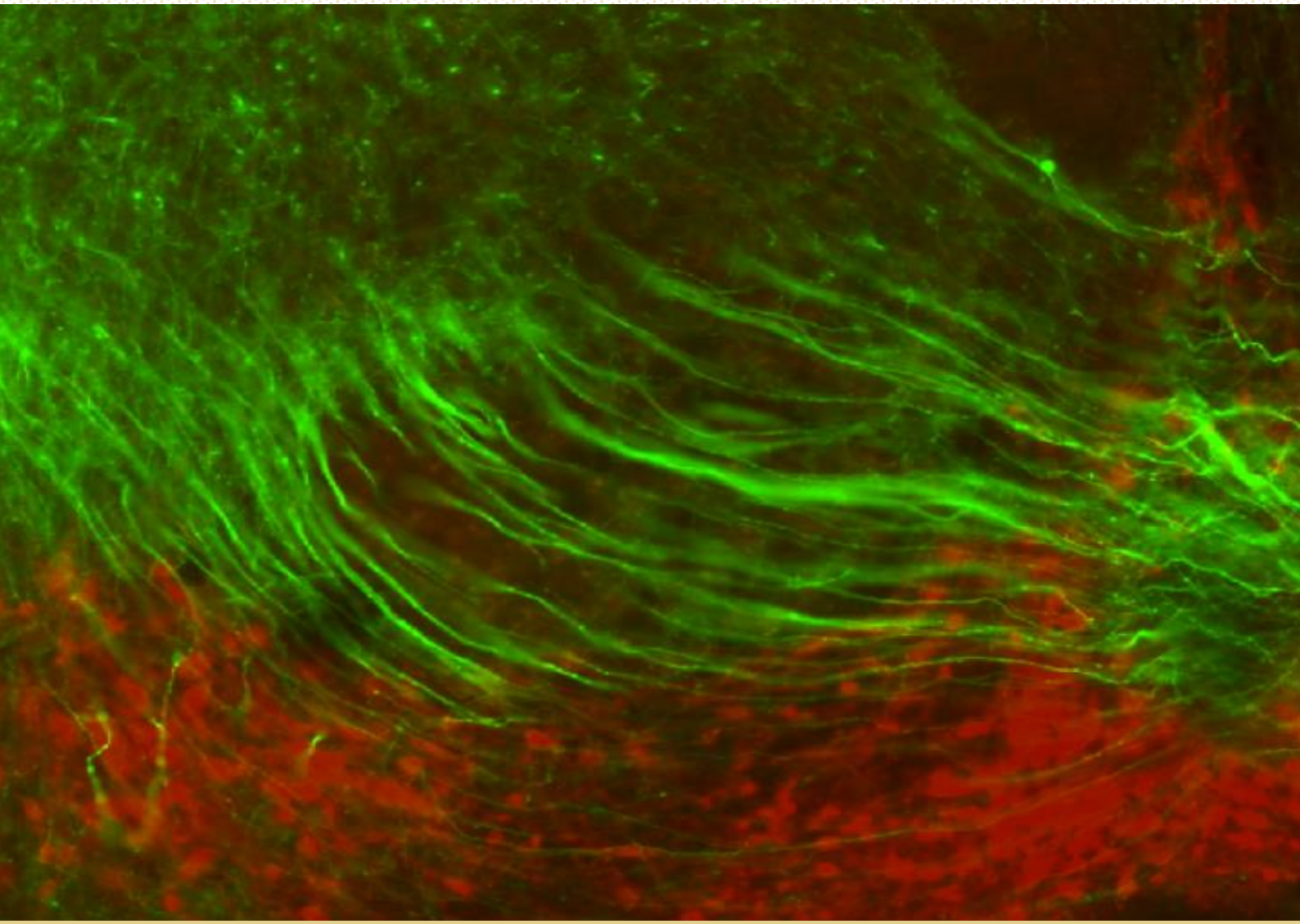

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The superior colliculus projection to midbrain dopaminergic neurons: a study on its anatomy and role during spatial orientation behavior

Dissertação de Mestrado em Biologia Celular e Molecular, orientada pela Doutora Ana Cruz e pelo Professor Doutor Carlos Duarte
a ser apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologias da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

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Master Dissertation on Cellular and Molecular Biology, Life Science Department, Science and technology Faculty of University of Coimbra

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Master dissertation preformed in the "Neurobiology of action" Lab, at Champalimaud Center for the Unknown, under the scientific orientation of Doctor Ana Cruz and Doctor Andreas Klaus, and under the orientation of Doctor Carlos Duarte (University of Coimbra)

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Cover image: Glutamatergic projection from superior colliculus (green) in the midbrain. Dopaminergic midbrain neurons are represented in red. Image acquired using the Zeiss AxioImager Fluorescence microscope with a 20x magnification objective

Imagem de capa: Projecções glutamatergicas do colliculus superior (verde) no meséncefalo. Neurónios dopanimérgicos estão respresentados a vermelho.Imagem adquirida usando o microscópio de fluorescencia Zeiss AxioImager com uma objectiva com magnificação de 20x

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Key Words: Superior Colliculus, Substantia Nigra pars compacta Dopaminergic Neurons, movement initiation, orientation

Abstract

The selection of suitable actions in response to external factors in the environment and given an internal state, is essential for the survival of any animal. Such selection is thought to be dependent on the activity of the cortex and basal ganglia circuitry. Specifically, the initiation of actions requires activity of Substantia Nigra pars compacta (SNc) dopaminergic neurons. A fundamental feature of this process is the ability to adjust the head and body orientation. Therefore, brain centers responsible for orientation and movement initiation should communicate. Superior Colliculus (SC), a well-studied midbrain area, is the structure that for a long time has been connected with orientation behavior, because it is a center of sensory information processing and motor relay, but also a point of convergence of multiple cortical projections associated with cognition. Although a direct projection between SC and SNc exists and has potentially a role in influencing dopaminergic neuronal activity during gait initiation, this possibility has not been explored so far.

We hypothesize that such connection modulates SNc dopaminergic neuron activity, contributing to the initiation of movements, in particular influencing orientation behavior. Our goal, with this works, was to characterize anatomically the SC-to-SNc pathway, in terms neuronal populations and its topographic distribution, and to begin to dissect the neuron-specific role of this path during action initiation and orientation. Using transgenic mouse lines and a combination of Cre-dependent viral injections and immunohistochemistry techniques, we found that the SC-to-SNc projection is strongly glutamatergic with few and no contribution from GABAergic and cholinergic neuronal populations, respectively. The glutamatergic projection is organized in a topographic manner, with the higher density of projections, although not exclusively, present in the dorso-lateral SNc.

We furthermore studied the functional role of this pathway in freely moving mice in the open field. Through electrophysiology recordings of SC activity, optogenetic manipulation of SC glutamatergic projections in SNc, and using detailed movement analysis of the mouse tracked by a head-mounted accelerometer and high-resolution video recordings we found that activity of SC-to- SNc projection Is sufficient for eliciting movement initiation, largely in the form of turning behavior. Such movements correspond to short-latency ipsilateral rotations. These behaviors were associated with both SNc and SC activity. Moreover, the neuronal activity in the deep layer of SC was found to be low during resting and to increase when movements are initiated or when these glutamatergic projections are stimulated, suggesting an involvement in movement initiation.

Overall, the results presented in this thesis suggest that the SC-to-SNc pathway is largely dominated by a glutamatergic projection, and that activity of these neurons is sufficient to initiate movements, mainly, rotations similar to orientation behavior. Therefore, this projection could be a critical modulator of SNc dopaminergic neurons activity, maybe influencing orientation behavior during the initiation of an action.

Palavras-chave: Colliculus Superior, Neurónios dopaminergicos da Substantia Nigra pars compacta, iniciação de movimento, orientação.

Resumo

A selecção de acções apropriadas em resposta a factores externos tendo em conta um dado estado interno, é essencial para a sobrevivência de todos os animais. Esta selecção é dependente da actividade do circuito entre o cortex e os ganglios basais. Especificamente, a iniciação de acções requer a actividade dos neurónios dopaminérgicos da substancia nigra pars compacta (SNc). Uma característica fundamental deste processo é a capacidade de ajustar a orientação da cabeça e do corpo. Portanto, os centros cerebrais responsáveis pela orientação e por iniciar movimentos devem comunicar entre si. O colliculus superior (SC), uma área do mesencéfalo, é a estrutura que se pensa estar relacionada com o comportamento de orientação, visto que é um centro de processamento de informação sensorial e de transmissão motora, para além de ser um ponto the convergência de múltiplas projecções corticais associadas à cognição.

A projecção directa entre o SC e o SNc poderá influenciar a actividade neuronal dopaminérgica durante a iniciação de movimentos, mas esta possibilidade nunca foi explorada.

Nós hipotetizamos que esta conecção pode modular os neurónios dopaminérgicos do SNc, contribuindo para a iniciação dos movimentos, influenciando o comportamento de orientação. O objectivo do presente estudo é caracterizar anatomicamente a projecção tecto-nigral, em termos das suas populações neuronais e da sua topografia, e começar a desvendar a função desta via durante a iniciação de acções e orientação. Utilizando linhas trangénicas de ratinho e uma combinação de injecções virais dependentes de cre e técnicas de imunohistoquímica, descobrimos que a projecção SC-SNc é fortemente glutamatérgica, com pouca e nenhuma contribução das populações neuronais GABAérgicas e colinérgicas, respectivamente.

Observámos que a projecção glutamatérgica está organizada topograficamente, sendo que a maior densidade de projecção, mas não a exclusiva, se encontra na região dorso-lateral do SNc.

Adicionalmente, estudámos a função desta projecção no movimento livre de ratinhos numa arena. Através de estudos electrofisiológicos da actividade do SC e da manipulação optogenética das projecções glutamatérgicas no SNc, enquanto o movimento detalhado do ratinho é monotorizado por meio de um acelerómetro e video-gravações de alta resolução, descobrimos que a actividade desta via é suficiente para desencadear a iniciação de movimento, em particular na forma de rotações ipsilaterais de curta latência. Ambos os comportamentos são associados à actividade do SNc e do SC, respectivamente. Ademais, a actividade neuronal das camadas profundas do SC é muito baixa durante períodos de imobilidade e aumenta significativamente quando o movimento é iniciado ou quando as projecções glutamatérgicas são estimuladas, sugerindo um possível envolvimento na iniciação da locomoção.

Estes resultados sugerem que a projecção SC-SNc é sobretudo glutamatérgica e que a actividade deste neurónios é suficiente para iniciar movimentos, principalmente rotações similares a comportamentos de orientação. Assim sendo, esta projecção pode ser um modulador crítico da actividade dos neurónios dopaminérgicos do SNc, em particular regulando a iniciação de movimento, talvez influenciando o comportamento de orientação durante a iniciação de uma acção.

Contents

List of abbreviations

(e)YFP: (enhanced) Yellow fluorescent protein

AP: Anterior-posterior

BA: Body acceleration

CD: Caudate nucleus

ChaT: Choline Acyl transferase

ChR2: Channel rhodopsin 2

CLS: Closed loop stimulation

CLS-B: Bilateral CLS

CLS-L: Left CLS

CLS-R: Right CLS

CNS: Central nervous sistem

DA: Dopamine

DV: Dorso-ventral

Fps: frames per second

GABA: γ-Aminobutyric acid

GAD: Glutamic acid decarboxylase

GPe: Globus pallidus externo

GPi: Globus pallidus interno

ML: Medio-lateral

mRt: mesencephalic reticular formation

MSN: Medium spiny neuron

OF: Open field

PAG: lateral periaqueductal gray

PhID: Photo identification

PPT: Pedunculopontine nucleus

SC: Superior colliculus

SEM: Standard error of the mean

SNc: Substantia nigra pars compacta

SNr: Substantia nigra pars reticulata

STN: Sub thalamic nucleus

TH: Tyrosine hydroxylase

VAch: Vesicular acetylcholine transporter

VGlut2: Vesicular glutamate transporter 2

VTA: Ventral Tegmental Area

Introduction

General Introduction:

In order to survive, animals navigate the environment to find food, reproductive partners, escape predators, etc. What at first seems to be a basic function, at a closer look is a process that requires highly complex integration of sensory stimuli, evaluation of changes in internal state and value of situations, and activation and control of musculature essential to produce the specific movement, which will generate the most desired outcome. Within all these process, the most ubiquitous and fundamental feature, is the need to initiate movement and to orient themselves before it. The brain circuit that has been identified as a major player in selecting and initiating movements, is the basal ganglia circuit, in specific, the activity of dopaminergic neurons in SNc and its connection to striatum, seems to be the key player in eliciting the initiation of movements.

Indeed, substantia nigra pars compacta (SNc) dopaminergic neurons are thought to control action initiation through the differential modulation of the synapses between Glutamatergic neurons from cortex and GABAergic medium spiny neurons (MSN) striatal neurons that are responsible for the inhibitory control of brainstem motor centers (Figure 1). The inhibitory MSN of the striatum, so called due to its high density of dendritic spines, are divided in two populations, depending on the differential expression of dopamine type 1 (D1) or type 2 (D2) G-protein coupled Dopamine receptors and targeted output nuclei. Dopamine acts on D1 receptors promoting an overall increase in neuronal excitability, these neurons project then to globus pallidus internus (GPi) where they synapse with GABAergic neurons directed to thalamus, resulting in a disinhibition of glutamatergic projections from thalamus to motor and

premotor cortex. On the other hand, activations of D2 receptor by dopamine leads to an overall decrease in excitability (Gerfen 2011) of (D2R)-expressing neurons. These neurons inhibit GPe GABAergic neurons that in turn inhibit STN. Therefore, action of dopamine leads to a decrease in STN activity, since there is a reduction on GPe inhibition. STN neurons are glutamatergic and project to GPi resulting in the increase of inhibitory effect of GPi over thalamus and consequently decreased excitation of premotor and motor cortex (review: Grillner 2013).

Several studies, which supported the classical view of the basal ganglia circuit function, proposed that these two pathways work in an opposite way: the movement would be promoted by the activity of the direct pathway (D1R expressing neurons), once it increases activity of motor and premotor cortex, while the activity of indirect pathway (D2R expressing neurons) would inhibit the movement, through inhibition of motor and premotor cortex activity (Albin 1989, DeLong 1990, Kravitz 2010). However, recent studies clearly demonstrated that concurrent activation of both pathways is essential for action initiation (Cui 2013, Jin 2014), suggesting that direct pathway could facilitate the expression of the required motor program while competing motor programs would be inhibited by the indirect pathway (Cui 2013, Isomura 2013, Tecuapetla 2014, Tecuapetla 2016, Mink 2003). In an integrative view of these models, dopamine plays the central role, since increase activity of SNc dopaminergic neurons is known, not only to precede initiation of movements, but also, to be required for the gait initiation (Alves da Silva, submitted).

This importance of DA signaling is highlighted by the symptomatology of Parkinson's disease. This pathology is characterized by the loss of dopaminergic neurons in the SNc (Hassler 1938), resulting in depletion of DA in the caudate and putamen (human subdivisions of striatum) (Ehringer 1960), which in turn leads to the known symptoms presented by Parkinson's disease patients, such as gait initiation difficulties or bradykinesia (slowness in the execution of movement).

Interestingly, DA neurons of SNc are not only known for its relation with the control of gait initiation and locomotion, but are also known to be implicated in reinforcement learning and habit formation mechanisms, which, in the long term, are used to maximize

reward acquisition. Indeed, these neurons are phasically activated (i.e, burst with short-latency, < 100 ms, and short-duration, ~100 ms) (Schultz 1986, Overton 1997, Schultz 1998) by salient stimuli or rewards. Moreover, if a reward is preceded by a predictive stimulus, through training, the phasic burst is shifted from the reward to the predictive stimulus itself, and if after the predictive stimulus the reward is omitted a decrease in firing occurs (Schultz 1997, review: Bromberg-Martin 2010). These finding lead to the emergence of the idea that DA signaling also works as a reward prediction error (Schultz 1997), coding the difference between what is expected and the real outcome and acting as a learning signal that reinforces rewarding behaviors. In fact, addictive drugs, which exaggeratedly enhance DA signaling (Di-Chiara 1988, review: Wise 2004), highlight the power of DA as reinforcement learning signal.

Nevertheless, reward prediction error should not be the only function of phasic DA neurons activity since these bursts are also elicited by aversive and alerting stimuli (attenuated if predicted), suggesting that phasic DA bursts also encodes salience (Matsumo 2009). Additionally, some authors have connected the role of SNc DA neurons to the concept of motivation (Niv 2007, Mazzoni 2007). This suggests that these neurons, and in particular their connection with the striatum, are fundamentally coding the motivation to perform a specific action, which seems to be congruent with the bradykinesia symptom of Parkinson's disease (Mazzoni 2007).

Initially, the key to dissociate the role of DA in these different functions appeared to be the firing profile of the neurons, while the phasic firing is more related with reward prediction (Schultz 2007), the tonic firing appeared to support movement and/or motivation (Niv 2005). Interestingly, phasic alterations in firing have also been clearly reported before action initiation (Jin 2010, Howe 2016), suggesting that this straightforward view is incomplete. Bromberg-Martin (Bromberg-Martin 2010) tried to integrate the different function attributed to SNc DA neurons by proposing that while one population of SNc DA neurons is excited by rewarding events and inhibited by aversive, coding values, a second one would be excited by both events, coding salience, besides, both populations would be activated by alerting signals (sensory cues of high importance) acting together in the modulation of motivation. Differences in the activity

and functions of the populations would represent the differences in the inputs that the neurons receive.

Indeed, one central question that arises from these observations and that is crucial for the understanding of basal ganglia functions, is how SNc dopaminergic neurons are themselves regulated. Since different inputs should be the responsible for the different functions associated with these neurons, a full understanding of which inputs regulate SNc DA neurons activity and how they do it, would be of extreme importance to unravel this circuit and for the comprehension of behavior itself. In fact, SNc receives a wide variety of inputs, which range from autonomic, motor, and somatosensory areas as was shown by monosynaptic retrograde tracing (Watabe-Uchida 2012), but not much is known about how they are integrated and their individual contributions for SNc function.

One of the inputs, is the projection from Superior Colliculus (SC) intermediate and deep layers (Comoli 2003, May 2009). Interestingly, such as SNc salience coding DA neurons, SC is known to respond to novel, noxious or intense sensory stimuli (Dean 1986). Besides, the main behavioral function attributed to SC is the induction of orientation behaviors, such as gaze shifts and head and/or bodies movements towards wanted objects or away from unwanted or threatening situations (Sahibzada 1986). Moreover, as referred in the beginning, in an even more fundamental approach, orientation is a crucial behavior before gait initiation, because animals need to orient themselves before initiation of locomotion.

Thus, the SC could be an important modulator of SNc DA neurons activity during movement initiation. The next sections provide a detailed overview about the physiological properties of SC that allow it to be a key brain area in controlling orientation as well as other more complex functions and how they might relate to SNc DA and basal ganglia activity.

1. The SC and orienting behavior

The orientation of an animal towards a stimulus involves the coordinated movement of eyes, head and trunk. Consequently, sensory information from the different senses has to be integrated so that the animal can perceive the origin of the stimulus and translate this information into the motor command that leads to the movement towards it. A brain area that receives projections from sensory systems like those for vision, audition and somatosensation, is the SC. The SC integrates these inputs in multisensory neurons and then translates them into outputs to motor neurons in spinal cord and brainstem. Although early studies on the SC described a relationship with orientation, it was not until later that basic mechanisms underlying this function have been revealed. The first study stating the connection between activity of SC and head orienting movements was from Hess 1946 (Hess 1946) where they observed that electrical stimulation of SC induces contraversive head movements in rats. Later studies performed microstimulation (Dean 1896, Mchaffie 1982, Freedman 1996) or microinjection of sodium L-glutamate to increase neuron firing, and corroborated this finding by inducing contralateral movements of head and body (Dean 1988). Moreover, Roucoux (Roucoux 1980) besides observing the coordinated eye and head movements elicited by the stimulation of the SC, also identified that stimulation in different areas would create different outcomes such as saccades with different directions or amplitude. These findings suggested the topographical organization of SC that has been described in other studies (Finlay 1978). The confirmation of the crucial role of the SC in orienting behavior came from lesion experiments in which a unilateral partial ablation led to a neglect of the contralateral visual field and to absence of eye, head and body movements towards the stimulus. Further support was provided by lesion studies of the deep layers in three shrew that blocked the orientation either to moving or static objects (Casagrande 1974). Studies that reported the neglect of stimuli in the visual field may also be interpreted as alterations in visual attention, besides inhibition of the orientation (see next chapter for more details).

Notwithstanding, some early studies involving stimulation of the SC presented contradictory results, since sometimes the stimulation evoked contradictory behaviors (Dean 1989). Redgrave and colleagues (Sahibzada 1986) clarified this question by electrically stimulating the rat SC in different locations. The authors showed that when

stimulating the SC medial optic layer, corresponding to the upper visual field, it produced avoidance behavior, while stimulation of the lateral optic layer, corresponding to the lower visual field, elicited orienting behavior. These findings have ethological significance since stimuli that elicit defensive response are more probable to appear in the upper visual field in rodents while approach or orienting responses will occur more frequently when the stimuli are presented in the lower visual field, ultimately these behaviors are the two sides of the same coin, since animals can orient towards a wanted object or away from unwanted or threatening situations.

The integration of the sensory and cognitive information into the expression of a motor output toward the exact location of the stimulus implies complex function such as motor preparation, shifting of attention or even target selection. In order to fully understand how activating the SC evokes orienting behavior we need to dissect these functions. The next sections will cover these topics.

2. The SC: an integration center

2.1 SC anatomy

The SC, also known as tectum (non-mammalian homolog), is a major component of the midbrain. Located on the roof of the brain stem (between thalamus and pineal gland) (figure 2) it is organized in seven alternating cellular and fibrous layers that can be functionally divided into three groups (Wurtz 1980): the superficial layers, which comprise the stratum zonale, stratum griseum superficiale, and stratum opticum, the intermediate layers comprising in the stratum griseum intermedium and stratum album intermedium and finally the deep layers consisting of the stratum griseum profundum, and album profundum. The superficial layers react to visual stimuli that show up at a given location in the contralateral hemifield. In contrast, the deep layers have sensitivity to different sensory stimuli such as vision, audition or somatosensation (Ganghi 2011). The intermediate and deep layers have been shown to be related to orienting behavior

in response to a stimulus (Dean 1986, Dean 1988), indeed a sub-set of neurons in this area responds with a premotor burst during orienting behavior (Ganghi 2011).

This functional division is also represented in terms of cellular structure. Indeed, the superficial layers are composed of horizontal cells presumably interneurons, vertical cells with dendrites that spread obliquely in the direction of the collicular surface forming a wide-ranging field, cells with multiple dendrites without orientation and small cells with dendritic field through all stratum griseum superficial (May 2006), a cell profile similar to other primary sensory nuclei, while the deep layer comprise multipolar cells of different types that don't appear to have a specific target, and some small interneurons that match the profile of brain stem reticular core (Wurts 1980). Interestingly it has been shown that wide-field cells in the superior layers are smaller in animals depending more on vision, suggesting a negative correlation between the cell size and the necessity of a more thorough scrutiny of the visual environment (Hilbig 2000). Also, it has been shown that multipolar cells of intermediate/deep layers project to the brainstem and spinal cord (Grantyn 1982, Rhoades 1987), but the overall knowledge of the relation between cellular structure and physiological function is still incomplete.

