Universidade de Aveiro
Ano 2019

Departamento de Biologia

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DISSEÇÃO DE CIRCUITOS ESTRIATO-NIGROESTRIATAIS

DISSECTION OF STRIATO-NIGROSTRIATAL CIRCUITS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Professor Doutor Ingo Willuhn, Professor Associado do Departamento de Psiquiatria do Centro Médico Académico da Universidade de Amesterdão e sob a co-orientação do Doutor Rudolf Faust, Pós-doutorado do Instituto Holandês de Neurociências e da Professora Doutora Maria de Lourdes Gomes Pereira, Professora Associada com Agregacão do Departamento de Ciências Médicas da Universidade de Aveiro

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agradecimentos

O trabalho aqui apresentado só foi possível graças à boa vontade e cooperação de diversas personalidades. Assim, começo por agradecer à Prof. Dra. Maria de Lourdes Pereira, por mais uma vez ter aceite ser minha orientadora e pelo seu acompanhamento e ajuda ao longo do ano. Seguidamente, agradeço ao Prof. Dr. Ingo Willuhn por me ter dado a oportunidade de realizar a minha tese de mestrado no seio do seu grupo de investigação. Agradeço ao Dr. Rudolf Faust por todos os conhecimentos que me transmitiu ao longo deste ano que trabalhámos juntos, assim como por toda a sua ajuda, paciência e disponibilidade. Agradeço, também, ao Ralph Hamelink e à Dra. Nicole Yee pela sua ajuda, em especial, com os elétrodos, perfusões e imunohistoquímica, ao Dr. Joop van Heerikhuize pela ajuda com o microscópio e análise de dados e à Elizabeth Godynyuk pela sua ajuda na minha integração no projeto desenvolvido. Deixo, também um agradecimento ao Dr. Marko Popovic e à Dra. Elena de Miguel, ambos da Universidade Vrije centro médico (VUmc), pela ajuda na análise de imagens. De modo geral, agradeço a todos os membros do grupo de Neuromodulação e Comportamento pela ótima receção e simpatia e por todos os momentos que partilhámos em conjunto. Agradeço muito aos meus pais por todo o apoio financeiro e emocional que me deram e que foi crucial, não só para tornar este ano fora de Portugal possível como, também, mais fácil e agradável. Por último, agradeço aos meus amigos, em especial à Ana Filipa Costa e Maria Helena Torres.

palavras-chave

Vício, Dopamina, Gânglios Basais, Circuito Estriato-Nigroestriatal, Toxina da Cólera b

resumo

A dependência de drogas é considerada uma doença neuropsiquiátrica que afeta cerca de 5.5% da população adulta global. O consumo de drogas, por norma, inicia-se tendo por base o alcance de um determinado objetivo (comportamento motivado), embora se possa tornar num comportamento habitual. Esta troca de comportamentos ocorre através de uma mudança gradual de controlo desde estriado ventral (ou nucleus accumbens; região limbíca), passando para o estriado dorsomedial (região associativa) e terminando no estriado dorsolateral (região sensório-motora), por meio do mesencéfalo. A descoberta deste circuito de passagem de informação foi realizada por Haber e colegas, quando analisaram estudos efetuados em primatas, pelo que pode ser designado de modelo de Haber. Contudo, ainda não está bem elucidado de que forma funciona este circuito. De modo a desvendar esse mistério foram realizadas cirurgias esterotáxicas, em ratos do tipo selvagem, com diferentes marcadores neuroanatómicos, tendo por base alguns diagramas experimentais. Fizeram, ainda, parte dos métodos utilizados, a marcação de células dopaminérgicas por imunohistoquímica, a visualização e obtenção dos resultados por microscopia de fluorescência e análise dos dados com utilização do programa FIJI. Os resultados obtidos mostraram que as projeções nigroestriatais para diferentes domínios funcionais do estriado são originadas de populações de neurónios dopaminérgicos distintas e as projeções estriatonigrais de diferentes domínios funcionais inervam regiões segregadas do mesencéfalo. Concluindo, vimos com este trabalho que existe a troca de informações, entre as três regiões estriatais referidas, através de áreas dopaminérgicas distintas.

keywords Addiction, Dopamine, Basal Ganglia, Striato-Nigrostriatal Circuit, Cholera Toxin b

abstract Drug addiction is considered a neuropsychiatric disease, affecting about 5.5% of the global adult population. Drug use usually begins based on the achievement of a particular goal (motivated behavior) but eventually may become habitual behavior. This change of behavior occurs through a gradual shift of control from the ventral striatum (or nucleus accumbens; limbic region), to the dorsomedial striatum (associative region) and ending in the dorsolateral striatum (sensorimotor region), through the midbrain. The discovery of this information passing circuit was made by Haber and colleagues, when they analyzed studies performed in primates, and may therefore be called the Haber model. However, it is not yet well understood how this circuit works. In order to unravel this mystery, stereotaxic surgeries were performed in wild-type rats, with different neuroanatomic tracers, based on some experimental diagrams. Also, part of the methods used consisted in the labelling of dopaminergic cells by immunohistochemistry, visualization and obtaining of results by fluorescence microscopy, and data analysis using FIJI program. The results showed that the nigrostriatal projections for different functional striatal domains originate from distinct dopaminergic neuron populations and the striatonigral projections of different functional domains innervate segregated midbrain regions. In conclusion, we have seen from this work that there is an exchange of information between the three striatal regions mentioned, through distinct dopaminergic areas.

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Abbreviations

AAV5 – adeno-associated virus serotype 5

aDLS – anterior dorsolateral striatum

BG - basal ganglia

CTb - cholera toxin b

DA – dopamine

DLS - dorsolateral striatum

DMS - dorsomedial striatum

EGFP – enhanced green fluorescent protein

GP - globus pallidus

GPe - external segment of the globus pallidus

GPi - internal segment of the globus pallidus

NAc - nucleus accumbens

pDMS – posterior dorsomedial striatum

PFC - prefrontal cortex

SN - substantia nigra

SNc - substantia nigra pars compacta

SNr - substancia nigra pars reticulata

SNS - striato-nigrostriatal circuit

STN - subthalamic nucleus

TH – tyrosine hydroxylase

VMS - ventromedial striatum

VTA - ventral tegmental area

1. Introduction

1.1. Drug addiction

Addiction, as described by the American Psychiatric Association, is a complex condition considered as a brain disease that manifests as compulsive substance use despite harmful consequences. Addicted people, who are characterized by severe substance use disorder, have a strong desire to use a certain substance(s) until the point that it takes over their life (Parekh, 2017).

According with the World Drug Report 2019 it is estimated that 35 million people around the world suffer from drug use disorders. Data from 2017 estimate that 271 million people, or 5.5% of the global adult population (between 15 and 64 years), used drugs of abuse in the previous year. Despite the fact that this estimate is similar to the one from 2016, a long-term view reveals that the number of people using drugs has increased by 30% compared to 2009 (Figure 1). Among all of the variety of drugs available, opioids remain the most dangerous, being responsible for two thirds of the 585 thousand deaths as a result of drug use in 2017 (UNODC, 2019).

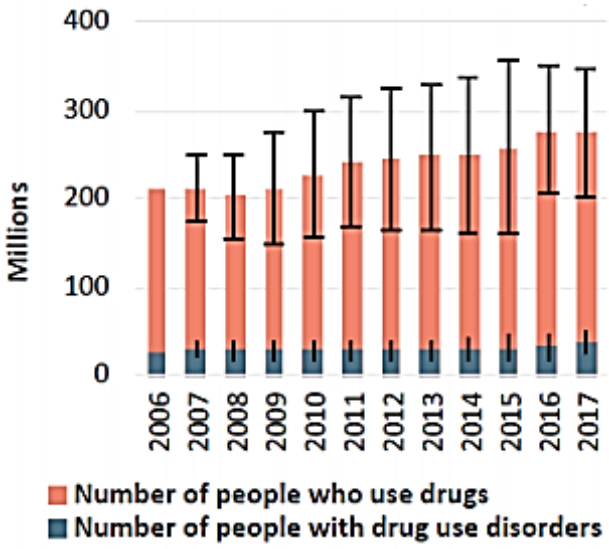


Figure 1 – Global trends in estimated numbers of people who used drugs and those with drug use disorders, between 2006 and 2017 (Taken from World Drug Report, 2019).

Drugs of abuse interact with various cerebral areas, especially with the ones related to the reward system. Immediate effects resulting from the action of these drugs induce long lasting changes in neuroplasticity. In this context, the designation *drug* applies to any substance that modifies, increases, inhibits or reinforces physiological and psychological functions of the organism in transitory or permanent ways. Despite having different effects, the use of these substances causes, generally, adaptive changes in the nervous system, which lead to tolerance, physical and/or psychological dependence and relapse in case of withdrawal. Usually, the initial consumption of drugs is performed to induce pleasurable feelings, relieve stress or depression, and/or increase cognitive or athletic performance. Over time, the addiction manifests itself through signals and symptoms as drug tolerance, consumption for relief of abstinence syndrome, loss of control over drug consumption, abandonment of usual activities, neglect of responsibilities and consumption of drugs even though it has negative consequences. These behavioral manifestations occur from modifications in cerebral areas associated with judgement, decision-making, memory, learning and behavioral control (Fardilha and Ferreira-Fernandes, 2016).

One of the neurotransmitters involved in this process is dopamine (DA). Its neurotransmission in areas such as the core of the nucleus accumbens (NAc core) and the dorsomedial striatum (DMS), among others, is responsible for goal-directed behaviors, which are associated with natural rewards. However, when a behavior becomes well established as a stimulus-response habit, its control is dependent instead upon the dorsolateral striatum (DLS) and its dopaminergic innervation from the substantia nigra pars compacta (SNc), among other areas. Thus, behaviors such as cocaine seeking and alcohol drinking, which in the beginning are sensitive to outcome devaluation, become immutable after a long period of drug taking. Notwithstanding, this shift from the DMS to DLS, when happens the transition between goal-directed and habitual control, should not be considered alone, but instead be integrated into a striatal circuit, based on a shift from the ventral to the dorsal striatum, like several groups already suggested (Nauta et al, 1978; Haber et al, 2000; Belin et al, 2013).

1.2. Dopamine (DA) and its pathways

1.2.1. DA synthesis

Commonly denominated DA, the chemical name of this decarboxylated amine is 3,4-dihydroxyphenylethylamine or, also known as 3-hydroxytyramine. Its synthesis proceeds from L-dopa, which is an abbreviation of L-3,4-dihydroxyphenylalanine, by the enzyme L-dopa decarboxylase. The synthesis of L-dopa occurs through the hydroxylation of tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) (Figure 2) (Fahn, 2008).

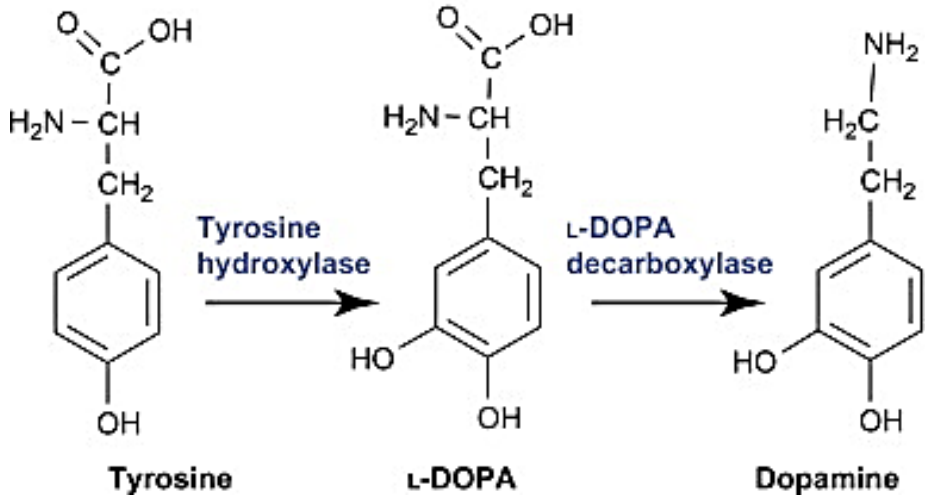


Figure 2 – Steps for the synthesis of DA. First, the amino acid tyrosine is transformed into L-dopa by the enzyme TH, followed by the transformation of L-dopa into DA by the enzyme L-dopa decarboxylase (Taken from Feher, 2012).

1.2.2. DA receptors and the dopaminergic pathways

In order to produce effects, DA has to bind to its receptors, which are located at the plasma membrane of respective target cells, both in peripheral and central nervous systems. Those receptors belong to the superfamily of G-protein coupled receptors, and five different subtypes have been described so far: D1, D2, D3, D4, and D5. All of them are metabotropic, leading to the formation of second messengers, which trigger or block the activation of specific cell signaling pathways (Baik, 2013). D1 and D2 are the most

abundantly expressed in the brain (D1 being the highest), and the two are rarely co-expressed in the same cells (Missale et al, 1998; Klein et al, 2019). This neurotransmitter is essential for daily brain functions such as reward, cognition and motor control, which are associated with the mesolimbic, mesocortical and nigrostriatal pathways, respectively. Besides these three pathways, there is, also, the tuberoinfundibular pathway (Figure 3) (Iversen et al, 2010).

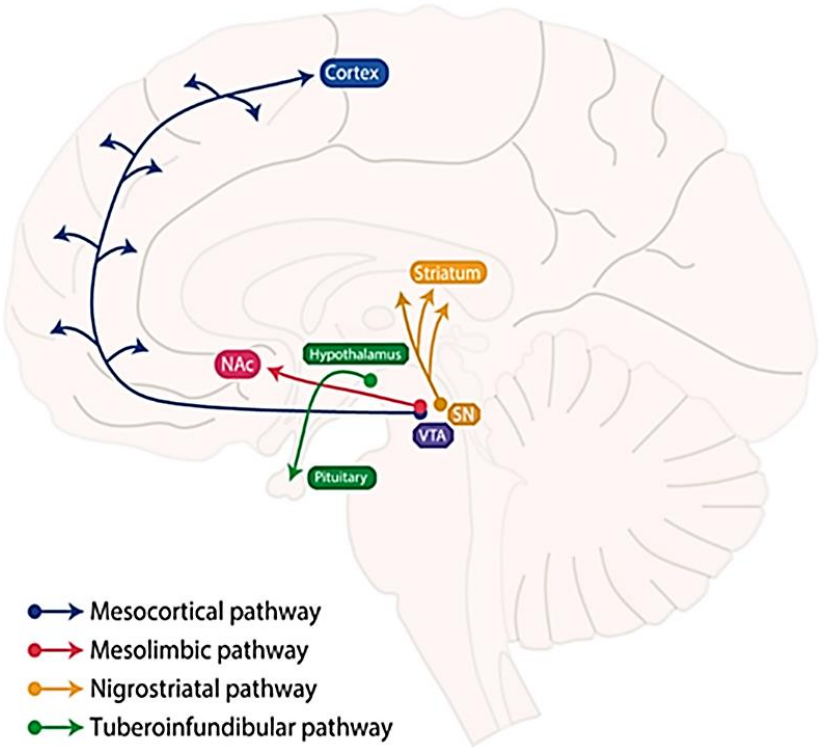


Figure 3 - Schematic of the right hemisphere (sagittal cut), showing the distribution of the four main dopaminergic pathways in the central nervous system. The VTA is the source of the mesocorticolimbic system: dopaminergic neurons project to cortex via mesocortical pathway (blue) and, to NAc via mesolimbic pathway (red). DA neurons in the SN project to the striatum and form the nigrostriatal pathway (yellow). The tuberoinfundibular pathway (green) is formed by dopaminergic neurons that project from hypothalamic nuclei (arcuate nucleus and periventricular nucleus) to the pituitary (Taken from Klein et al, 2019). Abbreviations correspond to: VTA – ventral tegmental area; NAc – nucleus accumbens; SN – substancia nigra.

- **The mesolimbic and mesocortical pathways (mesolimbocortical system)**

As previously mentioned, drugs of abuse interact mostly with the reward system, which is constituted by two principal neural pathways. The best described and characterized is the mesolimbic pathway, which includes the dopaminergic neurons of the ventral tegmental area (VTA) that project towards the NAc or ventromedial striatum (VMS), through the medial bundle of the forebrain (Figure 3). In addition, there are also minority dopaminergic projections from the VTA to the olfactory tubercle, amygdala and hippocampus. This pathway is important for the recognition of rewards and initiation of their consumption, and also responds to aversive stimuli. The other pathway is the mesocortical pathway, which also originates in the VTA but projects to the prefrontal cortex (PFC) (Figure 3). Other projections from this pathway include the ones from the VTA to the cingulate gyrus and perirhinal cortex. These projections from the VTA to cortical areas influence cognitive control, motivation and emotional responses. Although, these pathways can be studied separately, their targets are widely interconnected, being frequently designated as mesolimbocortical system. Dysregulation in these pathways have been implicated in pathological conditions such as schizophrenia and addiction (Fardilha and Ferreira-Fernandes, 2016; Luo, 2012).

