Charles University

Faculty of Science

Study Program: Biology

Branch of Study: Animal Physiology

Bc. Alexandra Tyshkevich

Význam cholinergní signalizace ve striatu pro řízení chování a kognitivní flexibility

Studying the role of striatal cholinergic signalling in control of behaviour and behavioural flexibility

Diploma thesis

Supervisor: **MUDr. Helena Janíčková, PhD**

Prague 2021

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze 26.04.2021

Abstract

 Cognitive flexibility is an important mechanism enabling organisms to adapt to their changing environment. Different brain structures are involved in this complex process. It has been repeatedly shown that the striatum is one of the key structures controlling cognitive flexibility. Striatum receives rich input from different brain regions while its output is rather uniform. Striatal functions and signalling are greatly modulated by dopamine and acetylcholine. A number of studies have shown involvement of striatal acetylcholine and its receptors in the control of cognitive flexibility but very little is known about the role of M4 muscarinic acetylcholine receptors. These receptors are inhibitory, and they have been shown to induce long-term depression in striatal medium spiny neurons, therefore opposing the action of the dopamine D1 receptors. We hypothesize that the inhibitory effect of M4 muscarinic acetylcholine receptors may supress spiny projection neurons coding for outdated and no longer effective behavioural strategy and thus they may be necessary for the flexible change of behaviour. In the present thesis, I investigated the effects of pharmacological antagonism of M4 receptors on cognitive flexibility of mice tested in a simple reversal learning paradigm.

Key words: striatum; cholinergic system; cognitive flexibility; mouse; behavioural experiments

Abstrakt

Kognitivní flexibilita je důležitým mechanismem umožňujícím organismům adaptovat se na neustále se měnící prostředí. Jednou z klíčových částí mozku podílejících se na řízení kognitivní flexibility je striatum. Striatum přijímá projekce neuronů z různých mozkových oblastí, zatímco jeho vlastní projekce jsou poměrně uniformní a podílejí se na výběru vhodných motorických odpovědí organismu. Signalizace ve striatu je modulována především dopaminem a acetylcholinem. Řada studií ukázala na význam acetylcholinu a jeho receptorů ve striatu pro správnou funkci kognitivní flexibility, ale konkrétně role M4 muskarinových receptorů v tomto kontextu je stále nejasná. M4 inhibiční receptory jsou schopné indukovat dlouhodobou depresi (LTD) na projekčních neuronech striata a působí tak proti účinku D1 dopaminových receptorů. Podle naší hypotézy může inhibiční účinek M4 receptorů potlačovat projekční neurony kódující již zastaralou a neefektivní behaviorální strategii, a tím přispívat ke flexibilní změně chování. Cílem této práce bylo otestovat účinek farmakologické inhibice M4 receptorů na kognitivní flexibilitu myší vystavených jednoduchému testu reverzního učení v operantním boxu.

Klicová slova: striatum; cholinergní systém; myš; behaviorální experimenty

Contents

List of abbreviations

[³H]-NMS	[³H]-N-methylscopolamine		LTD	long term depression
5-HT3A	5-hydroxitryptamine 3A		LTP	long term potentiation
ACh	acetylcholine		LTSI	low-threshold spiking interneurons
BSA	bovine albumin serum			
cAMP	cyclic adenosine monophosphate		mAChR	muscarinic acetylcholine receptor
CB2R	cannabinoid receptors 2		MSN	medium spiny neuron
CHO	Chinese hamster ovary		nAChR	nicotinic acetylcholine receptor
CIN	cholinergic interneuron			
CR	calretinin		NGS	normal goat serum
DA	dopamine		NPY	neuropeptide Y
DAB	3,3'-diaminobenzidine		OCD	obsessive compulsive disorder
DS	dorsal striatum			
dSPN	direct spiny projection neuron		PBS	phosphate-buffered saline
			PFA	paraformaldehyde
DYN	dynorphin		shRNA	small hairpin ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid			
			SNc	substantia nigra parc compacta
ENK	enkephalin			
fMRI	functional magnetic resonance imaging		SNr	substantia nigra pars reticulata
FSI	fast spiking interneurons		SP	substance P
GABA	gamma-aminobutyric acid		STN	subthalamic nuclei
GPe	globus pallidus external		VS	ventral striatum
GPi	globus pallidus internal		VTA	ventral tegmentum area
iSPN	indirect spiny projection neuron		WT	wild type
ITI	intertrial interval			

Introduction

Cognitive flexibility, an ability to change a behaviour or a behavioural strategy, allows organisms to adapt to the environment and therefore, it plays a crucial role in their daily lives and survival. An impairment of cognitive flexibility is observed in many neuropsychiatric disorders including Parkinson's disease, addiction, autism, obsessive-compulsive disorder (OCD) and eating disorders. Potential mechanisms underlying cognitive flexibility have been studied since the second half of the past century. So far, brain regions playing the crucial role in cognitive flexibility have been identified including the prefrontal cortex and the striatum. Previous studies have shown that acetylcholine (ACh) and cholinergic signalling in the dorsomedial striatum is necessary for cognitive flexibility in mice and primates. However, a mechanistic understanding of the processes involved is still missing and processes or signalling pathways that are recruited by acetylcholine to control cognitive flexibility remain elusive. In addition, it is not clear which acetylcholine receptors, expressed by striatal neurons and nerve terminals, are involved in this control. While several studies indicated a role for M1 muscarinic acetylcholine receptors (mAChRs) in the dorsomedial striatum playing a role in cognitive flexibility, a potential role of other receptors is still controversial or has been rarely investigated. In the present thesis, I am focusing on a role of M4 mAChRs in cognitive flexibility in mice. The M4 receptors have the ability to inhibit striatal projection neurons and thus, we hypothesized that they can inhibit motor programs and behavioural strategies that are no longer efficient in a given context. Currently, there is little studies available investigating a role of M4 mAChRs in the control of cognitive flexibility. Therefore, in this study, I decided to focus on M4 mAChRs expressed by striatal projection neurons and on their potential role in the control of cognitive flexibility. First, using pharmacological binding experiments and testing several different behavioural paradigms, I established a suitable pharmacological and a behavioural approach to study the role of M4 mAChRs in the cognitive flexibility in mice. Then I adopted a technique of stereotaxic surgeries to implant permanent cannulas into mouse dorsal striatum and test the effect of pharmacological M4 inhibition on performance in a cognitive flexibility-based behavioural task.

1. Cognitive flexibility

Cognitive flexibility is one of the key processes for successful adaptation to the changing environment. It is an ability to change old, no longer appropriate strategy to a new, suitable one; ability to switch attention from one concept to another or, in an animal model, to adjust behaviour according to the changing environmental context. Observed and described in 60's and developed as psychological phenomenon in 90's by Spiro, cognitive flexibility has been shown to act on different brain levels from cortex to basal ganglia (Scott, 1962; Spiro et al., 1988). The whole process involves a large net of connections and signals between different brain structures, such as prefrontal, anterior cingulate and posterior parietal cortexes and basal ganglia. Activation of these areas has been shown with functional magnetic resonance imaging (fMRI) during cognitive flexibility tasks (Leber et al., 2008). In addition, other brain structures are also involved in this mechanism, such as thalamus, limbic system and different cortical areas which all send projections to the basal ganglia and strongly influence their functioning. Involvement of subcortical structures such as basal ganglia tells us about the long existence of cognitive flexibility phenomenon, which makes it possible to study this mechanism effectively in animals such as mice and rats and suggest that findings are at least partially applicable to humans.

Impairment of cognitive flexibility, as in the case of other executive functions, can be often observed with aging as well as induced by pathological states or neurological diseases such as Huntington's, Parkinson's diseases or schizophrenia (Hanes et al., 1995). Inability to learn new strategy and repeating the old one or problems in decision making are behavioural markers of these diseases. For instance, in animal models of Parkinson's disease an impaired ability of reversal learning can be seen in performance of set-shifting test or T-maze test and connected with the striatum signalization (Grospe et al., 2018; Owen et al., 1992). Impairment of the attentional set-shifting task can be also observed in patients with OCD (Vaghi et al., 2017) and Huntington's disease (Langley et al., 2020).

2. Striatum, anatomy and function

2.1 Anatomy of the striatum

Anatomically, we can distinguish two main structural parts of the striatum – ventral and dorsal striatum. They can be further divided based on their glutamatergic input connections and specific functions.

In primates, the dorsal striatum consists of the caudate nucleus and the putamen. In rodents, it can be analogically subdivided into two regions – dorsolateral striatum (corresponding to putamen in primates), which receives input primarily from sensorimotor cortex, and dorsomedial striatum (corresponding to caudate nucleus in primates) with afferent projections from frontal and parietal association cortices. The ventral striatum consists of nucleus accumbens, which can be divided into shell (ventromedial) and core (ventrolateral) parts and the olfactory tubercle. It receives glutamatergic projections from limbic structures such as the amygdala (to the ventrolateral striatum), hippocampus (to the ventromedial striatum) and from the medial prefrontal and anterior cingulate cortices (Haber, 2003; Rusu and Pennartz, 2019).

The striatum also has a strong input from the thalamus, and it is indirectly connected with the thalamus through its output projections, the direct and indirect pathways. These projections form thalamo-cortico-striatal loops, where subareas of thalamus with input from basal ganglia are primarily interconnected with motor-related cortical areas. (Hunnicutt et al., 2016)

Another strong input to the striatum comes from the midbrain regions. Ventral tegmentum area (VTA) and substantia nigra pars compacta (SNc) dopaminergic neurons projecting to the nucleus accumbens form an important reinforcement pathway (Fiorino et al., 1993).

Activating effect on motor behaviour provided by the direct pathway and is mediated through the inhibition of two gamma-aminobutyric acid producing (GABAergic) output nuclei – global pallidus internal (GPi) and substantia nigra pars reticulata (SNr) – therefore disinhibiting thalamocortical and brainstem motor circuits. In opposite, indirect pathway activation is increasing the activity of basal ganglia's output nuclei and thus increasing their inhibitory effect on thalamocortical motor output. It has additional synapses in the global pallidus external (GPe) and subthalamic nuclei (STN). Inhibiting of GPe by indirect projection neurons leads to lack of inhibition of the STN which has excitatory projections to the SNr/GPi, therefore supporting GABAergic activity of this nuclei and suppressing motor functions.

While the direct pathway is required to initiate and sustain locomotion and motor action, the indirect pathway inhibits movement, and it is thought to suppress actions that would interfere with currently selected behaviour. The two pathways are the most crucial in the control of motor behaviours depended on the basal nuclei, nevertheless, there are other connections which add to the complexity of the whole structure (Albin et al., 1989; Gerfen and Surmeier, 2011; Lee et al., 2016). Among those connections, there are collaterals bridging of direct spiny projection neurons (dSPNs) to GPe (Cazorla et al., 2014), intranigral inhibitory connections (Mailly et al., 2003), projections from the penduculopontine and laterodorsal tegmental nucleus to the striatum (Dautan et al., 2014) and from GPe to the cortex (Saunders et al., 2015).

2.2 Cellular composition of the striatum

Fig. 1 Striatal neurons diversity (adapted from Hjorth et al., 2020). ChIN – cholinergic interneurons, CR – calretinin, dSPNs -direct spiny projection neurons, FS – fast spiking, IN-interneurons, iSPNs - indirect spiny projection neurons, LTS – low-threshold spiking, NGF – nerve growth factor, TH-IN – tyrosine hydroxylase expressing interneurons
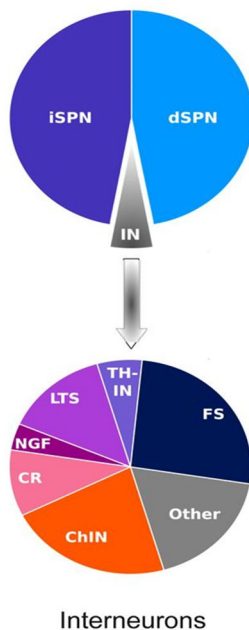
Various types of neurons can be found in the striatum. The majority of them are spiny projection neurons between which we can distinguish those who project through direct or indirect pathway (dSPNs or iSPNs, respectively). They are medium-sized cells with typical spiny dendrites, and they are all produce GABA as a main neurotransmitter. The rest of the striatal neurons consists of interneurons of different types.

Spiny projection neurons (SPNs) (also known as medium spiny neurons (MSNs)) are making 95% of striatal neuron population and can facilitate or inhibit motor behaviour in two pathways – direct and indirect one. Activating effect on motor behaviour is provided by dSPNs with projections to the SNr/GPi and forming the direct pathway. Indirect pathway is formed by iSPNs with projections to GPe and mediates suppression of motor actions.

Besides the connectivity distinction, direct and indirect SPNs also differ in their neurochemical profiles. While dSPNs are mostly expressing $G_{s/olf}$-coupled D1 type dopamine receptors and contain neuropeptides substance P (SP) a dynorphin (DYN), iSPNs are expressing $G_{i/o}$-coupled

D2 type and contain enkephalin (ENK) (Deng et al., 2006; Gagnon et al., 2017). Despite the fact that this expression is not exclusive for a certain type of neurons it is still clearly prevalent to be an important marker for distinguishing between dSPN and iSPN.

Interneurons in the striatum can be divided into two main groups: GABAergic and cholinergic interneurons (CINs). They are not having a big share in the whole striatal neuronal population but play an important role in the local inhibition and modulate striatal input as well as signalling by striatal microcircuits.

Cholinergic interneurons are large cells with ~1% share of the striatal cells. They make dense local connections within striatum and provide ongoing acetylcholine signal because of their tonic activity (Oorschot, 1996; Zhou et al., 2002).

GABAergic interneurons further have been divided into the three main subgroups based on their electrophysiological and neurochemical properties: fast spiking interneurons (FSI) also referred to as parvalbumin-expressing interneurons, somatostatin-expressing low-threshold spiking interneurons (LTSI) and calretinin-expressing (CR) interneurons. Nevertheless, there are other GABA interneuron types that have been discovered lately and added to the diversity of the population. In particular, 5-hydroxitryptamine 3 (5-HT3) receptor expressing interneurons which are classified as fast adapting (Faust et al., 2015), tyrosine hydroxylase expressing interneurons (Ibáñez-Sandoval et al., 2015) and neuropeptide Y (NPY)- expressing neurogliaform interneurons (Ibáñez-Sandoval et al., 2011). All these types show specific patterns of firing and connectivity and add-up to the complex system of striatal microcircuits.

In summary, there is a dense concentration of different neuronal types in the striatum with intertwined dopamine and acetylcholine circuits, which promote main modulative effects and are involved in the control of executive functions including cognitive flexibility. In this thesis, we will focus on acetylcholine, as one of the key striatal neurotransmitters, and try to clarify its' role in cognitive flexibility.

2.3 Behavioural functions of the striatum

The striatum is a major part of the basal ganglia and plays an important role in motor functions as well as being a part of reward system. It is an important regulation point for signals passing from the cortex to the motor neurons with conductive and regulatory functions (Redgrave et al., 1999). Its' functions in motivation and reinforcement learning of certain behaviour strategies are also have been shown. In contradistinction to prefrontal cortex, which mediates cognitive action planning and is responsible for model-based reinforcement learning, striatum is used to

be considered as responsible for model-free reinforcement learning, based on the direct behavioural outcome. However, last studies have shown more complex picture with a role for the striatum also in model-based reinforcement learning. (Daw et al., 2011, 2005; Deserno et al., 2015)

Dopamine (DA) neurons projecting to the striatum take part in the reinforcement learning processes. They have been shown to encode reward prediction error – the difference between experienced and expected reward (Montague et al., 1996). DA neurons are increasing their activity in response to an unexpected reward, cues that predict reward and distance of the animal from the reward (Cohen et al., 2012; Fiorillo et al., 2003; Roesch et al., 2007). The DA signal is positively modifying glutamatergic synapses in D1-expressing SPNs and increasing probability of repeating the action that lead to the unexpected reward (Bromberg-Martin et al., 2010). Decrease of DA release, in turn (caused by the omission of the reward), leads to the extinction of the previously conditioned response (Salinas-Hernández et al., 2018).

Besides the role of a neurotransmitter, dopamine also has modulative functions on the SPNs synapses. It modulates neuron's responsiveness to stimuli not only by increasing sensitivity of membrane but also by gene expression affection and long-term potentiation (LTP) induction which therefore leads to stronger nervous signal towards preferred type of behaviour. (Tritsch and Sabatini, 2012).

Different striatal regions have different roles in cognitive functions and behaviour. The dorsomedial striatum is important for goal-directed learning and behaviour, behavioural flexibility, reward associated motor learning and reaction time. The dorsolateral striatum is important for habit formation. Getting DA signal in response to the cue that leads to undirected movements (not cued), dorsal striatum is thought to be important for stimulus-response associations and response-outcome associations (Balleine et al., 2007; O'Doherty et al., 2004; Saunders et al., 2018).

