Experimental investigation into the role of the subthalamic nucleus (STN) in motor control using optogenetics in mice

Adriane Guillaumin a,1, Gian Pietro Serra a,1, François Georges b, Åsa Wallén-Mackenzie a,∗

a Department of Organism Biology, Uppsala University, SE-752 36 Uppsala, Sweden
b Université de Bordeaux, Institut des Maladies Neurodégénératives, UMR 5293, F-33000 Bordeaux, France

A R T I C L E   I N F O

Keywords:
Basal ganglia
Coordination
Locomotion
Movement
Optogenetics
Subthalamus

A B S T R A C T

The subthalamic nucleus (STN) is critical for the execution of intended movements. Loss of its normal function is strongly associated with several movement disorders, including Parkinson’s disease for which the STN is an important target area in deep brain stimulation (DBS) therapy. Classical basal ganglia models postulate that two parallel pathways, the direct and indirect pathways, exert opposing control over movement, with the STN acting within the indirect pathway. The STN is regulated by both inhibitory and excitatory input, and is itself excitatory. While most functional knowledge of this clinically relevant brain structure has been gained from pathological conditions and models, primarily parkinsonian, experimental evidence for its role in normal motor control has remained more sparse. The objective here was to tease out the selective impact of the STN on several motor parameters required to achieve intended movement, including locomotion, balance and motor coordination. Optogenetic excitation and inhibition using both bilateral and unilateral stimulations of the STN were implemented in freely-moving mice. The results demonstrate that selective optogenetic inhibition of the STN enhances locomotion while its excitation reduces locomotion. These findings lend experimental support to basal ganglia models of the STN in terms of locomotion. In addition, optogenetic excitation in freely-exploring mice induced self-grooming, disturbed gait and a jumping/escaping behavior, while causing reduced motor coordination in advanced motor tasks, independent of grooming and jumping. This study contributes experimentally validated evidence for a regulatory role of the STN in several aspects of motor control.

1. Introduction

The subthalamic nucleus (STN) is a small but highly influential brain structure which exerts prominent impact over voluntary movement. Consequently, damage or dysregulation of the STN is strongly associated with motor dysfunction and movement disorder. For example, unilateral damage to the STN causes strongly uncontrolled movements, so called hemiballismus (Hamada & DeLong, 1992), while degeneration of the STN is associated with supranuclear palsy and Huntington’s disease (Dickson et al., 2010; Lange et al., 1976). Further, hyperactivity of STN neurons is a pathological hallmark of Parkinson’s disease (PD) (Albin et al., 1989; DeLong, 1990). Surgical lesioning of the STN, so called subthalamotomy, improves motor symptoms in PD (Heywood & Gill, 1997; Parkin et al., 2001). So does deep brain stimulation (DBS), an electrical method in which high-frequency stimulation electrodes, when positioned in the STN, can correct its aberrant activity (Benazzouz et al., 2000; Filali et al., 2004). DBS of the STN (STN-DBS) successfully alleviates motor symptoms in advanced-stage PD (Benabid, 2003; Benabid et al., 2009). The high success rate of these clinical interventions confirms the critical role of the STN, yet its regulatory role over different motor parameters required to achieve willed movement remains to be fully established.

Abbreviations: 6-OHDA, 6-Hydroxydopamine; AAV2, adeno-associated virus 2; ALT, Alternated Light; BS, Baseline; ChR2, Channelrhodopsin 2; CONT, Continuous Light; DBS, deep brain stimulation; eArch3.0, Archaerhodopsin 3.0; eYFP, enhanced yellow fluorescent protein; EP, entopeduncular nucleus; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; OCD, obsessive compulsive disorder; PD, Parkinson’s disease; PRIOR, prior stimulation; PSTH, peri-stimulus time histograms; pSTN, para-subthalamic nucleus; SNC, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; Vglut2, Vesicular glutamate transporter 2.

* Corresponding author.
E-mail address: asa.mackenzie@ebc.uu.se (Wallén-Mackenzie).
1 Equal contribution, listed alphabetically.

https://doi.org/10.1016/j.brainres.2020.147226
Received 3 August 2020; Received in revised form 20 November 2020; Accepted 23 November 2020
Available online 23 December 2020
0006-8993/© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
The role of the STN in motor function is commonly explained by its connectivity on the neurocircuitry level in which it forms an integral part of the basal ganglia. According to the classical basal ganglia motor model, two pathways, the so-called direct and indirect pathways, exert opposing control over motor behavior via the thalamus (Albin et al., 1989; Alexander & Crutcher, 1990; DeLong, 1990; Graybiel et al., 1994; Smith et al., 1998). Via the direct path (cortex - striatum - internal segment of the globus pallidus (GPi) - thalamus), intended movements are promoted, while via the indirect path (cortex - striatum - external segment of the globus pallidus (GPe) - STN - GPi - thalamus), competing motor programs are suppressed and stop signals mediated (Nambu et al., 2002; Sano et al., 2013; Schmidt & Berke, 2017). In addition to the indirect pathway, the STN is directly regulated by the cortex in the so-called hyperdirect pathway. The STN itself is excitatory, using glutamate as neurotransmitter. Extensive STN projections reach the GPi and substantia nigra pars reticulata (SNr), and the STN is also reciprocally connected to the GPe. This organization effectively means that STN activity is regulated by both excitatory (cortex) and inhibitory (GPe) projections. When STN neurons are activated, they contribute to the suppression of movement by counteracting the movement-promoting activities of the direct path (Féger et al., 1994; Gradinaru et al., 2009; Mourgoux & Féger, 1993; Sanders & Jaeger, 2016; Sugimoto & Hattori, 1983).

This model of STN’s role in motor regulation via the basal ganglia pathways has been formed primarily based on anatomical projection patterns and functionally by lesion and high-frequency stimulation in experimental animals, as well as studies of human symptoms, primarily in PD (Alkemade et al., 2015; Benabid, 2003; Benabid et al., 2009; Benazzouz et al., 2000; Centonze et al., 2005; Hamada & DeLong, 1992; Hamani, 2004; Haynes & Haber, 2013; Heywood & Gill, 1997; Parent & Hazrati, 1995; Parkin et al., 2001; Rizelio et al., 2010). However, studies based on human pathological conditions suffer from complexity in terms of confounding factors, such as multiple symptom domains and pharmacological treatments. Another complicating issue is the fact that data derived from DBS-studies can be difficult to fully decipher since the underlying mechanisms of this method have remained unresolved (Chikin & Nambu, 2016; Florence et al., 2016; Vitek, 2002). Further, the STN has been demonstrated to play an important role in action inhibition in stop signals or during high-conflict decisions (Aron et al., 2007; Cavanagh et al., 2011; Pasquereau and Turner, 2017; Schmidt et al., 2013; Zavala et al., 2015). By exerting different roles, the selective impact of the STN on distinct motor parameters can be difficult to distinguish. Experimental animals in which carefully controlled conditions can be achieved and functional output reliably recorded are therefore essential to increase the understanding of the roles that distinct structures play in circuitry and behavioral regulation. Indeed, several studies in rodents have supported the hypothesis that removal of STN excitatory activity enhances movement. Both STN-lesions and STN-selective gene knockout of the Vesicular glutamate transporter 2 (Vglut2), encoding the transporter that packages the excitatory neurotransmitter glutamate for synaptic release, lead to behavioral phenotypes of enhanced locomotion (Centonze et al., 2005; Rizelio et al., 2010; Schweizer et al., 2014, 2016). However, lesioning and gene-targeting events suffer from methodological drawbacks and do not allow the analysis of how the intact STN, via excitation of its target areas, regulates movement. Further, gene-targeting during brain development might cause structural and functional adaptations that likely contribute to behavioral phenotypes in adulthood.

During the past decade, the use of optogenetics in freely-moving rodents has enabled more advanced possibilities for decoding the role of distinct neuronal structures and circuitries in behavioral regulation. By allowing spatially and temporally precise control over neural activity using excitatory and inhibitory opsins (Deisseroth, 2011; Kim et al., 2017), ground-breaking discoveries have been made, not least in terms of motor control. This includes the experimental validation of the classical model of the basal ganglia using excitatory and inhibitory opsins in striatal neurons, verifying the opposing roles of the direct and indirect pathways in regulation of movement (Kravitz et al., 2010). More recently, optogenetic dissection of neurons in the globus pallidus verified their role in motor control (Tian et al., 2018). In terms of optogenetics in the STN, the strong interest in understanding the mechanisms of STN-DBS has led to a focus on PD models. However, somewhat surprisingly, the first study addressing the STN using optogenetics did not find any effect on recovery in a PD model using either excitatory or inhibitory opsins in the STN itself (Gradinaru et al., 2009). Instead motor improvement was only achieved upon optogenetic excitation of the cortico-subthalamic pathway (Gradinaru et al., 2009), a finding subsequently confirmed (Sanders & Jaeger, 2016). In contrast, another study reported that optogenetic inhibition of the STN structure resulted in improved motor function in a rodent PD-model (Yoon et al., 2014). Thus, contradicting results have been obtained with optogenetic excitation and inhibition of the STN structure. Several factors might contribute to the differences in findings, including the inherent properties of the transgenic line implemented to drive the expression of the opsin constructs, the behavioral setup, the PD model, as well as additional experimental parameters.