Studies regarding the mesolimbic pathway showed that depletion of DA in the NAc induced by local 6-hydroxydopamine injections severely attenuates the rewarding effects of cocaine or amphetamine (Lyness et al, 1979). However, it is the shell portion of the accumbens, instead of the core, that seems to be more important for drug reward. For instance, rats learn to self-administer psychomotor stimulants into the accumbens shell or olfactory tubercle, but not the core (Carlezon et al, 1995; Ikemoto, 2007). In addition, lesions of DA terminals in the shell attenuated experiments of conditioned place preference induced by intravenous administration of cocaine or amphetamine (Sellings and Clarke, 2003). The VTA is also involved, at least partly, in mediating the rewarding effects of drugs, as showed by selective lesions in VTA DA neurons projecting to the ventral striatum, which attenuated the effects of intravenous self-administration of nicotine in rats (Corrigall et al, 1992). The studies also demonstrated that the posterior part of the VTA plays a more important role than the anterior one. In this way, the pattern of drug-reward

trigger zones appears to be explained by cellular connectivity between the ventral midbrain and the ventral striatum. Previous reports indicated that the dopaminergic neurons in the VTA project into the NAc with mediolateral topography (Fallon and Moore, 1978; Nauta et al, 1978). Therefore, the medial accumbens shell (drug-reward trigger zone in the striatum) receive strong dopaminergic innervation from the posteromedial VTA (drug-reward trigger zone in the ventral midbrain), but little innervation from anteromedial or lateral VTA (Ikemoto, 2007).

- **The nigrostriatal pathway**

In the nigrostriatal pathway, the substantia nigra (SN), which is located within the midbrain posterior to the crus cerebri fibers of the cerebral peduncle, is functionally and morphologically divided into two regions; the SNc that contains the dopaminergic neurons that project to the dorsal striatum (Figure 3), formed by the caudate nucleus and putamen neurons, and the pars reticulata (SNr), with inhibitor GABAergic neurons (Fabbri et al, 2017). This pathway is involved in the regulation of voluntary movement and its degeneration causes Parkinson's disease in humans (Luo, 2012).

In a study performed by Deniau et al, (1996) it was shown that projections from different regions of the striatum correspond to as many segregated areas in the SNr. The resulting striatonigral pathway presents a nigral mosaic that seems to be organized in curved layers centered on projections coming from the striatal territory, which means that exists a precise segregation in these projections. This concept was already previously accepted since it was known that the flow of cortical information through the basal ganglia (BG) is processed along segregated paths (Donoghue and Herkenham, 1986). Regarding the spatial distribution of the nigrostriatal neurons it is known that neurons innervating the caudate nucleus and putamen are distributed throughout the mediolateral extent of the SNc, while the ones innervating the NAc lie in the medial SNc and VTA. Laterally in the SNc, the nigrostriatal cells are mixed with the dopaminergic cells that project towards the amygdala (Maurin et al, 1999). The first contributions about the organization of the nigrostriatal pathway, were from Lynd-Balta and Haber (1994). Based on observations from retrograde axonal tracing, from primate´s brain, they distinguished two main populations

of nigrostriatal neurons, located in the ventral and dorsal tiers of the SN, respectively. The neurons from the ventral tier were mainly found to project into the sensorimotor region of the striatum, while neurons located in both the ventral and dorsal tiers mainly innervated the limbic and associative regions of the striatum. The current question is whether segregated populations of the SNc DA neurons project to different parts of the dorsal striatum.

1.3. The basal ganglia (BG) system

The BG system is a group of nuclei in the brain, formed by neurons that interconnect with the cerebral cortex, thalamus and brainstem. Although this system is best known for motor functions, nowadays, it is also recognized to mediate the emotions, motivation and cognition that drive voluntary movement (Iversen et al, 2010). The components of this system include the striatum (caudate-putamen and NAc), the internal and external segment of the globus pallidus (GP), the subthalamic nucleus (STN), and the SN (Figure 4) (Gerfen and Bolam, 2010).

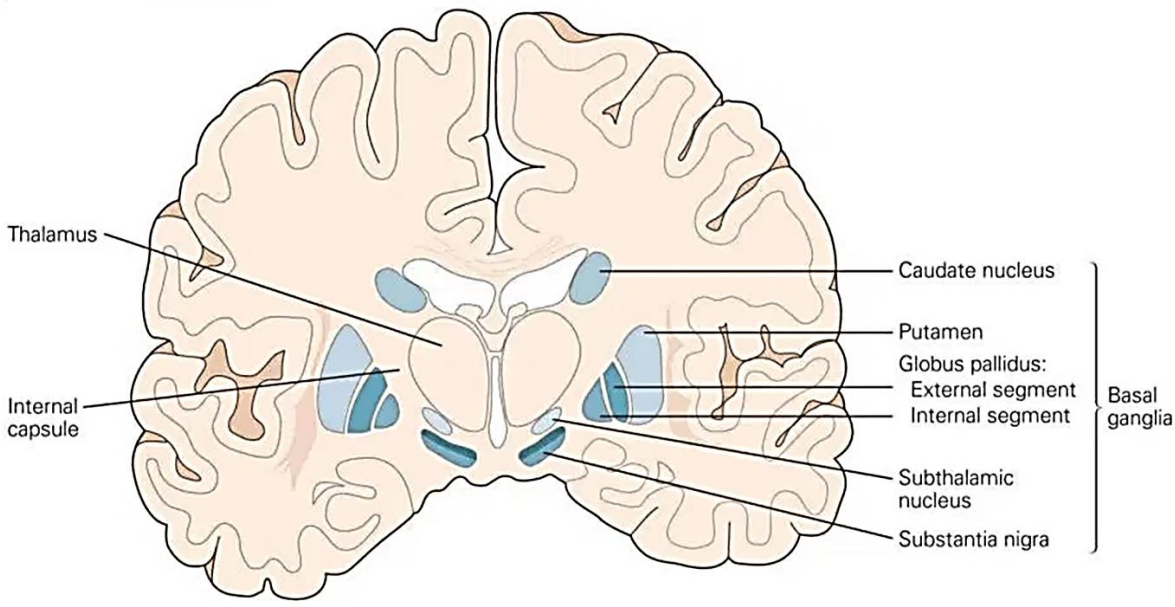


Figure 4 - Schematic of a coronal section representing most of the constituents of the BG system (Taken from Nieuwenhuys et al, 2007).

The striatum is the main input structure of the BG, which in its turn receives inputs from cortical neurons, mostly located in layer 5, and in some cases layer 3. All corticostriatal neurons are pyramidal and utilize glutamate, which is an excitatory neurotransmitter. Except for the part of the medial accumbens shell and the olfactory tubercle, all regions of the striatum as well as some striatal interneurons receive cortical input. The medium-sized spiny GABAergic projection neurons are inhibitory and account for about 95% of neurons in the striatum. They are divided into two types, the direct and indirect striatal projections (Figure 5) (Gerfen and Bolam, 2010).

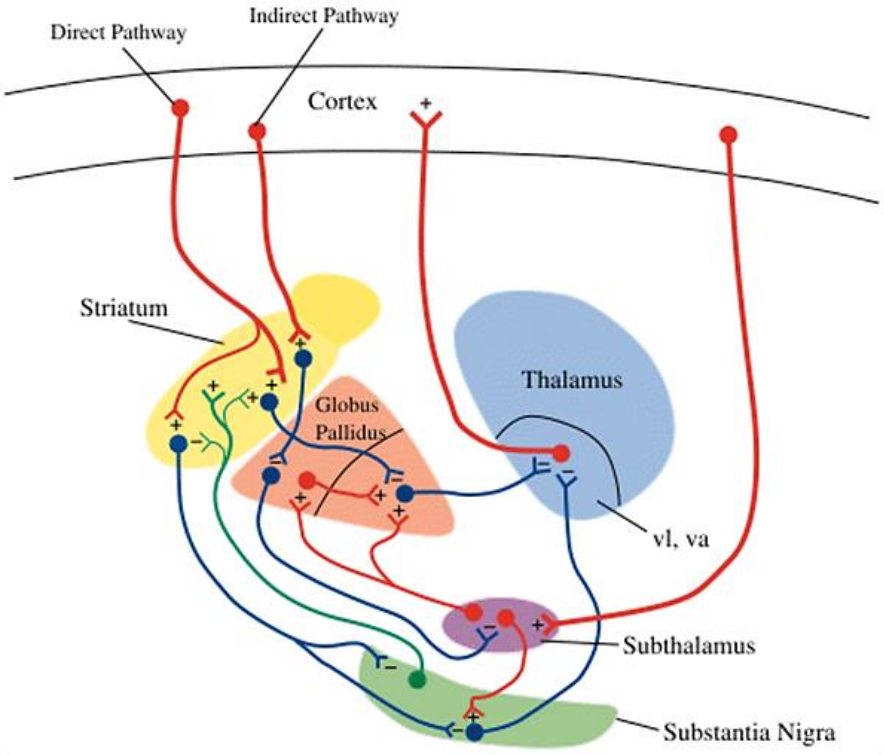


Figure 5 - Schematic of the direct and indirect pathways, and their targets (Taken from HumanPhysiology.Academy, 2015).

The direct projections provide direct inputs to the output neurons of the BG, in the internal segment of the globus pallidus (GPi) and the SNr. Projection neurons in these regions display a relatively high level of tonic activity, which is inhibited when the striatonigral pathway is activated. Contrarily, the indirect projections provide inputs to the external segment of the globus pallidus (GPe), which together with the STN compose the

major components of the indirect BG circuit. GABAergic neurons in the GPe project back to the striatum, to the output neurons of the BG (GPi and SNr) and to the STN. Thus, cortical excitation of this pathway inhibits the GABAergic pallidal output, resulting in disinhibition of the output neurons of the BG and the STN. This last one, which itself receives excitatory inputs from the cortex, provides excitatory projections, also, to the output neurons of the BG (Gerfen and Bolam, 2010).

1.4. Breaking into the striato-nigrostriatal (SNS) circuit

For some years several groups of researchers have been trying to decipher the functional organization of the BG system. The reasons for such a slow progress are due to the great complexity of this system, the difficulty to find suitable methods for its study and the contradictory results from different research groups (Hedreen and DeLong, 1991).

The story goes back to the early 1960s, when the first projections from the caudate nucleus and putamen neurons to both segments of the GP and to the SN, and from SNc neurons back to the striatum were established with certainty (Voneida, 1960; Szabo, 1962; Anden et al, 1964). Nonetheless, due to the techniques available at that time it was not possible to demonstrate the striatofugal or nigrostriatal axons and terminal fields. Even with the development of new methods, such as anterograde and retrograde axonal tracers, horseradish peroxidase or lectins, there were still questions about the topographic organization of these projections (Hedreen and DeLong, 1991). Only after more than a decade, already in the late 1970s, a bunch of studies, performed in rats, concluded that the VMS neurons, which receive dopaminergic afferents from the VTA, send axons to the SN, which in its turn, provides a dopaminergic projection to the dorsal striatum (Nauta et al, 1978; Fallon and Moore, 1978; Van der Kooy, 1979; Beckstead et al, 1979). Thus, in the 1980s, it was proposed the hypothesis that functional subdivisions within the striatum, created by projections from different cortical regions, are integrated in segregated cortico-basal ganglia-thalamocortical circuit and are organized in a parallel manner (DeLong and Georgopoulos, 1981; Alexander et al, 1986).

In order to test the proposed hypothesis, Haber and colleagues (2000) analyzed a collection of retrograde and anterograde tracing studies, in primates. By looking at the big picture, they were able to see that the VMS projects to a wide range of DA cells, in the midbrain and receives a relatively small DA input. On the other hand, the DLS is affected by a relatively large midbrain area, while influencing a limited one. In addition, the central striatum receives input from and projects to a relatively wide area of the midbrain. These results showed that exists an interface between different striatal regions via the midbrain DA cells, which forms an ascending spiral between regions. The basic mechanism of operation is that the shell influences the core, the core influences the central striatum, and this last one influences the DLS. This aspect creates a hierarchy of information flow that provides an anatomical basis for the limbic/cognitive/motor interface via the ventral midbrain (Figure 6) (Haber et al, 2000).

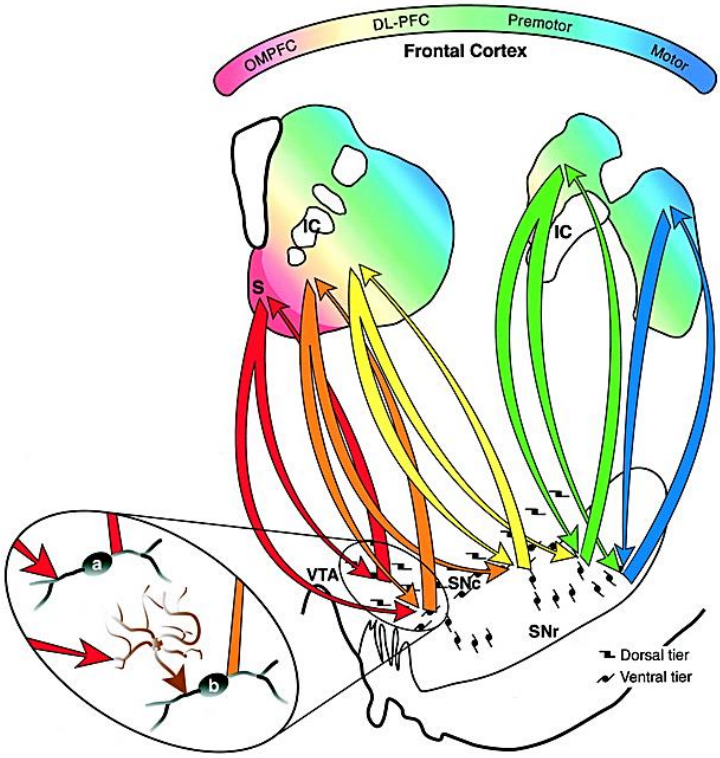


Figure 6 - Schematic of SNS circuit. The colored gradient shows the organization of functional corticostriatal inputs (red = limbic, green = associative, blue = motor), from rostral to caudal. In the NAc (ventral striatum), the shell receives forebrain input especially from the amygdala and hippocampus, whereas the core receives input from the entire OMPFC. The dorsolateral prefrontal cortex projects to the DMS, and premotor and

motor cortex projects to the DLS. Projections from the shell target both the VTA and ventromedial SNc (red arrows), while midbrain projections from the VTA to the shell form a "closed," reciprocal SNS loop (red arrow). From the medial SN are sent projections to the core forming the first part of a spiral (orange arrow). The spiral continues through the SNS circuit (yellow and green arrows) with pathways originating in the core and projecting more dorsally (blue arrows). A hypothetical model of the synaptic interactions of SNS projections in reciprocal versus feedforward loops is shown in the magnified oval region. The reciprocal component (red arrows) of each limb of the SNS projection terminates directly (a) on a dopamine cell, resulting in inhibition. The nonreciprocal, or feedforward, component (orange arrow) terminates indirectly (b) on a dopamine cell via a GABAergic interneuron (brown cell), resulting in disinhibition and facilitation of dopaminergic cell burst firing. (Taken from Haber et al, 2000). Abbreviations correspond to: DL-PFC - Dorsolateral prefrontal cortex; IC - internal capsule; OMPFC - orbital and medial prefrontal cortex; S - shell; SNc - substantia nigra, pars compacta; SNr - substantia nigra, pars reticulata; VTA - ventral tegmental area.

Even after all of these insights about the SNS circuit, there is still lack of information regarding the position of specific subpopulations of mesencephalic dopaminergic neurons (Wouterlood et al, 2018). In this respect, Somogyi and colleagues, (1981) presented an interesting study in which they used transmission electron microscopy and demonstrated, for the first time that exists synaptic contacts between terminals of ventral striatal efferents and dendrites of nigral neurons projecting to the dorsal striatum. More recently, with an optogenetic approach in ex vivo slice preparations, Xia et al, (2011) found evidence that non-dopaminergic VTA neurons might be involved in this circuitry, too. Nonetheless, further studies are necessary to fully understand how the SNS circuit function.

2. Aims

This project was based on the study performed by Haber et al, (2000).

- Because the mentioned group performed the experiments in primates, the first aim was to confirm the existence of SNS circuit in rats. To this end, wild-type rats were used in the experiments;

- The second aim was to clarify that the nigrostriatal projections to different functional domains of the striatum are originated from distinct populations of DA neurons. This aspect was assessed by the use of Cholera Toxin B conjugated with Alexa Fluor fluorescent dyes (CTb – A555 and CTb – A647), which is a retrograde axonal tracer, and labeled the neurons from the axon terminals present in the striatum until the neuron bodies present in the midbrain;

- The last aim was to elucidate if the striatonigral projection fields involved in the circuit innervate segregated regions of SNr or if they overlap. This aspect was assessed by the use of Adeno-Associated Virus serotype 5 conjugated with fluorescent proteins (AAV5 - EGFP and AAV5 – mCherry), which is an anterograde axonal tracer, and labeled the neurons from their neuron bodies present in the striatum until the axon terminals present in the SNr.

3. Materials and Methods

3.1. Animals

All procedures were conducted in accordance with governmental guidelines for care of laboratory animals and approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands. Adult female and male rats (Long Evans, 200-300g; bred-in-house and Janvier Labs, La Rochelle, France) were group-housed and maintained under a standard light/dark cycle, with food and water provided *al libitum*.

