

Optogenetics-based Neuromodulation of the Cortico-Basal-Ganglia Circuitry in a hemi-Parkinsonian Rat Model

by
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Contents

Acknowledgments	vii
1 Background	1
1.1 Introduction	2
1.2 Parkinson's Disease	2
1.2.1 History and epidemiology	2
1.2.2 Pathology and progression	4
1.2.3 Motor symptoms	6
1.2.4 Impacts on cognitive functions	8
1.2.5 Dopamine-replacement therapy	8
1.3 Animal Models of Parkinson's Disease	10
1.3.1 6-OHDA lesion model in rats	10
1.3.2 Other neurotoxin models and genetics models	12
1.4 Pathophysiology of the Cortico-basal-ganglia Motor Circuitry	14
1.4.1 A brief overview of anatomy and neurophysiology	14
1.4.2 The Rate model	16
1.4.3 Healthy versus pathological oscillatory activities in the motor cortex	18
1.5 Deep Brain Stimulation	20
1.5.1 Overview	20
1.5.2 Hypotheses on the mechanisms of action	21
1.5.3 On-demand stimulation based on ongoing pathophysiology	23
1.6 Optogenetics in the Study of Parkinsons Disease	24
1.6.1 An overview of optogenetics	24
1.6.2 Dissection of neural mechanisms by optogenetics	25
1.7 Goals of this Thesis	28

2	Optogenetic Modulations in the Cortico-basal-ganglia System of Non-transgenic Rats	30
2.1	Introduction	31
2.2	Achieving Optogenetic Inhibition of the Subthalamic Nucleus	32
2.2.1	Expression of inhibitory opsin in the STN achieved with electrophysiology-assisted targeting	33
2.2.2	Optogenetic inhibition of STN spiking activities	35
2.3	Achieving Optogenetic Modulation of the Globus Pallidus Externus	37
2.3.1	Expression of excitatory opsin in the GPe	38
2.3.2	Modulation of electrophysiology of GPe and the associated basal ganglia circuit by optogenetic excitation of GPe	39
2.4	Achieving Widespread Functional Opsin Expression in the Motor Cortex	47
2.4.1	Limitations of the diffusion-based delivery method	47
2.4.2	Development of the convection-enhanced delivery method	49
2.4.3	Widespread functional opsin expression achieved with CED-based opsin transduction	52
2.4.4	Functional opsin expression enabled modulation of electrophysiology of the motor cortex and motor cortical projection targets	59
2.4.5	Functional opsin expression enabled direct elicitation of motor response and optical mapping of the motor cortex	66
2.4.6	Section summary	70
2.5	Conclusion	70
2.6	Detailed Methods for Chapter 02	71
2.6.1	Subjects	71
2.6.2	Viral vectors	71
2.6.3	Design of CED cannula	72
2.6.4	Stereotaxic injection of viral vectors	73
	(a) Diffusion-based delivery in the STN, GPe, and the motor cortex	73
	(b) Convection-enhanced delivery in the motor cortex	74
2.6.5	Intraoperative electrophysiology of non-CED rats	74
	(a) Electrolytic lesion for marking of electrode location	75
2.6.6	Intraoperative electrophysiology of CED rats	75

(a)	Verification of opsin expression	75
(b)	Extracellular recording and optogenetic modulation	76
(c)	Local field potential mapping of the motor cortex	76
(d)	Functional mapping of the motor cortex	77
2.6.7	Intraoperative epifluorescence imaging	78
2.6.8	Histology and image analysis	78
(a)	Prussian blue, nuclear fast red, and Nissl staining	79
(b)	Immunohistology	79
2.6.9	Data analysis	80
(a)	Inclusion criteria	80
(b)	Extraction of LFP, MUA and isolation of spikes	81
(c)	Stimulus-triggered average (STA) of LFP and peri-stimulus time histograms (PSTH)	81
3	Rescue of Pathological Rotational Behaviors by Optogenetic Neuro- modulation in 6-OHDA hemi-Parkinsonian Rats	82
3.1	Introduction	83
3.2	Verification of the 6-OHDA Model	86
3.2.1	Dopaminergic depletion and hemi-Parkinsonian motor symptoms . .	86
3.3	Rotation Tests	89
3.3.1	Rotational behavior: stereotypes and mechanisms	89
3.3.2	Implementation of a custom magnorotamer	91
3.4	Summary of Hypotheses and Modalities of Modulation	95
3.4.1	Hypothesis 1: Inhibition of the principal neurons of STN is therapeutic for rescue of rotational bias	96
3.4.2	Hypothesis 2: With the premise inhibition of STN is therapeutic, excitation of GPe, which sends inhibitory efferents to STN, shall also rescue rotational behavior.	96
3.4.3	Hypothesis 3: Excitation of motor cortex rescues rotational bias . .	98
3.5	Electrical Stimulation of the Subthalamic Nucleus (STN-eStim), as the Gold- standard Modulation Paradigm, Rescued Rotational Behaviors in h-P Rats	100
3.6	Optogenetic Inhibition of the Subthalamic Nucleus (STN-oInhibit)	103
3.7	Optogenetic Excitation of the Globus Pallidus Externus (GPe-oStim)	106

3.7.1	Pan-neuron activation of GPe resulted in robust rescue of rotational bias	106
3.7.2	Electrical stimulation of the globus pallidus externus (GPe-eStim)	111
3.8	Optogenetic Excitation of the Motor Cortex (MC-oStim)	115
3.8.1	Single-site motor cortical stimulation (ssMC-oStim)	116
3.8.2	Spatially-extended motor cortical optical stimulation (seMCoS)	118
(a)	Cranial windows for optical access	119
(b)	Findings from rotation tests	121
(c)	Developing an LED-based seMCoS	124
3.8.3	Optogenetic excitation of the hyperdirect pathway (HD-oStim)	126
3.9	Simulation Verification of Stimulation Paradigms	128
3.9.1	Sufficient light coverage was achieved for deep brain light delivery without significant risk for photothermal effects	130
3.9.2	Suspended fiber illumination increased lateral coverage of light stimulation	131
3.10	Conclusions	136
3.10.1	Summary	136
3.10.2	Parallel mechanisms of actions of DBS	138
(a)	Therapeutic effects achieved through upstream and downstream modulations confirmed the important role the indirect pathway to motor symptoms.	138
(b)	Therapeutic effects of HD-oStim: orthodromic excitation of the STN or antidromic activation of the motor cortex?	139
(c)	Therapeutic effects from motor cortical stimulation: engagement of deeper layers versus superficial layers	140
3.10.3	Therapeutic effects of seMCoS suggest opportunities for non-invasive neuromodulation paradigms	141
(a)	An outlook for further improving optogenetic motor cortical stimulation	141
3.10.4	Apomorphine-induced rotations: therapeutic over-compensation or dyskinesia	142
3.11	Detailed Methods for Chapter 03	143
3.11.1	Subjects	143

3.11.2	Probe fabrication and assembly	144
(a)	Trident construct	144
(b)	Fixed array	144
(c)	Screw ECoG construct	145
(d)	MEAs	145
3.11.3	Surgical procedures	145
(a)	General surgical procedures	145
(b)	6-OHDA lesion procedure	147
(c)	General implantation procedure	148
(d)	Implantation and management of cranial windows	148
(e)	Implantation of recording and stimulation devices	149
3.11.4	Assessment of spontaneous and drug-induced rotations	149
3.11.5	Electrophysiological recording and stimulation in freely-behaving animal	151
(a)	Optical stimulation	152
(b)	Electrical stimulation	153
3.11.6	Experimental session control	153
3.11.7	Simulation of spatial light distribution and photothermal effects . .	154
3.11.8	Histology and image analysis	157
3.11.9	Statistics	158

4	Power Inhomogeneity and Phase Coherence of the Pathological Beta Oscillations in the Motor Cortex	159
4.1	Introduction	160
4.2	Findings	161
4.2.1	Spectral power of beta oscillations was elevated on the lesion motor cortex	161
4.2.2	Frequency variations of beta oscillations	161
4.2.3	Temporal dynamics of beta oscillations as individual events	163
4.2.4	Spatial variations of beta oscillations	167
4.2.5	Phase locking was elevated among the lesion motor cortex	170
4.3	Conclusions	174
4.3.1	Summary of results	174

4.3.2	An outlook for optogenetic motor cortical stimulation with spatial and temporal specificity	175
4.4	Detailed Methods for Ch04	177
4.4.1	Subjects	177
4.4.2	Data analysis	177
(a)	Recording and signal processing	177
(b)	Spectral analysis	178
(c)	Extraction of beta events	179
(d)	Cross-spectrogram	179
(e)	Phase-locking index	180
(f)	Classifications	181
(g)	Statistics	182
5	Discussions and Conclusions	183
5.1	Summary	184
5.2	What We Learned about the Basal Ganglia, the Parkinsonism, and the Deep Brain Stimulation	186
5.3	Remarks on Optogenetic Modulations for Potential Symptomatic Management	187
5.4	An Outlook Towards the Closed-loop, Adaptive Neuromodulation	189
A	REFERENCES	191

List of Tables

3.1	Parameters used in light and heat simulation.	157
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List of Figures

1.1	Dopaminergic depletion and aggregation of Lewy bodies in the substantia nigra pars compacta (SNc) of postmortem human patients.	5
1.2	Chemical structures of dopamine and its analogs, as well as schematics for unilateral 6-OHDA injection in MFB.	11
1.3	The rate model of the basal ganglia in the healthy and parkinsonian state.	16
2.1	Examples of STN targeting outcomes in the early phase of the study.	34
2.2	Opsin expression of STN achieved with the assistance of intraoperative electrophysiology.	35
2.3	Intraoperative validation of optogenetic inhibition of STN spiking activity with SwiChR _{CA}	36
2.4	Trident probe was implanted within opsin-expressing GPe in a h-P rat.	39
2.5	Electrophysiological modulations evoked by GPe-oStim in GPe and STN of healthy rats.	40
2.6	GPe-oStim evoked LFP responses in the motor cortex	42
2.7	Power-dose response of evoked LFP in GPe and motor cortex by GPe-oStim in awake h-P rat.	43
2.8	Trial-averaged response of spiking activity in the motor cortex to a 100 msec single-pulse GPe-oStim in awake h-P rat.	44
2.9	Dose-response characteristics of the cortically evoked spiking activity to a 10 msec single-pulse GPe-oStim in awake h-P rat transduced with ChR2.	45
2.10	Dose-response characteristics of the cortically evoked spiking activity to a 100 msec single-pulse GPe-oStim in awake h-P rat transduced with ChR2.	46
2.11	A summary of spatially limited expression in the motor cortex as an outcome of DBD-based virus injection observed in prior work of the lab.	49

