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Investigating the role of anterior ventro-lateral striatum direct pathway neurons in instrumental licking behaviour of mice using optogenetics in an operant behavioural task

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RESUMO

O movimento permite ao indivíduo interagir e reagir ao ambiente envolvente: desde cílios em bactérias, aos estomas em plantas, e finalmente, redes neurais complexas em animais. Durante o processo evolutivo, o desenvolvimento de um sistema nervoso central - um avanço significativo desde a rede neural difusa nos cnidários - serviu não apenas para integrar informação recolhida por órgãos sensoriais cada vez mais sofisticados, mas também para gerar comportamentos complexos que permitissem uma vantagem adaptativa ao indivíduo.

No seu dia a dia um animal depara-se com numerosas situações, por vezes repetidas, e noutras inesperadas, pelo que a capacidade de aprendizagem de comportamentos distintos é vantajosa para a sua sobrevivência. Adquirir e aprender estas acções intencionais é conseguido através da integração de circuitos sensoriais e motores que permitem a modulação e evolução dinâmicas dessas acções, com base nas suas consequências (rácio custo-benefício). Após a exposição e prática repetidas deste processo de aprendizagem, o resultado obtido é a criação de repertórios de acções que serão depois usadas no desempenho da tomada de decisões: a selecção da acção. Ao seleccionar uma acção, para qualquer circunstância e dentro de certos contextos, este repertório de informação é utilizado para salientar a acção a executar mais apropriada de uma variedade de alternativas possíveis. Contudo, uma mesma acção pode ter diferentes resultados a si associados, e recompensas com diferentes valores, dependendo do contexto em que é aplicada. Daí, a capacidade de reconhecer contextos diferentes é fundamental para a implementação de comportamentos bem sucedidos. Pensa-se que esta contextualização é levada a cabo por áreas do cérebro tais como o hipocampo e o córtex orbitofrontal, assim como por outros circuitos presentes nos gânglios da base.

Ainda assim, aprender e seleccionar as acções apropriadas para determinados contextos são apenas os primeiros passos na produção motora. É necessário que o sistema nervoso central opere de modo a implementar as acções selecionadas - a execução da acção - através de padrões de contracção muscular específicos, e com precisão temporal e espacial. A produção de movimento é obtida através da ligação de sequências (*chunks* (pedaços) ou "frases") de elementos de movimentos mais simples (primitivas motoras ou "sílabas"), de modo a atingir um programa motor unificado - uma acção - e a executar uma tarefa. Pensa-se que diferentes circuitos e grupos de células estarão implicados nas diferentes fases da produção de um movimento.

Os gânglios da base são um grupo de estruturas que estabelecem ligações com áreas que incluem o córtex, o tálamo e outras regiões motoras descendentes, e desempenham um papel na acção da selecção e na produção motora. Estas estruturas, que se localizam abaixo do córtex, são conservadas em todos os vertebrados e têm uma posição privilegiada para integrar informação e de seguida a retransmitir para vias motoras descendentes. Os gânglios da base estão organizados em áreas diferentes que possuem entradas e saídas axoniais distintas, assim como padrões de expressão génica específicos (ex. receptores dopaminérgicos D1/D2) - que permitem a integração e o processamento de informação relevante necessária à selecção e execução de movimentos específicos. No estriado - a mais proeminente estrutura nos gânglios da base - as vias estriado-nigral e estriado-palidal trabalham em conjunto para, respectivamente, facilitar ou suprimir uma acção com base no contexto e aprendizagens passadas. Em específico, a região anterior do estriado ventro-lateral (aVLS) foi implicada no controlo do movimento orofacial. O modo como este controlo é obtido e qual é o papel das vias estriado-nigral e estriado-palidal no movimento orofacial voluntário é ainda pouco compreendido.

Neste estudo, investigámos como os inputs estriatais na substância nigra pars reticulata (via estriado-nigral) afectam o movimento orofacial em murganhos com recurso à técnica de manipulação optogenética e utilizando uma tarefa comportamental operante de cabeça fixa dependente do olfacto.

A técnica utilizada, desenvolvida por Karl Deisseroth por volta do ano 2005, permite manipular alvos com especificidade ao nível celular - quer em tecido como em animais vivos - e controlar a actividade eléctrica celular com precisão espaço-temporal. A utilização deste procedimento em neurociência significa que é possível despolarizar ou hiperpolarizar um conjunto de neurónios específicos, numa determinada localização do cérebro, com grande precisão temporal, e em circuitos neurais intactos de animais vivos e com comportamento livre.

Para a aplicação da técnica de neuromodulação optogenética, injectámos bilateralmente 8 murganhos da linha transgénica D1-Cre com um vector viral para a expressão de uma opsina na região aVLS, e de seguida implantámos fibras ópticas no mesmo local. Depois, os animais foram treinados na tarefa comportamental, em 4 ensaios, nos quais aprenderam a associar 4 odores diferentes com as suas respectivas consequências: a) o odor "go", associado à acção de lamber para receber uma recompensa, b) o odor "nogo" associado à supressão da acção de lamber para evitar uma punição, c) o odor "wait" associado à supressão da acção de lamber para receber uma recompensa mais atrasada e d) o odor "neutral", ao qual não foi associada nenhuma consequência, independentemente do comportamento do animal. Após 25-45 sessões de treino para aprendizagem dos 4 tipos de ensaio, os animais foram submetidos a manipulação optogenética com diferentes frequências de estimulação (2Hz, 5HZ e 10Hz) e foi medida a frequência de lambidelas (protrusões de língua).

Confirmámos que a estimulação optogenética das populações estriado-nigrais desta área do estriado provocam movimentos orofaciais. Verificámos que a estimulação nas várias frequências utilizadas tem maior efeito na iniciação da acção de lamber, e não tanto no prolongamento da acção. Vimos também que, para a frequência de estimulação de 10Hz, o animal lambe para beber água em todos os ensaios, mesmo naqueles em que aprendeu a reprimir o comportamento de lamber. Para o ensaio "go", foi observado que a estimulação das células estriado-nigrais reduz a velocidade de reacção e aumenta o comportamento antecipatório dos murganhos. Mais ainda, foi detectada uma diferença entre os ensaios "nogo" e "wait" - nos quais o animal aprende a reprimir a acção de lamber - sendo que para os ensaios "nogo" é necessária uma frequência de estimulação mais elevada para despoletar a iniciação da acção (10Hz) enquanto que no ensaio "wait" as frequências de 2Hz e 5Hz foram suficientes para aumentar a iniciação da acção.

Em suma, a modulação optogenética da população neural estriado-nigral da região aVLS conduziu à produção de movimento orofacial em murganhos, de um modo que é dependente da fase de execução movimento e do contexto em que a acção é aplicada.

Sendo que há muito ainda desconhecido sobre a anatomia dos circuitos dos gânglios da base e a sua respectiva funcionalidade, mais estudos serão necessários para compreender a conectividade entre estes diferentes núcleos cerebrais, e o seu papel na selecção de acções, aprendizagem, contextualização e função motora. Uma vez que neste estudo mostrámos que este circuito pode ser modulado por contextos aprendidos, seria interessante estudar a actividade desta população ao longo do processo de aprendizagem de uma tarefa, e compreender se esta população neuronal - que parece estar implicada na iniciação de uma acção - estará mais activa numa fase mais cedo

da aprendizagem. Este estudo teve como foco a produção de movimentos orofaciais. Porém, é possível que outros movimentos paralelos estejam a ser produzidos durante a estimulação optogenética desta região, tanto a nível corporal como a nível facial, pelo que se poderia investigar esta possibilidade para, potencialmente, compreender se existe um gradiente de funcionalidade motora nestas regiões, e os seus movimentos distintos associados.

Palavras-chave : gânglios da base, selecção de acções, estriado, comportamento, circuitos.

ABSTRACT

The production of coordinated movements requires the synchronised activity of multiple neuronal networks that are distributed in different brain regions. In particular, the basal ganglia plays a critical role in deciding what action is selected in a given context, based on previous learned behaviour and on the sensorial information available. These domains are organised in functionally specialised basal ganglia areas with distinct axonal inputs and outputs, as well as specific gene expression patterns (e.g. D1/D2 dopamine receptor), - thus allowing to anatomically integrate and process the relevant information required to select and execute specific movements.

Recently, the functional and anatomical connectivity of basal ganglia has started to be investigated in a systematic manner. In the striatum - the most prominent structure in basal ganglia - striatonigral and striatopallidal pathways work together to facilitate or suppress an action based on past learning and present context. Therefore, the plasticity needed to generate a wide range of adaptive behaviours arises from dopaminergic neuromodulation of simultaneous activation or suppression of a combination of striatal circuits. By carefully dissecting this circuit board, we can begin to identify key clinically interventionable circuit nodes that go awry and give rise to maladaptive states like those observed in Parkinson's or Huntington's disease.

In this study, we investigated how the striatonigral pathway affects orofacial movement in mice. To this end, we trained mice in an headfixed olfactory-guided operant task to a) associate odours with the action of licking to receive a reward, b) suppress licking to avoid a punishment and c) suppress licking for a delayed reward. Then, we optogenetically stimulated striatonigral D1 receptor expressing neurons in anterior ventro-lateral striatum and explored the role of this circuit in licking behaviour. We found that D1 stimulation elicits licking that is dependent on light frequency, action phase and context.

Keywords: basal ganglia, action selection, striatum, behaviour, circuits.

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FIGURE LIST

ABREVIATION LIST

BG - Basal ganglia

SNr - Substantia nigra pars reticulata

SNc - Substantia nigra pars compacta

VTA - ventral tegmental area

GPe - Globus pallidus external

STN - subthalamic nucleus

aVLS - anterior ventro-lateral striatum

OCD – Obsessive-compulsive disorder

ITI - inter-trial interval

ChR2 – Channelrhodopsin 2

eYPF – enhanced yellow fluorescent protein

GFP – green fluorescent protein

PFA - Paraformaldehyde

BSA – bovine serum albumin

PBS – phosphate-buffered saline

1. INTRODUCTION

1.1. Movement

Movement is an intricate adaptive feature essential for the survival and proliferation of all species. It enables the individual to engage and react to the surrounding environment: from cilia in bacteria, to stomata in plants, and finally, complex neural networks in animals.

During evolution, the appearance of a central nervous system - a significant development from the simple neuron mesh in cnidaria – served not only to integrate information collected by sophisticated sensory organs, but also to generate complex behaviour output. Therefore, an individual was now able - in addition to mapping the rapidly changing physical features of the surrounding environment - to generate complex cognitive strategies and estimating cost-benefit ratios, thus maximising the likelihood of survival and reproduction [1].

Humans and animals share common cognitive domains that allow intricate social interactions, flexible learning strategies, playfulness, creativity, empathy. However, in order to implement these cognitive abilities into actions, the main behaviour output of the brain is movement.

1.2. Learning and Action selection

In its daily life, an animal will use actions, either to explore its environment (spontaneous actions) or to achieve a predicted outcome (intentional)[2]. Some actions are performed so frequently they become automated - or habitual - representing well-learned motor programs that are dispatched when the occasion is right (e.g., reaching and picking up a glass of water) [3]. This learning is achieved by associating a stimulus to a certain response. Other tasks are non-automated, designated as intentional or self-generated. Learning, on these occasions, depends on the resulting consequences of the action. Hence, an action is initially executed spontaneously and, if its consequence is positive or desirable, the execution of this action is reinforced – a process called reinforcement learning. These actions are generally most useful in novel scenarios or with higher uncertainty variables, and require a higher level of executive attention, or cognitive control [4,5].

Acquiring and learning these intentional actions is accomplished by integrating sensory and motor circuits to enable the dynamic evolution and modulation of those actions based on their consequences (cost-benefit ratio). After several iterations of this process, the ultimate result is the creation of repertoires of effective, goal-oriented actions[6].

For this learning to occur, several steps must be met: 1) the selection of the best action for a given context by comparing the expected value of each of the possible actions; 2) the execution of the selected action successfully, and 3) posterior evaluation of the result of the implemented action (outcome) [7]. Therefore, in the initial phase of motor learning, animals produce random exploratory actions, initially with poor performance. Next, during more insightful behaviour, the subject links an action to a goal by comparing appropriate and inappropriate ways to reach the goal and repeats the action accordingly. In a third phase of motor learning, motor activity is adjusted to optimise the outcome and lastly, the action is so optimised it becomes a skill or a habit, if the value of the outcome is kept the same [7].

There are two types of associative learning. Pavlovian learning, in which an initially neutral conditioned stimulus starts to elicit anticipatory reflexive behaviours, through repeated pairing with a positive event. The second type of associative learning is instrumental learning, or operant conditioning[8]. In this case, an animal learns that performing an action increases the probability of a desired outcome to occur or to avoid an undesired outcome [9].