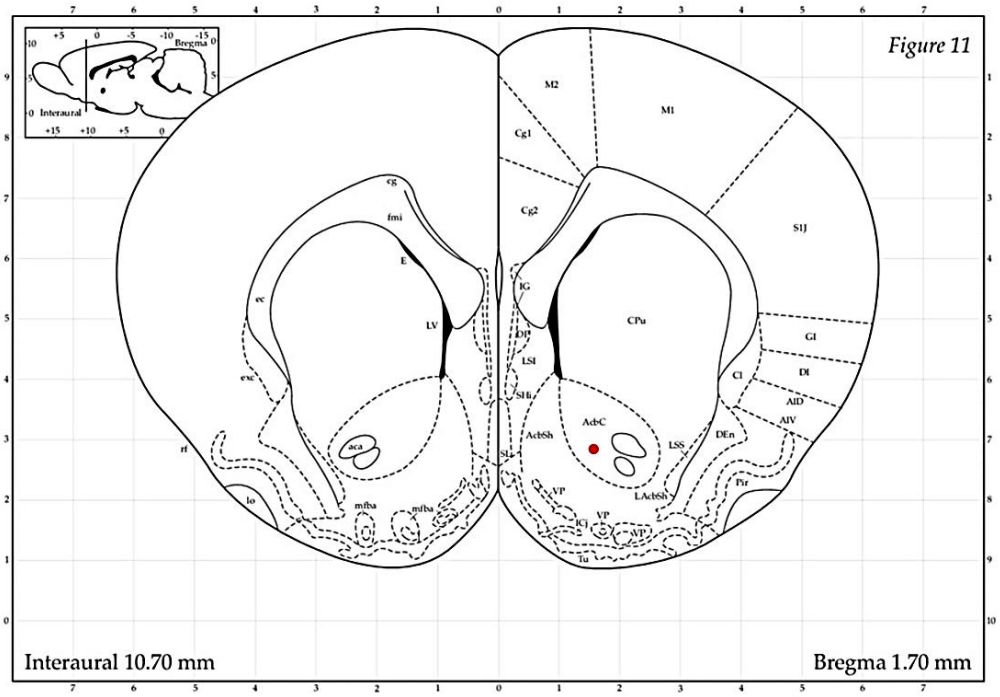
3.2. Surgical procedures

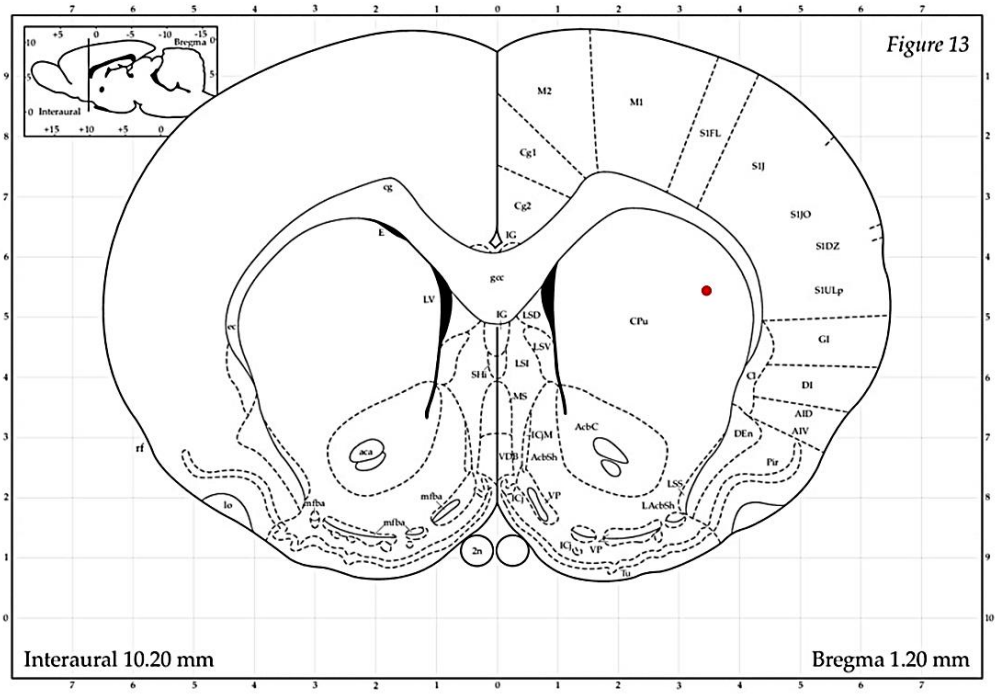
On the day of surgery, animals were first weighed, and then deeply anaesthetized, in an induction chamber, with 3% isoflurane-O_2 mixture. After stabilization, they were placed in a stereotaxic frame, with a continuous flow of 2.5-2.5% isoflurane-O_2 mixture during stereotaxic surgery (David Kopf Systems). Body temperature (36-37°C) was maintained with a homeothermic mat and local anesthesia (lidocaine 100 mg/ml) was applied after a midline skin incision was made and exposed the skull surface.

After proper head leveling, burr holes were made in the desired areas according with the coordinates from the rat brain atlas (6^{th} edition) by Paxinos and Watson (2007). Figure 7 and Table 1 present charts of injection sites and injection site coordinates, respectively. A Hamilton syringe (Hamilton Company, Reno, NV) controlled by a stereotactically mounted microdrive (WorldPrecision Instruments) was lowered into the holes at an approximate rate of 10 µm/sec and used to inject (in nL) the anterograde and retrograde tracers. Prior removing the needle, the tracers were allowed to diffuse for an additional 10 minutes.

After surgery, animals were injected with saline to prevent dehydration, and with Meloxicam to alleviate pain (1mg/kg). Each animal was allowed to recover in a heated

chamber until fully mobile and were then returned to social housing. During the next 2 days, animals were weighed and allowed to recover for at least 2 weeks before further experimentation.





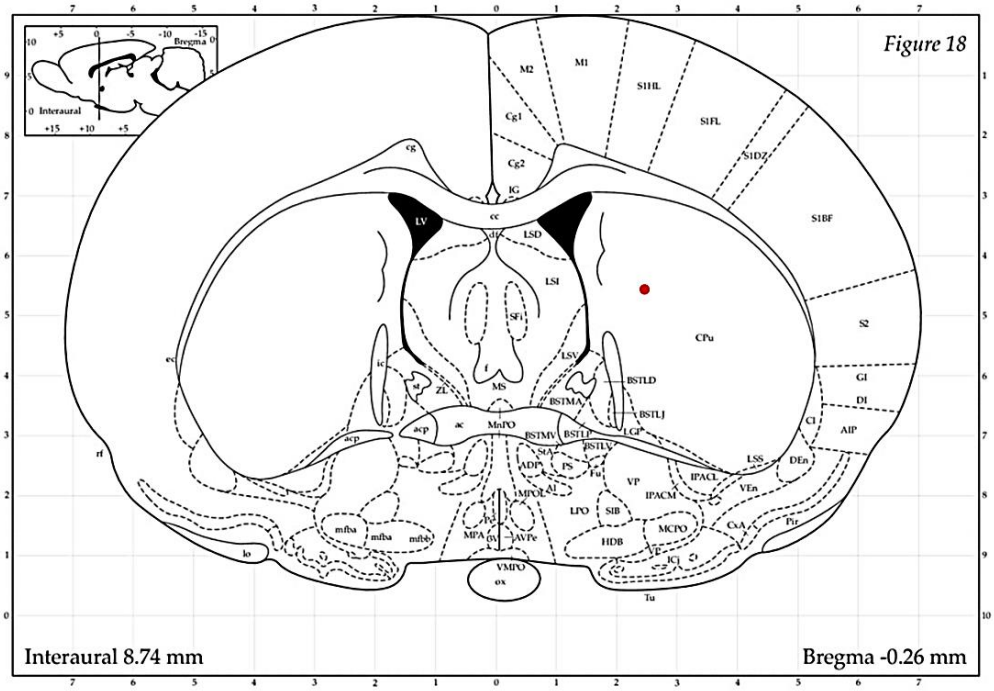


Figure 7 – Examples of chartings of the injection sites in the VMS (top), aDLS (middle) and pDMS (bottom). The red dot correspond to exact coordinates used, and even though it is only represented the injection sites in one hemisphere, the injections were bilateral (Taken from Rat Brain Atlas, a tool by Matt Gaidica, 2006).

Table 1 – Table with all coordinates of the injection sites, from bregma.

Striatal areas	Medial – Lateral (ML)	Anterior - Posterior (AP)	Dorsal – Ventral (DV)	Animals
VMS	+/- 1.6	1.8	-7.2	35663; 35664; 35665; 35667
aDLS	+/- 3.5 +/- 3.5 +/- 3.6	1.1 1.4 1.5	-4.6 -4.8 -5.0	12988 Janvier T 35666
pDMS	+/- 2.5 +/- 2.7 +/- 2.8	-0.2 -0.4 -0.4	-4.6 -4.8 -5.0	12988 Janvier T 35663; 35664; 35665; 35666; 35667

Abbreviations: VMS – ventromedial striatum; aDLS – anterior dorsolateral striatum; pDMS – posterior dorsomedial striatum.

3.3. Histological procedures

3.3.1. Fixation, sectioning and storage

Sixteen to forty-one days after the surgery, the subjects were weighed and prior to euthanasia, deeply anaesthetized, in an induction chamber, with 5% isoflurane-O_2 mixture, followed by an intraperitoneal injection of sodium pentobarbital (100mg/kg). Subsequently, animals were transcardially perfused with fresh solutions of PBS switching for 4% paraformaldehyde (PFA) in PBS, after the liver become white. The brains were subsequently removed and stored in at 4 degrees in 4% PFA. After a week, they were switched to 30% sucrose and, after infiltration with sucrose, they were snap frozen in isopentane. Coronal brain sections, 50µm thick, were cut using a cryostat (R1-156 3050, Leica Biosystems), and stored in free floating in a microplate, with 0.02% PBS azide.

3.3.2. Free-floating immunohistochemistry

Coronal slides from the striatum and the midbrain of animals injected with the anterograde tracer, AAV5-EGFP, were washed 3 times in PBS (10 min each wash) and blocked in a blocking solution (5% BSA, 5% NDS, 0.5% Triton-X 100 in PBS) for 1h, at RT. After, the sections were directly transferred and incubated overnight, at 4° C, in primary antibodies (1:1000 Chicken anti-TH and 1:1000 Rabbit anti-GFP), in antibody incubation buffer (5% BSA, 5% NDS, 0.1% Triton-X 100 in PBS). On the next day, sections were given three successive 10 min washes in PBS. Subsequently, they were moved onto the secondary antibodies (1:1000 Donkey anti-rabbit A488 and 1:1000 Donkey anti-chicken A594) in antibody incubation buffer (5% BSA, 5% NDS, 0.1% Triton-X 100 in PBS), where they were kept for another hour, at RT and with the wellplates covered with aluminum foil.

After this step, the sections were washed 3 times in PBS (10 min each wash), with DAPI added in the second wash (1:1000).

For the animals injected with the anterograde tracer, AAV5-mCherry, coronal slides from the striatum and the midbrain, were washed 3 times in PBS (10 min each wash) following a 4th wash of 10 min with 0.2% Triton-X 100 in PBS. Then, sections were blocked in a blocking solution (1% BSA, 5% NDS, 0.2% Triton-X 100 in PBS) for 1h, at RT. Thereafter, they were directly transferred and incubated overnight, at 4° C, in primary antibodies (1:1000 Mouse anti-TH; 1:1500 Chicken anti-GFP and 1:1000 Rabbit anti-RFP), in antibody incubation buffer (1% BSA, 5% NDS, 0.2% Triton-X 100 in PBS). On the next day, sections were given four successive 10 min washes in PBS. Posteriorly, they were moved onto the secondary antibodies (1:1000 Goat anti-mouse A532; 1:1000 Donkey anti-chicken A488 and 1:1000 Donkey anti-rabbit A594) in antibody incubation buffer (1% BSA, 5% NDS, 0.2% Triton-X 100 in PBS), where they were kept for another 1.5 hours, at RT and with the wellplates covered with aluminum foil. After this step, sections were, once again, washed 3 times in PBS for 15 min, with DAPI added in the second wash (1:1000). The last step was mounting the sections on slides, cover slipping them with mowiol, and sealing with nail polish.

Table 2 – Table with antibodies, reagents and tracers used and its source.

Name	Source
Chk anti-TH	Aves Labs (Davis, CA, USA)
Chk anti-GFP	Abcam (Cambridge, UK)
Rb anti-RFP	Rockland (Victoria, Canada)
Rb anti-GFP	Nacalai Tesque (Kyoto, Japan)
Gt anti-Ms A532; Dk anti-Chk A488; Dk anti-Rb A488	Life Tech (Waltham, Massachusetts, USA)

Dk anti-Chk A594; Dk anti-Rb A594; NDS	Jackson ImmunoResearch (Cambridgeshire, UK)
DAPI; BSA; Triton-X	Sigma (St Louis, MO, USA)
CTb - A555; CTb - A647	Invitrogen - Molecular probes (Waltham, Massachusetts, USA)
AAV5-EGFP; AAV5-mCherry	Vector (Malvern, PA, USA)

Abbreviations: Chk – chicken; TH – tyrosine hydroxylase; GFP – green fluorescent protein; Rb – rabbit; RFP – red fluorescent protein; Gt – goat; Ms – mouse; Dk – donkey; NDS – normal donkey serum; BSA – bovine serum albumin.

3.4. Imaging and data analysis

Striatal sections were imaged using a stereomicroscope (Leica Microsystems, M205 FA, Germany) and the consequent images were opened with the application Leica X (Las X software). For the midbrain, sections were imaged with a quantitative slide scanner (Vectra Polaris 1.0.9, PerkinElmer, USA). Each image contains seven different channels: DAPI, FITC, CY3, Texas Red, CY5, merged and autofluorescence. After a whole slide scan of the tissue, individual regions were selected for multispectral imaging (MSI), using Phenochart 1.0.10 (PerkinElmer, USA). The criteria used to choose the MSIs was the presence of retrogradely labelled cells and anterogradely labelled terminals from the two striatal regions injected. By the use of inForm 2.4.2 software (PerkinElmer, USA), it was performed the spectral separation of the collected images, which were then converted to TIF files.

The midbrain analysis consisted in verify the amount of overlapping area, in percentage (%), between the areas with retrogradely labelled cells from the striatum and TH positive cells. With the obtained data was even calculated (in the overlapping area) the % of retrogradely labelled cells that also contained TH, this is, the amount of cells that were both retrogradely labelled and dopaminergic. In addition, it was analyzed whether the retrogradely labelled cells or anterogradely labelled terminals (this last one was only performed in one set of experiments), from the striatum, correspond to the same or

segregated areas, in the midbrain. To do this, MSI files containing midbrain regions of interest were used, which were opened and analyzed with FIJI/ImageJ software. First, an individual threshold was set for each desired group of images; the images with TH positive cells and retrogradely labelled cells and/or anterogradely labelled terminals. The criteria used to set the threshold was selected by choosing an area with low signal, measuring the mean value and multiplying it by two, in order to avoid the background. The threshold was consistent for all of the images of each group and from the same animal. Despite the fact, that we used CTb as a retrograde tracer, we also observed anterograde labelling with it, and so, before setting the threshold in these images, the anterograde labelling was excluded. Next, the images with retrogradely labelled cells and/or anterogradely labelled terminals were superimposed with the images with TH positive cells, and the amount of overlapping area was calculated.

4. Results

Before starting with the results, three different theoretical brain circuit diagrams were designed for this project, which were the base for conducting the experiments presented here. Each diagram, represents the conjugation of toxin/virus and the targeted locations for the injections in the striatum (left side). The colored arrows show the direction of the axonal projections (anterograde or retrograde) labeled by the striatal injections (right side).

4.1. VMS (CTb – A647 + AAV5 – EGFP) and pDMS (CTb – A555)

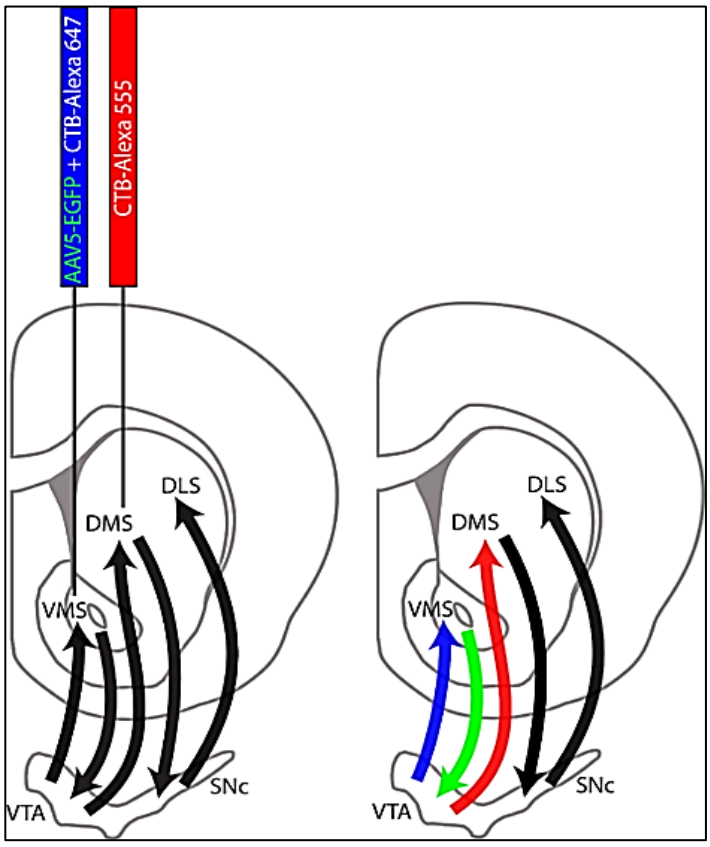


Figure 8 - Scheme of the injections on the VMS, with AAV5 – EGFP (anterograde direction) and CTb – A647 (retrograde direction), and on the pDMS, with CTb – A555 (retrograde direction). Abbreviations correspond to: VMS – ventromedial striatum; DMS – dorsomedial striatum; DLS – dorsolateral striatum; VTA – ventral tegmental area; SNc – substancia nigra pars compacta; AAV5 – EGFP – adeno-associated virus serotype 5 conjugated with enhanced green fluorescent protein; AAV5 – mCherry – adeno-associated virus serotype 5 conjugated with mCherry; CTb – Alexa 555 – cholera toxin b conjugated with alexa fluor 555; CTb – A647 – cholera toxin b conjugated with alexa fluor 647.