2.2 Neurons and neurotransmitters

The SC also contains glutamatergic neurons located in the superficial visual layer. These neurons are known to strongly excite premotor neurons in the intermediate layers that promote the already identified motor outcome of SC activity, as demonstrated using electrophysiology recordings and stimulation or glutamate uncaging techniques (Isa 2000, Helms 2016, Lee 1997). These visual neurons are activated regarding the specific location within the map of visual field of the superficial layers and seem to excite the underlying premotor neurons in the saccade vectors map of the intermediate layers, providing a way for the transfer of information from the sensory cells encoding the location of some stimulus to the cells that direct the movement to that particular area (Lee 1997).

Another major neurotransmitter involved in SC functions is gamma-aminobutyric acid (GABA), since SC is one of the central nervous system areas with higher concentration of this molecule and the overall machinery involved in its function, such as glutamic acid descarboxylase (GAD) and GABA uptake transporters (Okada 1974, Fosse 1989, Mize 1988, Kvale 1983, Houser 1983, Mize 1981). GABAergic neurons are distributed through all the layers of the SC, although with different densities: in the superficial visual layers, GABA neurons can compose up to 55% of all neuronal population, gradually decreases its density throughout the inferior layers, being the lowest concentration in the deep layers (Mize 1992). Morphologically these neurons present different shapes such as horizontal cells, piriform or multipolar neurons (Mize 1988). Functionally, GABAergic neurons in the intermediate layers are activated by the neighbor premotor cells, inhibiting then visuosensory neurons in the superficial layers (Lee 2007). Moreover, GABAergic neurons in the SC are implied, together with caudate nucleus, in inhibiting SNr GABAergic neurons that tonically inhibit output neurons in the SC, therefore, releasing this inhibition and promoting generation of saccades (Lee 2007, Meredith 1998). Nevertheless, the overall circuitry and its functions are not yet fully understood, and GABAergic neurons are likely to be involved in other SC functions.

While GABAergic and glutamatergic neurons of the SC have been studied in similar experimental contexts, little is known about the interaction between these two populations. Further studies are required in order to clarify this subject.

Several papers report the inexistence of Choline acetyltransferase (ChaT) and Vesicular acetylcholine transporter (VAch) positive neurons in any layer of the SC, in the rat (Ichikawa 1997). But In the cat, while some studies report also the inexistence of Chat positive neurons in the SC (Kimura 1981) others appear to have found the presence of few cholinergic neurons located in the superficial gray layer and even fewer in the optical layer (Hall 1989). Interestingly, both studies were based on ChaT immunoreactivity. Although acetylcholine seems not to be synthesized in the SC of rats, it seems to be a preponderant neurotransmitter in SC function as suggested by the multiple cholinergic inputs that it receives from other brain areas (e.g. PPT, parabigeminal nucleus) (Hall 1908, Woolf 1986).

2.3. Multisensorial integration

The superficial layers of the SC are known to receive their main inputs from the retina. The retinal ganglion cells project to the SC in an organized pattern that corresponds to a topographic map of the visual field, having each cluster of neurons a field of response that corresponds to a given area in the visual space (Dräger 1976). However, retinal ganglion cells are not the only input to superficial SC. Other regions involved in visual processing, such as visual cortex, also project to the superficial layers, highlighting the role of this area in processing visual stimuli. Nevertheless, not only the superficial layers, but also the intermediate and deep layers of the SC receive inputs from cortical areas (Hikosaka 2000), making the SC a point of convergence of multiple cortical projections. Moreover, the intermediate and deep layers are also a point of convergence of multimodal sensory information: they receive inputs from auditory and somatosensory cortices, as well as visual inputs, which also topographically organize preserving the spatial mapping of the sensory receptors, similar to the one in the superficial layer (Finlay 1978, Dräger 1976, Chapula 1977, Harris 1980, Donaldson 1980). This translates into a sensory stimulus (visual, auditory, somatic) coming from a given place being processed in the same collicular area and even by the same single neuron (Meredith 1986). Moreover, recently a honeycomb organization of afferents and efferents in the intermediate layers of the SC has been discovered. According to this model, each cylindrical module is an orientation unit, which receives its triggering information from a given location in the sensory space and contains the premotor neurons for the generation of an orienting movement to that location (Rene 2001).

Given all of these anatomical characteristics, the SC is thought to be a center of multisensory integration (Meredith 1986) that gathers ascending sensory pathways and descending projections from the cortex. This information converges at the level of a single neuron and is subsequently translated into motor commands for orienting behavior, such as saccades, head or body movements.

Indeed, SC is a brain area with a high density of multisensory neurons (Stein 2008), each of them having multiple excitatory receptive fields, which receive input from different sensory streams. These receptive fields correspond to a given spatial area in which some stimulus will induce activity in the corresponding neuron (Stein 2008). The receptive fields of each of the sensations overlap, resulting in the activation of the same single neuron if a visual and auditory stimulus occurs in the same spatial point.

If some stimuli are close in space and time the crossmodal integration of both modalities has a synergistic effect (multisensory enhancement). This enhancement is inversely correlated with salience of the modality-specific stimuli, therefore, larger increases in impulse frequency occur when the components are weakly effective (Meredith 1986). This is reflected in the additive and super-additive (i.e., the total response is larger than the sum of the individual responses) computations found in the multisensory neurons. Moreover, sub-additive computations (i.e., total response is smaller than the sum of the individual responses) also exist in the rare cases where the stimuli are the most effective and seem to approach the limit firing frequency of the neuron (Standford 2008). The behavioral expression of this mechanism is an increase in the capacity and speed of detecting the stimuli (Nozawa 1994).

Contrarily, when two stimuli arise from distinct locations, and the second stimulus is not inside the receptive field of the neuron activated, depression can occur in the activity of the multisensory neuron (Stein 2008). Such a profile could represent a “winner-takes-all” mechanism that, when presented with several disperse stimuli, would allow to enhance one stimulus at the expense of another.

Nevertheless, not all aspects of the multisensorial integration are yet fully understood. For example, it is known that cortical inputs are crucial for the multisensory enhancement (Jiang 2001) but how they interact with sensorial information is unknown.

3. SC functions: motor and non-motor functions

As mentioned above, the SC functions as an integration center of sensory information and cognitive factors involving descending pathways from the cortex. This information is then translated into motor commands associated to orienting behavior. Although motor functions of SC are the most conspicuous, others have been attributed to this brain area, such as orientation, attention, goal representation or reward related modulation (Ikeda 2003, Krauslis 2003, Hikosaka 2014). Nevertheless, there is an intrinsic relation between motor and non-motor functions of SC where the former often acts as representations of the latter. In the next sections, the known functions of SC will be further discussed.

3.1. Motor functions

It is well known that SC is implicated in generating eye, head and body movements that resemble orienting or approach (Dean 1988). Electrophysiological and anatomical studies showed that there are projections from the SC to motor neurons in spinal cord and brain stem that are implicated in contralateral movements elicited by stimulation of the SC. Nonetheless this crossed tecto-reticulo-spinal descending pathway is not the only one involved in SC's motor commands, since they were not abolished by the section of this path (although they were altered) (Dean 1986). Therefore, other motor systems must be implicated in motor functions of SC such as cerebellum and basal ganglia. Indeed, SC deep layers connect with cerebellum, especially saccade-related vermal visual area, via the dorsolateral pontine nucleus (DLPn) and nucleus reticularis tegmenti pontis (nRTP) (Harting 1977, Burne 1981). This is a reciprocal connection that can possibly have a role in correlating the different maps within SC (May 1990). Moreover, basal ganglia also connect with SC controlling its activity through SNr GABAergic projections (Lee 1988). Interestingly, the same neurons that are inhibited by SNr are also excited by cerebellar projections (Dean 1989), suggesting that these two brain areas act by modulating SC activity and therefore, the motor outcome that can be the movement of eyes, head or body.

Within the SC, excitatory neurons coming from the intermediate and deep layers seem to be the ones promoting contraversive movements, while the interaction among

different motor programs is controlled by inhibitory projections between the SC layers (Lee 2007, Phongphanphanee 2011).

3.1.1 Eye, head and body movements

When electrically stimulated, the intermediate layer of SC induces the formation of saccades, being dependent on direction and amplitude of the stimulus location but not on intensity or duration (Robinson 1972). If a stimulus appears in the visual field, the visual signal generated will activate visual neurons in the superficial layer in the corresponding zone of the topographic map that, in turn, will activate the below neurons in the intermediate/deep layer (Isa 2000, Mooney 1998). These neurons show a burst that is followed by a contraversive saccade towards the location of the stimulus. This burst acts as a command for saccade that is transmitted to the reticular formation that generates the movement (Grantyn 1985, Munoz 1986).

It is known that different types of SC neurons with different properties are associated with saccades (Munozz 1991, Munoz 1991(b), Munoz 1995): the ones previous described that have a high-frequency burst before the movement, neurons that gradually increase their activity from a low-frequency burst, which is associated with integration of sensory information and processes such as motor preparation, target selection and attention, to a high-frequency burst that generates the saccade (buildup neurons), and neurons that are active during fixation and inactive during most of the saccades (fixation neurons).

The firing rate determines the speed of the saccade while the location of the active population on the topographic maps may indicate the saccade vector (Sparks 1990).

SC is also involved in gaze shifts, besides the saccades. When the animal orients towards something, a coordinated movement of head and eyes occur (Roucoux 1980). So, when the animal is with his head free, stimulation of monkey SC provoked high-velocity eye and head movements of gaze shifts, which are similar to visually guided gaze shifts. The movement is dependent on the frequency of stimulation (Freedman). Moreover, the high frequency premotor burst that is seen before saccades is also present before head shifts supporting the gaze displacement hypothesis, which states that a single signal in SC codes amplitude and direction of eye and head movements in a gaze shift (Freedman 2016).

Furthermore, a visual stimulus in the ipsilateral hemifield (Corneil 2004) induces a transient response in the neck musculature as shown using electromyography (EMG). The SC seems to be the source of this activity, since when stimulated with low frequency, low level EMG signal is also elicited in the deep neck muscles (Corneil 2002). The signal increases during the time previous to the saccade, being correlated with motor preparation (Corneil 2006, Rezvani 2008).

At last, is also important to refer to the SC and its role in only head movements without gaze shifts, although the topographical organization of these neurons is not known (Corneil 2002 (b)).

In some cases, the movement of only the eyes and head is not enough to complete orientation, and therefore, some full body movements are essential. Indeed microstimulation of SC evokes whole body movements that are critical to orientation in several animals, such as circling and turns in tetrapodes (Freedman 1996, Rezvani 2008) or tail and swim movements in fish (Rodriguez 1998, Saitoh 2006). Moreover, in species that use whisker or pinnae as environmental sensors, SC's stimulation also induces the movement of these structures (Hemeit 2008, Stein 1981).

3.2 Motor preparation

Besides the generation of motor commands SC is also thought to be involved in the selection and planning of these commands. Indeed, Glimcher (Glimcher 1992) showed that the activity of the SC is predictive of saccade choice. This suggests SC involvement in saccade selection, and not just executing some formerly selected action. Some saccade related cells from the SC have low frequency activity during the period that precedes the appearance of the target (Munoz 1995), which could represent the process of motor preparation. Dorris (Dorris 1997) show that SC fixation related neurons decrease their activity between the initially fixation of some target and the fixation of accurate saccade target, which is coherent with the previous idea that release of ocular fixation facilitates premotor preparation. This process of motor preparation occurs in the intermediate layers of SC and is influenced by the external sensory signals and internal motor preparation signals (Dorris 2007).

3.3 Target selection

Several studies have been suggesting a role for SC in the selection of the target. Mcpeek (Mcpeek 2004), using chemical inactivation of SC and a visual search task, in which the saccades were misdirected after administration of the chemical depressor, demonstrated that SC activity correlates with target selection. This led to the confirmation of other studies that also suggested this hypothesis by showing that SC neurons have preference for the target stimulus before the onset of the movement. The activity of visuo-motor and motor neurons from the deep and intermediate layers is higher when the target, and not a distractor, is in the field of response and happen right before the saccade. This burst is not correlated with the saccade latency, but with the stimulus onset (Mcpeek 2002). Nevertheless, other neurons of SC from these populations and exclusively movement related neurons show activity related with the saccade latency, indicating groups of neurons within the SC with differential roles in target selection and triggering of saccades (Mcpeek 2002). Moreover, some SC neurons activity is selective to target stimuli, predicting the choice of saccade and the time of pursuit target choice (Krauzlis 2002).

The process of target selection by SC seems to involve more than just a motor decision based on the sensorial processing, but it also participates on a wider class of decisions. In an odor-cue orienting task associated with an auditory go-signal, which allows the dissociation of activity related with motor execution with the activity related with decision, the SC activity predicts the forthcoming choice way before the movement onset (Felsen 2013). This suggests that the SC is implicated in orienting decisions beyond the sensory modality they are based on. Moreover, Stubblefield (Stubblefield 2013) during a delayed response odor cue motor task that requires the mice to select and perform movement depended on the SC, showed that unilaterally optogenetic stimulation of deep layer neurons applied on the moment of the selection leads to a bias towards the contralateral side when the neurons were excited and towards the ipsiversive when inhibited.

3.4 Goal representation

Besides selecting the target, SC also represents the location of the goal, and not the specific movement that must be performed to obtain it (Krauzlis 2003, Stanford 1994). A study by Bergeron (Bergeron 2003), using memory guided gaze shifts paradigm and single unit recordings, showed that activity of a population of SC neurons in the cat encodes the distance to the target and not the amplitude of the saccade or any individual step to the get to the target. Actually, this had been suggested before by Keller (Keller 1996), where they showed that the amplitude of a saccade depends on the direction of the movement if the target is moving. Moreover, the activity of the neurons that signal the gaze displacement represents the overall saccade movement and not the specific eyes and head movements (Freedman 2016). Possibly, this activity represents a position-error signal instead of the motor command (Krauzlis 2000). This counter the idea of a map of saccades endpoints. Nevertheless, it is important to clarify that these studies were based on a remembered saccades paradigm, which probably implies additional mechanisms besides the ones of purely visual saccades.

3.5 Attention

The connection between SC and attention seems quite intuitive if one considers that a shift of attention requires orienting to a new stimulus, and that SC is a major player in orienting the eyes and head towards some stimuli. Moreover, this idea is reinforced by the already described role of SC in selecting the target of the saccade. Initially, attention, more specifically visual spatial attention, was associated with cortical brain networks but an additional function of SC in this process was early recognized by the observation that lesions in SC lead to a recovery in spatial orienting in cats with previous lesions in the contralateral occipital or parietal cortex – attention network – this was possible because loss spatial orienting in cortical lesions appears to be due to depression of the ipsilateral SC by the crossed SC, thus, removing it would abolish the inhibition and returned function of spatial orienting (Sprague 1966).

This awareness was reinforced by the observation that patients with lesions on the tectum appear to have problems in shifting attention (Posner 1982). Since that point several studies that relate SC function to attention had been performed.

Kustov (Kustov 1996) studying monkeys found that the eye movement preparation related with SC is associated with shifts of attention, information corroborated by Ignashchenkova (Ignashchenkova 2004) that showed that visuomotor neurons of intermediate layers of SC are active during covert shifts of attention. Additionally, other authors using microsimulation of the monkey SC in a paradigm of change-blindness, were a spatial cue was delivered in some trials to indicate a path that was about to change and SC stimulation in other trials, showed that the effect of the SC stimulations was similar to the one caused by the delivery of the cue: detection and reaction time improved, features of shifts of attention. An improvement in discrimination performance upon SC microstimulation was also demonstrated in by Philiastides (Philiastides 2004) using a more challenging task where distractors were also present.

Lately, Lovejoy 2010 (Lovejoy 2010) demonstrated that inactivation of the SC in monkeys that were performing a motion discrimination task caused neglect-like effects, since the

monkeys showed severe inattention to the stimulus in the visual field that was inactivated, although the distractors needed to be in the unaffected visual field, further stating the requirement of SC activity in attention.

However, it is fully accepted that SC is a central player in attention and that the communication with cortex and motor centers is of major importance to attentional functions, the underlying circuitry and cellular mechanisms are not fully understood. One lately discovered circuit involving SC that seems to be implicated in attention is the subcortical loop with basal ganglia, which involves projections to the intralaminar nuclei (thalamus), that in turn form a loop with caudate and putamen that then project to SNr and back to SC in a form of inhibitory input (Krauzlis 2013). Indeed, intralaminar nuclei activity is increased during covert attention tasks, an area that appears to have a concrete role in the processing of salient events (Smith 2004, Werf 2002). But essentially a connection with basal ganglia structures is likely to involve value-based selections as discussed below.

3.6 Reward related modulation

Animal behavior is thought to be guided by the reward value of actions, therefore orienting towards a high value object is a basic component of behavior and is critical for survival. Indeed, when an animal is presented with several objects it is more likely that it will orient its gaze to the one with more apparent reward (i.e., higher value) (Glimcher 2001, Lauwereyns 2002), which requires a motor output induced by the SC. In this case the driver of the orientation must be internal instead of an external stimulus, since the animal must have previous information about the situation in order to choose the more valuable target. Thus, SC activity, besides being modulated by sensory information, must also be modulated by brain areas processing reward-related information. Indeed Ikeda 2003 (Ikeda 2003), using a memory-guided saccade task showed that the visual response of monkey SC neurons was higher when the stimulus was an indicator of an upcoming reward. This reward associated activity is anticipated by activity of basal ganglia,

specifically in substantia nigra par reticulata (SNr) and caudate nucleus (CD) since they show pre-cue alterations in activity in a similar task to the one used by Ikeda (Sato 2002, Luwereyns 2002 (b)).

Indeed, the control of saccades by basal ganglia has been acknowledged before (Hikosaka 1983). Tonically active GABAergic projections from SNr to the intermediate layers of SC (Chevalier 1984, Graybiel 1978), where saccade related neurons are believed to be located, inhibit SC and the generation of saccades. This projection can be modulated by activity of inhibitory GABAergic neurons coming mainly from the CD to the SNr that results in disinhibition of SC neurons that induce saccades (Karabelas 1985). Actually, these tonically active neurons of SNr that are related with gaze show a pause in burst activity before each saccade (Hikosaka 1983, Joseph 1985).

This control can be direct, through SNr, as described before, or indirect through globus pallidus externus (GPe), where its GABAergic projections to SNr are inhibited leading to a decrease in activity of SC and an inhibition of saccades (Figure 3). The activity of one path, rather than the other, is hypothesized to depend on value (Hikosaka 2014).

4. SNc and SC

Neurons in SC are known to respond unexpected biological salient stimuli, such as novel, noxious or intense sensory stimuli, the same type of stimuli that elicit a short latency phasic signaling in SNc (short-latency (< 100 ms), short-duration (~100 ms)) (Schultz 1998, Guarraci 1999, Horvits 2000). Although, several lines of evidence link this type of burst with reward prediction errors (Overton 1997, Schultz 1998), currently it is known that beside it connection with reward, the phasic activity is also clearly related to initiation of moments (Jin 2010, Howe 2016).

Curiously, when SC is ipsilaterally lesioned, these short latency phasic bursts in SNc are abolished. On the other hand, local electrical collicular stimulation increases them, as reported by Redgrave and colleagues (Comoli 2003 (b)). In the same study, a direct projection from SC to SNc DA and non-DA neurons was described in rats. Accordingly, chemical stimulation of SC with GABA receptor antagonist, which has an overall

excitatory effect in SC, was found to induce phasic activity in SNc and ventral tegmental area (VTA), suggesting the SC as a source of control/modulation of DA and non-DA SNc and VTA neurons (Comoli 2003). This correlation between both activities can be important to perceptual discriminations of stimuli (Comoli 2003 (b)). Also, Haffie (Haffie 2007) and May (May 2009) identified a tectonigral projection in cats and monkeys, respectively, in which neurons from the deep layers of the SC synapse with DA neurons of SNc. All these studies suggest that this pathway is a route from which short latency sensory information can be transported to midbrain and therefore modulate basal ganglia activity. It is likely that the neurons that project to SNc are multisensory neurons, since few projections from only visual superficial layers were found, further supporting the transmission of short latency multisensory information. Another interesting question is how positive (i.e., rewarding) and negative (i.e., aversive) signals are differentially transmitted to SNc, since it is known that SNc DA neurons have differential burst activity regarding reward or aversive cues. May and colleagues (May 2009) reported that SC projections form both asymmetrical (presumably excitatory) and symmetrical (presumably inhibitory) synapses on DA and non-DA neurons of SNc and suggest they could be the cue to unravel this question. In addition, SNc GABA neurons could be activated during aversive stimuli, directly inhibiting SNc DA neurons.

All of these observations led to the emergence of the concept of “sensory prediction error”, which was proposed as an alternative to the “reward prediction error” theory, as a mechanism in which the SC provides a signal that causes phasic DA responses in the SNc which could support reinforcement learning (May 2009). In this theory, the information about value and identity of the stimulus would be provided by cortical areas related with perception.

Moreover the majority of the SC neurons that project to SNc are targeted to the dorsal tier (May 2009), the area that preferentially innervates ventral striatum, associated with reward processing. Nevertheless, some cells also innervate the ventral tier of SNc, which in turn innervates the dorsal striatum. Furthermore, this projection to dorsal striatum by SNc DA neurons is important to gait initiation, and locomotion (Figure 1) (Alves da Silva, submitted). The animals need to orient themselves before initiating locomotion, so, an important component of gait initiation is orientation. But so far, no studies have

systematically investigated the precise connection between mechanisms underlying gait initiation and orientation. An aspect that favors this view is the fact that Parkinson's disease patients with freezing of gait also present deficits in saccade performance (Nemanich 2016).

In the following work, we want to explore the function of the SC-SNc projection in behavior. Given all the anatomical and electrophysiological evidence presented, we hypothesize that the SC, through the tecto-nigral pathway, contributes to the increase in dopaminergic activity in the SNc, which has been shown to precede gait initiation. In order to test this hypothesis, we will characterize the neuronal populations in the SC that project to SNc, using a combination of tracing and immunohistochemistry techniques. Furthermore, we will study the neural dynamics of these tecto-nigral subpopulations during orienting behavior at gait onset. Taking advantage of transgenic tool available for mice, we will use mice expressing cre recombinase in glutamatergic neurons, which allow genetic manipulations in this specific population of neurons, to electrophysiologically record neuron-specific activity in the SC, in order to study neural dynamics, and to specifically stimulate the projections of SC neurons in the SNc during self-paced locomotion. The detailed movement of mice will be tracked using a head-mounted accelerometer and high-resolution video recordings while mice freely walk in an open field. After correlation between neuronal activity of SC-SNc projection neurons with movement and acceleration at gait initiation we expect to gain further insight into the function of this circuit during this phase of the behavior.