Within instrumental learning a further division is established, between habit learning and goal-directed learning. In habitual learning the stimulus ends up eliciting the learned response and becomes independent from the outcome. In goal-directed learning, representations of the outcome of actions are created in terms of their value and causal antecedents (action-outcome contingency)[9].

The resulting repository of information gathered by learning mechanisms is fundamental for the ensuing process of decision making - action selection. In selecting an action, for any given circumstance and within a certain context, this assembled repertoire of information is used to single out the most appropriate course of action to perform based upon an evaluation from a variety of possible value choice alternatives [10,11]. Furthermore, the same choice might have different outcomes associated with it, and different reward values, whether it is available for one context or another, hence the ability of recognizing contrasting contexts is fundamental. This contextualization is thought to be driven by areas of the brain such as the hippocampus and the orbitofrontal cortex [11]. Other circuits, in particular in basal ganglia's direct and indirect pathways of songbirds have also been found to take part in these computations [12,13].

However, learning and selecting appropriate actions encompass only the first steps in motor production. The central nervous system must then operate in a way to implement the selected actions - action execution - through specific muscle contraction patterns, and with temporal and spatial precision. Since many behaviours in the natural world entail a series of complex, intricate, orderly movements, motor implementation in the nervous system can be described through "sentence-syllable" analogy. Motor output is achieved by connecting sequences (chunks or "sentences") of more simple movement elements (motor primitives or "syllables"), to obtain a single motor program - an action - to execute a task [14,15]. These simple movement elements, or motor primitives, are encoded within the dynamics of neuronal sub-networks and their task-specific activation elicits repeatable, coordinated spatio-temporal patterns of muscle activity [16].

The ability to organise individual elements of action sequences in a single chunk allows for more reliable recall and more efficient performances, in particular on occasions where fast and precise control is required [14,17]. One brain region proposed to be implicated in the organisation of motor and cognitive actions into chunks is the basal ganglia, given that its dysfunction was noted to affect chunking ability in both animals and humans [18,19]. Moreover, neurons within basal ganglia circuits, in particular those belonging to direct and indirect pathways, have been found to have their activity related to neural start/stop signalling sequences, and to have contrasting roles at different points throughout the execution of a motor sequence, displaying either sustained or inhibited activity [17]. Nevertheless, it is not yet well understood how unitary action elements are concatenated into motor sequences, and how discrete sequences are translated within basal ganglia circuits. Hence, further exploration of these circuits could prove helpful in elucidating motor control and chunking within these structures.

1.3. **Movement Production**

Movement and dynamic three-dimensional posture changes represent the building blocks of common behaviours like spatial navigation, foraging, prey detection and escape, social interaction, all of which require flexible and rapid changes in body position [20]. In order to interact effectively with the environment, the brain has the ability to choose and initiate voluntary movements. From an ethological perspective, action selection can be driven by strong external stimuli (ex: danger avoidance behaviour cued by external stimuli, like the sighting of a predator) or internally triggered (self-paced) [21]. Given that in reality voluntary motor behaviour is a result of a combination of both internal states and external sensory input, trying to dissect the specific contribution of each branch to cognitive decision making remains challenging.

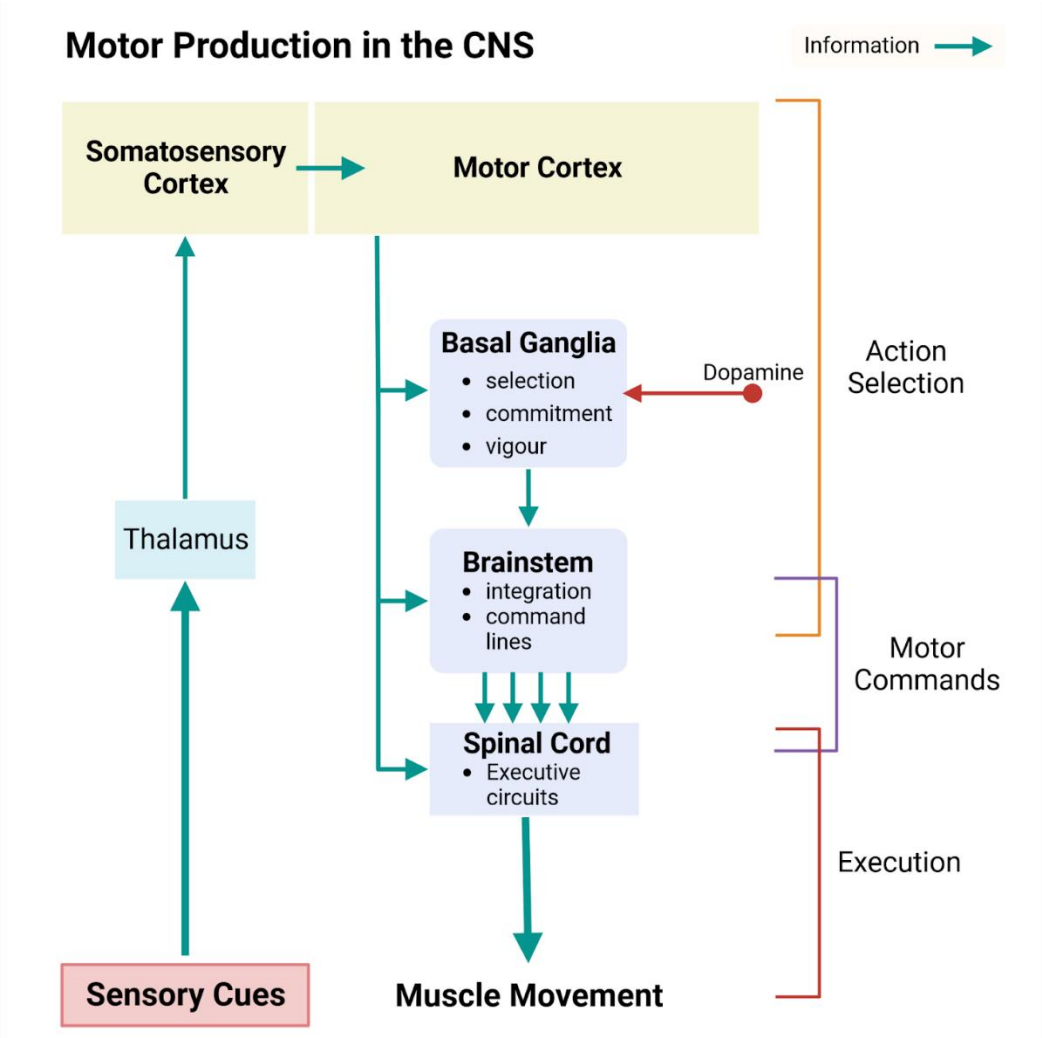

Figure 1.1 - **Motor production in the central nervous system (CNS).** Green arrows indicate the flow of information within the CNS. Sensory cues from somatosensory neurons arrive at the thalamus - a relay centre between the brain and the nervous system - where it is passed on to cortical areas. Modalities of sensory information are integrated and processed and eventually led to the motor cortex. The selection and planning of an appropriate action given the available sensory information is formulated between the motor cortex and other downstream structures - the basal ganglia and brainstem. Intricate planning of the selected action is followed by the deployment of relevant motor programs and corresponding activation of executive circuits, culminating in movement in the body (figure adapted from Arber 2018[22]).

Historically, rodents have been employed to investigate how body movements are initiated. The selection of a motor pattern in the central nervous system first begins with the arrival of sensory

cues to the thalamus. This brain structure functions as a relay to cortical areas, where incoming information is processed and the process of action selection starts. When a motor program is then selected, its correct downstream implementation occurs at descending pathways in spinal cord and ultimately, effector motor neurons innervating target muscles (Figure 1.1) [22]. Even a simple consummatory behaviour like feeding and drinking, essential for the survival of an animal, can be segmented in different phases driven by specific neuronal circuits. For example, in rodents, licking is a self-initiated stereotyped movement which repeats with a constant frequency of 6-9 Hz [23]. However, a simple protrusion of the tongue requires complex orofacial movements that rely on the coordinated activity of many facial muscles. A critical region in the brain that controls voluntary oromotor behavioural output is the basal ganglia.

1.3.1. Basal ganglia and orofacial movement

Sitting below the cortex, the basal ganglia (BG) are a set of nuclei conserved in all vertebrates that are uniquely positioned to receive input from motor, somatosensory and frontal cortex thus integrating sensory and cognitive information and subsequently relaying it downstream to major descending motor pathways. Therefore, they can effectively control movement execution of a voluntary action, as well as the dynamic refinement of action performance of a skilled motor output [5,24].

These central functions of the BG are supported by disease states (e.g., Parkinson) or artificial chronic manipulation of neuronal activity specifically in this area resulting in changes in movement kinematics[25–27]. In seminal experiments conducted in the 70s, cats and rats without intact BG show only reflexive licking, while cats lacking the cerebral cortex can still self-initiate licking, thus providing the first clues that BG is involved in the control of the voluntary aspect of motor behaviour [28–30]. Recent technological advances have allowed higher resolution in targeting specific neuronal populations in the BG (e.g., manipulation of neuronal activity by chemogenetic or optogenetic methods) and thus further dissecting the impact of BG in different aspects of movement like timescale, vigour, commitment, gain modulation, and variability [9,27,31].

1.3.2. Basal ganglia anatomy

The exploration of higher resolution examination in BG studies have permitted to look in detail at the sub-organisation of the neuronal populations of these structures. The segregation of neuron populations is seen at both anatomical and functional levels in well-defined nuclei. Most projections arriving to BG do so through the striatum, while the principal output pathway structures consist of the globus pallidus external (GPe) and substantia nigra reticulata (SNr) [27]. However, it is the complex interconnected pathways between these nuclei that generate the essential computations that are required for integration and execution of voluntary motor movement at the BG level[32].

There can be two perceptions on the organisation of these internal circuits and their role in motor production. First, different regions of BG nuclei are devoted to different functionalities (e.g., motor, associative and limbic functions), but are also somatotopic functionally discrete. Cortical topography (e.g., the sensory homunculus) is also translated into somatotopically distinct regions within the BG structures. It has been observed that the ventro-lateral portion of striatum (VLS) is involved in motor control of orofacial movement and licking behaviour. Lesions of the VLS have been found to hinder movements of the tongue in rats [33]. Additionally, it was found that VLS receives excitatory inputs from cortical motor and somatosensory regions related to the mouth [34].

Although licking action is produced by pattern generators in the brainstem, these studies and other studies suggest that the somatotopic location for orofacial motor control in striatum is the VLS, and that output from VLS and other BG structures may provide top-down regulation to downstream motor circuits [35]).

Secondly, there are two main classes of neuronal pathways going through BG that encompass biochemically and morphologically distinct projection neural populations and have distinct roles in the modulation of movement: the direct and the indirect pathways. Both these circuits originate in the striatum - the most prominent BG nuclei - and are led to output BG structures [36]. However, the neurons projecting these pathways express different receptors in their membranes: direct pathway neurons express dopaminergic type 1 receptors (D1), while the indirect pathway neurons express dopaminergic type 2 receptors (D2) [37].

Further differentiating these pathways is the circuit anatomy. The pattern in which the different nuclei of BG project and interact with one another defines how motor output in this area of the brain is either facilitated or suppressed. In a classical vision, direct and indirect pathways, respectively, inhibit and disinhibit SNr cell activity and therefore modulate the activation of its downstream structures [36,38]. Direct pathway neurons project from striatum directly to SNr - thus being named direct pathway, or striatonigral pathway - inhibiting its activity. SNr, as an output BG nuclei, exhibits high baseline firing and maintains its motor downstream structures - brainstem, thalamus, superior collicullus - under tonic inhibition, allowing animals to stay still and to choose and perform goal-directed actions. By inhibiting SNr, the direct pathway lifts inhibition in downstream motor areas and facilitates the production of voluntary movement by allowing the release of motor programs[39–41]. On the other hand, the indirect pathway projects from striatum to output nuclei SNr indirectly, via GPe and STN - thus being referred to as indirect pathway or striatopallidal pathway. The indirect pathway is thought to suppress the production of voluntary actions by means of SNr disinhibition. To understand this process, note that a) GPe, when active, projects both to SNr and to STN, decreasing activity in both nuclei, and b) activity in STN increases activity in SNr. Therefore, when activated D2 cells silence GPe activity, it ceases to inhibit SNr and STN. Increased activity in STN leads to more activity in SNr. Therefore, SNr sees its activation increased simultaneously by activation from a less suppressed STN and from less inhibition from an inhibited GPe (disinhibition) (Figure 1.2)[25,26,42].