While the use of optogenetics in various rodent PD models has begun to answer fundamental questions around STN-DBS mechanisms, the natural role of the STN in regulating different aspects of motor behavior during non-pathological conditions has remained more poorly explored. However, a basic understanding of STN’s regulatory role in motor control should prove useful for advancing both pre-clinical and clinical knowledge. The objective of the present study was to tease out the impact of the STN on several motor parameters required to achieve intended movement, including locomotion, balance and motor coordination. To ensure selectivity and efficiency, Pitx2-Cre mice were used based on previous validations demonstrating that the Pitx2-Cre transgene is a solid driver of floxed opsin constructs in the STN which induces glutamate release in basal ganglia target areas upon photostimulation (Schweizer et al., 2014; Viereckel et al., 2018). Mice expressing excitatory or inhibitory opsins selectively in the STN were analysed both upon bilateral and unilateral photostimulations. Further, spontaneous activity was analysed as well as trained behavior in advanced motor tasks. The findings reported contribute to increased understanding of motor control by both confirming and challenging the role of the STN as predicted by the basal ganglia motor model.

2. Material and methods

2.1. Animal housing and ethical permits

The Pitx2-Cre transgenic mouse line was originally imported from Dr Martinis laboratory, Texas, US (Martin et al., 2004; Skidmore et al., 2008). The line was maintained on a C57BL/6NTac background. Pitx2-Cre, C57BL/6NTac transgenic mice were bred in-house and housed at the animal facility of Uppsala University before and during behavioral experiments, and at University of Bordeaux for in vivo electrophysiological experiments. Mice had access to food and water ad libitum, and were housed under standard temperature and humidity conditions with a 12-hour dark/ light cycle. Both female and male mice were used throughout the study. PCR analyses were run to confirm the Pitx2-Cre genotype in DNA extracted from ear biopsies. All animal experimental procedures followed the European Union Legislation (Convention ETS 123 and Directive 2010/63/EU), and were approved by the Uppsala (ethical permit C13B/15) or Bordeaux (N°50120205-A) Local Ethical Committee.

2.2. Stereotaxic virus injection and optic cannula implantation

**Virus injection:** Stereotaxic injections were performed in anaesthetized Pitx2-Cre mice maintained at 1.4–1.8% (0.5–2 L/min, isoflurane-air mix v/v). Before starting the surgery, and 24 h post-surgery, mice received
subcutaneous injection of Carprofen (5 mg/kg, Norocarp). A topical analgesic, Marcain (1.5 mg/kg; AstraZeneca), was locally injected on the site of the incision. After exposing the skull, holes were drilled in the skull for virus injections. Mice were injected in the STN bilaterally with an AAV2 virus containing a DNA vector encoding either a Cre-dependent channelrhodopsin (ChR2) and a fluorescent reporter (eYFP) (rAAV2/EF1a-DIO-hChR2(H134R)-eYFP) (mice referred to as Pitx2/ChR2 mice), a Cre-dependent archaerhodopsin (Arch) (rAAV2/EF1a-DIO-eArch3.0-eYFP) and the eYFP reporter (mice referred to as Pitx2/Arch mice), or a control virus in which the vector encoded the eYFP reporter but no opsins (rAAV2/EF1a-DIO-eYFP) (mice referred to as Pitx2/eYFP-C for controls within the ChR2 group, and Pitx2/eYFP-A for controls within the Arch group) at concentrations of 3.8 × 10^{12} virus molecules/mL, 2.7 × 10^{12} virus molecules/mL, 4.6 × 10^{12} virus molecules/mL, respectively (UNC Vector Core, Chapel Hill, NC, USA). The following coordinates were used (adapted from Paxinos and Franklin, 2013): anteroposterior (AP) = -1.90 mm, mediolateral (ML) = +/-1.70 mm from the midline. 250 nL of virus was injected with a NanoFil syringe (World Precision Instruments, Sarasota, FL, USA) at two dorsoventral levels (DV) = -4.65 mm and –4.25 mm from the dura mater at 100 nL/min. All mice went through the same surgical procedure for virus injection, irrespective of whether subsequently assessed in behavior or electrophysiological analysis.

**Optical cannula implantation:** For behavior experiments, optical cannulas (MFC_200/245-0.37_5mm_ZF1.25_FLT, Doric Lenses) were implanted directly after the virus injections. Two skull screws were implanted in the skull to hold the optic cannula-cement-skull complex. Primers (OptibondTM FL, Kerr) were then applied and harden with ultraviolet light. Optical cannulas were implanted bilaterally above the STN and fixed with dental cement, coordinates: AP = -1.90 mm, ML = +/-1.70 mm from the midline DV = -4.30 mm. 1 mL of saline was injected subcutaneously at the end of the surgery.

### 2.3. Electrophysiology: Single-cell extracellular recordings

**Surgery (Optical fiber, optrode and micropipette):** Following a recovery period of at least four weeks after virus injections, Pitx2/ChR2 mice were prepared for analysis by in vivo single-cell extracellular recordings. Mice were anesthetized with a isoflurane-air mixture (0.8–1.8% v/v) and placed in a stereotaxic apparatus. Depending on the experiment, either an optrode or an optic fiber was placed in the STN and a recording micropipette was placed in the GP according to the following coordinates: STN: AP = -1.90 mm, ML = +/-1.70 mm and DV = -4.30 mm for the STN; Anterior GP: AP = -0.11 mm relative to bregma, ML = +/-1.50 mm from sagittal vein, DV = from -3.00 to -5.50 mm depth; Central GP: AP = -0.71 mm relative to bregma, ML = +/-1.80 mm, DV = -3.00/-4.20 mm.

**Light-stimulation protocols:** Two different light stimulation protocols were used in the electrophysiological experiments. These are referred to as the “0.5 Hz protocol” and the “20 Hz protocol” according to the stimulation frequency used. The 0.5 Hz protocol consisted of a 100 s long protocol at 0.5 Hz, 5 ms pulse duration of 5–8 mW, giving a total of 50 light pulses. This protocol was used to create a peri-stimulus time histogram (PSTH) which gives information of the effect (excitation, inhibition, or no response) of the photostimulation in the 500 ms period post-stimulation. Neurons are considered as excited during the 0.5 Hz protocol when, following the light pulses centered on 0, the number of spikes/Sms bin is higher than the baseline (-500 ms to 0 ms) plus two times the standard deviation.

In previous work, we have shown that 20 Hz optogenetic stimulation of the STN in Pitx2-Cre mice induces glutamate release and postsynaptic currents in basal ganglia target areas (Schweizer et al., 2014; Viereckel et al., 2018). Based on this, a 20 Hz protocol was used throughout the behavioral analyses, and confirmed electrophysiologically in separate cohorts of mice. The “20 Hz protocol” for electrophysiological confirmation consisted of a 100 s long protocol at 20 Hz, 5 ms pulse duration of 5–8 mW, giving a total of 2000 light pulses. Note: In the behavioral experiments, the total stimulation length varied depending on task and success rate of the mice to complete the task.

The stimulations were generated using a laser (MBL-III-473 nm-100 mW laser, CNI Lasers, Changchun, China). The laser power was measured before starting each experiment using a power meter (Thorlabs). Extracellular action potentials were collected online (CED 1401, SPIKE2; Cambridge Electronic Design), recorded and amplified with an Axoclamp-2B and filtered (300 Hz/0.5 kHz).

**STN optotagging:** This method was used for recordings of STN activity upon STN-photostimulation. A custom-made optrode was generated with an optic fiber (100 μm diameter, Thorlabs) connected to a laser (MBL-III-473 nm-100 mW laser, CNI Lasers, Changchun, China), mounted and glued on the recording glass micropipette which was filled with 2% pontamine sky blue in 0.5 μM sodium acetate (tip diameter 1–2 μm, resistance 10–15 MΩ). The distance between the end of the optical fiber and the tip of the recording pipette varied between 650 nm and 950 nm. Following baseline recording (100 s), the 0.5 Hz protocol was applied for at least 100 s and PSTHs were created. The photostimulation protocol was set and triggered with Spike2 software.

**GP recordings:** Here, an optic fiber was placed above the STN while a recording micropipette was placed in the GP. For each GP neuron, the 0.5 Hz protocol was applied to create a PSTH followed by the 20 Hz protocol once the activity returned to baseline.

**Confirmation of recording positions:** At the end of each experiment, a deposit was made at the last recording coordinate by pontamine sky blue electrophoresis (−20 μA, 25 min). Brains were collected following transcardial perfusion of 0.9% NaCl and 4% PFA. 60 μm brain sections were cut with a vibratome at the levels of the STN and the GP, mounted with Vectashield medium (Vector Laboratories), coverslipped and visualized with an epifluorescent microscope to confirm eYFP fluorescence as well as the position of the recording pipette and the optical fiber.

### 3. Behavioral analysis

#### 3.1. Experimental setup and photostimulation protocol

Following a recovery period of at least four weeks after virus injection and optic cannula implantation, Pitx2/ChR2, Pitx2/eYFP-C, Pitx2/Arch and Pitx2/eYFP-A mice were all analyzed in the open field (bilateral and unilateral stimulation). In addition, Pitx2/ChR2 and Pitx2/eYFP-C mice were analyzed in the rotarod and beam walk tests, in this order.