The ventral striatum is involved in reinforcement learning and in appetitive as well as aversive behaviours. It also contributes to the control of sensitivity to drugs of abuse and natural rewards, such as food intake. Ventral striatum is thought to be important for stimulus-outcome associations and DA projections there mediate a cue-approaching behaviour .(O'Doherty et al., 2004; Saunders et al., 2018)

Considering their involvement in the direct and indirect output pathway, respectively, it is not surprising that dSPNs and iSPNs can be also distinguished by their effect on behaviour. dSPNs' and iSPNs' influence on behavioural outcome is based on the go/no go system where direct

pathway promotes, and indirect pathway inhibits actions. For instance, optogenetic activation of the iSPN led to the increased freezing, bradykinesia and decreased locomotion, when activation of the dSPN has shown opposite effect with increased locomotion and reduced freezing (Kravitz et al., 2010). This effect has also been observed on the SNr, where after dSPN and iSPN optogenetic activation was induced excitation or inhibition that correlated with motor suppression or facilitation, respectively. This characterises SPNs types as opposite acting "switches" for output nuclei that allows to control motor behaviour (Freeze et al., 2013). Modulative functions of SPNs are observed as well upstream the signal, in the cortex. While dSPNs promote excitation through the striatonigral projections to the primary motor cortex, iSPNs act antagonistic to them through the striatopallidal projections. (Oldenburg and Sabatini, 2015; Alexander and Crutcher, 1990; Deniau and Chevalier, 1985; Gerfen et al., 1990)

However, this go/no go system cannot be simplified only to this function as both pathways are activated during the task performance (Wu et al., 2000). Latest studies suggest the role for dSPNs in selecting action, whereas iSPNs role is in avoiding it. This also supported by observations that dSPNs are more active in presence of the reward while iSPNs in its absence. (Shin et al., 2018) Optogenetic experiments also show the role for SPNs activity rather in decision making than movement output, with dSPNs responsible for repeating the action if it is rewarding and iSPNs responsible for avoiding non-rewarding action (Nonomura et al., 2018). Latest study, where inhibition of iSPNs, but not dSPNs leaded to the attenuation of the response flexibility, suggests the greater role for the first in the cognitive flexibility processes (Peak et al., 2020).

Cholinergic striatal system also plays a role in behavioural control. CINs have been shown to regulate reinforcement learning processes whet it occurs but not promoting reinforcement learning by themselves (Lee et al., 2016). CINs role is also confirmed to be rather in cognitive flexibility and reversal learning than in the initial learning (Aoki et al., 2015). Its influence is mediated through the direct synapses with SPNs as well as through the influence on glutamatergic synapses on SPNs. Mechanisms of these effects are described in further chapters of this thesis.

3. Cholinergic system, its structure and functioning in the striatum

3.1 Striatal cholinergic interneurons: morphology and physiological properties

In mouse brain, acetylcholine is produced by cholinergic projection neurons of midbrain and basal forebrain and by striatal interneurons. While cholinergic projection neurons reach various areas including neocortex and limbic cortex, hippocampus and amygdala or thalamus, striatal cholinergic interneurons are influencing local microcircuits in the striatum (Woolf, 1991).

Cholinergic interneurons (CINs) are also known as „tonically active neurons" for being able of the ongoing spontaneous activity, inducing action potentials at about 3-10 Hz. This tonic activity has been shown to be independent on the synaptic input with intrinsic origin and caused by afterhyperpolarization that follows single spikes. It is caused by calcium-dependent potassium currents and provides rhythmic single spiking of CINs (Wilson et al., 1990; Wilson and Goldberg, 2006). However, the tonic spiking pattern can be influenced by external stimuli, which can cause pauses in their activity. Those pauses in CINs tonic spiking have been shown to inhibit SPNs activity and influence the behavioural outcome (Kimura et al., 1984; Zucca et al., 2018).

Another remarkable characteristic of the CINs is their size, they are the largest cells in the striatum. Their large axons are spreading wide across the other neurons and affecting them through a variety of nicotinic and muscarinic acetylcholine receptors (Gonzales and Smith, 2015).

3.2 Striatal cholinergic interneurons: connectivity

CINs are primarily receiving their excitatory input from the cortex and thalamus. Main thalamic source of projections are intralaminar nuclei, in particular, parafascicular nucleus (Lapper and Bolam, 1992; Van der Werf et al., 2002). Thalamus, in turn, receives projections from the sensory cortex. These projections can influence the autonomous firing pattern of CINs and mediate the thalamic regulation of striatal responsiveness to the cortex stimuli (Ding et al., 2010; Schulz et al., 2011).

Another important input to the CINs is represented by DA afferents, which can directly affect them through the D2 dopamine receptors and provide inhibition of CINs, as well as modulate their responsiveness and excitatory input which leads to pauses in their tonic activity. This mechanism is possibly important for the reward learning and can be downregulated through

nAChRs expressed by DA terminals, forming a feedback loop (Schulz and Reynolds, 2013; Zhang et al., 2018).

Inhibition of the CINs is also provided by surrounding GABAergic interneurons, activity of which can be in turn regulated by acetylcholine through their nAChRs (Sullivan et al., 2008).

CINs projections are widespread within the striatum and make synapses with SPNs, releasing acetylcholine as a main neurotransmitter. Besides acetylcholine, CINs have also been shown to co-release glutamate (Gras et al., 2008; Guzman et al., 2011; Sakae et al., 2015).

3.3 Acetylcholine receptors in the brain and the striatum

CINs are the main source of acetylcholine in the striatum, with lesser contribution of the pedunculopontine and laterodorsal tegmental nuclei in the brainstem (Dautan et al., 2014). Striatal acetylcholine affects receptors on spiny projection neurons as well as on GABAergic interneurons and its own pre-synaptic receptors expressed by CINs.

Synthetized in the axonal end of neuron by choline acetyltransferase, acetylcholine is then released to the synapse where it binds to its receptors, which are heterogeneous in their structure and function, and it is also quickly degraded by acetylcholine esterase. Two main types of acetylcholine receptors are distinguished: nicotinic acetylcholine receptors (nAChRs) which are represented by ion channels activated by acetylcholine and cause membrane depolarization, and muscarinic acetylcholine receptors (mAChRs) represented by G-protein coupled metabotropic receptors, activation of which may lead both to inhibition or activation of neurons based on the type of mAChR.

nAChRs are widely distributed across the brain and peripheral nervous system and play a role on muscular synapses as well as in autonomous nervous system's synapses. They are also found in the brain where their function is more regulative and modulative rather than direct. nAChRs are typically made of five subunits which can be either all the same type in homopentameric receptors, or, more frequently, of different types in heteropentameric receptors. Different subunits of nicotinic receptors can be more widely expressed in some brain areas and rarely found in others. In the striatum, heteropentameric structure of nAChRs is characteristic, nicotinic receptors can be found on a variety of neuronal cells and are affected primarily by acetylcholine produced by CINs (Gotti et al., 2006).

Muscarinic receptors family is represented by transmembrane proteins coupled with intracellular G proteins. Subtypes are divided by their G protein type, binding site's structure

and therefore ligands, and location in the body. M1, M3 and M5 are providing neuronal excitation through the G_q protein, M2 and M4 are inhibiting neurons through $G_{i/o}$. Induced through G proteins, mAChR response is slower than nAChR but persists longer and have effects on gene expression. Activation of the muscarinic receptors M1, M3 and M5 by acetylcholine upregulates phospholipase C and increases Ca^{2+} influx, M2 and M4 activation decreases cAMP concentration in the cell. This second messenger's action leads to the changes in the cell functioning and particularly in gene expression (Caulfield, 1993).

Muscarinic receptors distribution is also rather heterogeneous. Typically, M1, M2 and M4 types can be found in the brain, mainly in cortex, basal forebrain and hippocampus. M5 presence is specifically observed in the midbrain (Moran et al., 2019, Vilaró et al., 1993).

Modulatory effect of acetylcholine on the striatal output is mediated through the both main types of acetylcholine receptors. There is not so much specific evidence for nAChRs distribution in the striatal neurons, but they are abundantly expressed by different types of projection terminals. In addition, they seem to have modulatory effect in presynaptic location on specific types of striatal interneurons (Matsubayashi et al., 2001; Luo et al., 2013; English et al., 2011). More evidence of direct postsynaptic action can be found for mAChRs, especially M1 and M4, it suggests their major role in striatal cholinergic signalization and therefore in regulating of the striatum output in cooperation with dopamine and GABA effects.
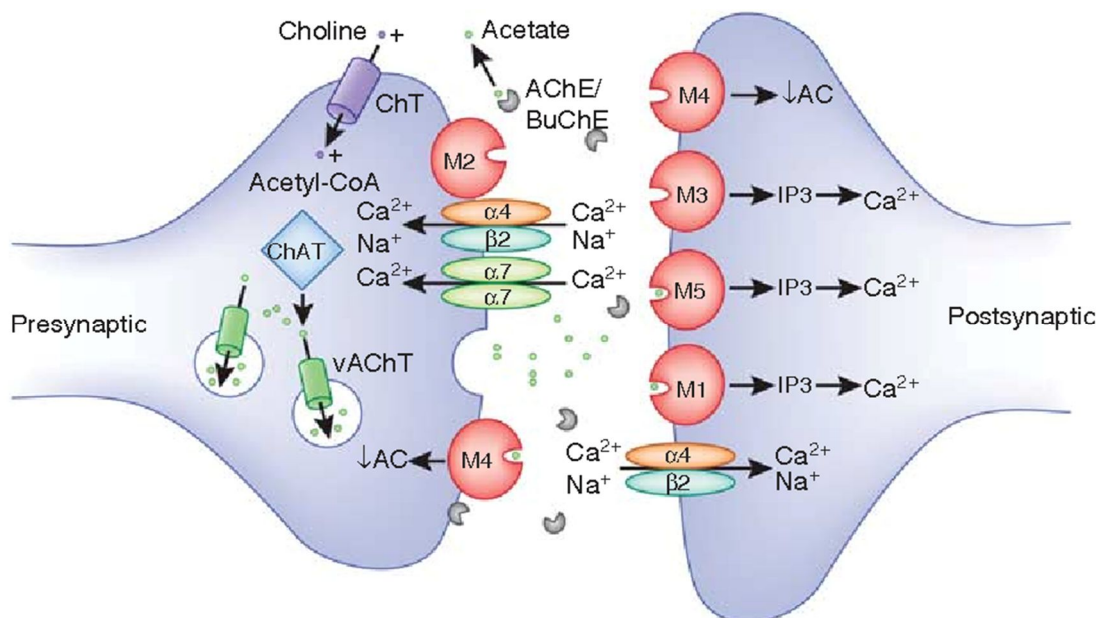

Fig.2. Schematic representation of a hypothetical cholinergic synapse illustrating general synaptic localization and function of cholinergic receptors. AC- adenylate cyclase, IP3 – inositol triphosphate, ChAT – cholinacetyltransferase, AChE – acetylcholine esterase, BuChE – butyrylcholinesterase, vAChT – vesicular acetylcholine transporter (adapted from Jones et al., 2012)

4. M4 muscarinic acetylcholine receptors in the striatum

M4 receptors are a subtype of muscarinic acetylcholine receptors with inhibitory function. Upon activation by acetylcholine, they activate mechanisms leading to lowering cAMP and Ca^{2+} concentration in the cell and therefore inhibiting gene transcription. M4 is a classical G-protein coupled receptor with seven transmembrane domains which endogenously binds acetylcholine as an agonistic ligand. Other agonists for this receptor are carbachol and oxotremorine. M4 has a variety of known antagonists among which are himbacine, mamba toxin 3 and tropicamide. Also, a variety of M4 positive allosteric modulators has been synthesized. (Goyal, 1989; Kargbo, 2018)

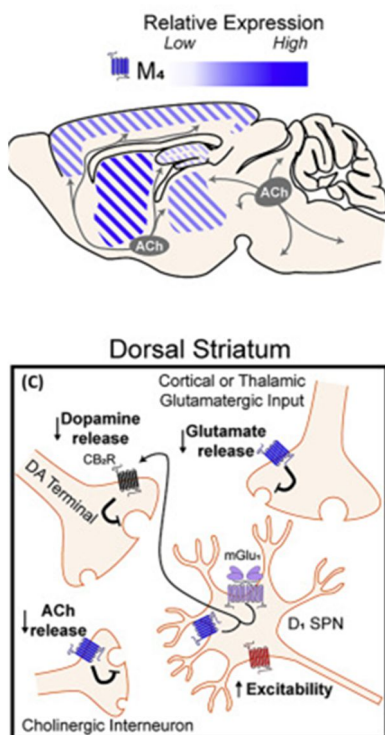## 4.1 Effect of M4 mAChRs on striatal signalling

Fig. 3. M4 receptor distribution in a mouse brain (upper picture) and its location on the striatal synapse (lower picture). (Moran et al., 2019)

While M1 receptors can be found on both dSPNs and iSPNs, M4 receptors with inhibiting function can be found preferentially on the direct pathway (D1) medium spiny neurons. As was mentioned before, those are GABAergic neurons, activated primarily by DA through the D1 dopamine receptors. Thus, inhibitory M4 receptors can counterbalance the activating effect of DA in dSPNs (Gomeza et al., 1999).

When expressed presynaptically, role of M4 receptors mostly consists of acetylcholine release regulation (Bubser et al., 2014), postsynaptically its effect extends broader and can influence cell activation and gene expression in a more sophisticated way. Acetylcholine does not have an action potential inducing effect on dSPNs synapses; however, it has an important modulative role, contributing to the striatal acetylcholine/dopamine intertwined and balanced system, both pre-and postsynaptically (Oldenburg and Ding, 2011).

One of this modulative roles consists in regulation of the dopamine release from dopaminergic neurons. Acetylcholine distribution from the CINs can inhibit or promote further dopamine release, depending on the targeted receptor type. In case of M4 receptors, this effect in inhibitory. One of the mechanisms responsible for this regulation was suggested to be through the activation of postsynaptic M4 receptors expressed by D1-SPNs that can promote releasing of 2-AG endocannabinoids,

substrates for the cannabinoid receptors 2 (CB2R) with inhibitory effect on the DA neurons endings (Foster et al., 2016). Another modulative role of M4 receptors is in its presynaptic inhibition of glutamate release from cortical/thalamic input projection to the striatum (Pancani et al., 2014).

Acetylcholine acting through the M4 receptors can also interfere with dopamine effect in dSPNs. Mechanism of this interaction consists in competition of D1 activating and M4 inhibiting signalling on adenylyl cyclase activity and further on production of cAMP (Dencker et al., 2011). Another recent study suggested that M4 mAChRs exert tonic inhibition on striatal SPNs and a pause in this inhibition is required for dopamine-induced LTP in dSPNs (Nair et al., 2019). Also, M4 receptors activation can directly promote LTD on glutamatergic synapses in SPNs (Shen et al., 2015). However, not only acetylcholine from CINs can influence M4 receptors expressed by dSPNs. Acetylcholine is also released from extra-striatal cholinergic projections to SNr and it was suggested that it inhibits dSPNs activity there, decreasing probability of GABA release on SNr synapses (Moehle et al., 2017).
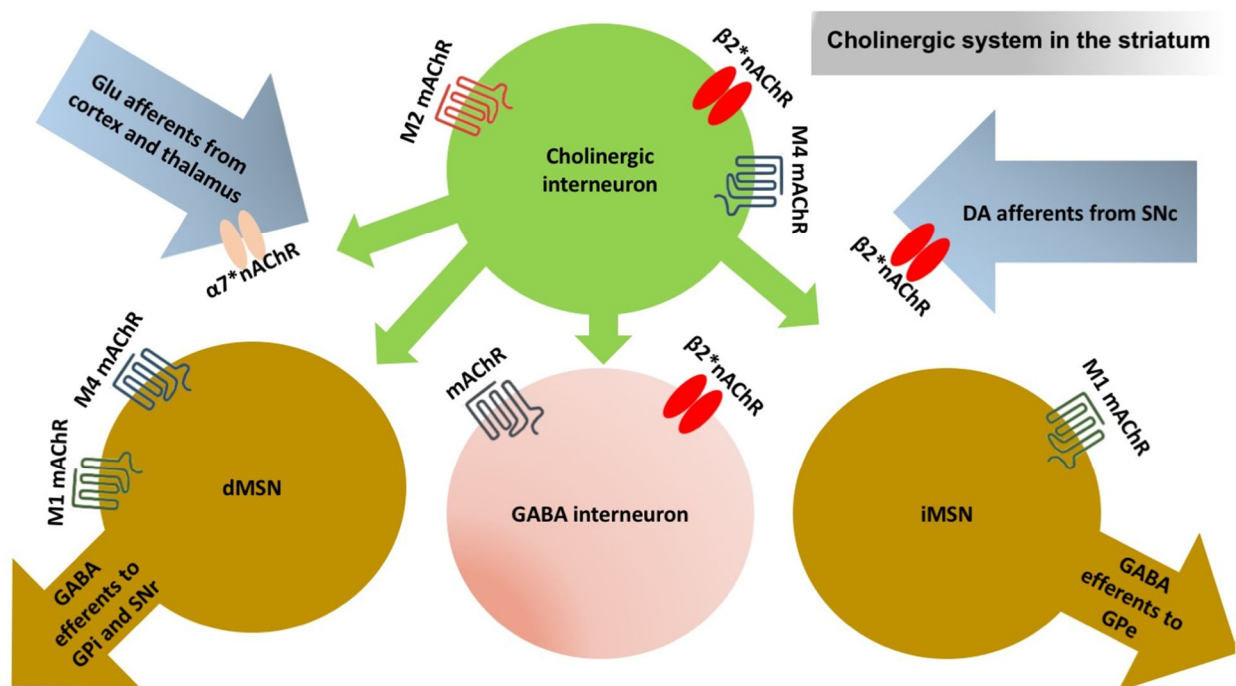

Fig. 4. Schematic of striatal cholinergic system. iMSN and dMSN – indirect and direct medium spiny neurons, respectively (Janickova, 2018)

4.2 Role of M4 mAChRs in the control of striatal-based behaviour

Not surprisingly, M4 receptors' role in modulation of dopaminergic system makes their functions tightly connected with dopamine-dependent behaviours. Behaviours such as increased motor activity and increased behavioural sensitization following treatment with psychostimulants can be observed in mice with M4 deletion on dSPNs (Jeon et al., 2010). There is evidence for M4 receptors' role in suppressing alcohol-addiction (Walker et al., 2020). In addition, antipsychotic effect (Foster et al., 2016; Hailwood et al., 2019; Yohn and Conn, 2018) can be induced by activation of M4 receptors which makes them potential target for treatment of several striatum-associated diseases such as addiction or schizophrenia. Deletion of M4 receptors was also connected with compulsive behaviour which adds up to list of the disorders, in mechanisms of which M4 receptors are involved (Justinussen et al., 2020). Furthermore, boosting M4 activity with specific allosteric positive modulator attenuated dyskinesia, a Parkinson's disease treatment consequence (Shen et al., 2015).

