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OPTOGENETIC STIMULATION OF CORTICO-SUBTHALAMIC PROJECTIONS IS SUFFICIENT TO AMELIORATE BRADYKINESIA IN 6-OHDA LESIONED MICE

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Abstract

Electrical deep brain stimulation (DBS) of the subthalamic nucleus (STN) is effective for ameliorating the motor symptoms of Parkinson's disease (PD) including bradykinesia. The STN receives its main excitatory input from cortex; however, the contribution of cortico-subthalamic projection neurons to the effects of DBS remains unclear. To isolate the consequences of stimulating layer 5 primary motor cortex (M1) projections to the STN, we used a dual virus transfection technique to selectively express opsins in these neurons in mice made parkinsonian by unilateral nigrostriatal 6-OHDA lesioning. AAVs containing WGA-Cre constructs were injected in the STN to retrogradely place Cre in STN afferents, while AAVs containing Cre-dependent ultrafast hChR2(E123T/T159C)-EYFP opsin constructs were injected in M1 layer 5, producing specific opsin expression in M1-STN projections. Under unstimulated conditions, lesioned mice showed bradykinesia and hypokinesia (decreased movement), along with electrophysiological changes similar to those observed in PD patients. Specifically, low frequency power (theta, alpha, low beta) was increased and gamma power was decreased, while M1/STN coherence and STN phase-amplitude-coupling (PAC) were increased. Optogenetic stimulation (100–130 Hz) of STN afferents in these mice ameliorated bradykinesia and hypokinesia and brought the neural dynamics closer to the non-parkinsonian state by reducing theta and alpha and increasing gamma power in M1, decreasing STN PAC, and reducing theta band coherence. Histological examination of the EYFP expression revealed that, in addition to orthodromic and antidromic effects, stimulation of cortico-subthalamic neurons may cause wide-spread increased glutamatergic activity due to collaterals that project to areas of the thalamus and other brain regions.

Keywords

subthalamic nucleus; Parkinson's disease; Deep Brain Stimulation; DBS; optogenetics; phase-amplitude coupling; PAC; local field potential; motor cortex; movement analysis

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Introduction

Treatment with the dopamine precursor L-DOPA can ameliorate bradykinesia (abnormal slowing of movement) in most Parkinson's disease (PD) patients for 10 years or more. However, many patients eventually require supplemental treatments due to the progressive nature of the disease and the dyskinesias (involuntary movements) that often occur after prolonged L-DOPA use. Deep brain stimulation (DBS) of the STN has been shown to be an effective treatment for bradykinesia in humans (Benabid et al., 2009; DeLong et al., 2007, Wichmann et al., 2011), and rats (Li et al., 2012), and can prolong the effective window of L-DOPA therapy. However, optimizing DBS treatment to subject-specific neural dysfunction and symptom profiles is often difficult due to a lack of understanding about the cellular and circuit activations (and/or deactivations) that lead to the DBS therapeutic effects. Previous studies have shown evidence that activation of layer 5 cortico-subthalamic projections and antidromic effects may play an important role in the DBS therapeutic mechanism (Gradinaru et al., 2009, Li et al., 2007). Additionally, recent discoveries about the role of STN in motor behavior have emphasized the importance of the STN in movement stopping and initiation. For example, STN activation in inhibiting ongoing movements has been demonstrated (Chu et al., 2015; Mallet et al., 2016), and increased STN neuron spiking has been observed immediately subsequent to stop cues and contralateral movement initiation cues (Schmidt et al., 2013).

Previously, Gradinaru et al., 2009 found that high frequency optogenetic stimulation of cortico-subthalamic projections ameliorated ipsilateral rotations in mice unilaterally lesioned with 6-OHDA. However, these initial studies were performed in Thy1::ChR2 line 18 transgenic mice that expressed first generation ChR2 opsins in many locations including layer 5 cortical neurons across the entire cortex, CA1 and CA3 pyramidal neurons of the hippocampus, and neurons in the thalamus (Arenkiel et al., 2007). The slower response kinetics of the ChR2 opsin and the broad expression of ChR2 under control of the Thy1 promoter led to concerns regarding the specificity and mechanism of action involved in the 2009 results. Motivated in part by these concerns, the study reported here examined whether specific optogenetic stimulation of second-generation ultrafast opsins [hChR2(E123T/T159C)] placed selectively in layer 5 motor cortical (M1) projections to the STN using a dual virus retrograde transfection approach in ordinary C57BL/6J mice would ameliorate behavioral signs of parkinsonism.

In addition to observing the effects of specific motor-cortico-subthalamic (M1-STN) stimulation on behavior in normal and parkinsonian mice, this study examined the histology of M1-STN projections and their collaterals, and the electrophysiological signs of parkinsonism. The electrophysiological questions explored include whether increased M1 and STN beta band power (Delaville et al., 2014; Engel et al., 2010; Leventhal et al., 2012), increased coherence (Goldberg et al., 2002; Hammond et al., 2007), and changes in phase-amplitude-coupling (Sanders et al., 2013b) observed in other species also occur in mice, and how optogenetic stimulation impacts the M1-STN electrophysiology.

Materials and Methods

Experimental procedures

All experimental procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, the PHS Policy on the Humane Care and Use of Laboratory Animals, and the American Physiological Society's Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training (updated 2014) and were approved by the Emory Animal Care and Use Committee.

Animals

Male C57BL/6J mice (Jackson Labs) were housed with free access to chow and water in environmentally controlled conditions with a reversed 12 hour light/dark cycle (lights on at 7 pm). Mice were gently handled daily for one week prior to surgery and habituated to an open-field arena used for freely moving recording in the study by placing them in the arena for 10–30 minutes each day. Video was recorded for the purpose of baseline movement assessment. All mice were aged 4 – 9 months at the time of testing.