For Pitx2/ChR2 and Pitx2/eYFP-C mice, the 20 Hz stimulation protocol was used: 473 nm light, 5 mW, 20 Hz, 5 ms pulse delivered by a MBL-III-473 nm-100 mW laser (CNI Lasers, Changchun, China). For Pitx2/Arch and Pitx2/eYFP-A mice, 532 nm continuous light, 10 mW, delivered by a MBL-III-532 nm-100 mW laser (CNI Lasers, Changchun, China). Duration of photo-stimulation (ON) and conditions were specified for each test. After completed behavioral tests, mice were sacrificed and brains analyzed histologically and the position of optic cannulas was validated. Mice in which the optical cannulas were not in correct position above the STN were excluded from further analysis.

#### 3.2. Habituation

Three weeks post-surgery and prior to the first behavioral test, all mice were handled and habituated to the experimental room, the experimenter and to the optical fiber to reduce stress. Before each behavioral test, mice were acclimatized for 30 min in the experimental room.

#### 3.3. The open field test

Following the connection of the two implanted optical cannulas to
optical fiber and light source, mice were individually placed in neutral cages for three minutes to recover, after which they were placed in the open field arena. The open field arena consisted of a 50x50 cm, transparent plastic chamber with white floor divided (virtually) into a central zone (center, 25% of the total area) and a peripheral zone (borders lining the center). Mice were positioned in the central zone and allowed to freely explore the entire arena for 5 min before starting the test. The open field test consisted of one 20 min long session divided into four alternating 5-min trials (OFF-ON-OFF-ON), during which bilateral photostimulation was either off (OFF) or on (ON). During ON trials, photostimulation was delivered according to the protocols described above, giving a total of 6000 light pulses for each 5-min long ON epoch. The optical fiber was connected to a rotary joint connected to a laser controlled by an Arduino Uno card. Total distance moved, speed, time spent and frequency in crossing to the center, and body elongation, were automatically documented by the EthoVision XT 12.0/13.0 tracking software (Noldus Information Technology, The Netherlands). Rearing, grooming and escape behaviors were manually recorded by an experimenter blind to the experimental identity of each mouse using the same software.

3.4. Unilateral STN-stimulation in the open field arena

The test was performed in the same testing arena used for the open field test and consisted of two ten-minute phases, each divided in two five-min trials during which photostimulation was either turned off or on (OFF-ON). In “Phase 1”, photostimulation was randomly paired to one hemisphere while in “Phase 2”, the photostimulation was delivered to the contralateral hemisphere compared to “Phase 1”. Before starting “Phase 1”, mice were allowed to recover in a neutral cage for five min, after which they were placed in the center of the arena and allowed to freely explore the entire arena for five minutes before starting the test. Between the “Phase 1” and “Phase 2”, the optical fiber was switched from one hemisphere to the contralateral one. Again mice were allowed to recover from handling in a neutral cage for five min and then placed in the center of the arena for free exploration during “Phase 2”. Body rotations were automatically recorded (EthoVision XT 13.0 tracking software; Noldus Information Technology, The Netherlands).

3.5. The rotarod test

Following the bilateral and unilateral stimulations in the open field, Pitx2/ChR2 and Pitx2/eYFP-C mice were assessed in a fixed-speed rotarod assay to investigate the ability of the mice to maintain balance on a rotating rod, the rotarod (BIOSEB, Vitrolles, France). Before testing, the mice were trained for four days (“Training”) in multiple trials with a fixed-speed protocol (6, 8, 12, 16 rpm). Each trial consisted of a maximum of three attempts. Upon connecting the two implanted optical cannuas to optical fibers and light source, mice were individually placed in neutral cages for 15 min to recover, after which they were placed on the rotating rod. The start of each trial was set to five seconds after the mouse was placed on the rotating rod, and ended either when the mouse fell from the rotating rod, or after a total time of 120 s had elapsed. Resting time between trials was five min. During “Training”, once a mouse succeeded to stay on the rod for 120 s, it was approved to proceed to the next trial according to the fixed-speed protocol. Next day followed a “Pre-test” consisting of four trials at a fixed speed of 16 rpm. The “Pre-test” served to assess balance skills in absence of optogenetic stimulation.

Only mice that passed the “Pre-test” were allowed to proceed on to the “Test” in which they were assessed for their response to photostimulation. The “Test” was performed the day after the “Pre-test”. The “Test” was performed throughout four trials at a fixed speed of 16 rpm, during which OFF trials (no photostimulation) alternated with ON trials (photostimulation). The laser was started five seconds after the mouse was placed on the rod, in conjunction with the start of each ON trial, and turned off either when the mouse fell off the rod or when the full 120 s has passed. Latency to fall was automatically scored by the EthoVision XT13.0 tracking software (Noldus Information Technology, The Netherlands). When more than one attempt was needed, or when a mouse was not able to stay on the rod for 120 s, the best performance among the three attempts was selected for statistical analysis.

3.6. The beam walk test

Mice were trained to walk on round wooden beams (80 cm of total length, and either 25, 21 or 15 mm in diameter) to reach a goal cage into which they could escape the exposed beam. The beam was placed horizontally, elevated 40 cm from a lab bench, with one end held by a metal holder and the other one leaned against the neutral goal cage. The beam was divided into three sections: “Starting Area” (SA, 10 cm length) which was brightly lit with a 60 W lamp serving as an aversive stimulus motivating the mouse to move forward on the beam; “Recording Area” (RA, 60 cm segment); “Goal Zone” (GZ, 10 cm) attached to the goal cage (supplied with clean bedding and home-cage nesting material). Mice were individually placed in the goal cage for 15 min before starting the training in order to recover from optical cable connection and to habituate to the goal cage. On “Training” days (TD), trials were performed on the 25, 21 (TD 1) and 15 mm (TD 2) diameter beams until two consecutive trials were completed without stopping. Mice were individually positioned in the SA section and allowed to walk through the RA to reach the GZ and the goal cage.

On the “Test” day, four different testing conditions were applied as mice were positioned on a 15 mm diameter beam: “Baseline” (BS), to measure balance skills in absence of photostimulation; “Alternate Light” (ALT), photostimulation applied in alternating 15 cm segments along the RA section (OFF and ON zones); “Continuous Light” (CONT), photostimulation applied across the whole RA section, turned on as the mouse entered the RA, and turned off as the mouse reached the GZ or fell from the beam; “Prior Stimulation” (PRIOR), photostimulation delivered 15 s prior to positioning the mouse on the SA section and maintained until the GZ section was reached, or the mouse fell. For each condition, two trials in which the mouse did not stall on the beam were averaged.

Each trial session started when the mouse entered the RA section and ended either when the mouse reached the GZ section, or when the mouse fell off the beam (failure to complete the task). Resting time in the goal cage lasted three mins between each session. The time to cross the RA section was recorded (EthoVision XT 13) and the number of hind-foot slips when crossing this section was observed by manual counting. A “neuroscore” rating scale, based on Korenova et al (Korenova et al., 2009) and adapted for mice, was used to grade the performance of each mouse in each of the four testing conditions, and to allow their comparison: 1–5 points was scored for time to cross + 1–5 points for hind-foot slips (Supplementary Table 2). The higher the score, the lower the performance on the beam. The neuroscore was implemented to enable scoring also of mice that failed to complete the test.

3.7. c-Fos activation

After behavioral testing, a group of Pitx2/ChR2 and Pitx2/eYFP-C control mice were again connected to the optical fiber and placed in a neutral cage for 20 min before receiving a two min long photostimulation using the 20 Hz protocol to allow detection of c-Fos, a marker of neuronal activity. 90 min post-stimulation, mice were perfused. Detection of c-Fos is described under Histological analysis below.

3.8. Histological analysis

Fluorescent immunohistochemistry for eYFP (to enhance the fluorescent signal) was performed on sections from each mouse to validate
the efficiency of the expression of the ChR2-eYFP, Arch-eYFP or control eYFP construct, and for c-Fos on randomly selected mice. Following behavioral tests, mice were deeply anesthetized and perfused transcardially with phosphate-buffered-saline (PBS) followed by ice-cold 4% formaldehyde. Brains were extracted and 60 μm sections were cut with a vibratome. After rinsing in PBS, sections were incubated for 90 min in PBS containing 0.3% X-100 Triton and 10% blocking solution (normal donkey serum) followed by incubation with primary antibodies diluted in 1% normal donkey serum in PBS, overnight at 4 °C (chicken anti-GFP 1:1000, cat. no. ab13970, Abcam; rabbit anti-c-Fos 1:800, cat. no. 226003, Synaptic System). Next day, sections were rinsed in PBS containing 0.1% Tween-20, and incubated for 90 min with secondary antibodies diluted in PBS (Cy3 donkey anti-rabbit 1:1000; A488 donkey anti-chicken 1:1000, cat. no. 703–545–155, Jackson Immunoresearch). After rinsing in 0.1% Tween-20/PBS solution, sections were incubated for 30 min with DAPI diluted in distilled water (1:5000). Sections were mounted with Fluoromount Aqueous mounting medium (Sigma, USA) and cover-slipped. Analysis was performed upon slide scanning using the NanoZoomer 2-0-HT.0 (Hamamatsu) scanner followed by visualization using the NDPView2 software (Hamamatsu).