2.12	Strong, but spatially limited, expression in the pyramidal neurons of motor cortex was achieved with the DBD-based method in the preliminary studies.	50
2.13	Example of cortically evoked responses to a 500 msec single-pulse ssM1-oStim in an awake h-P rat.	51
2.14	Mechanisms, design of cannula, and selection of injection parameters for CED	53
2.15	Injection procedures for DBD and CED	55
2.16	Intraoperative and post-mortem epifluorescence imaging of the CED transduced cortex.	56
2.17	Comparisons of the expression patterns associated with CED and DBD-1 μ L.	58
2.18	Optogenetic modulations of spiking activity sampled from various sites of the transduced (with C1V1 or Chr2) cortices.	59
2.19	Optogenetic modulations of LFP in CED and DBD transduced cortices.	61
2.20	Opsin expression in the terminals of cortical projection targets in a healthy rat.	63
2.21	Modulation of LFP activities of cortical projection targets by motor cortical stimulation in two h-P rats.	65
2.22	Modulation of LFP activities in the STN and bilateral motor cortices by optogenetic-stimulation of the cortico-subthalamic projections in two h-P rats.	67
2.23	Functional motor maps acquired intraoperatively with electrical and optogenetic stimulations.	69
3.1	Tyrosine-hydroxylase immunohistology of dopamine depletion.	86
3.2	Forelimb-use asymmetry in wall touching, standstill, and food manipulation.	88
3.3	Mechanisms and behavioral consequences of drug challenges.	91
3.4	Custom magnorotameter for continuous rotation measurement.	93
3.5	Schematic illustrations of modulation modalities investigated in this thesis.	95
3.6	Rotational bias modulated by high-frequency electrical stimulation of STN.	101

3.7	Rotational bias induced by amphetamine (Amph-IR) was reduced by optogenetic inhibition of STN.	104
3.8	Example of hysteresis in the effect of STN-oInhibit.	105
3.9	Rotational bias alleviated by optogenetic stimulation of GPe (GPe-oStim).	109
3.10	Rotational bias modulated by electrical stimulation of GPe (GPe-eStim).	114
3.11	Rotational bias reduced by intracortical single-site stimulation of the motor cortex (ssMC-oStim).	117
3.12	PDMS and glass cranial windows.	120
3.13	Rotational bias reduced by spatially-extended stimulation of the motor cortex (seMCoS).	123
3.14	The LED approach for epicortical illumination: light output profile and thermal management.	125
3.15	Rotational bias reduced by stimulation of the hyperdirect pathway (HD-oStim).	127
3.16	Light distribution from implanted optical fiber in relation to the geometry of th STN and GPe.	132
3.17	Spatial temporal temperature increase profile in relation to taper geometry and input power	133
3.18	Simulated light output and effects of heating from an intracortical implanted optical fiber.	134
3.19	Improved lateral spread of light using a suspended fiber for seMCoS.	135
3.20	Bar graph summary of the rescue of rotational biase.	136
3.21	Probes and their implantation.	146
3.22	Temporal structures of optical and electrical stimulation protocols.	152
4.1	Elevated beta power on the lesion motor cortex.	162
4.2	Variations in the peak center frequencies of beta activities.	163
4.3	Amplitudes of beta events.	165
4.4	Durations of beta events.	166
4.5	Comparisons of amplitudes, durations, and occurrence rates of beta events.	167
4.6	Spatiotemporal variations of beta power.	169
4.7	Increased phase synchrony over time within the lesion motor cortex.	172

4.8	Phase-locking analyses in other animals.	173
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Chapter 1

Background

1.1 Introduction

Parkinson's disease is the most common movement disorder and the second most common neurodegenerative disorder (after Alzheimer's disease), affecting 10-15 million people worldwide. Yet, further understanding in the symptom generation, and the development of more effective and resilient treatment are necessary and are made possible by the recent development of new paradigms of neuromodulation.

In this chapter, I will first review the history of Parkinson's disease since its official documentation 200 years ago. I will briefly discuss the associated pathology, motor and non-motor symptoms, as well as the current gold-standard pharmacological treatments. I will then describe animal models of Parkinson's disease, specifically the one that was chosen for this thesis. I will next discuss the pathophysiology occurred in the motor cortico-basal-ganglia circuitry and motivate the hypotheses established in this study. Afterwards, I will introduce deep brain stimulation, the most effective neuromodulation paradigm known to medicine, and present its limitations. Subsequently, I will provide background on optogenetics, which could potentially overcome many aspects of its limitations, and review its applications in Parkinson's disease research.

Finally I will present the three goals of this thesis: to achieve functional optogenetic modulations of multiple loci of the cortico-basal-ganglia circuit; to investigate whether the said neuromodulation paradigms could lead to therapeutic symptomatic improvements; and to characterize cortical neural features as potential biomarkers for Parkinson's disease.

1.2 Parkinson's Disease

1.2.1 History and epidemiology

Two hundred years ago, a neurological syndrome hallmarked by tremor in motions, weakness in motor vigor, and instability in posture was medically described and separated from other

similar diseases by neurologist Dr. James Parkinson, after whom this disease was named (Charcot, 1872), in his seminal work “*An Essay on the Shaking Palsy*” (Parkinson 1817, reprinted (Parkinson 2002)). In fact, early written evidence could be found in civilizations across the world, including in the ancient Chinese texts on medicine.

In as early as the 6th Century, Sun Simiao, one of the most famous doctors/physicians /medical scientist in the ancient Chinese history described a syndrome in the book “*Essential Prescriptions Worth a Thousand Gold*” (Sun 1982): “[Those] who for several years feel heavy, just as if being pulled by a string when raising an arm, a foot or the body, . Who find it difficult to turn around, to walk without dragging the legs ” (Z.-X. Zhang, Dong, and Román 2006). A more holistic description of a case bearing great resemblance with Parkinsonism was reported by Zhang Zihe in the 11th Century: “... a man of 59 years of age had tremor for 3 years. He failed to hold things and to eat by himself. He attempted suicide, but he was unable to hang himself because of his [tremorous] hands” (reviewed by (Z.-X. Zhang, Dong, and Román 2006)). Zhang Zihe also reported the presence of stiffness, difficulty in common motor tasks, loss of finger dexterity, and cognitive symptoms such as depression and suicidal tendency.

Perhaps the ample availability of ancient literature on this disease worldwide corroborates the high prevalence of the Parkinson's disease. It is the most common movement disorder and the second most common neurodegenerative disorder (first being the Alzheimer's disease), affecting about 1 million people in US, 2.5 million people China (S. Chen et al. 2016), 10-15 million people worldwide (C. M. Tanner and Goldman 1996; Tysnes and Storstein 2017). Current estimates give a global prevalence rate of 0.1-0.3% (10-30 in 10,000 people) across all ages (von Campenhausen et al. 2005; Werner Poewe et al. 2017). However, the prevalence of the disease is highly age-dependent: after the age of 60 the likelihood increased exponentially to more than 3% in those >80 years of age (Werner Poewe et al. 2017). The prevalence and incidence is also about 1-2 times higher in the male population than in females (von Campenhausen et al. 2005; Ma et al. 2014; Tysnes and Storstein

2017; Van Den Eeden et al. 2003). With an aging global demographics, Parkinson's disease will exert further socioeconomic impact in the near future (Kowal et al. 2013; J.-X. Yang and Chen 2017).

1.2.2 Pathology and progression

The degenerative aspect of Parkinson's disease is hallmarked by the loss of dopaminergic neurons and processes in the midbrain region including the substantia nigra pars compacta (SNc) (Fig. 1.1 A), which was correlated with the widespread accumulation of alpha-synuclein protein in the neurons. Alpha-synuclein tends to aggregate and polymerize to form neuronal inclusions in the cytoplasm known as the Lewy bodies (Wakabayashi et al. 2013; LEWY and FH 1912) (Fig. 1.1 B).

While its functional significance is not fully appreciated, a-Synuclein does occur normally in the neurons in a soluble format. Pathological accumulation and failure of timely degradation of a-synuclein, however, could initiate polymerization and form more and more insoluble agglomerates. Moreover, it has been shown that protein misfolding and the prion-like propagation of misfolding could play important roles in the progress and spread of Lewy pathology (Desplats et al. 2009; Olanow 2014). Even so, exactly how Lewy bodies lead to cellular toxicity and cell death was not clear, though possible mechanisms through mitochondrial dysfunction, oxidative stress, and neuroinflammation have been demonstrated (Wakabayashi et al. 2013; Ingelsson 2016; Werner Poewe et al. 2017). On the note of neuroinflammation, a fair amount of study focused on establishing dietary risk factors of PD and has for example linked erroneous glucose metabolism to neuroinflammation (Dunn et al. 2014; Backes et al. 2016); in fact, some diabetic patients have a higher risk to PD (Cereda et al. 2011; Yue et al. 2016). The deposition of a-synuclein and Lewy bodies occur before the manifestation of symptoms. In fact, Braak et al. established a multi-stage progression model that indicated the spread of Lewy body

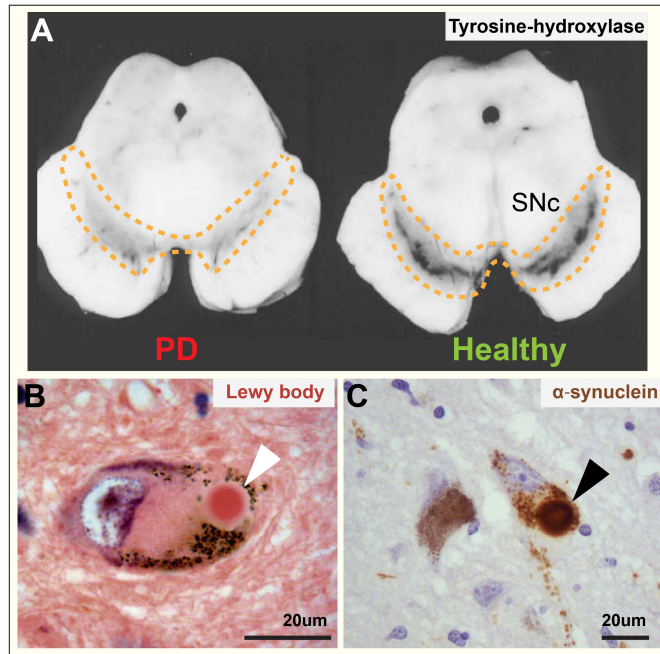


Figure 1.1: Dopaminergic depletion and aggregation of Lewy bodies in the substantia nigra pars compacta (SNc) of postmortem human patients. **A.** Significant reduction of tyrosine-hydroxylase (TH) labeling in the diseased sample. Adopted with permission from (Mackenzie 2001). **B-C.** Human midbrain tissue showing Lewy body in the dopaminergic neurons: haematoxylin and eosin (H&E)-staining showed Lewy body in pink color (white arrowhead, B), and immunostaining of synuclein within the Lewy body (black arrowhead, C). Figure courtesy of neupsykey.com and Prof. Steve Gentleman, Imperial College of London.

from lower brain stem region, through the midbrain, to throughout the cortex (at which stage symptoms associated with dementia became more pronounced) (Braak et al. 2003, 2006; Halliday and McCann 2010).