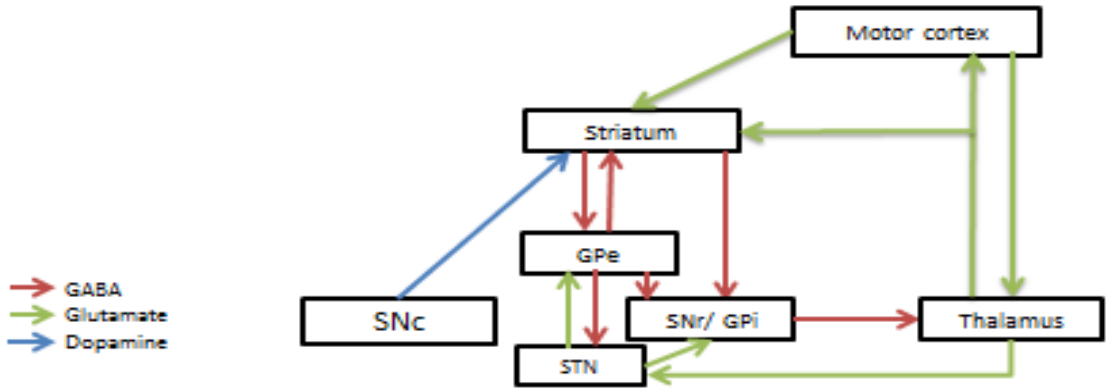

Figure 1. SNc DA neurons control gait initiation by modulating the synapse between Glutamatergic neurons from cortex and GABAergic neurons of the striatum, the medium spiny neurons (MSN). DA potentiate the activity of MSN that express D1 receptors and decrease the activity of MSN expressing D2 Receptors, that then project directly or indirectly to the thalamus which modulates motor and premotor cortex activity: D1R expressing neurons project to GPi where they synapse with GABAergic neurons directed to thalamus, resulting in a disinhibition of glutamatergic projections from thalamus to motor and premotor cortex, while D2R expressing neurons disinhibit STN through inhibition of GPe GABAergic neurons that project to the STN. STN neurons are glutamatergic and project to GPi resulting in the increase of inhibitory effect of GPi over thalamus and therefore decreased excitation of Premotor and motor cortex. Activity of D1R pathway seems to be connected with an increase in locomotion, while D2R pathway with a decrease (Kravitz 2010). Nevertheless, D1 and D2R need to be phasically active during gait initiation (Cui 2013).

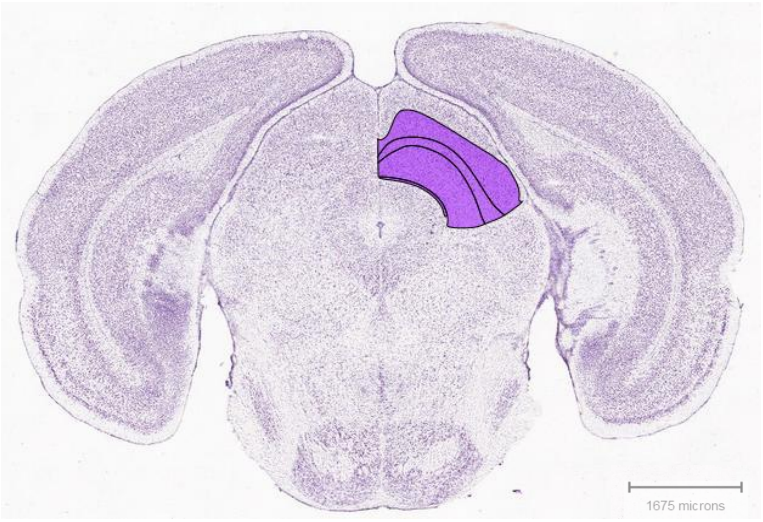

Figure 2: P 56 Coronal section of the mouse brain. SC intermediate and deep layers are highlighted in purple. Allen Brain Atlas (Paxinos 2004)

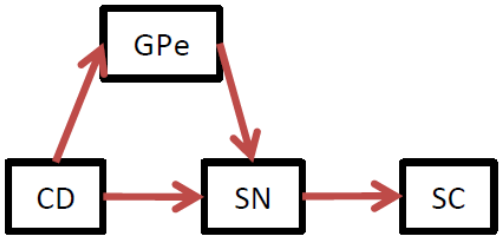

Figure 3: GABAergic circuit controlling SC activity. This circuit seems to be modulated by reward

Objectives

With the presented work, we propose to:

1) Characterize anatomically the tecto-nigral projection in terms of neuronal populations (Glutamatergic, Gabaergic and Cholinergic) and of topographic distribution, through the use of transgenic mice lines and a combination of cre dependent viral injections and immunohistochemistry techniques.

2) Begun to dissect the role of the tecto-nigral pathway, in particular the glutamatergic subpopulation, during action initiation and orientation. Through optogenetic manipulation of the SC-SNc projecting neurons and neuron-specific electrophysiological recordings in Cre transgenic mice during self-paced locomotion, we intend to gain further insight on the function of this projection.

This work is part of a larger ongoing project aimed to unravel the contribution of the tecto-nigral pathway in animal behavior and its physiological properties. This work was done with the collaboration with Doctor Ana Cruz.

Methodology and Results

Acknowledgments:

I would like to thank Doctor Sevinc Mutlu for the help with the cloning of the tdTomato virus used for tracing and Doctor Ana Cruz and Doctor Andreas Klaus for providing some of the custom Matlab scripts used for the analyses.

Animals:

Mice (*Mus musculus*) were kept on a 12:12 hour light/dark cycle, set for 8am-8pm light. Before surgery, mice were housed in groups of 5-6, after surgery they were single-housed, with food and water *ad libitum* in both situations. Animals were approximately 3 months old before surgery and had not been used in any prior experiments. All animal procedures were reviewed and performed in accordance with the Champalimaud Center for the Unknown Ethics committee guidelines and approved by the Portuguese Veterinary General Board (Direcção Geral de Veterinária, Ref. No. 0421/000/000/2014).

Section 1: Retrograde tracing of SC-to-SNc projection

Our first attempt to dissect the tecto-nigral projection was through the injection of retrobeads into the SNc. Our goal was to find the extent and localization of retrogradely labeled SC-to-SNc projecting neurons. We wanted to use this information to guide our anterograde tracing experiments (see below).

Methods:

For these experiments 10 wild-type black 6 male mice, approximately 3 months old, were used.

Stereotaxic surgery:

Animals were deeply anesthetized through inhalation of isoflurane (Vetflurane - Virbac) (2.0 – 4.0 %) and placed in the stereotaxic head holder. After alignment of the head, a craniotomy was performed using the anterior-posterior (AP) and mediolateral (ML) coordinates optimized for SNc[1]. 30 nl of green retrobeads (Lumafluor) in a total volume of 60 nl (1:1 retrobeads : Evans Blue) were injected into the left SNc (target coordinates: AP -3.16 mm, ML 1.4 mm, DV -4.2 mm), at a rate of 4.6 nl every 10 seconds, using a glass micropipette attached to a nanoject II auto-nanoliter injector (Drummond scientific) (14 injections sites (2 per mouse), 7 mice). In order to control the efficacy of the beads we also injected, under the same conditions, the same volume of red retrobeads (Lumafluor) into the right dorsal striatum using the target coordinates of AP 0.5 mm, ML -2.0 mm and DV -2.3 mm (3 injection sites, 3 mice). Following this procedure, the scalp was closed with vetbond (3M) and carprofen (0.1 ml/g subcutaneously) was injected as post-surgical analgesic. Throughout the surgery, the temperature of the mice was maintained at 36^{o}C by a thermostatically controlled heating pad, and the eyes were covered with a gel (Vidisic gel, Bauch&Lomb) to prevent drying. Animals were allowed to recover under supervision in the home cage placed on a heading pad.

The injected animals were kept for 3 days, in order to allow the transport of the beads through the axonal projections (following manufacturer recommendations).

Perfusion and histology:

After this period, the animals were sedated by inhalation of isoflurane and deeply anesthetized with a Ketamine-Xylazine-PBS (8-12-80%) solution (0.1ml/g intraperitoneal). Once completely insensitive, they were perfused through the heart with cold 1x, pH 7.2 phosphate-buffered saline (PBS), to clear all the blood from the circulatory system, followed by a fixative (4% Paraformaldehyde pH 7.3-7.4). The brains

[1] Coordinates of SNc were optimized through previous injections of Evans Blue dye following the same procedures described above (2 wild type Black 6 mice, 4 injection sites).

were collected and post fixed in the same fixative overnight at 4 °C. For further detail see (Gage 2012).

The collected brains were cryopreserved in 20% sucrose solution and cut in 50 µm serial coronal sections in the freezing microtome. The sections acquired between 1.42 mm and -4.72mm from bregma, corresponding to the volumes that contain the entire SC, SNc and striatum structures, were rinsed in PBS and then mounted in a glucose-free mounting medium (Fluoro-gel - EMS) and coverslipped.

Sequential images of SC, SNc and striatum were acquired using the Zeiss AxioImager Fluorescence microscope with a 10x magnification objective.

Analysis:

The acquired images were overlaid with the corresponding section from the *Paxinos Brain Atlas* (Paxinos 2004), using the Inkscape software, in order to determine location of the injection, and to qualitatively analyze the presence or absence of retrogradely labelled cell bodies in the different areas. Only brains with injections in the correct location were used for the analysis.

Results:

As shown in figure 4, when injected in the dorsal striatum, the retrobeads appear in the cell bodies of cortical (Figs. 4 c and 4 d) and striatal neurons (Fig. 4 b). This is in line with the known major inputs of dorsal striatal neurons arriving from cortex (Pan 2010), and confirms the efficacy of the retrobeads in the retrograde tracing.

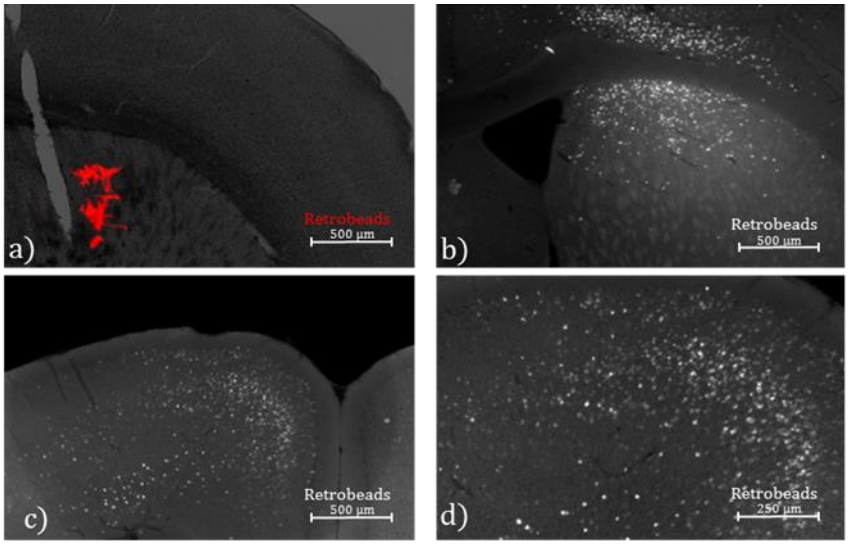

Figure 4 – **Retrobeads injected in the dorsal striatum label cortical neurons**; a) Brightfield (gray scale) and retrobead fluorescence (red) showing the injection site. b, c) Fluorescence images showing examples of retrogradely labelled cells in dorsal striatum (b) and cortex (b, c). d) Higher magnification image of the cortical labelled cells shown in (c). All images were acquired using the Zeiss AxioImage fluorescence microscope with the 10x magnification objective

When we targeted the SNc, the location of the injection was not completely circumscribed to SNc, but some of the total volume was indeed inside SNc boundaries (figure 5 a). The analysis of all SC containing brain slices revealed little to no retrograde labeling of cell bodies through all AP, ML and DV axes of SC. That is, some dim labeling was observed in 2 coronal sections of the same brain (figure 5 b and c), bregma -4.24 mm) in which the injection site was in the lateral SNc (figure 5 a). What we observe in

figure 5 b and c), could indeed represent cell bodies retrogradely labelled with green retrobeads, through all intermediate gray layer of SC. The labelling is also only present ipsilateral to the injection site, congruent to what is thought about SC-SNc projection (May 2009).

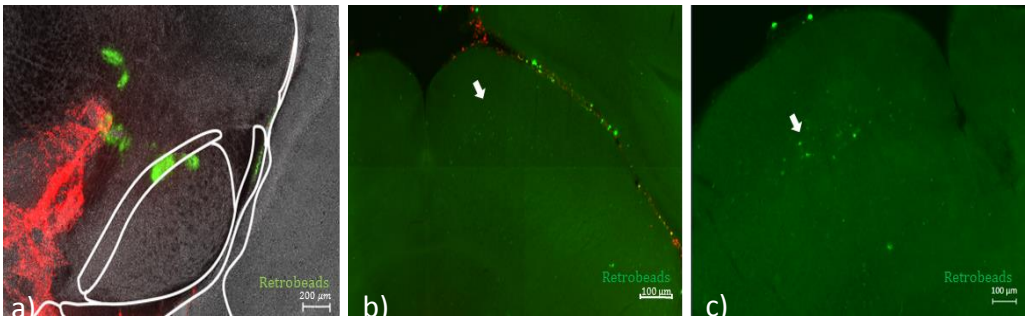

Figure 5 – **Almost no retrogradely cell bodies were found in SC**. a) Example of the injection sites of green retrobeads into the SNc with overlay with the corresponding figures in the Paxinos Brain Atlas (Paxinos 2004). (Red staining belongs to red retrobeads injected in the lateral ventricle). Images were acquired in the Zeiss AxioImage fluorescence microscope using a tile scan with a 20x magnification objective. b) and c) Only images of SC where we were able to see possible retrogradely labelled cell bodies with green retrobeads. Images acquired in the Zeiss AxioImage fluorescence microscope using a tile scan with the 10x magnification objective for b) and a 20x magnification objective for c). These images were taken from the brain from which the injection site is showed in a).

Section 2: Anterograde tracing of SC-to-SNc projection

Although retrograde experiments where not very successful in identifying the specific localization of neurons in SC projecting to SNc (section 1), we wanted to determine which was the neuronal type involved in this connection. We tested for the presence of glutamatergic, gabaergic and cholinergic neurons, since precious studies report the presence of these neuronal types within SC.

section 2a: Glutamatergic and GABAergic neurons

Glutamatergic neurons were found through several layers of the SC, mainly in the superficial and intermediate layers as previously shown by in situ hybridization analysis of the vesicular glutamate transporter 2 (VGlut2) mRNA expression in the primate (Hackett 2012, Balaram 2012). Moreover, neurons with morphologies consistent with projection neurons within the deep layers were found to contain glutamate, further supporting the presence of glutamatergic neurons in SC (Jeon 1997). Furthermore, several studies reported the presence of a large density of GABA-containing neurons with different morphologies in the superficial gray layers and in the deep layers of the rat, mouse (Ottersen 1984), cat (Mize 1988), monkey (Mize 1991), rabbit (Mize 1994) and primate SC (Mize 1992). In addition, several neurons in SC also express glutamic acid decarboxylase (GAD), the enzyme responsible for the conversion of glutamate to GABA (Lee 2007). Interestingly, SC is one of the CNS regions (Dean 1988) with higher concentration of GABA and GAD, suggesting an important role of this neurotransmitter for the SC function (Mize 1992). In order to test whether these neurons project to the SNc, we performed tracing experiments using Cre-dependent anterograde virus.

Methods:

For these experiments, we used 7 male Vglut2-IRES-Cre[2] and 1 female Gad-IRES-Cre mice with approximately 3 months old.

Stereotaxic surgery:

Animals were deeply anesthetized through inhalation of isoflurane (Vetflurane - Virbac) (2.0 – 4.0 %) and placed in the stereotaxic head holder. After alignment of the head, a craniotomy was performed using the AP and ML coordinates optimized for SC[3] (AP -4.0 mm, ML +/- 1.1 mm, DV -2.1 mm). 100-250 nl of anterograde Cre-dependent virus (rAAv2.EF1a.DIO.TdTomato or rAAV2(xx2). EF1a.DIO.eYFP) were injected in SC, at 4.6 nl per pulse every 10 seconds, using a glass micropipette attached to a nanoject II auto-nanoliter injector (Drummond scientific). Following this procedure, the scalp was closed with vetbond (3M) and carprofen (0.1ml/g subcutaneously) was injected as post-surgical analgesic. Throughout the surgery, the temperature of the mice was maintained at 36^{o}C by a thermostatically controlled heating pad, and the eyes were covered with a gel (Vidisic gel- Bauch&Lomb) to prevent drying.

The injected animals were kept for 3 weeks, in order to allow the replication and transport of the virus through the axonal projections.

Perfusion and histology:

After this period, the animals were sedated by inhalation of isoflurane and deeply anesthetized with a Ketamine-Xylazine-PBS (8-12-80%) solution (0.1ml/g intra peritoneal). Once completely insensitive, they were perfused as described above (section 1).

The collected brains were cryopreserved in 20% sucrose solution and cut in 50 µm serial coronal sections in the freezing microtome. The sections acquired between -2.54 mm

[2] The Vglut2cre line was chosen because in SC there is high expression of VGlut2 and not VGlut1(Hackett 2012, Balaram 2012).

[3] Coordinates of SC were optimized through previous injections of Evans Blue dye following the same procedures described above (2 wild type Black 6 mice, 4 injection sites).

and -4.72mm from bregma, corresponding to the volumes that contain the all SC and SNc structures, were permeabilized in PBS-Triton 0,4% solution and incubated overnight at 4^{o}C in mouse antibody to Tyrosine hydroxylase (1: 5000 dilution in PBS-Triton 0.4% solution) (ImmunoStar 22941), the enzyme responsible for the conversion of tyrosine to L-Dopa the precursor of catecholamines (mainly dopamine and (nor)adrenaline), diluted in PBS-Triton 0,4%. A secondary incubation for 2h, at room temperature, was performed with different secondary antibodies (1: 1000 dilution in PBS-Triton 0.4% solution), depending on the reporter of the virus injected (Alexa 488, Molecular Probes/Invitrogen A11029, for TdTomato virus injections; Alexa 594, Molecular Probes/Invitrogen A11032, for eYFP virus injections). After rinse in PBS-T 0.4%, the sections were then mounted in Mowiol (Merk) and coverslipped.

Sequential images of SC and SNc were collected using a Zeiss AxioImager Fluorescence microscope, with a 20x magnification objective. For some slides, higher magnification images of the injection site and the SNc were acquired with a confocal laser point-scanning microscope (Zeiss LSM 710).

Analysis:

Injection sites were determined through identification of the location with higher viral infection (qualitative analysis) and comparison with the corresponding brain section of the *Paxinos Brain Atlas* (Paxinos 2004). Cell density in the center of the injection was quantified using the cell counting plugin in FIJI software (Schindelin 2012) and results were analyzed in Microsoft Excel (Microsoft Office) and Matlab (MathWorks). Normal distribution of data was tested using one-sample Kolmogorov-Smirnov test, with a 5% significance level. Differences between the different viruses were compared by a two-sample t-test, with a 5% significance level (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

The density of projections from SC to SNC was analyzed using a custom Matlab (MathWorks) script, in which the intensity of fluorescence within the contralateral and ipsilateral SNc area was quantified. For that, the acquired images were overlaid with the corresponding *Paxinos Brain Atlas* (Paxinos 2004) figure, and the contralateral and ipsilateral SNc regions were highlighted in different colors for better distinction using the Inkscape software.

Statistical analyses were performed using Matlab (MathWorks). Normal distribution of data was tested using one-sample Kolmogorov-Smirnov test, with a 5% significance level. Due to small sample size and non-normal distribution of data, a non-parametric two-sample Kolmogorov-Smirnov test was used to compare the different conditions (ipsilateral vs. contralateral SNc intensity of projections and medial vs. lateral SNc intensity of projections), with a 5% significance level. After, a Bonferroni-Holm correction for multiple comparisons was used with an 0.05 α value (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

Results:

- **Anterograde Cre-dependent virus efficiently infected SC cell bodies**

The SC neurons were efficiently infected by the anterograde virus used. Indeed, for both virus (rAAv2.EF1a.DIO.TdTomato and rAAV2(xx2).EF1a.DIO.eYFP) and cell lines (VGlut2Cre and GAD-Cre) used, a large density of infected cell bodies was found surrounding the injection site coordinates and the pipette track (when visible; 6 a). We found no differences (two sample t-test, p= 0.6247) in the density of infected cell bodies in Vglut2cre mice for both YFP and TdTomato containing virus (Figure 6 b), assuring that the further results should not be biased due to different rates of infection. For the GAD-Cre line only TdTomato-containing virus was used. The brain volume showing infected neurons spread radially through approximately 200 to 300 µm in the AP axis around the center of the injection.

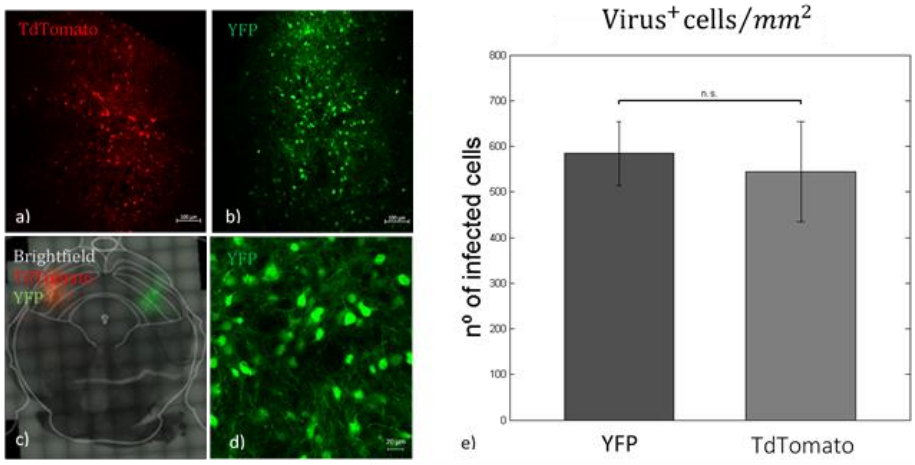

Figure 6 – **YFP and TdTomato virus efficiently infect cell bodies in superior colliculus of Vglut2cre mice.** a) Tdtomato-virus infected cell bodies in the center of the injection site in the right SC. b) eYFP-virus infected cell bodies in the center of the injection site in the left SC (5x5 tile scan). c) Example of the injection sites of the Td tomato and YFP anterograde virus with the overlay with the Paxinos brain atlas, -3.88mm from bregma. Axioscan image acquired with the 20x magnification objective d) Image of eYFP-virus infected cell bodies in the center of the injection site in the left SC (2x2 tile scan). Images acquired in the confocal microscope using the 40x magnification objective. e) Quantification of YFP and TdTomato virus infected cell bodies in superior colliculus of Vglut2cre mice**,** by cell counting using FIJI (n=3 injections per virus type, mean ± standard deviation).