4.3 Striatal M4 mAChRs and cognitive flexibility

Striatal acetylcholine has been shown to be important in reversal learning and cognitive flexibility in general (Prado et al., 2017). CINs activity is shown to be important for behavioural flexibility tasks and its absence decrease set-shifting tasks performance (Aoki et al., 2015). However, there are also evidence of the opposite action of CINs on the reversal learning (Okada et al., 2018, 2014). Higher acetylcholine levels in some striatal region, in particular in dorsomedial striatum can be observed during the reversal learning tasks, but not during the acquisition (Kitabatake et al., 2003; Ragozzino et al., 2009). It confirms a role for acetylcholine in cognitive flexibility processes and its influence on striatum-depended memory.

The possible mechanism for the mediating attention shift might be through the enhancing activity of iSPNs by acting on its M1 receptors and suppressing non-rewarded action (Tzavos et al., 2004). However, role of the dSPNs and M4 muscarinic receptors can be distinguished here as well. Specifically, they were suggested to mediate attenuative effect of acetylcholine on behavioural flexibility and stabilize previously used strategy in order to obtain the reward. These effects were observed after CINs elimination or M4 gene-specific silencing in the dorsomedial striatum, which both led to the enhancing of the reversal learning ability (Okada et al., 2014). This influence on cognitive flexibility has been also shown to be dependent on the duration of interatrial intervals, with longer intervals needed to detect enhancing effect of CINs elimination (Okada et al., 2018).

Another study proposed that the effect of dorsomedial striatum acetylcholine on cognitive flexibility is mediated primarily by M1 receptors. Their inhibition led to the impairment of reversal learning with increased number of regressive but not perseverative errors. This suggests the importance of acetylcholine in mechanisms maintaining a new way of action but not in initial strategy switching processes. Importantly, blocking the M4 receptors by specific antagonist did not show any effect on the cognitive flexibility in this study (McCool et al., 2008).

Considering general function of dSPNs to promote an action and support a strategy leading to the reward and D1 LTP-promoting effect, which is controverted by M4 LTD induction, acetylcholine may have a regulative function here as well as during the behavioural strategy switch. It can suppress activity of the dSPNs responsible for the old strategy and increase cognitive flexibility. Therefore, we hypothesize that inhibition of M4 receptors could lead to cognitive flexibility impairment.

There is only limited number of studies investigating this topic which is making it relevant and intriguing for studying.

Aims

Based on the available literature, the role of striatal acetylcholine and striatal CINs in the control of cognitive flexibility is quite clear. In contrast, roles of individual acetylcholine receptors in cognitive flexibility needs to be clarified. In particular, there is little information on the role of M4 mAChR expressed by dSPNs. It has been shown that the inhibitory effect of M4 mAChRs on dSPNs is in opposition to the activating effect of D1 dopamine receptors and in contrast to D1-induced LTP, M4 mAChRs were shown to induce LTD in dSPNs (Shen et al., 2015). In addition, one of the two available studies reported that knock-down of M4 mAChRs in the striatum leads to improvement in cognitive flexibility (Okada et al., 2014). Therefore, the main goal of the present study was to test a hypothesis that inhibition of M4 mAChRs in the dorsal striatum would affect mice performance in a behavioural task where an intact cognitive flexibility is required. To test this hypothesis, I set up the following aims:

1. Find a suitable M4-selective or -preferential antagonist to inhibit M4 mAChRs in the DS in mice *in vivo* and test its pharmacological properties.

2. Test several behavioural paradigms commonly used to test cognitive flexibility in rodents in order to select the optimal paradigm to be used in mice with permanent intra-cerebral cannula placement. Both male and female mice should be behaviourally tested in order to detect potential differences between sexes and select the more suitable sex (or both) for the final testing.

3. Establish stereotaxic coordinates for the DS in mice and optimize the stereotaxic placement of intra-cerebral cannula that would allow a microinfusion of the selected M4 antagonist into the DS.

4. Test our hypothesis using the selected behavioural paradigm. The task will be tested in mice with cannulas placed in the DS and administered with M4 antagonist before being subjected to the behavioural task.

Materials and methods

1. Binding assays with M4-preferential antagonist tropicamide

After extensive search of literature, I selected M4-preferential antagonist tropicamide to be used in my behavioural experiments for blocking M4 receptors in the DS and testing their role in cognitive flexibility. I selected tropicamide based on its reported preference for M4 mAChRs *and* because it has been previously used *in vivo* in rodents (Betz et al., 2007; Ztaou et al., 2016). Then, to confirm the presence of mAChRs in the mouse striatum as well as the preferential binding of tropicamide to M4 mAChRs, I performed two types of binding experiments: saturation experiments to measure expression of all types mAChRs in the mouse striatum and competitive binding experiments to measure the tropicamide affinity for M1 versus M4 mAChRs. M1 mAChRs were selected for the comparison because, according to the data in literature, M1 affinity to the tropicamide is close to M4 and it is one of the most common mAChR in the striatum. It is also expressed on the same neurons as M4 receptors and its' antagonism may potentially interfere with the effect of tropicamide on the M4 receptors (Betz et al., 2007) (Table1.). For the saturation experiments, I used membrane fraction of striatal homogenates. For the competitive assays, I used membranes prepared of two different cell lines with heterogeneous expression of either M1 or M4 mAChRs.

Table 1 pK_i values (±standard error) for binding affinity of tropicamide to four subtypes of muscarinic receptor

pK_i (μM)				
	M1	M2	M3	M4
Tropicamide	7.18 (0.04)	7.30 (0.05)	7.42 (0.05)	7.85 (0.07)

Table 1.Tropicamide affinity for the mAChRs subtypes (adapted from Betz et al., 2007).

1.1 Samples' preparation and membranes isolation

Mouse was sacrificed by decapitation and brain was quickly extracted. Striata from both hemispheres were dissected on ice, homogenized in homogenization medium (100 mM NaCl, 10 mM $MgCl_2$, 20 mM Hepes, 10 mM ethylenediaminetetraacetic acid (EDTA), pH=7.4) and

used for membrane isolation as further described. All samples were homogenized with Ultra-Turrax homogenizer for approximately 1 min. Large fragments (sediment) were discarded after centrifugation at 300 g and 4 °C for 3 min. 1.4 ml of supernatant was then distributed to microtubes and centrifuged for 30 min at 30 000 g and 4 °C. Supernatant was discarded and sediment washed with 1 ml of incubation medium (100 mM NaCl, 10 mM MgCl$_2$, 20 mM Hepes, pH=7.4). Samples were centrifuged again for 30 min at 30 000 g and 4 °C. Isolated membranes were stored at − 80 °C until use.

1.2. Saturation binding assay

In saturation experiments, a radioactively labelled ligand of increasing concentrations is added to the membrane sample and after incubation, bound fraction of radioactivity is separated by filtration and measured with a scintillation counter. For mAChRs, [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) is commonly used as a radioactive ligand. For eliminating the effect of non-specific binding, an extra sample is prepared with addition of a high concentration of non-labelled ligand (atropine) that is supposed to displace the labelled [^{3}H]-NMS from its specific binding. The measured value of the non-specific binding is then deducted from the specific binding values. The saturation binding assay allows to quantify a maximum number of binding sites (maximum binding, B$_{max}$) and affinity of the receptors to the ligand (equilibrium dissociation constant, K$_d$). To measure the expression of mAChRs in the mouse striatum, we prepared a sample of striatal membranes (50 ug protein/well) and loaded on 96-well plate in triplicates. The total incubation volume was 400 ul. To individual samples, a radioactively labeled [^{3}H]-NMS was added in the following concentrations: 4000-2000-1000-500-250-125-62,5 pM. In addition, 8 ul of 1 mM atropine was added to the non-specific binding wells. Samples were incubated for 3 hours at the room temperature. After the incubation, plates were filtered (Brandel Cell Harvestor) and membranes with radioactive ligand were fixed on the filtration paper, previously soaked in polyethylenimine to lower non-specific binding of [^{3}H]-NMS. Dried filters were fixed in scintillation wax and measured for radioactivity with scintillation plate counter (Microbeta, Perkin Elmer). Saturation assay was repeated in three independent experiments.

1.3. Competitive binding assay

For these experiments, membranes prepared from Chinese hamster ovary (CHO) cells with heterologous expression of either M1 or M4 mAChRs were used. 100 µl of membrane samples were prepared and loaded in the 96-well plate in quadruplicates (50µg of protein/well). To the membranes, I added 50 µl of 2 mM [^{3}H]-NMS and 50 µl of tropicamide diluted according to

the Table 2 or 50 µl of buffer in control samples and non-specific (NS) binding wells. The total incubation volume was 200 ul. 5 µl of 1mM atropine was added to the non-specific binding wells.

			Tropicamide concentration 10 $^{(...)}$									
M1	Ctrl	Ctrl	-8	-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	NS
M4	Ctrl	Ctrl	-8	-7.5	-7	-6.5	-6	-5.5	-5	-4.5	-4	NS

Table 2. Scheme of the wells filling with tropicamide dilution stage. Ctrl -control sample, NS -non-specific binding.

Samples were incubated for 3 hours at the room temperature. Filtration and radioactivity measurement were performed as described in the previous paragraph.

2. Animals

In the following behavioural experiments, 41 wild type (WT) inbred mice of mixed background were used, 31 males and 10 females. 19 mice (9 males, 10 females) were used in the initial experiments in order to select an optimal behavioural paradigm and compare performance of both sexes. 22 male mice were implanted with intra-cerebral cannulas used to test the effect of tropicamide on cognitive flexibility. All experimental procedures complied with the directive of the European Community Council on the use of laboratory animals (2010/63/EU) and were approved by the Czech Central Commission for Animal Welfare. Mice were housed in a temperature and humidity-controlled room with a 12-hour light/dark cycle (lights ON at 6 AM). Mice were provided with standard rodent chow and water *ad libitum*. For the food-motivated tasks (T-maze and instrumental task in the operant box), mice were food-restricted to 85-90 % of their original (adult, free-food) weight. As a food reward, we used condensed milk (Mlekarna Hlinsko) diluted 1:1 with water and chocolate-flavoured cereal pellets (BioServ) for the T-maze and operant box task, respectively.

3. Behavioural tests

To test the effect of M4-preferential antagonist tropicamide on cognitive flexibility, I had to select an optimal behavioural paradigm. The ideal task should be 1/ commonly used for investigating cognitive flexibility in rodents and widely reported in literature, 2/ relatively easily acquired by animals so the number of microinfusions would be minimized as well as the

risk of cannula damage during the experimental period, 3/ feasible in mice with permanent cannulas avoiding a risk of cannula damage or contamination, 4/ consisting of relatively short individual sessions so the effect of the microinfused tropicamide can be assumed during the whole testing period. Initially, I chose three candidate tests that I optimized while comparing performance of drug-naive male and female mice. The same cohort of mice was subjected to all three tests in the following order: response-based T-maze (plus maze), Morris water maze with reversal and instrumental task in the operant box.
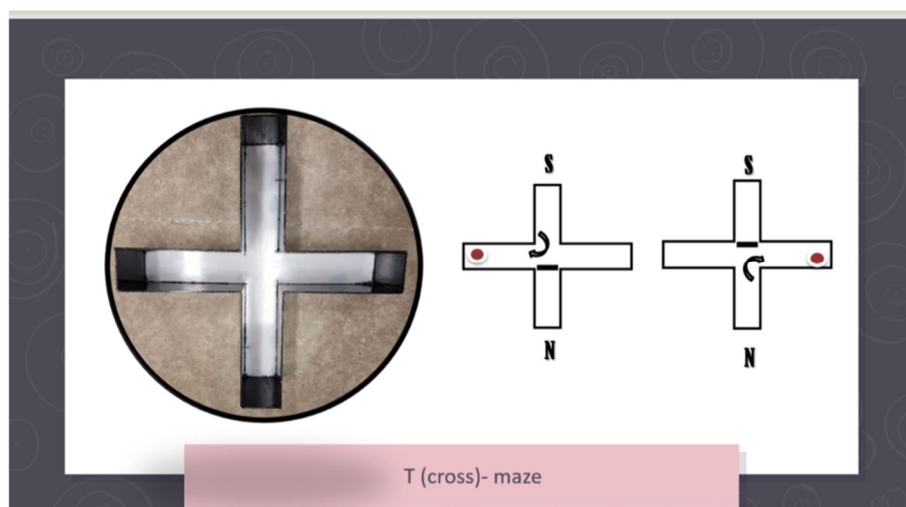
3.1. T-maze task

Fig. 5 Cross maze design scheme

This behavioural task is based on the ability of rodents to learn the direction to a reward regardless on their cognitive space map made in the hippocampus. Instead, it relies on the egocentric navigation, that is, on body turn. In the experiment, mouse is placed into a starting arm of a cross-maze with the opposite arm blocked, so the maze is in the shape of T-maze. A reward is placed in the correct direction, either left or right (the correct direction is pseudo-randomly assigned to each animal). The starting position of the animal is alternated in two opposite starting arms (Fig. 5) so the position of the reward depends solely on the correct body turn and cannot be deduced from the external spatial cues. The experiment consists of the acquisition and reversal phase and the correct direction is switched to the opposite side during the reversal. For the whole experiment duration, mice were kept on the food restriction at 85-90% of their normal weight (Deacon and Rawlins, 2006; Juszczak and Stryjek, 2019; Okada et al., 2018).

Pre-habituation took place every day before starting the experiment. Mice were left in the experimental room for 30 minutes to habituate to the environment. Mice have been also held in hands and habituated to a human presence.

1st phase of the experiment consisted of the habituation of the mice to the apparatus and the experimenter. On the first day of habituation mice were given a little of reward (condensed milk diluted with water in the proportion 1:1) and were placed in the maze apparatus together with their cage mates. On the second day and the following days, mice were taught to look for and consume the reward regardless of the direction as it could be found in both side arms. One session consisted of ten trials and was held once a day. Sessions were started from south arm and north arm, five times each, and were limited to the time of 90s, reward amount set to 40μl. Mouse was taken out of the maze after the session time elapsed or after consuming the reward. When all mice were reliably searching for the reward and consumed it 10 times in a row, they have been moved to the second (acquisition) phase.

2nd phase was the acquisition phase during which mice were toughed to find the reward in only one of the arms and reliably choose the correct body turn (direction). Direction for each mouse was chosen based on their original preference during the habituation in favour of the non-preferred direction. Design of this phase sessions was the same as in the first phase, only now there was the reward only in the correct arm and starting arm (north or south) choice was randomized. Mice were given maximum 10 days to learn, however, after reaching 70% accuracy threshold on 2 days in a row they were passed to the 3rd phase of experiment.

3rd phase of the experiment investigated the ability of mice to abandon the original and now inefficient strategy and make a new correct choice. They were tested by the same protocol as in the 2nd phase, only now the correct direction was switched to the opposite side. Mice completed the experiment after they reached 70 % accuracy on two days in a row or spent 10 days in the reversal.

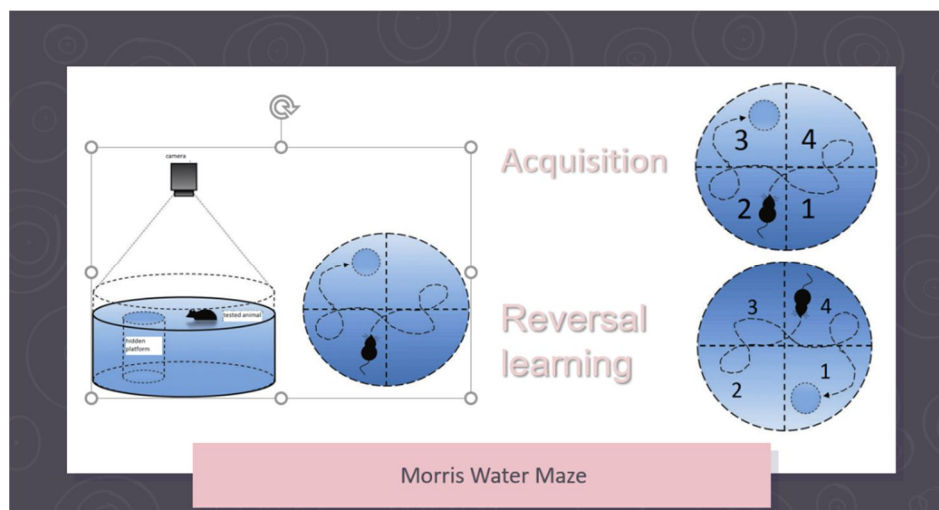## 3.2. Morris Water Maze (MWM) with reversal phase

Fig. 6. MWM design scheme (adapted from Kazantsev et al., 2017)

Morris water maze is a behavioural memory task commonly used in mice and rats. Animals need to find a hidden platform in a pool with filled water. The starting position of the animal is altered during the training while the position of the hidden platform is constant and must be found using external spatial cues such as furniture in the room or highly visible black and white patterns attached to the walls for that purpose (Morris, 1981). A little stress caused by water environment makes the learning process faster and the whole method convenient for short-term experiment. For testing cognitive flexibility, a reversal stage is included in the task where the position of the hidden platform is changed and animals are therefore forced to switch the previously acquired strategy (Vorhees and Williams, 2006).