Nonetheless, where do Lewy bodies first occur (or where does the first mis-folding event occur) is a valuable questions to the search of preventive methods for PD, which do not exist at this moment. A particularly worrisome and frightening view has been portrayed that neurodegenerative disease of the CNS can in fact be acquired from outside the brain and transmit back into the CNS. This is supported by the discovery of early signs of α -synuclein in the olfactory areas (the only part of the CNS that interacts directly with the environment, (Hawkes, Shephard, and Daniel 1997; Desplats et al. 2009; Haehner,

Hummel, and Reichmann 2011; Doty 2012; Dehay et al. 2015)) and in the enteric nervous system (a PNS neuronal network that controls the activity of the gastrointestinal system, e.g. intestinal motility, and was connected to the CNS through the spinal cord (Lebouvier et al. 2009)) (Houser and Tansey 2017). I was completely intrigued by the implications, as loss of olfactory acuity and experience of gastrointestinal problems (e.g. constipation and nausea) (Poirier et al. 2016) are also common in the healthy aging population.

Why are the dopaminergic neurons targeted by the pathology? As an engineer, one perspective caught my eyes in particular. Recent studies discovered inherent vulnerabilities of dopaminergic neurons to mitochondrial dysfunctions and oxidative stress. Dopaminergic neurons have high metabolic demands: they send out long, widespread, and divergent unmyelinated processes over great volume of the brain; their action potentials were large and broad (contribution of Ca channels) and spontaneously showing tonic activities (Blesa and Przedborski 2014). To make the matter worse, the concentrated cytosolic dopamine itself increases oxidative stress on the neurons (Blesa and Przedborski 2014).

Besides dopaminergic neurons, significant neuronal loss was also found, to various degrees, for noradrenergic (NE) neurons in the locus coeruleus (LC) and serotonergic (5-HT) neurons in the dorsal raphe nucleus (Jellinger 2001). These non-dopaminergic losses have been implicated with the cognitive impairments in PD. Lesion in the locus coeruleus was found to correlate with rigidity, akinesia, autonomic dysfunctions, and dementia; while loss of 5-HT neurons was associated with cognitive symptoms like depression.

1.2.3 Motor symptoms

Diagnostic motor symptoms of PD include akinesia/bradykinesia, muscle rigidity, and tremor, as well as postural instability, freeze-of-gait (FOG) and etc. Many cortical and subcortical areas may play a role in these motor behaviors. A network of brain regions of particular importance is the cortico-basal-ganglia motor circuitry, the involvement of which

will be discussed in the following sections separately. In this section, I will provide brief background on each motor symptoms.

Tremor, despite being among one of the first historically discovered symptoms and the most publicized in the mainstream conception, is not unique to PD and is not even the most prevalent symptom (occurs in only about 75% of all patients (W. Chen et al. 2017)). In fact, tremor in PD manifests mainly at rest (hence named resting tremor), while tremorous motion during voluntary movement (active tremor) is more prevalent in essential tremor (Bhidayasiri 2005; W. Chen et al. 2017). The kinematics of the resting tremor is also characteristic: having a 4 to 6 Hz frequency and resembling a pill-rolling action. More interestingly, this tremor is suppressed during purposeful movements (Deuschl, Papengut, and Hellriegel 2012) and can be provoked by stress or during cognitively demanding tasks (Raethjen et al. 2008).

Akinesia refers to the lack of motion and the difficulty in initiating motion; and bradykinesia describes the slowness of movement. Unlike tremor at rest, hypokinetic motor signs were universally present (eventually) in all PD patients (Mazzoni, Shabbott, and Cortés 2012). Somewhat related to akinesia and bradykinesia are muscle rigidity (increased muscle tone) and postural instability (inability to recover from disturbance, e.g. tripping over) (Mazzoni, Shabbott, and Corts 2012).

Freeze of gait (FOG) describes the brief and episodic breakdown of movement spontaneously or in response to environment cues (Vercruysse et al. 2014). For example, an FOG episode may be triggered as patients are walking through spatial transitions, e.g. a door, and the patient would either stop or switch into small shuffling steps. On the other hand, FOG can be reduced or eliminated with the presence of visual aid (painted step indicators on the floor)(Vercruysse et al. 2014). The possible modulatory effects of cognition and perception suggested possible interference or competition with the functions of the motor area (Nieuwboer and Giladi 2013).

1.2.4 Impacts on cognitive functions

While not the primary focus of my thesis, it is worth noting that PD is associated with a broad-spectrum of non-motor symptoms including anhedonia, depression, sensory dysfunctions, and autonomic dysfunctions (W. Poewe 2008). Symptoms like apathy, depression, and cognitive dysfunctions have a strong link with the role of dopamine in the reward system. In addition, as mentioned previously, the loss of noradrenergic and serotonergic neurons impact many aspects of the cognitive functions, in particular depression and autonomic dysfunctions. Other non-motor signs may reflect the cellular toxicity of the synucleinopathy over the limbic, association, temporal, and olfactory cortical areas (Halliday and McCann 2010; Haehner, Hummel, and Reichmann 2011; Werner Poewe et al. 2017).

1.2.5 Dopamine-replacement therapy

After determination of dopamine depletion as the key neuropathology in patients with PD (Hornykiewicz 1966) (see review by (Hornykiewicz 2002)), the rationally derived therapy concept was introduced to pharmacologically replenish dopamine, stimulate dopamine release, or mimic the function of dopamine (Hornykiewicz 2002; LeWitt 2008; Connolly and Lang 2014; J. A. Obeso et al. 2017a; Ovallath and Sulthana 2017; Werner Poewe et al. 2017).

Dopamine itself cannot pass the blood-brain barrier, however, rather fortunately, its precursor, the aromatic amino acid L-3,4-dihydroxy phenylalanine (L-DOPA or levodopa) does. Naturally in the cytosol catecholaminergic (dopaminergic and noradrenergic) and serotonergic (5-HT) neurons, levodopa can be formed from tyrosine; it is then made into dopamine by the action of aromatic L-amino acid decarboxylase enzyme (AAAD) (Prediger et al. 2014). In PD, the exogenous levodopa is taken up by serotonergic neurons via the L-type amino acid transporters; serotonergic neurons also have AAAD and can convert L-DOPA into dopamine, in the absence of dopaminergic neurons that were lost due to the

pathology (Reed, Nijhout, and Best 2012).

The levodopa therapy lead to significant improvement of the motor symptoms, especially in bradykinesia, rigidity, and gait, while less effectiveness was seen for tremor (Yahr et al. 1969; LeWitt 2008; Ovallath and Sulthana 2017). However this treatment loses its effectiveness and becomes less reliable (e.g. motor fluctuations, the exaggerated on-off state differences) after 2 to 5 years of treatment; and complications headed by L-Dopa-induced dyskinesia (LID) and impulse control disorders also develop over the course (LeWitt 2008; Hickey and Stacy 2016; Werner Poewe et al. 2017). Similar therapeutic effects can also be achieved by administration of dopamine agonist (mimicking the function of dopamine) and by chemicals that increase dopamine release from residual neurons (Calipari and Ferris 2013) and inhibit dopamine clearance (see review by (Werner Poewe et al. 2017)). These approaches are often used in conjunction with L-Dopa to extend the therapeutic course of the treatment.

1.3 Animal Models of Parkinson’s Disease

Experimental animal models are essential to the understanding of the disease and investigation of treatment. In this section, I will introduce the 6-hydroxydopamine lesion model used in this thesis, as well as several other animal models of PD that are frequently adopted in research.

1.3.1 6-OHDA lesion model in rats

The key rationale behind animal models of PD is to create dopaminergic depletion and mimic the main pathology of PD. We used the 6-hydroxydopamine (6-OHDA), a reactive catecholamine neurotoxin, as well as a close analog of dopamine in molecular structure (Heikkila, Sonsalla, and Duvoisin 1989) (Fig. 1.2 A). When intracerebrally injected near the terminals, axon, or cell body of a dopaminergic neuron, 6-OHDA is taken into the cytosol by the dopamine reuptake transporter proteins; once inside, it can be transported back to the soma region through existing cellular mechanisms and cause lesion across the neuron. As a reactive oxygen species (ROS), 6-OHDA destroys normal cellular function by oxidation-reduction reactions and blockade of mitochondrial respiration, and thus leads to cellular death (Glinka, Gassen, and Youdim 1997; Rodriguez-Pallares et al. 2007). In fact, this process shares great resemblance to the proposed actions of cellular toxicity by Lewy bodies.