Opsin placement

Opsin expression in layer 5 M1-STN projections in C57BL/6J mice ($N = 10$) was induced by a dual-virus retrograde transfection technique (Figure 1A) previously used to transfect projections in other brain regions (Gradinaru et al., 2010; Dautan et al., 2014). Two AAVs containing viral constructs (MTA from Karl Deisseroth at Stanford University; purchased from UNC Vector Core) were stereotactically injected as described in detail in the surgical procedures. 0.2 μ l of the solution of AAVs containing WGA-Cre genetic constructs [AAV-Ef1a-mCherry-IRES-WGA-Cre, serotype 2, titer 10^{12} vg/ml] were injected in the STN. WGA retrograde transport activity then placed Cre in STN afferents. 0.5 μ l of the solution of AAVs containing Cre-dependent ultrafast channelrhodopsin constructs [AAV-EF1a-DIO-hChR2(E123T/T159C)-EYFP, serotype 5, titer 10^{12} vg/ml] were injected in layer 5 M1. This led to opsin expression in layer 5 M1-STN projections where Cre-dependent opsin constructs intersected with retrogradely placed Cre. As described above, the ultrafast opsin used in this study was the ET/TC mutation of ChR2. The newer ET/TC mutation was selected because of its shorter τ_{off} (8 ms) (Yizhar et al., 2011) and its large stable photocurrents (~ 1400 pA) (Berndt et al., 2011) as compared to the wild-type ChR2 used in the Gradinaru et al., 2009 study ($\tau_{\text{off}} = 10$ ms; stationary photocurrent = ~ 950 pA). The decreased τ_{off} was important for enabling the opsins to respond at a faster stimulation frequency while the large stable photocurrents were important for maximizing the activation of cortico-subthalamic projections transfected using the dual virus approach (presumably fewer neurons than would be observed in the Thy1 transgenic mouse).

Surgical procedures

Mice were anesthetized with isoflurane (3% for initial sedation, then 1.5–2.0% delivered via nose cone throughout surgery), administered subcutaneous (s.c.) buprenorphine (0.1 mg/kg), and placed in a stereotaxic frame. Ophthalmic ointment was applied to prevent corneal dehydration and a heating pad was used to maintain temperature at 37° C. Anesthesia levels

were adjusted as needed to ensure ongoing deep anesthesia (assessed by visual monitoring and toe pinches). Two surgeries were performed, separated by a minimum one month interval. In the first surgery, an incision was made that allowed 0.9 mm diameter craniotomies above the medial forebrain bundle, M1, and STN. For lesioned mice (N = 9), 1 μ l of 6-OHDA solution was injected in the medial forebrain bundle (−1.2 AP, −1.2 ML, −4.75 DV) using the procedure described in Cenci and Lundblad, 2007 [sterile saline solution (NaCl, 0.9% w/v) with 0.02% ascorbic acid combined with 6-OHDA powder (Sigma) to produce a concentration of 3.6 mg/ml]. This method produced strong nigrostriatal lesioning as verified histologically through TH staining (see *Analysis of Histology* section). AAV5s containing Cre-dependent opsin constructs were injected in left M1 (2.0 AP, −1.56 ML, −1.0 DV; Figure 1B) and AAV2s containing WGA-Cre constructs were injected in the left STN (−1.76 AP, −1.56 ML, −4.2 DV; Figure 1C) (coordinates calculated based on Paxinos and Franklin, 2004).

In the second surgery, two (or four) 50 micron tungsten or Pt/Ir microwires were implanted; one (one pair) was placed in M1 (2.0 AP, −1.56 ML, −1.0 DV) and one (one pair) in STN (−1.76 AP, −1.56 ML, −4.2 DV). An additional wire was attached to a skull screw above the cerebellum to serve as an instrument ground. All wires were then attached to a Neuralynx electronic interface board (EIB-16) with gold pins and affixed to the skull using dental cement. Additionally, each mouse was stereotactically implanted with a 0.22 NA optical fiber with tip located approximately 150 microns above the transfection site either in layer 5 M1 or STN. Fibers were 200 microns in diameter and were terminated with small (1.25 mm) stainless steel fiber ferrules (Thorlabs; CFML21U-20). Dental cement was used to secure the fiber ferrule to the skull.

Immediately after surgery, bacitracin ointment was applied to the region around the incision. Mice were weighed twice daily and assessed for overall health and comfort for 1 week post-surgery. S.c. buprenorphine injections (0.1 mg/kg) were administered (up to twice daily) if the animal was visibly uncomfortable.

Confirmation of parkinsonism following 6-OHDA lesioning

Parkinsonism was confirmed behaviorally (Figure 3A–B) and histologically (see *Analysis of Histology* section). Mice were placed in an open-field arena consisting of a 50.8 × 30.5 × 24.1 cm opaque bin for 5 minutes for each behavioral trial. Video was captured at 30 frames per second with a camera placed directly above the arena. Automated video analysis of each experiment was used to determine the mouse's centroid and orientation for each frame (adapted from a MATLAB script created by Gomez-Marin et al., 2012). Parkinsonism was confirmed in each mouse by calculating the distance traveled and net number of rotations (in the direction ipsilateral to the lesioned side) during bi-weekly video recording sessions beginning 5 days after the lesioning surgery. Prior to 6-OHDA lesioning, mice traveled from 5,118 – 13,311 mm per trial. After 6-OHDA injections, the distance traveled decreased. The distance traveled per trial ranged from 1,645 – 4,589 mm by 2 months post-lesion and from 1,545 – 3,984 mm by 4 months post-lesion. Prior to 6-OHDA lesioning, all mice rotated from 0 to a maximum of 6 net turns in either the clockwise or counter clockwise direction per 5 minute open field trials. After 6-OHDA injections, the net ipsilateral (counter-

clockwise) rotations increased over time reaching a maximum of 35 – 54 rotations at 10 – 30 days post-lesion. These behavioral measures were found to represent a greater than 80% nigrostriatal dopamine lesion as confirmed by histological analysis. Similar behavioral and histological results were reported in Cenci and Lundblad, 2007.

Behavioral Analysis

During the stimulation and recording test sequences, the mice were again placed in the open-field arena described in the previous section. For each mouse, a total of 5 ± 1 consecutive behavioral trials were performed per testing day (Figure 5A shows the timeline for behavioral trials). Experiments for which locomotion occurred were analyzed for movement speed and fraction of total seconds with movement calculated as follows (post hoc data analysis performed with MATLAB; The MathWorks, Inc.; Natick, Massachusetts). In cases where the mouse did not move for the initial unstimulated condition, a second unstimulated trial was recorded before proceeding with the stimulated conditions.