During the acquisition phase, mice were released in the pool (Ø 90cm) filled with water (25 °C) and given 60s to find the hidden platform. If the mouse failed to find the platform, it was gently guided to it, let stand there for several seconds and then withdrawn from the pool and left in cage until the next trial. Each mouse had four trials per day with intertrial intervals of approximately 10 - 15 minutes. After four days of training mice had a test day when they were given 60s in the pool with no platform to check if they spend most of their time looking for the platform in the correct location (quadrant). After completing the acquisition, reversal phase was performed according to the same protocol, only the platform was moved to a different position in the pool. Reversal part lasted only two days as the mice were already familiar with the task and they acquired the new position quickly. The third day was the test day as in the acquisition phase. The experiment was recorded by a camera and the mice path to the platform was tracked and analysed by a Tracker software (Biosignal Group). Average speed, distance and time for

reaching the platform were the primary outcomes of the task during training and on the test days, I analysed time spent in individual quadrants of the pool.

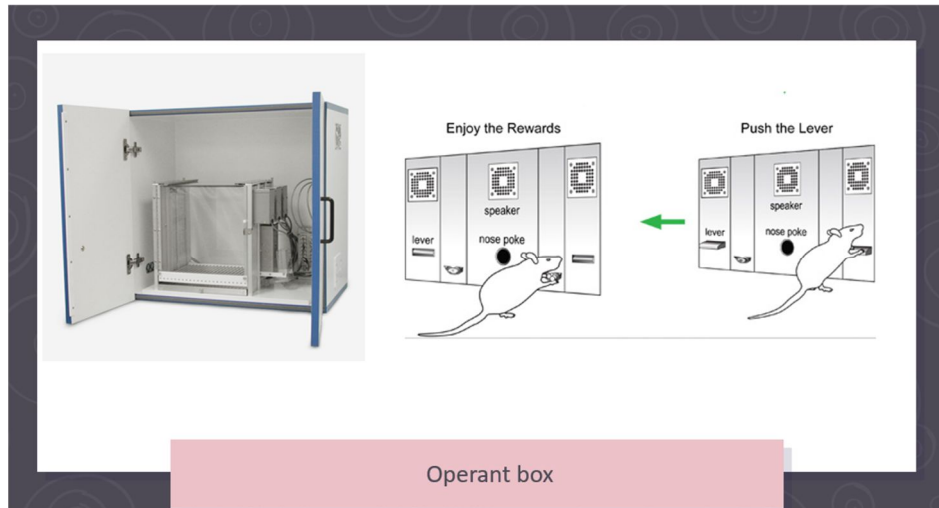## 3.3 Instrumental task in the operant box

Fig. 7. Operant box experiment design scheme (adapted from Med associates Inc.)

Operant boxes allow us to test mice in a variety of instrumental tasks and thanks to the flexible and programmable control software, those tasks can be adjusted to the experimenter's needs. I have selected and programmed a simple instrumental task where mice had to choose a correct lever (left or right) and pressing the correct lever resulted in a reward delivery in a ratio 1:1. All mice were food-restricted and maintained at 85-90 % of their normal weight before the beginning of the experiment to ensure their motivation to earn the food reward (Floresco et al., 2009). Operant box task was programmed in Pascal according to the manufacturer's manual (Med Associates). The full code can be found it attachments (attachment 1.) The experiment consisted of several phases including individual steps of habituation, operant training, acquisition and reversal learning. 1st day of habituation consisted of 10 minutes spent in the operant box chamber with the house light on. 2nd day of habituation was a 40 min session with the reward falling out automatically every two minutes. All the mice were checked for consuming the reward and in case of successful reward consumption were passed to the next step. After the habituation, an operant training was initiated. Mice had to learn to press any lever (left or right) in order to obtain the reward. Session time was set to 30 minutes and maximum of rewards to be earned was 40. After successful completion of the session with all the maximum rewards obtained and consumed, mice proceeded to the acquisition phase. In this phase only one lever was correct, opposite from the one that was naturally preferred during the initial training sessions for each mouse. Criterium for passing to the reversal phase was to obtain and consume 40 rewards with at least 80% of correct presses. After completing the acquisition

23

phase, mouse had to complete two baseline sessions to ensure that task had been learned and to assess baseline performance. The final, reversal phase was identical to the acquisition, only the correct lever has been switched to the opposite side.

4. Stereotactic surgeries

Stereotactic frame (Stoelting) was used for surgeries and implantation of permanent intra-striatal cannulas. Coordinates were adjusted before to target the dorsal striatum.
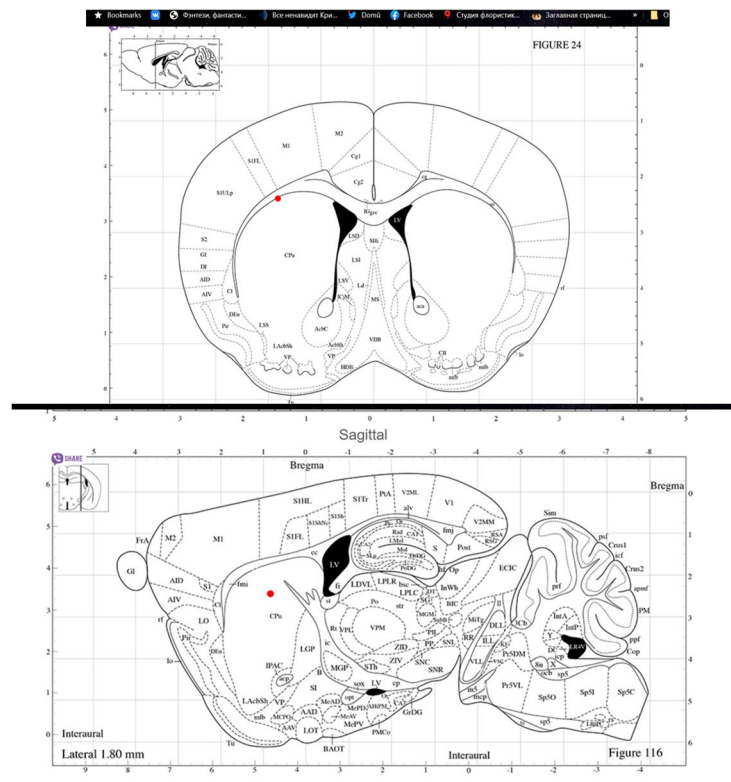

Fig. 8. schematic of target coordinates for the DS. Red dot represents placement of the cannula tip

Mice were deeply anaesthetised with mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg; both Vetoquinol), and eye gel was applied for preventing eyes from drying. Mouse's head was fixed in the frame with metal bars inserted into ears to prevent any head movements during the surgery. After the skin incision, hydrogen peroxide was carefully applied on the skull surface to make the sutures clearly visible. In the next step, I ensured that bregma and lambda points of the scull were laying in one plane and they were both placed in the midline. For ensuring better cannula fixing two holes were drilled and two screws of 1,6 mm diameter (PlasticsOne) were placed approximately 2 mm posterior from the cannula holes. Then two holes were drilled in

the skull with the following coordinates: AP: 0.8 mm; ML: ±1.8 mm from bregma. Cannulas (PlasticsOne; RWD Life Science) were placed in depth of 2.4 mm DV from bregma. Cannulas were fixed with a small amount of dental cement and let dry for approximately 30 minutes. Then the mouse was released from the frame and moved to a home cage for recovery from anaesthesia. Mice were held in separate cages after the surgery to prevent them interacting with mate's cannulas and decrease the possibility of cannulas damage.

5. Microinfusions into the dorsal striatum

After evaluation of the three different behavioural paradigms, we chose the instrumental task in the operant box as the final task for testing the role of M4 mAChRs in cognitive flexibility. The stereotaxic surgery and cannula placement was either done after completion of the acquisition session of the task or at the beginning of the task before the training was initiated. After the surgery, mice were given recovery time of 2-7 days. Before each session of the reversal phase, tropicamide or saline was infused through the cannula into the dorsal striatum. For the microinfusion, mice were gently restrained, and an injector (PlasticsOne) was inserted into the cannula, attached to a syringe held by a microinfusion pump. The infusion rate was set at 0,5μl/min. Mice were infused with 1μl of tropicamide in concentration of 1μg/μl or the same amount of saline in each hemisphere and then left for 20-30 mins before initiating the task. Then they were placed into the operant box and tested according to the protocol described above. Immediately after completion of the last session, mice were anaesthetised, transcardially perfused with 4 % paraformaldehyde (PFA) and their brains were extracted and post-fixed in 4% PFA for 24 hours. After that brains were put into 30% sucrose solution, cut with the vibratome in sections of 40 μm and stored in cryoprotective solution at -20 °C as free floating sections.

6. c-Fos staining

Stored brain slices were taken out and moved to the phosphate buffer saline (PBS) (0.1M) solution at the room temperature. For c-Fos imagining, slices were stained with anti-Fos antibody according to the following protocol: Free floating brain sections were rinsed three times in PBS 0.1M + 0,15% Triton-X solution (pH 7,4; washing buffer) for 5 minutes each time. For antigen retrieval, brain slices were incubated at 95 °C in citrate buffer (0.1M, pH 6) for 10 minutes then left for 10 more minutes to cool down at the room temperature and washed three times in washing buffer for 5 minutes each time. To remove endogenous peroxidase activity, slices were pre-incubated for 30 minutes with 1% hydrogen peroxide in PBS and then

washed for 5 minutes with washing buffer. To avoid unspecific binding of the antibody, free-floating sections were also incubated in a solution containing 5% bovine albumine (BSA), washing buffer and 5% normal goat serum (NGS) for one hour. Then sections were incubated overnight with primary antibody (Abcam, ab190289, anti-c-Fos rabbit, 1:1000) in washing buffer with 2% NGS. After the primary antibody incubation, sections were successively washed three times in washing buffer 5 minutes each and incubated with an anti-rabbit HRP-conjugated secondary antibody (Santa Cruz, 1:500) in washing buffer plus 2% NGS for 2 hours and then washed three times at PBS 0.1M for 5 minutes each time. Sections were placed into solution for 3,3'-diaminobenzidine (DAB) reaction prepared with the DAB staining kit according to manufacturer's instructions (Abcam) and developed for 2-10 minutes. Stained slices were put into PBS to stop reaction and then washed in PBS 2 more times. Sections then were mounted with DPX mountant (Sigma), coverslipped and after drying analysed under a microscope.

7. Statistical analysis

Behavioural and binding experiments data were analysed using GraphPad Prism versions 7 and 8 (GraphPad Software, SanDiego, CA). We used two-tailed Student's t-test for comparison of two experimental groups. Two-way ANOVA or repeated measures two-way ANOVA followed by Sidak's or Tukey's post-hoc tests was used to evaluate effects of two variables. Significance was set at $p < 0.05$. Data are reported as means ±SEM and statistical analysis includes p values and 95 % confidence intervals (C]I) for the differences between means.

Results

1. Affinity tests

1.1. Saturation binding assay

Initial experiments consisted of measuring and calculating saturation curve for the [^{3}H]-NMS in the striatum to prove that it has a high expression of the muscarinic receptor. For this aim we used radioactively labelled ligand - [^{3}H]-NMS, that is non-specifically binding to all five subtypes of muscarinic receptors. Because of the lack of M4 specific radioactive ligand I was not able to evaluate M4 receptors expression specifically. Nevertheless, during the experiment I defined a value indicating the maximum expression of receptors that could bind the ligand (B_{max}) and equilibrium dissociation constant, which indicates concentration of ligand occupying 50% of binding sites (K_d). Experiment was repeated three times and analysed in GraphPad Prism software (Fig.9).
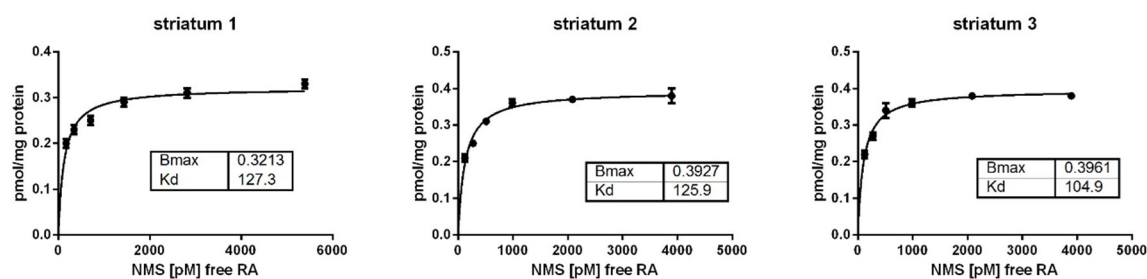

Fig. 9 Saturation binding curves in striatal homogenates, RA – radioactivity

Saturation experiments have shown high concentration of the muscarinic receptors in the striatum with average B_{max} of 0,3700 pmol/mg and average K_d of 119,4 pM (Fig.9).

1.2. Competitive binding assay

For determining whether tropicamide has a higher preference to M4 receptors comparing to M1, another set of binding experiments was done. For this experiment we used cell membranes from the CHO cell lines, which exclusively expressed either M1 or M4 subtype of muscarinic receptors. These binding experiments consisted of measuring competition between [^{3}H]-NMS and tropicamide, to determine which concentration of the latter is sufficient to displace the radioactive ligand from the binding site of M1 and M4 receptor i.e., the affinity of the tropicamide to the receptor. Experiment was repeated 5 times.
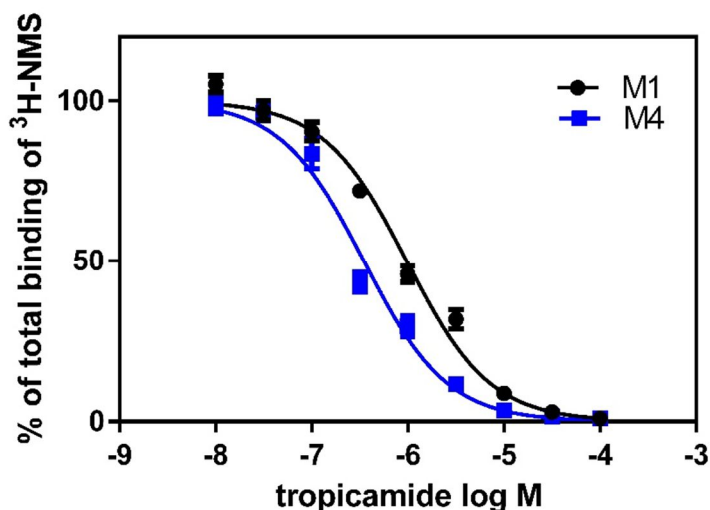
Fig. 10 Curves of the competitive binding of tropicamide to M1 and M4 receptors. values are averaged from 5 independent experiments. Difference between two receptor affinity is significant with p = 0.0179 (two-way ANOVA, unpaired t-test).

affinity expressed as Ki (nM)

	M1	M4
Number of values	5	5
Mean	195.1	68.95
Std. Deviation	102.7	42.17
Std. Error of Mean	45.95	18.86

Table 3. Ki (nM) average parameters for competitive binding experiments.

Tropicamide has a relatively strong affinity to both M1 and M4 receptor. However, the M4 binding curve is slightly left-shifted, indicating that lower tropicamide concentration is needed for the [³H]-NMS displacement and receptor binding, which proves tropicamide preference towards M4 receptors. Figure 10 is representing results averaged from five competition experiments.

2. Behavioural tests

Three types of behavioural tests were done to select the optimal one to use with mice after the microinfusion of tropicamide. Criteria we considered to select the optimal test were 1/ total time of testing, 2/ inter-trial interval (if any) and 3/ mice motivation during task performance. Before choosing the optimal test for our future experiments with tropicamide, we tested and

optimized our behavioural tasks in wild type mice (C57BL/6 background), both males and females. Mice were separated into 2 groups – male (n=9) and female (n=10) - and the two groups were tested separately. Performance of both sexes was also compared to decide whether males or females will be used for future testing the effect of tropicamide.