3.9. Statistics

Results from all statistical analyses are shown in Supplementary Table 1. Data from behavioral test are expressed in the plots as means ± SEM and when necessary were averaged for the two ON and OFF trials. Normal distribution of residuals was checked by using QQ plot and Shapiro-Wilk W test. Rank based z score transformation was applied prior the Two-way ANOVA analysis when more than two outliers were detected. For tests in the open field, RM Two-way ANOVA was performed. Given that the experimental question was focused on the comparison between specific conditions (ON vs. OFF for example), Bonferroni’s or Tukey’s multiple comparisons always followed the Two-way ANOVA analysis. For number of jumps, the statistical analysis Two-tailed Wilcoxon matched-pairs test was performed. For analysis of in vivo extracellular recordings, the Friedman test was performed followed by Dunn’s multiple comparisons. For the rotated and beam walk tests, the Friedman test was performed followed by Dunn’s multiple comparisons (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA).

4. Results

4.1. Expression of optogenetic constructs in the STN of Pitx2-Cre mice with c-Fos induced upon photoexcitation confirms experimental setup

The Pitx2 gene encodes a transcription factor essential for STN development and function (Martin et al., 2004; Schweizer et al., 2016; Skidmore et al., 2008). We previously verified that expression of the optogenetic ion channel Channelrhodopsin (ChR2) in the STN of Pitx2-Cre mice causes post-synaptic currents and glutamate release in STN target areas upon photostimulation (Schweizer et al., 2014; Vierckel et al., 2018), thus validating the use of optogenetics in Pitx2-Cre mice for the study of STN.

Here, to allow excitatory and inhibitory control of STN neurons, Pitx2-Cre mice were bilaterally injected into the STN with an adeno-associated virus (AAV2) containing a DNA-construct encoding either the excitatory ion channel hChR2 or the inhibitory proton pump eArch3.0, each opsin construct fused with the enhanced yellow fluorescent protein, eYFP (Fig. 1A). These mice are hereafter referred to as Pitx2/ChR2 mice and Pitx2/Arch mice, respectively. As controls, separate cohorts of Pitx2-Cre mice were injected with a control AAV2 expressing the fluorescent reporter eYFP in absence of any opsin. Control mice are referred to as Pitx2/eYFP-C mice representing the controls to Pitx2/ChR2 mice, and Pitx2/eYFP-A for the controls to Pitx2/Arch mice. For behavioral experiments, optical cannulas were placed above the STN allowing for photostimulation at 473 nm for Pitx2/ChR2 mice and 532 nm for Pitx2/Arch mice (Fig. 1A).

In vivo electrophysiological recordings and behavioral analyses were performed on separate cohorts of mice (experimental outline, Fig. 1A; statistical analysis, Supplementary Table 1). After completed behavioral analysis, a group of Pitx2/ChR2 and Pitx2/eYFP-C mice (N = 9) underwent prolonged photostimulation and brains were dissected for histological validation of c-Fos, an indicator of induced neuronal activity. All mice in the study were sacrificed upon completed analysis, brains sectioned and the distribution of the eYFP fluorescent reporter, virus injection site and cannula position were validated. Mice that displayed strong cellular eYFP labeling throughout the extent of the STN and optical cannulas placed immediately above the STN were included in the statistical analyses while displaced cannula and/or lack of eYFP were set as criteria for exclusion.

 Histological analysis of eYFP fluorescence in the STN and projection target areas (Fig. 1B) verified the selectivity of eYFP labeling in the STN structure over surrounding structures (Fig. 1C-D). The entire extent of the STN was labeled, and within STN neurons, eYFP was detected throughout the cell body. No eYFP was detected in adjacent structures, apart from weakly labeled fibers in the close associated para-STN (pSTN) (Fig. 1C-D). No cell bodies of the pSTN were labeled with eYFP, in accordance with highly limited Pitx2 gene expression in this area (Wallen-Mackenzie et al., 2020). Strong eYFP labeling was identified in STN-projections reaching the ventral pallidum, entopeduncular nucleus (EP, corresponding to GPi in primates), GP (GPe in primates), SNr and substantia nigra pars compacta (SNc) (Fig. 1E-H).

In terms of c-Fos, no c-Fos-positive neurons were detected in the STN of control mice, i.e. Pitx2/ChR2 mice that had not received photostimulation (N = 3) (Fig. 1I-I’). Pitx2/eYFP-C control mice that had received photostimulation (N = 3) (Fig. 1J-J’). In contrast, Pitx2/ChR2 mice that had received photostimulation showed c-Fos labeling in neurons throughout the STN structure (N = 3) (Fig. 1K-K’). The labeling was most prominently detected in neurons located immediately below the optic cannula, showing 90 to 100% overlap with eYFP. This finding verified the presence of induced neuronal activity upon optogenetic stimulation of the STN. Thus, the STN and its target areas express the optogenetic constructs and allow neuronal activation upon photostimulation.

4.2. Optogenetic activation excites STN neurons and induces post-synaptic responses

To ensure firing and assess connectivity upon optogenetic activation of the STN, Pitx2/ChR2 mice were analyzed in two electrophysiological paradigms. Light-evoked responses of both the STN itself and one of its main target areas in motor control, the GP, were studied by in vivo single cell electrophysiological recordings upon optogenetic stimulation of the STN. First, an optotagging protocol (Fig. 2A) was used to stimulate and record within the STN. To observe the reaction of STN neurons to photostimulation, peri-stimulus time histograms (PSTHs) were created by applying a 0.5 Hz stimulation protocol for at least 100 s (referred to as the 0.5 Hz protocol: 0.5 Hz, 5 ms pulse width, 100 s). Action potentials in ChR2-positive STN cells were successfully evoked by STN photostimulation (Fig. 2B).

Next, recordings were performed within the GP structure upon two different stimulation paradigms applied in the STN (Fig. 2C). Firstly, the 0.5 Hz protocol was implemented which evoked action potentials in the majority of the recorded GP neurons (Fig. 2D-E). This finding verified functional connectivity between the STN and GP. Based on our previous confirmation of in vivo glutamate release upon optogenetic excitation of the STN using a 20 Hz photostimulation protocol in anaesthetized Pitx2/ChR2 mice (Vierckel et al., 2018), a 20 Hz photostimulation protocol with the purpose of driving STN excitation in freely-moving animals was established. For validation, such a 20 Hz photostimulation was here applied (0.5 Hz, 5–8 ms pulse width) for 100 s in anaesthetized Pitx2/
Experimental timeline

A. Guillaumin et al.

Histological eYFP validation

C-Fos

(caption on next page)
due to the low spontaneous activity of STN neurons, upon photoexcitation, characterized by sliding or slipping on the floor or (Fig. 3F). In addition, some Pitx2/ChR2 mice displayed abnormal gait.

Having confirmed optogenetics-driven excitation of STN neurons and their post-synaptic responses, behavioral motor effects upon optogenetic STN activation and inhibition were next assessed.

4.3. Optogenetic excitation and inhibition of the STN induce opposite effects on both horizontal and vertical locomotion

First, Pitx2/ChR2 and Pitx2/Arch mice and their respective controls were analyzed in the open field test, allowing a qualitative and quantitative measurement of motor activity. The open field test also allows analysis of additional functions such as exploration and anxiety. Mice were allowed to explore the apparatus for five minutes before starting the experimental protocol. This habituation period serves to avoid effects due to separation stress and agoraphobia that can induce thigmotaxis. Following habituation, mice were tested in a single 20-minute session composed of four alternating five-minute long photostimulation-off (OFF) and photostimulation-on (ON) protocols using laser sources providing blue (for Pitx2/ChR2 mice and controls) and green (for Pitx2/Arch mice and controls) light. Photostimulation (ON) for Pitx2/ChR2 and Pitx2/eYFP-C mice corresponded to the 20 Hz protocol; Pitx2/Arch and Pitx2/eYFP-A mice corresponded to continuous light stimulation.

First addressing Pitx2/ChR2 mice, during OFF epochs, primarily horizontal and vertical movements were observed, with short periods of self-grooming. No differences between Pitx2/eYFP-C and Pitx2/ChR2 mice were observed during OFF epochs (Figure A-G). In response to STN-photostimulation (ON), Pitx2/ChR2 mice responded with a significant reduction in vertical activity, also known as rearing (Fig. 3A). The parameters horizontal activity, measured as distance moved, speed and time spent moving were all decreased upon STN-photostimulation (Fig. 3B-D). By decreasing these motor parameters upon optogenetic activation of the STN (ON compared to OFF), the expected role of STN excitation in suppressing locomotion could be verified experimentally.

The time spent exploring the center of the arena, a measure of anxiety, was not affected by the stimulation (Fig. 3E), but the number of exploratory visits to the center was significantly lower during ON epochs (Fig. 3F). In addition, some Pitx2/ChR2 mice displayed abnormal gait upon photoexcitation, characterized by sliding or slipping on the floor or backwards movements as indication of a lack of balance, while some showed a prominent jumping behavior, often directed towards the walls of the arena as if trying to escape the apparatus (Fig. 3H). No differences were detected for any of the measured parameters in Pitx2/eYFP-C control mice between OFF and ON epochs (Fig. 3A-H).

