days with probe trials. See Figure 12 for the results.

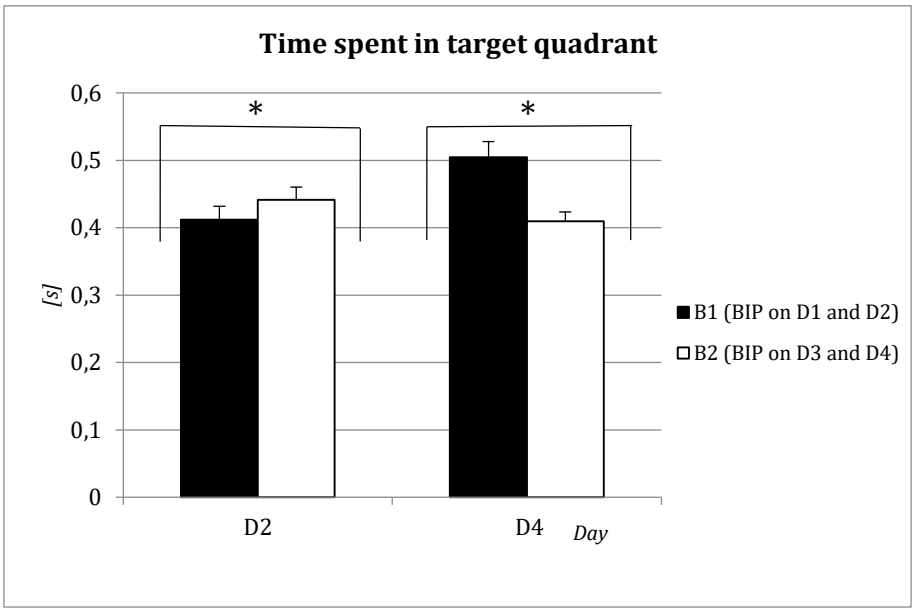

Figure 12: The graph presents the time spent in the target quadrant during the probe trials held on the second and fourth day of testing. The respective biperiden-treated group spent significantly shorter time in the target quadrant.

6. DISCUSSION

The rapid rise of incidence of neurodegenerative diseases in the aging population with no effective therapy available to date presses the need for the development of better animal models to be used in preclinical research. Here, we investigated memory-impairment capacities of biperiden, an M1 selective antimuscarinic compound used in treatment of Parkinson's disease. This drug has been proposed as a potentially superior alternative to the non-selective scopolamine in generating fast screening animal models of neurodegeneration and dementia in rodents (Klinkenberg & Blokland, 2011).

Klinkenberg et al. (2012) published a study which compared the effects of biperiden and scopolamine on various tasks using Skinner boxes, from operational conditioning to attention tests, and delayed-non-matching-to-sample. Thus short-term memory, as well as food motivation and sensorimotor responsiveness could be evaluated, whilst enabling the authors to measure any potential attention deficit. Having tested three different doses of both drugs, the authors found short-term memory disruption following biperiden treatment (at doses of 1 and 3 mg/kg), whereas no significant changes in food motivation and attention were observed. Sensorimotor responding was affected only after the highest dose of 10 mg/kg. In contrast, scopolamine-treatment was followed by attention and sensorimotor deficits and lowered food motivation at both middle and high doses (0.3 and 1 mg/kg). Short-term memory was also affected; however, the authors argue the impairment may have been in a larger part of non-mnemonic nature. Thus, the authors conclude by recommending biperiden for future study (Klinkenberg & Blokland, 2011). In 2014, Klinkenberg et al. published another study assessing the effects of biperiden in human volunteers, and validating biperiden as a translational modelling tool for research of cognition (Sambeth et al., 2014).

In contrast, the study of Szcodry et al. (2014) reached a virtually opposite verdict: a cone-field test revealed no significant differences between rats treated with biperiden (at both 3 and 10 mg/kg doses) versus controls, thus suggesting little or no influence on either working or reference memory. Furthermore, side effects were observed following the higher dose injections in the form of increased latency to start the task and lower number of food rewards collected, which might indicate possible xerostomia. Hence, in conclusion, the authors do not support the validity of this model for research of neurodegenerative diseases (Szczodry et al., 2014).