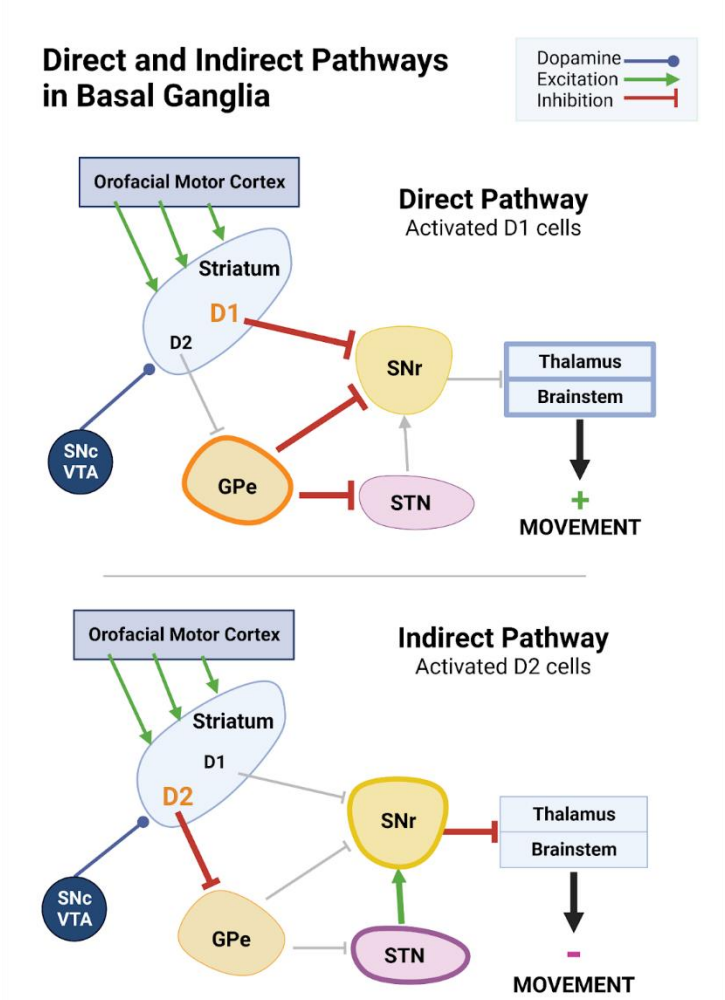

Figure 1.2 - **Direct and Indirect Pathways in Basal Ganglia.** Activation of D1 receptor cells of direct pathway in striatum increases inhibition of SNr, reducing silencing in downstream motor areas and facilitating motor production. Activation of indirect pathway D2 expressing neurons in striatum reduces inhibition in SNr, leading to inhibited downstream motor areas and suppressed motor production.

However, this classical model of direct and indirect pathways in BG for inhibition and disinhibition is simplistic, as multiple new functional connections between BG nuclei have been described. These connections interact with both striatopallidal and striatonigral pathways and within striatum itself [43–45], hinting that BG circuits might be more complex that initially thought and implicating new roles of these pathways for motor production and action selection.

Nevertheless, these models of inhibition and disinhibition have been helpful in the study of BG disorders and dysfunctions.

1.4. Clinical Significance

Historically, neurological studies relied on loss of function observations, where patients exhibiting abnormal behaviours would later be found to have suffered lesions in particular brain areas. Such is also the case with diseases that affect motor control. Parkinson's disease, Huntington's disease, hemiballism and obsessive-compulsive disorder, have been found to be

related to lesions or loss of function in brain areas thought to be responsible for control of motor function: the basal ganglia[25,46]. Below, I briefly review these pathologies.

Parkinson's disease is a progressive neurodegenerative disease that is linked to the degeneration of neurons critical for the production of dopamine in substantia nigra pars compacta [47,48]. The causes for this loss are still unclear, as patients that suffer from this disorder exhibit a vast range of motor abnormalities that result in difficulties in controlling unwanted movements. Primary symptoms characterising this condition include akinesia (difficulty in initiating movements, poor quality of action produced), bradykinesia (slow movement), rigidity (resistance of muscles to change tone from passive stretching) and tremor (at rest, in posture or during voluntary movement), all of which greatly affect daily life and prevent patients from conducting normal behaviour [49].

Huntington's disease affects not just motor control but also cognition and other aspects of behaviour. It is a neurological disorder brought about by a single gene that encodes for a mutant form of the protein huntingtin, leading to neuron dysfunction and death. Striatal medium spiny neurons are particularly affected by mutant huntingtin related harm, resulting in involuntary, uncontrollable movements (chorea) and impaired voluntary movements (incoordination, rigidity, bradykinesia) [50].

Hemiballism is a hyperkinetic movement disorder characterised by acute onset of involuntary, high amplitude, often abrupt movements of the limbs affecting only one side of the body (hemichorea). The emergence of these movements is associated with damage to corresponding contralateral BG and subthalamic nucleus (STN) [46].

Obsessive Compulsive Disorder (OCD) is considered a chronic neuropsychiatric disorder where the patients suffer from recurrent persistent thoughts or/and repetitive compulsive behaviours [51]. The motor aspect of this disorder - compulsions - is thought to arise when cognitive processes and motivation to act persist because feedback from motor behaviour fails to reset said cognitive thinking and motivation, leading to repetitive action despite the outcome [51]. Examples of this are excessive washing, or repeatedly checking for something. Although several brain areas are implicated in this disorder, the dopamine system in BG is thought to be involved in OCD. In particular, the striatum is central to establish ritualization and lesions occurring in this area have been reported to produce acquired OCD [52,53].

Because these conditions affect patients' ability to control movements and are related to damage in BG, this group of structures are thought to be composed of the key circuits involved in voluntary movement control. Understanding them and further dissecting their connectivity could be useful for therapies that bypass the malfunction of these structures and restore functional motor control in affected patients.

Recent developments in technology have increased researchers' ability to manipulate neural cells in vivo. In fact, developments in implantable electrodes have been much useful in animal studies but have also represented a major advancement in clinical therapies, as is the case with deep brain stimulation and its positive effects in patients suffering from Parkinson's disease [54]. Other methods of in vivo brain manipulation arised to allow more precise control of specific neuronal activation, and have become hallmarks in neuroscience studies, in particular methods in optogenetics, which I will approach next.

1.5. **Optogenetics**

The optogenetic neuromodulation technique integrates optical and genetic methods within specific targeted cell types - both in living tissue and in living animals - to achieve precise spatio-temporal control of cellular electrical activity [55,56]. Implementation of this technique encompasses three different disciplines: a) genetic approaches - to induce the expression of light sensitive proteins in the cells of interest; b) technologies for delivering light at precise time points and specific frequencies - to manipulate the cells; and c) methods that enable useful readouts from the manipulations - such as quantitative behavioural analysis [57].

Optogenetics uses light to either hyperpolarize or depolarize a designated set of neurons, with great time precision and within intact neural circuits of living, moving animals. To achieve this, the Cre-Lox system is used, where transgenic mouse lines are engineered to express the Cre recombinase in association with cell specific promoters. The cells are then infected, through injection in a particular area with a viral vector (often an adeno associated viral vector, AAV) that includes a floxed sequence for an opsin[58]. Cre proteins recombine with the region of interest in the vector, and the opsin is expressed in the desired cells. Microbial opsins are light-responsive proteins that suffer conformational changes by absorbing photons, subsequently allowing the flow of ions through a membrane [59]. There is now a plethora of opsins available, from microbial and animal origins, including several channelrhodopsins (ChR2, ChR1), and halorhodopsins, which can induce hyper or depolarization in nervous cells. In addition, different opsins are activated by different light frequencies and can be used simultaneously in the same place[60].

In deep brain optogenetic manipulation, an optic fibre is implanted in specific coordinates to match the exact region where the opsin-carrying virus was injected. It is then possible to control light intensity and light delivery patterns, to simulate different excitation levels in neurons, with tonic and phasic firing [61]. More recently, developments have been achieved to allow not only for the general somatodendritic spiking evoked by light stimulation, but also to specifically target the presynaptic membranes of the desired neurons [62].

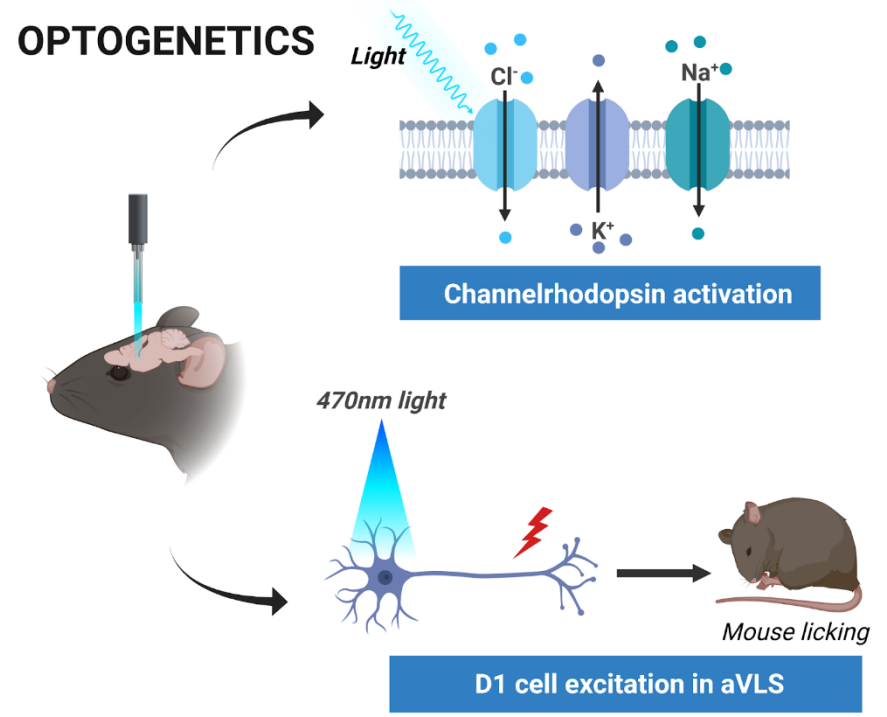

Figure 1.3 - **Optogenetics.** Optogenetic technique enables the targeting of specific neuronal populations with precise spatiotemporal control. Light induced channelrhodopsin activation in the membrane allows for the change of conductivity and neuron excitability, leading to behavioural outputs (figure adapted from BioRender.com).

As with any technique, optogenetics is faced with its own constraints. For instance, after repeated stimulation opsins might turn less responsive and require a recovery period, becoming desensitised in a process referred to as bleaching [63]. Furthermore, heat derived from illumination during optogenetic manipulations could impact temperature dependent processes, such as ion channel conductance and synaptic transmission, and therefore interfere with interpretation of experiments [64]. Nevertheless, the possibilities for application and adaptation of this technique are vast[65], and in a recent application in medicine, optogenetics was successfully used therapeutically to partially restore vision in a human patient with neurodegenerative retinitis pigmentosa [66].

In the present work we used an optogenetic manipulation approach to modulate electrical activity of dopaminergic receptor D1 expressing neurons present in the anterior ventro-lateral striatum of mice, to observe its impact in the orofacial motor behaviour in these animals.

1.6. **Previous Work by the Group**

The study from which the experiments described in this dissertation take part was conducted in Dr. Rui Costa lab with the collaboration of Dr. Megan Carey lab and Dr. Zachary Mainen lab, during the years between 2013 and 2019. The study's main purpose was to investigate the anatomy and function of striatal downstream circuits, namely the striatonigral and striatopallidal pathways, and its involvement in initiation and execution of voluntary motor behaviour in mice, and thus further expand the knowledge of these important neural circuits[2].

In order to assign a specific behaviour to a certain circuit, it is necessary to ascertain anatomical (e.g., histological evidence of neuronal projections), functional (e.g., behavioural output from said

circuit manipulation) and physiological information (e.g., electrophysiological responses of the neurons) that are complementary. Initially, an anatomical approach was taken to understand how different areas of striatum project onto their output structures, and whether functionality in this structure is somatotopically organised. Striatonigral and striatopallidal output projections of aVLS were mapped onto their output nuclei, SNr, and GPe respectively, by labelling different striatal neuron populations simultaneously. It was shown that D2 (striatopallidal) populations project directly to corresponding areas of GPe (dorsal striatum to dorsal GPe, lateral striatum to lateral GPe, etc.). Further, D1 (striatonigral) populations projected onto SNr correspondingly in the mediolateral axis (medial to medial and lateral to lateral), but in the dorso-ventral axis the projections target inversely (dorsal to ventral and ventral to dorsal). This could indicate spatial and functional selectivity between corresponding areas of different structures[2].