Contrary to STN activation in Pitx2/ChR2 mice, but in accordance with the anticipated role of the STN in motor control, continuous optogenetic STN inhibition in Pitx2/Arch mice resulted in increased horizontal and vertical movement (Fig. 3I-L). In contrast to STN excitation in Pitx2/ChR2 mice, while not affecting the time spent exploring the center (Fig. 3M), STN inhibition increased exploratory activity, visible by a higher number of visits to the center during ON epochs (Fig. 3N). No differences were detected for any of the measured parameters in Pitx2/eYFP-A control mice between OFF and ON epochs (Fig. 3I-N).

Further pinpointing the opposite effects on motor parameters by STN excitation vs inhibition, also self-grooming behavior was altered in opposite directions by the optogenetic manipulations. Excitation of the STN induced significant face-grooming behavior in the Pitx2/ChR2 mice (Fig. 3G, Video clip 1). In contrast, the naturally occurring grooming behavior was lower in Pitx2/Arch mice during ON epochs than during OFF epochs (Fig. 3O). No difference in the time spent grooming was observed in Pitx2/eYFP-C (Fig. 3G) and Pitx2/eYFP-A (Fig. 3O) controls in response to optogenetic manipulation. In rodents, self-grooming is an innate behavior which follows a distinct pattern, referred to as the cephalo-caudal rule, covering the extent of the body but starting in the face region (Berridge et al., 2005; Fentress, 1988). The self-grooming displayed by Pitx2/ChR2 mice was directly associated with STN-photostimulation. While grooming was not continuous throughout each stimulation phase in all mice, it was initiated shortly upon STN-photostimulation to most often be observed in numerous episodes of separate activity. Further, the observed behavior did not follow the cephalo-caudal grooming rule, but was displayed as strongly repetitive strikes by both front paws tightly in the face area, primarily around the nose (Video clip 1).

By plotting the behavioral activity patterns for each cohort of mice, their comparison identifies clear differences (Fig. 3P-R). Compared to control mice (Fig. 3P), when receiving photostimulation in the STN, Pitx2/ChR2 mice (Fig. 3R) increased their time spent self-grooming at the expense of the time spent moving forward, rearing or engaged in other activities (Fig. 3P, R; Supplementary Fig. 1). In contrast, upon STN-photostimulation, Pitx2/Arch mice (Fig. 3Q) increased their time spent moving and rearing at the expense of other activities (Fig. 3Q; Supplementary Fig. 1). Together, these results confirm the importance of the activational level of the STN in horizontal and vertical movement. The data also identify a direct causality between STN excitation and stereotyped self-grooming.

4.4. Unilateral excitation and inhibition of the STN induce opposite rotations

To further assess the impact of optogenetic manipulations on motor activity, unilateral STN photostimulation was performed in Pitx2/ChR2 and Pitx2/Arch mice and their respective controls during two ten-
Optotagging in the STN

A AV2-hChR2-eYFP

Injections

4 weeks

Optotagging

light stimulation

STN recordings

B

0.5Hz, 5ms, 8 mW

40

30

20

10

0

-500

0

500 ms

In vivo recordings in the globus pallidus, GP

C

Injections

4 weeks

GP recordings

AAV2-hChR2-eYFP

In vivo

light stimulation

GP recordings

D

0.5Hz, 5ms, 8 mW

60

50

40

30

20

10

0

-500

0

500 ms

E

STN light stimulation ON

5 ms

F

PSTH z-score

Z-score (% baseline)

Baseline

Excitation

G

100 sec

Frequency (Hz)

Membrane potential (mV)

H

Firing rate (% baseline)

Baseline

Stimulation

Post-stimulation

I

eYFP

Fontamings

J

Bregma: -0.10 mm

-0.22 mm

-0.34 mm

35%

20%

45%

Not responding

Excited with both protocols

Excited only with one protocol

(caption on next page)
Results showed a significant difference in the firing rate between levels (N = 20 Hz protocol with the frequency and the recording trace. (H) Firing rate of excited GP neurons before, during and after 100 s of light stimulation as shown in g. Third of GP neurons were excited upon STN photostimulation. Blue arrow in B, D and E shows STN photostimulation. Abbreviations: STN, subthalamic nucleus; GP, substantia nigra pars compacta (SNpc). (F) z-score of 12 GP neurons excited during the 0.5 Hz protocol. (G) Example of an excited GP neuron during the two different protocols (0.5 Hz and 20 Hz protocols), light blue dots indicate neurons excited during one of the two protocols and white dots correspond to neurons which did not respond to the light. The pie chart shows that about two third of GP neurons were excited upon STN photostimulation. Blue arrow in B, D and E shows STN photostimulation. Abbreviations: STN, subthalamic nucleus; GP, globus pallidus.

minute long sessions divided into two five-minute long OFF-ON sessions of photostimulation (20 Hz protocol; continuous stimulation, respectively) (Fig. 4A). Since unilateral activation or inhibition of the STN is expected to give rise to rotational rather than forward movement, behavior was scored by comparing ipsilateral and contralateral rotations. Indeed, STN-photostimulation induced a strong rotational behavior. Pitx2/ChR2 mice made a significantly higher number of ipsilateral than contralateral rotations during ON epochs (Fig. 4B-C). In contrast, Pitx2/Arch mice made significantly more contralateral than ipsilateral rotations during ON epochs (Fig. 4E-F). No rotational behavior was detected for control mice during either ON or OFF epochs (Fig. 4D and 4G). The results confirm the importance of the STN for coordinated motor output by demonstrating that manipulation of one of the two STN structures is sufficient to produce measurable rotation effects.

4.5. Optogenetic STN-activation disrupts motor coordination in the rotarod and beam walk tests

The strong reduction in locomotion together with gait abnormality and prominent grooming behavior observed during the open field test of Pitx2/ChR2 mice, but not Pitx2/Arch mice, prompted further analysis of the impact of STN excitation on motor coordination. Pitx2/ChR2 and control mice were therefore next analyzed in both the rotarod and the beam walk tests. These tests are commonly used for assessment of crude and fine motor coordination, respectively (Brooks & Dunnett, 2009; Deacon, 2013; Luong et al., 2011). Photostimulation (ON) for Pitx2/ChR2 and Pitx2/eYFP-C mice again corresponded to the 20 Hz protocol.

First, the rotating rotarod was implemented to assess the ability of Pitx2/ChR2 mice to sustain an on-going motor coordination task when STN excitation is induced by optogenetic stimulation. To enable this test, mice are first trained during several sessions until they solidly learn to master a coordinated walking behavior on the rotating rod. Once training to maintain walking throughout a full two-minute rotarod session was completed, mice progressed to the actual test in which they were exposed to STN-photostimulation in four OFF/ON trials, each trial corresponding to maximum of 120 s (Fig. 5A-B). The latency to fall off the rod (before the maximum time reached) was scored.

During the test, Pitx2/ChR2 and control mice all managed to stay on the rod throughout the entire length of 120 s during the OFF trials. During ON trials, photostimulation was applied five seconds after the mouse was properly walking on the rod to ensure they were already stable once the stimulation started. The effect of STN-photostimulation was almost immediate. All Pitx2/ChR2 mice fell off the rod within seconds after STN excitation was initiated, giving rise to a strikingly short latency to fall (Fig. 5C, Video clip 2). The average latency to fall was 13.5 s ± 4.66 SEM during the first ON trial and 16.7 s ± 6.95 SEM during the last ON trial. In contrast, all control mice maintained their rod-walking undisturbed throughout each 120 s long session (Fig. 5D).

The disrupting effect on motor coordination in Pitx2/ChR2 mice was transient and displayed specifically upon photostimulation, with completely restored coordination during subsequent OFF trial (Fig. 5C).

Further, since bilateral excitation in the open field test had been observed as associated with self-grooming, specific attention was directed to observe if this behavior was manifested while walking on the rotating rod. However, it was evident that no grooming behavior was displayed while the mice were walking on the rotarod (Video clip 2).

Thus, mice did not fall off the rotarod because they started grooming. Instead, the observation of immediate falling behavior upon optogenetic STN stimulation suggested that STN activation induces loss of motor coordination. However, given the well established role of the STN in stopping or interrupting on-going motor tasks (Aron et al., 2007; Cavagnan et al., 2011; Fife et al., 2017; Pasquerneau and Turner, 2017; Schmidt et al., 2013; Zavala et al., 2015) and in response inhibition (Green et al., 2013; Jahanshahi et al., 2000; Witt et al., 2004), the STN-photostimulated Pitx2/ChR2 mice might have fallen off the rod due to stopping or response inhibition rather than loss of motor coordination. To investigate this possibility and also to better understand any effects that STN excitation might impose on motor coordination, the beam walk test was next applied.

Upon training to master walking on three different sized beams (increasingly small diameter) passing from a starting area (SA) through a recording area (RA) to reach a goal zone (GZ), mice were tested on the thinnest beam. Photostimulation was applied as mice passed the RA section of the beam, and their behavior (time to pass RA and number of hind-foot slips) was recorded (Fig. 5E-F). Three protocols were tested in which photostimulation was activated during varying lengths of time as the mice passed the RA: Alternated OFF and ON epochs along RA (referred to as ALT), continuous stimulation along RA (referred to as CONT), or pre-set photostimulation starting a 15 s before the mice were placed in the SA and subsequently continuous as they crossed the RA (referred to as PRIOR) (Fig. 5E). 15 s prior stimulation was implemented to mimic the result observed during the rotarod test when the average latency to fall was 13.5 s during the first ON trial and 16.7 s during the last ON trial.