2.1. T-maze task

During the T-maze experiment mice were required to learn the correct turn in the maze in order to obtain the reward. After they reached the learning criteria in the acquisition phase, they had to re-learn the opposite turn in the reversal part of the experiment. Percentage of correct choices was analysed for each day of training as well as the number of days needed to reach the learning criterion (70% of correct choices). The mice received maximum 10 days of training for each phase of the experiment.
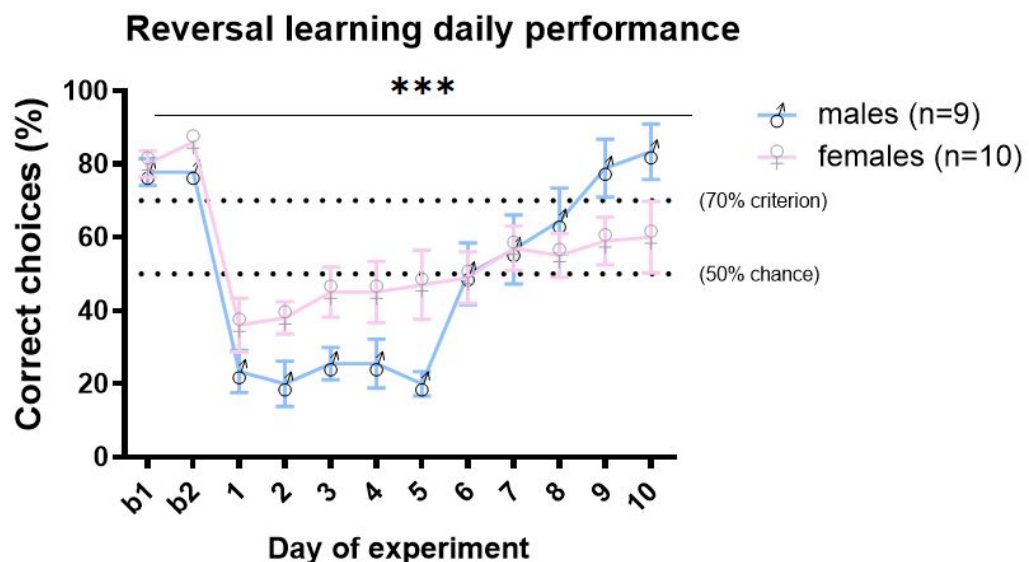

Fig. 11. Comparison of the daily performance of the male (blue) and female (pink) cohort during the reversal phase of the T-maze task. b1 and b2 for 2 days of baseline, before entering the reversal phase (*, effect of time on task performance, p <0.0001, two-way ANOVA)**

When we analysed performance (percentage of correct choices) of mice on individual days of the reversal phase of the task, we found that time had a highly significant effect on the performance (p <0.0001, repeated measures two-way ANOVA). This confirms that both males and females were able to re-learn task and after the initial drop in performance, their accuracy improved with time. However, there is also a significant effect of sex vs. time interaction (p <0.0001, repeated measures two-way ANOVA). Specifically, while males had lower

performance at the beginning of the reversal phase and in the end, they reached the same accuracy as in the baseline, females did not drop their performance as much but after 10 days of reversal training, they were not able to reach the baseline performance.
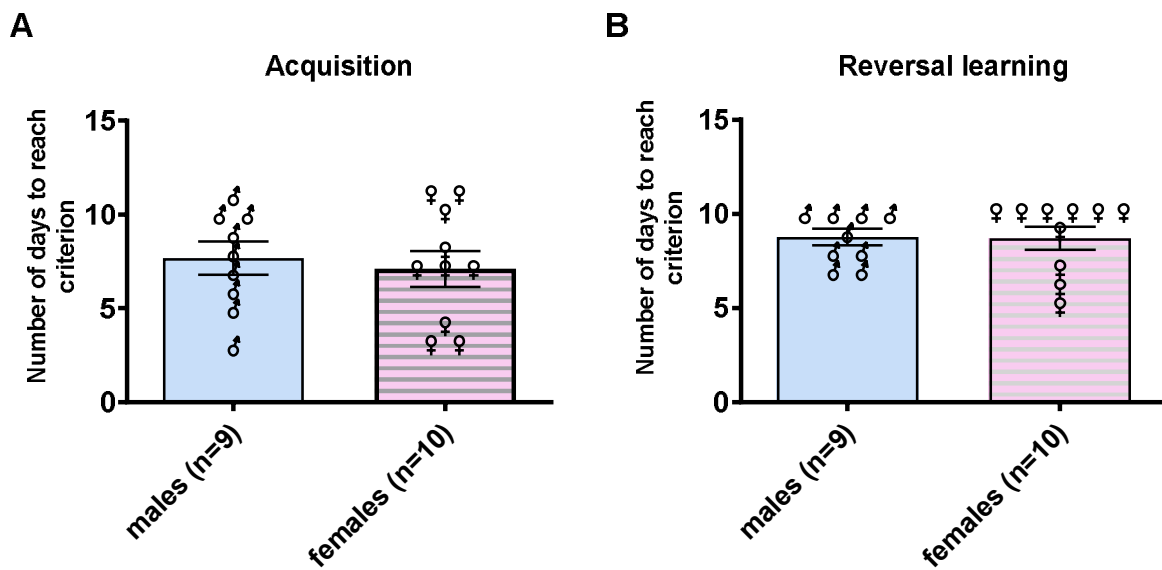

Fig. 12. Comparison of the days number to reach criterion for the acquisition (A) and reversal (B) learning

In contrast to accuracy, there was no difference in the number of days needed for reaching the learning criteria in the acquisition or reversal stage (acquisition: mean ±SEM of days to reach the criterion in males and females, respectively: 7.667 ±0.8819, 7.100 ±0.9597; 95 % CI for the difference between means in males and females [-3.317 to 2.183], p = 0.6692, unpaired t-test; reversal: mean ±SEM of days to reach the criterion in males and females, respectively: 8.778 ± 0.4339 and 8.700±0.6155; 95 % CI for the difference between means [-1.521 to 1.676], p = 0.9190, unpaired t-test) (Fig.12). The discrepancy with the accuracy data from the reversal phase may be caused by the fact that both sexes required close to 10 days or more to reach the accuracy criteria and the even in animals that did not reach it, the training was terminated after 10 days of training. Therefore, the difference between sexes in the number of days to reach the criterion could manifest only after longer training.

2.2. MWM with reversal phase

Next behavioural test we performed was Morris water maze with reversal phase, where mice were required to learn a position of the hidden platform in the water pool. Learning parameters including time and distance travelled to reach the platform and speed of swimming were

evaluated. This task also consisted of acquisition and reversal phase where mice had to learn and re-learn two different positions of the platform. After each acquisition and reversal phase of the task, a probe test was performed, to confirm successful learning in the absence of the platform. During probe sessions, time spent in the correct (target) quadrant was the main evaluated parameter.

Statistical analysis of MWM data showed highly significant effect of time on the performance, as both males and females have shown ability to learn platform location and decreased the time and distance needed to reach the platform over the training (fig. 13A, 13B) ($p < 0.0001$, two-way ANOVA). The maximum performance was reached already on the second day of the training and then it was maintained at the same level during the following acquisition days. On the first day of the reversal phase, we can see increasing of these parameters as the platform was moved to a new location. However, both groups were able to return to the previous level of performance on the second day of the reversal training.

In the MWM experiment we did not detect a significant difference between sexes in either time or distance needed to reach the platform. However, the average speed of swimming was higher in the female cohort during the acquisition phase but not during reversal. Bonferroni post-test has shown significant difference between sexes in the second, third and fourth day of the acquisition phase (Fig. 13 C)

Probe tests show that both cohorts tended to stay in the quadrant of the maze, where the platform was previously located (Fig.14). That confirms effective spatial learning for male and female mice both in acquisition and reversal phase of the experiment. In some sector comparisons the difference is lower due to the limited pool size, where mice tend to cross the sectors border. However, this effect is seen only in case of nearby sectors. This effect can be seen, for example, in the reversal learning probe test for the females. Another explanation might be in the worse reversal learning performance of the female's cohort. We see the trend for females in the reversal to not differ to the same extent in spending time in the target sector versus other sectors as for the males. In case of the opposite sector difference is clearly seen for both acquisition (95 % CI for the difference between mean target and opposite target (%) [-38,83 to -17,97] and [-28,48 to -8,684] for males and females respectively, multiple comparison two-way ANOVA) and reversal (95 % CI for the difference between mean target and opposite target (%) [14,09 to 38,71] and [4,812 to 28,17] for males and females respectively, two-way ANOVA) learning phase.
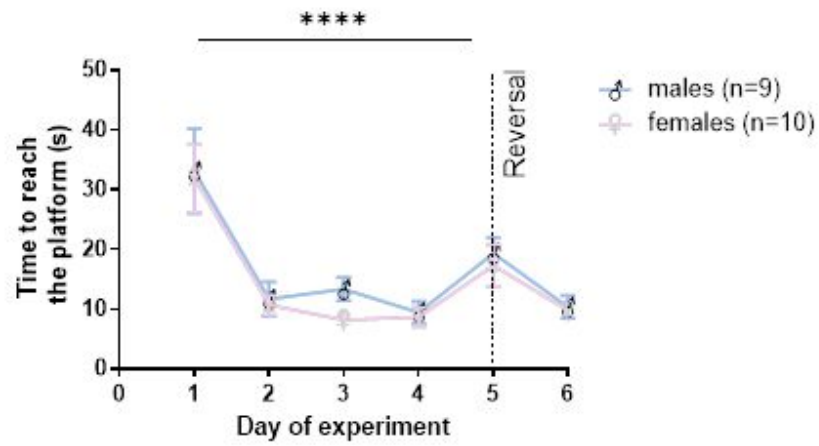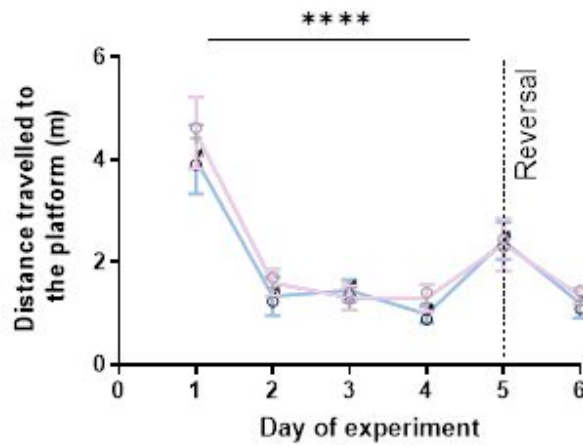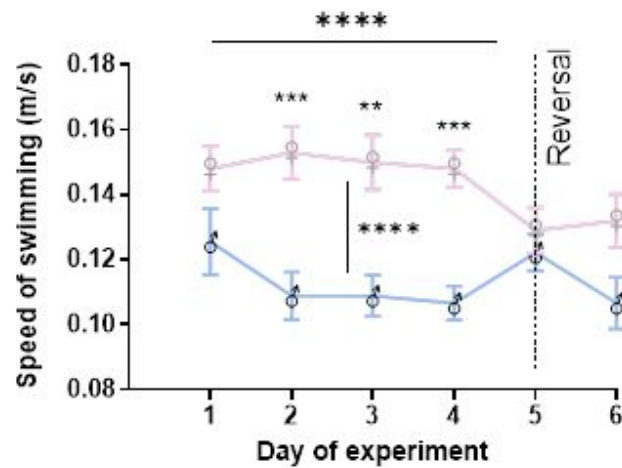
Fig. 13 **Performance of the male and female cohort during the training days of MWM: average time to reach the platform (A); in average distance swam to the platform (B) and average speed of swimming (C). (****, *** p < 0.0001; ** p < 0.005)**
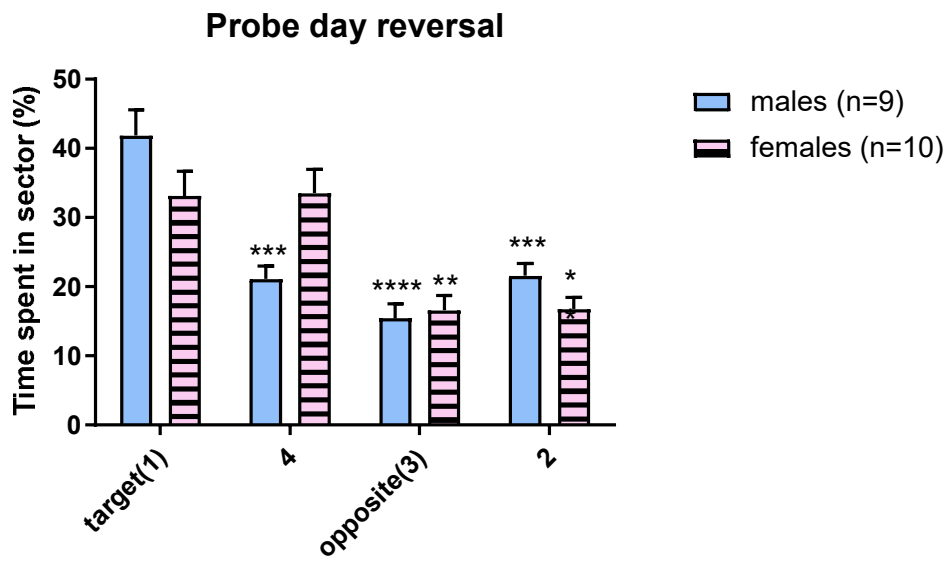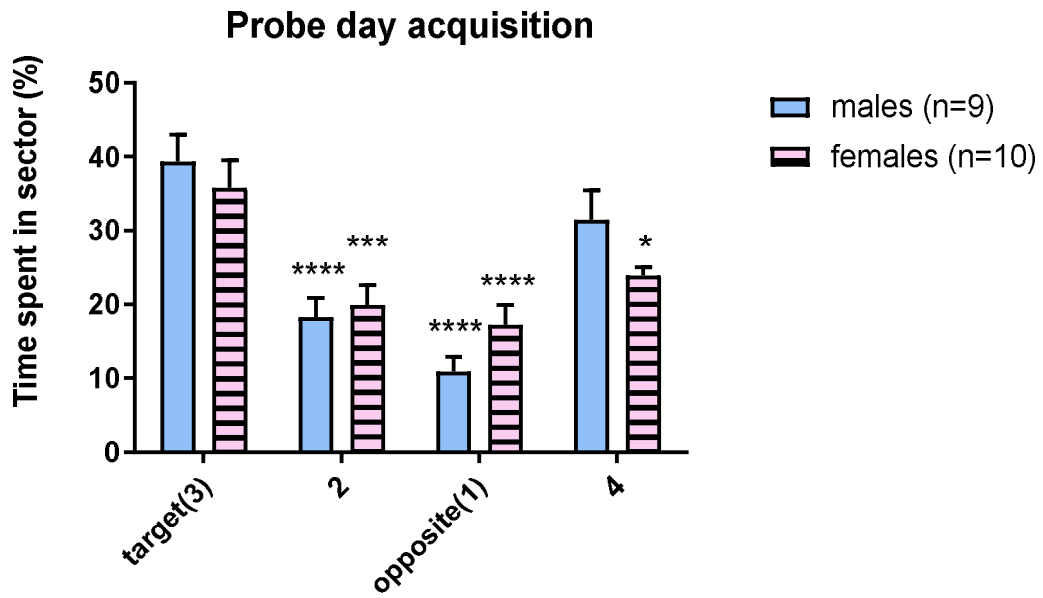
Fig. 14. Acquisition learning probe test (upper graph) and reversal learning probe test (lower graph) results for the male and female group. Sector numeration is described in methods (Fig. 6)

2.3. Instrumental task in the operant box

The third behavioural test was performed in the operant box (Med Associates Inc.) where mice were taught to press one of the levers in order to get a reward. The main parameter to evaluate was the percentage of correct presses. This parameter is important in order to see if mice were able to associate the correct lever with the reward. Learning criterion was set at 80% accuracy. Number of training days needed to reach the learning acquisition criterion was also evaluated, to see if there is any difference in the initial learning between the groups.

Fig. 15A is showing daily performance of the male and female mice. Both groups were able to reach criterion for the acquisition phase and they did not differ in their performance. On the 1st day of the reversal phase the performance decreased in both groups to a similar extent. On the second day of the reversal, returning to the same level, reaching the criterion of 80% success again. This is proven by significant effect of the time on the performance (p <0.0001, two-way ANOVA) and also by significant difference between the referred days (Bonferroni post-test). There is, however, no significant difference between sexes (p= 0.5166, 95 % CI for the difference between mean of males and female performance (%) [-8,190 to 4,426], two-way ANOVA).