In our model, 6-OHDA is injected on one hemisphere to the medial forebrain bundle (MFB) (Fig. 1.2 B), a collection of axonal fibers at the base of rat brain connecting basal ganglia nuclei in the anterior-posterior axis, among which are the projecting axons of dopaminergic neurons. Since 6-OHDA lesion is non-specific to neurons with catecholamine reuptake transporters, we use a drug, desipramine, to block 6-OHDA uptake by noradrenergic transporters and protect noradrenergic neurons (Deumens, Blokland, and Prickaerts 2002). As a result, near complete depletion of dopamine and loss of dopaminergic neurons

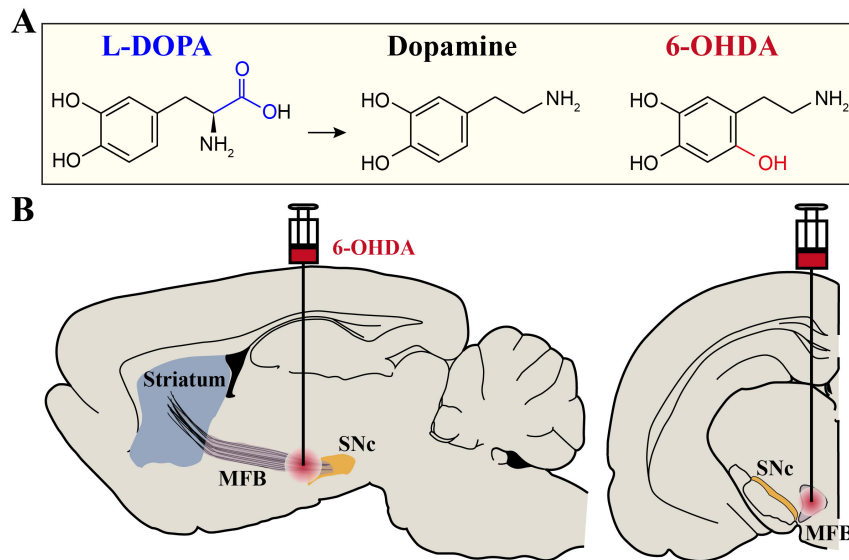


Figure 1.2: Chemical structures of dopamine and its analogs, as well as schematics for unilateral 6-OHDA injection in MFB. **A.** The structural similarities of the dopamine, L-DOPA, and 6-OHDA allow their transportation into the dopaminergic neurons, where L-DOPA is transformed into dopamine by DOPA decarboxylase. The extra hydroxyl group in 6-OHDA gives rise to the generation of reactive oxygen species. **B.** 6-OHDA was injected unilaterally within the posterior portion of the MFB. [Abbreviations: SNc, substantia nigra pars compacta; MFB: medial forebrain bundle; 6-OHDA: 6-hydroxydopamine; L-DOPA: levodopa.]

occur on only one side of the brain, both in literature (Ungerstedt 1968; Deumens, Blokland, and Prickaerts 2002; Iancu et al. 2005; Da Cunha et al. 2008) and in our experiment.

These animals were referred to have hemi-Parkinsonism or to be hemi-Parkinsonian (h-P) for the presence of Parkinsonian syndrome on one hemisphere. Due to the laterality of the lesion, these h-P animals showed the motor symptoms similar to that of human idiopathic PD on the contralateral side of their body (contralateral hemibody). As a result, asymmetry in motor behavior developed (which is advantageous from the experimental point-of-view for the contralateral control). h-P rats displayed impairments in the use of contralateral forelimb, impairments in the sensorimotor responses on the contralateral body, and a striking rotational bias. Despite the hemispheric lesion, h-P rats showed clear behavioral signs of akinesia and bradykinesia (Deumens, Blokland, and Prickaerts 2002).

Tremor of the limbs were also occasionally reported (Jolicoeur, Rivest, and Drumheller 1991; Metz et al. 2005).

The 6-OHDA model mimics the late, severe stage of PD particularly well (Deumens, Blokland, and Prickaerts 2002). In patients with PD, pathology progressed over years to reach the threshold for motor symptoms to manifest. On the contrary, MFB lesion can lead to extensive dopaminergic loss within the first week of lesion and animals manifest severe motor symptoms. Compensatory mechanisms may gradually develop and slightly ameliorate some symptoms after peak lesion (Blanchard et al. 1996), especially in the case of unilateral lesion. The unilateral lesion is further justified by the frequent observation of asymmetrical development of dopaminergic depletion onset of unilateral symptoms in human patients with PD (Kempster et al. 1989; Djaldetti, Ziv, and Melamed 2006; Werner Poewe et al. 2017).

In this thesis, I focused on rotational bias as a key measure of hemi-Parkinsonian motor symptoms, and its functional significance will be discussed in more details in Section 3.3. Overall, the rotation behaviors have been one of the goldstandard motor assessment to predict the antiparkinsonian effects of drugs, gene therapy, and neuromodulation paradigms (J. A. Obeso et al. 2017a).

1.3.2 Other neurotoxin models and genetics models

Another commonly used neurotoxin model of PD uses 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) through mechanisms of toxicity similar to that of 6-OHDA, relying on the interruption of mitochondria functions and induction of oxidative stress (Sian et al. 1999; Przedborski et al. 2000). Yet unlike 6-OHDA, MPTP readily crosses BBB and can be administered orally (bilateral lesion) or intrajugularly/intraventricular (unilateral lesion) (Meredith, Sonsalla, and Chesselet 2008). The model is also capable of mimicking most

of the clinical and neuropathological hallmarks of PD and is used more frequently in non-human primate research (Przedborski et al. 2000; J. A. Obeso et al. 2017a). MPTP model in non-human primates has played essential roles in the understanding of pathophysiology and, for example, in the discovery of deep brain stimulation targets (Bergman, Wichmann, and DeLong 1990).

Scientific discoveries are in many cases full of surprise, serendipity, and sadness. The connection of MPTP to PD was discovered due to its abuse as a substitute for heroin (see details of the story in the bibliographical account by (Langston 2017)). The discovery of MPTP also led to attentions on other structurally analogous chemical commonly used in farming as herbicide, pesticide, insecticide, and piscicide (killing fish): paraquat (Berry, La Vecchia, and Nicotera 2010) and rotenone. Rotenone, in particular, gained wide use due to the believed low toxicity to humans; a fish poison tree containing rotenone is used by tribal people in Amazon to catch fish. Yet later, the link to PD was confirmed through animal experiments and in human patients (Priyadarshi et al. 2000; Sherer et al. 2003; Sindhu, Saravanan, and Mohanakumar 2005; Caroline M. Tanner et al. 2011).

On the other hand, the clear neuropathology (dopamine depletion and synucleinopathy) also provides opportunities to develop targeted transgenic animal models of PD (Meredith, Sonsalla, and Chesselet 2008; Werner Poewe et al. 2017): 1) mutations to genes associated with familial PD, e.g. Parkin (Rial et al. 2014) and alpha-synuclein (V. M.-Y. Lee and Trojanowski 2006; Fernagut and Chesselet 2004; Giraldo et al. 2018); and 2) modifications of crucial functions of DA neurons that cause degeneration in adulthood, e.g. MitoPark mice in which mitochondrial functions are compromised (Ekstrand et al. 2007).

Given the multitude of mechanisms of actions and variable associated manifestation of pathology and symptoms, while none of which being perfect, these animal models of PD could continue to facilitate the understanding of the disease and the search of therapies if used purposefully and interpreted carefully for the targeted PD pathology.

1.4 Pathophysiology of the Cortico-basal-ganglia Motor Circuitry

1.4.1 A brief overview of anatomy and neurophysiology

The basal ganglia, situated at the base of the forebrain, mainly consist of four interconnected nuclei: the striatum (caudate/putamen, which is not well distinguished in rodents), the globus pallidus internus (GPi) and externus (GPe), the subthalamic nucleus (STN), and the substantia nigra (pars reticulata, SNr, pars compacta, SNc). The motor aspect of basal ganglia connects with the motor cortex, the motor thalamus, and the downstream brainstem motor nuclei to form the cortico-basal-ganglia-thalamo-cortical system, which acts as a crucial processing stage for the control of motor actions (Steiner and Tseng 2016): popular theories suggest key roles in action selection based on context where motor commands from motor cortex are filtered and feedback for smooth and precise motor outputs (Jose A. Obeso et al. 2008). In this thesis, we will focus on a subset of this system, the cortico-basal-ganglia circuitry. A simplified yet powerful canonical model of basal ganglia is illustrated in Fig. 1.3 Left.

First, let's look at the output of the basal ganglia (BG): SNr and GPi send inhibitory GABAergic (GABA: gamma-Aminobutyric acid) projections to the motor thalamic nuclei, which have excitatory connections to the cortex, and to downstream brainstem premotor nuclei. The inhibitory neurons in GPi/SNr spontaneously maintain a high firing rate and thus exert continuous inhibition; this overall inhibitory effect of BG output gave rise to theories on focusing of motor output by the suppression of unwanted movement (Hikosaka, Takikawa, and Kawagoe 2000; Hikosaka 2007).

As for the inputs, the striatum (specifically the dorsolateral striatum) is the primary input nucleus of the motor basal ganglia; two main sources of input are the glutamatergic corticostriatal projections from the motor cortex and the dopaminergic nigrostriatal projections from the SNc. The striatum outputs GABAergic efferents to downstream targets

through two types of medium spiny neurons (MSNs) (Alexxai V. Kravitz et al. 2010):

- neurons expressing D1 dopamine receptors (DARs), D1-MSNs, project directly to GPi and SNr. The activation of D1-MSNs hence decreases the overall inhibitory output of BG (disinhibition) and is hence regarded “pro-kinetic.” This pathway is named the striatonigral “direct pathway.”
- neurons expressing D2 DARs, D2-MSNs, project firstly to GPe; then GPe send inhibitory efferents to the STN; finally STN outputs excitatory drive to GPi and SNr. Hence activation of D2-MSNs would increase the inhibitory tone to motor output and is regarded “anti-kinetic.” This multistage pathway is named the striatopallidal “indirect pathway.”

While certain level of segregation is found between subcompartments of striatum, i.e. the striosome (more D1-MSNs) and the matrix (more D2-MSNs), the distributions of D1-MSNs and D2-MSNs are well interspersed and roughly equal (Brimblecombe and Cragg 2017). While cortical inputs will excite both D1-MSNs and D2-MSNs and lead to divergent effects on motor output, dopamine (DA) increases D1-MSN activity and suppresses D2-MSN activity hence leading to convergent pro-kinetic effects.

Another form of input to BG is the direct projection from motor cortex to the STN, which is termed the corticosubthalamic “hyperdirect pathway” (Degos et al. 2008a; Turner and Desmurget 2010). Also the cortical inputs of hyperdirect pathway are divergent and distributed (Milardi et al. 2015). The outcome of its action is similar to that of the indirect pathway, i.e. increasing inhibition on motor output, yet faster due to the monosynaptic connection. The fast hyperdirect pathway may set the stage for slower effects through the striatum (Atsushi Nambu, Tokuno, and Takada 2002; Mathai and Smith 2011).

1.4.2 The Rate model

How do malfunction of BG correlate to motor symptoms of PD? Hypotheses based on the changes in firing rates of BG nuclei has been verified in rodents, non-human primates, and patients and are summarized in the “rate” model (Atsushi Nambu, Tachibana, and Chiken 2015; Herrington, Briscoe, and Eskandar 2017; Werner Poewe et al. 2017) (Fig. 1.3 Right). Here I will briefly introduce this model, based on which are the hypotheses for treatment options proposed in this thesis.