Two 6-OHDA mice could not be automatically tracked due to degradation of the video data caused by the Faraday cage used in early experiments. For the remaining mice, the speed for each 1 second video segment was calculated from mouse position data output by the tracking script. Next, a speed threshold (4 mm/s) was applied to remove non-moving and non-locomotion segments such as head bobs and grooming. The remaining segments were then counted as locomotion segments. Bradykinesia was assessed by analyzing the distributions of movement speed in control, 6-OHDA, and stimulated conditions (Table 1; Figures 3A and 5E).

For the purposes of this study, the degree of hypokinesia was estimated by determining the fraction of seconds with movement (smaller values correspond to greater hypokinesia). The “fraction of seconds with movement” measure was calculated by dividing the total number of locomotion segments by the total number of seconds in the trial. This measure was assessed for all 9 mice. For the two 6-OHDA mice that could not be automatically tracked due to the reduced visibility caused by the Faraday cage, the measure was obtained by manually counting video frames with mouse movement.

Power Analysis and Statistical Methods

The primary goal of this study was to determine whether high frequency optogenetic stimulation (HFOS) of specifically transfected cortico-subthalamic projections would ameliorate signs of parkinsonism in unilaterally lesioned 6-OHDA mice. In order to accomplish this goal, we chose to examine the effect of HFOS on bradykinesia and hypokinesia since these measures of parkinsonism reached steady state within the desired time (2 – 4 months post lesion) in C57BL/6J mice after 6-OHDA lesioning (see *Confirmation of parkinsonism following 6-OHDA lesioning*). The effect size chosen was 0.58 since this represented a 20% change in the mean time in motion per trial based on the mean (49 s) and standard deviation (17 s) for the 6-OHDA mice observed in the preliminary analysis. In order to achieve this effect at a power of 0.8 and significance level of 0.05 with a paired t-test, a minimum of $n = 26$ paired data samples were required (“n” determined using R power analysis package, “pwr”). To reduce the likelihood that results were not due to

individual variations in mice, data were collected over a minimum of $N = 3$ mice. Statistical analyses for paired measures were performed only if a minimum of $n = 26$ paired samples across a minimum of $N = 3$ mice were collected. For unpaired measures, the effect size and power remained the same. However, more data is required to reach a given power in unpaired tests. A minimum of $n = 48$ samples from each data group were necessary to achieve a power of 0.8. Statistical analyses for unpaired measures were performed only if a minimum of 48 samples from each group across a minimum of 3 mice were collected. Note that for both paired and unpaired tests, the minimum “ n ” was greatly exceeded in most analyses.

In order to collect sufficient data to power the analysis necessary to disprove the null hypothesis, 78 5-minute open field trials (33 unstimulated; 45 stimulated) were collected over $N = 9$ mice (six 6-OHDA lesioned and three controls) at multiple time points (2–4 months post lesion for 6-OHDA mice; age matched controls). For the 5-minute stimulated trials, 2 (out of 45) trials (one 30 Hz; one 100 Hz) could not be analyzed due to complete lack of mouse locomotion (electrophysiological analysis suggests the mice were asleep during these trials).

All stimulated trials were started at the initiation of stimulation and ended after 4 minutes; all unstimulated trials were started after a delay corresponding to the minimum stimulation initiation time for that recording session and ended after 4 minutes. Thus, each analyzed trial contains 240 seconds of data. Bradykinesia was assessed primarily with the Kolmogorov-Smirnov test since calculation of cumulative distribution statistics was possible due to the large quantity of accurate velocities (1 second samples) obtained from the automated movement analysis scripts (Table 1 and Figure 5E). Measures taken from the same subjects before and after manipulation were assessed using the paired t-test [e.g., the fraction of time in motion (used to quantify hypokinesia) before and after stimulation; Figure 5F]. For non-paired measures that were pooled across mice, two sample t-tests (for Gaussian-like distributions) or Wilcoxon ranksum tests (for non-normally distributed data) were used. The chi-squared goodness of fit test was used to evaluate whether or not data were normally distributed.

Recordings

Electrophysiological recordings were made with chronic electrodes implanted as described in the surgical procedures. Recordings were captured at 20 kHz using an Intan system (Intan Technologies; Los Angeles, California) with 16 or 32 channel (with accelerometer) digitizing head stage amplifiers. Since changes in electrophysiology may occur transiently soon after lesioning (Brazhnik et al., 2012), we waited a minimum of 2 months post-lesion before electrophysiological recording to reduce the likelihood of studying transient effects.

In order to facilitate free movement, electronic cables and fibers connected to the mouse were passed through a commutator (Doric Assisted Optic and Electric rotary joint; AHRJ-OE_12_HRW-HRW).

Stimulation

Light stimulation was provided by a 473 nm laser with laser power set to 12 ± 4 mW as measured at the tip of the fiber (0.22 NA; Doric) to be connected to the ferrule on the mouse's head. Laser power was calibrated at the beginning of each recording week using a Thorlabs Optical Power Meter (PM100D). Stimulation was controlled by TTL output from a Cambridge Electronic Design (CED) Power 1401 and controlled by custom Spike 2 scripts (CED, Ltd). Three stimulation frequencies were tested (30 Hz, 100 Hz, 130 Hz). For each 5 minute stimulation trial, stimulation was administered at a single frequency with a 4 ms pulse width throughout the trial. The 4 ms pulse width was chosen since it created an approximately 50% duty cycle for the 130 Hz stimulation.

Results from previous studies suggest that high frequency electrical or optical stimulation reduces signs of parkinsonism while low frequency stimulation (< 50 Hz) does not (Eusebio et al., 2008; Garcia et al., 2005; Gradinaru et al., 2009; Moro et al., 2002). Based on these results from the literature and the statistical similarity between 100 Hz and 130 Hz results in the current study, the high frequency optical stimulation (HFOS) trials were pooled for analysis (see results).

Analysis of Histology

After the last recording session, mice were perfused transcardially with a 4% paraformaldehyde solution in phosphate-buffered saline, and the brains were removed for histological processing. Coronal, sagittal, or horizontal sections of fixed brains (50 μ m thick) were cut on a microtome and mounted onto slides.