Moreover, injections effectively targeted the intermediate and deep layers of SC and were mainly circumscribe to this area. Although, in some cases, minor infection of SC optical layers, lateral periaqueductal gray (PAG) neurons and superior mesencephalic reticular formation (mRt) occurred. In figure 2.2 the higher infection density area for each injection are represented in different colors, for both cell lines (Figure 7).

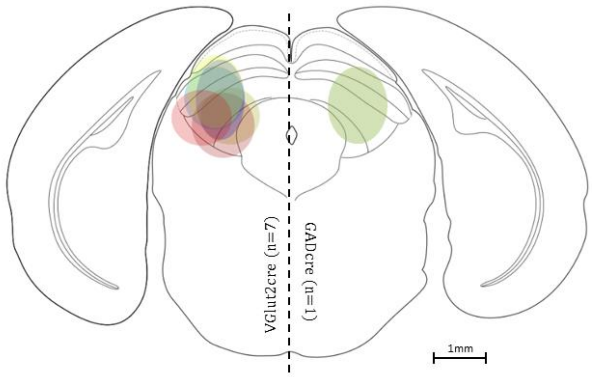

Figure 7 – Representation of all the virus injection sites. Bregma -4.24 mm, Paxinos Mouse brain atlas (Paxinos 2004)

- **SC glutamatergic neuronal population has projections to SNc**

SC is known to have direct-projecting neurons to SNc (Haffie 2006). Here we show that at least part of the projection is through glutamatergic SC neurons, as shown in figure 8. Independently of the SC layers or position targeted by the injection, for all the extension of the SNc (identified by the presence of dopaminergic neurons) the presence of labeled neuronal projections was found. Furthermore, several presumable synapses (labelled boutons – dot-like structures) are also found within the SNc area, as shown in figure 8 a'') to g'' – images scaled linearly for better visualization of the projections). It is important to notice, nevertheless, that the density of labeled projections within the SNc seems lower than the ones observed in the medio-dorsal nucleus of the thalamus, a long-known target of SC deep layers projecting neurons (Universiteit 1988).

The distribution of SC glutamatergic projections within the SNc area for each AP distance analyzed is not homogenous, (Figure 9), patches of higher and lower density of projections are found throughout all AP distances.

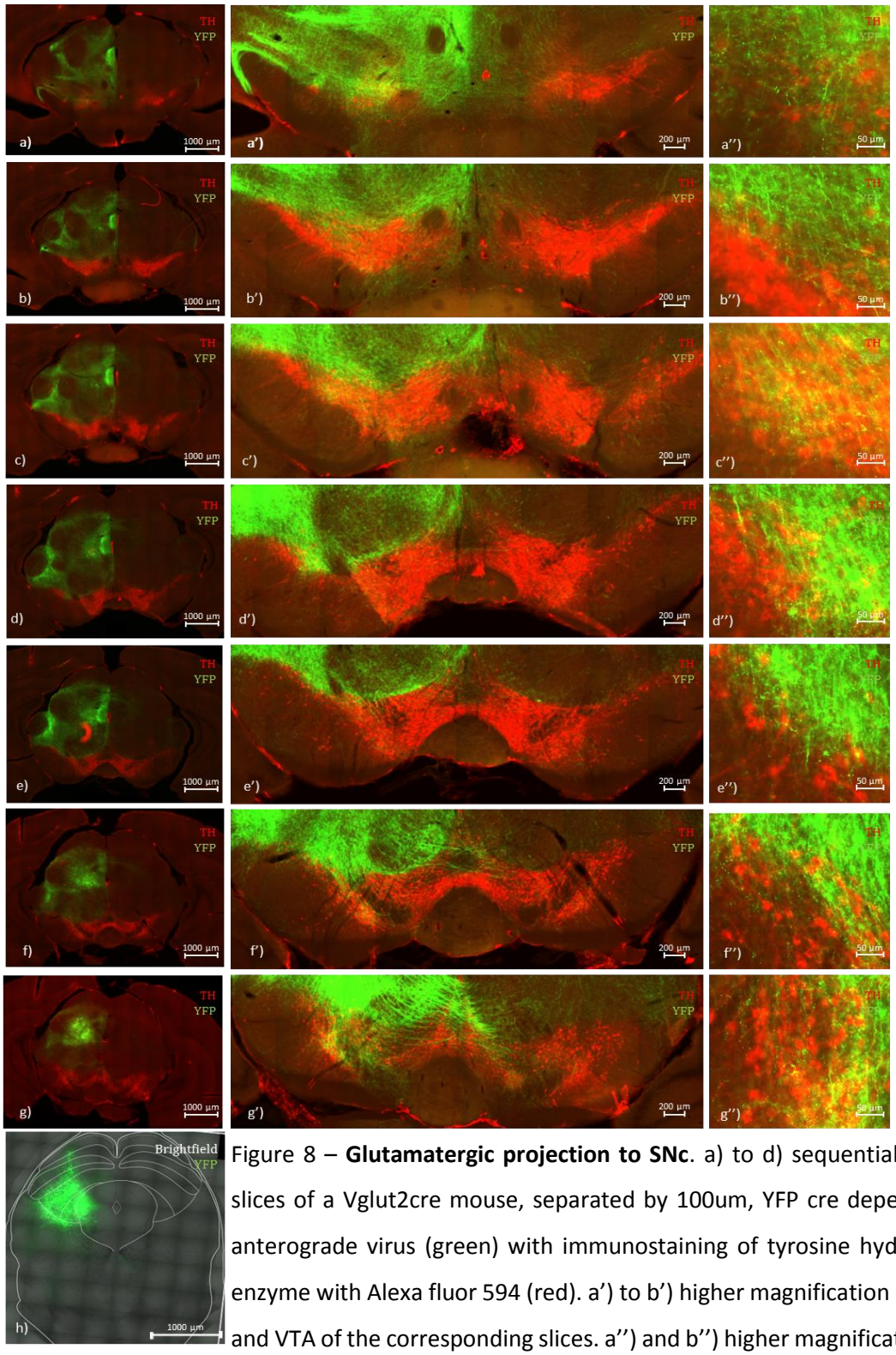

Figure 8 – **Glutamatergic projection to SNc**. a) to d) sequential brain slices of a Vglut2cre mouse, separated by 100um, YFP cre dependent anterograde virus (green) with immunostaining of tyrosine hydrolase enzyme with Alexa fluor 594 (red). a') to b') higher magnification of SNc and VTA of the corresponding slices. a'') and b'') higher magnification of of an area of the SNc with labeled projections. h) Injection site of the YFP cre dependent anterograde virus (green) in the deep and intermediate layers of the left Superior Colliculus (Bregma -4.24). Images acquired with a brightfield and fluorescence slide scanner microscope using a 20x magnification objective.

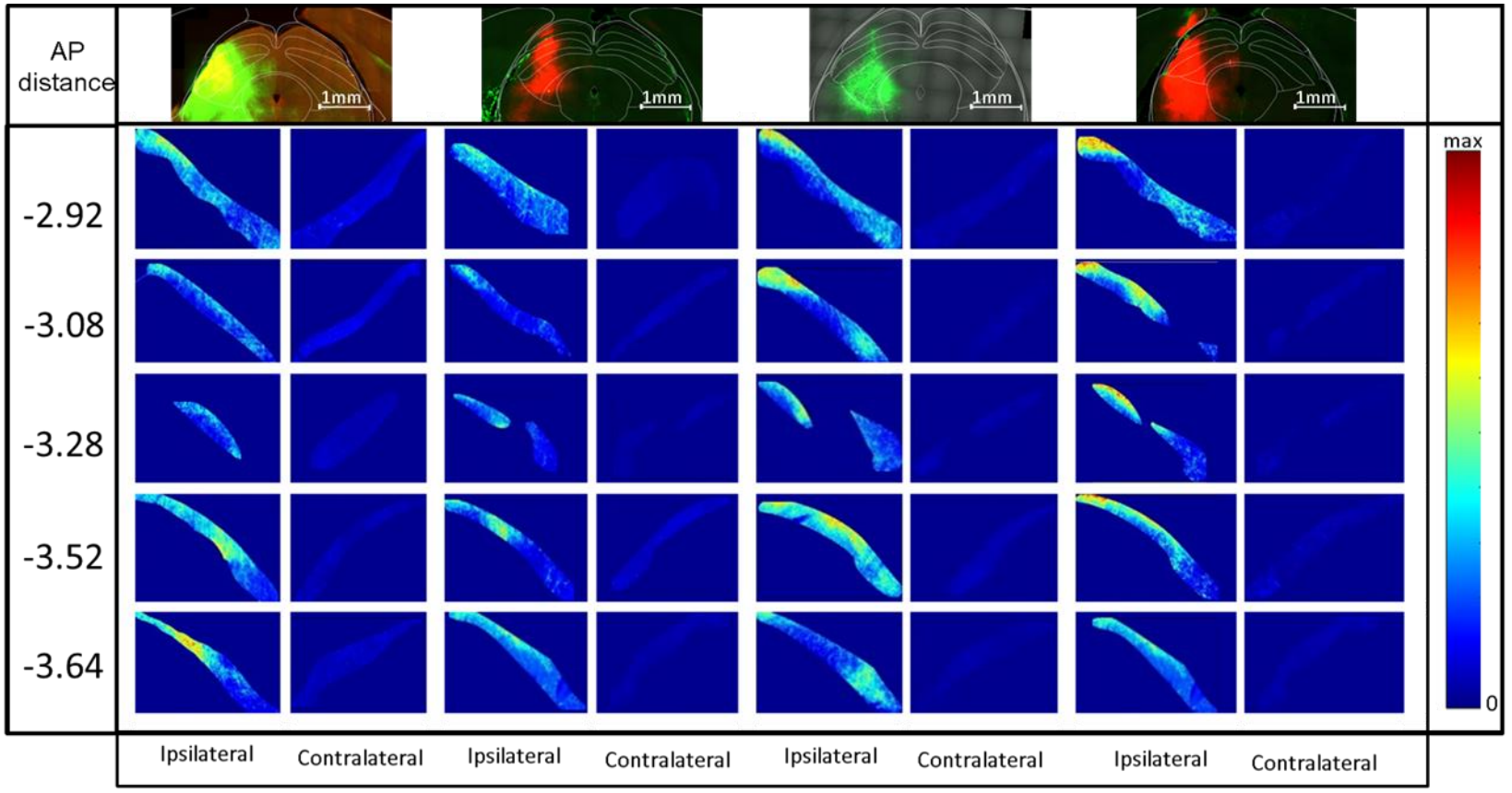

Figure 9 – **Heat-map of the SC projections in ipsilateral and contralateral SNc.** Intensity projections restricted to the ipsilateral and contralateral SNc area, represented as a logarimic heat-map of intensity, normalized to the minimum intensity in SNc, througout the anterior-porterior coordinates represented. Injection site of the YFP or Tdtomato cre dependent anterograde virus (green and red respectively) in the deep and intermediate layers of the left SC are represented on the top. This images were acquired with a brightfield and fluorescence slide scanner microscope using a 20x magnification objective and were overlaied with the corresponding overlay with the Paxinos brain atlas figure. Each two colums belong to the brain which the injection is shown on the top, being the left column the ipsilateral profiles and the right column the contralateral profiles.

In figures 8 and 9, almost no labelled projections were visible in contralateral SNc. Accordingly, statistical analysis showed that the density of projections from SC to the SNc was significantly different for the ipsilateral and contralateral site to the injection (Figure 10). For all AP distances analyzed, contralateral SNc has a lower intensity of projection than ipsilateral SNc (Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, for AP coordinate between -2.5 and -2.75 p=0.0361, AP coordinate between -2.75 and -3 p=4.2970e^{-5}, AP coordinate between -3 and -3.25 p=0.0016, AP coordinate between -3.25 and -3.5 p=0.001, AP coordinate between -3.5 and -3.75 p=0.0036 and AP coordinate between -3.75 and -4 p=0.0196).

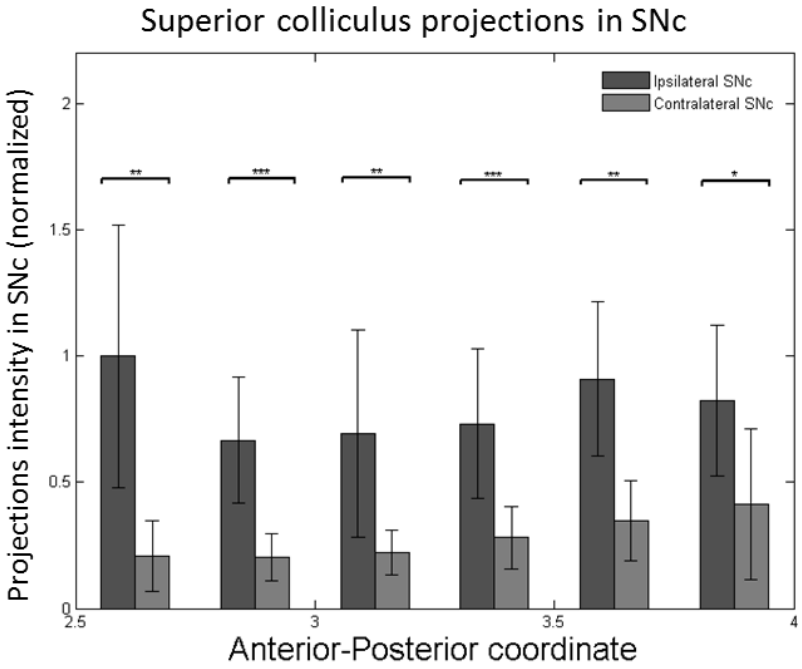

Figure 10 – **Intensity of superior colliculus axonal projections is higher in ipsilateral SNc.** Intensity of projections within ipsilateral and contralateral SNc area normalized to the unity [n=7 mice, (mean ± standard deviation)/maximum value of intensity in SNc], (* - p<0.05, ** - p<0.01, *** - p<0.001).

Moreover, when analyzing the differences between medial and lateral SNc, a higher density of projections is found in the lateral area for the AP distance between -3.25 and -3.5 (Two-sample Kolmogorov-Smirnov test, p=0.0012) in ipsilateral SNc (Figure 11). For the remaining AP distances, although not significant (Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, for AP coordinate between -2.5 and -2.75 p=1.0378, AP coordinate between -2.75 and -3 p=0.9056, AP coordinate between -3 and -3.25 p=1.2699, AP coordinate between -3.5 and -3.75 p=0.5540 and AP coordinate between -3.75 and -4 p=1.2699), it seems to be a tendency for higher density of projection in the lateral area, which could be a consequence of injection site target.

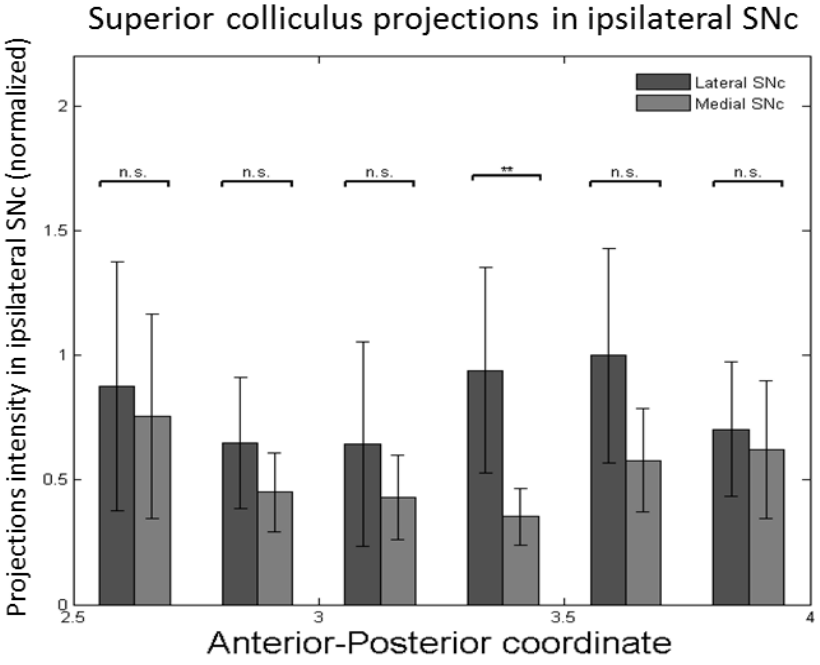

Figure 11 –**Lateral and medial ipsilateral SNc have similar projection intensities.** Intensity of projections within lateral and medial ipsilateral SNc area normalized to the unity (n=7). ((mean ± Standard deviation)/maximum value of intensity in ipsilateral SNc).

- **GABA neurons appear not to be a major player in tecto-nigral projection**

Next, we tested the possible contribution of GABAergic neurons to the SC-to-SNc projection. We found almost no labeling of terminal in SNc, suggesting that GABAergic neurons are not a major player in the direct SC-SNc projection. When analyzing the presence of labelled projections within SNc area of GAD-Cre mice we found a generalized absence of GABAergic projections (Figure 12), although, very sparse and rare, bouton-like structures were found throughout the all structure (Figure 12 a'') to g'')). Moreover, although, a high density of cell bodies was infected with the virus, the labelling seems to be mainly circumscribed to the SC area, and few GABAergic projections were found anywhere in the brain.

It is important to mention that for this experiment only one mouse was obtained, and obtaining more samples is part of ongoing experiments. Therefore, no quantifications and few conclusions regarding the distributions of the projections can be made at the current stage. Nevertheless, the lack of strong GABAergic input from SC to SNc seems to be evident.

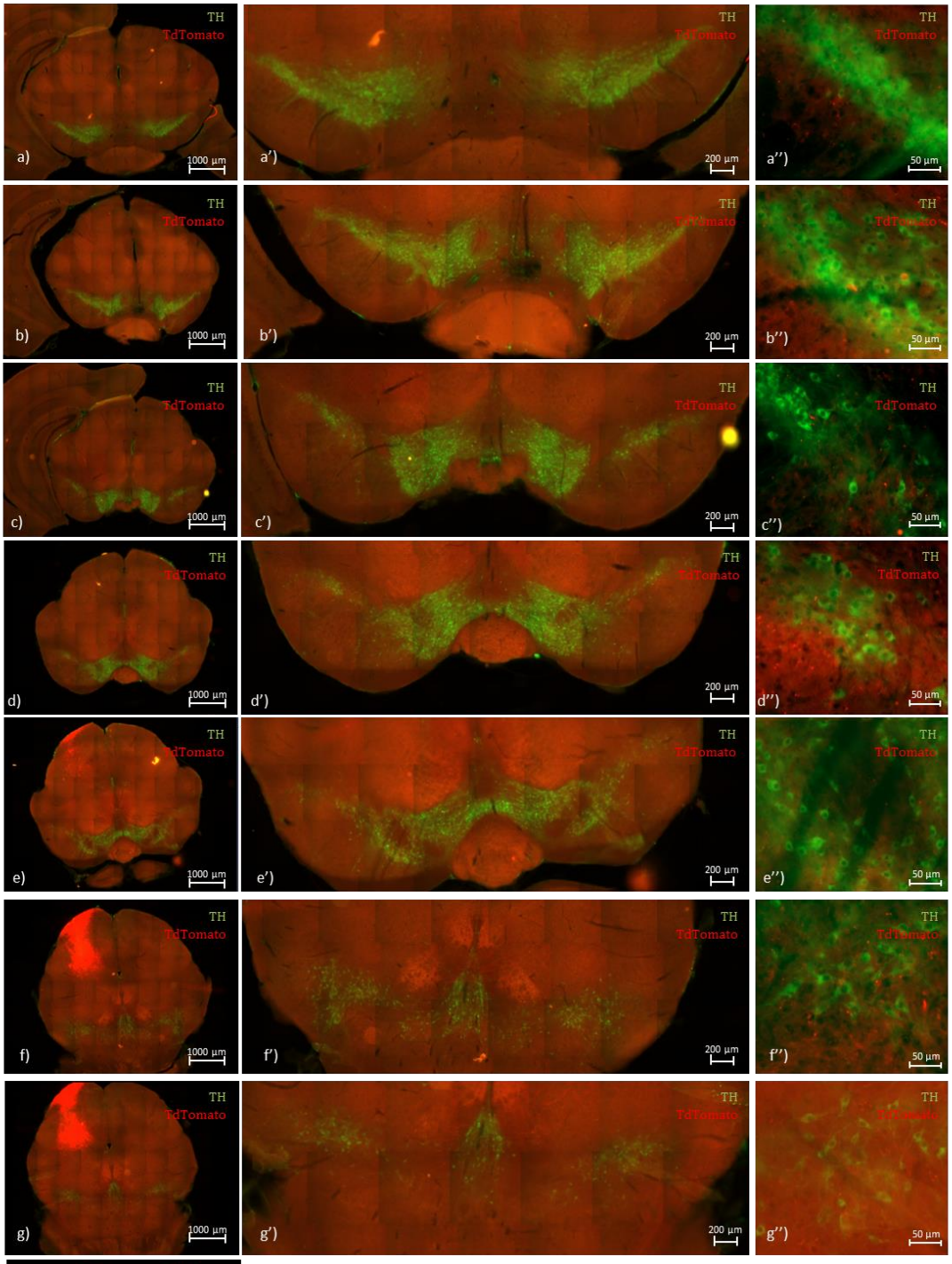

Figure 12 - **GABAergic projection to SNc** a) to g) sequential brain slices of a Vglut2-Cre mouse, separated by 100um, tdTomato Cre-dependent anterograde virus (red) with immunostaining of tyrosine hydroxylase enzyme with Alexa fluor 488 (green). a') to g') higher magnification of ipsilateral and contralateral SNc and VTA of the corresponding slices. a'') to g'') higher magnification of an area of the SNc with labeled projections. h) Injection site of the tdTomato Cre-dependent anterograde virus (red) in the deep and intermediate layers of the left SC (bregma -4.16). Images acquired with a fluorescence slide scanner microscope using a 20x magnification objective.

Section 2b: Cholinergic neurons

The inexistence of choline acetyltransferase (ChaT) and vesicular acetylcholine transporter (VAch) positive neurons in any layer of the SC,was resported in the rat (Ichikawa 1997). But In the cat, while some studies report also the inexistence of Chat positive neurons in the SC (Kimura 1981) others report the presence of few cholinergic neurons located in the superficial gray layer and even fewer in the optical layer (Hall 1989). Interestingly, both studies were based on ChaT immunoreactivity. Although Acetylcholine seems not to be synthesized in the SC of rats, it reaches the deep layers of the rat's SC (Schafer 1998) and the intermediate gray layer of cat's SC in a patched distribution (Bickford 1993) possibly via afferent inputs from the pons and peduncle pontine nucleus(PPN) (Sparks 1989).

Since no study, so far, reported the distribution of cholinergic neurons in the mouse SC, we decided to further clarify this issue before trying to explore the possibility of a cholinergic input from SC to the SNc.