Figure 9 displays images of the injection sites in the VMS and pDMS, of the animal 35664. The VMS injection, with the anterograde tracer (AAV5 – EGFP), which is labelling cells, spread on the NAc and along the lateral ventricle (Figure 9 – left image), while the retrograde tracer (CTb – A647) is not visible due to lack of appropriate filter on the microscope used. In its turn, the pDMS injection, with the retrograde tracer (CTb – A555) is visibly on target (Figure 9 – right image).

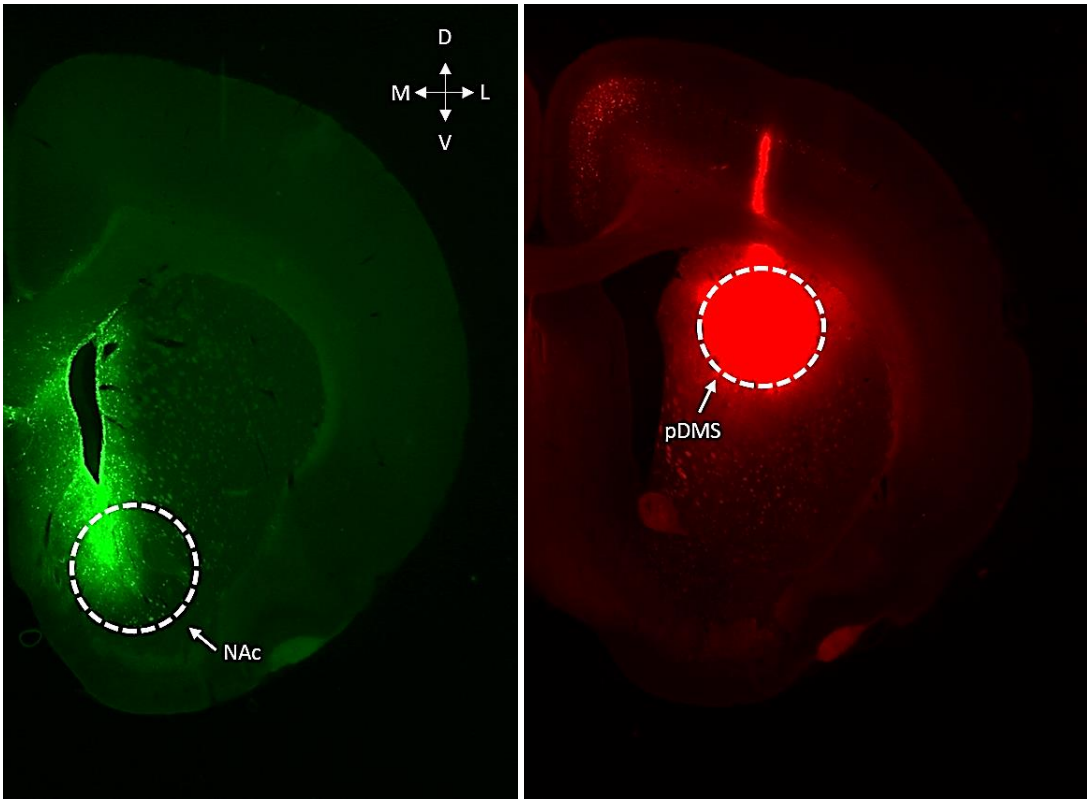
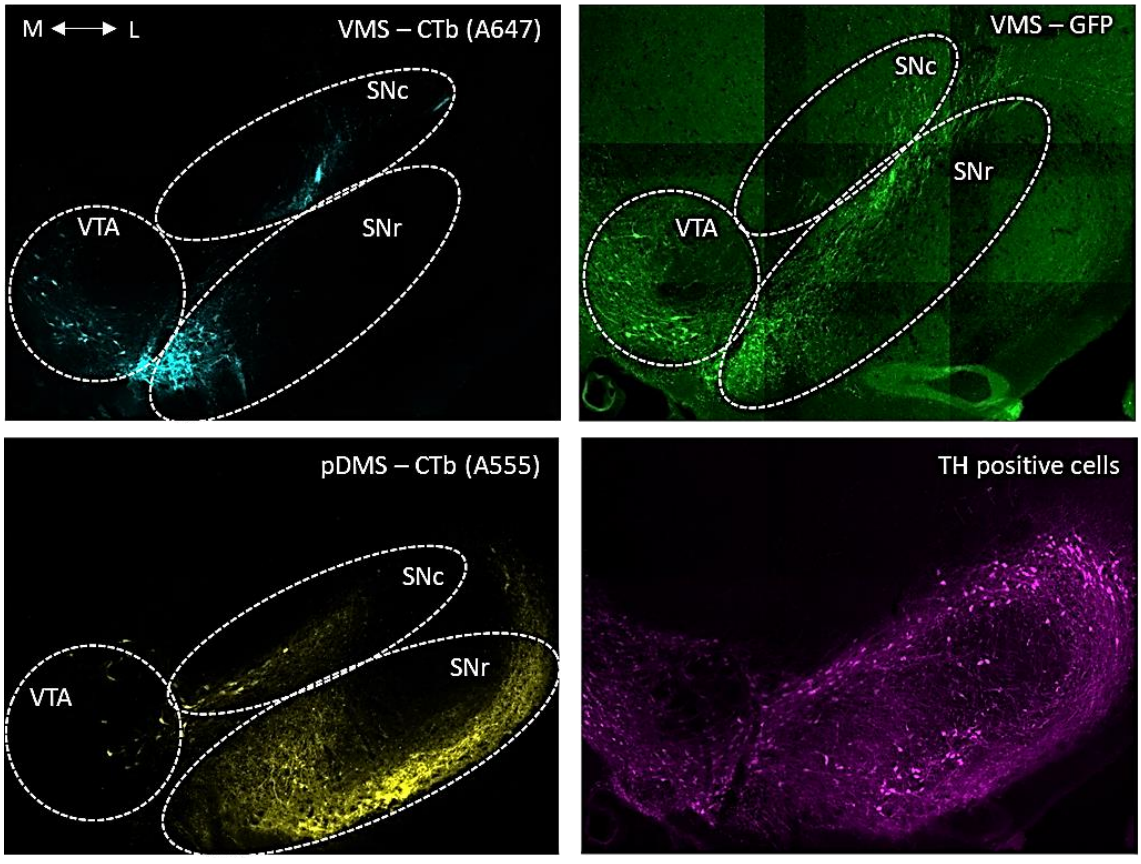


Figure 9 – Striatal results of the animal 35664. Left: Histology for the injection on the VMS (right hemisphere). The surrounded area (white dashed circle) corresponds to the Nac, both core and shell. Right: Histology for the injection on the pDMS (right hemisphere). The surrounded area (white dashed circle) corresponds to the region of interest. Presence of the needle track on the cortex. Both images have the same directions. Abbreviations correspond to: Nac – nucleus accumbens; pDMS – posterior dorsonedial striatum; D – dorsal; V – ventral; M – medial; L – lateral.

In Figure 10 are shown images of the midbrain results from the striatal injections showed previously (Figure 9), as well as from the immunohistochemistry. For both tracers

injected into the VMS, in this animal, resulted some retrogradely labelled cells on the VTA and anterogradely labelled terminals, especially, on the SNr but also on the SNc (Figure 10 – top). This is something that should have not happen, since the retrograde tracer was supposed to only label cells, while the anterograde tracer should only label terminals. From the pDMS injection, also retrogradely labelled cells, predominantly present on the SNc, and anterogradely labelled terminals, on the SNr, are visible (Figure 10 – middle left). Looking at the labelled terminals from the two injections, the ones from the pDMS are located in a more ventrolateral position than the ones from the VMS (Figure 10 – top right and middle left).



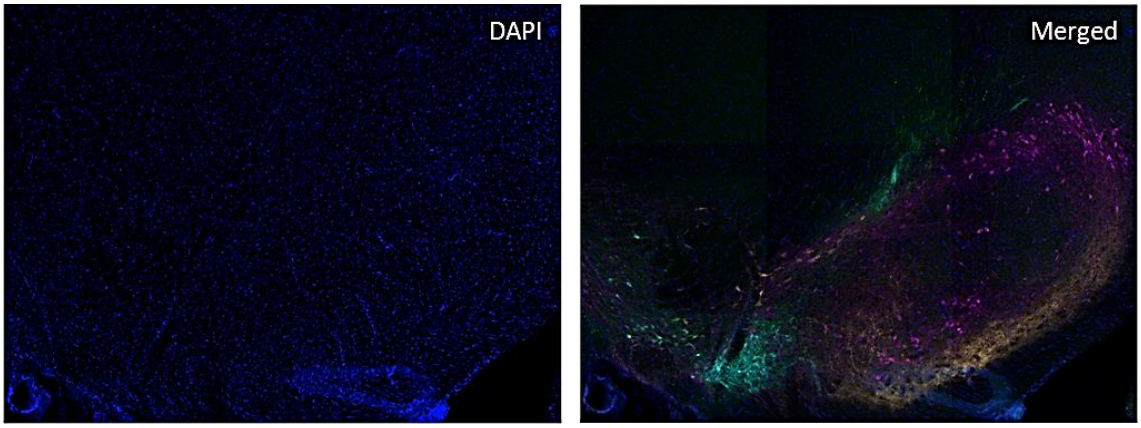


Figure 10 – Midbrain results of the animal 35664. Top left: Retrogradely labelled cells (VTA) and anterogradely labelled terminals (SN), with CTb - A647, resulting from VMS injection. Top right: Anterogradely labelled terminals (SN) and retrogradely labelled cells (VTA), with AAV5 - EGFP, resulting from VMS injection. Middle left: Retrogradely labelled cells (VTA and SNc) and anterogradely labelled terminals (SNr), with CTb – A555, resulting from pDMS injection. Middle right: Location of the dopaminergic cells in the midbrain (corresponds to the VTA and SN), resulting from TH staining. Bottom left: All cell bodies in this particular area, resulting from DAPI staining. Bottom right: Merged image. All images have the same direction. Abbreviations correspond to: VTA – ventral tegmental area; SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

In Figure 11 are shown images of the injection sites, on the animal 35665. The VMS injection resulted very similar to the previous animal, with the anterograde tracer spreading not only on the NAc, but also along the lateral ventricle (Figure 11 – left image). The pDMS injection fell over the corpus callosum (Figure 11 – right image).

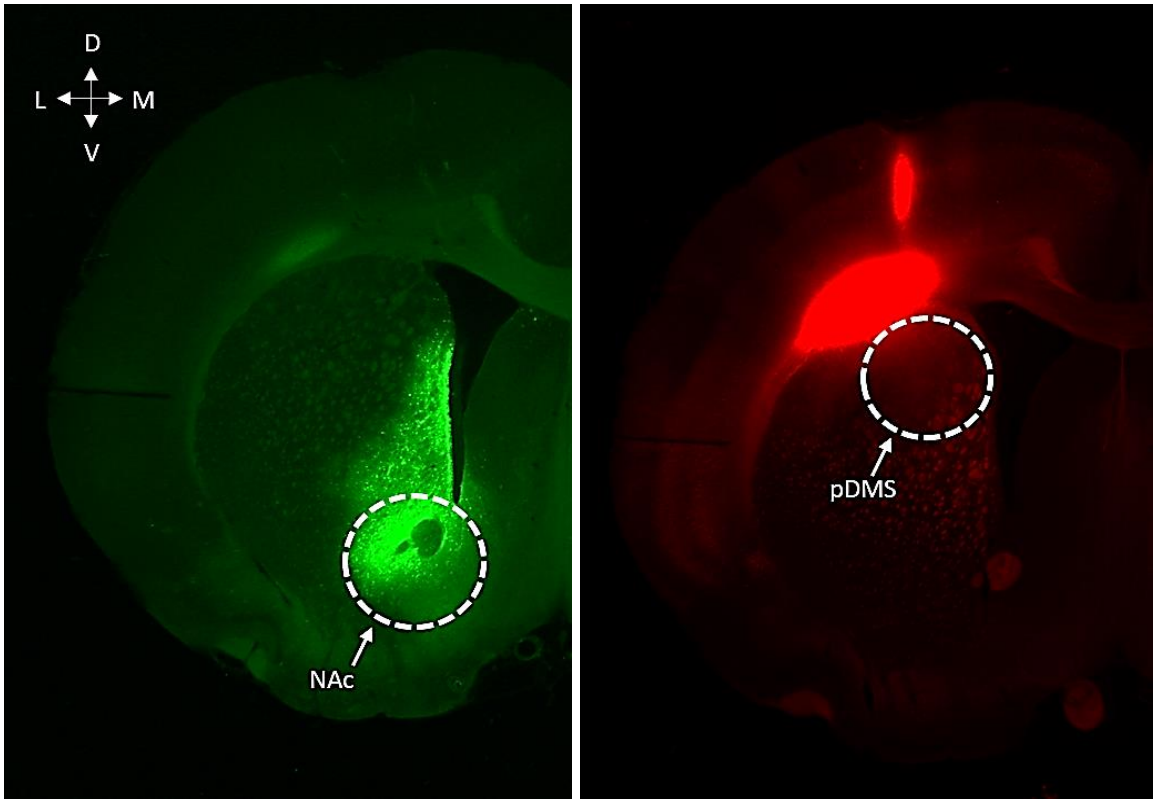


Figure 11 – Striatal results of the animal 35665. Left: Histology for the injection on the VMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the Nac, both core and shell, and it is also visible the anterior comissure (black dot surrounded by fluorescence). Right: Histology for the injection on the pDMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the region of interest. Presence of the needle track on the cortex. Both images have the same directions. Abbreviations correspond to: Nac – nucleus accumbens; pDMS – posterior dorsonedial striatum; D – dorsal; V – ventral; M – medial; L – lateral.

In Figure 12 are shown images of the midbrain results from the animal 35665, as well as from the immunohistochemistry. Starting with the injection in the VMS, only a few retrogradely labelled cells are present on the VTA, and even less on medial SNc. Anterogradely labelled terminals are visible on the SNr (majority) and SNc (Figure 12 – top left). With the anterograde tracer, were also obtained labelled cells on the VTA and medial SNc, and anterogradely labelled terminals, predominantly, in the same position of the ones labelled with the retrograde tracer, on the SNr. Moreover, some terminals are visible on the VTA and SNc (Figure 12 – top right). From the injection in the pDMS, the retrogradely labelled cells are present on the SNc, as well as terminals on the SNr, even though the terminals were not expected. Like the results from the previous animal (Figure 10 – middle

left), the labelled terminals from the pDMS are found on the ventrolateral part of the SNr (Figure 12 – middle left).

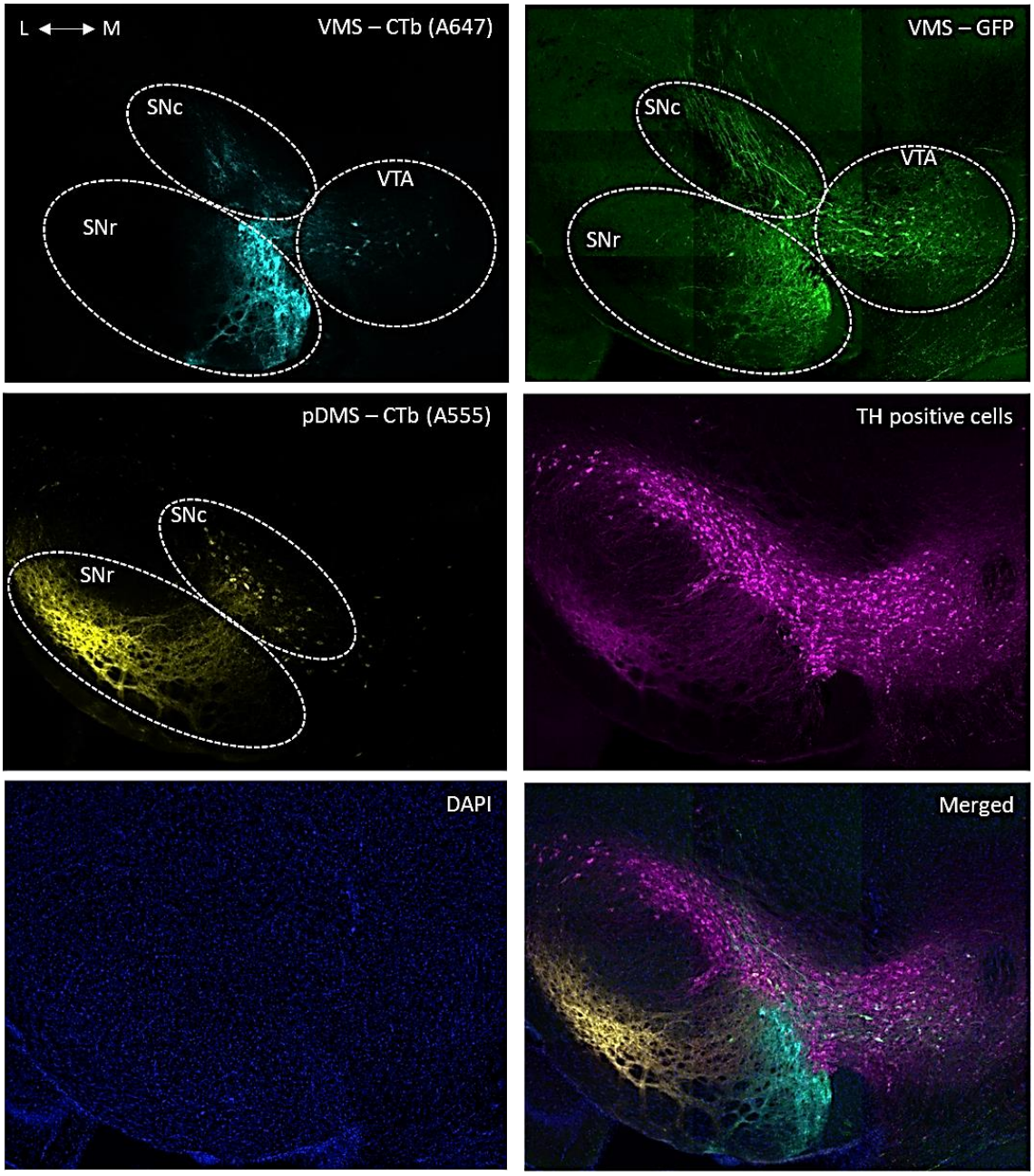
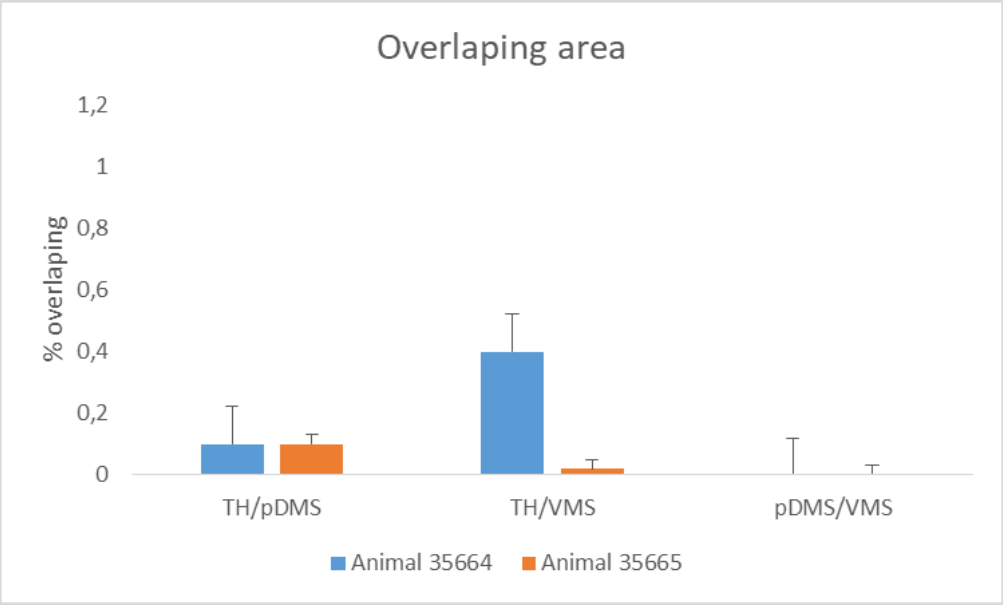