In succeeding experiments, the team then focused on a specific region: aVLS, and studied its upstream projections, observing that primary and secondary orofacial motor cortices project onto aVLS, further suggesting this area of striatum is implied in this type of movement [67]. To explore this idea, D1 populations in aVLS were optogenetically stimulated in freely moving mice, evoking head and jaw movements and licking. It was also demonstrated that aVLS activation was sufficient to induce head, jaw and licking movements, but activation of other areas did not evoke the same results. Physiology results indicate that both D1 and D2 pathway neurons show activity during initiation of instrumental licking, but they do it differently, indicating they are needed alternatively for successful initiation and execution of this behaviour.

Given that the striatum is related to learning and motivation, the team developed a behavioural, head fixed operant olfactory guided task to further explore the role of aVLS in motor control and action initiation/suppression. In this set of experiments, D1 and D2 cells were studied both through optogenetic and through calcium imaging techniques, and observations were performed at different timepoints of the behavioural task and using different light frequencies.

For brevity, in this dissertation I will focus on the stimulation experiments of D1 neuronal populations in aVLS of Cre-D1 mice, using excitatory opsin ChR2 and with optogenetic light frequencies of 2 Hz, 5 Hz and 10 Hz in a particular time period of the behavioural task: during 4s, from 1s after odour delivery to 2s after reward delivery.

1.7. **Objectives and Outline of the Experiment**

The main purpose in the study described in this dissertation is to investigate the contribution of the striatonigral pathway cells in aVLS in the initiation and execution of instrumental licking movement in mice.

To conduct this, 8 D1-Cre transgenic mice were cranially injected with a viral vector for opsin expression in aVLS, and optic fibres were implanted in the same area, bilaterally. Subsequently, mice were trained to learn an operant olfactory guided task. Once the task was learnt, optogenetic stimulation was conducted and behavioural output recorded.

We propose that optogenetic manipulation of striatonigral pathway cells in aVLS will induce orofacial movement that will change the licking responses in mice during the stimulation window, in a way that is context dependent and that changes differently in separate aspects of the motor response.

2. METHODS

2.1. Animals

All procedures were performed according to the European Union Directive 2010/63/EU and the Champalimaud Centre for the Unknown Ethics Committee guidelines and were approved by the Portuguese Veterinary General board. Approval 0421/000/000/2014.

D1-Cre (FK150-Cre) transgenic C57BL/6 mouse line was used, expressing Cre recombinase in cells that express dopamine receptors type 1 (D1). Male mice (n=8 and n=3 for control mice) of 10 to 24 weeks housed under normal light cycle (white light from 8am to 8pm) were used for all experiments. Training of mice and experiments were performed during the normal light cycle. Animals were housed in groups prior to surgery and individually housed post-surgery.

To induce expression of channelrhodopsin ChR2 in D1 cells, the following adeno-associated viral vector was used: AAV2/1-EF1a-ChR2(H134R)-EYFP-WPRE (titre $1.4x10^{13}$, University of North Carolina). Viral vector aliquots (5µL) were stored at -80^{o}C, and were thawed on ice to be used on the day of the injection.

2.2. Stereotaxic viral injections and optical fibre implantation

Animals were anaesthetized using isoflurane and given a mix of oxygen and anaesthetic throughout the procedure to ensure correct depth of anaesthesia (4% isoflurane for induction of anaesthesia inside an anaesthesia chamber and 1 to 2% for maintenance in a mask, at a flow of 1 litre/min) and were placed in a stereotaxic instrument (David Kopf Instruments), over a heating pad. Eye ointment was provided to protect eyes from drying out. Aseptic practices were kept throughout the procedure to reduce infection risk. Butorphanol (0.4 mg/kg) was injected subcutaneously for analgesia. The hair on the scalp of the animal was shaved and the skin disinfected with iodine solution and ethanol 70% prior to the incision. The incision was made longitudinally along the skull of the animal, and forceps placed to keep skin away from the working area. With the use of the stereotaxic instrument the head was positioned to be properly levelled. Next, H2O2 solution at 10% was dabbed with a cotton tip to remove any connective tissue from the skull surface. The coordinates for the fibre implantation were mapped and marked on the skull using the stereotax and a craniotomy of around 1 mm in diameter was subsequently drilled. Coordinates relative to Bregma for targeting of aVLS were: AP:+1.0mm, ML: 1.85mm, DV: 3.0mm, bilaterally.
Bilateral injections of 1µL of AAV2/1-EF1a-ChR2(H134R)-EYFP-WPRE were performed using injection system Nanoject 2 (Drummond Scientific) and glass micropipettes with 25-35um tip size. The injections are done through 4,6 nL pulses with 5 second intervals. The injection pipete was pulled out slowly about 10 to 15 minutes after injection completion[68].
Control mice were injected with EYPF expressing viral vector AAV2.2-EF1a-DIO-EYFP-WPRE (University of North Carolina, titer 1.85x1012).

Optical fibres (230 µm diameter and 10 mm in length, with zirconia ferrule) were produced in-house according to a previously published protocol [69](FMT200 EMT, NA 0.39, Thorlabs) and were chronically implanted bilaterally 100um above the injection site (DV: 2.9mm depth).

After correct positioning of fibres, the craniostomy was filled with silicone. The skull surface was cleaned and dried and covered with a layer of Super Bond C and B (Morita). A metal bar was positioned posteriorly to the fibres to be used for head fixing during training and experiments. The implants and the bar were secured with dental acrylic (Pi-Ku-Plast HP 36, Bredent). Post-surgery care was provided to ensure proper healing of the wound around the implant and to reduce infection risk.

2.3. **The behavioural task**

The setup for the behaviour experiments (see Figure 3.1-A in Results) consisted of a box with a headstand in which the mice's head was fixed, and an acrylic tube where the mouse would stay during training. Setup box included a camera for video recording of the training and data collection sessions. A tube for odour delivery was placed roughly 2 cm away from the animal's nose, and a second tube for air puff delivery (punishment, see below) was placed about 3cm away from the left eye area. Mice licking movements were detected using a lickometer (007120.0002, Island Motion Corporation) where an infra-red photodetector was used to measure licking at 1 KHz, located on two sides of the water delivery port. Each time the mouse licked to reach for the water port, the infra-red beam was broken, and the licking time was recorded. The sound tones that signal the beginning of the trial and the different outcomes were amplified (PCA1, PYLE Audio Inc.) and delivered through speakers (Neo3 PDRW W/BC, Bohlender-Graebener)[70]. Water valves (LHDA1233115H, The Lee Company) were calibrated and used for the delivery of water. The delivery of odours was achieved through a custom made olfactometer designed by Z.F.M. (Island Motion). Odours were diluted in mineral oil (Sigma-Aldrich) at 1:10 and 25 µL of each diluted odour was placed inside a syringe filter (2.7 mm pore size, 6823–1327, GE Healthcare), placed in the olfactometer, and used in two sessions (up to 200 trials for each odour) and replenished as needed[70]. Odorized air was delivered at 1000 ml/min. Odours used were cuminaldehyde (for go-trials), octanol(S)-(+) (for nogo trials), carvone (R)-(-) (for wait trials) and limonene R-(+) for neutral trials. These odours were novel to mice, having no previous associations.
Licking data from the optical lickometer was acquired using a behavioural control program (Bcontrol), developed by Carlos Brody (Princeton University), Calin Culianu, Tony Zador (Cold Spring Harbor Laboratory) and Z.F.M.

In previous work done by Zachary Mainen's and Rui Costa's research goups, a behavioural task was developed to further examine the role of aVLS in licking movement, after it was established that the stimulation of this brain region elicited orofacial and licking movements in free moving mice. The task developed is a head fixed olfactory guided instrumental conditioning model[2].

In this task (see Figure 3.1 in Results), mice were conditioned to modulate licking behaviour differently, in response to different odours delivered which are then associated with different conditions during training: go, nogo, wait and neutral. Each of these odours correspond to a trial type.

A trial for the task consisted of different time periods. First, a pre-trial period ranging from 1.5s to 3s (with a uniform probability distribution within those limits) where white noise was delivered. One of the 4 odours was then delivered for 1 second, followed by a 2 second trace (delay) period,

to allow time for decision making. A decision period followed, ranging from 1 to 7s (with a Gaussian probability distribution with a 4s mean). During this time licking behaviour had outcomes depending on the trial type, either a water reward, or a punishment in the form of an air-puff to the eye region. Inter trial periods (ITI) were between 3 to 5 seconds (with a uniform probability distribution within those borders). A task session would involve around 100-300 trials with randomization in trial types, depending on the learning stage of each individual mouse. The variability within time for each period in the task was set to prevent mice from learning the timing of the task and to be more attentive to the given cues.

Go-odour (cuminaldehyde) indicated the delivery of water after licking. Go trial has a tone (go tone) of 100ms 6KHz that is delivered simultaneously with a 3μL water drop when the mouse licks during the decision period. As they learned the task and for this odour in specific, mice start licking during the trace period immediately after odour delivery, in anticipatory licking behaviour. For a correct go trial the mouse licks for water after it receives the go-odour.

The nogo odour (octanol) indicates a punishment (an air puff near the face) would occur if the animal licked during the decision period. Licking during the decision period for this trial prompts the delivery of a 100ms 10khz nogo tone paired with a 100ms air puff. In a correct nogo trial the mouse withholds licking movement after odour delivery, avoiding punishment.

Wait odour (carvone) indicates a 3μL water reward is delivered simultaneously with a go-tone if the mouse successfully withholds licking and waits for a variable period of time (ranging between 3-5s).

Wait trial is the most challenging to learn because it requires active withholding of licking to receive a reward, unlike nogo, which is active withholding to avoid a punishment. In case the mouse was unsuccessful in withholding licking during wait trial decision time, the inter trial period started and no other outcome would occur.

Lastly, the neutral odour (limonene) indicates no outcome regarding mice behaviour.

2.4. **Training for olfactory guided operant task**

Water deprivation was used to increase mice's motivation during learning (by increasing the value of water as a reward). In this process, water was only available to the animals during the training sessions, which occurred daily.

Water deprivation started once the animals were fully recovered from implant surgery, around 2-3 days post-surgery. Mice were weighted daily during water deprivation and their health was monitored to ensure their body weight would not drop under 80% of original value. Additional water was given if mice performance during training did not allow for sufficient water to be consumed.

At an initial point the training consisted of classical conditioning where the mouse had to associate the setup and the go odour with a reward. Later it is shifted to operant conditioning in which the mouse needs to learn to perform different behaviours (licking or withholding licking) in order to obtain or to avoid reinforcing or punishing stimuli.

In the first 2-3 days of training mice were given light anaesthesia to reduce stress during habituation to the head fixed setup. Optical lickometer was slowly introduced to mice by gently presenting it near the mouth area with free flowing water. For the following 2 to 3 days mice were given water drops (4µL) through the optical lickometer at regular intervals between 1-2 seconds until they licked continuously and with ease in the head-fixed setup. After this pre-training phase the mice were started on the training for the olfactory guided operant task.

Go trials were presented to the mice gradually and with short initial (trace) times on the first few days of the training period. Task duration times were slowly increased as the individual learned the go-odour association to water during the first few days of training.
Once mice were performing correctly >50% go-trials, which took about 2 days, nogo odour was introduced progressively. It took about 2-4 days for mice to stop licking to avoid air-puff.
Wait trials were introduced when the mouse performed more than 50% correct nogo trials, successfully withholding licking after the odour. Wait trial was initially introduced with a fixed waiting time of 2s and gradually increased to 2.5 and 3s. After mice learned to discriminate wait trials from go and nogo trials we introduced variable waiting time that changed between 1-7s (Gaussian distribution with 4±1s). If performance of waiting dropped, the decision time was set back to 2.5 and the previous steps were repeated, until the mouse performs about 50% of correct wait-trials.
During training for the wait trial, mice achieved performance of around 100% in both go and nogo trials, and said performance stayed at those values for 1-3 weeks, depending on the performance of the mice.
Neutral odour was introduced shortly after wait performance started to increase.

Mice were trained using this protocol everyday sometimes with one day a week brake, in which they were given a limited amount of water (around 0.5mL). The duration of the training period ranged from 2 to 4 weeks. Optogenetic manipulations started after mice performed correctly 50% of wait trials for two consecutive days, and showed good performance for all trial types. We recorded 2 days baseline performance before the optogenetic manipulation days.

2.5. **Optogenetic manipulations**

Optogenetic manipulation started once mice exhibited stable performance for all trial types.