Notably, there is a significant difference between sexes in number of days needed to reach the learning criterion in the acquisition phase, with shorter time needed for the female cohort (mean ±SEM of days to reach the criterion by males and females, respectively: 3,333 ± 0,6455, 2,000 ± 0,1491, 95 % CI for the difference between means (days) [-2,663 to -0.003326], p = 0,0495, unpaired t-test) (Fig.15B). However, analysis of the performance during the whole task showed no significant difference between the sexes (95 % CI for the difference between mean of males' and females' performance (% accuracy) [-1.743;15.43], p = 0.1049, two-way ANOVA) (Fig.15B).
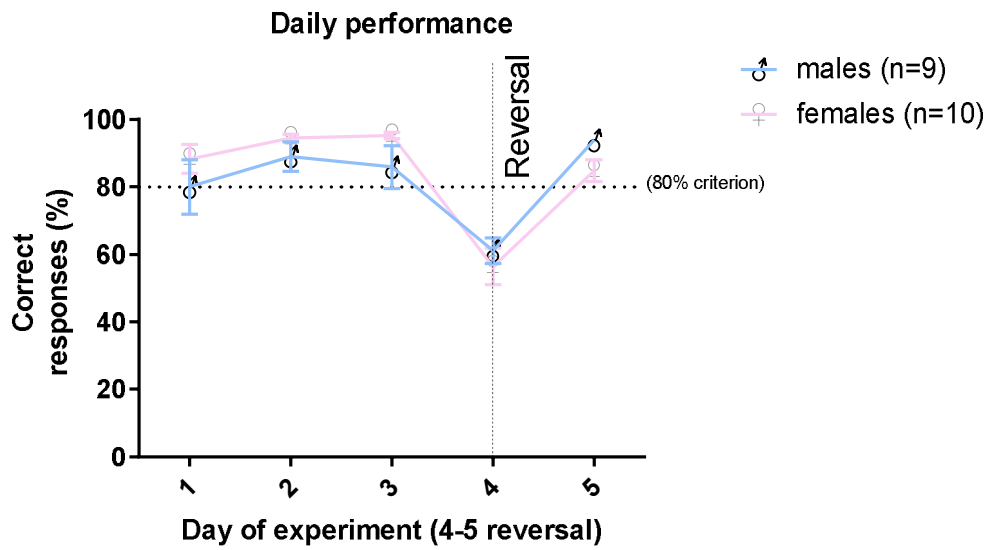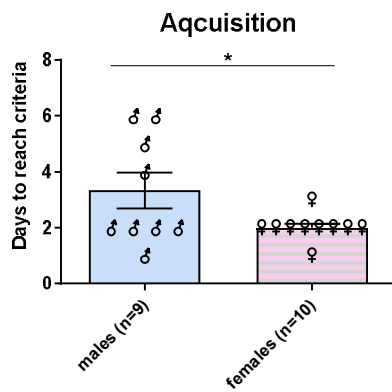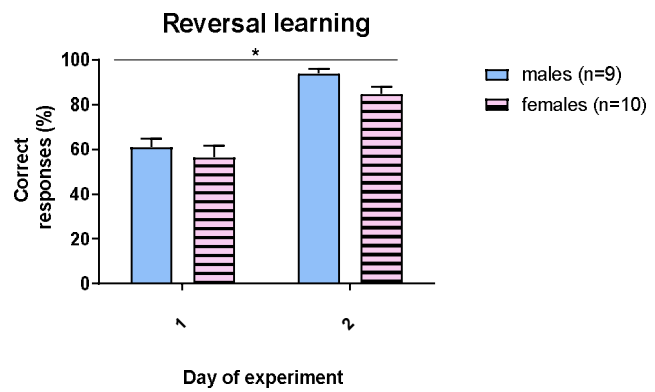
**Fig. 15** Daily performance of the male and female cohort in the operant box task (A); number of days needed to reach criteria during the acquisition phase (B); performance of the male and female cohort during the reversal phase of the operant task (the last two days in graph A) (C).; * p < 0.05

3. Effect of tropicamide on the performance in the operant box

Finally, instrumental task in the operant box was chosen as the optimal task for testing the effect of M4 antagonism on cognitive flexibility in mice. Learning criterion of 80% accuracy and the experimental setting remained the same as in the previously described instrumental task in the operant box.

A
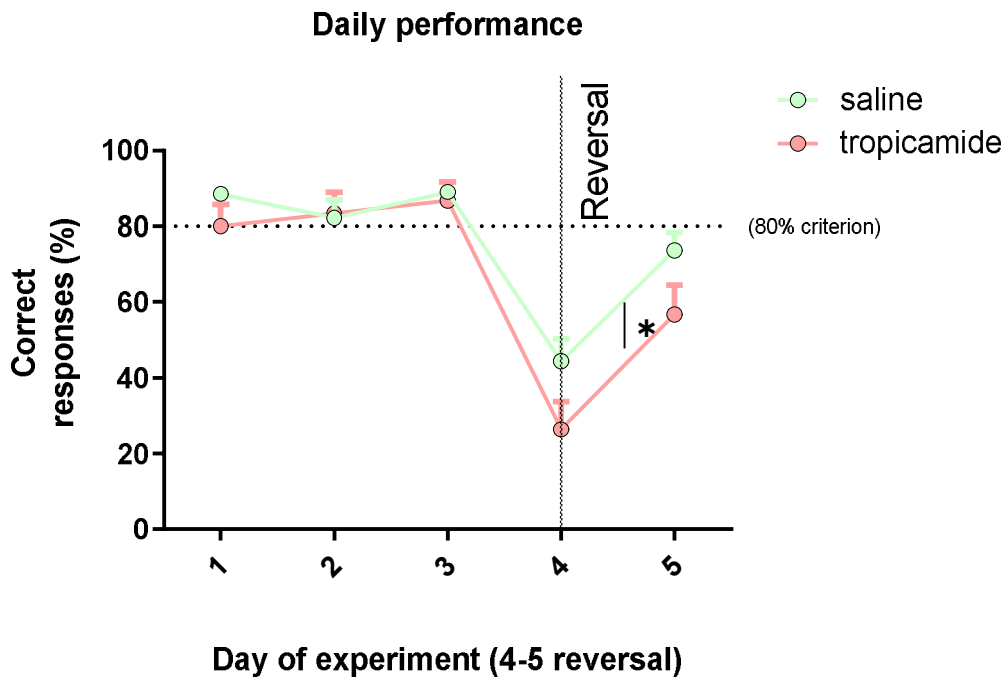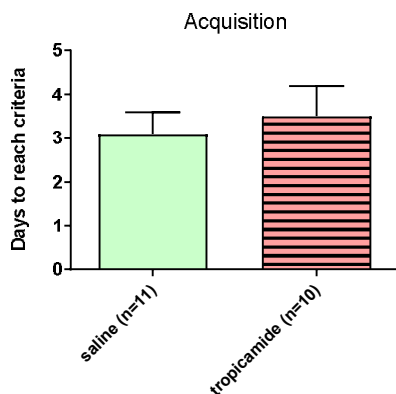
B **C**
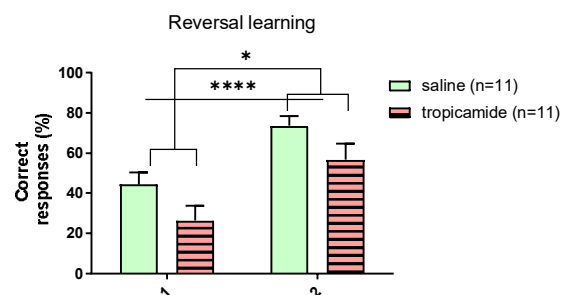
Fig. 16 Daily performance of the saline-(n=11) and tropicamide (n=11)-injected groups during the operant box task (A)(two-way ANOVA); days needed to reach the acquisition learning criterion for the saline and tropicamide to-be-injected mice (B) (unpaired t-test); performance during the

reversal learning phase only in the saline- and tropicamide-injected group (C) (unpaired t-test, two-way ANOVA).(** p < 0.0001; *p < 0.05)**

In the Fig. 16A, a significant effect of time on the daily performance is shown (p <0.0001, two-way ANOVA). Both groups have learned the correct lever position during the acquisition phase, they decreased their performance on the 1st day of reversal learning and then improved on the 2nd day. There is also a significant difference in performance between the mice injected with the saline and tropicamide, specifically during the reversal phase of the task. While the two groups show very similar performance during acquisition, injection of tropicamide impaired performance of mice during reversal phase (mice were not injected during acquisition) (p = 0.0106, 95 % CI for the difference between mean of performance during the reversal (%) [2.122 to 15.70], two-way ANOVA). In the Fig. 16B, number of days needed for reaching the learning criterium during the acquisition phase are shown. As expected, there is no difference between the groups (mean ±SEM of saline and tropicamide to-be-injected mice, respectively: 3.091± 0.4946, 3.500±0.6872, p = 0.6298, unpaired t-test). In Fig. 16C reversal learning performance is specifically compared between the saline- and tropicamide-injected mice. The analysis shows a significant effect of time on the performance as both groups improved their performance with time (p <0.0001, two-way ANOVA) (Fig.16A) and on the saline or tropicamide injection (p=0.0118, 95 % CI for the difference between mean of performance (%) [4.091 to 30.82], two-way ANOVA) (Fig. 16C). However, no significant difference was shown between groups on individual days.

4. Immunohistochemical analysis of c-Fos expression

To see the effect of tropicamide on neuronal activity in the striatum, we wanted to detect expression of an immediate early gene c-Fos with immunohistochemistry. Unfortunately, we were not able to detect a sufficient c-Fos expression in the striatum. However, in the same sections, strong c-Fos expression was detected in the lateral septum and to the less extent also in the cortex. For the lack of strong striatal expression, we did not perform any quantitative analysis of c-Fos expression.

Discussion

The main goal of this thesis was to test the hypothesis that the M4-preferential antagonist tropicamide affects cognitive flexibility in mice. Based on the current literature, it is unclear if M4 muscarinic receptors are involved in the control of cognitive flexibility (McCool et al., 2008; Okada et al., 2014) as available data are scarce and contradictory. The results of the thesis support the notion that at least in certain tasks and conditions, blocking the activity of M4 receptors in the dorsal striatum impairs cognitive flexibility in mice.

Before arriving to the final experiment that allowed us to test the initial hypothesis, I performed a range of pilot experiments to select the optimal behavioral paradigm and establish the optimal conditions for stereotaxic surgery and microinfusion of tropicamide in the dorsal striatum.

In the first series of experiments, I confirmed that tropicamide is a suitable preferential antagonist of the M4 mAChRs. However, the final concentration used in the microinfusing experiments was adapted from the literature (Ztaou et al., 2016), rather than calculated from the experiments.

The next experimental block consisting of three different behavioral tests suggested that in general, all three tests are suitable for testing cognitive flexibility mice. Both males and females were able to acquire the tasks and they showed expected behavioural pattern during the acquisition and reversal phase. Nevertheless, based on the other criteria such as the duration and easiness of each test, the instrumental task in the operant box was chosen for the microinfusion experiments in mice with intracerebral cannulas, testing the effect of tropicamide.

In the pilot behavioral experiments, I tested both sexes and compared their performance to decide which sex (or both) should be used in the final test with tropicamide. Both males' and females' performance were sufficient to use them for the test. However, they showed several significant differences in their performance, which suggests that the sexes should be tested and analyzed separately in future experiments. Specifically, females have shown less accuracy in reaching the required criteria in the reversal phase (see T-maze) and in MWM probe tests, where their time spent in the target sector does not differ to the same extent from the other sectors as for the male's cohort. There is a trend for females in all three experiments to perform better rather in acquisition phase and, relatively to this success, show less successful performance in the reversal phase. In literature, there is an evidence for the sex influencing performance in the cognitive flexibility tasks. The striatal dopaminergic system is affected by the estrogen and

female's performance in cognitive flexibility tasks differ depending on the actual phase of their estrous cycle (Korol et al., 2004; Shams et al., 2016). This behaviour is seen not only in rodents but also in primates (LaClair et al., 2019; Schoenberg et al., 2019). Overall, the results of my pilot experiments are supporting these observations from the literature and the lower performance during reversal phase in females was the main reason for choosing the male as the only sex for the following experiments with tropicamide. Female mice could be tested in these experiments as well, however, the difference between sexes should be considered here and the results should not be mixed. As well as conclusions and observation should consider the sex bias in the cognitive flexibility testing experiments.

Results of the instrumental task in the operant box show the suitability of this task for testing the cognitive flexibility. Also, due to the time needed for the experiment (2 reversal days) it is the most convenient task for testing larger mice cohort and choose the mice with the same level of acquisition performance for the future surgeries and tropicamide effect testing. In comparison, T-maze task lasts longer, and environment is more complex which allows to observe more behavioral details than in the easier and faster paradigm in the operant box. Due to this complexity, there is also higher variability in performance during the acquisition which can influence the reversal phase performance. To avoid this variability influence, larger mice cohorts may be needed in this task. The last tested task, the MWM, was not chosen primarily because of the fact that multiple trials with inter-trial intervals are run within a single session which would complicate precise timing of tropicamide microinfusion in individual animals that need to be administered consistently the same time before starting the experiment. Additionally, the risk of water filling the cannulas makes this task less suitable for the experiment with the tropicamide despite its time convenience relatively to T-maze.

In literature, T-maze task was previously used for testing the effect of M4 blockade on cognitive flexibility. However, those experiments were based on the place discrimination task, which is highly dependent on hippocampus activity and may be not that suitable to test the effect of M4 receptors in the striatum. (McCool et al., 2008; Okada et al., 2014) In the place discrimination task, rodents are required not to remember a turn direction, but the fixed position of the reward in the maze using external navigation cues. Direction of the turn is therefore changing with the change of the starting position. Such task is corresponding more with the cognitive map formation, whereas response discrimination task requires to remember the turn direction and therefore is better suited to test striatal alterations.

The crucial results of my thesis obtained in the final experiment using tropicamide microinfusion, are supporting our hypothesis that M4 receptors in the dorsal striatum are involved in the control of cognitive flexibility. Currently, there are only two studies examining the role of M4 receptors in the cognitive flexibility. The two studies are contradictory, and my own results are in partial contradiction with both of these studies.

In the McCool study (McCool et al., 2008), the authors are showing a role for acetylcholine in regulation of the striatal SPNs responsible for the acquisition of a new strategy. Using specific antagonists, they confirmed a role of M1 but not M4 muscarinic receptors in this regulation. In contrast, in the Okada study M4 receptors small hairpin ribonucleic acid (shRNA) block has been shown to increase reversal learning performance, whereas analogical M1 block had no effect (Okada et al., 2014). One of the key differences between these two studies, besides the block method is intertrial interval (ITI), which was set at 15s in the first study and 20 min in the second one. 15s might just be not enough for the seeing M4 block effect on the reversal learning. On the other hand, 20 min might be just too much, so another compensatory mechanisms are getting involved into this regulation.

Importance on the timing is also has been shown in another Okada studying (Okada et al., 2018). Cholinergic interneurons elimination during the response discrimination task has been shown to impair cognitive flexibility in experiment with 15s ITI but has an opposite effect in the experiments with 10- and 20-min ITIs. That might mean that acetylcholine contributes to the cognitive flexibility shortly after the trial, but later has rather regulative function and contributes to the maintaining of the old strategy. The operant box, used in our case, does not have an ITI problem, as the experiment is running continually, and it depends on the mouse when it chooses to press the lever in order to obtain the reward. M4 receptors block effect, therefore, will be generally noticeable. I would predict more accuracy in this case for the short intervals between lever presses and less accuracy for the longer intervals. However, additional box data analysis should be done for getting more information about the presses timing.

Besides the test choice and ITIs there is also a few very important differences between these three experiments. First are the animals. When I was using mice during my experiments, other two studies were using rats, which are having more complex brain and higher variety of the compensatory mechanisms. Together with the choice of the place discrimination task it gives higher chances that subtle changes in the striatum neurochemistry can be obscured by compensatory mechanisms, also due to the hippocampal involvement. There is also a difference in the way of blocking the targeted receptors. Also, the toxin used for block in this thesis and

McCool experiments are different. Presence of the tropicamide affinity to the M1 receptors could influence the results as well.

Besides the differences described above, time awaited after the drug application was 10 and 60 min in McCool study (McCool et al., 2008). 20-30 minutes awaited in our case might be more suitable, as it's enough for the animal to calm down and not too much for drug extinction. Also, relatively short duration of the experiment in the operant box (30 min) also ensures time sufficiency for the tropicamide action.

As it could be observed in results, surgery and microinfusion itself cause impairment of the general performance in the reversal learning task, so the time for recovering for the mouse is also crucial for it's representee performance. The chosen time of 30 min was sufficient in this case for recover after the injection. The timeframe for the surgery recovery, however, was not set immediately. The first half of the cohorts which only had 2 days for recover has shown worse performance and no significant difference between the vehicle and tropicamide injected mice. Another half was given 7 days for the recovery and their performance was so significantly different that this effect is observed even in the mixed results.

The mice housing after the surgery may also influence the final performance, as they are separated into individual cages and therefore have no social interaction during the recovery stage and reversal tests. It is done to prevent the cannula losing by mice grooming each other or other interactions and commonly used in experiments with cannula injections. However, the experiment should be held as soon as possible after the recovery time to prevent social isolation impact on the mice performance.

Another important parameter is the drug concentration which is shown to be reliable as mice performance was sufficiently affected by it. It also has worked for the other studies from which the final concentration has been taken (Ztaou et al., 2016).

Results described above are showing that M4 blocking impair cognitive flexibility in mice. However, the effects described above might be affected by other factors. Such as sensitivity of the tropicamide to the M1 muscarinic receptors or expression of the M4 receptors on CINs. First can lead to the lack of activation of iSPNs responsible for the old strategy which would lead to the worse performance in the reversal learning and higher chance of regressive errors.

M4 receptors expressed by CINs after tropicamide block might have an effect of increased acetylcholine release from the CINs and therefore lead to the enhanced effect on M1 receptors.

This might also be a case for the described studying where targeted genetical block of M4 receptors could also affect CINs.(Betz et al., 2007; Yan and Surmeier, 1996)

Further studying of this subject are certainly needed, as the whole picture of the striatal M4 receptors functioning in the cognitive flexibility is not clear and the studies in this area are having contradictory data.

More specific manipulation with M4 receptors such as positive allosteric modulation or genetic approach can be used to follow their effects on behavior.

Genetical block of the M4 receptors with the shRNA delivered to the target area with help of lentivirus vector can be repeated similar to the experiments described in the literature (Okada et al., 2014), but other behavioral experiments should be done to observe cognitive flexibility change. The most precise one in this case would be T-maze response discriminative task, in which is involvement of the striatal motor neurons memory is higher and has a key role comparing to the place discrimination task, where hippocampal spatial map is formed. Operant box can be also suitable for this aim, especially with some additions such as light stimuli dependent learning, where the response discrimination task will be in choosing the lever under the light regardless of the left/right position.