Methods:

In order to confirm the location and/or presence of cholinergic neurons within the SC we used 3 wild type Black 6 mice approximately 3 months old.

Perfusion and Histology:

These animals were perfused following the same procedures described above (see section 1).

The collected brains were cryopreserved in 20% sucrose solution and cut in 50 µm serial coronal sections in the freezing microtome. The sections acquired were permeabilized in PBS-Triton 0.4% solution and incubated 48h at 4^{o}C in goat antibody to choline acetyltransferase (ChaT) 1: 500 (Chemicon/Millipore AB144P), diluted in PBS-T 0.4%. A secondary incubation with Alexa 594 anti-goat (Molecular Probes/Invitrogen A11058) 1:1000, diluted in PBS-T 0.4% solution, was performed for 2 hours at room temperature. After rinse in PBS-T 0.4%, the sections were then mounted in Mowiol (Merk) and coverslipped.

Images of brain areas that are known to contain cholinergic neurons, and of the all volume of SC were acquired in a confocal laser point-scanning microscope (Zeiss LSM 710) using a 6x4 tile scan with the 63x magnification objective at 20µm from the surface.

Analysis:

Cell bodies with clear ChaT staining were quantified using the cell counting plugin in FIJI software (Schindelin 2012) and results analyzed in Microsoft Excel (Microsoft Office) and MATLAB (MathWorks). Distribution of Number of cells with clear staining, for the different areas, was tested for normality using One-sample Kolmogorov-Smirnov test, with a 5% significance level. Due to small sample size and non-normal distribution of data, a non-parametric two-sample Kolmogorov-Smirnov test was used to compare the density of cells in the different areas to the SC, with a 5% significance level (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

Results:

In line with previous reports, we found strong staining of ChaT in Pedunculopontine Nucleus (PPN) (Spann 1992), Latero Dorsal Tegmental Area (LDTg) (Satoh 1986), and OculoMotor Nucleus (OCM) (Dos Santos 2016). Importantly, we also found ChAT-immunopositive cell bodies in the striatum, a structure known for the presence of a small percentage (~1%) of cholinergic neurons (Macintosh 1940, Lim 2014). In contrast, we found no immunopositive ChAT cell bodies in the entire extension of the AP, ML and DV axes of the mouse SC (n=3), as was assessed by counting the cell bodies with clear staining of ChaT (figure 13 a) to f)). Some focal staining (<5 µm in diameter) is visible in all imagens, especially in SC, which should be the aggregation of cholinergic terminals around cell bodies, therefore, not representing cholinergic cell bodies (confirmed by higher magnification confocal microscopy). To better demonstrate the inexistence of ChaT neurons in SC we compared the staining in SC with the staining in other brain areas known to have this type of neurons, in different densities (two-sample Kolmogorov-Smirnov test), such as (PPN) ($p=0.0326$), (LDTg) ($p=0.0326$), Striatum ($p=0.0326$) and (OCM) ($p=0.0326$) (figure 13 g). Our results indicate that there are no cholinergic

neurons in the mouse SC, similar to what was observed in the rat. Thus, we can conclude that the direct tectal input to SNc is not cholinergic.

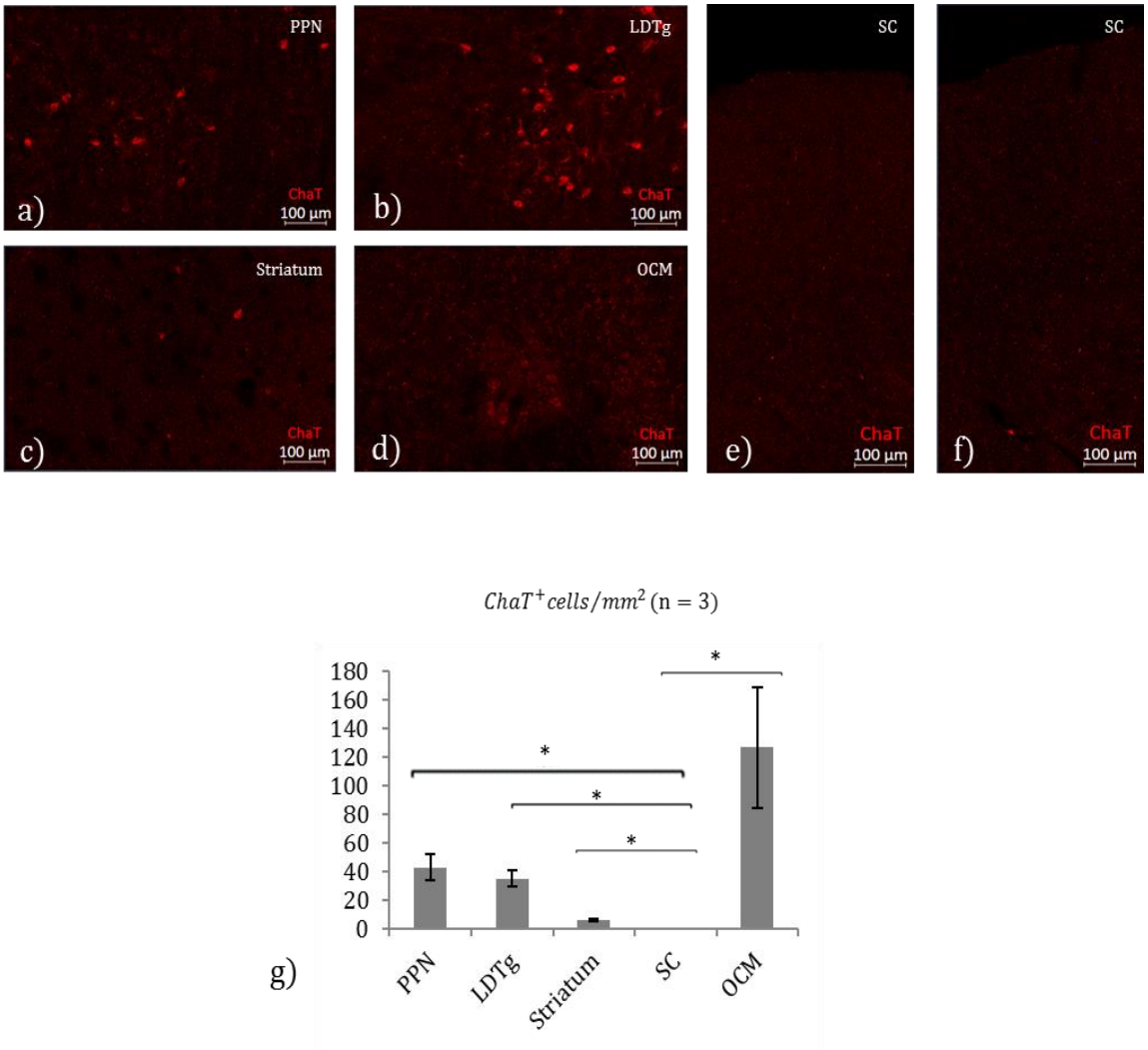

Figure 13 - **Cholinergic neurons are absent in SC.** Quantification of cholinergic neurons in different brain areas known to have this type of neurons and in the superior colliculus. Sections with anti-ChAT staining: a) Pedunculopontine nucleus (PPN) b) Latero dorsal tegmental nucleus (LDTg) c) Striatum d) Oculomotor nucleus (OCM) e) Superior colliculus (SC): bregma -4.48 f) Superior colliculus (SC): bregma -4.96. All images were acquired in the confocal microscope using a 6x4 for a) to d) and a 3x7 tile scan for e) and f) with the 63x magnification objective at 20µm from the surface. g) Quantification of cholinergic neurons in the above referred areas by anti-Chat staining and cell counting using FIJI (mean ± Standard deviation).

Section 3: Behavior implications of SC to SNc projection

In the previous sections we have shown that the majority of the SC to SNc projections are glutamatergic. Therefore, we next wanted to study their neural activity and dissect the behavioral role of this projection. To this end, we used mice expressing Cre recombinase in glutamatergic neurons, electrophysiologically recorded neuron-specific activity in the SC, and specifically stimulated the projections of SC neurons in the SNc during self-paced locomotion.

Methods:

For these experiments, we used 4 Vglut2-ires-cre male mice with approximately 3 months age. Two were used for the experimental condition [channelrhodopsin 2 (ChR2) virus injections] and 2 for the control condition [enchanced yellow fluorescent protein (eYFP) virus injections].

Stereotaxic Surgery:

Animals were deeply anesthetized through inhalation of isoflurane (Vetflurane - Virbac) (1.5% – 4.0 %) and placed in the stereotaxic head holder. After alignment of the head, a craniotomy was performed using the AP and ML coordinates optimized for SC (AP -4.0 mm, ML +/- 1.1 mm), SNc (AP -0.8 mm, ML +/- 1.40 mm) and 3 additional craniotomies were opened to place 2 holding screws for support and one for grounding. 750 nl of Cre-dependent virus that expresses ChR2 (AAV1.EF1a.DIO.hChR2(H134R)-eYFP.WPRE.hGH) or eYFP (AAV1.EF1a.DIO.eYFP.WPRE.hGH) were injected in the SC (AP -4.0 mm, ML +/- 1.1 mm, DV -2.1 mm), at 4.6 nl per pulse every 10 seconds, using a glass micropipette attached to a Nanoject II auto-nanoliter injector (Drummond Scientific). Afterwards, we bilaterally implanted an optic fiber[4] in the SNc using the target coordinates AP -0.8 mm, ML +/- 1.40 mm, DV -4.5 mm. The fibers were lowered at a 25° angle, in order to avoid any possible damage to SC by bypassing it, therefore achieving the final coordinates of

[4] All the fibers were made using the (Sparta 2012) specifications, and only fibers with efficiencies higher than 65% were used for these experiments. Moreover, fibers in both sides were matched to have similar efficiency.

AP -2.75 mm, ML +/- 1.40 mm, DV -4.5 mm[5] (Figure 3.2). Additionally, a movable recording electrode with a 16-channel array was implanted in the top of the intermediate layers of SC (AP -4.0 mm, ML +/- 1.1 mm, DV -1.0 mm) and connected with one of the screws in order to electrically ground the system. Fixation of the fibers and electrode to the skull was achieved through application of superglue and dark dental cement (Lang Dental).

Following this procedure, the scalp was closed with Vetbond (3M) and carprofen (0.1ml/g sub-cutaneous) was injected as post-surgical analgesic. Throughout the surgery, the temperature of the mice was maintained at 36^{o}C by a thermostatically controlled heating pad, and the eyes were covered with a gel (Vidisic gel- Bauch&Lomb) to prevent drying.

The injected animals were given 5 weeks, in order to allow the replication and transport of the virus through the axonal projections.

Setup:

The experimental setup was designed in order to allow a self-placed movement of the mice and its detailed tracking. Avoidance of external sensory stimuli that could activate SC and perturb mice behavior was also a requirement. The experimental setup consists of an open field arena in transparent acrylic (48cm x 48cm) placed inside of a noise and light isolated box. We captured video (150 frames per second) from two dimensions using a PointGrey Grasshoper3 camera mounted below the transparent flooring of the behavior box. The side dimension was imaged by using a mirror placed on the side of the box. This allowed us to capture x, y and z axis of the movement of the mice. Illumination was achieved through an infrared lamp also placed in the bottom of the open field.

Additionally, when the mouse is in the setup, body acceleration, on the three axes, was also recorded by an accelerometer attached to the head-stage (Figure 3.0), and the movement of the animal captured by the video camera at 150 frames per second (fps).

[5] These coordinates were previously used in (Alves da Silva, submitted) and overlap with an area with glutamatergic projections from SC, as shown in section 2.

Besides, the accelerometer had a structure with bilateral led lights attached to it to allow better tracking of the mice's movements. Moreover, electrophysiology signal from in vivo neural activity was amplified, band-pass filtered and recorded.

Behavioral protocol:

The behavioral experiments consist of 4 sessions with 30 or 20 min of self-paced movements on the open field arena, preceded by 1-minute (min) session of unilateral photo identification[6] (left and right side separately). At the end, a control session with the duration of 10 min was run, during which a visual stimulus (external LED light) was delivered as a control for the light visible at the coupling point of the patch cord and implanted fibers. During all sessions, SC extracellular activity was obtained via electrophysiology recordings and unilateral or bilateral laser stimulations were delivered.

During the photoidentification (PhID) session, a 1 Hz, 200 ms pulse of blue light was unilaterally delivered (right and left stimulation separately). The PhID sessions were performed in the home cage of the animals in order to avoid habituation to the experimental setup. For the second session, the first 30 min were free of any manipulation, consisting only in recording the extracellular spike activity and the animal's self-placed behavior in the open filed. During the next 90 min, ChR2 or eYFP infected terminals in SNc were unilaterally (left and right fibers attached individually) and bilaterally stimulated with a 10 pulse, 20 Hz, 500 ms train of blue light when the animal was immobile. The stimulation timings were calculated based on real-time information from the accelerometer placed on top of the recording electrode. Every time the animal remained immobile for a period of 900 ms, the laser was triggered with a probability of 50% (positive/ negative stimulation).

To synchronize all time series (accelerometer, video and electrophysiology signals and Multipower generator/Laser frequency driver) we used a WEAR controller board (Scientific Hardware Platform, Champalimaud Foundation), and BONSAI software

[6] To try to identify which SC neurons are activated by the light.

framework (Lopes 2015) to store all respective time stamps and data. The triggering of all components was done by the board, which in turn was triggered by BONSAI.

In the interval between the different conditions both the electrode and accelerometer remained connected. The laser power applied in these experiments was between 3.5 mW and 6.5 mW depending of the mouse. We used the lowest power within this range that produced a behavior phenotype, in order to avoid excitotoxicity due to over activation of neurons. After each session, the electrode was lowered 0.104 or 0.156 mm, so that at the time of the next session (>24 h) the tissue around the electrode tip was already stabilized.

Sessions were run once per day, in a total of 9 sessions per animal, corresponding to the number of times the electrode needed to be lowered in order to cross all dorsoventral extension of the intermediate and deep layers of SC. The order of unilateral stimulations was alternated in every session.

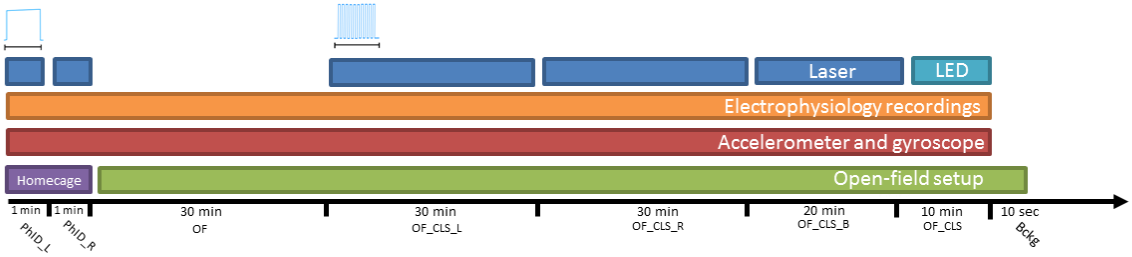

Figure 14 – **Schematic of one behavior session.** PhID_L: unilateral photo identification on the left side; PhID_R: unilateral photo identification on the right side; OF: Open field; OF_CLS_L: Open field with closed loop unilateral stimulation on the left hemisphere when immobile; OF_CLS_R: Open field with closed loop unilateral stimulation on the right hemisphere when immobile; OF_CLS_B: Open field with closed loop bilateral stimulation when immobile. OF_CLS_LED: Open field with closed loop activation of a LED light when immobile. Bckg: video recording of empty open field arena for background subtraction. . : 1 Hz, 200 ms pulse of blue light every 1 second. : 20 Hz, 500 ms burst of blue light after a period of immobility.

Perfusion and histology:

After completion of the behavior experiments the animals were perfused following procedures described above (see section 1).

The collected brains were cryopreserved in 20% sucrose solution and cut in 50 µm serial coronal sections in the freezing microtome. The sections acquired between -2.54 mm and -4.72mm from bregma, corresponding to the volumes that contain the all SC and SNc structures, were permeabilized in PBS-Triton 0,4% solution and incubated overnight at 4^{o}C in mouse antibody to Tyrosine hydroxylase (1: 5000 dilution in PBS-Triton 0,4% solution) (ImmunoStar 22941), diluted in PBS-Triton 0,4%. A secondary incubation for 2h, at room temperature, was performed with an Alexa 594 anti-mouse secondary antibody (1: 1000 dilution in PBS-Triton 0,4% solution). After rinse in PBS-T 0,4%, the sections were then mounted in Mowiol (Merk) and coverslipped.

Sequential images of SC and SNc were collected using a Zeiss AxioImage Fluorescence microscope, with the 10x magnification objective. The acquired images were overlaid with the corresponding Paxinos Brain Atlas figure in order to confirm the virus injection site and position of the electrode and fibers.

Localization of fiber and electrodes was determined through identification of the respective track in the brain and comparison with the corresponding brain section of the *Paxinos Brain Atlas* (Paxinos 2004).

Analysis:

Data analysis was done using custom Matlab (MathWorks) scripts.

Behavioral analysis:

Acceleration (g) and angular velocity (measured in degrees per second, dps), were recorded during the all duration of experiments. This allowed us to have detailed information about animal all body movement in the 3 axes of acceleration (x: right/left body movement; y: font/back body movement; z: up/down body movement), and head rotation, in the 3-axis (left/right; up/down rotations and left/right head leaning).

To study the relationship between total body acceleration (the squared sum of all individual accelerations (high-pass filtered at 0.5 Hz), which represents all movement irrespective of direction), head angular velocity (head rotations) and stimulation or gait

initiation time points were done using custom Matlab (MathWorks) scripts. Peak amplitudes for each axis of acceleration and rotation was calculated as the maximum amplitude above baseline in the 2 seconds period after stimulation. (Baseline: Root mean square for the -2 seconds to 2 seconds around the start of the stimulus). Normal distribution of data was tested using one-sample Kolmogorov-Smirnov test, with a 5% significance level. Due to non-normal distribution of data, a non-parametric two-sample Kolmogorov-Smirnov test, with a 5% significance level, was used to compare the peak amplitudes and motor response times (time between the start of stimulation and the occurrence of a peak) for all stimulation paradigms and axis of acceleration and rotation, between ChR2 and YFP mice. Then, a Bonferroni-Holm correction for multiple comparisons was used with a 0.05 α value (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$). Difference between groups, in ChR2 and YFP mice were compared using the Kruskal-Wallis test. Percentage of left and right rotations upon stimulation or spontaneous gait initiation for ChR2 and YFP mice were also calculated and compared by the two-sample Kolmogorov-Smirnov test, with a 5% significance level. After, a Bonferroni-Holm correction for multiple comparisons was used with an 0.05 α value (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

Electrophysiology analysis:

Offline spike sorting of the continuous electrophysiology data was performed using *Spike2* software (CED). Continuous electrophysiology data files from the different sets within each session were collated in the following manner: PhID_L+OF_CLS_L+OF; PhID_R+ OF_CLS_L +OF; PhID_L+PhID_R+ OF_CLS_B + OF_CLS_LED.[7]This was done so that the files were analyzed as a single set in order to unambiguously identify the same single unit spiking across sets. After high-pass filtering the data with with a 300 Hz cut-off, spike detection was performed using a -0.4 to 0.8 ms window. For better extraction of spikes, and to separate by amplitude, spike detection was performed in two steps:

[7] PhID_L: unilateral photo identification on the left side; PhID_R: unilateral photo identification on the right side; OF: Open field; OF_CLS_L: Open field with closed loop unilateral stimulation on the left hemisphere when immobile; OF_CLS_R: Open field with closed loop unilateral stimulation on the right hemisphere when immobile; OF_CLS_B: Open field with closed loop bilateral stimulation when immobile. OF_CLS_LED: Open field with closed loop activation of a LED light when immobile.

first with a negative double threshold set at 3*Root mean square of the signal recorded in the open field condition, and second with a single negative threshold of 15*Root mean square of the same signal. Afterwards, spike clusters were created using the k-means method and manually adjusted. Single unit classification was accepted if the percentage of inter spike intervals higher than 1.5 milliseconds was lower than 0.001%. When this criterion was not fulfilled, spikes were treated as multiunit activity.
Correlations between spike activity, stimulation time points and acceleration and angular velocity were done using custom Matlab (MathWorks) script.
Spike activity was calculated as the average activity across trials around stimulation. The spike activity was measured as rate, i.e., spikes/s, by dividing the average rate per bin by the bin duration (0.05 s).

Normal distribution of spike activity during the -2 seconds and 2 seconds around events (spontaneous movement initiation or the different stimulation paradigms) and of spike response times after the events was tested using one-sample Kolmogorov-Smirnov test, with a 5% significance level. Due to non-normal distribution of data, a non-parametric two-sample Kolmogorov-Smirnov test, with a 5% significance level, was used to compare the distribution of spike activity and response times for all stimulation paradigms, between ChR2 and YFP mice. After that, a Bonferroni-Holm correction for multiple comparisons was used with an 0.05 α value (* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$).

Results:

- **Stimulation of SC glutamatergic projections in SNc elicit gait initiation and turning behavior**

SC glutamatergic projections to SNc were stimulated, in an open-field task, for 500 ms with a 20-Hz burst, during periods of immobility, in order to assess the consequences of the activity of such pathway (Figure 16 a). Placement of the fibers and electrodes in SNc and SC Intermediate/deep layers, respectively, was successfully achieved (Figure 15). Optic fibers are placed slightly above SNc so that the blue light could spread through a bigger area of projections in agreement with the work of (Alves da Silva, submitted).

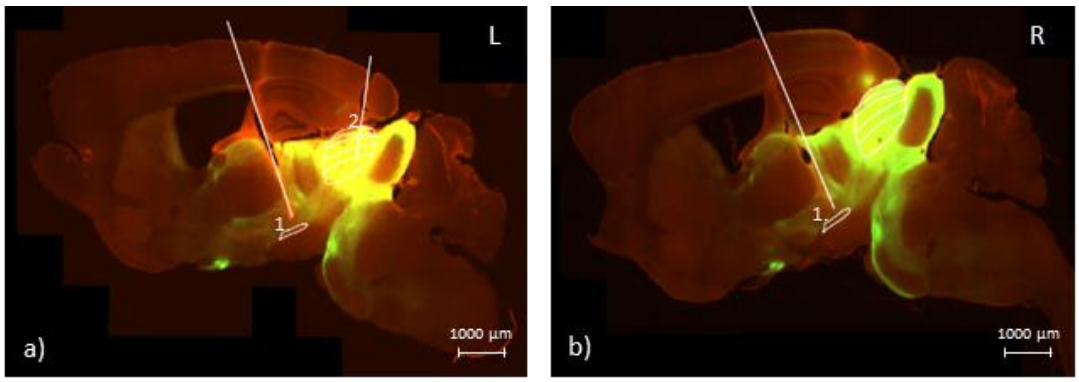

Figure 15 – **Example of fibers and electrode positions in the brain.** Sagittal sections of one of the brains from the mice used in the following experiments as an example of the position of the fiber and electrode implanted, and virus injection (green). a) Left hemisphere with fiber and electrode implanted (lateral 1.4 mm). b) Right hemisphere with only the fiber implanted (lateral -1.4 mm). 1. SNc and track of implanted fiber 2. SC and track of electrode implanted in the intermediate and deep layers. Images acquired with a fluorescence slide scanner microscope using a 10x magnification objective.