The firing rate of D1 neurons at baseline in awake mice is lower than 5Hz [17,71]. Hence, we applied 3 different frequencies: 2Hz (below baseline), 5Hz (similar to baseline) and 10Hz (higher than baseline). Stimulations with 2Hz, 5Hz and 10Hz were delivered with 10 ms pulses of blue light (473nm) and with 2.5-2.6 mW of power at the tip of the fibre. To achieve this, we used an acousto-optic modulator (to control the transmitted power of the laser beam) and fixed frequency drivers (AOM, AAOptoelectronic). Master-8 stimulator was used to define precise stimulation times.

Sessions of optogenetic manipulations were performed for 5 days, with a session per day. Only 15-20% of trials were stimulated. During each session stimulated trials were delivered randomly. Stimulations were performed 1s after the odour onset with a duration of 4s, overlapping trace and decision period. We delivered stimulation 1s post odour presentation to allow animals to make a decision, to lick or to wait.

2.6. **Brain processing**

To confirm the positioning of the fibres and the successful expression and location of the virus, the brains of the animals were imaged using fluorescence microscopy. Brains were collected post optogenetic manipulations. Brains were fixed through perfusion, sliced, immunostained and mounted in microscopy slides in preparation for imaging.

2.6.1. Perfusions

For perfusion, mice were injected intraperitoneally with a lethal dose of Ketamine/Xylazine (100µL/10g of body weight) and further anaesthetised in an isoflurane induction chamber. The mouse was secured to a board with the abdominal area facing up, and cuts were made to expose the heart. A syringe connected to a pump was inserted in the left ventricle. A cut was then done in the right atrium and the pump switched on, letting PBS 1X (about 30mL) flow into the left ventricle and be pumped into the circulatory system of the mouse, replacing the blood. When the the organs appeared clear PBS was switched for PFA 4% (P6148, Sigma-Aldrich, 50ml) and PFA was pumped into the circulatory system of mice to fix the tissues, for a minimum of 5 minutes. The brain was extracted carefully from the skull to a glass vial and kept 24h in PFA 4% at 4ºC. After the 24h PFA was substituted for PBS 1x.

2.6.2. Slicing

Brains were stored at 4ºC. Before slicing they were left at room temperature for 15-20 min to avoid condensation with agarose at the later steps. Agarose 2.4%, which is similar in density to that of the perfused brain, was prepared and poured into a small container, leaving some time to slightly cool off. When it cooled to around 37ºC the brain was lightly dried with kim-wipes to remove excess PBS and placed in an upright position in the agarose. The extremity where we begin to slice should be touching the bottom of the container and some agarose should be above the other extremity. After agarose solidified it was removed from the container and excess was cut out.

Agarose with the included brain was fixed to the vibratome plate with superglue and placed in the vibratome (Leica VT1000S). The 50µm thick slices were placed in 12 well plates with 2ml PBS1x each and afterwards plates were isolated with parafilm, wrapped in tinfoil and kept at 4ºC.

2.6.3. Immunostaining

To detect GFP fluorescence corresponding with viral expression in the brains of the animals, brain slices were incubated with blocking buffer (20% BSA, 0.5% Triton X-100 in PBS) for 2h at RT (room temperature) and afterwards incubated with antibody (goat antiGFP conjugated 488, Molecular probes/Invitrogen, 1:1000 dilution) diluted in blocking buffer for 2h, RT. Slices were then rinsed with PBS and mounted onto glass slides.

2.6.4. Mounting brain slices

The slices from one well were placed in a PBS1x bath. Slides were dipped in the bath and then gently pushed on top of the treated surface of a positively charged glass slide with a brush, carefully not to distort the shape of the slice and arrange them evenly apart (6-10 slices per slide). Next, the slide is retrieved from the bath and excess PBS removed with Kim wipes. About 140µL Mowiol (Sigma-Aldrich) was placed along the slide and a coverslip is placed on top. The preparation was let sit overnight at 4ºC after which the edges were sealed with clear varnish, dried for 30 minutes and stored in a slide box at 4ºC.

2.6.5. Imaging

Wide field fluorescence scanning microscope (Zeiss Axioimager M2) at 10X was used for imaging.

Brightfield imaging was used to illuminate the structure of the brain slice based on its density. Fluorescent signals from GFP and EYFP were acquired using specific illumination wavelengths (485 nm-517 nm). Resulting images were analysed using ImageJ (NIH, US).

2.7. **Data analysis**

Data was processed using BControl and all the analysis was performed using MATLAB 2014a or GraphPad Prism (GraphPad Software, Inc).

Three-way ANOVA paired analysis was used, in which the frequency of licking was compared among the stimulation frequencies, the individual animals, and the trial type.

ANOVA analysis was applied to the dataset as a whole (all animals, sessions, trials, and frequencies) and Tuckey-Kramer post-hoc tests were performed for pairwise comparisons.

3. RESULTS

Stimulations of striatonigral populations (D1 receptor expressing cells) in aVLS facilitated or induced licking in all trial types for 10Hz stimulations. Stimulations induced liking that affected the trial performance and changed the median lick rate during stimulated trials compared to non-stimulated trials.

3.1. Mice learned the olfactory guided operant task

Using the training procedures described in Methods, the animals learned the task and the action-outcome associations for all 4 trial types in 25-45 sessions. Animals were considered as having learned the task when they performed 100% correct trials in go and nogo trials and 50% in wait trials.

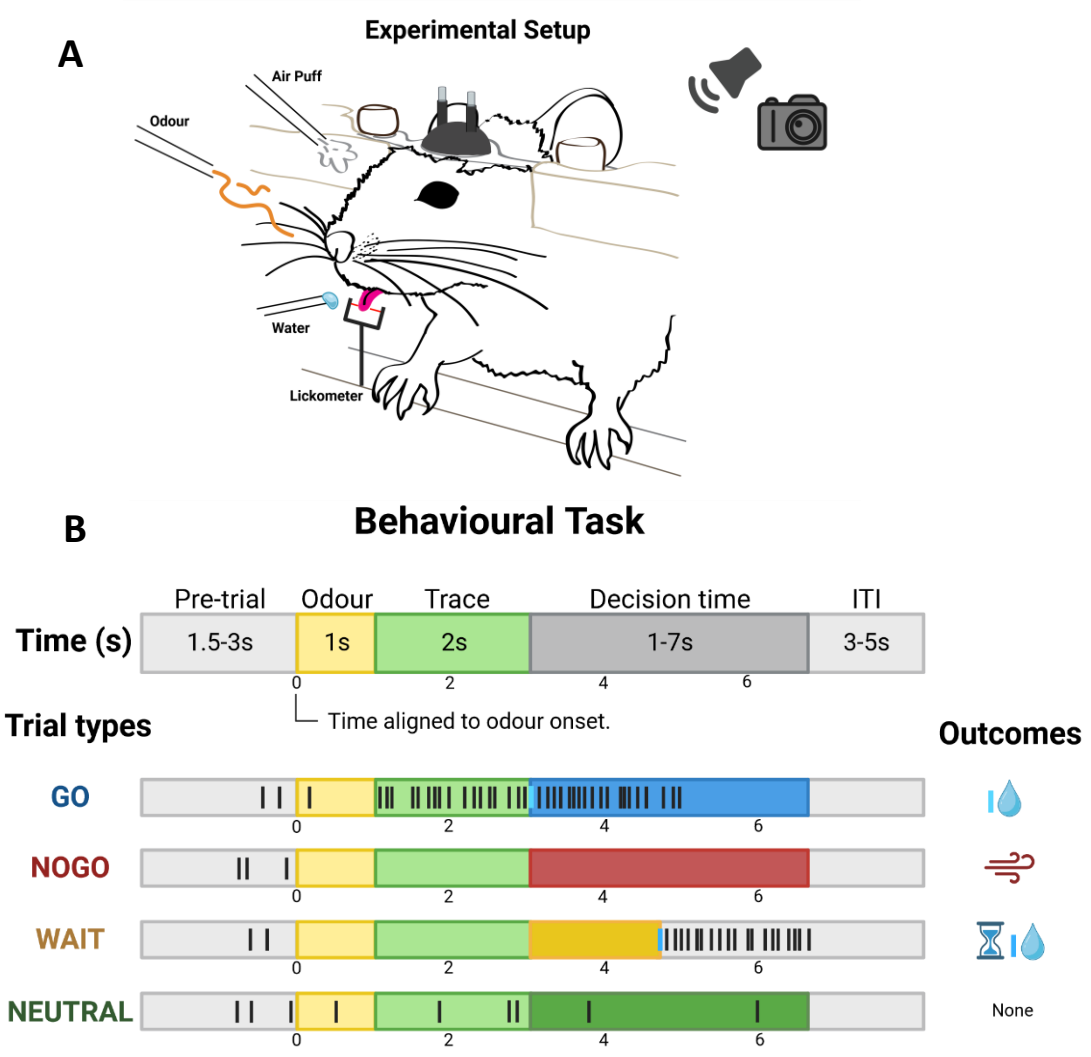

Figure 3.1 – **Experimental Setup, Behavioural Task and Baseline lick histograms. A - Experimental setup.** General schematic of the experimental setup, including sources of water, odour and air puff, optical lickometer, head fixing bar, a speaker and a camera (Figure adapted from SciDraw.com). **B - Behavioural task scheme.** Each trial is organised into different periods with variable times (s): pre-trial, odour, trace, decision time and inter-trial interval (ITI). The 4 different trial types evoke different outcomes after odour based on the animal's behaviour: Go - water reward when the animal licks; Nogo - punishment (air puff) when the animal licks; Wait - water reward if the animal waits for a variable time for the go tone; Neutral - no outcome regardless of animal's behaviour. Short black vertical bars represent one licking movement (break in the infrared beam of the lickometer).

In go trials, mice learned to expect a reward after the delivery of the go odour. This resulted in the exhibition of anticipatory licking behaviour after the onset of the odour and throughout the trace period, with lick frequency rising up to 5 licks/s. Lick rate peaked at the time of water delivery (3s after odour) and subsequently diminished (Figure 3.2, grey curves). For nogo trials animals learned to suppress licking after the nogo odour, avoiding punishment in the form of an air-puff near the eye. As a consequence, baseline lick rate histograms show lick rate to be close to 0 licks/s throughout the nogo trial (Figure 3.5, grey curves). During wait trials, the mouse learned to wait for a variable period of time, withholding licking, until a go tone and a water reward were delivered. In baseline lick histogram for wait trial, lick rate remained near 0 licks/s until 5s after odour onset (2s after trace period), after which it rises up to 3 licks/s, matching the variable period for reward delivery (Figure 3.7, grey curves). In neutral trials mice learned that no reward or punishment would be delivered regardless of licking behaviour, hence the animals learned not to lick for this trial type. Baseline lick histogram shows lick rate close to 0 licks/s throughout the trial (Figure 3.9, grey curves).

3.2. Histology data validated coordinates for optic fibres and viral expression

Data from imaging brain tissue slices confirmed the correct coordinate positioning both for bilateral fibres and for viral vector expression pattern, on all animals used for this essay.

3.3. The effect of optogenetic stimulations on Go trials

Both in non-stimulated and stimulated go trials, animals started licking after odour delivery, anticipating the reward. Lick rate increased after odour delivery and rose to 6 licks/s at delivery of water (3s after odour delivery), after which it decreased. Lick rate histograms show the change in lick rate responses of mice across a trial with and without optogenetic stimulation (Figure 3.2).

When we stimulated striatonigral pathway, during go trials, we did not see a significant change in the median lick rate or in the fraction of trials with licking when comparing 2Hz, 5Hz and 10Hz stimulated trials with non-stimulated trials (Figure 3.3, three-way ANOVA, Tukey-Kramer post-hoc tests, $p>0.05$).