Also, more precise analysis of the behavioral data and errors type (either perseverative – with repeating of the old strategy or regressive – coming back to the old strategy after acquiring a new one) can give more information of the origin of the observed effects.

Conclusion

Aims of this thesis were achieved and the main hypothesis was confirmed. However, it brought more information about the possible subtleness of the underlying mechanisms and more sophisticated methods needed for its revealing. Also, the obtained results are somehow controversial to the existing studying around this topic and show how easily the final results can be influenced by the experimental setting. As most of the research works, it answers some questions but revealing much more to ask and explore.

Literature

Albin, R.L., Young, A.B., Penney, J.B., 1989. The functional anatomy of basal ganglia disorders. Trends Neurosci. 12, 366–375. https://doi.org/10.1016/0166-2236(89)90074-X

Alexander, G.E., Crutcher, M.D., 1990. Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci. 13, 266–271. https://doi.org/10.1016/0166-2236(90)90107-l

Aoki, S., Liu, A.W., Zucca, A., Zucca, S., Wickens, J.R., 2015. Role of Striatal Cholinergic Interneurons in Set-Shifting in the Rat. J. Neurosci. 35, 9424–9431. https://doi.org/10.1523/JNEUROSCI.0490-15.2015

Balleine, B.W., Delgado, M.R., Hikosaka, O., 2007. The role of the dorsal striatum in reward and decision-making. J. Neurosci. Off. J. Soc. Neurosci. 27, 8161–8165. https://doi.org/10.1523/JNEUROSCI.1554-07.2007

Betz, A., McLaughlin, P., Burgos, M., Weber, S., Salamone, J., 2007. The muscarinic receptor antagonist tropicamide suppresses tremulous jaw movements in a rodent model of parkinsonian tremor: Possible role of M4 receptors. Psychopharmacology (Berl.) 194, 347–59. https://doi.org/10.1007/s00213-007-0844-6

Brimblecombe, K.R., Threlfell, S., Dautan, D., Kosillo, P., Mena-Segovia, J., Cragg, S.J., 2018. Targeted Activation of Cholinergic Interneurons Accounts for the Modulation of Dopamine by Striatal Nicotinic Receptors. eNeuro 5. https://doi.org/10.1523/ENEURO.0397-17.2018

Bromberg-Martin, E.S., Matsumoto, M., Hikosaka, O., 2010. Dopamine in motivational control: rewarding, aversive, and alerting. Neuron 68, 815–834. https://doi.org/10.1016/j.neuron.2010.11.022

Bubser, M., Bridges, T.M., Dencker, D., Gould, R.W., Grannan, M., Noetzel, M.J., Lamsal, A., Niswender, C.M., Daniels, J.S., Poslusney, M.S., Melancon, B.J., Tarr, J.C., Byers, F.W., Wess, J., Duggan, M.E., Dunlop, J., Wood, M.W., Brandon, N.J., Wood, M.R., Lindsley, C.W., Conn, P.J., Jones, C.K., 2014. Selective Activation of M4 Muscarinic Acetylcholine Receptors Reverses MK-801-Induced Behavioral Impairments and Enhances Associative Learning in Rodents. ACS Chem. Neurosci. 5, 920–942. https://doi.org/10.1021/cn500128b

Burke, D.A., Rotstein, H.G., Alvarez, V.A., 2017. Striatal Local Circuitry: A New Framework for Lateral Inhibition. Neuron 96, 267–284. https://doi.org/10.1016/j.neuron.2017.09.019

Caulfield, M.P., 1993. Muscarinic receptors--characterization, coupling and function. Pharmacol. Ther. 58, 319–379. https://doi.org/10.1016/0163-7258(93)90027-b

Cazorla, M., de Carvalho, F.D., Chohan, M.O., Shegda, M., Chuhma, N., Rayport, S., Ahmari, S.E., Moore, H., Kellendonk, C., 2014. Dopamine D2 receptors regulate the anatomical balance of basal ganglia circuitry. Neuron 81, 153–164. https://doi.org/10.1016/j.neuron.2013.10.041

Cohen, J.Y., Haesler, S., Vong, L., Lowell, B.B., Uchida, N., 2012. Neuron-type specific signals for reward and punishment in the ventral tegmental area. Nature 482, 85–88. https://doi.org/10.1038/nature10754

Cox, J., Witten, I.B., 2019. Striatal circuits for reward learning and decision-making. Nat. Rev. Neurosci. 20, 482–494. https://doi.org/10.1038/s41583-019-0189-2

Daw, N.D., Gershman, S.J., Seymour, B., Dayan, P., Dolan, R.J., 2011. Model-based influences on humans' choices and striatal prediction errors. Neuron 69, 1204–1215. https://doi.org/10.1016/j.neuron.2011.02.027

Daw, N.D., Niv, Y., Dayan, P., 2005. Uncertainty-based competition between prefrontal and dorsolateral striatal systems for behavioral control. Nat. Neurosci. 8, 1704–1711. https://doi.org/10.1038/nn1560

Deacon, R.M.J., Rawlins, J.N.P., 2006. T-maze alternation in the rodent. Nat. Protoc. 1, 7–12. https://doi.org/10.1038/nprot.2006.2

Deng, Y.-P., Lei, W.-L., Reiner, A., 2006. Differential perikaryal localization in rats of D1 and D2 dopamine receptors on striatal projection neuron types identified by retrograde labeling. J. Chem. Neuroanat. 32, 101–116. https://doi.org/10.1016/j.jchemneu.2006.07.001

Deniau, J.M., Chevalier, G., 1985. Disinhibition as a basic process in the expression of striatal functions. II. The striato-nigral influence on thalamocortical cells of the ventromedial thalamic nucleus. Brain Res. 334, 227–233. https://doi.org/10.1016/0006-8993(85)90214-8

Deserno, L., Huys, Q.J.M., Boehme, R., Buchert, R., Heinze, H.-J., Grace, A.A., Dolan, R.J., Heinz, A., Schlagenhauf, F., 2015. Ventral striatal dopamine reflects behavioral and neural signatures of model-based control during sequential decision making. Proc. Natl. Acad. Sci. 112, 1595–1600. https://doi.org/10.1073/pnas.1417219112

Faust, T.W., Assous, M., Shah, F., Tepper, J.M., Koós, T., 2015. Novel fast adapting interneurons mediate cholinergic-induced fast GABAA inhibitory postsynaptic currents in striatal spiny neurons. Eur. J. Neurosci. 42, 1764–1774. https://doi.org/10.1111/ejn.12915

Fiorillo, C.D., Tobler, P.N., Schultz, W., 2003. Discrete coding of reward probability and uncertainty by dopamine neurons. Science 299, 1898–1902. https://doi.org/10.1126/science.1077349

Fiorino, D.F., Coury, A., Fibiger, H.C., Phillips, A.G., 1993. Electrical stimulation of reward sites in the ventral tegmental area increases dopamine transmission in the nucleus accumbens of the rat. Behav. Brain Res. 55, 131–141. https://doi.org/10.1016/0166-4328(93)90109-4

Floresco, S.B., Zhang, Y., Enomoto, T., 2009. Neural circuits subserving behavioral flexibility and their relevance to schizophrenia. Behav. Brain Res. 204, 396–409. https://doi.org/10.1016/j.bbr.2008.12.001

Foster, D.J., Wilson, J.M., Remke, D.H., Mahmood, M.S., Uddin, M.J., Wess, J., Patel, S., Marnett, L.J., Niswender, C.M., Jones, C.K., Xiang, Z., Lindsley, C.W., Rook, J.M., Conn, P.J., 2016. Antipsychotic-like Effects of M4 Positive Allosteric Modulators Are Mediated by CB2 Receptor-Dependent Inhibition of Dopamine Release. Neuron 91, 1244–1252. https://doi.org/10.1016/j.neuron.2016.08.017

Freeze, B.S., Kravitz, A.V., Hammack, N., Berke, J.D., Kreitzer, A.C., 2013. Control of Basal Ganglia Output by Direct and Indirect Pathway Projection Neurons. J. Neurosci. 33, 18531–18539. https://doi.org/10.1523/JNEUROSCI.1278-13.2013

Gagnon, D., Petryszyn, S., Sanchez, M.G., Bories, C., Beaulieu, J.M., De Koninck, Y., Parent, A., Parent, M., 2017. Striatal Neurons Expressing D1 and D2 Receptors are Morphologically Distinct and Differently Affected by Dopamine Denervation in Mice. Sci. Rep. 7. https://doi.org/10.1038/srep41432

Gerfen, C.R., Engber, T.M., Mahan, L.C., Susel, Z., Chase, T.N., Monsma, F.J., Sibley, D.R., 1990. D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science 250, 1429–1432. https://doi.org/10.1126/science.2147780

Gerfen, C.R., Surmeier, D.J., 2011. Modulation of striatal projection systems by dopamine. Annu. Rev. Neurosci. 34, 441–466. https://doi.org/10.1146/annurev-neuro-061010-113641

Gomeza, J., Zhang, L., Kostenis, E., Felder, C., Bymaster, F., Brodkin, J., Shannon, H., Xia, B., Deng, C., Wess, J., 1999. Enhancement of D1 dopamine receptor-mediated locomotor stimulation in M4 muscarinic acetylcholine receptor knockout mice. Proc. Natl. Acad. Sci. U. S. A. 96, 10483–10488.

Gonzales, K.K., Smith, Y., 2015. Cholinergic interneurons in the dorsal and ventral striatum: anatomical and functional considerations in normal and diseased conditions. Ann. N. Y. Acad. Sci. 1349, 1–45. https://doi.org/10.1111/nyas.12762

Gotti, C., Zoli, M., Clementi, F., 2006. Brain nicotinic acetylcholine receptors: native subtypes and their relevance. Trends Pharmacol. Sci. 27, 482–491. https://doi.org/10.1016/j.tips.2006.07.004

Goyal, R.K., 1989. Muscarinic Receptor Subtypes. N. Engl. J. Med. 321, 1022–1029. https://doi.org/10.1056/NEJM198910123211506

Gras, C., Amilhon, B., Lepicard, E.M., Poirel, O., Vinatier, J., Herbin, M., Dumas, S., Tzavara, E.T., Wade, M.R., Nomikos, G.G., Hanoun, N., Saurini, F., Kemel, M.-L., Gasnier, B., Giros, B., El Mestikawy, S., 2008. The vesicular glutamate transporter VGLUT3 synergizes striatal acetylcholine tone. Nat. Neurosci. 11, 292–300. https://doi.org/10.1038/nn2052

Grospe, G.M., Baker, P.M., Ragozzino, M.E., 2018. Cognitive Flexibility Deficits Following 6-OHDA Lesions of the Rat Dorsomedial Striatum. Neuroscience 374, 80–90. https://doi.org/10.1016/j.neuroscience.2018.01.032

Guzman, M.S., Jaeger, X.D., Raulic, S., Souza, I.A., Li, A.X., Schmid, S., Menon, R.S., Gainetdinov, R.R., Caron, M.G., Bartha, R., Prado, V.F., Prado, M.A.M., 2011. Elimination of the Vesicular Acetylcholine Transporter in the Striatum Reveals Regulation of Behaviour by Cholinergic-Glutamatergic Co-Transmission. PLOS Biol. 9, e1001194. https://doi.org/10.1371/journal.pbio.1001194

Hanes, K.R., Andrewes, D.G., Pantelis, C., 1995. Cognitive flexibility and complex integration in Parkinson's disease, Huntington's disease, and schizophrenia. J. Int. Neuropsychol. Soc. JINS 1, 545–553. https://doi.org/10.1017/s1355617700000679

He, N., Mao, L.-M., Sturich, A.W., Jin, D.-Z., Wang, J.Q., 2018. Inhibition of basal and amphetamine-stimulated extracellular signal-regulated kinase (ERK) phosphorylation in the rat forebrain by muscarinic acetylcholine M4 receptors. Brain Res. 1688, 103–112. https://doi.org/10.1016/j.brainres.2018.03.019

Hjorth, J.J.J., Kozlov, A., Carannante, I., Nylén, J.F., Lindroos, R., Johansson, Y., Tokarska, A., Dorst, M.C., Suryanarayana, S.M., Silberberg, G., Kotaleski, J.H., Grillner, S., 2020. The microcircuits of striatum in silico. Proc. Natl. Acad. Sci. 117, 9554–9565. https://doi.org/10.1073/pnas.2000671117

Ibáñez-Sandoval, O., Tecuapetla, F., Unal, B., Shah, F., Koós, T., Tepper, J.M., 2011. A Novel Functionally Distinct Subtype of Striatal Neuropeptide Y Interneuron. J. Neurosci. 31, 16757–16769. https://doi.org/10.1523/JNEUROSCI.2628-11.2011

Ibáñez-Sandoval, O., Xenias, H.S., Tepper, J.M., Koós, T., 2015. Dopaminergic and cholinergic modulation of striatal tyrosine hydroxylase interneurons. Neuropharmacology 95, 468–476. https://doi.org/10.1016/j.neuropharm.2015.03.036

Jeon, J., Dencker, D., Wörtwein, G., Woldbye, D.P.D., Cui, Y., Davis, A.A., Levey, A.I., Schütz, G., Sager, T.N., Mørk, A., Li, C., Deng, C.-X., Fink-Jensen, A., Wess, J., 2010. A Subpopulation of Neuronal M4 Muscarinic Acetylcholine Receptors Plays a Critical Role in Modulating Dopamine-Dependent Behaviors. J. Neurosci. 30, 2396–2405. https://doi.org/10.1523/JNEUROSCI.3843-09.2010

Jones, C., Byun, N., Bubser, M., 2012. Muscarinic and Nicotinic Acetylcholine Receptor Agonists and Allosteric Modulators for the Treatment of Schizophrenia. Neuropsychopharmacology. https://doi.org/10.1038/npp.2011.199

Juszczak, G.R., Stryjek, R., 2019. Scopolamine increases perseveration in mice subjected to the detour test. Behav. Brain Res. 356, 71–77. https://doi.org/10.1016/j.bbr.2018.07.028

Kargbo, R.B., 2018. Allosteric Modulators of the M4 Muscarinic Acetylcholine Receptor. ACS Med. Chem. Lett. 9, 783–784. https://doi.org/10.1021/acsmedchemlett.8b00330

Kazantsev, A., BURKOV, N.N., ANOUFRIEV, A.I., Tarasov, R., 2017. DEBAKEY TYPE I AORTIC DISSECTION EXTENDING TO RENAL AND ILIAC ARTERIES IN A PATIENT WITH MECHANICAL PROSTHETIC AORTIC VALVE: AN ORIGINAL SURGICAL APPROACH. Fundam. Clin. Med. 2, 93–99. https://doi.org/10.23946/2500-0764-2017-2-2-93-99

Kitabatake, Y., Hikida, T., Watanabe, D., Pastan, I., Nakanishi, S., 2003. Impairment of reward-related learning by cholinergic cell ablation in the striatum. Proc. Natl. Acad. Sci. U. S. A. 100, 7965–7970. https://doi.org/10.1073/pnas.1032899100

Korol, D.L., Malin, E.L., Borden, K.A., Busby, R.A., Couper-Leo, J., 2004. Shifts in preferred learning strategy across the estrous cycle in female rats. Horm. Behav. 45, 330–338. https://doi.org/10.1016/j.yhbeh.2004.01.005

Kravitz, A.V., Freeze, B.S., Parker, P.R.L., Kay, K., Thwin, M.T., Deisseroth, K., Kreitzer, A.C., 2010. Regulation of parkinsonian motor behaviors by optogenetic control of basal ganglia circuitry. Nature 466, 622–626. https://doi.org/10.1038/nature09159

LaClair, M., Febo, M., Nephew, B., Gervais, N.J., Poirier, G., Workman, K., Chumachenko, S., Payne, L., Moore, M.C., King, J.A., Lacreuse, A., 2019. Sex Differences in Cognitive Flexibility and Resting Brain Networks in Middle-Aged Marmosets. eNeuro 6. https://doi.org/10.1523/ENEURO.0154-19.2019

Leber, A.B., Turk-Browne, N.B., Chun, M.M., 2008. Neural predictors of moment-to-moment fluctuations in cognitive flexibility. Proc. Natl. Acad. Sci. U. S. A. 105, 13592–13597. https://doi.org/10.1073/pnas.0805423105

Lee, H.J., Weitz, A.J., Bernal-Casas, D., Duffy, B.A., Choy, M., Kravitz, A.V., Kreitzer, A.C., Lee, J.H., 2016. Activation of direct and indirect pathway medium spiny neurons drives distinct brain-wide responses. Neuron 91, 412–424. https://doi.org/10.1016/j.neuron.2016.06.010

Lee, J., Finkelstein, J., Choi, J.Y., Witten, I.B., 2016. Linking Cholinergic Interneurons, Synaptic Plasticity, and Behavior during the Extinction of a Cocaine-Context Association. Neuron 90, 1071–1085. https://doi.org/10.1016/j.neuron.2016.05.001

Luo, R., Janssen, M.J., Partridge, J.G., Vicini, S., 2013. Direct and GABA-mediated indirect effects of nicotinic ACh receptor agonists on striatal neurones. J. Physiol. 591, 203–217. https://doi.org/10.1113/jphysiol.2012.241786