In this study, we hoped to shed light on the matter of usability of biperiden as a cognitive impairer and help resolve the conflicting observations reported by other authors, using several design variants of the Morris water maze task to assess cognitive flexibility (reversal), working memory (DMP), memory acquisition (CA), and memory retention (probe trials included in reversal and CA experiments). We also conducted one session of visible platform paradigm to test for visual and/or sensorimotor impairment. Significant differences between the two experimental groups were found in the probe trials of both reversal and CA tasks, as well as in the first two days of the CA. Some differences were observed in the DMP as well; however, these were not clearly attributable to a working memory deficit. No significant differences were revealed in the reversal task. These results thus put our study somewhere in between the conflicting reports from other authors (Klinkenberg and Blokland, 2011; Szczodry et al., 2014).

In the *reversal* task, all rats successfully re-learned the new location of the hidden platform, suggesting no detrimental effect of biperiden on cognitive flexibility and adjusting to changed conditions once the principle of the task has been learnt. To the best of our knowledge, this is the first case of biperiden assessment in this task. Within the design of this paradigm, three probe trials were also conducted: (1) on the last day of acquisition phase, when no drugs had been administered, (2) and (3) at the end of the two days of reversal learning (under drug treatment). No differences in performance were found during the first (treatment-free) probe trial, however in the very last probe trial (following drug injections), the biperiden-treated group was found to spend significantly less time in the quadrant where the platform had been previously positioned, hence suggesting reference memory impairment. These results were further confirmed in the probe trials conducted within the *counter-balanced* acquisition paradigm. The memory-retention impairment findings are in line with those of Gieling et al. (2013), who investigated the effects of biperiden in Gottingen minipigs in a hole-board task (Gieling et al., 2013), and the study of Kimura et al. (1999), focusing on alteration of performance in a step-down passive avoidance task (Kimura et al., 1999).

The delayed-matching-to-position task was used to test for disruption of working memory. In agreement with findings of Szczodry et al. (2014) and partially of Gieling et al. (2013), we observed no markedly significant difference in performance between the biperiden-treated group and the control group, especially when comparing the rats' performance in the first two trials and regardless of the length of the first inter-trial

interval. Although, the biperiden group did exhibit a smaller decrease in escape latency times within a session. This can be attributed to the control animals *a priori* displaying worse performance. A more detailed analysis revealed that the biperiden-treated animals performed as good as, or even better, than the control group in the first two trials. However, in the third and in the fourth trial, their escape latencies were higher than those of the control group (the difference being significant in the last trial) which might hint at a compromised memory processes.

In the *counter-balanced acquisition*, biperiden was found to significantly increase escape latency times when administered in the first two days, but not when administered in the last two days. In agreement with the work of (Kimura et al., 1999) and (Asth et al., 2012), these results suggest a disruptive effect of biperiden on memory acquisition. Although, having investigated the binding properties of biperiden, Kimura et al. (1999) also reported a possible partial irreversibility of binding of this compound, which would explain longer-lasting effects observed in their study (Kimura et al., 1999). This might explain the lack of differences in performance between the two experimental groups during the last two days; possibly, the performance of the B1 group (who received biperiden injections for the first two days) was still compromised on the third and fourth day in spite of biperiden treatment cessation, whereas the B2 group (who were treated with biperiden for the last two days) worsened in their performance due to the biperiden injections.

Taken together, our findings suggest only a minor effect of biperiden on spatial learning and memory; any disruption being perceptible only in memory retention and acquisition. However, in light of other studies reporting well-pronounced cognitive impairment following biperiden treatment, this compound cannot be simply ruled out as ineffective. There are many possible reasons for the contradictory results of our experiment and the work of Klinkenberg and Blokland (2011) and others. For example, Klinkenberg et al. (2011) reported using biperiden lactate which they dissolved in purified Milli-Q water and injected the animals intraperitoneally, whereas here we used biperiden hydrochloride dissolved in DMSO (with saline added to reach the required concentration) and we administered the drug subcutaneously. Szcodry et al (2014) also argued their negative results may be due to the rat strain used; they chose Lister-Hooded rats for their experiment whereas Klinkenberg et al. (2011) used Wistar rats, who are known to be more sensitive to pharmacological interventions (Szczodry et al., 2014).

Despite having used the Wistar strain as well, our findings are more in line with those of Szcodry et al. (2014). However, this does not entirely exclude the rat strain as one of the possible reasons for the differing results as long-term breeding in a single institution might over time generate differences even within a single strain. Other than that, the discrepancies in results may also be in part due to the particular behavioral tests employed, as each of them exhibits different sensitivity in revealing specific cognitive impairments.