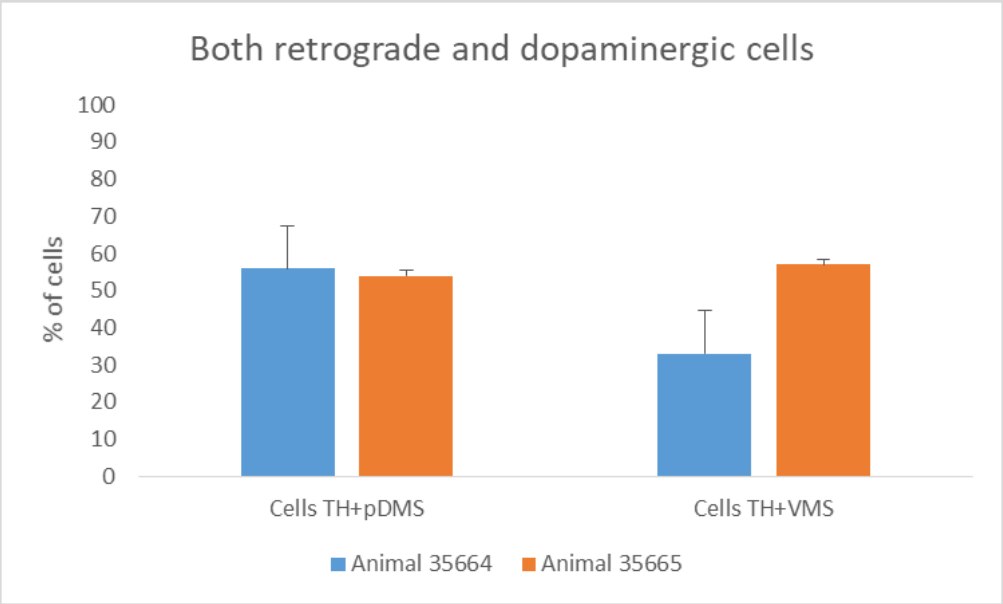
Figure 12 – Midbrain results of the animal 35665. Top left: Retrogradely labelled cells (VTA and SNc) and anterogradely labelled terminals (SN), with CTb - A647, resulting from VMS injection. Top right: Anterogradely labelled terminals (VTA and SN) and retrogradely labelled cells (VTA and SNc), with AAV5 - EGFP, resulting from VMS injection. Middle left: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A555, resulting from pDMS injection. Middle right: Location of the dopaminergic

cells in the midbrain (corresponds to the VTA and SNc), resulting from TH staining. Bottom left: All cell bodies in this particular area, resulting from DAPI staining. Bottom right: Merged image. All images have the same direction. Abbreviations correspond to: VTA – ventral tegmental area; SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

Graphics 1 and 2 represent the results from the data analysis for this set of experiments (or brain circuit diagram) (n=8). Graphic 1 corresponds to the overlapping area (%) of three different combinations of images. In these image overlay analysis, only the areas containing cells were considered. As shown, none of the combinations reach more than 0.5% of overlap, and the last combination has even 0% overlap. The first combination is similar between the two animals analyzed, whereas the second one shows a big discrepancy. Graphic 2 displays the results (%) of the cells that contain both CTb – A555 + TH and CTb – A647 + TH, respectively, on the overlapping area previously calculated. For the cells with CTb – A555 + TH, the results are, once again, similar between animals. In the cells with CTb – A647 + TH, besides from the variably between animals, there is also the opposite observed in Graphic 1. Even though the animal 35664 showed a bigger overlapping area, between TH/VMS, comparatively to the animal 35665 (Graphic 1), the % of cells TH+VMS is higher for the animal 35665 (Graphic 2).



Graphic 1 - Results, in %, of overlaping area between the images with: TH positive cells and retrogradely labelled cells from the pDMS, TH positive cells and retrogradely labelled cells from the VMS, retrogradely labelled cells from the pDMS and retrogradely labelled cells from the VMS. The vertical bars represent ± standard error (SE).



Graphic 2 - Results, in %, of the cells that are both retrogradely labelled from the pDMS or VMS and TH positive. The results present in this graphic are in accordence with the overlaping area previously obtained. The vertical bars represent ± SE.

4.2. VMS (AAV5 – mCherry) and pDMS (CTb – A647 + AAV5 – EGFP)

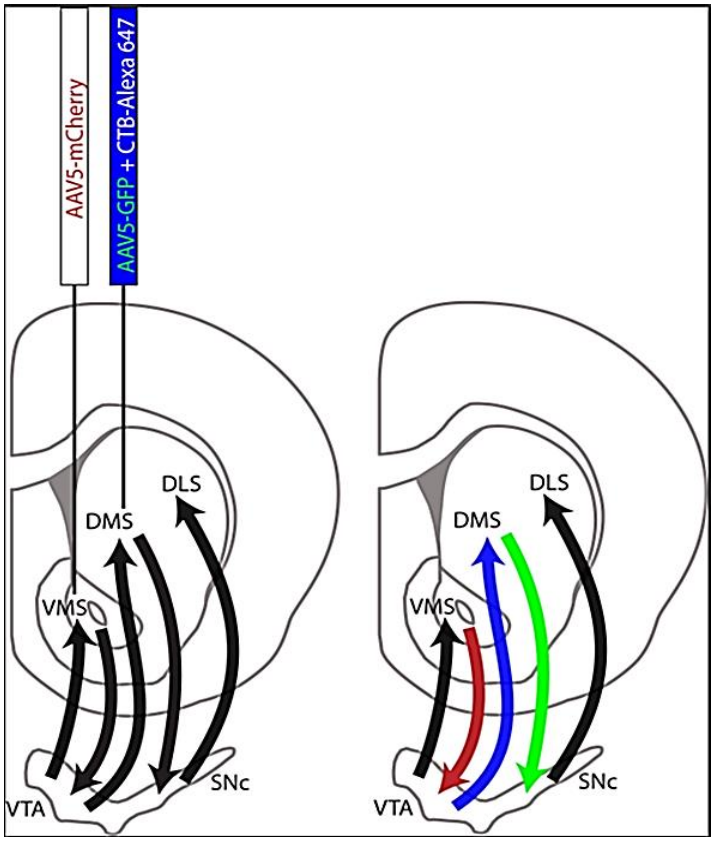


Figure 13 – Scheme of the injections on the VMS, with AAV5 – mCherry (anterograde direction), and on the pDMS, with AAV5 – GFP (anterograde direction) and CTb – A647 (retrograde direction). Abbreviations correspond to: VMS – ventromedial striatum; DMS – dorsomedial striatum; DLS – dorsolateral striatum; VTA – ventral tegmental area; SNc – substancia nigra pars compacta; AAV5 – EGFP – adeno-associated virus serotype 5 conjugated with enhanced green fluorescent protein; AAV5 – mCherry – adeno-associated virus serotype 5 conjugated with mCherry; CTb – Alexa 555 – cholera toxin b conjugated with alexa fluor 555; CTb – A647 – cholera toxin b conjugated with alexa fluor 647.

Figure 14 displays images of the injection sites in the VMS and pDMS, of the animal 35663. The VMS injection spread mostly outside the region of interest (Figure 14 – left side), while for the pDMS injection were not obtained any results (Figure 14 – right side).

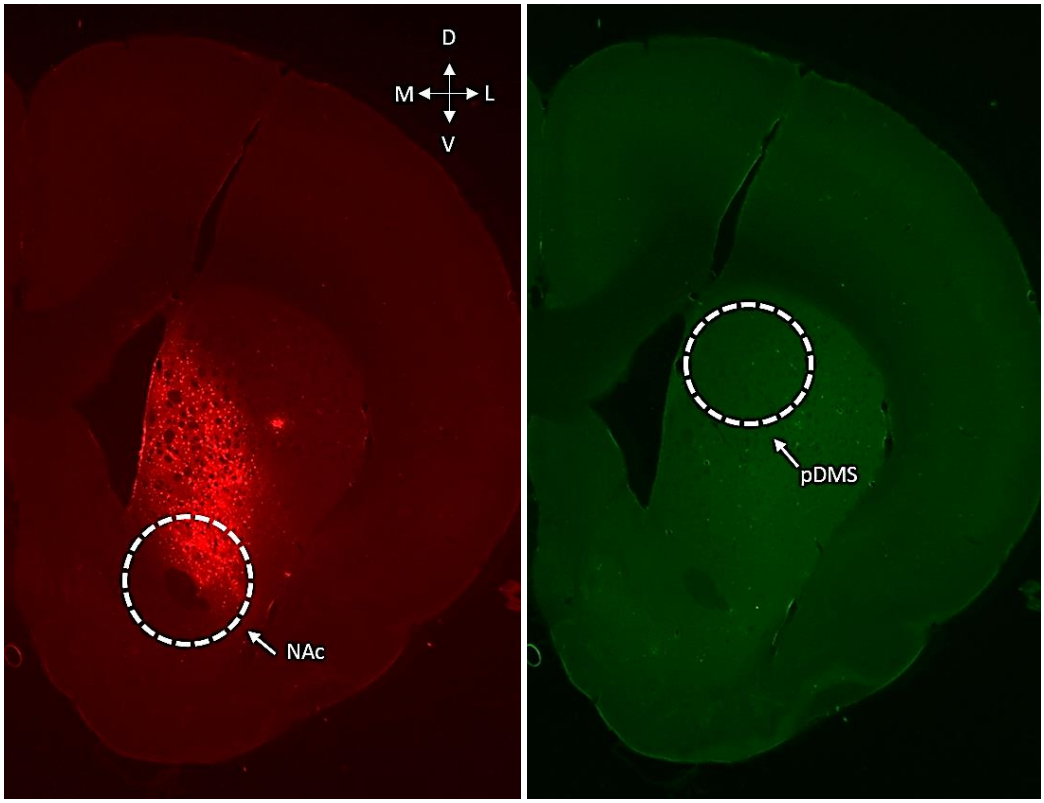


Figure 14 – Striatal results of the animal 35663. Left: Histology for the injection on the VMS (right hemisphere). The surrounded area (white dashed circle) corresponds to the Nac, both core and shell, and it is also visible the anterior comissure (black dot surrounded by fluorescence on top). Right: Histology for the injection on the pDMS (right hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. Both images have the same directions. Abbreviations correspond to: NAc – nucleus accumbens; pDMS – posterior dorsomedial striatum; D – dorsal; V – ventral; M – medial; L – lateral.

In Figure 15 are shown images of the midbrain results, for the animal 35663, as well as from the immunohistochemistry. Even though the expected results were only terminals, for the injection on the VMS, retrogradely labelled cells are, also, visible on the VTA and medial SNc, whilst the terminals are mainly found on the SNr, but also on the VTA (Figure 15 – top left). From the injection on the pDMS, resulted retrogradely labelled cells on the SNc, and terminals on the SNr (Figure 15 – top right). More or less the same results were obtained with the anterograde tracer also injected on the pDMS (Figure 15 – middle left). One more time, the labelled terminals from the pDMS are located more laterally and

ventrally, comparatively with the labelled terminals from the VMS (Figure 15 – top left and middle left).

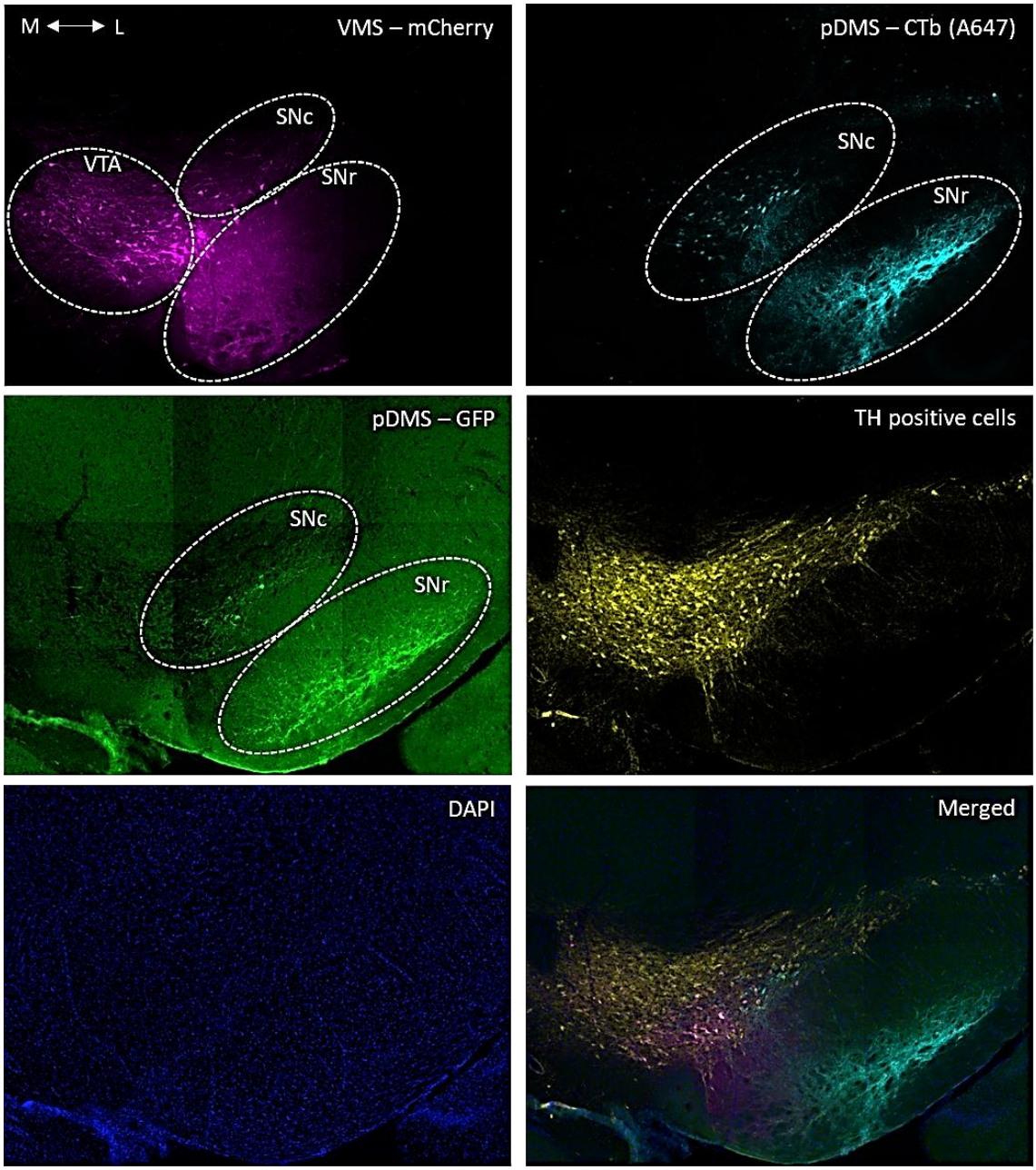


Figure 15 – Midbrain results of the animal 35663. Top left: Anterogradely labelled terminals (SNr and VTA) and retrogradely labelled cells (VTA and SNc), with AAV5 - mCherry, resulting from VMS injection. Top right: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A647, resulting from pDMS injection. Middle left: Anterogradely labelled terminals (SNr) and retrogradely labelled cells (SNc), with AAV5 – EGFP, resulting from pDMS injection. Middle right: Location of the dopaminergic cells in the midbrain (corresponds to the VTA and SNc), resulting from TH staining. Bottom left: All cell bodies in this particular

area, resulting from DAPI staining. Bottom right: Merged image. All images have the same direction. Abbreviations correspond to: VTA – ventral tegmental area; SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

Figure 16 displays images with the injection sites for the animal 35667. On the VMS, the anterograde tracer (AAV5 – mCherry) spread outside the area of interest (Figure 16 – left image), as well as the anterograde tracer (AAV5 – EGFP) on the pDMS (Figure 16 – right image).