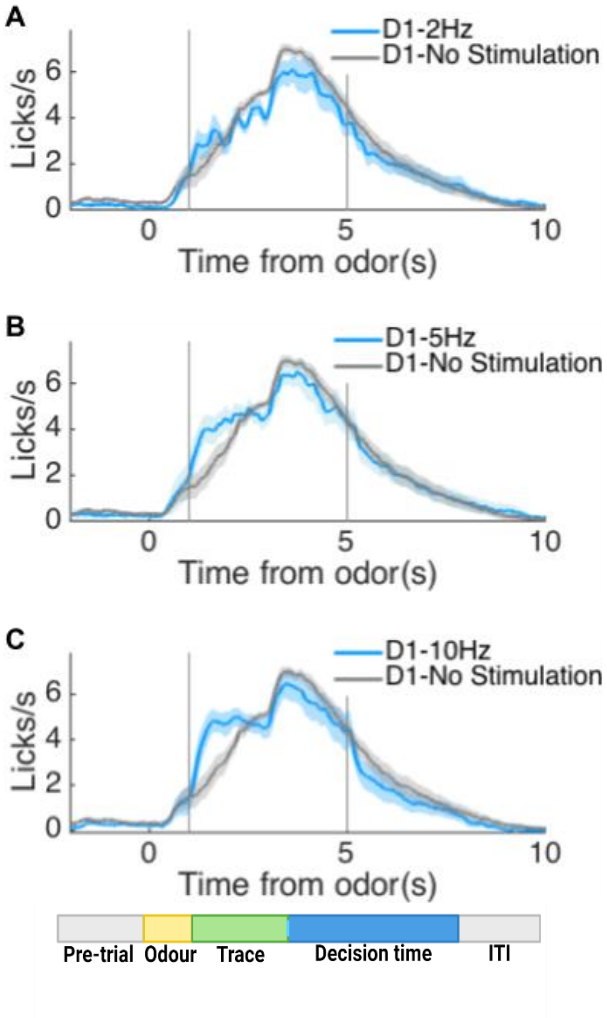

Figure 3.2 - **Lick rate histograms (licks per second) in function of trial time (time from odour, s) in go trials.** The two vertical grey lines represent the stimulation interval of 4 seconds, beginning 1s after odour delivery. Grey trace represents non-stimulated trials. Blue trace represents stimulated trials with A - 2Hz stimulation frequency, B - 5Hz stimulation frequency and C - 10Hz stimulation frequency. Lighter grey and lighter blue areas represent standard error of the mean.

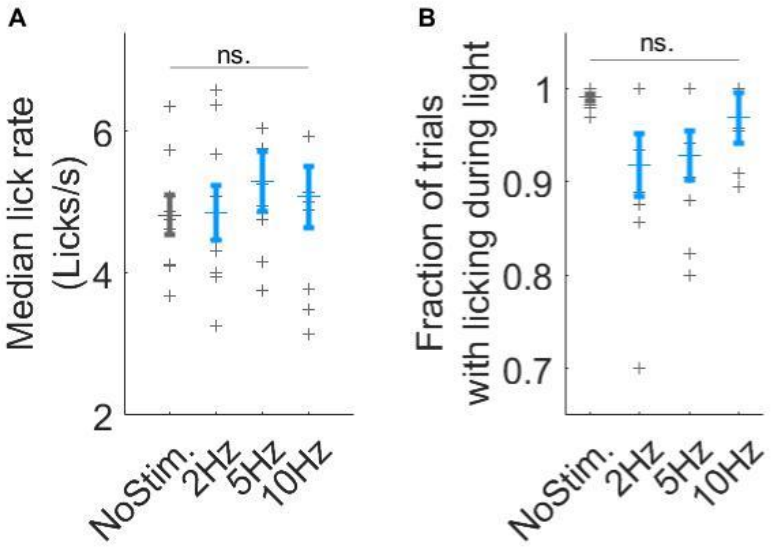

Figure 3.3 - **Median lick rate for go trials and Fraction of trials with licking during light.** A - Median lick rate (licks/s) for go trials during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. No significant difference was found between stimulation types. Error bars show standard error of the mean. B - Fraction of go trials where licking occurred during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. No significant difference was found between stimulation types. Error bars show standard error of the mean.

However, we found that 2Hz, 5Hz and 10Hz stimulations reduced the reaction time in go trials compared to the non-stimulated go trials, during the delay period (Figure 3.4-A, three-way ANOVA Tukey-Kramer post-hoc tests; no-stim. vs all stim. types: $p<0.001$). Therefore, all three stimulation frequencies facilitated initiation of licking.

Additionally, we observed that 5Hz and 10Hz stimulations - but not 2Hz stimulations - increased anticipatory lick rate (licking from odour onset to end of trace period, 3s) in go trials compared to non-stimulated trials (Figure 3.4-B, three-way ANOVA Tukey-Kramer post-hoc tests; no-stim. and 2Hz vs 5Hz and 10Hz: $p<0.001$).

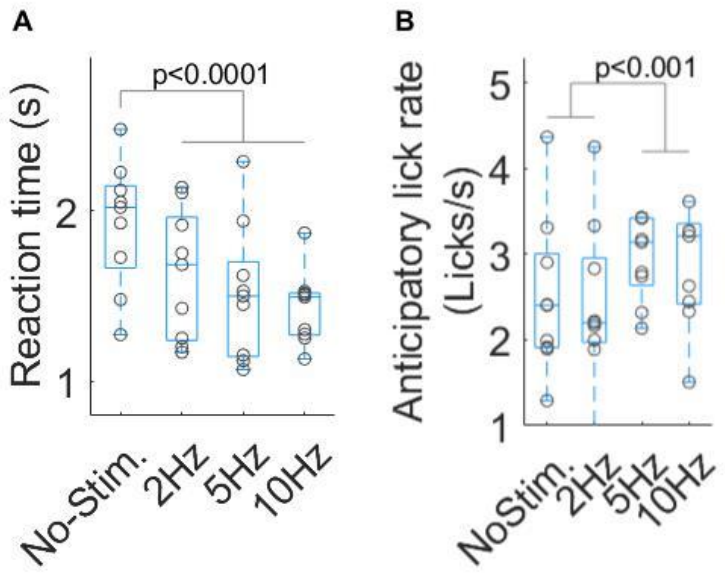

Figure 3.4 - **Reaction time and Anticipatory lick rate in go trials.** A - Reaction time in seconds shows the speed of licking initiation in a 2s period (starting 1s after odour onset). Stimulated go trials with 2Hz, 5Hz and 10Hz showed significantly reduced reaction times compared with non-stimulated trials. Error bars show standard error of the mean. B - Anticipatory lick rate (licks/s) during 2s period of stimulation (starting 1s after odour onset). Stimulation with 5Hz and 10Hz significantly increased the amount of licking before reward compared with no-stimulation and 2Hz stimulation. Error bars show standard error of the mean.

In go trials effects of stimulation were non-significant for any stimulation frequency in median lick rate and fraction of trials without licking. Stimulation had an effect in reaction time and in anticipatory licking. In the first, all stimulation frequencies led to decreased reaction times. In the second, only 5Hz and 10Hz frequencies significantly increased anticipatory licks.

For go trials, stimulation of all frequencies had no significant effect for median lick rate or fraction of trials with licking, but it reduced reaction time (all frequencies) and increased anticipatory licking (5Hz and 10Hz).

3.4. **The effect of optogenetic stimulations on Nogo trials**

In nogo trials only 10Hz stimulation showed noticeable change in lick rate during the 4s stimulation period, as seen in lick rate histograms (Figure 3.5).

In stimulated nogo trials only 10Hz stimulation was sufficient to increase median lick rate during stimulation period. 2Hz and 5Hz stimulation did not change median lick rate compared to non-stimulated trials (Figure 3.6-A, three-way ANOVA Tukey-Kramer post-hoc tests: 10Hz vs no-stim., 2Hz and 5Hz: $p<0.0001$). However, both 5Hz and 10Hz stimulation were sufficient to increase the fraction of trials with licking. Therefore 5Hz stimulations increased the fraction of trials with licking while not affecting the median lick rate during stimulation, while 10 Hz stimulations increased both fraction of trials with licking and median lick rate during stimulation (Figure 3.6-B, three-way ANOVA Tukey-Kramer post-hoc tests; 5Hz vs no-stim. and 2Hz: $p<0.0001$; 10Hz vs no-stim, 2Hz and 5Hz: $p<0.0001$).

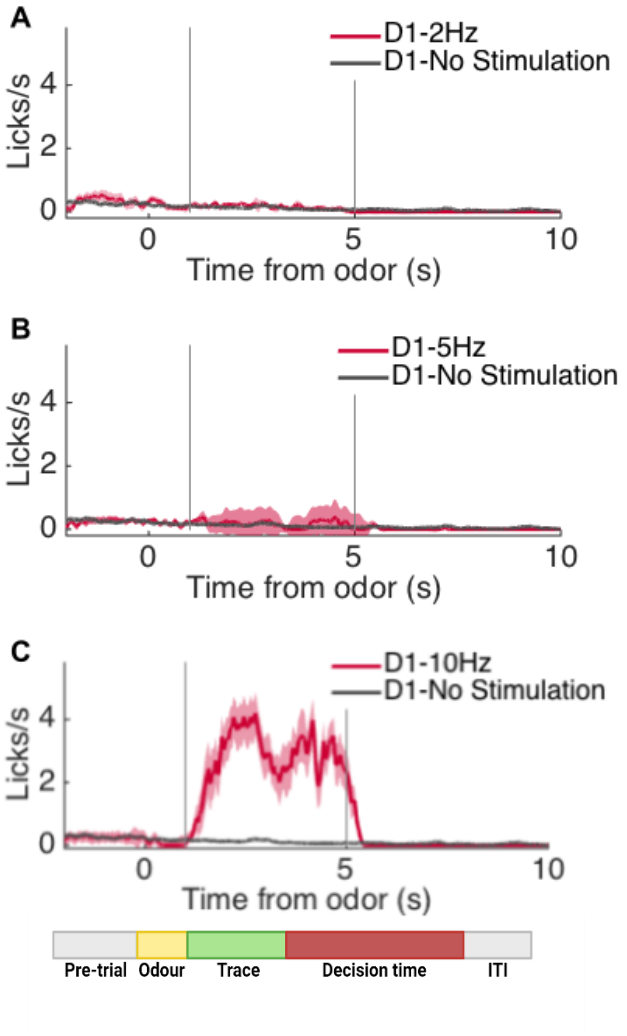

Figure 3.5 - **Lick rate histograms (licks per second) in function of trial time (time from odour, s) in nogo trials.** The two vertical lines represent the stimulation interval of 4 seconds, beginning 1s after odour delivery. Grey curve represents non-stimulated trials. Red curve represents stimulated trials with A - 2Hz stimulation frequency, B - 5Hz stimulation frequency and C - 10Hz stimulation frequency. Lighter grey and lighter red areas represent deviation from the mean. 10Hz stimulation evoked a higher rate of licking from stimulation onset throughout the stimulation period compared to 5Hz, 2Hz and non-stimulated trials.

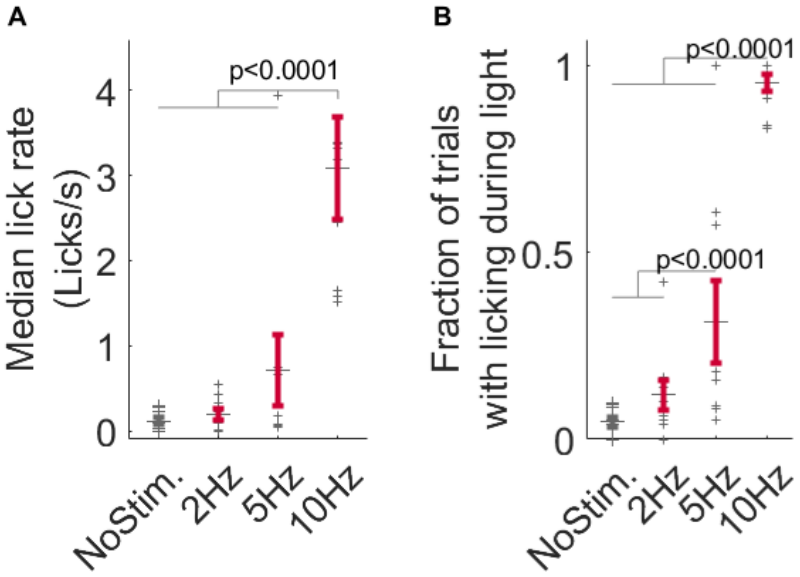

Figure 3.6 - **Median lick rate and fraction of trials with licking during light for nogo trials. A** - Median lick rate (licks/s) for nogo trials during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. 10Hz stimulation showed significant difference from other stimulation conditions ($p<0.0001$). Error bars show standard error of the mean. **B** - Fraction of nogo trials where licking occurred during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. 5Hz stimulation significantly increased the probability of licking to occur when compared to non-stimulated and 2Hz stimulated trials ($p<0.001$). 10Hz stimulation significantly increased the probability of licking to occur during stimulation compared to all other stimulation conditions ($p<0.0001$). Error bars show standard error of the mean.

3.5. The effect of optogenetic stimulations on Wait trials

Lick rate histograms for wait trials show only 10Hz stimulation produced change in the median lick rate responses of mice (Figure 3.7).

During wait trials when we stimulated striatonigral pathway cells only 10Hz stimulation frequency induced significant change in median lick rate during stimulation (Figure 3.8-A, three-way ANOVA Tukey-Kramer post-hoc tests; 10Hz vs no-stim., 2Hz and 5Hz: $p<0.0001$). Conversely, all stimulation conditions of 2Hz, 5Hz and 10Hz were sufficient to increase the fraction of trials with licking during light compared to non-stimulated trials (Figure 3.8-B, three-way ANOVA Tukey-Kramer post-hoc tests; 5Hz vs no-stim. and 2Hz: $p<0.0001$; 10Hz vs no-stim., 2Hz and 5Hz: $p<0.0001$).