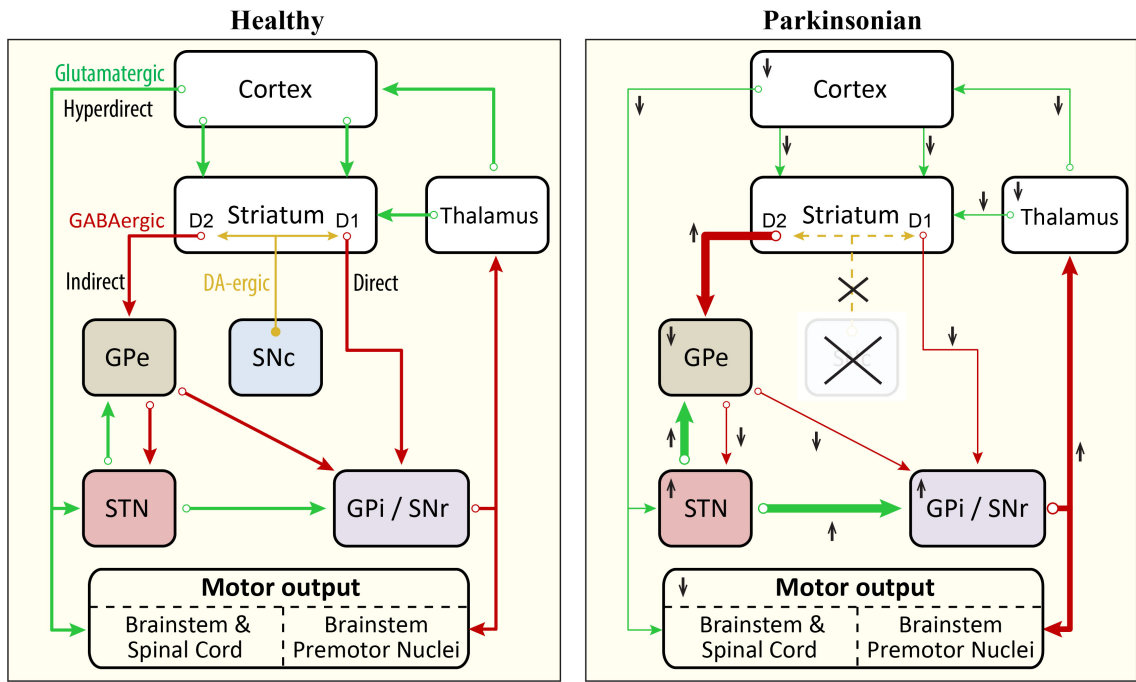


Figure 1.3: The rate model of the basal ganglia in the healthy and parkinsonian state. Only the major glutamatergic (green), GABAergic (red), and dopaminergic (yellow) projections were shown. The black arrows indicated the hypoactivity (downward) or hyperactivity (upward) of each projection or nucleus predicted by the model.

Nigral loss of DA neurons and the following depletion of striatal DA lead to reduced activation of the direct pathway and increased activity in the indirect pathway, both of which increase the inhibitory output of BG and lead to anti-kinetic effects observed in motor

symptoms. In this regard, GPe would experience overall hypoactivity and STN would be in a state of hyperactivity, which was corroborated in a number of studies (Filion and Tremblay 1991; Boraud et al. 1998; Hamani et al. 2004; Mallet et al. 2006; Galvan and Wichmann 2008; Hegeman et al. 2016; Hamani et al. 2017). Further evidence from neurosurgery and neuromodulation studies also validated the significance of these predictions, which will be discussed in later sections.

Despite its usefulness and relevance in PD research and clinical treatment, the rate model may only provide an oversimplified view of the pathophysiology. For example, dopaminergic projections indeed existed in other BG nuclei (e.g. GPe, STN, and motor cortex), however the effects of degeneration of these terminals were not captured. Depletion of dopamine in cortical areas in PD causes additional impairment to motor functions (Valencia et al. 2014; M. Wang et al. 2015). In addition, the model also meets criticisms for overlooking critical interactions between subpopulations of neurons within each nucleus: e.g. the existence of multiple subgroups of GPe neurons forming local inhibitory network (Mastro et al. 2014). Furthermore, symptoms like tremor cannot be explained by the rate model (Atsushi Nambu, Tachibana, and Chiken 2015). Finally, electrophysiological evidence in animal models and human patients found abnormally bursty, oscillatory and synchronous firing of neurons within and across BG nuclei, hence leading to the establishment of the “firing pattern” (or the “oscillation”) model (Ni et al. 2000; Magill, Bolam, and Bevan 2000; P. Brown et al. 2001; Levy et al. 2002; Weinberger et al. 2006; Hammond, Bergman, and Brown 2007; Galvan and Wichmann 2008; Mallet et al. 2008; Guo et al. 2012; Pan et al. 2016). Increased synchronies may reduce the information capacity of the basal ganglia (Schneidman et al. 2003), which is in particular a problem for the motor cortex, limiting the flexibility to generate the necessary activity patterns for a motor command, i.e. reduce the dimensionality of the motor cortical state space (Lindenbach and Bishop 2013).

1.4.3 Healthy versus pathological oscillatory activities in the motor cortex

Until this point, we have been focusing on the unit spiking activity of individual neurons; a wealth of evidence has pointed to an increased synchrony in spiking within and across the cortico-basal-ganglia system (Magill, Bolam, and Bevan 2000; P. Brown et al. 2001; Mallet et al. 2008; Pollok et al. 2012). Bursting and synchronous spiking activities (with spectral contents $>300\text{Hz}$) can manifest in the local field potentials (LFP, electrophysiology at lower portion of spectral frequencies (1-100Hz)) (Buzsáki, Anastassiou, and Koch 2012; Herreras 2016), in the form of oscillatory activities around the dominant spiking/bursting frequencies (spike-LFP coherence) (Teleczuk and Destexhe 2013; Pan et al. 2016). When synchronous activities happen over even greater spatial scale and near the cortical surface, they could contribute to oscillatory waveforms in the electrocorticogram, ECoG and electroencephalogram, EEG.

Indeed, abnormally exaggerated oscillations at the beta band (13-30Hz in human (P. Brown et al. 2001; Kühn et al. 2005; Sharott et al. 2005) and 20-45Hz in rats (Sharott et al. 2005; Pan et al. 2016; T. O. West et al. 2018)) are widely associated with symptoms of bradykinesia and rigidity (Peter Brown 2003; Kühn et al. 2009) and are normalized during effective treatments with L-dopa and deep brain stimulation (Meissner et al. 2005; Weinberger et al. 2006; Moran et al. 2011; Trager et al. 2016; T. West et al. 2016; Beudel et al. 2017).

Do oscillations and synchronous activities occur during normal functions? In the healthy functions of motor cortex in movement planning and execution, oscillations in the beta and gamma ($>40\text{ Hz}$) range do occur. Specifically, in non-human primates and humans, beta oscillations are attenuated during voluntary movement or motor imagery (during which gamma power increases) (Pogosyan et al. 2009), and are most prominent during attentive holdings of movement (isometric force generation) (Witte et al. 2007; Chakarov et al. 2009) or before motor initiation and after completion of movement (Murthy and Fetz 1996;

Pfurtscheller and Lopes da Silva 1999; Engel and Fries 2010). This process is referred to as the event-related desynchronization and synchronization (van Wijk, Beek, and Daffertshofer 2012), and is found also in rodents based on evidence showing shifts from low beta band during resting to high beta/gamma band during mobility in h-P rats (Avila et al. 2010; Delaville et al. 2014). Therefore, it is thought that the dominance of beta activity in the cortex helps maintain the status quo (Engel and Fries 2010) and that motor cortex in the parkinsonian state may have difficulty in switching from the beta state (anti-kinetic) to the gamma state (prokinetic) during manifestation of akinetic symptoms (Peter Brown 2003) (for a thorough review, the reader is referred to (Brittain et al. 2014; Brittain and Brown 2014)).

1.5 Deep Brain Stimulation

1.5.1 Overview

Deep brain stimulation (DBS) refers to the local delivery of high frequency electrical pulses (100-180Hz) through implanted electrodes to the deep brain nuclei including the subthalamic nucleus (STN) and globus pallidus internus (GPi). In mid/late stage PD, especially when patients respond poorly to dopamine-replacement therapies, DBS has become the most effective treatment in managing motor symptoms (Hickey and Stacy 2016). A range of PD symptoms have been shown to improve by DBS, including reduced rigidity, decreased bradykinesia, reduced dosages in L-DOPA-like therapy (hence decrease in motor fluctuations and dyskinesia), as well as improvement in the overall quality of life (Alim Louis Benabid 2003; McIntyre, Savasta, et al. 2004; Tan et al. 2016; Hickey and Stacy 2016; Fang and Tolleson 2017; Werner Poewe et al. 2017; Xu et al. 2017; Mansouri et al. 2018). Two BG nuclei are commonly selected as targets for DBS, the STN and the GPi, with some distinction in symptomatic benefits: STN-DBS has better efficacy in the reduction of medication; and GPi-DBS may allow better improvement in mood dysfunctions, e.g. depression, verbal fluency, and overall quality of life (Tan et al. 2016; Mansouri et al. 2018). Other less common targets for DBS include the thalamic ventralis intermedius nucleus (Vim) specifically for the management of tremor (Parihar et al. 2015; Cury et al. 2017) and the brainstem pedunculopontine nucleus (PPN) for reducing fall and freeze-of-gait (Pereira et al. 2008; Hamani et al. 2016; French and Muthusamy 2018). Despite the increasing popularity (more than 150,000 cases (J. A. Obeso et al. 2017a)) and the drastic improvements in many motor symptoms of PD, DBS does not provide complete relief of all motor deficits nor does it eliminate the necessity of supplementary DA therapy (Werner Poewe et al. 2017; French and Muthusamy 2018).

Just like any other surgical procedures, DBS is not without side-effects or complications. Putting aside surgery-related complications, there have been reports of abnormal

tingling sensations (paresthesias, especially in thalamic DBS), speech problems (dysarthria, especially in thalamic DBS), involuntary movements (dyskinesias) (S. Zhang et al. 2016; Akram et al. 2017) and impulse control problems (e.g., pathological gambling, compulsive eating) (Hälbig et al. 2009; Dodd et al. 2005; Broen et al. 2011; Merola et al. 2017).