When the laser was activated, immediate transition from resting state movement was observed in ChR2 mice, in a form of a complete or incomplete turn. In figure 16 d an example of the behavior of a ChR2 positive mouse, upon unilateral stimulation of glutamatergic projections in left SNc, is shown for the 500 ms during blue light laser burst

stimulation. In this example, the animal performs a nearly complete turn within this time window. The movement elicited has components in the 3 spatial axes (Figure 16 b) (x: right/left body movement; y: font/back body movement; z: up/down body movement), after the beginning of the stimulation (time = 0) an immediate increase of modular acceleration (g) occurs, which persists during the total time of stimulation (500ms) and stabilizes afterwards. Real acceleration (g) oscillates between negative and positive values before returning to near zero values (stabilization) after the stimulation. Leaning and rotations of the head in all the directions (left/right; up/down) are also elicited (Figure 16 c)) and likewise occur with a higher amplitude during light-on period. For control animals (YFP positive) no alteration on resting state occurred.

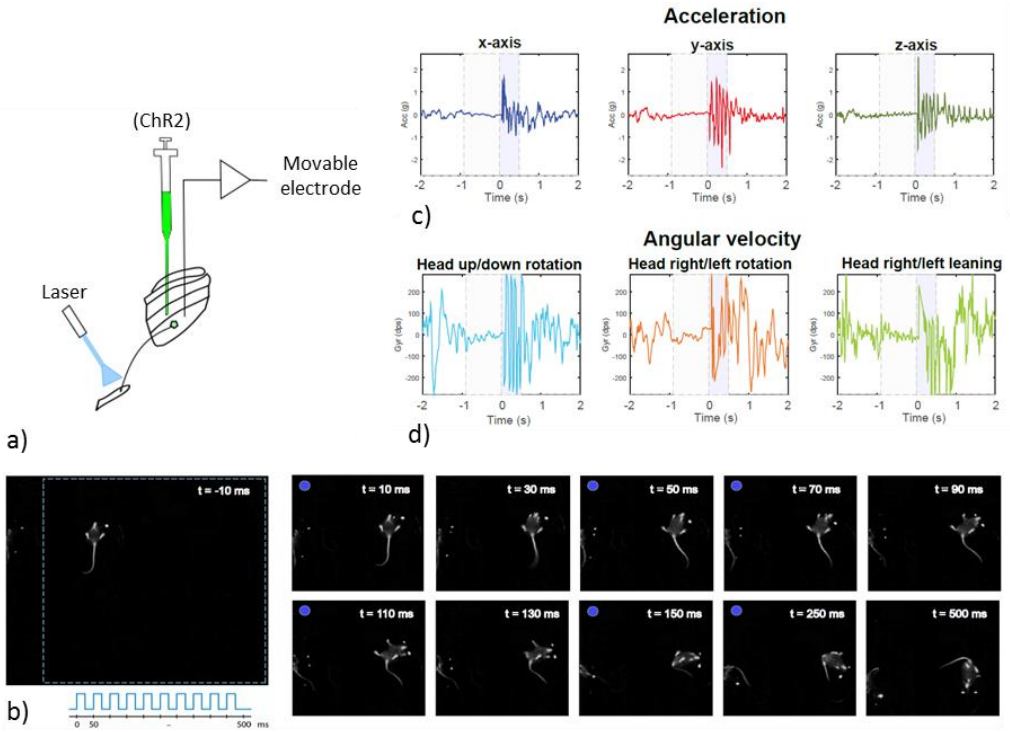

Figure 16 – **Stimulation of SC glutamatergic projections in SNc elicit turning behavior**. a) Schematic of simultaneous SC glutamatergic projections stimulation in SNc and SC recording. b) example of the behavior of a ChR2 positive mouse upon unilateral stimulation of glutamatergic projections in left SNc, for the 500ms that the blue light burst last (represented in the bottom) c) corresponding acceleration (*g)* profiles in the 3 axes for the 2 seconds before and after stimulation. Beginning of stimulation corresponds to time = 0, and duration to the darker window. d) corresponding angular velocity, rotation in both directions and leaning, axes for the 2 seconds before and after stimulation. Beginning of stimulation corresponds to time = 0, and duration to the darker window. : Light-on.

This behavior was elicited both by unilateral (left and right side separately) and bilateral stimulation (Figure 17). Indeed, all these protocols in ChR2 mice, generated a similar acceleration and angular velocity pattern in all axes, which reaches a maximum value between 0 and 0.05 seconds in both situations (Figure 17 a, a'). Activation of an LED, in the same conditions that the laser was activated, which would induce optical responses and was used to control for possible outcomes of SC activation by visual stimuli, failed to produce major changes in acceleration and angular velocity, suggesting that the light elicited by a laser stimulation would not affect our results. A low amplitude peak is observed, but its latency exceeds the 0.1 seconds (Figure 17 b, b'). For the control animals (YFP), no major alterations in acceleration and angular velocity were registered upon laser stimulation (Figure 17 c, c'). Interestingly, also LED activation did not create any low amplitude peak in YFP mice, contrarily to what was observed in ChR2 animals (Figure 17 d, d'). Therefore, behavioral result of the stimulation could be attributed to the effect of blue light, and thus to the activity of this pathway, since control animals presented no alterations in behavior upon stimulation.

Accordingly, the amplitude of the peak[8] generated by the stimulation in the different axes of acceleration and angular velocity in the ChR2 mice is significantly higher than the peak generated in YFP mice (two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, p-values in supplementary material – Figure S1) (Figure 18). It is important to notice that CLS-L and CLS-R look similar because in this analysis only absolute values were used. Interestingly, differences in peak amplitude between different stimulation protocols also occurred (Kruskal-Wallis test, differences in supplementary material – Figure S2), being the clearest difference to LED stimulation. In this case the amplitude of the peak generated is evidently smaller than the one for neuronal stimulation, consistent to what was expected. Additionally, situations in which stimulation failed to produce a peak that reached threshold where only visible in YFP (CLS-B: 3.1%, CLS-L: 4.1%, CLS-R:3.5% and CLS-LED:2.1% of stimulations) mice and ChR2 when stimulation was with the external LED (1.1% of stimulations). Differences in the time delay for a motor response after stimulation is also significantly smaller in ChR2

[8] maximum amplitude above baseline in the 2 seconds period after stimulation. Baseline: RMS for the -2 seconds to 2 seconds after stimuli period

mice (two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, p-values in supplementary material – Figure S2). For ChR2 mice, as showed in figure 19, an obvious motor response is generated in the 0.05 seconds after stimulation. The exception, again, lays in the LED stimulation, in which the motor response times are superior to the 0.15 seconds period after stimulation (also verified for the YFP mice). Interestingly, differences in peak amplitude between different stimulation protocols also occurred (Kruskal-Wallis test, differences in supplementary material – Figure S3).

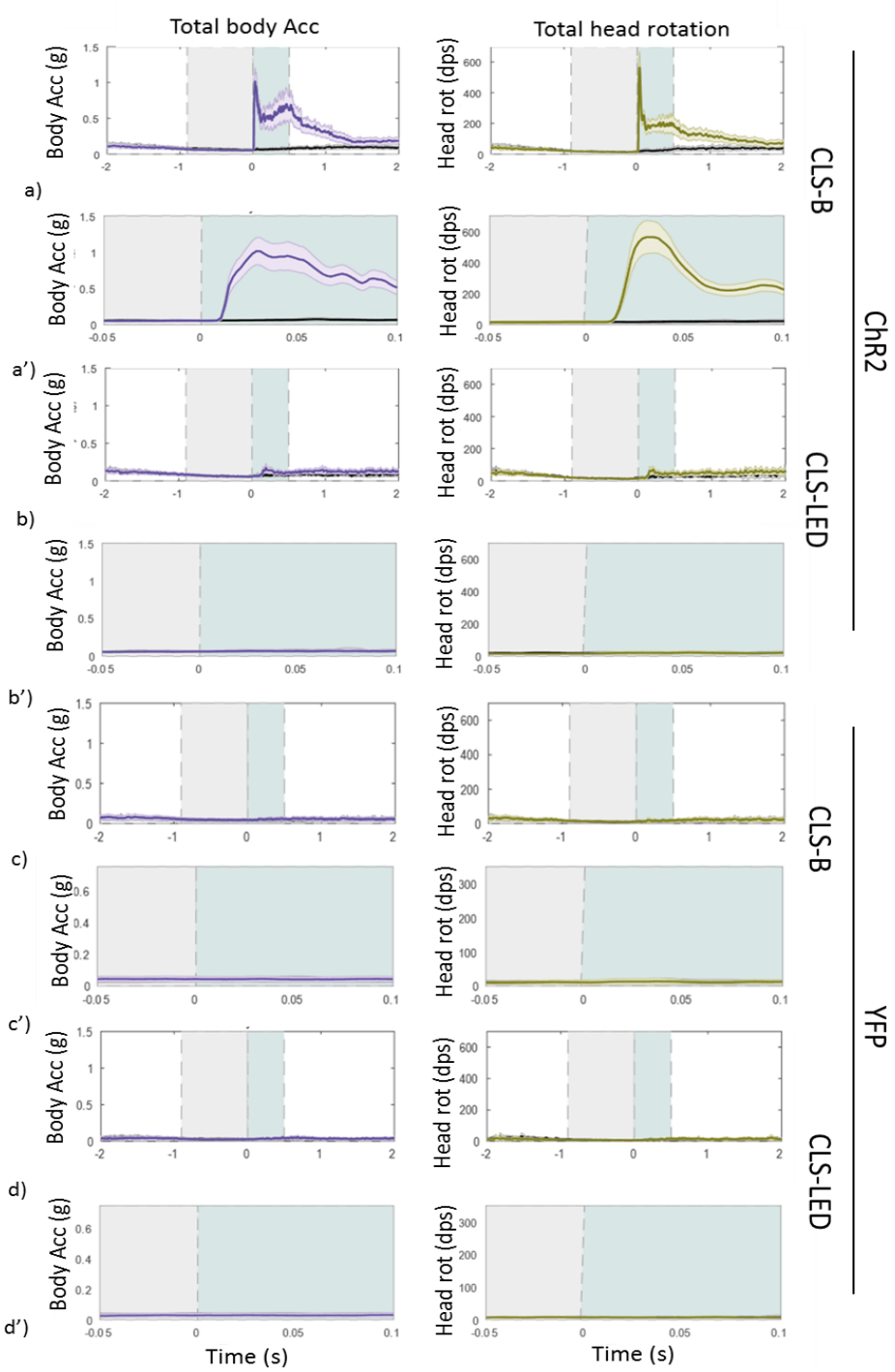

Figure 17**: Stimulation of terminal in ChR2 mice creates an amplitude peak in the firsts 0.05 seconds after stimulation.** Profiles of total body acceleration (g) and total head rotation (dps) for different stimulation paradigms in Chr2 mice (n=2, 3 sessions each) and YFP mice (n=2, 3 sessions each) for the -2 to 2 seconds period around stimulation (a , b, c, d) and for the -0.05 to 0.1 seconds around stimulation (a', b', c', d'). (CLS-B: Bilateral closed loop stimulation, CLS-LED: Closed loop stimulation with an external LED) (Mean ± SEM).

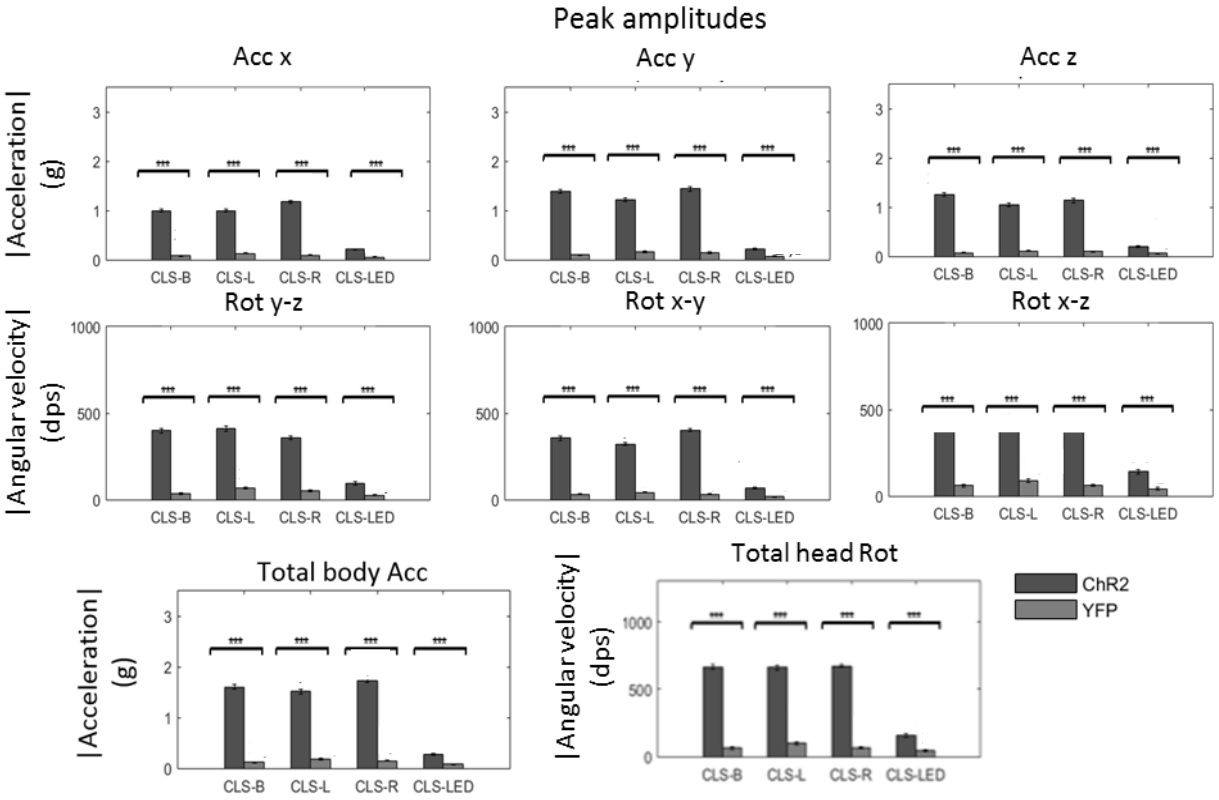

Figure 18 – **Peak amplitude is higher in ChR2 than in YFP mice**. Maximum peak amplitude during the 500ms of stimulation, for ChR2 and YFP mice, in the different axis of acceleration (g) and direction of rotation (dps) for the different stimulation paradigms (CLS-B: Bilateral Closed loop stimulation, CLS-L: Unilateral Closed loop stimulation in the left SNc, CLS-R: Unilateral Closed loop stimulation in the right SNc, CLS-LED: Closed loop stimulation with an external LED). a) Peak amplitude in the three axis of acceleration (g) b) Peak amplitude in the three planes of rotation (dps) c) Peak amplitude in the total body acceleration (g) d) Peak amplitude in the total head rotation (dps) (mean± standard error of the mean (SEM)).

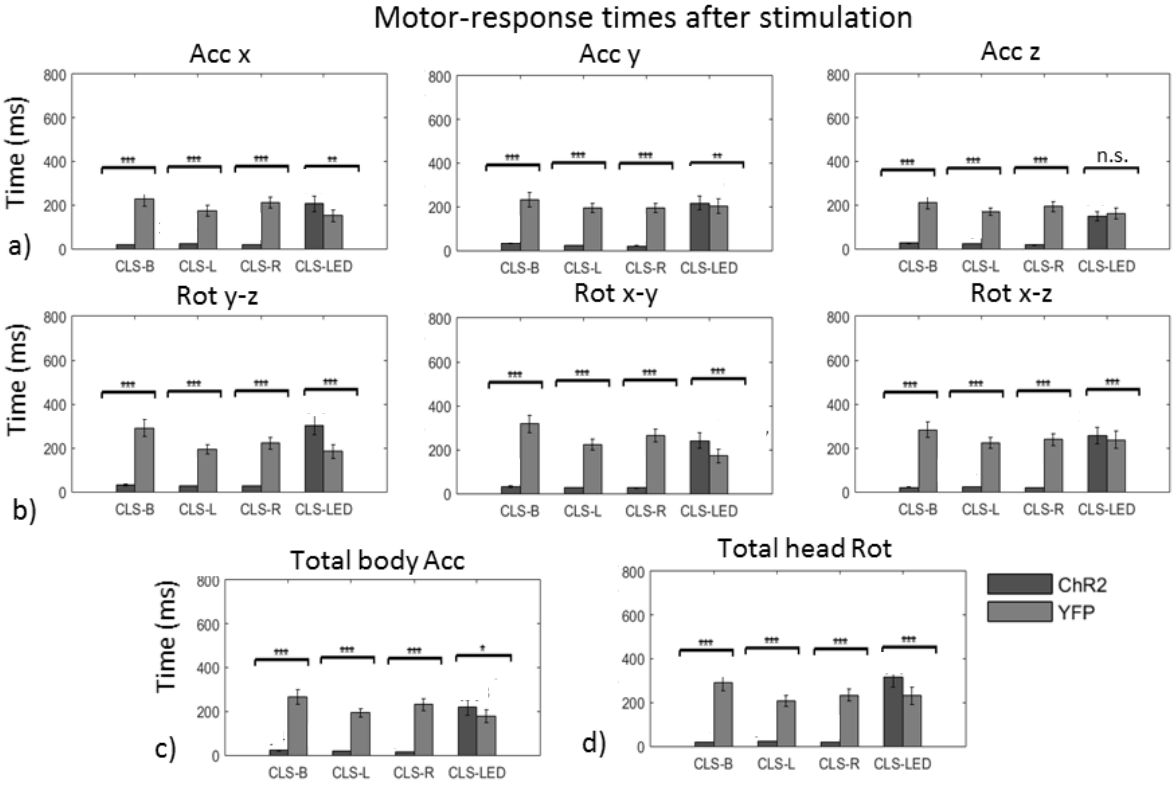

Figure 19 – **Motor-response times after laser stimulation are lower in ChR2 than in YFP mice**. Motor-response times after stimulation, for ChR2 and YFP mice, in the different axis of acceleration and planes of rotation for the different stimulation paradigms (CLS-B: Bilateral Closed loop stimulation, CLS-L: Unilateral Closed loop stimulation in the left SNc, CLS-R: Unilateral Closed loop stimulation in the right SNc, CLS-LED: Closed loop stimulation with an external LED). a) Motor-responses times in the three axis of acceleration (ms) b) Motor-responses times in the three planes of rotation (ms) c) Motor-responses times in the total body acceleration (ms) d) Motor-responses times in the total head rotation (ms) (n= 2 mice per condition, 3 sessions each. Mean± SEM).

An example of the original traces recorded, of body acceleration and cumulative rotation, and respective interpretation, is shown in (Figure 20). The profile of angular velocity (cumulative rotation) generated by a spontaneous movement initiation appear similar to what is generated by the bilateral and unilateral in the right-side stimulation of terminals in ChR2 mice. Stimulation in YFP mice generated no visible rotation (Figure 21 b, b' and d). A rapid oscillation between a negative and positive peak occurs around the time=0 which does not occur in YFP mice. Interestingly, amplitude of the oscillation is higher during stimulations in ChR2 mice. The alternation between negative and positive peaks is consistent for all the events recorded in ChR2 mice (Figure 21 a, a' and c).

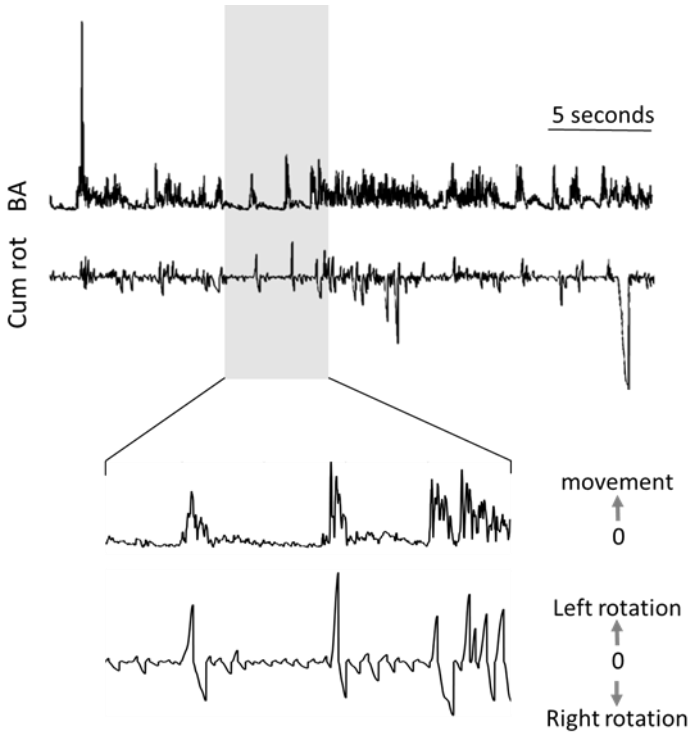

Figure 20: **Positive peaks of Body acceleration represent movement, positive peaks of cumulative rotation represent left head rotation and negative peaks right head rotation.** Original traces of body acceleration (BA) and cumulative head rotation (Cum rot) for 30 seconds of self-placed movement of a mouse.