Every sixth section was stained for tyrosine hydroxylase (TH) and the lesioned side was compared to the non-lesioned side with light microscopy to confirm successful lesioning (Figure 2A). Lesioning was considered successful if the number of TH-stained pixels above a noise threshold in the lesioned dorsal striatum was reduced by greater than 80% compared to the non-lesioned side (noise threshold applied and pixels counted using ImageJ; Rasband et al., 1997–2015; <http://imagej.nih.gov/ij/>). The mean number of TH-stained pixels for the 6 mice used in this study was 358349 ± 17917 pixels on the non-lesioned side as compared to 14072 ± 9833 on the lesioned side. The number of TH-stained pixels on the lesioned side ranged from 1% – 7% of the number of TH-stained pixels on the non-lesioned side (reduced by 93% – 99%). Successful electrode and fiber placements were verified by viewing all brain sections (both TH-stained and non-stained) with light microscopy to identify electrode and fiber tract locations (Figure 2B).

EYFP and mCherry expression was assessed visually using confocal microscopy. Successful dual-virus transfection was judged by confirming mCherry expression at the STN (Figure 1C), and EYFP expression in both layer 5 motor cortex and STN (Figures 1B, 4A, and 4D). Cortico-subthalamic neuron morphology was assessed by visual inspection of EYFP observed in confocal microscopy from all sections. In particular, EYFP expression was traced from cortical cell bodies to STN terminals (e.g., Figure 4D) and to collaterally connected terminals in other structures (Figure 2C).

Basal ganglia nuclei, substantia nigra, brain stem nuclei, all layers of cortex, STN-adjacent nuclei, and thalamus were identified and checked for presence or absence of fluorescence in all mice. After fluorescence was observed in the superior colliculus in one mouse, slides for all mice were re-examined to confirm the presence or absence of fluorescence in this structure (Figure 4G).

Analysis of Electrophysiological Data

In order to reduce the potential for movement artifacts and ensure that all electrophysiology was assessed in a common state, only non-moving 10 second epochs of data with acceptable noise levels captured during wakeful periods were analyzed (e.g. Figure 3C). Wakefulness was assessed by grooming, head movements, and/or locomotion occurring within the same minute as the non-moving epoch and by visually assessing the electrophysiology for increased slow wave activity (0.5 – 4 Hz) and sleep spindles (10 – 16 Hz). Noise variance was assessed for all epochs using MATLAB scripts. Data segments with noise standard deviation > 75 microvolts were rejected. Movement was assessed by automated detection of accelerometer magnitudes for mice recordings acquired using the Intan headstage with on-board accelerometer, followed by visual verification. Any 100 ms segments with accelerometer standard deviation > 0.033 m/s² were discarded. Non-moving segments for mice recordings acquired with the Intan headstage without accelerometer were selected by visual video analysis. Data meeting the above requirements for each mouse included in the analysis exceeded 100 seconds. Mice that did not meet this minimum required data size were not included in the electrophysiological analysis. Notch filters were applied to recorded data at 60 Hz, optical stimulation frequencies (100 Hz ± 3 Hz or 130 Hz ± 3 Hz), and harmonics (200 Hz ± 3 Hz or 260 Hz ± 3 Hz).

Calculation of phase-amplitude-coupling and power measures

All 20 kHz recorded signals were band-pass filtered between 3 Hz and 500 Hz, then downsampled to 1000 Hz. Power spectral densities (psd) were calculated with the standard MATLAB Welch spectrogram. Coherence was calculated using the MATLAB mscohere function (Figure 3D). Psd and mscohere calculations were performed for each 10 second epoch that met the quality criteria, then log(power) and coherence statistics were calculated from this population of 10 second epoch measures. Band power measures were normalized to the total power within the analysis range (4–58 Hz). Band power and coherence measures were averaged over all frequencies in the band before statistics were calculated.

Phase-amplitude-coupling (PAC) was calculated using EEGfilt subroutines (Delorme and Makeig, 2004) with a Morlet wavelet as described in Sanders et al., 2013b and the Modulation Index (MI) measure proposed by Tort et al., 2010. Briefly, the LFPs were filtered into 2 Hz sub-bands from which the amplitude and phase was extracted. The coupling between sub-bands was then quantified by the average of the amplitudes in each sub-band from 50–300 Hz that co-occurred with the phase in each subband from 3–50 Hz. Finally, the MI measure was used to assign a single value to the degree of coupling by comparing the phase-amplitude quantification curve to a uniform distribution (the expected distribution is uniform if no coupling is present). PAC is typically displayed on a heat map with the x-axis corresponding to the phase frequency bands (3–50 Hz), and the y-axis

corresponding to amplitude frequency bands (50–300 Hz), and intensity reflecting the MI measure (degree of coupling). For this study, the peak PAC magnitude was identified from the heat map in an area of interest comprising phase frequencies from 9 to 36 Hz and amplitude frequencies from 130 to 295 Hz (Figure 6D). For each 10 second epoch, PAC magnitudes were averaged for 4×10 Hz “pixels” within the area of interest, and the peak PAC magnitude was obtained from the averaged pixels. The peak PAC magnitudes for all epochs were then histogrammed and compared for the control, 6-OHDA lesioned, and stimulated recordings (Figure 6E–F).

Results

Thirteen mice were examined. Four mice were included in the baseline electrophysiological measures or histological analysis but were not optogenetically stimulated. Six mice unilaterally lesioned with 6-OHDA (males; aged 4 – 9 months at the time of testing) and three age and gender matched unlesioned control mice (with matched electrode and fiber implants) were behaviorally tested in an open-field arena using the protocol shown in Figure 5A. Each mouse served as its own unstimulated behavioral baseline.

All lesioned mice were transfected with opsins in descending layer 5 projections. Since there were no significant time-related changes in the measures recorded between 2 and 4 months post-lesion (verified by evaluation of correlations between the time since lesioning and behavioral / electrophysiological measures), measures were pooled over this time window.

6-OHDA lesioned mice were bradykinetic and hypokinetic

All 6-OHDA lesioned mice whose video quality permitted quantitative analysis with automated tracking scripts displayed bradykinesia. These lesioned mice ($N = 4$; $n = 3840$ s) moved significantly slower than controls ($N = 3$; $n = 1680$ s) (Table 1, $p = 1.0\text{e-}20$, significance assessed for cumulative distributions of speeds on 1 s intervals with Kolmogorov Smirnov (K-S) test). The mean speed was 5.7 ± 0.3 mm/s for lesioned mice as compared to 16.0 ± 0.6 mm/s for control mice (Figure 3A; means indicated by black horizontal lines; $p = 2.24\text{e-}21$; two sample t-test).