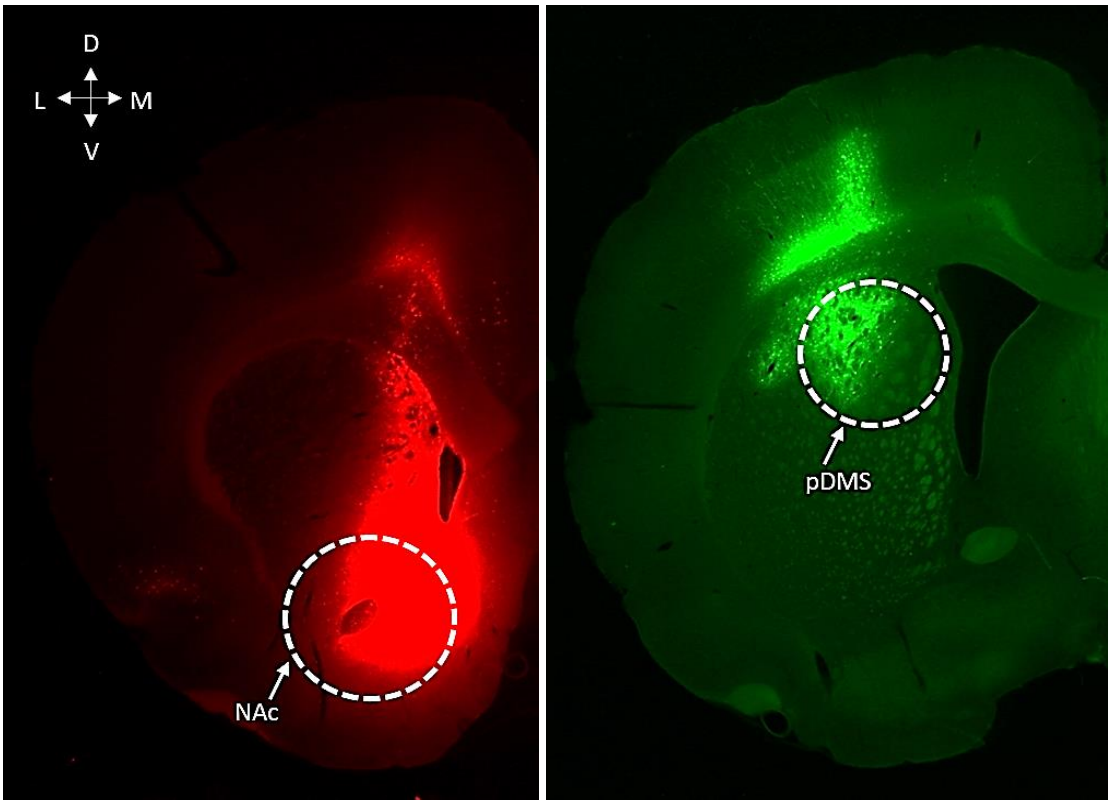
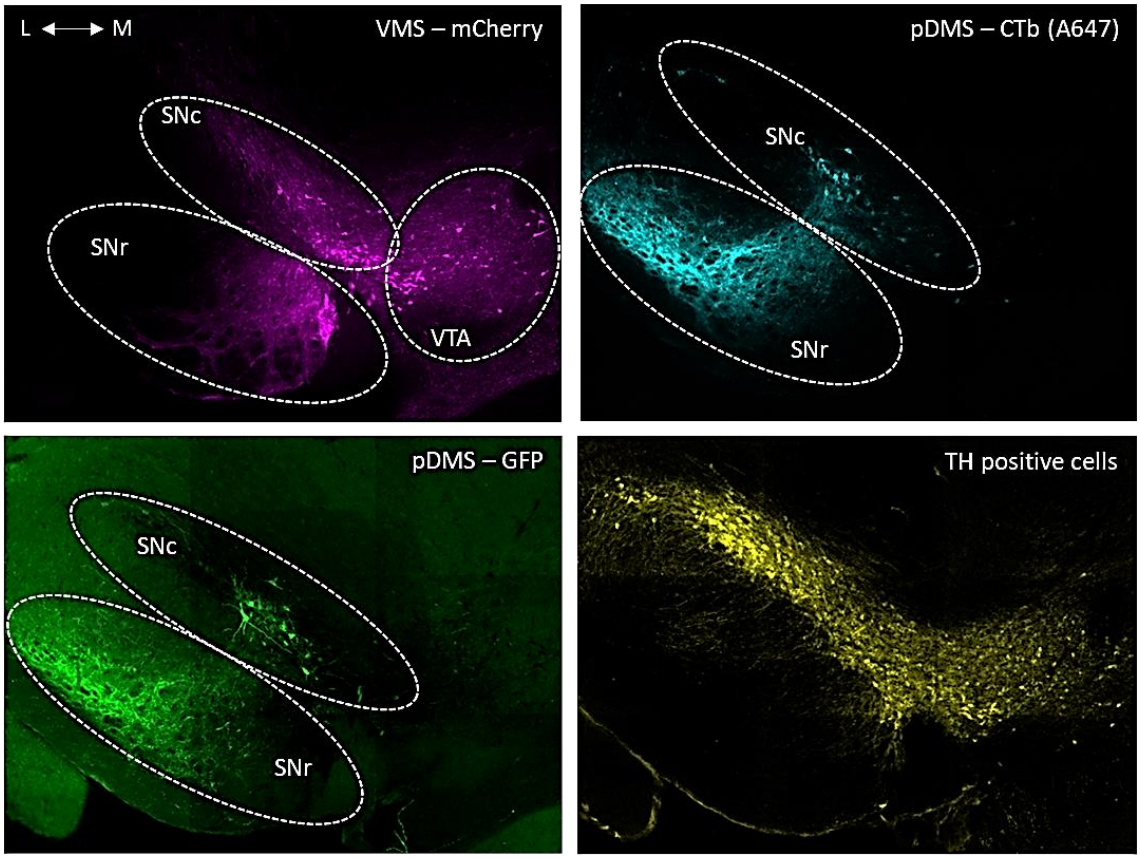


Figure 16 – Striatal results for the animal 35667. Left: Histology for the injection on the VMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the Nac, both core and shell, and it is also visible the anterior comissure (black dot surrounded by fluorescence). The needle track is visible along the lateral ventricle, corpus callosum and cortex. Right: Histology for the injection on the pDMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. Presence of the needle track on the cortex. Both images have the same directions. Abbreviations correspond to: NAc – nucleus accumbens; pDMS – posterior dorsomedial striatum; D – dorsal; V – ventral; M – medial; L – lateral.

In Figure 17 are shown images of the midbrain results from the striatal injections showed previously (Figure 16), as well as from the immunohistochemistry. As shown, from the VMS injection resulted retrogradely labelled cells on the VTA and SNc, whereas the terminals are located on the VTA and SN (Figure 17 – top left). From both tracers injected on the pDMS, it is visible retrogradely labelled cells on the SNc, and terminals on the SNr (Figure 17 – top right and middle left). The labelled terminals from the pDMS are visibly ventrolateral, comparatively with the labelled terminals from the VMS (Figure 17 – top left and middle left).



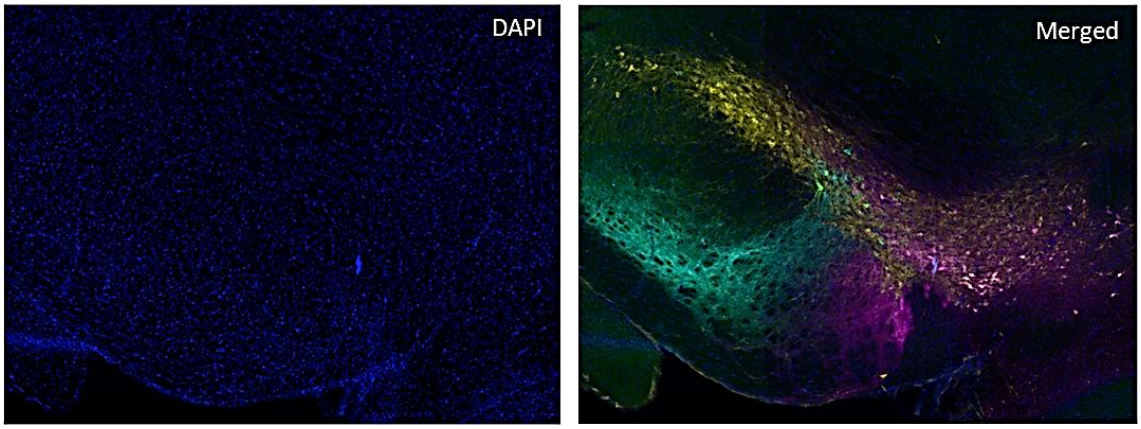
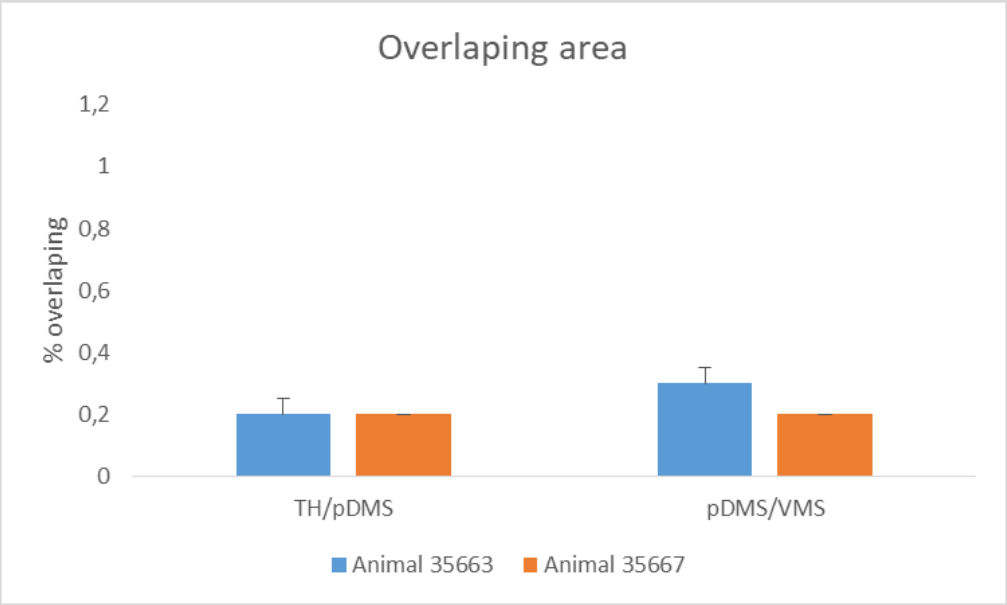
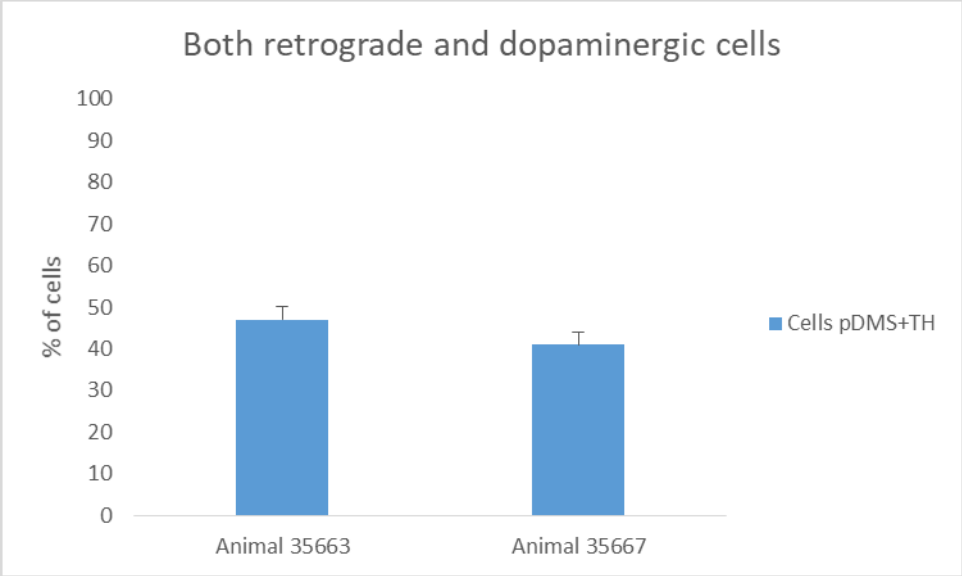


Figure 17 – Midbrain results of the animal 35667. Top left: Anterogradely labelled terminals (VTA and medial SN) and retrogradely labelled cells (VTA and SNc), with AAV5 - mCherry, resulting from VMS injection. Top right: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A647, resulting from pDMS injection. Middle left: Anterogradely labelled terminals (SNr) and retrogradely labelled cells (SNc), with AAV5 – EGFP, resulting from pDMS injection. Middle right: Location of the dopaminergic cells in the midbrain (corresponds to the VTA and SNc), resulting from TH staining. Bottom left: All cell bodies in this particular area, resulting from DAPI staining. Bottom right: Merged image. All images have the same direction. Abbreviations correspond to: VTA – ventral tegmental area; SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

Results from the data analysis for this set of experiments (n=9) are presented in graphics 3 and 4. Graphic 3 corresponds to the overlaping area (%) of two different combinations of images. For these image overlay analysis, areas containing only cells were considered, for the first combination (TH/pDMS). For the second combination (pDMS/VMS) the area only containing cells (pDMS) and the area containing terminals (VMS) were considered. Despite the fact that the percentages are not to high, either between animals or between areas, the results are very similar, and the existence of overlap between the areas with retrogradely labelled cells from the pDMS and anterogradely labelled terminals from the VMS suggests a pathway of communication between the VMS and pDMS, in this way. In Graphic 4 are displayed the results (%) of the cells that contain both CTb – A647 + TH, on the overlaping area previously calculated. The obtained results are almost the same for both animals analyzed.



Graphic 3 - Results, in %, of overlaping area between the images with: TH positive cells and retrogradely labelled cells from the pDMS, retrogradely labelled cells from the pDMS and anterogradely labelled terminals from the VMS. The vertical bars represent ± SE.



Graphic 4 - Results, in %, of the cells that are both retrogradely labelled from the pDMS and TH positive. The results present in this graphic are in accordence with the overlaping area previously obtained. The vertical bars represent ± SE.

4.3. pDMS (CTb – A647 + AAV5 – EGFP) and aDLS (CTb – A555)

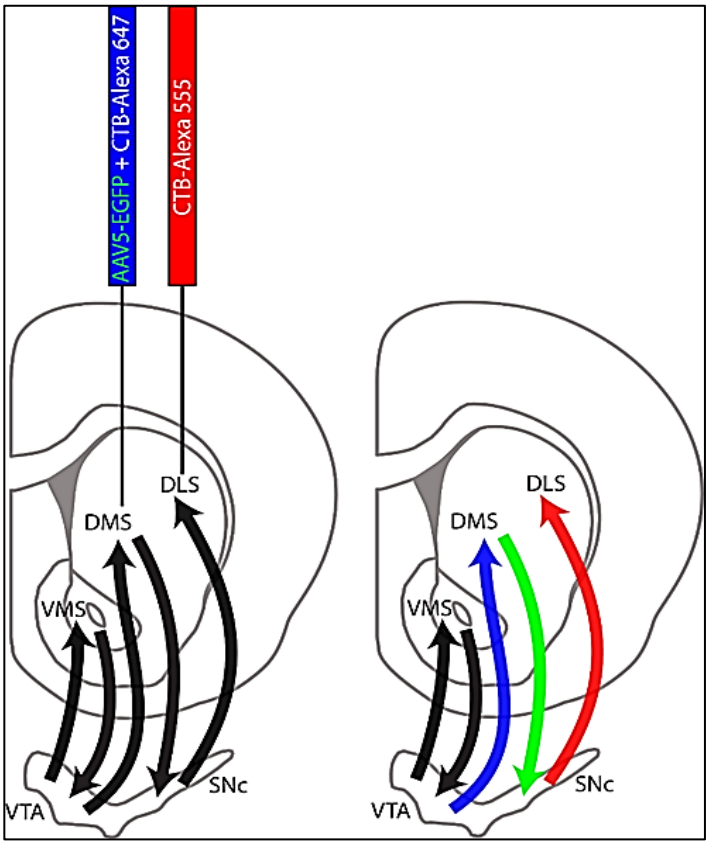


Figure 18 – Scheme of the injections on the aDLS, with CTb – A555 (retrograde direction), and on the pDMS, with CTb - A647 (retrograde direction) and AAV5 - EGFP (anterograde direction). Abbreviations correspond to: VMS – ventromedial striatum; DMS – dorsomedial striatum; DLS – dorsolateral striatum; VTA – ventral tegmental area; SNc – substancia nigra pars compacta; AAV5 – EGFP – adeno-associated virus serotype 5 conjugated with enhanced green fluorescent protein; AAV5 – mCherry – adeno-associated virus serotype 5 conjugated with mCherry; CTb – Alexa 555 – cholera toxin b conjugated with alexa fluor 555; CTb – A647 – cholera toxin b conjugated with alexa fluor 647.