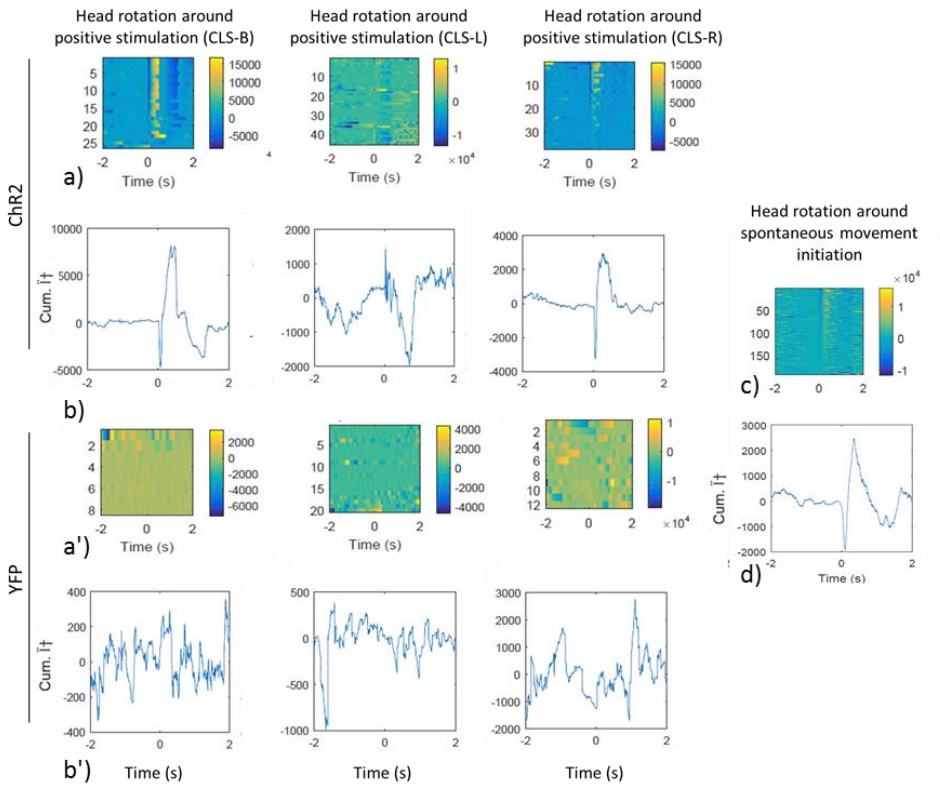

Figure 21: **Head rotation around stimulation (CLS-B and CLS-R) is similar to head rotation around spontaneous movement initiation in ChR2 mice.** Heat map of cumulative intensity of head rotation for each event of positive stimulation in ChR2(a) and YFP (a') mice for the bilateral stimulation (CLS-B), unilateral stimulation in the left (CLS-L) and in right (CLS-R) hemisphere. Cumulative trace of head rotation for one event of positive stimulation in ChR2(b) and YFP (b') mice for the CLS-B, CLS-L and CLS-R. c) Heat map of cumulative intensity of head rotation for each event of spontaneous movement initiation. d) Cumulative trace of head rotation for one event of spontaneous movement initiation.

Moreover, when a mouse spontaneously initiates movement, the direction of the rotation of its head is equally distributed between both left and right side (Figure 22) but when the terminals in SNc are stimulated unilaterally, the side with a higher percentage of rotation appear to be dependent on the side of stimulation, and indeed, a bias to the side of the stimulation is observed. For the left stimulation, the percentage of left rotations is significantly higher than the percentage of right rotation, and for the right stimulation, although not significantly different a tendency for a higher percentage of right rotations is observed (two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, for OF p=1.000, for CLS-L p=0.005, for CLS-R p=0.954 and for CLS-B p=0.954). When the stimulation is bilateral, the direction of the rotations is not significantly different, however a tendency to higher percentage of rotations to right side is observed, such tendency will be discussed later. Regarding the YFP mice, directions of rotations upon stimulation is consistently similar for both side (two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, for OF p=2.430, for CLS-L p=2.430, for CLS-R p=1.240 and for CLS-B p=1.908), however, the bias to the right side is present (Figure 23).

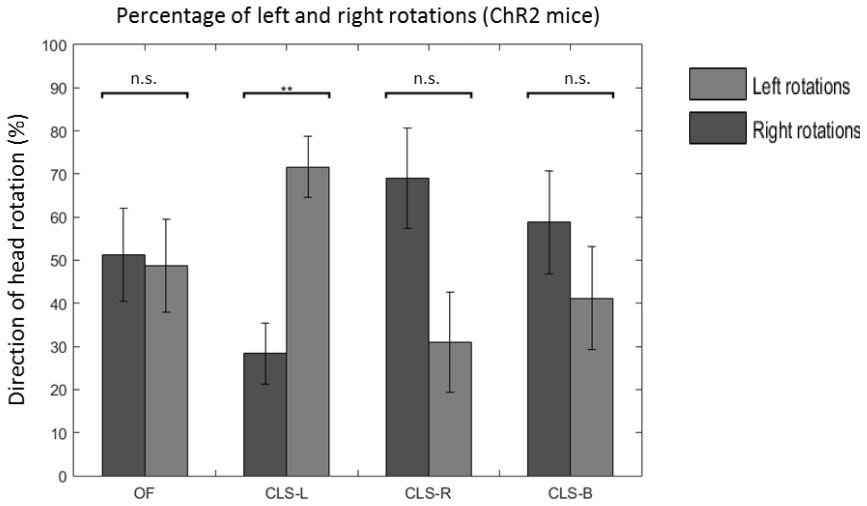

Figure 22**: Percentage of rotations seems higher for the side of the stimulation in ChR2 mice.** Percentage of head rotations for both side and for the different stimulation paradigms in ChR2 mice (OF: open field, CLS-L: Unilateral Closed loop stimulation in the left SNc, CLS-R: Unilateral Closed loop stimulation in the right SNc, CLS-B: Bilateral Closed loop stimulation). OF rotations calculated for rotations at the moment of spontaneous movement initiation. (n= 2 mice, 3 sessions each. Mean ± SEM)

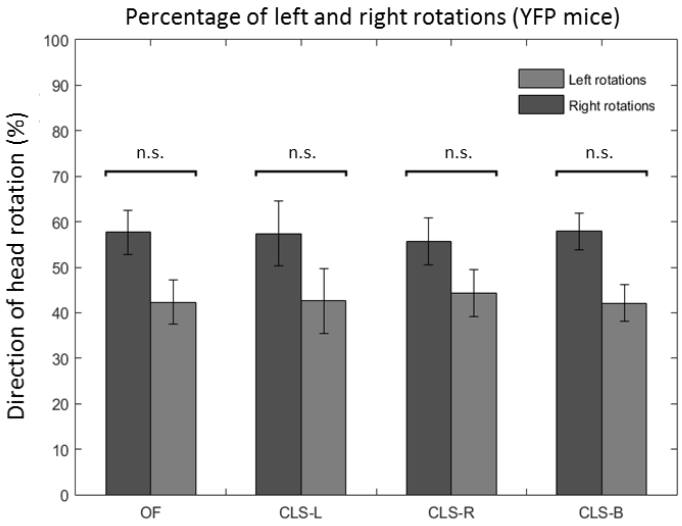

Figure 23**: Percentage of rotations for both sides is similar in YFP mice.** Percentage of head rotations for both side and for the different stimulation paradigms in YFP mice (OF: open field, CLS-L: Unilateral Closed loop stimulation in the left SNc, CLS-R: Unilateral Closed loop stimulation in the right SNc, CLS-B: Bilateral Closed loop stimulation). OF rotations calculated for rotations at the moment of spontaneous movement initiation. (n= 2 mice, 3 sessions each. Mean ± SEM)

- **Stimulation of SC glutamatergic projections in SNc activates neurons in SC deep layers**

At the same time that SC glutamatergic projections to SNc were stimulated, during periods of immobility, neuronal activity in the intermediate and deep layer of SC was being recorded (Figure 16 a). During resting periods, without delivering of any sensory stimulus, SC deep layer[9] neuronal activity is low (Figure 24, -1 to 0 seconds), but when the mouse initiates a movement, an immediate increase of neuronal activity (number of spikes per second) is observed (Figure 24, a, a', 0 to 2 seconds). When a positive stimulation occurred, i.e. laser activation after 900 ms of resting, an immediate increase in neuronal activity in the SC deep layer is also observed for all stimulation paradigms in the ChR2 mice (Figure 25, a to c) indicating that SC neurons were activated by backpropagating action potentials from the terminals in the SNc. In contrast to ChR2 mice, YFP mice did not show the fast transient at stimulation onset but showed a similar profile as observed for the negative stimulation under ChR2, that is, increases in firing reflected approach to baseline firing after the period of resting (Figure 25, a' to c'). At the time of a negative stimulation, i.e. 900 ms of resting were identified but there was no laser activation, a small increase of neuronal activity was also registered, both for ChR2 and YFP mice (Figure 24, b to d and b' to d'). To control for sensory effects of blue light (i.e., undesired stimulation of retinal cells) we used an external LED. This external LED stimulation created a small increase in neuronal activity with a longer delay (>0.15 s) compared to ChR2 stimulation (Figure 25, d). This delay may correspond to the time sensory information takes to go through the visual paths and reach SC. Curiously, the same stimulation in the YFP mice elicited no increase in neuronal activity. Number of spikes/s recruited in a laser stimulation (in ChR2 mice) is larger than the ones recruited in a spontaneous movement initiation. Interestingly, external LED stimulation recruitment of spikes is similar to a spontaneous movement initiation, with a small delay. Accordingly, spike activity generated by the positive stimulation is significantly

[9] Only 3 sessions, for the ChR2 mice and 2 for the YFP mice, with 3 channels each were used for this analysis, therefore, further analyses will be required and the results presented are preliminary.

higher in ChR2 mice than in YFP (Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, p-values in supplementary material, Figure S3), and spike activity generated by spontaneous movement initiation is similar between ChR2 and YFP mice. The spikes response time for spontaneous movement initiation and to the positive stimulation (both laser and LED) are also significative different between ChR2 and YFP mice. Curiously, differences were also found between negative external LED stimulations (Figure 26). (Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms, p-values in supplementary material, Figure S4).

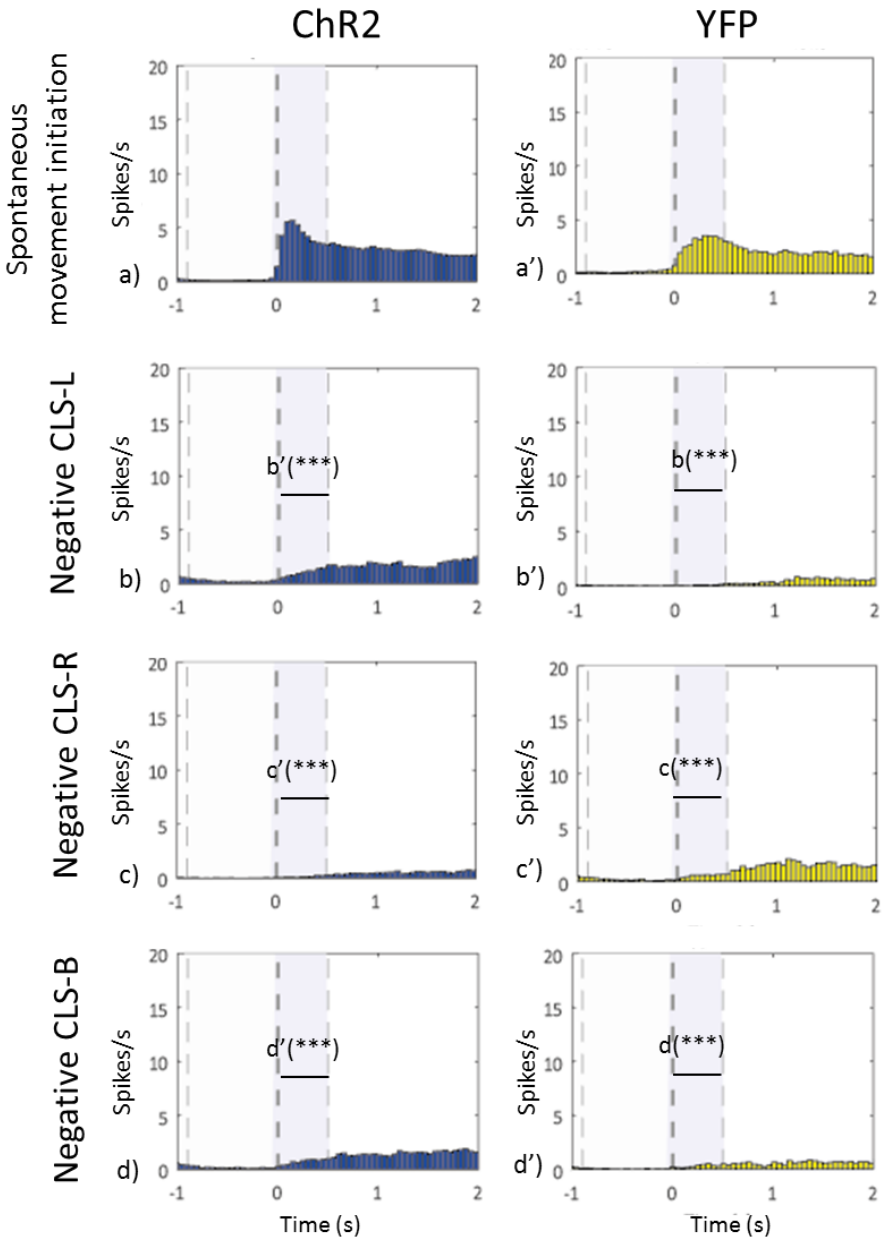

Figure 24: **Spontaneous movement initiation creates an increase in SC deep layers spike activity**. Peristimulus time histogram of spike activity (spikes/s) for the Chr2 and YFP mice in a spontaneous movement initiation. Time zero corresponds to onset of total body acceleration. (a) ChR2, a') YFP) and during negative stimulations for the different stimulation paradigms. CLS-L- unilateral closed loop stimulation in the left side (b) ChR2, b') YFP), CLS-R- unilateral closed loop stimulation in the right side (c) ChR2, c') YFP), CLS-B- Bilateral closed loop stimulation side (d) ChR2, d') YFP).) (n=2 mice per condition, 3 sessions each). a) nº events= 1051, nº units=134 a)' nº events= 213, nº units= 152 b) nº events= 230, nº units=134 b)' nº events= 137, nº units= 152 c) nº events= 235113, nº units=201 c)' nº events= 143, nº units= 94 d) nº events= 151, nº units=201 d)' nº events= 109, nº units= 127.

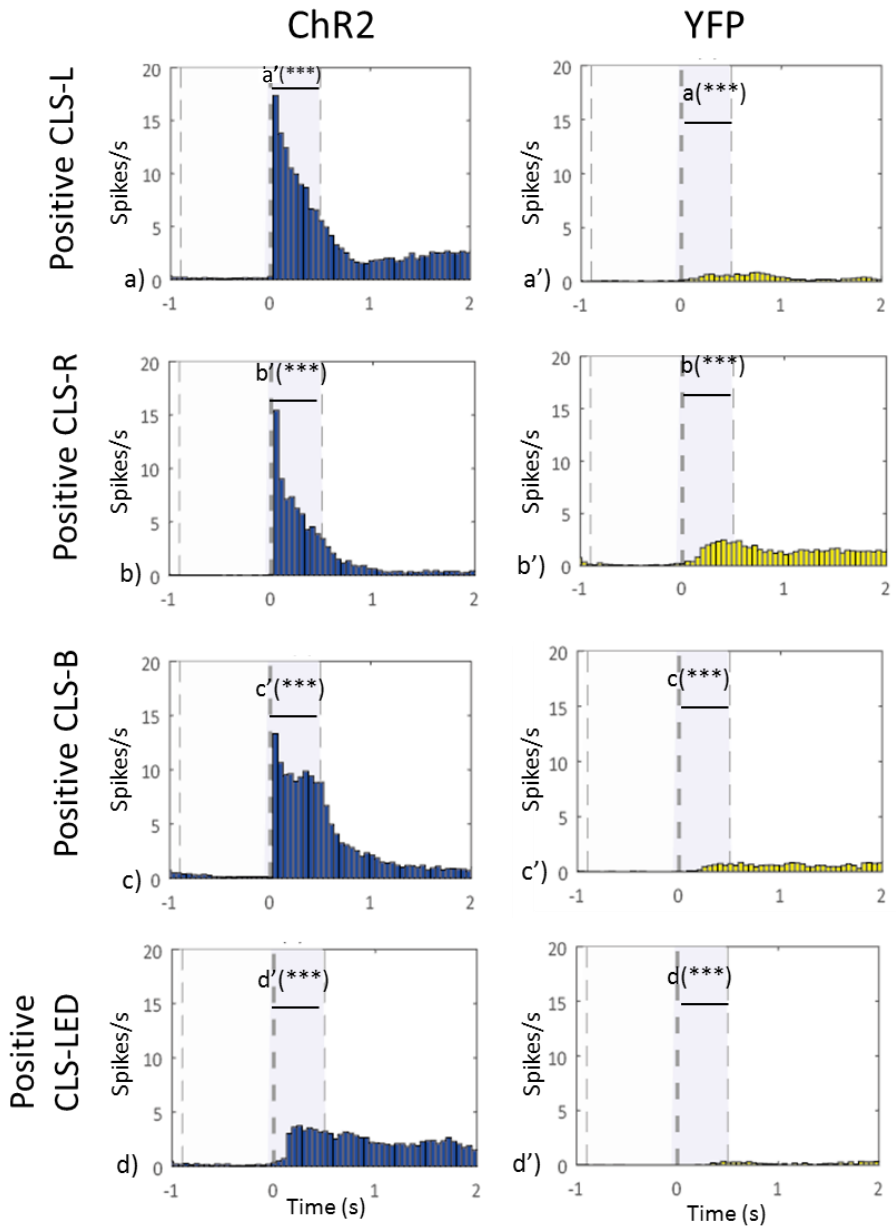

Figure 25: **Laser stimulation creates an increase in SC deep layers spike activity of ChR2 mice**. Peristimulus time histogram of spike activity (spikes/s) for the Chr2 and YFP mice during positive stimulations for the different stimulation paradigms. CLS-L- unilateral closed loop stimulation in the left side (a) ChR2, a') YFP), CLS-R- unilateral closed loop stimulation in the right side (b) ChR2, b') YFP), CLS-B- Bilateral closed loop stimulation (c) ChR2, c') YFP), CLS-LED – closed loop stimulation with an external LED (d) ChR2, d') YFP). (n=2 mice per condition, 3 sessions for the ChR2 and 2 sessions for the YFP). a) nº events= 237, nº units=134 a)' nº events= 119, nº units= 152 b) nº events= 216, nº units=113 b)' nº events= 144, nº units= 94 c) nº events= 134, nº units=201 c)' nº events= 108, nº units= 127 d) nº events= 56, nº units=201 d)' nº events= 91, nº units= 127.

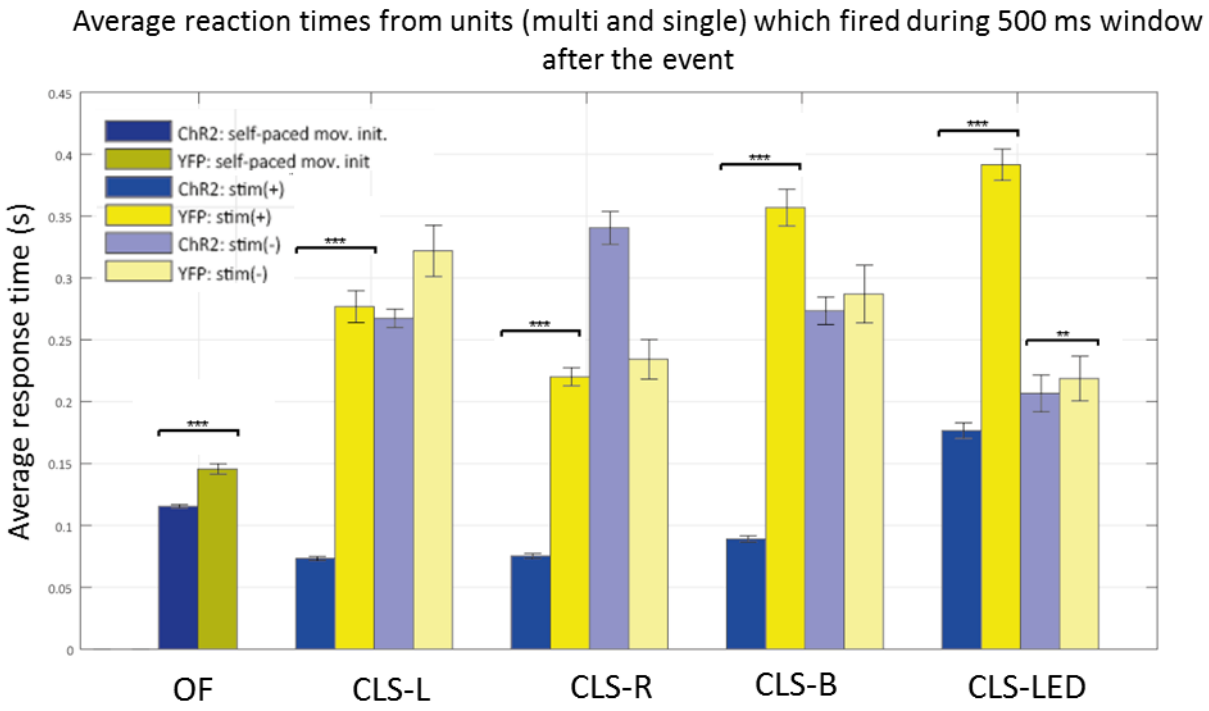

Figure 26: **Average reaction times for spike activity after the event are higher for YFP mice.** Average response times (s) for spike activity from multi and single units fired during the 500ms window after the event for spontaneous movement initiations and each stimulation paradigm. (CLS-B: Bilateral Closed loop stimulation, CLS-L: Unilateral Closed loop stimulation in the left SNc, CLS-R: Unilateral Closed loop stimulation in the right SNc, CLS-LED: Closed loop stimulation with an external LED). (n=2 mice per condition, 3 sessions for the ChR2 and 2 sessions for the YFP, Mean ± SEM).

In summary, evoked activity of SC glutamatergic terminals in SNc elicits short-latency movements and turning behavior, preferentially to the side of stimulation (when unilateral), with components in all axis of acceleration and rotation. Moreover, rotation profiles of these moments are consistently similar to the ones generated by spontaneous gait initiation but with a higher amplitude, although further analysis is still required to better understand how evoked and spontaneous movement initiation differ. Additionally, stimulation of these terminal activates neurons in the deep layers of SC,

which have low activity in resting behavior and increase activity during the initiation of spontaneous movements and in response to an external stimulus.

Discussion

SC-to-SNc projections have been previous described but not much was known about its detailed anatomy and their implications in animal behavior. In this work, we studied such questions by performing tracing experiments in cre-specific mice and by studying animal self-placed behavior when such projections were over-activated.

In order to investigate the correct location of neurons projecting to the SNc within the SC, we did retrograde tracing experiments using retrobeads. Although previous studies (McHaffie 2006, Comoli 2003) already reported the presence of retrogradely labelled cells in the SC after the injection of biotinylated dextran amines (BDA) and cholera toxin into the SNc of the cat and rat, respectively, we only found few cell bodies containing retrobeads in all the SC volume of one mouse out of 10, although they were found in the cortex after injection in the striatum as a control experiment. Our inability to replicate the results with retrobeads could be due to a series of factors: First, despite phylogenetic similarities between species, there could be differences in mouse compared to previously studied animal models (cat and rat) (Dean 1986, McHaffie 1982, Freedman, 1996, Dean 1988). For instance, the terminations may not be equally distributed through the SNc as it was shown in the rat (Dean 1988). Second, the variability in the location of the injection sites, in our study, might have resulted in low loading with retrobeads, or even no load at all, if the previous argument is indeed true. Indeed, for the one mouse with a positive result, injection was more circumscribed to the lateral SNc. Our next anterograde experiments suggested that lateral SNc are tendentially enervated with more projections from SC, which could explain the result here obtained. Third, the density of projections, or the number of up taking terminals, from each SC neuron to the SNc may be extremely low comparing to the cortico-striatal projections, and the beads transported to the projecting cell bodies may not be enough to visualize them. Therefore, the low effectiveness of retrobead labeling obtained in these experiments does not prove the inexistence of such projections and may be due to methodologic limitations. Nevertheless, in the only case where possible retrogradely labelled cell bodies were found, the location of such cells was in the intermediate gray

layer of SC and ipsilateral to the injection site, consistent with what was shown before (May 2009, McHaffie 2006). It is important to notice, however, that, in this animal, small volumes of retrobeads appeared to be injected also in the ventral part of the mRt and SNr (figure 1.2 a). Both ventral mRt and SNr are areas known to be a target of SC motor layers ipsilateral efferents in the mouse (Allen Brain Atlas Connectivity 2014). Since the volume injected in these areas appears really smaller than what was injected in the SNc, it is possible that the dim labelling could be indeed due to retrobead load by SNc terminals, but one cannot exclude the possibility of contamination by projections from these other areas.