Time to cross the RA and the number of hind-foot slips were scored and jointly analyzed using a neuroscore pre-defined rating scale (Supplementary table 2). By assigning a maximum of 5 points for time to cross and 5 points for hind-foot slips, the maximum score was 10 points for each photostimulation condition. The lower is the score, the better is the motor performance. Overall, no grooming or jumping behavior was observed in any mouse during the beam walk test, and no effect upon photostimulation was observed in control mice (Fig. 5H).

On average, during the BS condition, the time to cross the beam was 4.85 s ± 0.18 SEM for Pitx2/ChR2 mice and 5.40 ± 0.35 SEM for Pitx2/eYFP-C mice while the number of hind-foot slips was 0.92 ± 0.21 SEM for Pitx2/ChR2 and 0.77 ± 0.20 SEM for Pitx2/eYFP-C mice. This resulted in a total neuroscore of 1.9 points for Pitx2/ChR2 and 1.5 for Pitx2/eYFP-C calculated from the pre-defined rating scale, where the maximum score was 10 points (Supplementary table 2).

During the ALT condition, the total neuroscore was 2.6 for Pitx2/ChR2 mice and 1.6 for Pitx2/eYFP-C mice, while during the CONT condition the total neuroscore was 2.8 for Pitx2/ChR2 and 1.6 for Pitx2/eYFP-C mice, resulting in no significant difference from the BS condition in both groups.

In the PRIOR condition, the total score was 6.1 for Pitx2/ChR2 mice and 1.5 for Pitx2/eYFP-C mice. Here, motor coordination was significantly decreased among Pitx2/ChR2 mice, with some mice falling off the
Fig. 3. Optogenetic activation and inhibition of the STN induce opposite effects on both horizontal and vertical locomotion. (A-E) In the open field test, several behaviors representative of vertical and horizontal locomotion were recorded in Pitx2/ChR2 mice and their corresponding Pitx2/eYFP-C control mice: rearing (A), distance moved (B), speed (C), time spent moving (D), times spent exploring the center (E) and center visits (F). (G-H) Graphs showing the time spent grooming (G) and the number of jumps (H). (I-N) The same behavioral parameters were recorded in Pitx2/Arch and Pitx2/eYFP-A control mice: rearing (I), distance moved (J), speed (K), time spent moving (L), time spent exploring the center (M), visits to the center (N), time spent grooming (O). (P-R) Pie charts illustrating the proportion of each behavior (time spent grooming, rearing and moving) displayed in the open field test; Pitx2/eYFP control mice (Pitx2/eYFP-C and Pitx2/eYFP-A control mice pooled together) (P), Pitx2/ChR2 (Q), Pitx2/Arch (R). “Other” was calculated by subtracting the time spent engaged in the aforementioned behaviors from the total time of recorded epoch (300 s). Behaviors for which the parameter time spent is not available are also included in “Other”, such as jumping. All data are presented as average for the two ON and OFF epochs ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.
beam, unable to complete the crossing (Fig. 5G). Overall, no strong motor impairments were observed during the conditions ALT and CONT when STN-photostimulation was provided for a total estimated period of 2.5 and 5 s, considering the time to cross the beam during the BS condition. Instead, only when STN-photostimulation was applied 15 s prior to actually crossing the RA (PRIOR) did Pitx2/ChR2 mice mice show a deficiency in motor performance.

These findings suggest that prolonged STN excitation of 15 s is necessary and also sufficient to impair performance in the beam walk test. The results suggest that loss of motor performance in advanced tasks is not the result of inhibitory response or a stopping signal derived from a brief pulse of activation of the STN, but that motor coordination is impaired when the STN is excited for about 15 s. Taken together, the rotarod and beam walk tests identify a direct association between STN excitation and impairment of on-going motor coordination when mice are engaged in performing challenging motor tasks.

5. Discussion

The role of the STN in normal motor regulation has long been assumed as established. However, experimental evidence has remained somewhat sparse, not least in terms of motor effects upon STN excitation under non-pathological conditions. This is mainly due to the strong experimental focus on parkinsonian conditions and models with a primary focus to enhance movement during pathological conditions. But since the execution of intended movement depends on several different parameters to be fully functional, we reasoned that a solid identification of how each of these are regulated under normal conditions should contribute to decoding the complexity of motor function. Forward locomotion, gait, balance and motor coordination are all of critical importance to achieve intended movement, and their disturbance manifested in motor disorders, including PD, but also supranuclear palsy, Huntington’s disease and hemiballismus, all in which STN dysfunction is implicated. In this study, we found that optogenetic STN excitation was generally correlated with significant reduction in locomotor activity, while in contrast, optogenetic STN inhibition enhanced locomotion, just as the classical motor model of the basal ganglia would predict. Optogenetic excitation vs inhibition of the STN thus verify its role as predicted by the basal ganglia model in terms of locomotion. However, we also found that these correlations were not true for all types of movement. Upon optogenetic excitation of the STN in the open field test, jumping and self-grooming behaviors were induced, not reduced. A forceful jumping was displayed which appeared to the observer as attempts to escape. This type of jumping was observed in some but not all mice upon STN-photostimulation, and it ceased when stimulation ended. An even more potent behavior induced upon STN-photostimulation was the repetitive self-grooming, intimately correlated with the presence of STN-photostimulation. Curiously, jumping and self-grooming behaviors were only observed when mice were freely exploring a non-challenging environment, not when mice were engaged in advanced motor tasks. There instead, trained mice lost their motor coordination and failed to complete the challenging tasks. Together, these findings indicate that detailed analysis of the behavioral roles driven by the STN is likely to enhance knowledge of motor control and its disease, beyond what might be expected based on classical models.

In a recent study, we showed that in vivo optogenetic stimulation of the STN at 20 Hz, which is within the range of stimulation frequency that excites rodent STN neurons during normal conditions, in Pitx2/ChR2 mice was sufficient to release measurable amounts of glutamate in the GP (Viereckel et al., 2018). Based on this finding, a 20 Hz protocol for photoexcitation of the STN was used throughout the behavior analyses of Pitx2/ChR2 mice. This photoexcitation protocol was also validated by in vivo electrophysiological recordings performed in intact anaesthetized mice. Further, to ensure firing and assess connectivity upon optogenetic activation of the STN, Pitx2/ChR2 mice were also analyzed for optogenetically evoked responses by applying a 0.5 Hz stimulation protocol. The results firmly demonstrated that action potentials were reliably evoked in both the STN and the GP, a critical STN target area. Electrophysiological recordings showed that GP neurons were excited upon...
Fig. 5. Optogenetic STN-activation disrupts motor coordination (A) Schematic representation of the fixed speed rotarod assay timeline. (B) Graphical representation of the rotarod assay, with the mouse receiving bilateral optical stimulation during ON trials. (C) Latency to fall from the rotating rod for Pitx2/ChR2 is expressed as mean ± SEM, **p < 0.01, ***p < 0.001 and (D) for Pitx2/eYFP-C control mice, ± SEM, p = 0.1989. (E) Schematic representation of the beam walk test timeline. (F) Schematic representation of the tested conditions: "Baseline" (BS), "Alternated Light" (ALT), "Continuous Light" (CONT) and "Prior Stimulation" (PRIOR). (G-H) The neuroscore is calculated from a scoring scale (see Supplementary Table 2) and is expressed as mean ± SEM, *p < 0.05, **p < 0.01.
photo-stimulation of the STN structure. Evoked action potentials were observed in GP neurons following both the 0.5 Hz and 20 Hz protocols. With these validations, the behavioral assessments using ChR2 to excite the STN neurons in their natural brain network rest upon electrophysiological confirmation within both the STN itself and GP. However, additional recordings would be required to fully pinpoint the range of target areas that mediate the different behavioral responses observed, as additional brain structures beyond the GP are likely involved. Verifying the innervation pattern, STN-derived eYFP-positive projections were identified in the expected target areas, the GP, EP, SNr and SNC, structures that may all be involved in the observed behaviors.

While recordings in target areas beyond the GP should be of interest in terms of neurocircuitry analysis of the observed behaviors, another limitation of the study is the lack of electrophysiological recordings upon STN inhibition in Pitx2/Arch mice. These were not performed in the current setup due to the low spontaneous activity of the STN in vivo. Further, while separate cohorts of mice were tested in electrophysiological and behavioral experiments, it should be noted that behavioral mice were tested in more than one behavioral paradigm. All behavioral mice were assessed in the open field test, and were thereby naïve to photostimulation only in this test. In the rotarod and beam walk tests that followed the open field, and that were implemented to specifically assess motor coordination upon STN excitation, mice had thereby already experienced photostimulation. This could be an important factor to bear in mind, as repeated photostimulations might give rise to synaptic plasticity. However, in none of the behavioral tests following the open field test were any significant differences detected between Pitx2/ChR2 and Pitx2/eYFP-C mice under non-stimulation conditions, suggesting that circuitry adaptations had not been induced. While several behavioral testing paradigms might be a putative limitation to this study, the 3R’s in laboratory animal science (replace, reduce, refine) is a factor of considerable importance out of which reduce and refine were taken into special consideration. Beyond these putative limitations, the findings that STN excitation causes disruption in motor coordination and induces repetitive self-grooming are important revelations, and should be worthwhile to investigate further.