Figure 19 reveal images of the injection sites on the pDMS and aDLS, for the animal Janvier T. The injection on the pDMS, spread along the lateral ventricle (Figure 19 – left side), whereas the injection on the aDLS, even though it was on target, it is also visible spread on the corpus callosum (Figure 19 – right side).

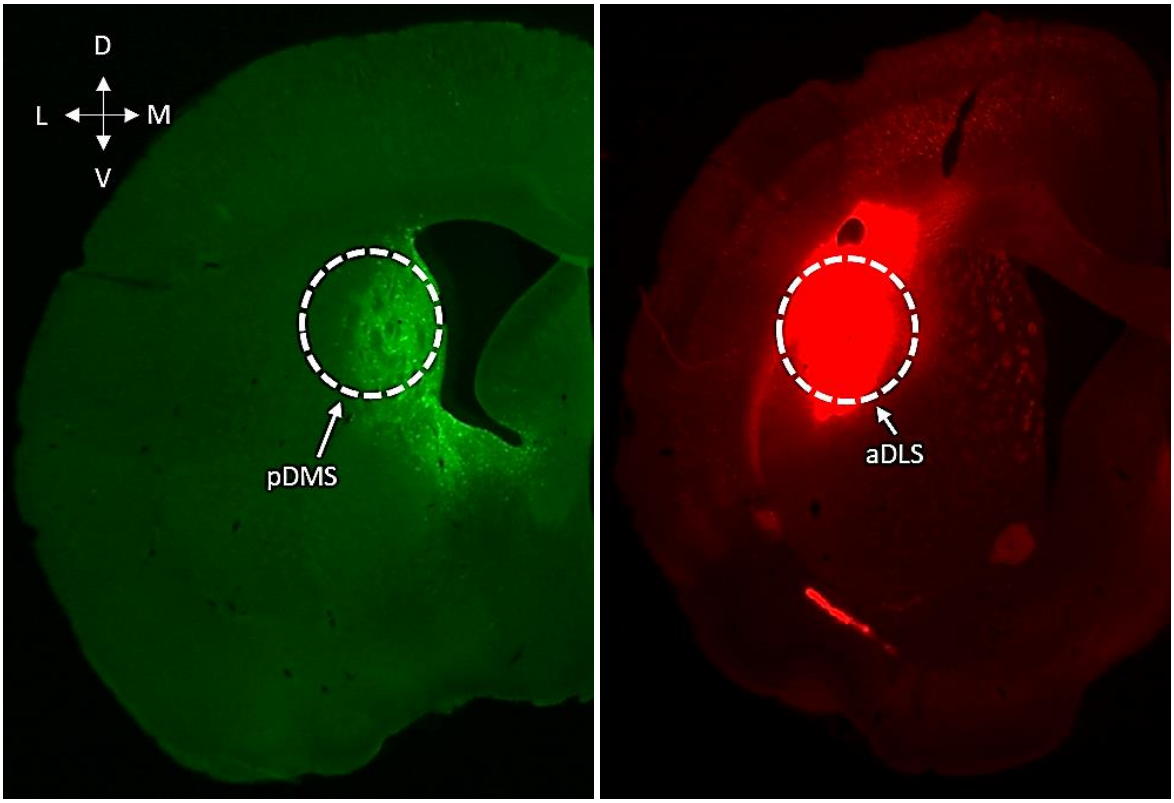


Figure 19 – Striatal results of the animal Janvier T. Left: Histology for the injection on the pDMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. Right: Histology for the injection on the aDLS (left hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. The hole above the corpus callosum corresponds to the needle site. Both images have the same directions. Abbreviations correspond to: pDMS – posterior dorsomedial striatum; aDLS – anterior dorsolateral striatum; D – dorsal; V – ventral; M – medial; L – lateral.

Images of the midbrain results from the striatal injections, on the animal Janvier T, are shown in Figure 20, as well as from the immunohistochemistry. From the injection in the pDMS, the retrograde tracer labelled cells on the SNc (few cells are also visible on the VTA) and terminals on the SNr (Figure 20 – top left). For the first time, with the anterograde tracer was only obtained labelled terminals on the SNr (Figure 20 – top right). From the injection in the aDLS, resulted retrogradely labelled cells on the SNc, and very few on the VTA. Anterogradely labelled terminals are visible on the SNr (Figure 20 – middle left). The position of the terminals labelled from the aDLS is visibly dorsolateral comparatively with the position of the terminals labelled from the pDMS (Figure 20 – top right and middle left).

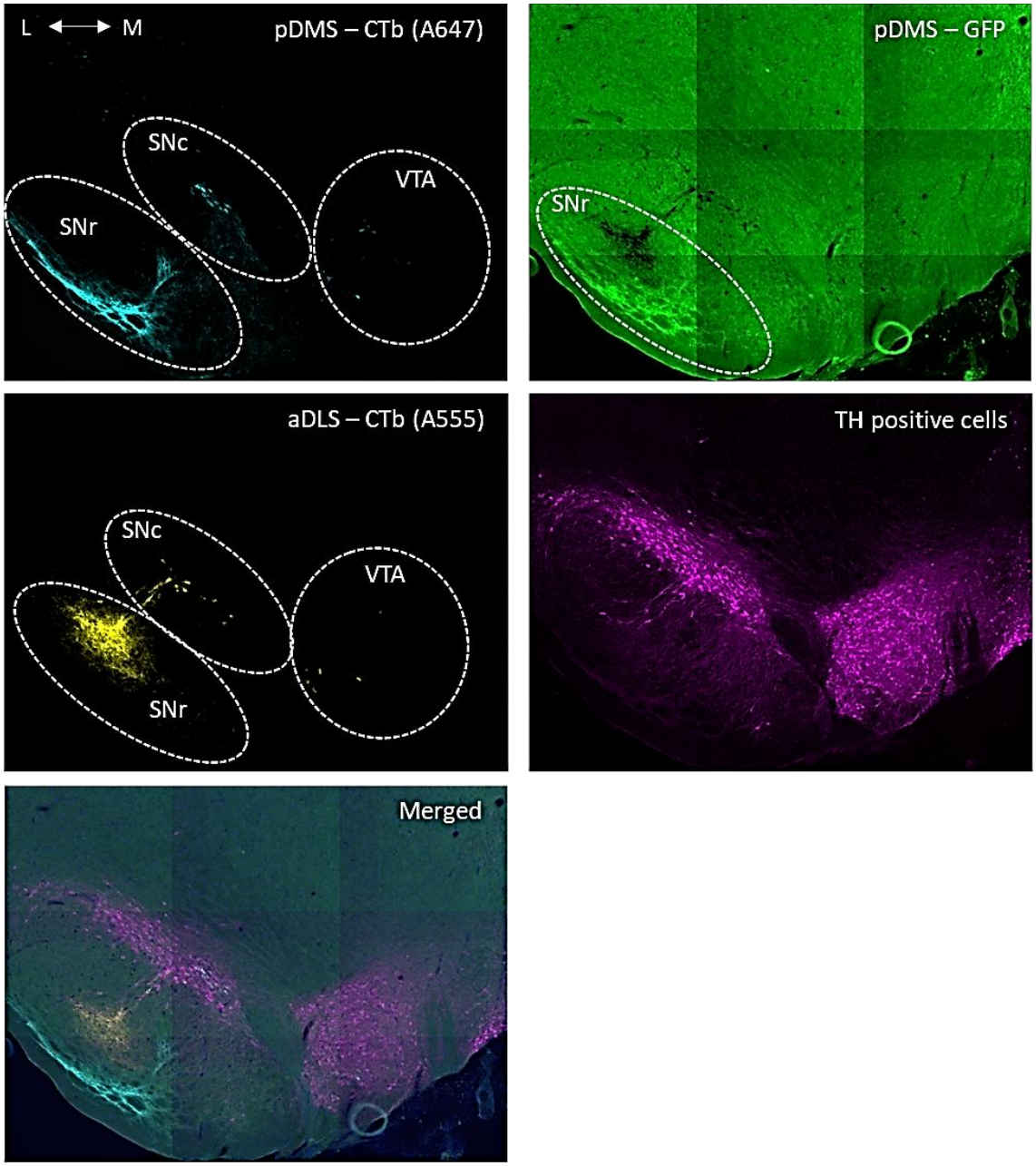


Figure 20 – Midbrain results of the animal Janvier T. Top left: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A647, resulting from pDMS injection. Top right: Anterogradely labelled terminals (SNr), with AAV5 – EGFP, resulting from pDMS injection. Middle left: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A555, resulting from aDLS injection. Middle right: Location of the dopaminergic cells in the midbrain (corresponds to the VTA and SNc), resulting from TH staining. Bottom left: Merged image. All images have the same direction. Abbreviations correspond to: SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

Figure 21 displays images of the injection sites on the animal 35666. As visible, the injection on the pDMS was noted on target and not much spread occurred outside the region of interest (Figure 21 – left image). The injection on the aDLS was also on target (Figure 21 – right image).

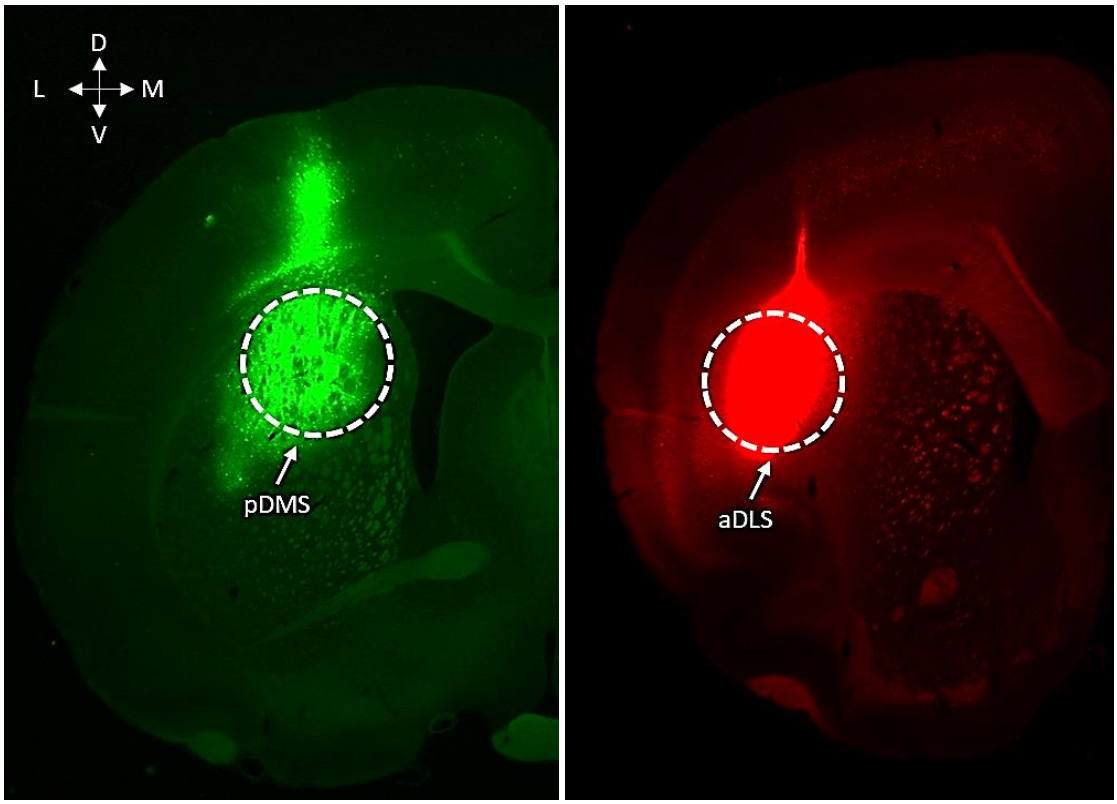


Figure 21 – Striatal results of the animal 35666. Left: Histology for the injection on the pDMS (left hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. The needle track is visible along the cortex. Right: Histology for the injection on the aDLS (left hemisphere). The surrounded area (white dashed circle) corresponds to the area of interest. The little horn on the cortex evidences the needle track. Both images have the same directions. Abbreviations correspond to: pDMS – posterior dorsomedial striatum; aDLS – anterior dorsolateral striatum; D – dorsal; V – ventral; M – medial; L – lateral.

In Figure 22 are shown the midbrain results from the striatal injections showed previously (Figure 21), as well as from the immunohistochemistry. From the injection on the pDMS with the retrograde tracer, resulted retrogradely labelled cells on the SNc and anterogradely labelled terminals on the SNr (Figure 22 – top left). The same results were

obtained with the anterograde tracer also injected on the pDMS (Figure 22 – top right). From the injection on the aDLS, resulted retrogradely labelled cells in a small extention of the SNc, that intermingled with the terminals connected to the SNr (Figure 22 – middle left). Once again, it is visible that the terminals from the pDMS occupy a wide area along the SNr compared to the ones labelled from the aDLS, which are found in a more dorsolateral position (Figure 22 – top left and middle left).

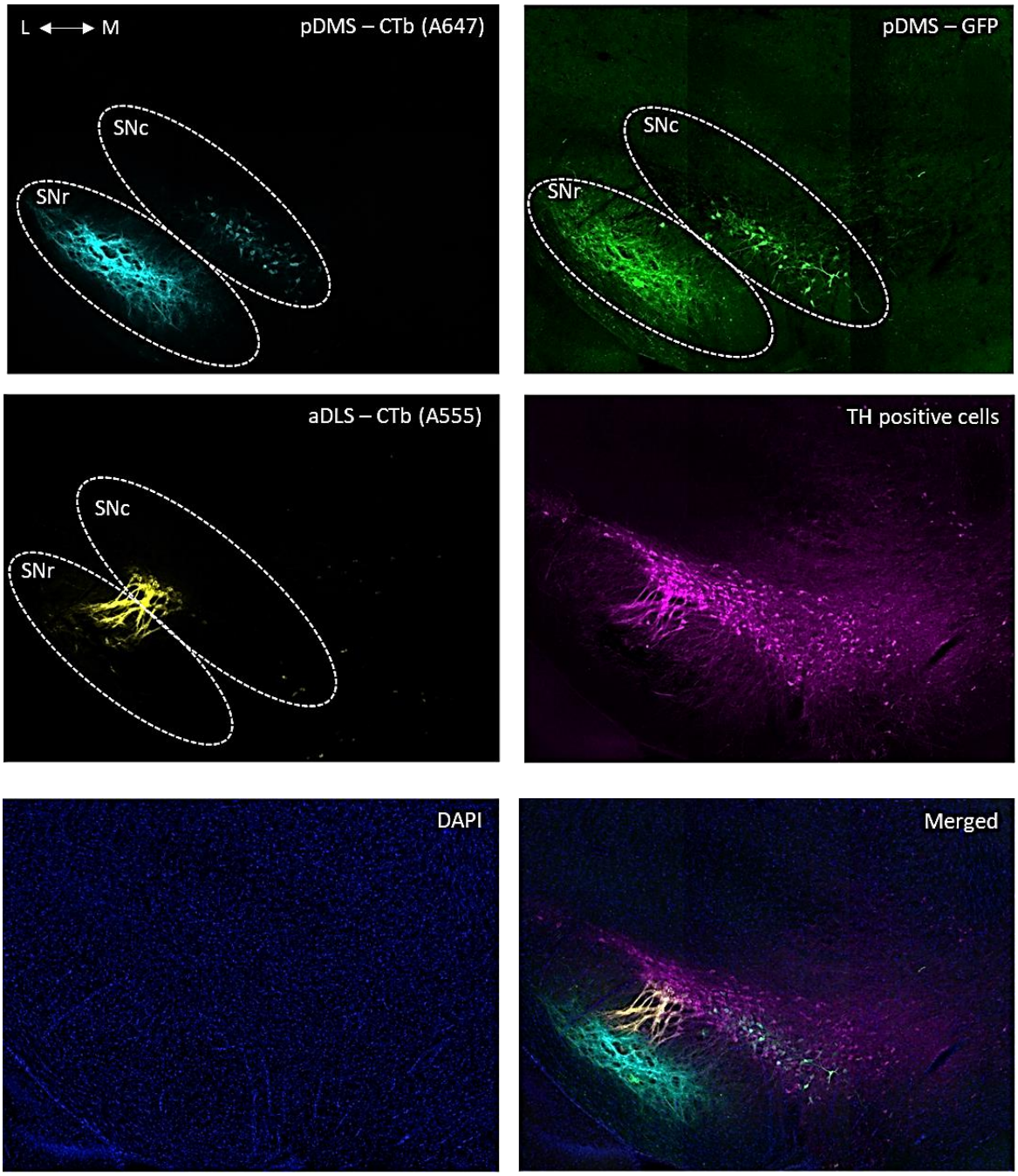
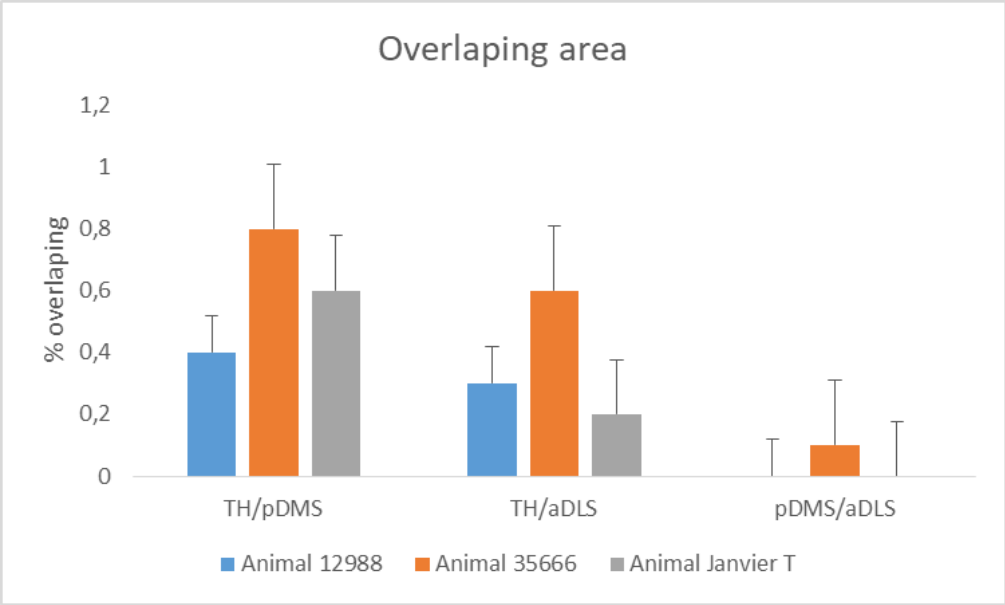
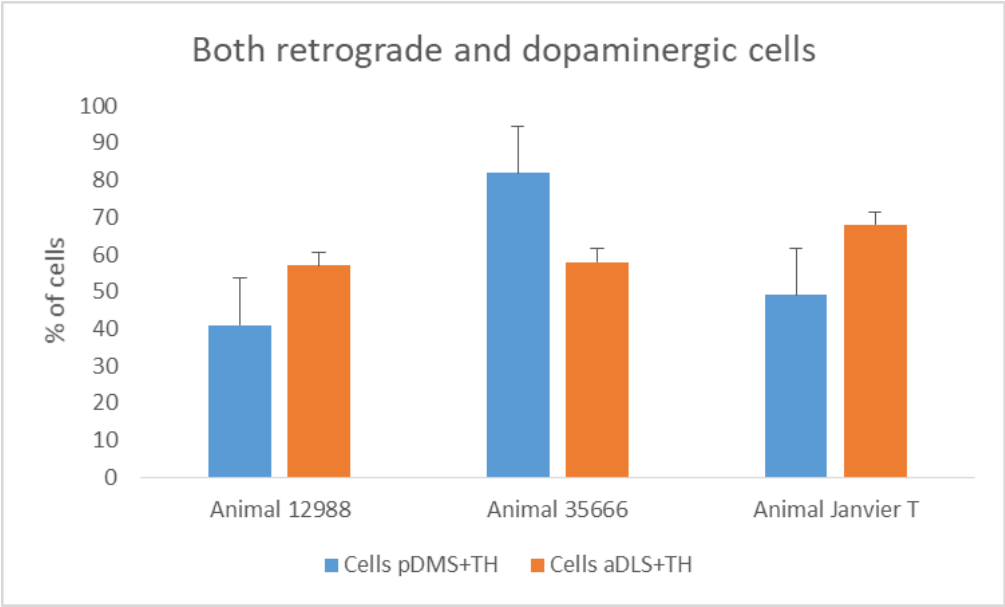