When injected in the SC, generic anterograde tracers (BDA and cholera toxin) were found in ipsilateral nerve terminals of the SNc (May 2009, McHaffie 2006). Labeled fibers leave the SC ventrally and seem to be running rostrally and ventrally through the SNc, being the higher density of terminals in dorsal SNc. Moreover, it is known that this projection has boutons that lay in close proximity to TH-positive neurons in the SNc, which could be an indication of active synapses. Indeed, Comoli (Comoli 2003), using electron microscopy in rats, found that labeled axon terminals from the SC form symmetric (presumably inhibitory) and asymmetric (presumably excitatory) synapses with TH positive and negative neurons. In this work, through cre-dependent anterograde labelling in transgenic mouse lines (VGlut2cre and GADcre), we showed that the SC-SNc projection is strongly glutamatergic. Consistent with what was described by Comoli (Comoli 2003), for the non-cell-type specific projection, glutamatergic terminals were found throughout the whole extension of the ipsilateral SNc (figure 2.3), although they were not homogenously distributed, with only few or no isolated boutons found in the contralateral side. A high density of glutamatergic projections was also seen in the dorsal part of the SNc, although not exclusively. Accordingly, boutons like structures were found within the SNc. A tendency for higher density of glutamatergic projections in lateral SNc was also reported in this study. Comoli (Comoli 2003) described a preponderance to more lateral staining in SNc when injection of a generic tracer was in more lateral SC (also verified for medial injections/labelling), this report corroborates our findings, since injections of the cre-dependent virus, in our study, were mainly restricted to more lateral SC. Nevertheless, in cases where the generic tracer was

extensively distributed in SC, denser labeling was also found in more lateral SNc (Comoli 2003).

Interestingly, although symmetric synapses (presumably inhibitory) were found throughout the SNc (Comoli 2003), we found through anterograde tracer injection in the SC of one Gad-cre mouse, almost no labelling of terminals in both ipsilateral and contralateral SNc. Moreover, although a lot of cell bodies were infected in the SC, the labelling seems mainly circumscribed to the SC structure, what is consistent with the idea that a majority of GABAergic SC neurons of the intermediate layers are interneurons that receive inputs from neighboring premotor cells and inhibit visuosensory neurons in the SC superficial layers (Lee 2007, Meredith 1998). It is important to mention that for these experiments only one mouse was obtained, and therefore few conclusions about possible distributions of GABAergic SC terminals in SNc can be made. Nevertheless, due to consistency with what is known about GABAergic neurons in SC, the lack of strong GABAergic input from SC to SNc seems to be probable.

Using immunohistochemistry technics (anti-ChaT staining), we found that the contribution of cholinergic neurons for this projection was clearly inexistent, since this type of neurons was absent in the SC of mice (figure 2.8), as it has been shown in the rat (Ichikawa 1997). Although cholinergic neurons are not present in the SC of the mice, this neurotransmitter must have a preponderant role in modulating SC neuronal activity, since high density of cholinergic terminals was found in the SC (Hall 1989, Woolf 1986).

Therefore, the SC-to-SNc projection appears to be mainly represented by glutamatergic neurons from the intermediate and deep layers of the SC that reach the SNc in a specific topographical organization. This topographical organization of projections in SNc has several implications. The dorsal tier of the SNc, which has the higher density of projections, preferentially enervates the ventral-medial striatum, an area thought to be implicated in reward and motivation. On the other hand, the ventral tier of the SNc, which has lower, but still wide, distribution of projections, preferentially enervates dorsal-lateral striatum, which is related to motor and cognitive control (O'doherty 2004). Moreover, it has been proposed that the dorsolateral SNc contain mainly salience coding neurons (Matsumoto 2009). These neurons have been shown to receive sensory

information relative to salient events, such information might come from SC multisensory neurons. These multisensory neurons appear to be glutamatergic (Burnett 2007), what is consistent with the laterality of glutamatergic projection found in our study.

However, it is still not clear if the boutons found in the SNc form active or silent synapses, and the electrophysiological properties of these connections. In addition, since (Comoli 2003) showed that several projections from the SC would form synapses with dopaminergic and non-dopaminergic neurons, it is unknown if the glutamatergic projections synapse preferentially to one or the other cell types. I*n vitro* patch clamping of dopamine and non-dopamine neurons in the SNc of cre-specific mice (Vglut2cre or GADcre) with a ChR2 cre-dependent injection in the SC, combined with optogenetic stimulation could be a useful experiment to unravel this question. Another set of experiments that could be done to study this matter would be to perform cre-dependent retrograde monosynaptic tracer injections in the SNc of TH-cre mice in combination with in situ hybridization of different neuronal type markers. Such information would be highly relevant for the deep understating of the circuit and its behavioral implications.

In previous studies, the SC-to-SNc direct projection has been regarded mainly as a reward-related phenomenon. This is due to evidence that electrical or chemical stimulation of the SC increases short latency phasic bursts in the SNc (Comoli 2003, Comoli 2003(b)), which are connected to reward prediction error, and to the fact that the dorsal SNc, which is known to modulate the ventral striatum, receives higher density of projections. This pathway was identified as the most likely, and maybe exclusive, source of short latency visual input to the SNc and VTA (Comoli 2003, Dommett 2005 .may 2009). In addition,it has been reported that the SC-SNc pathway is responsible for the short-latency (< 100 ms) phasic visual signals expressed by DA neurons, as visual activity is present in the intermediate layer of the SC at latencies of < 100 ms (May 2009). It is then possible that the neurons that project to the SNc are glutamatergic multisensory neurons, since few projections from only visual superficial layers were found, further supporting the transmission of short latency multisensory information that could modulate basal ganglia activity. This would provide the sensory signal

required for reinforcement and primarily carry information about the occurrence of a salient event (May 2009).

Phasic activity of DA neurons has been shown to also play a critical role in movement initiation (Howe 2016, Alves da Silva, submitted). However, it hasn't yet been investigated the contribution of the SC to this phasic activity required for motor control. Considering the anatomical evidence discussed above, and the known role of the SC in spatial orientation, we believe that SC glutamatergic projections to the SNc may modulate the activity of these DA neurons, probably through excitation, and influence its contribution in gait initiation and in the orientation required at this stage. An aspect that suggests that this connection is present is the fact that Parkinson's disease patients with freezing of gait, a condition that is characterized by the loss of DA neurons, in particular from the ventral lateral SNc (Hassler 1938, German 1989), present deficits in saccade performance and difficulties in directing gaze upward (Nemanich 2016), functions attributed to SC. In this work, data that support this view are presented, although a small sample size was obtain, and therefore all conclusions are very preliminary. More experiments are still required to unequivocally state what is the role of this projection.

In order to investigate the effect of the SC-to-SNc pathway in behavior, we optogenetically stimulated the SC glutamatergic terminals in the SNc infected with ChR2 (or YFP for control) and recorded the neuronal activity in SC deep layers, while the mouse was in a self-placed open field task. Activation of the SC glutamatergic terminals in the SNc when the mouse was resting, in a self-paced situation, elicited initiation of movement with head and eventually body rotation. The direction of the rotation showed a tendency to be ipsilateral to the activated hemisphere when unilaterally stimulated. This difference was significant for the stimulation in the left hemisphere, but not for the right, probably due to greater variation (high SEM) in the ChR2 animals. We believe, however, that an increase in N would result in a significant difference. Initiation of movement was consistent from what is expected from increased phasic activity of SNc DA neurons (Howe 2016, Alves da Silva, submitted) and rotations are a known motor output of SC activity (Dean 1988, McHaffie 1982). However, this tendency for rotation in the ipsilateral direction is especially interesting since, it is contradictory to what has

been observed with SNc DA neurons. When the later are more active in one side of the brain, for example in a compensating mechanism for unilateral lesions in the SNc (Ungerstedt 1971, Schwarting 1996), rotation behavior has been shown to occur contralateral to the side with higher activity. Furthermore, direct electrical or chemical stimulations of SC also induce contralateral movements of head and body resembling orientation behaviors (rotations). In accordance to what occurred to unilateral stimulations, the small bias, but not significantly different, to the right side observed in bilateral stimulation, in ChR2 mice, may be due to several factors such as unbalanced laser power reaching the ChR2 infected terminals, which, with a higher number of samples should be dissolved. Interestingly, the head rotation movements produced by a spontaneous movement initiation appeared similar to the one produced by the stimulation of SC glutamatergic terminals in SNc, mainly when the stimulation was bilateral or unilateral on the right side. This may suggest that activity of the path is acting to produce a normal initiation of movement, but further analysis is still required to confirm such hypothesis.

Additionally, laser stimulation of ChR2 infected terminals in the SNc produced a stronger and longer lasting effect in behavior that an external LED. The external LED produced a visual stimulus that, in agreement to what is known about SC, should activate primarily SC superficial visual layers and consequently the neurons in intermediate and deep layers in order to promote an orientation to the light (Isa 2000, Mooney 52). Such stimuli should only elicit a response in the first times it is activated, since habituation to the stimuli is a known characteristic of the system. These experiments allow comparing the latency of motor response of an external stimuli to the latency of our laser stimulation, and indeed the external signal produces a motor response with a bigger latency than the laser stimulation.

The neuronal activity in the SC intermediate and deep layers is known to be associated to orientation movements, multisensory integration and motor-preparation (see introduction). In this study, we showed that the neuronal activity in the deep layers of the SC is very low during periods of resting and in the absence of sensorial stimuli and immediately increases when a mouse spontaneously initiates movement, spiking in average around 100 – 150 ms after movement initiation. This data suggests that activity

of SC neurons may also be related to mechanisms involved in initiation of movements, but further research is still required to assess such possibility. One speculative hypothesis, is that the pre-motor neurons might be activated to promote the orientation required before an action.

In addition, when SC glutamatergic terminals in the SNc are stimulated, neuronal activity in the deep layers of the SC increases with a short latency. When the external LED is activated, a rise in neuronal activity is also elicited, very similar to a spontaneous movement initiation, although with a longer latency. This could mean that the sensorial stimulus is received and consequent orientation is promoted, what requires the initiation of movement. Curiously, when the external LED is activated in an YFP mice no increase in neuronal activity is observed. Higher neuronal activity in ChR2 mice in response to a sensory stimulus, may be a consequence of a previous increased activity of SC due to laser activation, which may increase neuronal excitability and therefore making it easier to cross action potential threshold. In addition, SC activity has been connected with attention (see introduction), and improvements in discrimination task were observed upon SC microsimulation (Philiastides 2004), so, increased neuronal excitably prompted by previous laser stimulation might mean that the animal is more attentive, and therefore more responsive to external stimuli.

One factor that cannot be ignored is the possibility that the behavior elicited by optical axonal stimulation of the terminals in the SNc may be due to the activation, through backpropagation of collaterals, of other motor pathways. Also, when DA neurons in the SNc were directly stimulated under the same protocol (Alves da Silva, submitted) the behavior generated was less evident, further suggesting that other factors besides the activations of SC glutamatergic terminal in SNc are contributing for the behavior outcome. Indeed, pre-motor neurons in SC intermediate and deep layers have projections to motor neurons in the brain stem and spinal cord that are implicated in the contralateral movements elicited by stimulation of the SC (see introduction). These pre-motor neurons could be activated by the glutamatergic neurons that project to the SNc or even be the same ones. Nonetheless, this crossed tecto-reticulo-spinal descending pathway is not the only one involved in SC's motor commands, since they were not abolished by the sectioning of this pathway (although they were altered) (Dean

1986), highlighting the possibility of an involvement of other motor systems, like the basal ganglia.

In some cases, stimulation of SC elicits an avoidance behavior, in a form of an ipsilateral rotation, instead of an orientation one (Sahibzada 1986, see introduction). The differences in elicited behavior are thought to depend on which part of the SC was activated (Sahibzada 1986), due to its organization as a topographic map of the environment (Isa 2000). One speculative possibility, is that when SNc terminals were activated by the optical stimulation, backpropagation could be activating neurons in a specific way and place that mimic a stimulus that should elicit avoidance. Therefore, this stimulation would produce an avoidance response instead of an orientation one, resulting in an rotation in the direction ipsilateral to the side of the stimulation.

In order to unambiguously dissect the specific role of SC-to-SNc glutamatergic synapses, the effect of backpropagation into other motor pathways should be excluded. Further work includes experiments in which SC local activity is inhibited at the same time that SC-SNc synapses are photo-stimulated. To do so, we will locally inject muscimol, a GABA A receptor selective agonist that is known to depress neuronal activity in the SC (Hikosaka 1985).

Since long ago that the role of basal ganglia in controlling SC activity was acknowledged. The basal ganglia, through inhibitory output from the SNr (Chevalier 1984), is thought to control saccade generation in a selective way, depending on behavioral context. New evidence suggests that primarily it may be the SC, through its outputs, that could be modulating basal ganglia activity, forming a loop. SC receives inputs from sensory systems, such as vision, audition and somatosensation, and integrates them in the multisensory neurons in its intermediate and deep layers (see introduction). These multisensory neurons, which appear to be glutamatergic (Burnett 2007), could be sending information about salient events that happen in the surrounding environment to SNc, generating the characteristic phasic burst. Phasic activity in ventral SNc will allow the gating of movements, through its modulation of cortico-striatal synapses in caudate/striatum (Gerfen 2011). The caudate/striatum integrates information about value of stimuli, probably through its inputs from association cortices and the

associational part of the thalamus (Selemon 1985, Yeterian 1991), and, probably, when concomitantly activated by the SNc, it inhibits the SNr (GABAergic projection), which tonically inhibits the SC (Lee 1988), resulting in the disinhibition of pre-motor neurons in the SC intermediate and deep layers. The later are known to communicate with brainstem and spinal cord motor centers and promote orientation behavior (Rene 2001). When a sensory cue contributes to a disinhibition of SC pre-motor neurons by SNr, a saccade and/or orientation response occurs to the location of the stimulus. This means that the SC-to-SNc projection could be modulating SNc activity to allow gait initiation in response to a sensory stimulus, in particular to generate an orientation or avoidance of that specific stimulus. This is consistent with the fact that Parkinson's disease patients may present saccade deficits (Nemanich 2016). Loss of DA neurons in SNc would prevent the activity of such loop. Since its communication with caudate was deficient, no disinhibition of SNr would happen and therefor the disinhibition of SC premotor neurons responsible for saccades would not occur, resulting in the deficits.

Moreover, the SC-to-SNc pathway could be transmitting a general alerting signal, when something sensory relevant happens in the environment, increasing motivation of the animal to perform an action in response to such stimulus, such as orientation. Alerting signals are hypothesized to be sent to the all-area of SNc (Hikosaka 2010), congruent to what was found about SC-to-SNc projection.

To further clarify the contribution of this SC-to-SNc projection to movement initiation, additional experiments should be done. Further work includes the inhibition of the SC-to-SNc glutamatergic projection, in similar conditions as the ones used in this study. This will allow better understanding if/which features of the self-paced behavior are impaired when this connection is not functional. A way to fulfill these requirements is to inject an Arch opsin in the SC, instead of ChR2, and follow the same protocols used here.

To assess the effect of stimulation or inhibition of such terminals in a situation in which the animal needs to orient itself in response to a sensory stimulus would be of great interest to further elucidate the function here proposed. Stimulation of the terminals at

the moment of orientation may bias the side of the orientation if stimulus and stimulations are not ipsilateral, while inhibition might suppress a correct orientation.

Moreover, although we found almost no SC GABAergic projections to the SNc, a few bouton-like structures were present in SNc. Considering the Comoli (Comoli 2003) findings of both SC symmetric (presumably inhibitory) and asymmetric synapses (presumably excitatory) with SNc neurons, it is likely that inhibitory synapses contribute in some way to the behavior. Therefore, further work will include accessing the effect of the activity of such synapses, by optically stimulating GABAergic projections to the SNC, using a Gadcre line and following the same protocols used in this study.

Conclusions

The basal ganglia circuit is critical for the selection and initiation of movements. In particular SNc dopaminergic neurons are known to be a key player in eliciting the initiation of movements, however not much is known about how these neurons are regulated and how specific movements are selected and promoted by its activity. In this work, we present anatomical and behavioral data that suggests that the SC, a known center involved in multisensorial integration and in eliciting orientation behaviors might be a critical modulator of the SNc dopaminergic neuronal activity, specifically in regulating gait initiation, for the orientation behavior required before the beginning of an action.

The main findings presented in this study suggest that the direct projection from the SC intermediate and deep layers to the SNc is mainly glutamatergic and is organized in a topographic way with high density of projections in dorsolateral SNc, although they were also found throughout all the SNc area. Bouton-like structures were also found in all the TH-positive area, suggesting the presence of glutamatergic synapses within SNc, which could be both to dopaminergic or non-dopaminergic neurons. Activity of these SC glutamatergic terminals in the SNc elicited head rotation that could prompt gait initiation, both behaviors associated with the SC and the SNc, respectively. Additionally, neuronal activity in the deep layers of the SC was mainly silent during rest and it increased with spontaneous movement initiation or if glutamatergic terminals in the SC were optically stimulated, suggesting that this structure might play a role in gait initiation. Further research is still required in order to deeply understand how this neural circuit works and modulates behavior. The recent development of fine genetic and molecular tools, such as optogenetics, will allow a much more detailed investigation of these questions.

At last, this work helped to clarify the nature of the SC-to-SNc projection, and is part of an ongoing project to test the hypothesis that its function is important for the control of animal behavior. This study gave new insights on how basal ganglia circuits could be modulated by and connects with circuits that interact with the sensory world, in order to select and initiate actions that better suit the requirements of the animal.

Supplementary material

Peak amplitude	CLS-B	CLS-L	CLS-R	CLS-LED
Acc x	2.72E-55	1.12E-49	4.29E-77	1.83E-16
Acc y	3.65E-54	1.22E-49	7.82E-73	1.82E-13
Acc z	1.37E-58	3.45E-53	1.12E-75	7.14E-14
Gyr yz	7.39E-52	1.85E-45	4.00E-60	1.35E-13
Gyr xy	2.09E-50	1.44E-49	7.88E-80	1.71E-14
Gyr xz	4.71E-51	4.15E-48	2.97E-74	2.07E-12
Total body Acc	8.88E-55	1.66E-50	1.50E-81	1.87E-14
Total head rot	1.31E-50	1.74E-49	1.01E-75	3.50E-12

Figure S1 – Adjusted p-values for the comparison between ChR2 and YFP mice peak amplitudes for all stimulation paradigms and axis of acceleration and rotation, using the Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms.

ChR2					YFP			
		CLS-B	CLS-L	CLS-R		CLS-B	CLS-L	CLS-R
Acc x	CLS-L	ns	-	-	CLS-L	*	-	-
	CLS-R	**	***	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	**
Acc y	CLS-L	**	-	-	CLS-L	**	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	***
Acc z	CLS-L	***	-	-	CLS-L	*	-	-
	CLS-R	*	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	***
Gyr xy	CLS-L	ns	-	-	CLS-L	ns	-	-
	CLS-R	*	***	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	*
Gyr xz	CLS-L	ns	-	-	CLS-L	*	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	ns
Gyr yz	CLS-L	ns	-	-	CLS-L	**	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	***	***
Total body Acc	CLS-L	ns	-	-	CLS-L	*	-	-
	CLS-R	ns	*	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	*	***	***
Total head Rot	CLS-L	ns	-	-	CLS-L	**	-	-
	CLS-R	ns	ns	-	CLS-R	ns	*	-
	CLS-LED	***	***	***	CLS-LED	ns	***	*

Figure S2 – Differences for the multiple comparison Kruskal Wallis test for the peak amplitudes. (* - p<0.05, ** - p<0.01, *** - p<0.001, ns – not significant)

ChR2					YFP			
		CLS-B	CLS-L	CLS-R		CLS-B	CLS-L	CLS-R
Acc x	CLS-L	***	-	-	CLS-L	ns	-	-
	CLS-R	ns	***	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Acc y	CLS-L	ns	-	-	CLS-L	ns	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Acc z	CLS-L	ns	-	-	CLS-L	ns	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Gyr xy	CLS-L	**	-	-	CLS-L	ns	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Gyr xz	CLS-L	*	-	-	CLS-L	ns	-	-
	CLS-R	ns	**	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Gyr yz	CLS-L	ns	-	-	CLS-L	ns	-	-
	CLS-R	ns	*	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	**	**	*
Total body	CLS-L	ns	-	-	CLS-L	ns	-	-
	CLS-R	*	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns
Total head	CLS-L	*	-	-	CLS-L	ns	-	-
	CLS-R	ns	ns	-	CLS-R	ns	ns	-
	CLS-LED	***	***	***	CLS-LED	ns	ns	ns

Figure S3 – Differences for the multiple comparison Kruskal Wallis test. (* - p<0.05, ** - p<0.01, *** - p<0.001, ns – not significant)

Motor-response time	CLS-B	CLS-L	CLS-R	CLS-LED
Acc x	3.10E-24	1.47E-27	1.78E-40	0.00437
Acc y	1.13E-20	1.96E-33	2.50E-46	0.006219
Acc z	2.54E-21	9.88E-34	1.39E-44	0.075224
Gyr yz	1.14E-23	1.19E-33	2.18E-27	6.82E-06
Gyr xy	1.19E-23	5.94E-34	8.25E-36	1.90E-06
Gyr xz	6.26E-31	1.34E-37	6.27E-44	0.000376
Total body Acc	2.78E-28	4.69E-37	1.35E-56	0.013299
Total head rot	9.12E-34	2.09E-35	2.23E-42	7.18E-05

Figure S4 – Adjusted p-values for the comparison between ChR2 and YFP mice motor-response times, for all stimulation paradigms and axis of acceleration and rotation, using the Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms.

Condition	CLS-B_N	CLS-B_P	CLS-L_N	CLS-L_P	CLS-R_N	CLS-R_P	CLS-LED-N	CLS-LED_P	BA
p-value	0.088863	2.33E-51	0.000561	1.06E-52	7.08E-08	1.34E-283	0.222176	6.71E-20	1.28E-119

Figure S5– Adjusted p-values for the comparison between ChR2 and YFP mice spike activity, for all stimulation paradigms, using the Two-sample Kolmogorov-Smirnov test, corrected with Bonferroni-Holms. CLS_N: negative stimulation, CLS_P: positive stimulation.

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