All lesioned mice displayed hypokinesia (Figure 3B). Lesioned mice ($N = 6$; $n = 4560$ seconds) moved significantly less than controls ($N = 3$ mice; $n = 1680$ seconds) ($p = 1.0\text{e-}30$; two-sample t-test). The mean number of seconds in motion was 48.6 ± 4.3 s for lesioned mice as compared to 102.0 ± 10.5 s for controls.

Lesioned mice had frequency band power changes and increased M1/STN coherence

Sample voltage traces for M1 and STN local field potentials in control and lesioned mice during open field trials are shown in Figure 3C. Coherence and power were assessed in 5 frequency bands: theta (4 – 7 Hz), alpha (8 – 12 Hz), low beta (13 – 19 Hz), high beta (20 – 30 Hz), and gamma (30 – 60 Hz). Coherence was higher in the lesioned mice than in controls in all 5 frequency bands assessed ($N = 4$ mice; $n = 580$ seconds; Wilcoxon ranksum test). In one narrow band (46–49 Hz), the control coherence was higher than that of the lesioned mice (Figure 3D).

We did not find significantly increased high beta power in the STN or ipsilateral cortex for recordings from non-moving lesioned mice as compared to controls (STN: N = 6 mice; n = 1140 seconds; M1: N = 5 mice; n = 680 seconds; lack of significance assessed with the two-sample t-test). However, we did find increased low frequency power (theta, alpha, low beta) and decreased gamma as reported in other animal model studies of parkinsonism (Figure 6A and B; Sharott et al., 2005).

Phase-amplitude-coupling was increased in lesioned mice

The average peak magnitude of PAC, calculated as described in the methods (Figure 6D), was increased in lesioned mice compared to controls. The increase was significant for the STN recordings (Figure 6E; $p = 0.03$; Wilcoxon ranksum test), but not for the M1 recordings. The number of mice with M1 recordings that met the quality criteria described in the methods (N = 5; n = 680 seconds) was less than the number with acceptable quality STN recordings (N = 6; n = 1140 seconds). This resulted in fewer M1 data samples and possibly contributed to the lack of M1 statistical significance.

All mice showed evidence of opsins in cortico-subthalamic projections

By visual tracing of opsin conjugated-EYFP staining, we located evidence of opsins in cortex and STN in all mice used in the study (7 mice used in the behavioral/electrophysiological experiments and 2 mice examined prior to experimentation to verify opsin placement; Figure 4A, B, D, and E). Presence of mCherry was localized to STN with some spreading to zona incerta. Despite the presence of mCherry in zona incerta, evidence of opsin transfection (EYFP) in that structure was limited to fibers of passage. All observed terminals were located in the STN or collaterals (as indicated in Figures 2C and 4E).

Cortico-subthalamic connectivity was verified by visually identifying EYFP passage through all structures from cortex to STN (Figure 4A, B, and D). The traced outline of the EYFP staining for all 7 mice with opsins used in the behavioral/electrophysiological experiments shows similar patterns of expression in all mice (Figure 2C). Note that fibers pass through the internal capsule and cerebral peduncle en route to collateral structures.

Possible plaques were observed in cortical dendrites in some mice (Figure 1B). However, despite the potential for plaques to alter the function of affected individual neurons, no significant changes were observed in the behavior or population electrophysiology in these mice. Histological examination did not show signs of cell death or dysplasia due to opsin expression or presence of plaques, even in mice expressing opsins for 6 months or longer.

EYFP opsin expression in collaterals was visible in mice transfected 3 months or longer

Although some differences in the patterns of transfection were observed, visual tracing revealed cortico-subthalamic axon passage through the striatum in all mice (Figure 4C). Collaterals were observed in ventro-lateral thalamus (Figure 4F; N = 5 mice) and superior colliculus/MRF (Figure 4G; N = 3 mice). Expression patterns shown in Figure 4 were typical except in the case of the thalamus and superior colliculus images (F, G). These images were collected from the mouse with the longest expression time (7 months) and show greater than typical expression.

In lesioned mice, hypokinesia and bradykinesia were significantly ameliorated by high frequency optical stimulation of M1 layer 5 projections

High frequency optical stimulation (HFOS; 100 or 130 Hz; 4 ms pulses as described in the methods) significantly increased the fraction of seconds with movement (indicating reduced hypokinesia) in lesioned mice with opsins in descending cortical projections [Figure 5 B, C, and F; fractional increase of 0.7, $p = 0.0023$; paired t-test; $N = 6$ mice (T, E, S, H, D, and R); $n = 4560$ s]. The mean number of seconds in motion per trial (240 s) increased from 48.6 ± 4.3 s in unstimulated to 83.0 ± 8.7 s in stimulated lesioned mice.

Movement speed was also significantly increased (indicating reduced bradykinesia) in the 4 mice analyzed with automatic movement scripts [Table 1; $p = 1.0\text{e-}16$; K-S test; $N = 4$ mice (mice E, R, S, and T); $n = 3840$ s]. The (mean, maximum) speeds averaged across mice were (5.7, 79.2) mm/s for unstimulated as compared to (6.6, 107.9) mm/s for stimulated lesioned mice. Figure 5E shows representative movement speed distributions (Mouse E).

In five lesioned mice and all controls the light stimulation was applied at the STN to activate M1 terminals. One lesioned mouse was stimulated in M1 to activate M1 cells directly (Mouse R; Figure 5D left).

In control mice, the fraction of seconds with movement was significantly decreased during HFOS intervals (Figure 5D right and 5F; fractional decrease of 0.3; $p = 0.0245$; paired t-test; $N = 3$; $n = 1680$ s). The mean number of seconds in motion per trial decreased from 102.0 ± 10.5 s in unstimulated to 70.3 ± 12.8 s in stimulated control mice. Movement speed was significantly decreased in these mice as well (Table 1; $p = 5.44\text{e-}8$; K-S Test). The (mean, maximum) speeds averaged across mice were (16.0, 125.2) mm/s for unstimulated as compared to (9.3, 103.9) mm/s for stimulated control mice.