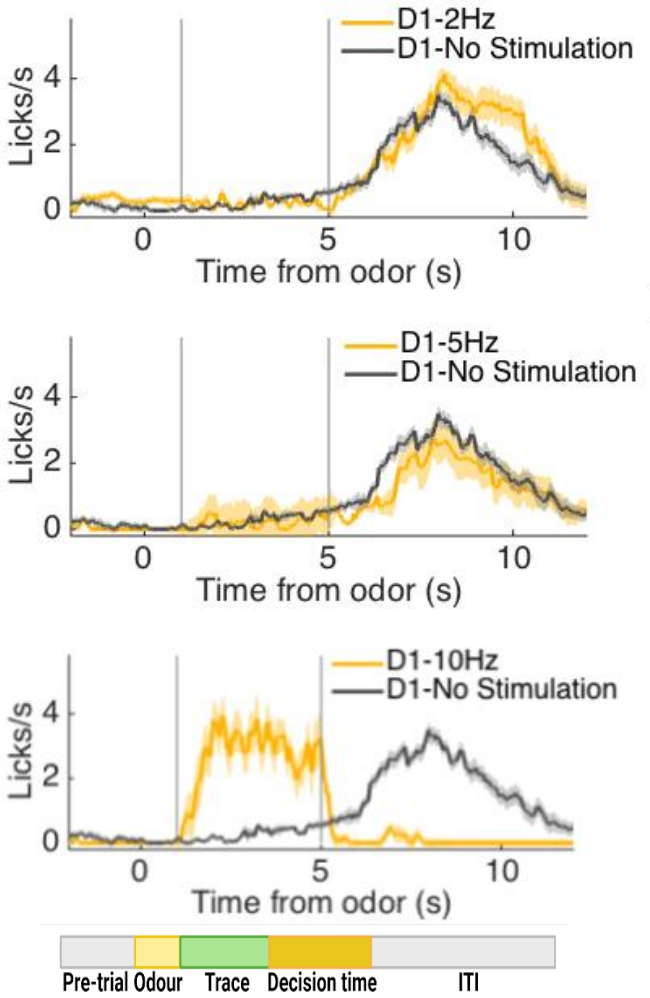

Figure 3.7 - **Lick rate histograms (licks per second) in function of trial time (time from odour, s) in wait trials.** The two vertical lines represent the stimulation interval of 4 seconds, beginning 1s after odour delivery. Grey curve represents non-stimulated trials. Yellow curve represents stimulated trials with A - 2Hz stimulation frequency, B - 5Hz stimulation frequency and C - 10Hz stimulation frequency. Lighter grey and lighter yellow curves represent deviation from the mean. 10Hz stimulation evoked a high rate of licking from stimulation onset throughout the stimulation period as compared to non-stimulated, 2Hz and 5Hz stimulation.

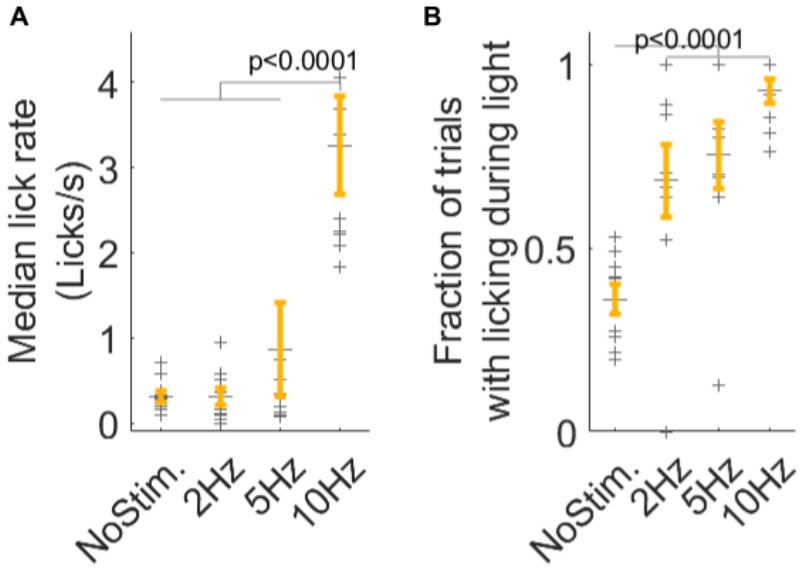

Figure 3.8 - **Median lick rate and Fraction of trials with licking during light for WAIT trials. A** - Median lick rate (licks/s) for WAIT trials during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. 10Hz stimulation showed significant difference from other stimulation conditions ($p<0.0001$). Error bars show standard error of the mean. **B** - Fraction of WAIT trials where licking occurred during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. All stimulation conditions significantly increased the probability of licking to occur when compared to non-stimulated trials ($p<0.0001$). Error bars show standard error of the mean.

3.6. **The effect of optogenetic stimulations on Neutral trials**

Lick rate histograms for neutral trials show the change in lick rate responses of mice across a trial with and without optogenetic stimulation (Figure 3.9).

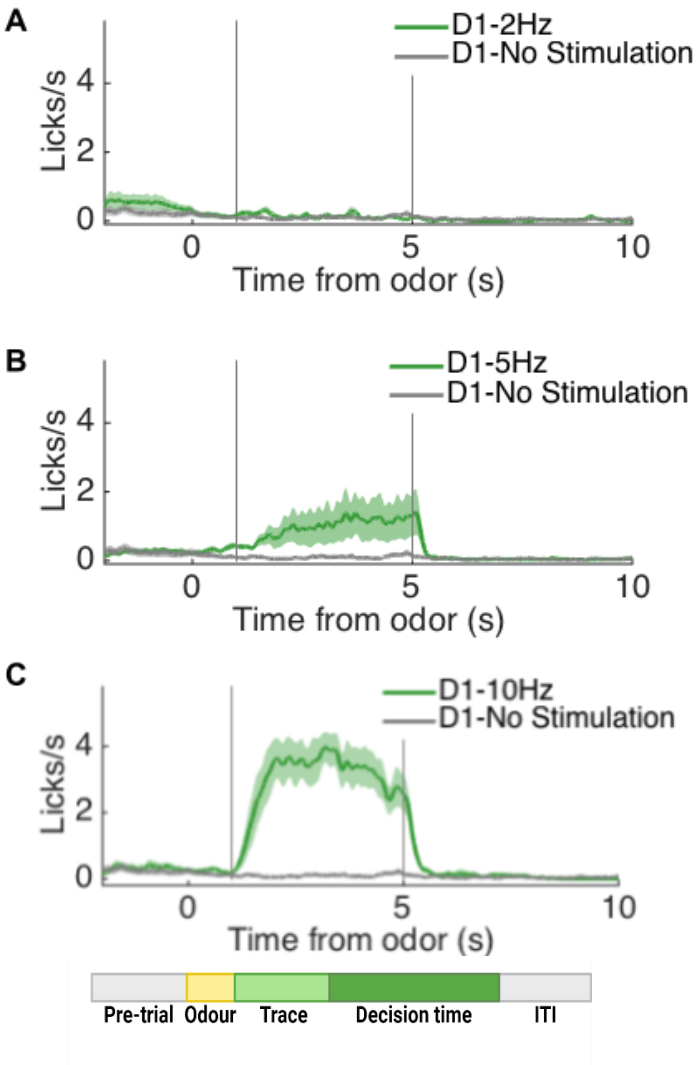

Figure 3.9 - **Lick rate histograms (licks per second) in function of trial time (time from odour, s) in neutral trials.** The two vertical lines represent the stimulation interval of 4 seconds, beginning 1s after odour delivery. Grey curve represents non-stimulated trials. Green curve represents stimulated trials with A - 2Hz stimulation frequency, B - 5Hz stimulation frequency and C - 10Hz stimulation frequency. Lighter grey and lighter green curves represent deviation from the mean. 10Hz stimulation evoked a high rate (above 3 licks/s) of licking from stimulation onset throughout the stimulation period as compared to non-stimulated and 2Hz, while 5Hz stimulation produced an increase to 1 licks/s in lick rate compared to non-stimulated trials.

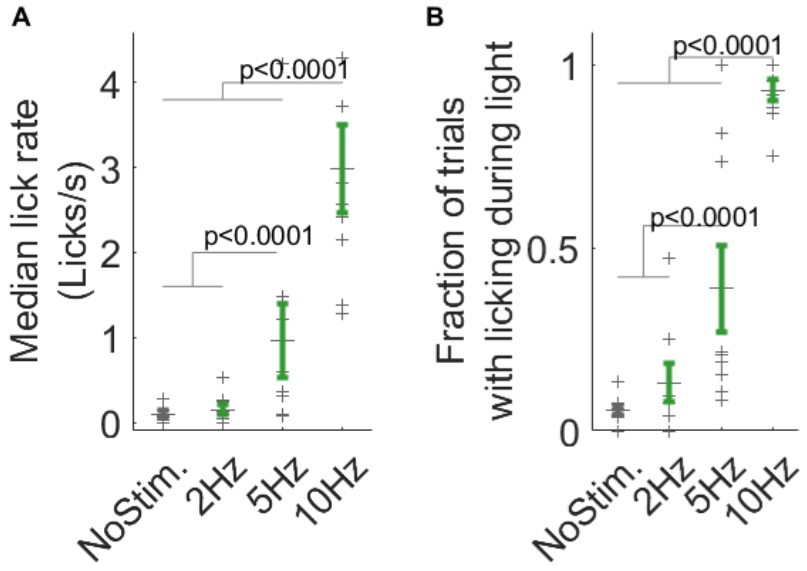

Figure 3.10 - **Median lick rate and Fraction of trials with licking during light for Neutral trials. A** - Median lick rate (licks/s) for Neutral trials during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. 10Hz stimulation showed significant difference from other stimulation conditions ($p<0.0001$) and 5Hz stimulation was significantly different from 2Hz stimulation and no stimulation ($p<0.0001$). Error bars show standard error of the mean. **B** - Fraction of Neutral trials where licking occurred during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. 5Hz stimulation produced significant differences when compared to non-stimulated and 2Hz stimulation ($p<0.0001$). 10Hz stimulation evoked significant differences when compared with non-stimulated and other stimulation types ($p<0.0001$). Error bars show standard error of the mean.

In striatonigral pathway stimulation during neutral trials, both 5Hz and 10Hz stimulation frequencies were sufficient to significantly increase the median lick rate and the fraction of trials with licking during stimulation compared to non-stimulated neutral trials and 2Hz stimulated neutral trials (Figure 3.10-A: three-way ANOVA Tukey-Kramer post-hoc tests: 10Hz vs no-stim., 2Hz and 5Hz, $p<0.0001$; 5Hz vs no-stim. and 2Hz, $p<0.0001$) (Figure 3.10-B: three-way ANOVA Tukey-Kramer post-hoc tests: 10Hz vs no-stim., 2Hz and 5Hz, $p<0.0001$; 5Hz vs no-stim. and 2Hz, $p<0.0001$).

3.7. The effect of optogenetic stimulations on all trial types

Median lick rates between nogo, wait and neutral trials were not different for 2Hz, 5Hz and 10Hz stimulations and no-stimulation conditions (Figure 3.11). For these 3 trials types, median lick rate changed similarly between the different stimulation conditions. Only 10Hz stimulation frequency increased median lick rates in an equal manner for nogo, wait and neutral trials, when compared with the same trials with no stimulation, 2Hz and 5Hz stimulation frequencies. Nevertheless, the amount of licking for nogo, wait and neutral trial types did not reach comparable levels to go trial median lick rate value, even when using higher 10Hz stimulation (Figure 3.11).
For median lick rate, stimulation only had a significant effect when higher frequencies were used, and the effect was not different between different trial types. Median lick rate remained the same for go trials in all stimulation conditions.