When addressing behavior, we used both optogenetic STN excitation (ChR2) and inhibition (Arch) to enable the comparison of results obtained in the open field test. This distinct experimental separation into STN excitation vs STN inhibition clearly caused different effects on several aspects of motor function with reduced forward locomotion and rearing upon STN excitation, and enhancement of both parameters upon inhibition. These findings firmly verify the opposing roles executed upon STN excitation and inhibition, with a mirror effect displayed by these opposite types of stimulations. Beyond the comparison with STN inhibition, the analyses also identified several unexpected behaviors upon STN excitation in the non-challenging environment that deserved additional attention. In contrast to the long-standing history of STN inactivation by surgical lesioning, optogenetics offers the benefit of controlled excitation. This is a critical experimental advantage in the study of the STN under non-pathological conditions, since in its natural network, the STN is excited by cortical projections via the hyperdirect pathway and is more or less inhibited via the indirect pathway, and in turn, exerts excitatory influence over its targets. While STN inactivation and inhibition are of major interest in PD-related research in order to find ways to override pathological STN hyperactivity and promote movement, the naturally occurring regulatory role of the STN upon excitation has been far less explored. Here we could observe that photogenetic excitation of the STN in Pitx2/ChR2 mice caused self-grooming behavior in the open field test. When explored further in more advanced motor tests, Pitx2/ChR2 mice displayed loss of balance and motor coordination. These behavioral displays were observed almost immediately upon STN excitation in Pitx2/ChR2 mice. Notably, the mice did not lose balance or coordination due to induced jumping or self-grooming, as these types of behavior were not detected when mice were performing in the more advanced tests, the rotating rotarod and the beam walk.

Thus, it seems that when mice are engaged in challenging motor tasks, STN excitation causes loss of coordination, while when they are freely exploring, STN excitation causes self-grooming, jumping and/or loss of normal gait. In the non-challenging environment, mice behaved as if the optogenetic STN excitation was perceived as unpleasant, even aversive. Some mice even seemed to display jumping as an attempt to escape the test arena. In contrast, in the challenging conditions of the rotarod and beam walk, the strong impact of STN excitation on balance and coordination likely reflects the reduced movement causing a disturbed ability to maintain position in tasks where flexible motor output is required.

While the rotarod and beam walk tests have been designed to address gross and fine motor coordination, respectively, these tests are not operant. Once mice have learnt to master the challenge, they sustain walking behavior. Thus, these types of motor tasks are not identical to instrumental tasks in which animals engage in learnt behavior to gain some type of reward. However, recent findings have shown that also these can be stopped by optogenetic excitation of the STN. Indeed, one study identified that on-going licking behavior in mice was interrupted by optogenetic excitation of STN neurons (Pile et al., 2017). While licking behavior is distinct from the type of motor coordination required to master the rotating rotarod, it might be noteworthy that behaviors that ought to require substantial motivation or engagement are disturbed when the STN is excited. Beyond this observation, many studies have demonstrated the role of the STN in response inhibition (Aron et al., 2007; Cavagnah et al., 2011; Pasquereau and Turner, 2017; Schmidt et al., 2013; Zavala et al., 2015) in particular during conflict to prevent impulsive actions. This has been evident also upon STN-DBS in PD. PD patients treated with STN-DBS have been shown to respond with higher degree of impulsivity in some impulse control tasks (Green et al., 2013; Jahnshahi et al., 2000; Witt et al., 2004). On the other hand, other STN-DBS studies did not find impairments in response inhibition, and some even showed improvements in response inhibition induced by DBS (Mirabella et al., 2012; van den Wildenberg et al., 2006). During a situation in which action selection is needed, the premotor cortex activates the STN through the hyperdirect pathway. The activated STN is turn excites the Gpi (EP in rodents) with a resulting thalamic inhibition and disinhibition of movements in a condition defined as “hold your horses” necessary to gather more evidences before taking a decision. Shortly after, the inhibitory feedback from the GPe (GP in rodents) overpower the stopping effect on movements induced by STN activation while a cortico-striatal activation inhibits the Gpi through the direct pathway resulting in thalamic disinhibition and facilitation of movement (Albin et al., 1989; Bogacz & Gurney, 2007; DeLong, 1990; Frank, 2006). In the present study, mice fell from both the rotarod and the beam (beam walk test) when optogenetic STN-stimulation proceeded for about 15 s. This was evident upon testing different lengths of stimulation. To distinguish between short and longer stimulations, the time of the photostimulation varied between three different protocols in the beam walk test. Indeed, the results of this test showed that a brief activation of the STN was not sufficient to induce falling off the beam, but a more sustained excitation was necessary to impede a normal completion of the motor task. These results suggest that prolonged optogenetic activation of the STN is more likely to mimic a condition of sustained hyperactivation induced by circuitry disruption typical of a pathological condition, rather than a momentary excitation representative of the “hold your horses” model of response inhibition.

Previous reports have shown that activation of STN neurons, either by surgical STN lesioning (Centonze et al., 2005; Heywood & Gill, 1997; Parkin et al., 2001; Rizell et al., 2010) or by conditional knockout of the Vglut2 gene in the STN of Pitx2-Cre mice (Schweizer et al., 2014) significantly elevated the level of locomotion. These findings, which provided support to the basal ganglia model in which STN inhibition is postulated to increase movement, are in accordance with the current study in which we identify that optogenetic inhibition of the STN causes hyperkinesia while optogenetic excitation causes hypokinesia during
non-pathological conditions. In addition, the opposite effects of STN excitation and inhibition observed here also lend indirect support for the inhibition hypothesis of DBS, albeit during non-parkinsonian conditions. Despite the current application of STN-DBS as treatment of PD, the mechanisms underlying its symptom-alleviating effects are still not entirely understood. It is even currently debated whether the neural elements are excited or inhibited by the stimulations (Chiken & Nambu, 2016; Deniau et al., 2010). One model of DBS mechanism involves the depolarization of axons and inhibition of cell bodies (Florence et al., 2016; Vitek, 2002), while another model proposes excitation of afferent, efferent and passing fibers (Kringelbach et al., 2007) with increased firing (Hashimoto et al., 2003) and glutamate release in basal ganglia output areas (Windels et al., 2008). Studies based on optogenetics in parkinsonian animal models have provided support to both the inhibition and excitation hypothesis of STN-DBS (Gradinaru et al., 2009; Sanders & Jaeger, 2016; Yoon et al., 2014). Recently, a study using the ultrafast opsin CHRONOS, aiming at creating an animal model of optogenetic STN-DBS, showed that parkinsonian movement disability was improved to a similar degree as when electrical STN-DBS was applied, further strengthening the correlation between the STN and motor output in the parkinsonian model (Yu et al., 2020). In PD and DBS contexts, our results showing opposite locomotor effects upon optogenetic STN excitation and inhibition, with enhanced locomotion upon STN inhibition, thus provide indirect support for the inhibition hypothesis of STN-DBS. However, the current results also identify strong impact of STN manipulation beyond locomotion, since gait, balance and motor coordination parameters are all affected. Future studies might be of interest in which a parkinsonian model such as 6-OHDA toxicity is applied, further strengthening the correlation between the STN and motor output in the parkinsonian model (Yu et al., 2020). In PD and DBS contexts, our results showing opposite locomotor effects upon optogenetic STN excitation and inhibition, with enhanced locomotion upon STN inhibition, thus provide indirect support for the inhibition hypothesis of STN-DBS. However, the current results also identify strong impact of STN manipulation beyond locomotion, since gait, balance and motor coordination parameters are all affected. Future studies might be of interest in which a parkinsonian model such as 6-OHDA toxicity is applied in the Pitx2-ChR2 and Pitx2/Arch mice in order to unravel the regulatory roles of the STN in compulsive-like behaviors. The neurological underpinnings of the STN in compulsive behavior will need further attention. However, it is evident from the present demonstration that STN excitation drives self-grooming of the face area should be worthwhile to investigate further.

6. Conclusion

Locomotion, gait, balance and coordination are critical motor features required to enable and sustain voluntary movement. The experimental approach used in this study allowed the firm identification of a clear correlation between the STN and regulation of each of these pivotal motor functions. The results presented provide experimental evidence supporting the predicted role of the STN in locomotion according to classical basal ganglia motor models, and identify an unexpected role of the STN in self-grooming behavior. A clear association between STN activation and loss of sustained motor control in advanced motor tasks is also found. Further studies will be required to pinpoint mechanistic underpinnings and circuitry components involved in mediating each of these distinct behaviors.

Funding

This work was funded by Uppsala University and by grants to Å.W.M. from the Swedish Research Council (SMRC 2017-02039), the Swedish Brain Foundation (Hjärnfonden), Parkinsonfonden, and the Research Foundations of Bertil Hållsten, Zoologiska stiftelsen and Åhlén.
Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Professor James Martin, Baylor College of Medicine, Houston, Texas, USA, for generously providing the Pitx2-Cre transgenic mouse line. Members of the Mackenzie Lab are thanked for constructive feedback throughout this study.

Author contributions

A.G: Investigation, Formal analysis, Visualization, Writing – original draft; G.P.S: Investigation, Formal analysis, Methodology, Visualization, Writing - original draft; F.G: Supervision; Å.W.M: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, Writing - review & editing, Formal analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2020.147226.