Note that although HFOS increased locomotion in the lesioned mice, their average speed and fraction of time spent moving was less than that of unstimulated controls (Table 1; Figure 5D).

We verified that the motor effects were not transient by comparing the movement during each minute of stimulation. Neither control nor lesioned mice showed a significant difference in movement between the first minute and the remaining minutes (paired t-test; controls: $N = 3$; $n = 1680$ s; lesioned: $N = 4$; $n = 3840$ s).

Low frequency optogenetic stimulation did not significantly change movement

Low frequency optogenetic stimulation (LFOS; 30 Hz; 4 ms pulsewidth) did not significantly change the fraction of seconds with movement in the 6-OHDA lesioned mice as assessed by the (unstimulated, stimulated) paired t-test ($p = 0.0648$; $N = 6$; $n = 3120$ s). However, in 75% of the LFOS trials in 6-OHDA lesioned mice, the fraction of seconds with movement increased over the unstimulated baseline. In comparison, the fraction of seconds with movement increased in 83% of 100 Hz HFOS trials in lesioned mice and 85% of 130 Hz HFOS trials in lesioned mice.

M1 Low frequency power was reduced and high frequency power was increased during HFOS

On the side ipsilateral to the lesion, M1 theta and alpha power were significantly reduced while high beta and gamma power were significantly increased by HFOS (Figure 6A; $p = \{0.0076, 0.0430, 0.6689, 0.009, 0.0415\}$ for {theta, alpha, low beta, high beta, gamma}, two sample t-test; $N = 5$; $n = 580$ s). However, STN power changes failed to reach significance in these power bands (Figure 6B; two sample t-test, $N = 6$; $n = 810$ s).

Theta band M1/STN coherence was reduced in lesioned mice during HFOS

There was a significant decrease in theta band M1/STN coherence during HFOS in 6-OHDA lesioned mice (Figure 6C; $p = 0.01$; Wilcoxon ranksum test; $N = 3$; $n = 490$ s). The other 4 frequency bands examined showed no difference in M1/STN coherence for lesioned mice between non-stimulated and stimulated conditions.

STN phase-amplitude-coupling was decreased in lesioned mice during HFOS

The average peak magnitude of PAC, calculated as described in the methods (Figure 6D), was decreased in lesioned mice optogenetically stimulated at high frequencies (100 or 130 Hz). The decrease was significant for the STN recordings (Figure 6F; $p = 0.04$; Wilcoxon ranksum test; $N = 6$; $n = 810$ s), but not for the M1 recordings ($p = 0.07$; Wilcoxon ranksum test; $N = 5$; $n = 580$ s). Note that the decrease in PAC during HFOS in the lesioned mice returned the PAC levels toward levels seen in unlesioned mice (Figure 6E).

Discussion

The importance of the STN in Parkinson's disease (PD) pathology and DBS treatment has been characterized extensively in animal models and human patients (Benabid et al., 2009; Bergman et al., 1990; Bevan et al., 2002; DeLong et al. 1985; Wichmann et al., 1996). However, selective exploration of pathways that connect to the STN was not possible until the recent development of tools such as optogenetics that allow in vivo activation of specific neuron populations (Deisseroth, 2014; Gradinaru et al., 2007; Kravitz et al., 2010; Yizhar et al., 2011).

Previous work demonstrated that electrophysiological signs of parkinsonism can be observed in the motor cortex (Goldberg et al., 2002; Li et al., 2012; Mallet et al., 2008; Sanders et al., 2013b) and that projections from the cortex to the STN in the hyperdirect pathway are highly involved in the DBS mechanism of action through antidromic (Li et al., 2007) and circuit activity (Gradinaru et al., 2009). Here, we transfected M1-STN projections with a dual virus transfection technique in order to determine whether specific activation of these projections alone is sufficient to ameliorate signs of parkinsonism in mice unilaterally lesioned with 6-OHDA. Our findings indeed identified pro-kinetic effects of stimulating this pathway and also showed that electrophysiological changes previously reported in PD patients were similarly changed in lesioned mice. We further showed that these electrophysiological measures returned to levels closer to those seen in non-lesioned controls during HFOS.

Three possible explanations for the pro-kinetic effect of cortico-subthalamic stimulation are as follows. First, the cortico-subthalamic projections could be prodromically stimulating STN neurons and thereby modulating downstream populations (Mallet et al., 2012). Homogeneous prodromic activation is not supported by the Gradinaru, et al., 2009 result that direct optogenetic stimulation of the STN did not produce amelioration of parkinsonism. However, the lack of preferential synapsing, or experimental differences such as the slower kinetics of the channel rhodopsin or the amount of opsin transfection present could have prevented direct STN stimulation from producing movement effects in that earlier study. It is also possible that in comparison to direct STN neuron activation, prodromic optogenetic cortical input stimulation more selectively targets subpopulations of STN neurons that respond strongly to cortical input such as the recently identified $\alpha 4\beta 2$ nicotinic receptor containing neurons (Xiao et al., 2015).

A second explanation regarding the increased locomotion in optically stimulated 6-OHDA mice in this study is that antidromic effects activated microcircuits within the cortex (as shown during electrical stimulation in rats; Li et al., 2007). This result is consistent with studies indicating that the therapeutic effects of STN DBS may be due to activation of non-local structures (Miocinovic et al., 2006) and/or antidromic activation of cortical neurons (Gradinaru et al., 2009; Hammond et al., 2008). A reduction in pathological synchronization in cortical microcircuits via the antidromic effect could also explain the reduced theta M1/STN coherence found in the HFOS lesioned mice.

Finally, the increased movement in lesioned mice in our study could have resulted from increased glutamatergic activity in descending collaterals of STN projecting M1 neurons via activation of the axons in STN. Previous work has found widespread descending collaterals of STN projecting motor cortical neurons (Kita and Kita, 2012). In agreement with this, we found opsin expression in collaterals in thalamus and other brain locations. In lesioned mice, stimulating these glutamatergic collateral projections may overcome a parkinsonian no-go bias (Mallet et al., 2016) and result in increased activation of multiple structures in the motor-impaired side thereby allowing increased locomotion.