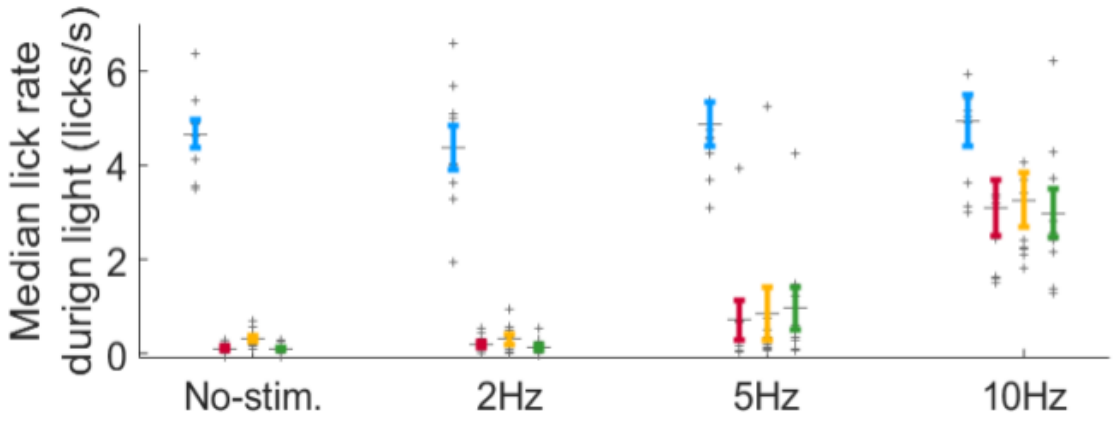

Figure 3.11 - **Median lick rate for all trial types and all stimulation conditions.** Median lick rate during the optogenetic stimulation period for all trial types in function of stimulation condition: without stimulation and with 2Hz, 5Hz and 10Hz stimulation frequencies. Only 10Hz stimulation frequency appears to be sufficient to have an impact on the amount of licking produced regardless of trial type. Error bars show standard error of the mean.

For non-stimulated trials, mice licked during the 4s stimulation period for 99.02%±1.22 in go trials, 4.61% ±4.0 in nogo trials, 36.06%±12.59 in wait trials and for 5.63%±3.71 in neutral trials. Mice licked more frequently for wait trials when compared to nogo and neutral trials.
With 2Hz stimulation frequency the fraction of trials with licking during light increased in wait trials from 36.06%±12.59 to 68.28%±29.67. However, this increase was not observed during nogo or neutral trials (11.82%±12.43 and 13.16±16.10 respectively, three-way ANOVA, Tukey-Kramer post-hoc tests, $p>0.05$). During 5Hz stimulation, the fraction of trials with licking increased for nogo, wait and neutral when compared with non-stimulated trials, with wait trials maintaining higher values as seen with non-stimulated and 2Hz stimulation (Figure 3.12). With 10Hz stimulation, values in fraction of trials with licking for nogo, wait and neutral were not different than of go trials (Figure 3.12).

For fraction of trials with licking during light, behaviour changed for both trial type and stimulation condition. Wait trials suffered more change with stimulation compared to nogo and neutral trials. In wait trials the fraction of trials with licking increased significantly with all stimulation frequencies. In nogo and neutral trials, fraction of trials with licking changed only with higher stimulation frequencies (5Hz and 10Hz). Fraction of trials with licking remained the same for go trials in all stimulation conditions.

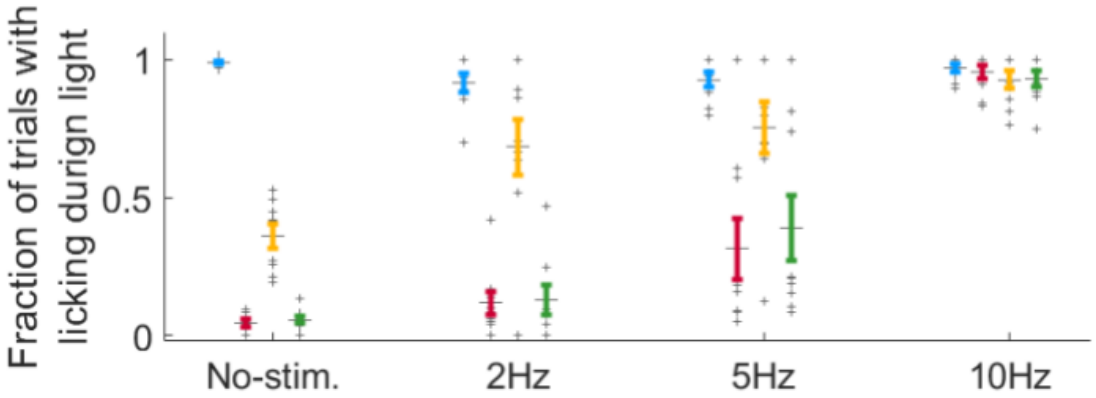

Figure 3.12 - **Fraction of trials with licking during light for all trial types and all stimulation conditions.** Fraction of trials for all trial types where licking occurred during optogenetic stimulation period: without stimulation and with 2, 5 and 10Hz. The effect of stimulation condition in the probability of licking initiation is dependent on trial type, except for 10Hz stimulation where this effect is not observed. Error bars show standard error of the mean.

In summary, stimulation had different effects on the two main parameters observed (median lick rate and fraction of trials with licking during light). In the first, stimulation effect was modulated by stimulation frequency but not by trial type. In the second, behaviour was modulated during stimulation both by different trial types and by different stimulation frequencies.

4. DISCUSSION

The present study aimed at investigating the role of striatonigral pathway cells of anterior ventro-lateral striatum, in the initiation and execution of instrumental licking behaviour.

To this effect, a viral vector for the expression of ChR2 was injected in D1-Cre transgenic mice in the aVLS region, and subsequently bilateral optic fibres were implanted in the same coordinates. Animals were trained to learn an olfactory guided operant task and optogenetic stimulation to manipulate D1 aVLS cells was then applied for 4s (1s after odour onset) and with three different frequencies of light: 2Hz, 5Hz and 10Hz. Behavioural output was recorded as licks per second.

In go trials we found that stimulation did not affect median lick rate (that can be understood as the amount of licking, as in longer bouts of licking) (Figure 3.2-A) or fraction of trials with licking during light (that can be understood as the probability of the action being initiated, and includes tentative, or check licks) (Figure 3.2-B). This was expected, as the non-stimulated, learned behaviour of the animal for this trial reinforced licking activity. Differences seen in the graphs can be attributed to variability between animals and between sessions of stimulation.
Nevertheless, the lick rate histograms indicated that an effect could be seen on the onset of stimulation, 1s after odour delivery. To further explore this phase of the behaviour, we tested the effect of striatonigral stimulation on two parameters during this time (from 1s to 3s after odour onset, a 2 second period): a) reaction time: time of first lick after odour delivery, indicating the speed of licking initiation, and b) anticipatory lick rate: number of licks per second, after odour delivery and before water reward, indicating the amount of licking before the reward is given. We found that even 2Hz stimulation is sufficient to decrease reaction time, but not to increase the amount of anticipatory licking (mouse licks earlier, but not more frequently once he starts licking). Stronger stimulation (5hz and 10hz) induced earlier reaction time and increased anticipatory licking (mouse licks earlier and licks more). The observed increase in reaction time under stimulation points to striatonigral pathway cells' classical role in facilitating the initiation of movement, in this case, licking [36,72–74].
In nogo trials both 5Hz and 10Hz produce an effect by increasing the fraction of trials with licking, but 10Hz does so with a greater magnitude. While 5Hz stimulation frequency is enough to increase fraction of trials with licking, it is not enough to increase median lick rate, as only 10Hz stimulation was sufficient to increase median lick rate. Given these results, it appears that a stronger stimulation frequency (10hz) is necessary to circumvent the previously learned behaviour of licking suppression for nogo trials, perhaps meaning that other concurrent circuits - like the indirect pathway - might be more active when the animals learns to suppress the action [25,42,75]. Additionally, because striatonigral cells appear to be more susceptible to change in fraction of trials with licking rather than in median lick rate, we can say that these cell populations might be involved in initiation rather than in maintenance of execution of the action. This finding supports the idea that different basal ganglia nuclei and their varied cell populations have distinct roles in the chunking organisation of whole functional movements [14,17,57,76].
In wait trials, striatonigral cell stimulation increased the fraction of trials with licking for all stimulation frequencies. However, to increase median lick rate, a stronger stimulation, with 10Hz, was necessary. Stimulation with 2Hz and 5Hz is sufficient to initiate action, but not to increase lick rate (Figure 3.7). A difference was noted when comparing the effect of the stimulation frequency on fraction of trials with licking, between nogo and wait trials. Despite the learned behaviour for both trials resulting in a similar suppression of licking, during stimulated wait trials

even a low stimulation was enough to significantly change the probability of initiating licking in contrast with stimulated nogo trials (3.10).
In neutral trials the animal did not learn to actively suppress licking, nor was it predisposed to lick, but rather would not lick for lack of reward or punishment. This could explain why there was not a response similar to wait, as the animal previously learned that, in that context, licking induced no reward, and there was no predisposition for licking.

Across all trials, stimulation of striatonigral cells had a bigger impact in the probability of initiating licking (fraction of trials with licking, Figure 3.10), rather than impacting the maintenance of execution of the action (median lick rate, Figure 3.11). Thus, the fraction of trials with licking was modulated by trial type for 2Hz and 5Hz stimulations but not for 10Hz stimulations. With 10Hz stimulation, values of fraction of trials with licking for nogo, wait and neutral increased to meet the value of go trials. On the contrary, the median lick rate for nogo, wait and neutral trial types did not reach comparable levels to go trial median lick rate value, even when using higher 10Hz stimulation. Hence, the amount of licking was not modulated by trial type for any of the stimulation conditions, meaning that differences in context are less perceptible in this stage of the action.
Overall, when studying the probability of initiation of licking we noticed that lower stimulation (2Hz) elicits lower levels of response, and that higher amount of stimulation (10Hz) elicited much stronger responses. However, an intermediate level of stimulation frequency (5Hz), and closer to the natural baseline activity for these neurons, enabled to best distinguish between different contexts, as exemplified by the comparison between wait and nogo trials. This difference in the fraction of trials with licking, between these two trial types might indicate that striatonigral activity can be dependent on the context of the behaviour, since the animal learned that in the nogo odour context suppression was needed in order to avoid punishment, but in the context of wait trial, suppression of licking meant a later retrieval of a reward. Thus, we can say that striatonigral pathway might be one of the circuits in basal ganglia that is involved in the process of contextualization [11,77,78].

In summary, through these experiments, we confirmed that the stimulation of the striatonigral neuron population of aVLS is sufficient to elicit licking movement, in accordance with previous studies that suggested that this area was responsible for orofacial movement[33,79,80]. As expected in view of the classical model, it appears that by increasing D1 activity, SNr output may be reduced, and downstream motor areas are enabled to produce movement. Thus, these neural populations could be able to modulate downstream circuits for orofacial motor control. Further, we have showed there are different effects, brought about by stimulation of striatonigral cells, between initiation and execution of the action, showcasing a bigger impact in the probability of initiation of licking than in the maintenance of longer bouts of licking. We suggest that these populations of striatum might be involved in the bracketing or chunking of the movement in study, being perhaps dedicated to a start signal for the action, as previously suggested[14,17,76]. And finally, we saw that the effect of stimulation in the striatonigral pathway of aVLS is different, depending on the context where the action is being executed.

5. CONCLUSION AND FUTURE PERSPECTIVES

With this experiment we confirmed that the striatonigral pathway is involved in orofacial motor control and is sufficient to generate instrumental licking behaviour. We further observed that D1 populations in aVLS have a greater impact in the initiation of instrumental licking than in the sustaining of movement execution, and that they take part in the process of contextualization.

Since there is still much to be known about the anatomy and circuitry of BG and their functionality, further studies will be necessary to understand the connectivity between these brain nuclei and their role in action selection, learning, contextualization, and motor function. As we showed in this study that striatonigral pathway could be modulated by learned contexts, it would be interesting to investigate its activity at different stages of learning of the task and understand if this neuronal population - that seems to be implicated in initiating actions - is more active at an earlier stage of learning. How does the significance of this pathway change throughout the learning process, and how does it change from early learning to established motor response, or even into habitual learning?

Our study focused on the production of voluntary orofacial movement. However, it is possible that parallel movements might be produced during optogenetic stimulation in this population, both along the body and the face of the animal. It could be valuable to investigate this possibility and to further explore a possible gradient of motor functionality in this area and its distinct associated movements. Additionally, new analysis could be done through more developed machine learning techniques - with DeepLabCut for instance - to further investigate possible parallel motor responses.

Other behavioural paradigms, for instance, ones that change the value of the reward and punishment, could be applied to increase our knowledge of this populations' contributions in context distinction and action selection.

The basal ganglia structures share an incredible number of neural connections, not just between themselves but also to many other areas of the brain. They are also implicated in fundamental aspects of human and animal behaviour: motor control, motivation, action selection and learning. There is much yet to be discovered. Hence, their study is paramount for the development of our understanding of the brain, and it is necessary for the establishment of possible therapies for the debilitating disease states that affect these regions, such as Parkingson's, Huntington's and OCD.

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