1.5.2 Hypotheses on the mechanisms of action

How does DBS affect the pathophysiology of the basal ganglia system to generate the symptomatic relief? How can we further improve its efficacy while reducing the risks of side effects? Unfortunately after more than two decades of clinical application and continuous efforts in electrophysiological studies, the exact mechanisms of the therapeutic actions of DBS had remained unclear and largely controversial (Deniau et al. 2010b; Chiken and Nambu 2015; Hickey and Stacy 2016; J. A. Obeso et al. 2017a; Werner Poewe et al. 2017). Here I will introduce several perspectives.

First, let's trace back to the history of DBS, especially that of the STN-DBS (see reviews by (Sironi 2011; Gardner 2013)). Through a series of serendipitous discoveries in ablative neurosurgeries (Svennilson et al. 1960) from 1940s to 1990s, it was gradually established that lesion of STN (subthalamotomy) and GPi (pallidotomy) could improve parkinsonian symptoms. On the other hand, deep brain stimulation of other targets, as well as electrical stimulation overall, had been experimented long before (Schwalb and Hamani 2008). Thus the attempts of using high frequency electrical stimulation as a form of transient and reversible lesion was made, which indeed resulted in anti-parkinsonian effects in MPTP non-human primates (Bergman et al. 1990). Shortly, demonstration of the drastic therapeutic efficacy was demonstrated intraoperatively in patients (A. L. Benabid et al. 1991; Pollak et al. 1993), which ultimately led to the approval of DBS for PD by the Food and Drug Administration (FDA) in 2002 (Gardner 2013).

It is because of this close history with ablation, action through local inhibition was

first proposed. This perspective was well received also because: 1) it matched well with the observed increase in STN firing rate in animal models and in patients (Miller and DeLong 1987; Fillion and Tremblay 1991; Welter et al. 2004; Remple et al. 2011), corroborated also by metabolic evidence (Lin et al. 2008); 2) high-frequency electrical stimulation may achieve local inhibition of STN activity through depolarization blocks (Benazzouz and Hallett 2000; Meissner et al. 2005). However, HFS could also cause excitation of fibers (McIntyre, Savasta, et al. 2004; Tehovnik and Slocum 2013), (Deniau et al. 2010b; Gunalan et al. 2017), and both STN and GPi reside in a space closely packed by neighboring structures and fibers of passage (f.o.b.). Complex responses could be elicited from the activation of fibers: on one hand activation of the GABAergic GPe-STN projections could lead to inhibition of STN (Chiken and Nambu 2013); excitation of STN output fibers, on the contrary, effectively excites STN (Hashimoto et al. 2003); and much evidence has also demonstrated the activation of corticosubthalamic hyperdirect pathway and the antidromic activation of motor cortex (Tai et al. 2003; McIntyre, Savasta, et al. 2004); (Paz, Deniau, and Charpier 2005; S. Li et al. 2007; Q. Li et al. 2012; Kita and Kita 2012). Further challenges also come from conflicting reports of the lack of rate modulation in STN (Welter et al. 2004).

Based on the mixed evidence for inhibitory and excitatory system level effects, mechanisms through normalization of errant firing patterns was also suggested (S. Li et al. 2007; Q. Li et al. 2012; Chiken and Nambu 2015). Also supporting this hypothesis, abnormal synchrony in the motor cortex and STN can be ameliorated by effective and exacerbated by certain ineffective DBS (Tinkhauser et al. 2017; Weinberger et al. 2006).

Overall, this elusiveness of DBS comes mainly from the lack of specificity in effects of electrical stimulation and the complexity of the basal ganglia circuitry. Understanding of the mechanism of DBS and identification of new targets for improved efficacy could benefit from pathway-specific and cell-type specific modulation methods.

1.5.3 On-demand stimulation based on ongoing pathophysiology

Adaptive DBS, using STN beta activity to inform and modify DBS amplitude and ON/OFF has been shown to provide as effective treatment as continuous DBS, while the power consumption and risks to dyskinesia are significantly reduced (Little et al. 2013; Rosa et al. 2015; Piña-Fuentes et al. 2017; Rosa et al. 2017). Demonstration of the feedback signals using motor cortical oscillations also begin to gain traction (Swann et al. 2018), as the cortex has the advantages of bigger size, the easier accessibility to less invasive monitoring methods (e.g. ECoG, EEG, MEG, fNIRS), and the manifestation cortical fine-tune gamma as biomarkers for dyskinesia (Dupre et al. 2016; Swann et al. 2016).

Both approaches are premised on the phasic, not tonic, presence of pathological (or particularly pathological) beta activities (Tinkhauser et al. 2017). Nevertheless, a common issue is how to discern pathological beta activity from the normally occurring ones in the absence of true reference point; currently both the determination of beta threshold and the mapping from beta power to stimulation amplitude were determined arbitrarily. To shed light on this issue, part of this thesis will investigate the spatial variations and the temporal dynamics of the cortical beta activity recorded in high spatial resolution with microelectrode array (MEAs).

1.6 Optogenetics in the Study of Parkinsons Disease

1.6.1 An overview of optogenetics

Optogenetics generally refers to a range of methods and tools that genetically modify neurons to express light-sensitive protein channels, named opsins, for the control of neural activities (or other cellular biochemical processes) with light (see review by (Yizhar et al. 2011; Delbeke et al. 2017)). A more specific definition was used in this entire thesis, *i.e.* the use of light-sensitive opsins to directly modulate membrane potentials of neurons and generation of action potentials.

Optogenetics brings many favorable features over electrical stimulation: 1) optical stimulation is more compatible with electrophysiological recording with much reduced stimulus-related artifacts; 2) cellular specificity of stimulation effect is greatly improved with building upon the genetic targeting principles; 3) existing anatomical connections can be utilized by optogenetics, such that by transducing opsins in the soma of neurons and delivery of light at the axonal terminal area, modulations can be achieved in the projection targets (Yizhar et al. 2011; A. V. Kravitz, Devarakonda, and Kreitzer 2017); and 4) by choosing the appropriate opsins, modulations of a variety of nature can be achieved in a combinatorial manner, including inhibition versus excitation, as well as the various temporal course of response (“momentary response” - opsin is turned on/off only during the period of illumination; “step-function”/“bistable” - opsin remains in its operational state for a period of time after light offset (Berndt et al. 2014)).

Genetics modifications *in vivo* in mammals are generally achieved in two ways: either through virus-based transduction or by the use of transgenic animals (Cre-recombinase driver lines; readers are referred to the review by Yizhar et.al. for details (Yizhar et al. 2011)). For studies requiring more complex behaviors or outlooking towards translational applications, transgenic mice models would not be appropriate. Despite ongoing efforts towards transgenesis (Oh et al. 2017), virus transduction based approaches are still essential

for optogenetics in higher mammalian species. Briefly, I will discuss several key factors in viral transduction with adeno-associated viral vectors (AAV). First, AAVs and especially the recombinant AAVs (rAAVs) are highly engineered for safety: 1) while they are relatively less immunogenic, they do not cause known human pathology; 2) while they retain invasiveness to the cells, they cannot replicate in the host cell; 3) only 10% of their DNAs integrate into host chromosomes (Delbeke et al. 2017).

The tissue and cell specificity is achieved through both the tropism of the specific AAV serotype and the choice of promoters. Promoters are special sequences on the viral vector that help recruit the transcriptional amplification mechanisms of the host neurons to enable mass production of the opsins together (Daya and Berns, 2008). Commonly used promoters include Human Synapsin1 (hSyn1), which has pan-neuron specificity and Ca²⁺/calmodulin-dependent protein kinase II- α (CaMKII α), which targets glutamatergic excitatory neurons. Nonetheless, reliable promoters suitable for targeting of inhibitory neurons has not been well established, independent of transgenesis.

In addition, genes for reporter fluorophores are included in the vector. Co-expression of the reporter fluorophore with opsin allows histological confirmation and assessment of expression level. Commonly used fluorophores include GFP (green), YFP (yellow), and mCherry (red). Finally, other sequences can be included to enhance transcription, modify packaging, and affect membrane insertion, as well as localization of the opsin.

1.6.2 Dissection of neural mechanisms by optogenetics

Optogenetics has been a powerful tool for study of brain functions. Most of the optogenetic studies in the context of PD was performed in transgenic mice expressing Cre-recombinase. Here I will summarize key literature pertinent to this thesis.

First, a seminal work by Gradinaru et al. (Gradinaru et al. 2009a) in hemi-Parkinsonian rodents demonstrated therapeutic effects (improvement in overall mobility and

reduction of rotational bias) by optogenetic excitation of the hyperdirect pathway in transgenic mice; on the contrary, no significant benefits were found for excitation or inhibition of the principal STN neurons in non-transgenic rats. Subsequently, Yoon et al. ((Gradinaru et al. 2009b; Yoon et al. 2014) reported conflicting observations that pan-neuron inhibition of STN indeed improved certain aspects of motor function (forelimb asymmetry) in non-transgenic rats.

More recently, using a variant targeting scheme, yet still relying on the transgenic mice, the efficacy of the excitation of the hyperdirect pathway in improving bradykinesia was further confirmed (Sanders and Jaeger 2016). Furthermore, in a combined optogenetic and chemogenetic study, reduction in the strength of the hyperdirect pathway (transmission effectiveness and the number of synapses) after dopamine depletion was causally linked to symptoms of akinesia (Chu et al. 2017).

Optogenetic studies in transgenic mice (with bilateral partial DA-depletion or healthy) also looked at the dichotomous functions of D1- and D2-MSNs in the striatum and showed amelioration of akinesia (also increase in contralateral rotation) by the excitation of D1-MSNs (DA-like action on the direct pathway) and exacerbation of akinesia by the excitation of D2-MSNs (DA-loss-like action on the indirect pathway) (Alexxai V. Kravitz et al. 2010; Freeze et al. 2013; Tecuapetla et al. 2014)). A later study in unilaterally partially DA-depleted transgenic mice showed increased ipsilateral rotations by the inhibition of D1- and D2-MSNs independently or simultaneously ((Alexxai V. Kravitz et al. 2010; Freeze et al. 2013; Tecuapetla et al. 2014), providing a new perspective on the importance of coordination between the two pathways.