It is important to note that these mechanisms are not mutually exclusive, and in fact may act in concert. Our results do not rule out any of the three mechanisms proposed above, but rather show that several other pathways and mechanisms such as GPe fiber stimulation or zona incerta neural stimulation are not necessary for the therapeutic effects demonstrated in this study. Nevertheless the overall effect of electrical DBS in human patients is likely the network effect of many of these mechanisms combined (McIntyre and Hahn, 2010).

The pro-kinetic effect of stimulation in lesioned mice was particularly notable in comparison to the reduced movement observed in the control mice with the same stimulation. However, the reduced movement in control mice following the initial unstimulated period may be explained by decreasing interest in exploration over time, since opsins were not present in all controls.

Observed power changes in parkinsonian animals can be difficult to interpret since the power spectral density is dependent on the state of the animal (Avila et al., 2010; Levy et al.,

2002). Sleep states are particularly problematic since PD and parkinsonism can lead to increased sleepiness during awake periods (Abe et al., 2005) and the frequency bands affected by parkinsonism overlap with those changed during sleep. For this reason, we only analyzed epochs that were free from visible signs of sleep such as increased slow wave activity or sleep spindles as described in the methods. Additionally, accelerometry and video analysis were used to restrict the analysis to non-moving periods dispersed throughout freely moving trials.

The 6-OHDA mice in our study showed frequency band power changes consistent with previous findings in PD patients and parkinsonian rats, specifically increased low frequency power (< 20 Hz) and decreased gamma (30 – 60 Hz) (Brown, 2003; Engel et al., 2010; Mallet et al., 2008; Sharott et al., 2005). During HFOS, low frequency power (theta and alpha) was returned to more normal levels and high beta power (20 – 30 Hz) and gamma were increased.

Associations between increased cortical and basal ganglia coherence and anti-kinetic activity have been observed in parkinsonian humans (Hammond et al., 2007), non-human primates (Goldberg et al., 2004), and rats (Sharott et al., 2005). Similarly, elevated PAC has been observed previously in parkinsonian humans (Lopez-Azcarate et al., 2010) and non-human primates (Sanders et al., 2013b). A recent modeling study (Sanders, 2016) demonstrated that elevated STN-PAC may be directly related to synchronization and increased neuronal bursting in parkinsonism (Bergman et al., 1994; Galvan et al., 2008; Sanders et al., 2013a, Walters et al., 2005). The observed increased PAC and coherence in parkinsonian mice compared to controls in the current study is consistent with these previous studies. Accordingly, the reduced PAC and coherence that occurred during HFOS in this study suggests that such stimulation may ameliorate motor deficits via the mechanism of reducing pathological synchronization in the M1/STN pathway.

The significant decrease in STN (but not M1) PAC and the significance of M1 (but not STN) power changes during HFOS suggest that the primary population response to stimulation may be different in the two structures. The reduced STN PAC suggests that the primary effect of HFOS in the STN may be decreased bursting and synchronization while the decreased low / increased high frequency power in M1 suggest the primary effect may be a shift toward enhanced circuit performance (Sohal et al., 2009) and active motor planning in that structure (Engel et al., 2010).

High frequency electrical stimulation of the STN has been shown to reduce signs of parkinsonism while low frequency stimulation (< 50 Hz) typically does not ameliorate parkinsonism, and in some cases worsens it (Eusebio et al., 2008; Garcia et al., 2005; Moro et al., 2002). Our own findings indicated that HFOS of M1-STN projections alone significantly reduced parkinsonism in 6-OHDA mice while LFOS had nonsignificant effects also in the direction of motor improvement. This finding differs from the Gradinaru et al. 2009 finding that LFOS at M1-STN terminals worsened parkinsonism (increased ipsilateral rotations) in Thy1::ChR2 6-OHDA mice. Reasons for this discrepancy include the possibility that different circuit effects occurred in our more specifically placed single hemisphere opsins versus the widely distributed bilateral opsins in the Thy1 transgenic mice,

the different stimulation protocols used (30 Hz, 4 ms pulsewidth vs. 20 Hz, 5 ms pulsewidth in the previous study), the different behavioral readouts (bradykinesia and hypokinesia vs. ipsilateral rotations in the previous study), or, again, slower kinetics of the channel rhodopsin used in the earlier study.

We conclude that high frequency optogenetic stimulation of cortico-subthalamic axons is sufficient to ameliorate bradykinesia and hypokinesia in 6-OHDA lesioned mice. This is consistent with the result shown by Gradinaru et al., 2009, that HFOS of these projections reduced parkinsonian signs (ipsilateral rotations and head position bias) in Thy1::Chr2 6-OHDA mice. The precise mechanism of action for these pro-kinetic changes is not discernable from the data collected in this study, however the reduced cortico-subthalamic synchronization indicated by decreased M1-STN coherence in our study suggests that antidromic effects play a role, while our histological finding of far-reaching motor-cortico-subthalamic collaterals suggest a role for widespread increased glutamatergic activity in the lesioned side as well. Overall, the electrophysiological changes show that HFOS in the lesioned mice restores power, PAC, and coherence measures to levels more indicative of normal circuit function. Finally, since our results were achieved through AAV injections in non-transgenic mice, we conclude that ameliorating parkinsonism with specific targeting of M1-STN projections may be feasible in patients.

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Highlights

- Dual virus transfection enables selective targeting of layer 5 neurons that project to the subthalamic nucleus
- High frequency (100 or 130 Hz) optogenetic stimulation of cortico-subthalamic projections increases locomotion in 6-OHDA lesioned mice but not in controls
- Cortico-subthalamic coherence and subthalamic nucleus phase-amplitude coupling are increased in the unilateral 6-OHDA mouse model of Parkinson's disease
- High frequency optogenetic stimulation of cortico-subthalamic projections returns electrophysiologic measures from lesioned mice toward levels observed in controls
- Extensive collaterals branch from cortico-subthalamic axons

Figure 1.