Mailly, P., Charpier, S., Menetrey, A., Deniau, J.-M., 2003. Three-Dimensional Organization of the Recurrent Axon Collateral Network of the Substantia Nigra Pars Reticulata Neurons in the Rat. J. Neurosci. 23, 5247–5257. https://doi.org/10.1523/JNEUROSCI.23-12-05247.2003

Mao, L.-M., He, N., Jin, D.-Z., Wang, J.Q., 2018. Regulation of Phosphorylation of AMPA Glutamate Receptors by Muscarinic M4 Receptors in the Striatum In vivo. Neuroscience 375, 84–93. https://doi.org/10.1016/j.neuroscience.2018.01.063

Matsubayashi, H., Amano, T., Amano, H., Sasa, M., 2001. Excitation of rat striatal large neurons by dopamine and/or glutamate released from nerve terminals via presynaptic nicotinic receptor (A4beta2 type) stimulation. Jpn. J. Pharmacol. 86, 429–436. https://doi.org/10.1254/jjp.86.429

McCool, M.F., Patel, S., Talati, R., Ragozzino, M.E., 2008. Differential Involvement of M1-type and M4-type Muscarinic Cholinergic Receptors in the Dorsomedial Striatum in Task Switching. Neurobiol. Learn. Mem. 89, 114–124. https://doi.org/10.1016/j.nlm.2007.06.005

Moehle, M.S., Pancani, T., Byun, N., Yohn, S.E., Wilson, G.H., Dickerson, J.W., Remke, D.H., Xiang, Z., Niswender, C.M., Wess, J., Jones, C.K., Lindsley, C.W., Rook, J.M., Conn, P.J., 2017. Cholinergic projections to the substantia nigra reticulata inhibit dopamine modulation of basal ganglia through the M4 muscarinic receptor. Neuron 96, 1358-1372.e4. https://doi.org/10.1016/j.neuron.2017.12.008

Montague, P., Dayan, P., Sejnowski, T., 1996. A framework for mesencephalic dopamine systems based on predictive Hebbian learning. J. Neurosci. 16, 1936–1947. https://doi.org/10.1523/JNEUROSCI.16-05-01936.1996

Moran, S.P., Maksymetz, J., Conn, P.J., 2019. Targeting Muscarinic Acetylcholine Receptors for the Treatment of Psychiatric and Neurological Disorders. Trends Pharmacol. Sci. 40, 1006–1020. https://doi.org/10.1016/j.tips.2019.10.007

Morris, R.G.M., 1981. Spatial localization does not require the presence of local cues. Learn. Motiv. 12, 239–260. https://doi.org/10.1016/0023-9690(81)90020-5

Nair, A.G., Castro, L.R.V., El Khoury, M., Gorgievski, V., Giros, B., Tzavara, E.T., Hellgren-Kotaleski, J., Vincent, P., 2019. The high efficacy of muscarinic M4 receptor in D1 medium spiny neurons reverses striatal hyperdopaminergia. Neuropharmacology 146, 74–83. https://doi.org/10.1016/j.neuropharm.2018.11.029

Nair, A.G., Gutierrez-Arenas, O., Eriksson, O., Vincent, P., Hellgren Kotaleski, J., 2015. Sensing Positive versus Negative Reward Signals through Adenylyl Cyclase-Coupled GPCRs in Direct and Indirect Pathway Striatal Medium Spiny Neurons. J. Neurosci. Off. J. Soc. Neurosci. 35, 14017–14030. https://doi.org/10.1523/JNEUROSCI.0730-15.2015

Nonomura, S., Nishizawa, K., Sakai, Y., Kawaguchi, Y., Kato, S., Uchigashima, M., Watanabe, M., Yamanaka, K., Enomoto, K., Chiken, S., Sano, H., Soma, S., Yoshida, J., Samejima, K., Ogawa, M., Kobayashi, K., Nambu, A., Isomura, Y., Kimura, M., 2018. Monitoring and Updating of Action Selection for Goal-Directed Behavior through the Striatal Direct and Indirect Pathways. Neuron 99, 1302-1314.e5. https://doi.org/10.1016/j.neuron.2018.08.002

O'Doherty, J., Dayan, P., Schultz, J., Deichmann, R., Friston, K., Dolan, R.J., 2004. Dissociable roles of ventral and dorsal striatum in instrumental conditioning. Science 304, 452–454. https://doi.org/10.1126/science.1094285

Okada, K., Nishizawa, K., Fukabori, R., Kai, N., Shiota, A., Ueda, M., Tsutsui, Y., Sakata, S., Matsushita, N., Kobayashi, K., 2014. Enhanced flexibility of place discrimination learning by targeting striatal cholinergic interneurons. Nat. Commun. 5, 3778. https://doi.org/10.1038/ncomms4778

Okada, K., Nishizawa, K., Setogawa, S., Hashimoto, K., Kobayashi, K., 2018. Task-dependent function of striatal cholinergic interneurons in behavioural flexibility. Eur. J. Neurosci. 47, 1174–1183. https://doi.org/10.1111/ejn.13768

Oldenburg, I.A., Ding, J.B., 2011. Cholinergic modulation of synaptic integration and dendritic excitability in the striatum. Curr. Opin. Neurobiol. 21, 425–432. https://doi.org/10.1016/j.conb.2011.04.004

Oldenburg, I.A., Sabatini, B.L., 2015. Antagonistic but not symmetric regulation of primary motor cortex by basal ganglia direct and indirect pathways. Neuron 86, 1174–1181. https://doi.org/10.1016/j.neuron.2015.05.008

Oorschot, D.E., 1996. Total number of neurons in the neostriatal, pallidal, subthalamic, and substantia nigral nuclei of the rat basal ganglia: a stereological study using the cavalieri and optical disector methods. J. Comp. Neurol. 366, 580–599. https://doi.org/10.1002/(SICI)1096-9861(19960318)366:4<580::AID-CNE3>3.0.CO;2-0

Owen, A.M., James, M., Leigh, P.N., Summers, B.A., Marsden, C.D., Quinn, N.P., Lange, K.W., Robbins, T.W., 1992. Fronto-striatal cognitive deficits at different stages of Parkinson's disease. Brain J. Neurol. 115 (Pt 6), 1727–1751. https://doi.org/10.1093/brain/115.6.1727

Peak, J., Chieng, B., Hart, G., Balleine, B.W., 2020. Striatal direct and indirect pathway neurons differentially control the encoding and updating of goal-directed learning. bioRxiv 2020.02.18.955385. https://doi.org/10.1101/2020.02.18.955385

Prado, V.F., Janickova, H., Al-Onaizi, M.A., Prado, M.A.M., 2017. Cholinergic circuits in cognitive flexibility. Neuroscience 345, 130–141. https://doi.org/10.1016/j.neuroscience.2016.09.013

Ragozzino, M.E., Mohler, E.G., Prior, M., Palencia, C.A., Rozman, S., 2009. Acetylcholine activity in selective striatal regions supports behavioral flexibility. Neurobiol. Learn. Mem. 91, 13–22. https://doi.org/10.1016/j.nlm.2008.09.008

Redgrave, P., Prescott, T.J., Gurney, K., 1999. The basal ganglia: a vertebrate solution to the selection problem? Neuroscience 89, 1009–1023. https://doi.org/10.1016/S0306-4522(98)00319-4

Roesch, M.R., Calu, D.J., Schoenbaum, G., 2007. Dopamine neurons encode the better option in rats deciding between differently delayed or sized rewards. Nat. Neurosci. 10, 1615–1624. https://doi.org/10.1038/nn2013

Sakae, D.Y., Marti, F., Lecca, S., Vorspan, F., Martín-García, E., Morel, L.J., Henrion, A., Gutiérrez-Cuesta, J., Besnard, A., Heck, N., Herzog, E., Bolte, S., Prado, V.F., Prado, M.A.M., Bellivier, F., Eap, C.B., Crettol, S., Vanhoutte, P., Caboche, J., Gratton, A., Moquin, L., Giros, B., Maldonado, R., Daumas, S., Mameli, M., Jamain, S., El Mestikawy, S., 2015. The absence of VGLUT3 predisposes to cocaine abuse by increasing dopamine and glutamate signaling in the nucleus accumbens. Mol. Psychiatry 20, 1448–1459. https://doi.org/10.1038/mp.2015.104

Salinas-Hernández, X.I., Vogel, P., Betz, S., Kalisch, R., Sigurdsson, T., Duvarci, S., n.d. Dopamine neurons drive fear extinction learning by signaling the omission of expected aversive outcomes. eLife 7. https://doi.org/10.7554/eLife.38818

Saunders, A., Oldenburg, I.A., Berezovskii, V.K., Johnson, C.A., Kingery, N.D., Elliott, H.L., Xie, T., Gerfen, C.R., Sabatini, B.L., 2015. A direct GABAergic output from the basal ganglia to frontal cortex. Nature 521, 85–89. https://doi.org/10.1038/nature14179

Saunders, B.T., Richard, J.M., Margolis, E.B., Janak, P.H., 2018. Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. Nat. Neurosci. 21, 1072–1083. https://doi.org/10.1038/s41593-018-0191-4

Scott, W.A., 1962. Cognitive Complexity and Cognitive Flexibility. Sociometry 25, 405–414. https://doi.org/10.2307/2785779

Shams, W.M., Sanio, C., Quinlan, M.G., Brake, W.G., 2016. 17β-Estradiol infusions into the dorsal striatum rapidly increase dorsal striatal dopamine release in vivo. Neuroscience 330, 162–170. https://doi.org/10.1016/j.neuroscience.2016.05.049

Shen, W., Plotkin, J.L., Francardo, V., Ko, W.K.D., Xie, Z., Li, Q., Fieblinger, T., Wess, J., Neubig, R.R., Lindsley, C.W., Conn, P.J., Greengard, P., Bezard, E., Cenci, M.A., Surmeier, D.J., 2015. M4 muscarinic receptor signaling ameliorates striatal plasticity deficits in models of L-DOPA-induced dyskinesia. Neuron 88, 762–773. https://doi.org/10.1016/j.neuron.2015.10.039

Shin, J.H., Adrover, M.F., Wess, J., Alvarez, V.A., 2015. Muscarinic regulation of dopamine and glutamate transmission in the nucleus accumbens. Proc. Natl. Acad. Sci. U. S. A. 112, 8124–8129. https://doi.org/10.1073/pnas.1508846112

Shin, J.H., Kim, D., Jung, M.W., 2018. Differential coding of reward and movement information in the dorsomedial striatal direct and indirect pathways. Nat. Commun. 9, 404. https://doi.org/10.1038/s41467-017-02817-1

Schoenberg, H.L., Sola, E.X., Seyller, E., Kelberman, M., Toufexis, D.J., 2019. Female rats express habitual behavior earlier in operant training than males. Behav. Neurosci. 133, 110–120. https://doi.org/10.1037/bne0000282

Smith, Y., Raju, D.V., Pare, J.-F., Sidibe, M., 2004. The thalamostriatal system: a highly specific network of the basal ganglia circuitry. Trends Neurosci. 27, 520–527. https://doi.org/10.1016/j.tins.2004.07.004

Tritsch, N.X., Sabatini, B.L., 2012. Dopaminergic modulation of synaptic transmission in cortex and striatum. Neuron 76, 33–50. https://doi.org/10.1016/j.neuron.2012.09.023

Tzavos, A., Jih, J., Ragozzino, M.E., 2004. Differential effects of M1 muscarinic receptor blockade and nicotinic receptor blockade in the dorsomedial striatum on response reversal learning. Behav. Brain Res. 154, 245–253. https://doi.org/10.1016/j.bbr.2004.02.011

Vilaró, M.T., Mengod, G., Palacios, J.M., 1993. Advances and limitations of the molecular neuroanatomy of cholinergic receptors: the example of multiple muscarinic receptors. Prog. Brain Res. 98, 95–101. https://doi.org/10.1016/s0079-6123(08)62385-7

Vorhees, C.V., Williams, M.T., 2006. Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat. Protoc. 1, 848–858. https://doi.org/10.1038/nprot.2006.116

Wess, J., Eglen, R., Gautam, D., 2007. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat. Rev. Drug Discov. https://doi.org/10.1038/nrd2379

Wilson, C.J., 2006. Striatal D2 Receptors and LTD: Yes, but Not Where You Thought They Were. Neuron 50, 347–348. https://doi.org/10.1016/j.neuron.2006.04.023

Wu, Y., Richard, S., Parent, A., 2000. The organization of the striatal output system: a single-cell juxtacellular labeling study in the rat. Neurosci. Res. 38, 49–62. https://doi.org/10.1016/s0168-0102(00)00140-1

Yan, Z., Surmeier, D.J., 1996. Muscarinic (m2/m4) receptors reduce N- and P-type Ca2+ currents in rat neostriatal cholinergic interneurons through a fast, membrane- delimited, G-protein pathway. J. Neurosci. 16, 2592–2604. https://doi.org/10.1523/JNEUROSCI.16-08-02592.1996

Zhou, F.-M., Wilson, C.J., Dani, J.A., 2002. Cholinergic interneuron characteristics and nicotinic properties in the striatum. J. Neurobiol. 53, 590–605. https://doi.org/10.1002/neu.10150

Ztaou, S., Maurice, N., Camon, J., Guiraudie-Capraz, G., Kerkerian-Le Goff, L., Beurrier, C., Liberge, M., Amalric, M., 2016. Involvement of Striatal Cholinergic Interneurons and M1 and M4 Muscarinic Receptors in Motor Symptoms of Parkinson's Disease. J. Neurosci. Off. J. Soc. Neurosci. 36, 9161–9172. https://doi.org/10.1523/JNEUROSCI.0873-16.2016

Book sources:

Spiro, R.J., And Others, 1988. Cognitive Flexibility Theory: Advanced Knowledge Acquisition in Ill-Structured Domains. Technical Report No. 441.

Appendix

Attachment 1. Representative version of the code used for the operant box experiments

```
" \ 30min aquisition
\ reward with left lever
\ session time = 30 min



\inputs
^leftlever = 1
^rightlever = 2



\outputs
^reinforcer = 3 \pellet dispenser
^HouseLight = 7
^leftstimuluslight = 4
^rightstimuluslight = 5
```

VAR_ALIAS Session time = M \default = 30 minute

VAR_ALIAS maximum number of reinforcers = N \ Default = 50

\variables

\ M = session time

\ F = fixed ratio

\ N = max of reinforcers

\ R = reinforcer counter

\ S = session timer

DIM C = 999

DIM E = 999

LIST Z = 1, 5, 2, 4, 3

PRINTORIENTATION = LANDSCAPE

PRINTCOLUMNS = 5

PRINTOPTIONS = FULLHEADERS, FORMFEEDS

PRINTVARS = C, E, L, P, R

S.S.1,

S1,

 1": SET F = 1, M = 30, N = 50 ---> S2

S2,

  #START : ON ^houselight ---> S3

S3,

 #R^leftlever : Z1 ---> SX

S.S.2,

S1,

  1": RANDD F = Z; SHOW 5, VR =, F ---> S2

  #Z32: ---> S1

S2,

F#R^right lever: ON ^rightstimuluslight ---> S3

S3,

 1": OFF ^rightstimuluslight ---> S1

S.S.3,

S1,

 1": RANDD F = Z; SHOW 5, VR =, F ---> S2

 #Z32: ---> S1

S2,

F#R^leftlever: ON ^leftstimuluslight ---> S3

S3,

 1": OFF ^leftstimuluslight ---> S1

S.S.4,

S1,

 #START : SHOW 3, Left responses, L ---> S2

S2,

 #R^leftlever : ADD L; SHOW 3, Left Responses, L ---> SX

S.S.5,

S1,

 #START : SHOW 4, Right responses, P ---> S2

S2,

#R^rightlever: ADD P; SHOW 4, Right Responses, P ---> SX

S.S.6,

 \reinforcment timer

 \ Z1=input

S1,

 #Z1: ON ^Reinforcer; ADD R; SHOW 2, Reinforcers, R ---> S2

S2,

 2": OFF ^Reinforcer ---> S1

 S.S.7, \ Increment time (T) in 0.01 second intervals

S1,

 #START: ---> S2

S2,

  0.01": SET T = T + 0.01 ---> SX

  #Z32: ---> S1

S.S.8, \Recording IRT's left

S1,

  #START: ---> S2

S2,

  #R^Leftlever: IF I > 999 [@arrayfull, @continue]

           @full: ---> S1

           @cont: SET C(I)=T, T=0; ADD I;

          IF I> 999 [@array, @sealarray]

            @full: ---> S1

            @seal: SET C(I) = -987.987 ---> SX

  #Z32: ---> S1

  S.S.9, \Recording IRT's right

S1,

  #START: ---> S2

S2,

  #R^rightlever: IF K > 999 [@arrayfull, @continue]

           @full: ---> S1

           @cont: SET E(K)=T, T=0; ADD K;

          IF K> 999 [@array, @sealarray]

            @full: ---> S1

            @seal: SET E(K) = -987.987 ---> SX

  #Z32: ---> S1

S.S.10, \session timer & max reinforcer limiter

S1,

 #START: SHOW 1, Session minutes, S/60 ---> S2

S2,

 0.01": SET S = S+0.01;

    SHOW 1, session minutes, S/60;

    IF (S/60 >= M) OR (R>=N) [@True, @false]

@true: Z32 ---> S3

@false:---> SX

S3,

2": ---> STOPSAVE   "