Figure 22 – Midbrain results of the animal 35666. Top left: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A647, resulting from pDMS injection. Top right: Anterogradely labelled terminals (SNr) and retrogradely labelled cells (SNc), with AAV5 – EGFP, resulting from pDMS injection. Middle left: Retrogradely labelled cells (SNc) and anterogradely labelled terminals (SNr), with CTb – A555, resulting from aDLS injection. Middle right: Location of the dopaminergic cells in the midbrain (corresponds to the VTA and SNc), resulting from TH staining. Bottom left: All cell bodies in this particular area, resulting from DAPI staining. Bottom right: Merged image. All images have the same direction. Abbreviations correspond to: SNc – substancia nigra pars compacta; SNr – substancia nigra pars reticulata; L – lateral; M – medial.

Graphics 5 and 6 represent the results from the data analysis for this set of experiments (n=7). Graphic 5 corresponds to the overlaping area (%) of three different combinations of images. For these image overlay analysis, only the areas containing cells were considered. As evidenced there is some variance between the three animals analyzed, for all combinations of images. Only on the last combination (pDMS/aDLS), the animals 12988 and Janvier T are in accordence, with 0% overlap. Graphic 6 displays the results (%) of the cells that contain both CTb – A647 + TH and CTb – A555 + TH, respectively, on the overlaping area previously calculated. The results from the cells that are both retrogradely labelled from the aDLS and TH positive are quite the same among all animals, while the results from the cells that are retrogradely labelled from the pDMS and TH positive are variable.



Graphic 5 - Results, in %, of overlaping area between the images with: TH positive cells and retrogradely labelled cells from the pDMS, TH positive cells and retrogradely labelled cells from the aDLS, retrogradely labelled cells from the pDMS and retrogradely labelled cells from the aDLS. The vertical bars represent ± SE.



Graphic 6 - Results, in %, of the cells that are both retrogradely labelled from the pDMS or aDLS and TH positive. The results present in this graphic are in accordence with the overlaping area previously obtained. The vertical bars represent ± SE.

5. Discussion

In the present study, we collected anatomical evidences that distinct dopaminergic populations in the midbrain project to different functional domains of the striatum and different striatal functional domains innervate segregated regions of the midbrain, too. First, the methodological approaches will be briefly discussed, and subsequently, the obtained data in the context of existing knowledge. Finally, some functional considerations will be provided.

5.1. Methodological approaches

Complex anatomical experiments of combined anterograde and retrograde neuroanatomical tracing and TH immunofluorescence phenotyping were performed in this study. The big difference between the number of animals used for each set of experiments and the number of animals used for the data analysis was due to lack of results for most animals. One of the explanations for this fact is related with wrong coordinates, mainly, for the pDMS, being that most injections ended up in the lateral ventricles. For this reason, different coordinates for this area as well as for the aDLS are shown in Table 1. Bad calibration of the stereotaxic frames used, human errors, freezing and defrosting of the neuroanatomical tracing aliquots and/or not enough incubation time, among other possible reasons, also, contributed to the lack of results.

By definition, anterograde tracing defines neurons from their cell bodies to the terminals of their axons, whilst retrograde tracing outlines neurons in the opposite direction, from the axon terminals to their cell bodies (Wanisch, 2018). Nonetheless, in this study we observed bidirectional tracing for both neuroanatomical tracers used (CTb and AAV5). Cholera toxin b (CTb), binds the ganglioside GM1 at the plasma membrane of the nerve ending. Its internalization occurs via clathrin-dependent or –independent mechanisms and traffics through the trans-Golgi network into the endoplasmic reticulum in a retrograde manner (Torgersen et al, 2001; Chinnapen et al, 2007; Vandeweerd et al, 2018). Despite being considered a monosynaptic tracer, experimental data have shown transneural passage (Lai et al, 2015). Moreover, in some studies, CTb has been used as an

anterograde tracer (Mikkelsen, 1992; Reiner et al, 1996; Ling et al, 1998; Derobert et al, 1999; Matteau et al, 2003). The adeno-associated virus serotype 5 (AAV5) is one of many existent serotypes (AAV1 – 9) from these type of virus and is characterized for binding *N*-linked sialic acid (SA) and performing efficient neuronal and some glial transduction. Nevertheless, successful transduction is dependent on many key steps such as cell surface receptor binding, endocytic uptake, endosomal escape, subsequent nuclear entry, capsid uncoating, genome release, second strand synthesis, and subsequent transcription (Murlidharan et al, 2014). On the other hand, other parameters seem to influence cellular tropism of AAV vectors, from direct brain injections. For instance, it is known that surface exposed regions on the AAV capsids influence the interactions with the host cell surface (Huang et al, 2014). Glycans are one of the cell surface components, identified as the preferred primary receptors for many natural AAVs (Asokan et al, 2012), so, differences in its architecture correlate to variations in the efficiency of gene transfer by AAV capsids in different organs. The age of the animal is also an important factor. Chakrabarty et al, (2013) showed that injections of AAVs 1, 8, and 9, performed on postnatal day 0 (P0), led to preferential neuronal tropism. The same vectors, showed neuronal and astrocytic transduction profiles from injections performed on P1 or later. In addition, reports of both anterograde (McFarland et al, 2009) and retrograde (Taymans et al, 2007) intracellular transport for AAV5, as well as other AAVs, was observed. Thus, even though most neuroanatomical tracers are sold as specific anterograde or retrograde tracers, in practice, this is very variable and depends on many factors.

5.2. Nigrostriatal projections

Through almost 60 years many investigator groups around the world conducted studies in order to try to decipher the mysteries behind the BG system. Still, after all these years, even with some discoveries along the way, important knowledge for its fully comprehension is lacking. The position of the mesencephalic dopaminergic neurons involved in the SNS circuit in rats is not yet known. From our experiments we observed that dopaminergic neurons in the VTA (Figures 10 and 12 – top left) and medial SNc (Figure 12

– top left) project to the ventral striatum, and many previous studies have reported the same observations (Nauta et al, 1978; Fallon and Moore, 1978; Corrigall et al, 1992; Maurin et al, 1999). In this project we did not focus to which part of the VMS, the VTA was projecting, but other groups have worked on that (for review see: Ikemoto, 2007). From the data analysis, the amount of overlapping area (Graphic 1 – TH/VMS) was quite variable between the animals; still the amount of cells on the area previously analyzed ranged between 30 and 60% (Graphic 2 – Cells TH+VMS). Reviews from Morales and Margolis, (2017), and Sesack and Grace (2010), reported that both VTA and SN are composed of a mixture of dopaminergic, GABAergic and even glutamatergic (not on the SNc) cellular elements, and within the VTA, the dopaminergic neurons account for about 60-65% of the total population, being the remaining fraction predominantly GABAergic. Thus, considering that the percentage of DA cells on the VTA is circa 60% and for one of the animals analyzed it was obtained a percentage of 30% of both retrogradely labelled and dopaminergic cells, it means that, in this particular animal, at least 50% of the dopaminergic cells on the VTA project towards the VMS. Since these findings are new, or at least, I was not able to find literature to compare these numbers with, it still seems to be a good number.

From the pDMS injections, we can state that the dopaminergic cells that project into this region of the striatum are mostly distributed in the medial SNc, since the results are consistent across all sets of experiments (Figures 10 and 12 – middle left; 15 and 17 – top right; 20 and 22 – top left). On the other hand, even though it is visible a distinct dopaminergic area, in the lateral part of the SNc, projecting to the aDLS (Figures 20 and 22 – middle left), this hypothesis is still uncertain, since the data analysis, for one animal, showed overlap between the dopaminergic areas projecting to the pDMS and aDLS (Graphic 5 – animal 35666). It may happen to be an isolated case, even so further experiments are necessary to firmly establish where the dopaminergic projections to this striatal region come from. Notwithstanding, our results present new findings, and are corroborated by previous literature. For instance, it is known that the SNc sends dopaminergic projections to the striatum, the putamen and the caudate nuclei, while the SNr works together with the GPi as the final output of the BG´s direct and indirect pathways (Fabbri et al, 2017). This knowledge is in favor of our observations, in the sense that the

dopaminergic projections, to the dorsal striatum (here subdivided as pDMS and aDLS), observed both start from the SNc. In addition, the overall data analysis, for all sets of experiments, showed no overlap between the dopaminergic areas projecting to the different striatal domains in study. In conclusion, the obtained results suggest that the nigrostriatal projections to different functional domains of the striatum originate from distinct populations of DA neurons.

5.3. Striatonigral projections

The NAc, which is part of the ventral striatum, is divided into two major territories; the core, that is the central portion directly beneath and continuous with the dorsal striatum and surrounding the anterior commissure, and the shell, which occupies the most ventral and medial parts (Zahm and Brog, 1992). From the core are known projections primarily to the dorsolateral portion of the ventral pallidum, the entopeduncular nucleus, and the SNr. In its turn, the shell mainly innervates the lateral hypothalamic and lateral preoptic areas, SNc, VTA, periaqueductal gray, pedunculopontine tegmentum, among other areas (Usuda et al, 1998). Even though in our study, the territories that make up the NAc were not considered independently, as mentioned before, our results are still in accordance with this knowledge. This is, projections from the VMS (or NAc) innervate mainly the VTA (innervated from the shell of the NAc), but also the medial part of both SNc (innervated from the shell of the NAc), and SNr (innervated from the core of the NAc), as illustrated in Figures 10 and 12 – top right and 15 and 17 – top left. At this respect, in a recent study performed by Wouterlood and colleagues, (2018) documented that the NAc shell exert an inhibitory influence on dopaminergic neurons that are located in the lateral portion of the VTA and medial SNc and that project to the sensorimotor territory of the ventrolateral striatum.

Regarding the projections from the pDMS the results were consistent along all of the experimental groups, being located always in the SNr and occupying a large area. From the set of experiments in which were injected AAV5 – mCherry in the VMS and CTb – A647 in the pDMS was visible overlap between the terminals labelled from the VMS and the cells

labelled from the pDMS (Figures 15 and 17 – bottom right), something that was confirmed through the data analysis (Graphic 3 – pDMS/VMS). In 1981, a study by Somogyi and colleagues also demonstrated the existence of synaptic contacts between terminals of ventral striatal efferents and dendrites of nigral neurons projecting to the dorsal striatum. Together, these observations suggest an exchange of feedback between the ventral and dorsal striatum, in this way. As explained before, the retrograde tracer labelled also terminals, and so taking advantage from that, in Figures 20 and 22 – middle left, we observed that the terminals from the aDLS are only found in the SNr, in the most dorsolateral part of it. In conclusion, these results show that the striatonigral projection fields from different functional domains innervate segregated regions of the midbrain.

5.4. From ventral to dorsal striatum and drug addition

Our present results confirm and extend previous neuroanatomical studies (Nauta et al, 1978; Haber et al, 2000). As in primates the organization of SNS pathways, which has been interpreted as a spiraling system, imply that the influence from ventral to dorsal striatum via the ventral midbrain shifts gradually (Haber et al, 2000). Indeed, and as hypothesized, also in rats we observed that small groups of dopaminergic neurons in the midbrain provide a projection to a striatal region located more dorsally, as we go more laterally in the midbrain. Whether this dopaminergic groups project only to a more dorsal region of the striatum or also to the same striatal region of origin we cannot conclude with the present study. In addition, Maurin et al, (1999) described a “proximal” neuron subpopulation located in SNc that occupied a position in register with the striatonigral projections in the subjacente SNr. Such relationship suggests that “proximal” neurons are involved in reciprocal SNS feedback circuits, while neurons belonging to a “distal” subpopulation located, for instance, medially and dorsally in SNc and VTA might be involved in nonreciprocal connections with the dorsal striatum. From our results, we can infer that it might exist both open and closed loops, since there are cells intermingled with terminals, in the borders between the VTA and SNr, and SNc and SNr, as well as cells located further away. In this cenario, the first cells, would be the so called “proximal” subpopulation and

be involved in the open loop, whereas the cells farthest from the terminals would be part of the "distal" subpopulation and the closed loop. In any case, this study provides anatomical evidences of a step-by-step mediolateral shift in the midbrain and that leads to the conclusion that functionally different striatal regions influence each other in a transition from motivational via cognitive to sensorimotor related functions.

Considering all of this, it is important to add few notes here. It is known that the striatum has an important role in mediating two different forms of associative learning. The Pavlovian learning (stimulus-response) describes the process by which an initially neutral conditioned stimulus, through repeated pairing with an unconditioned stimulus evoking an unconditioned response, acquires the capability to elicit the same, now conditioned, response. In instrumental learning (action-outcome), the likelihood of performance of a specific behavior is dependent on the appetitive or aversive outcome (unconditioned stimulus) which is associated with. Whereas stimulus-response and the early stages of action-outcome contingencies are sensitive to devaluation of the unconditioned stimulus, following repeated training action-outcome associations become habitual, regardless of changes to the outcome (Holland and Rescorla, 1975; Balleine and Dickinson, 1998). In detail into the striatal domains, the NAc, especially the core region and its inputs from the basolateral amygdala are implicated in the mediation of Pavlovian and instrumental conditioning (Cardinal and Everitt, 2004), while specific "hotspots" within the NAc shell appears to mediate hedonic reactions or "liking" for food and drug reward (Castro and Berridge, 2014). On the other hand, the dorsal striatum is implicated in the control of instrumental behavior by stimulus-action associations (Balleine et al, 2007). It was demonstrated that lesions in the DMS inhibit goal-directed instrumental conditioning, and lesions on the DLS disrupt habit formation, indicating that the DMS and DLS mediate the initial acquisition and later consolidation phases of skill learning, respectively (Yin et al, 2009). In essence, this means that the switch from voluntary to habitual and compulsive drug use in addiction is represented by a neural transition in the control of behavior from VMS to DMS and DLS striatal regions, via different dopaminergic population neurons in the midbrain.

6. Future directions

As the reader may have noticed, in the present study there are not many results, due to some issues that occurred during the experiment (as mentioned in the methodological approaches) and that needed to be optimized in order to improve it. In this way, the suggestions for the future are to stain and image additional tissue from animals that generated insufficient data for analysis. At this respect, it may happen that the fluorescent dyes degraded over time, and so, a better option is to inject new subjects. When the sample sizes reaches at least three animals per experimental group, then the data can also be analyzed statistically. Regarding the data analysis, it would be good to find a way to standardize the criteria used to choose the threshold, since the method used is subjective and dependent on the person that is doing it. Still on this point, it would be equally good to find a method to count the real number of retrogradely labelled cells. Finally, to create a more interesting and complete study, additional striato-nigrostriatal circuits, for instance between the pDMS and aDLS, but with the tracers switched or between the VMS and aDLS, could be traced.

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