For another nucleus in the indirect pathway, the GPe, a very recent report demonstrated rescue of bradykinesia by selective modulation of subpopulations of its GABAergic neurons (Mastro et al. 2017). Finally, several target structures of basal ganglia were also studied with optogenetics in relation to motor functions, for example the motor thalamus (Seeger-Armbruster et al. 2015) and the pedunculopontine nucleus (PPN) (Roseberry et

al. 2016).

Overall, there is no doubt that optogenetic approaches are crucial in the study of Parkinsonian pathophysiology. However, translation towards human therapy need to overcome the use of transgenic animal models. Furthermore, there has not been a unified study that tests the therapeutic effects of modulations at multiple locations of the basal ganglia circuitry (cortex and subcortical nuclei).

1.7 Goals of this Thesis

As seen in this chapter, how basal ganglia pathophysiology leads to the manifestation of Parkinsonian motor symptoms and how deep brain stimulation exerts its therapeutic effects are not completely understood. In particular, it is not clear whether rescue of motor symptoms can be achieved at multiple stages of the cortico-basal-ganglia circuitry, specifically the motor cortex and the indirect pathway. Additionally, most prior success of optogenetics-based symptomatic relief had been performed in the transgenic mice model. From a clinical translation point of view, it is pertinent to validate whether therapeutic effects can be reliably achieved through non-transgenic virus injection methods. Hence it is necessary to develop and validate methodological approaches to achieve precise and functional opsin expression in the basal ganglia nuclei. Last but not least, potential future translation of the principles of these new stimulation paradigms to the treatment of PD would demand a continuous, short latency feedback neural biomarker that correlates well with the motor symptoms. Studying fine-scale spatiotemporal neural dynamics during Parkinsonian motor behavior and optogenetic perturbations provides a great opportunity. Motivated by these scientific and technological considerations, the goals of this thesis are to:

1. Develop and validate experimental methods to achieve functionally robust, spatially precise, and geometrically appropriate opsin expression in the cortico-basal-ganglia system of non-transgenic rats (chapter 2).
2. Investigate the therapeutic effects on motor symptoms in 6-OHDA-induced hemi-Parkinsonian rats by optogenetic modulations of multiple loci of the cortico-basal-ganglia circuit, in particular focusing on the hypotheses that inhibition of the subthalamic nucleus, excitation of the globus pallidus externus, and excitation of the motor cortex could rescue the pathological rotational behaviors (chapter 3).
3. Characterize distinguishing motor cortical population activity patterns and features

as reporters for the neural and motor pathology in freely behaving hemi-Parkinsonian rats, in particular to study the spatial and temporal inhomogeneity in the power and synchrony of the 25-40 Hz beta-band oscillatory activities (chapter 4).

Chapter 2

Optogenetic Modulations in the Cortico-basal-ganglia System of Non-transgenic Rats

2.1 Introduction

Optogenetics is a crucial tool to the causal interrogation of brain functions, granting deterministic control, millisecond precision, and cellular specificity of the stimulation (Boyden et al. 2005). Indeed, optogenetics has been employed in the study of basal ganglia functions in the context of Parkinson's disease (PD). As mentioned previously, these attempts include inhibition of the subthalamic nucleus (STN) (Gradinaru et al. 2009; Yoon et al. 2014), stimulation of the motor cortex (MC) (Gradinaru et al. 2009), stimulation of the cortico-subthalamic projections (Gradinaru et al. 2009a; Chu et al. 2017), as well as manipulations of striatal medium-spiny neurons (MSNs) (Alexxai V. Kravitz et al. 2010; Tecuapetla et al. 2014; H. J. Lee et al. 2016) in the striatum and GABAergic neurons in globus pallidus externus (GPe) (Mastro et al. 2017).

However, a majority of these studies relied on transgenic mice in whole or in part to achieve the targeted optogenetic modulations. In this thesis, in rats, for which transgenic methods were not readily available, I aimed to achieve reliable, robust, and functional opsin expression in the STN, GPe, and the motor cortex (choice of these targets was briefly motivated in the previous chapter and will be explained in detail in the next chapter). A non-transgenic rat model was important because 1) rats have larger brain and body sizes allowing accommodation of large implants without significantly constraining their movements and they are also capable of more complex behaviors (Iannaccone and Jacob 2009), however transgenic methods have not been well-established in rats; 2) aiming towards potential clinical translation of optogenetic modulations, relying on transgenic methods would not be feasible for humans. Therefore, non-transgenic methods need to be evaluated for their feasibility and efficacy.

In this chapter, I will report, respectively for STN, GPe, and motor cortex, the methodological approaches we developed to achieve targeted and robust opsin expression, when facing drastically different constraints associated with each nucleus. I will also discuss

the results from series of validation experiments and show that in each case opsin expression in the target area was functional: enabling modulation of neural activities locally and in the projection targets and even elicitation of direct motor responses in the case of motor cortex optogenetic modulation. Results from this chapter are the foundation for the interrogation of the therapeutic rescue of rotational behaviors in a hemi-Parkinsonian rat model by the various optogenetic modulation paradigms.

I want to emphasize that most of the work in the chapter was done either together with Dr. Ilker Ozden or under his direct supervision.

2.2 Achieving Optogenetic Inhibition of the Subthalamic Nucleus

In non-transgenic rats, cell-specific opsin expression in the STN is a non-trivial task. On one hand, as STN houses predominantly excitatory neurons, opsin expression in these neurons can be achieved with the control of the Ca^{2+} /calmodulin-dependent protein kinase II- α (CaMKII) promoter (Gradinaru et al. 2009a). However, this is complicated by the richness of other Glutamatergic neurons in the vicinity of STN, especially in the area medial, i.e. the lateral hypothalamus (Stamatakis et al. 2016), and dorsal, i.e. the zona incerta (Heise and Mitrofanis 2004), to the STN. Further complication comes from the geometry of the STN: STN is a disk-shaped structure of about 0.8mm^3 in volume (Hardman et al. 2002), with a vertical dimension of about 0.5 mm and lateral dimension of about 1mm in rats (Fig. 2.1 A). Therefore, accurate targeting is required to ensure maximization of opsin expression specificity within the STN.

In this section, I will first show results from a STN targeting study performed in the early phase of my thesis and then show histology and electrophysiology evidence of successful STN inhibition by optical stimulation. Results from this section pave the path towards optogenetic modulation of hemi-Parkinsonian motor symptoms discussed in the

next chapter.

2.2.1 Expression of inhibitory opsin in the STN achieved with electrophysiology-assisted targeting

Although the stereotaxic coordinates of STN have long been established, the depth of the nucleus (-7.8 mm DV from dural surface) as well as the limited lateral extent poses low tolerance in the track angle ($< 4^\circ$ w.r.t. the DV axis). Fortunately, STN has rather distinct and characteristic features in extracellular electrophysiology: a high level of background activity as well as symmetric, long duration, and large amplitude morphologies in spiking, compared to the two quieter regions above (zona incerta, ZI) and beneath (internal capsule, i.c.).

Here I will briefly describe the characteristic of each nucleus along the dorsal-ventral axis: firstly abundant spiking activity was first observed in the ventral part of the thalamus (under Ketamine/Xylazine anesthesia, auditorized MUA activity showed characteristic “stepping-on-the-snow” phenotype across the thalamus; when exiting the thalamus, the background activity decreased, followed by sharp and rapid spiking passing the medial lemniscus bundle; afterwards, the electrode travelled through zona incerta (ZI), which showed much suppressed background activity with occasional high-amplitude short-duration spiking. STN was identified by the increased background and spiking activity after the ZI. The discrimination of the ventral part of ZI from the dorsal border of STN was initially difficult, as both structure may display high-amplitude spiking. A key point of differentiation was that spiking neurons were sparse in ZI, but dense in STN: STN was confirmed if elevated high-amplitude symmetrical spiking phenotypes persisted over a range of at least 200-300 μm . The internal capsule (or cerebral peduncle, which carries fiber bundles including the corticospinal tract) bordered STN ventrally, and displayed much reduced background activity and small amplitude, very rapid spiking. Our learning process was greatly accelerated by using electrolytic lesions to mark the location of the electrophysiologically determined

STN sites in four healthy rats (Fig. 2.1). With establish experience and confidence, we proceeded to validate opsin expression in STN.

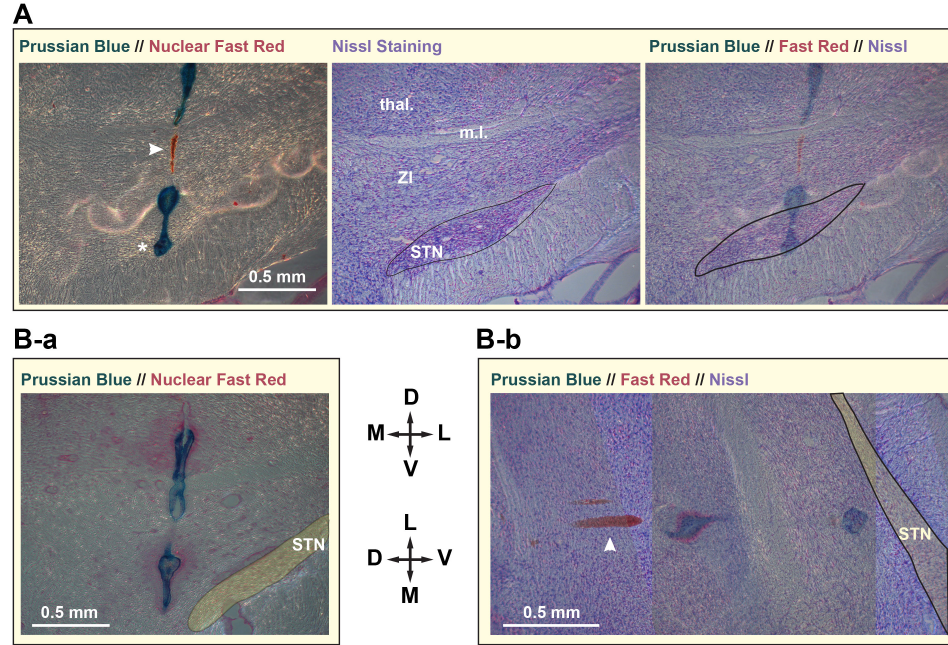


Figure 2.1: Examples of STN targeting outcomes in the early phase of the study. Electrolytic lesion sites, i.e. electrode tip locations, were indicated by Prussian Blue staining (dark blue) and were counterstained with Nuclear Fast Red (red) or Nissl staining (violet). Images were obtained from phase-contrast and brightfield microscopy. Multiple electrolytic lesions were performed to visualize the track; the deepest sites corresponded to the suspected STN location based on electrophysiology. **A.** Histology confirmed electrophysiologically identified STN location. **B.** Two examples where lack of characteristic STN electrophysiology corresponded to lesions sites outside of the STN. B-a: electrode missed STN medially; B-b: despite on the track to the STN, the depth of the STN was not successfully identified. When Prussian Blue//Nuclear Fast Red images were overlaid on Nissl images, the former was made 70% transparent. The white arrowhead indicated residual blood carried by the electrode insertion from the brain surface. The anatomical contour of the STN was indicated by a black outline with or without the shade. In the lower middle portion of the figure, orientation of images in A and B-a was indicated in the top compass, and that of B-b was indicated in the bottom compass. Abbreviations: thal., thalamus; ZI: zona incerta.