Regarding non-cognitive effects of biperiden, no differences were found in the visible platform paradigm, which suggests no visual impairment following biperiden injections. Average speed was also calculated for both experimental groups (data not shown), and again, no changes were revealed, pointing to little or no effect on motor skills. This is in contrast to the work of Asth et al. (2012) who reported the occurrence of hyperlocomotion in mice following biperiden treatment (Asth et al., 2012). The only observation of non-cognitive changes following biperiden treatment was: when performing the experiment, the experimenter noticed a slightly increased anxiety-related behaviour in the form of more frequent distress vocalization. This observation is similar to that of Szcodry et al. (2014), although they report increased fearfulness at a higher dose (10 mg/kg) (Szczodry et al., 2014).

Another aspect that might possibly play a role in the varying and sometimes conflicting results obtained by different laboratories is the previously mentioned complexity of the cholinergic system in the brain; mAChR are expressed both pre- and postsynaptically on various types of cells, hence their activation might lead to diverse ends depending on timing and localization. In spite of being labelled as a predominantly postsynaptic receptor, in some cells the M1-receptor may be found presynaptically as well, where it modulates activity of the given neuron (Bell et al., 2013; Kremin et al., 2006; Muller et al., 2013). For example, the M1 receptor (in cooperation with M2) has been shown to influence neurotransmission in the CA1 region of the hippocampus, where it suppresses glutamatergic signalling. It was suggested that this cholinergic activity probably forestalls older engrams from interfering during learning, and thus strengthen encoding and pattern discrimination (Kremin et al., 2006). Furthermore, presynaptic modulation by the M1-receptor has been hypothesized to be involved in processes of learning and memory, as it may stimulate glutamatergic transmission in hippocampal pyramidal cells (co-expressing NMDA receptors), consequently positively affecting long-

term potentiation (LTP). A similar mechanism might also be employed in basolateral amygdala in fear conditioning (Muller et al., 2013).

An interesting hypothesis, which might be very relevant to this particular study, was proposed by Kremin et al. (2006). The authors argue that the M1 receptor may not be crucial to all tasks that are hippocampus-dependent; following a blockage of signalling via M1 receptor, the disrupted inhibition of interference of previously acquired memories might be perceivable only under certain conditions. For example, M1 knock-out mice have been shown to exhibit impaired performance in the radial-arm maze, possibly owing to the animals' inability to distinguish which arm they had already visited, and these circumstances change with each trial. In contrast, in the MWM every trial contains the same, unchanging information (external cues, hidden platform) (Kremin et al., 2006). Hence, in our case it may be possible that the MWM was not a sensitive enough task to reveal impairment caused by the biperiden M1 blockage.

7. CONCLUSION

In this study, we investigated the effects of biperiden, an M1-selective muscarinic antagonist, which has been proposed as a potential tool for modelling cognitive impairment in rodents for the research of neurodegenerative diseases and pre-clinical testing in drug development. To this end, we used several variants of the Morris water maze, which assess different components of learning and memory: (1) cognitive flexibility, tested in reversal learning, as well as (2) working memory, vital for the DMP task, were unimpaired in the biperiden-treated animals. An increase in escape latency following biperiden injections was observed during the first two days in (3) acquisition learning (in the CA task). A significant impairment of (4) reference memory was revealed in the probe trials of the reversal and CA tasks. Also, the biperiden-treated rats displayed smaller improvement within the four trials each day in the DMP which may have been either due to the worse performance of the control group in the first two trials, or possibly due to a memory impairment. Based on our results, biperiden seems to exert some influence on cognitive processes involved in spatial navigation, however these were not markedly clear with the given number of subjects It is possible, given the complexity of the muscarinic cholinergic system in the brain, that the MWM is not a task well-suited to assessment of the consequences of this particular M1 blockade. The effects might be more perceptible and clear-cut if a larger number of experimental subjects was used. However, taking into account the ethics of working with laboratory animals, such a course of action would be at the very least questionable. Notwithstanding, the varying results reported by different laboratories make it rather unreliable as a research tool. As a number of other means of modelling neurodegeneration in rodents may be employed, we would thus not recommend biperiden as a useful cognitive impairer for research of neurodegeneration.

8. BIBLIOGRAPHY

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