References

Ahmari, S.E., Spellman, T., Douglass, N.L., Kheirbek, M.A., Simpson, H.B., Deisseroth, K., Gordon, A.J., Han, R., 2013. Repeated cortico-striatal stimulation generates persistent OCD-like behavior. Science 340 (6137), 1234-1239. https://doi.org/10.1126/science.1234733.

Albin, R.L., Young, A.B., Penney, J.B., 1989. The functional anatomy of basal ganglia disorders. Trends Neurosci. 12 (10), 366-375. https://doi.org/10.1016/0166-2236(89)90074-8.

Alexander, G.E., Crutcher, M.D., 1990. Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci. 13 (7), 266-271. https://doi.org/10.1016/0166-2236(90)90107-L.

Alkemade, A., Schnitzler, A., Forstmann, B.U., 2015. Topographic organization of the human and non-human primate subthalamic nucleus. Brain Struct. Funct. 220 (6), 3075-3086. https://doi.org/10.1007/s00429-015-1047-2.

Aron, A.R., Behrens, T.E., Smith, S., Frank, M.J., Poldrack, R.A., 2007. Triangulating a cognitive control network using diffusion-weighted magnetic resonance imaging (MRI) and functional MRI. J. Neurosci. 27 (14), 3743-3752. https://doi.org/10.1523/JNEUROSCI.0519-07.2007.

Baup, N., Grabi, D., Karachi, C., Mounayar, S., Francois, C., Yelnik, J., Feger, J., Tremblay, L., 2006. High-frequency stimulation of the anterior subthalamic nucleus reduces stereotyped behaviors in primates. J. Neurosci. 28 (35), 8785-8788. https://doi.org/10.1523/JNEUROSCI.2883-08.2008.

Benabid, A.L., 2003. Deep brain stimulation for Parkinson’s disease. Curr. Opin. Neurol. 16 (3), 696-706. https://doi.org/10.1097/00006123-200307000-00001.

Benabid, A.L., Chabardes, S., Mitrofanis, J., Pollak, P., 2009. Deep brain stimulation of the subthalamic nucleus for the treatment of Parkinson’s disease. Lancet Neurol. 8 (1), 67-81. https://doi.org/10.1016/S1474-4422(08)70291-9.

Benazzouz, A., Gao, D.M., Ni, Z.G., Piailat, B., Bouali-Benazzouz, R., Benabid, A.L., 2000. Effect of high-frequency stimulation of the subthalamic nucleus on the neuronal activities of the substantia nigra pars reticulata and ventrolateral nucleus of the thalamus in the rat. Neuroscience 99 (2), 289-295. https://doi.org/10.1016/S0306-4522(00)00109-6.

Berridge, K.C., Aldridge, J.W., Houchard, K.R., Zhuang, X., 2005. Sequential stereotypy of an instinctive fixed action pattern in hyper-dopaminergic mutant mice: A model of obsessive compulsive disorder and Tourette’s. BMC Biol. 16, 147226.
Expression of the cortico-subthalamo-pallidal 'hyperdirect' pathway. Neurosci. Res. 43 (2), 111–176.

Parente, A., Hassani, L., 1995. Functional anatomy of the basal ganglia. II. The place of the subthalamic nucleus and external pallidum in basal ganglia circuitry. Brain Res. Rev. 20 (1), 128–154. https://doi.org/10.1016/0169-1688(95)0000-B.

Parkin, S., Nandi, D., Giladi, N., Joint, C., Gregory, R., Bain, P., Scott, R., Aziz, T.Z., 2001. Functional significance of the subthalamic nucleus reverses motor deficits but not death of nigrostriatal projections is sufficient to ameliorate bradykinesia in 6-ohda lesioned mice. Neurology 51 (2), 384–390. https://doi.org/10.1016/S0028-3932(00)00863-4.

Baroni, J., Buhler, S., de Oliveira Bueno, M., Grassi, T., Huber, D., 2012. Evidence that the parafascicular nucleus projection to the thalamus is modulated by the subthalamic nucleus. J. Neurosci. 32 (47), 16153–16161. https://doi.org/10.1523/JNEUROSCI.1755-12.2013.

Schmidt, R., Berke, J.D., 2017. A Pause-Then-Cancel model of stopping: evidence from basal ganglia neurophysiology. Phil. Trans. R. Soc. B 372 (1718), 20160202. https://doi.org/10.1098/rstb.2016.0202.

Schmidt, R., Leventhal, D.K., Mallet, N., Chen, F., Berke, J.D., 2013. Canceling actions involves a race between basal ganglia pathways. Nat. Neurosci. 16 (8), 1118–1124. https://doi.org/10.1038/nn.3605.

Schweizer, N., Puge, S., Arvidsson, E., Nordenkand, K., Smith-Antilla, C.J.A., Mahmoudi, S., Andren, A., Dumas, S., Rajagopalan, A., Levesque, D., Leao, R.N., Wallen-Mackenzie, Å., 2014. Limiting glutamate transmission in a Vglut2-expressing subpopulation of the subthalamic nucleus is sufficient to cause hyperlocomotion. Proc. Nat. Acad. Sci. 111 (21), 7837–7842. https://doi.org/10.1073/pnas.1323499111.

Schweizer, N., Vierbeck, T., Smith-Antilla, C.J.A., Nordenkand, K., Arvidsson, E., Mahmoudi, S., Zapatera, A., Warner Jonsson, H., Bergquist, J., Levesque, D., Konradsson-Geuken, A., Andersson, M., Dumas, S., Wallen-Mackenzie, Å., 2016. Reduced Vglut2 in Slc17a6 gene expression levels throughout the mouse subthalamic nucleus cause cell loss and structural disorganization followed by increased motor activity and decreased sugar consumption. eNeuro 3 (6). https://doi.org/10.1523/ENEURO.0826-16.2016.

Skidmore, J.M., Cramer, J.D., Martin, J.F., Martin, D.M., 2008. Cre fate mapping reveals lineage specific deficits in neuronal migration with loss of Pitx2 function in the developing mouse hypothalamus and subthalamic nucleus. Mol. Cell. Neurosci. 37 (4), 697–707. https://doi.org/10.1016/j.mcn.2007.12.015.

Smolinsky, A.N., Berger, C.L., LaPorte, J.L., & Kalchev, A.V. (2009). Analysis of Grooming Behavior and Its Utility in Studying Motor Stress, Anxiety, and Depression in Mice. J Neurosci. 29 (4), 128–138. https://doi.org/10.1002/jnr.20670.

Song, C., Kim, I.H., Berridge, K.C., Graybiel, A.M., Fentress, J.C., 2016. Dopamine, Opioid, and Other Neuropathological Changes in Choreatic Diseases. J. Neurol. Sci. 28 (4), 401–406. https://doi.org/10.1016/j.jns.2005.12.005.

Speelman, J.D., Brunia, C.H.M., 2006. Stimulation of the Subthalamic Region in the Treatment of Parkinson Disease. Clin. Rev. 20 (1), 128–136. https://doi.org/10.1016/j.clinrev.2006.02.005.

Stebbings, N., Ricketts, C., Kiang, N., Bjornel, L.R., 2011. Spontaneous spike-and-wave discharges in the subthalamic nucleus in rats with dystonia. J. Neurosci. 31 (22), 8541–8550. https://doi.org/10.1523/JNEUROSCI.3532-10.2011.

Strassman, M., Pytlik, J., 1976. Morphometric studies of the nigrostriatal dopaminergic neurons in the rat. J. Neurosci. 4 (4), 138. https://doi.org/10.1002/jnr.4600404138.

Suo, Y. (2008). Toward understanding the functional anatomy of the cortico-thalamo-pallidal 'hyperdirect' pathway. Neurosci. Res. 52 (4), 112–117. https://doi.org/10.1016/j.neures.2008.03.007.

Skelton, M., Jackson, T., 2005. Evidence that the parafascicular nucleus projection to the subthalamic nucleus is sufficient to cause hyperlocomotion. J. Neurosci. 25 (43), 10026–10031. https://doi.org/10.1523/JNEUROSCI.2543-05.2005.

Speelman, J.D., Brunia, C.H.M., 2006. Stimulation of the Subthalamic Region in the Treatment of Parkinson Disease. Clin. Rev. 20 (1), 128–136. https://doi.org/10.1016/j.clinrev.2006.02.005.

Stebbings, N., Ricketts, C., Kiang, N., Bjornel, L.R., 2011. Spontaneous spike-and-wave discharges in the subthalamic nucleus in rats with dystonia. J. Neurosci. 31 (22), 8541–8550. https://doi.org/10.1523/JNEUROSCI.3532-10.2011.

Strassman, M., Pytlik, J., 1976. Morphometric studies of the nigrostriatal dopaminergic neurons in the rat. J. Neurosci. 4 (4), 138. https://doi.org/10.1002/jnr.4600404138.

Suo, Y. (2008). Toward understanding the functional anatomy of the cortico-thalamo-pallidal 'hyperdirect' pathway. Neurosci. Res. 52 (4), 112–117. https://doi.org/10.1016/j.neures.2008.03.007.

Skelton, M., Jackson, T., 2005. Evidence that the parafascicular nucleus projection to the subthalamic nucleus is sufficient to cause hyperlocomotion. J. Neurosci. 25 (43), 10026–10031. https://doi.org/10.1523/JNEUROSCI.2543-05.2005.