Dual AAV injection strategy to place opsins in layer 5 cortico-subthalamic projection neurons. A) Location of primary motor cortex (M1) and subthalamic nucleus (STN) in sagittal (left) and coronal (right) sections. Ultrafast AAV-EF1a-DIO-hChR2(E123T/T159C)-EYFP AAVs were injected in M1. Ef1a-mCherry-IRES-WGA-Cre AAVs (Cre attached to retrograde labeling agent WGA) were injected in STN during the same surgery. Orange scale bar = 0.5 mm. B) M1 region of sagittal brain section (−1.56 mm) with cortical layers indicated (left); layer 5 EYFP expression marking the location of the M1 layer 5 opsins (right). C) STN region of sagittal brain section (−1.56 mm; left) and corresponding sample of the mCherry (red) expression indicating the location of the Ef1a-mCherry-IRES-WGA-Cre AAV injection (right). White scale bars = 100 μ m. cp = cerebral peduncle; ZIV = zona incerta, ventral part

Figure 2.

Verification of 6-OHDA lesioning, recording electrode and stimulating fiber placement, and successful transfection of cortico-subthalamic axons. A) Representative coronal section showing tyrosine hydroxylase staining in lesioned (left) and control (right) hemispheres. B) Representative electrode tracts (left and right) and fiber tract (right) for recording and optical stimulation, respectively. Arrows indicate approximate vertical location of electrode tips. Dark spots indicate marking with current injection. C) Tracing of EYFP in cortico-subthalamic neurons as described in the *Analysis of Histology Methods* for all 7 mice used in stimulation experiments. Legend indicates the mouse letter corresponding to the colored traces, cell bodies (triangles), and terminals (circles). VL = ventro-lateral thalamus, ZI = zona incerta. Scale bars = 0.5 mm.

Figure 3. Behavioral and electrophysiological characteristics of control and 6-OHDA lesioned mice. A) Control mice moved faster than 6-OHDA lesioned mice as indicated by the 10 second average speed for control (black) and lesioned (red) mice in an open field arena ($p = 2.24 \times 10^{-21}$; two sample t-test). B) Example 5 minute movement traces for control (black) and lesioned (red) mice. C) Example M1 (top) and STN (bottom) local field potentials (LFPs) for control (left; black/gray) and lesioned (right; red/pink) mice. D) M1/STN coherence is increased in lesioned mice (red) as compared to controls (black). Symbols correspond to

significance between indicated populations based on Wilcoxon ranksum test. * indicates $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$

Figure 4.

Location of WGA-Cre injections (red) and opsin expression (green). Lettered boxes indicate the approximate location of each section shown in A, D, F, G. All images are from sagittal sections at 1.56 mm unless otherwise indicated. Scale bars = 100 microns. A) EYFP (green) shows the location of cortical opsin transfection resulting from the intersection of the M1 injected constructs with the retrogradely transported Cre. B) Cortico-subthalamic axon passage from cortex to striatum. C) Cortico-subthalamic axon passage through striatum. D) STN mCherry indicates WGA-Cre AAV injection site while EYFP indicates cortico-

subthalamic projections and terminals at STN. E) Number of mice with fluorophore expression suggesting cell bodies (cortex) or terminals in the major brain regions where fluorophores were present (out of 9 total mice). F) Collaterals in ventro-lateral thalamus (VL); Left: VM = ventro-medial thalamus; VPM = ventral posteromedial thalamic nucleus; Po = posterior thalamic nuclear group. G) Collaterals in superior colliculus/ mesencephalic reticular formation (MRF). Left: InWh = intermediate white layer of the superior colliculus; DpG = deep gray layer of the superior colliculus; C = colliculus (inferior); PrCnF = precuneiform area

Figure 5.

Behavioral effects of optogenetic stimulation. A) After acclimatization, the mice received three periods of stimulation, each with a different frequency. Stimulation periods were separated by 5 minute rest periods. B) Lesioned mouse movement traces during 5 minute unstimulated (gray), high frequency optogenetically stimulated (HFOS; red), and post-stimulation unstimulated (gray) periods (top row). Control mouse movement traces corresponding to the same periods (bottom row). C) Fraction of time spent in motion during unstimulated, HFOS, and post-stimulation unstimulated periods for all lesioned mice. D)

Box plots showing fraction of time spent in motion during unstimulated and HFOS periods for individual 6-OHDA lesioned mice (left) and controls grouped by whether or not they had cortico-subthalamic opsins (right). Note that data from Mice H and D were combined due to small sample numbers. E) Locomotion speed cumulative distribution functions (cdfs) showed faster speeds during 100 Hz stim as compared to no stim. ($p = 3.25 \times 10^{-15}$; K-S test; representative cdfs from Mouse E). F) Summary results showing the fractional change in locomotion during HFOS. Locomotion was increased for lesioned mice ($p = 0.0023$), and decreased for control mice ($p = 0.0245$) (paired t-test).

Figure 6.

Electrophysiological effects of 6-OHDA lesioning and HFOS. A) Theta, alpha, and gamma band M1 power changes that occurred in lesioned mice (red) as compared to controls (gray) were reversed during HFOS. B) HFOS effects on STN band powers were not statistically significant. C) M1/STN coherence was increased in lesioned mice as compared to controls. Theta band M1/STN coherence was decreased during HFOS. D) For each 10 s epoch, the averaged PAC in 4×10 Hz pixels was calculated in the area of interest (AOI; black square), the peak value was then extracted from the averaged PAC map for each epoch. E) STN PAC

was increased in 6-OHDA lesioned mice (red) as compared to controls (gray). (left; $p = 0.03$). F) Peak STN PAC was reduced during HFOS in 6-OHDA lesioned mice (right; $p = 0.04$). Significance was assessed with two-sample t-tests in A, B; Wilcoxon ranksum in C, E, F. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.

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Table 1

Bradykinesia measures

	Average speed mm/s 1 second intervals	Maximum speed max speed on 1 second intervals	Maximum speed mm/s over all trials	
Controls (1680 s collected over multiple days from N = 3 mice)	15.98 ± 0.61	125.18 ± 12.43	180.69	++
	9.29 ± 0.50	103.82 ± 15.94	119.73	
6-OHDA lesioned (3840 s collected over multiple days from N = 4 mice)	5.69 ± 0.29	79.21 ± 8.33	113.05	###
lesioned mice with HFOS (corresponds to above time, days, mice)	6.64 ± 0.33	107.89 ± 13.76	177.21	

Symbols correspond to significance between indicated population distributions based on Kolmogorov-Smirnov test.

++
p = 5.44e-8 (control > HFOS)
+++
p = 1.0e-20 (control > 6-OHDA)

p = 1.0e-16 (6-OHDA < 6-OHDA HFOS)