Fluorescence images confirmed that virus injection assisted with electrophysiology successfully transduced the STN with excitatory (ChR2, data not shown) and inhibitory opsins (eNpHR3.0 and SwiChRCA) (Fig. 2.2 A-B). The functional significance of these opsins will be discussed in the next chapter.

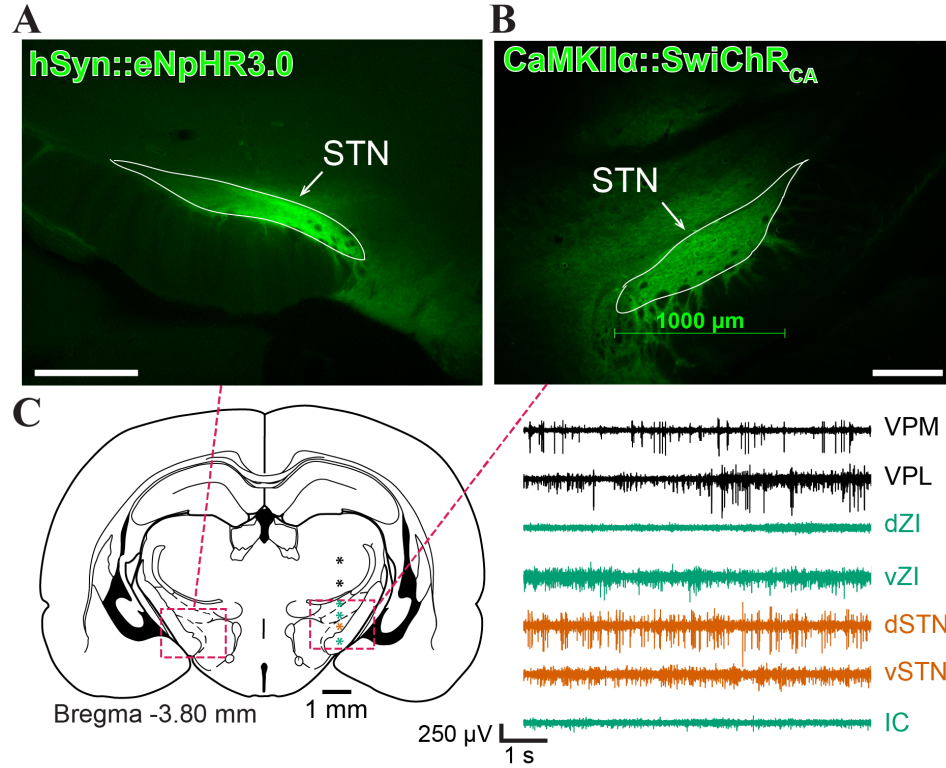


Figure 2.2: Opsin expression of STN achieved with the assistance of intraoperative electrophysiology. **A-B.** Expression of opsin (A: hSyn::eNpHR3.0; and B: CaMKIIα::SwiChR_{CA}) in the STN. The white outline indicated the location of STN. **C.** Electrophysiological characteristics along the STN track. Abbreviations: VPM, thalamic ventral posteromedial nucleus; VPL, thalamic ventral posterolateral nucleus; dZI/vZI: dorsal and ventral part of the zona incerta; IC, internal capsule. Reprinted from (Yu et al. 2017).

2.2.2 Optogenetic inhibition of STN spiking activities

After allowing 6 weeks for opsin transduction and maturation after virus injection, optogenetic inhibition of STN spiking activities was verified during intraoperative electrophysiology. Unlike the excitatory counterpart, validation of inhibitory opsin expression required optical stimuli of long pulse duration (Fig. 2.3); this is because spontaneous spiking activity was generally reduced during anesthesia and often rebound effects (post-stimulus increase in firing rate) were observed only after prolonged inhibition. In fact optogenetic inhibition *in vivo* did not always silence the modulated neurons and in many cases only a suppression

of firing rate was found (Fig. 2.3 B, right). This is likely because the increased hyperpolarizing membrane current from the activation of inhibitory opsins could be partially offset by other excitatory inputs through the network (even the local recurrent network).

In summary, electrophysiology-assisted targeting method allowed functional opsin expression within the STN.

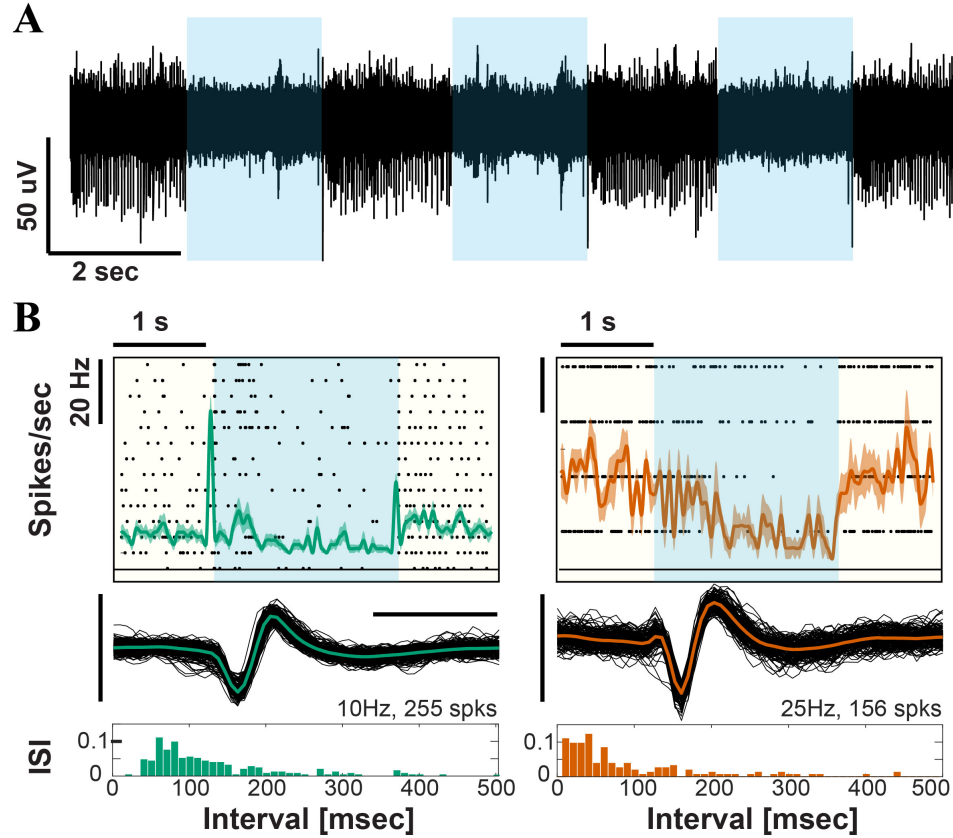


Figure 2.3: Intraoperative validation of optogenetic inhibition of STN spiking activity with SwiChR_{CA}. **A.** An example trace of MUA activity showing inhibition of spiking activity during optical stimulation (473 nm, light green shaded areas). **B.** Two isolated STN units with their trial-averaged peri-stimulus firing rate traces, spike waveforms, and interspike-interval (ISI) plots. The green unit was extracted from A; and the yellow unit was extract at a different STN recording site.

2.3 Achieving Optogenetic Modulation of the Globus Pallidus Externus

GPe is a key component of the indirect pathway (receiving GABAergic D2-MSNs efferents), which houses predominantly inhibitory neurons (subdivided into two major populations, see discussion in the next chapter). GPe neurons synapse strongly onto the STN, and at the same time receive excitatory afferents from the STN. In the healthy state, firing rate of GPe neurons correlated with bodily movements (D. Jaeger, Gilman, and Aldridge 1995; Mushiake and Strick 1995; Arkadir et al. 2004; Adler et al. 2010; Schmidt et al. 2013). Through disinhibition, GPe is suggested as an important relay station to oppose or focus the effects of the general inhibitory actions of the indirect pathway on motor output (Atsushi Nambu 2007). Disruption of the overall network, including the loss of dopamine alone, leads to plastic changes in the electrophysiology of GPe. A number of studies confirmed the prediction of the rate model that the activity of GPe was indeed reduced in a Parkinsonian state (Miller and DeLong 1987; Fillion and Tremblay 1991). Changed patterns of firing, increased synchronous bursting activities and increased functional connectivity was also reported in the GPe across several mammalian models (Bergman et al. 1998; Magill, Bolam, and Bevan 2000, 2001; Peter Brown 2003; Mallet et al. 2008; Tachibana et al. 2011; McConnell et al. 2012). Recent accumulation of anatomical, electrophysiological, and computational evidence points to even greater functional importance of the GPe, as efferent projections from GPe were found in essentially all basal ganglia nuclei (Hegeman et al. 2016).

Given the anatomical and functional complexity of the GPe, optogenetic methods had been highly anticipated to provide better dissection of the involved circuitry. Unlike the case for the excitatory Glutamatergic neurons in the STN, there has not been a virus serotype or promoter (engineered into a viral vector) that specifically targets GABAergic neurons. Until very recently have several groups successfully demonstrated cell-specific

optogenetic modulation of different subpopulations of GPe neurons (Mastro et al. 2017), though still relying on Cre-recombinase transgenic mice. In this section, I discuss how we achieved and verified functional opsin expression in the GPe, laying foundations for optogenetic modulation of hemi-Parkinsonian motor symptoms discussed in the next chapter.

2.3.1 Expression of excitatory opsin in the GPe

Viral vector injection in the GPe was preceded with a similar method of electrophysiology-assisted targeting, although much more straightforward compared to that of STN: GPe has a volume of about 10.3 mm^3 , (Hardman et al. 2002), a lateral dimension of about 1.5-2.0 mm, and a vertical dimension of about 2.0-2.5 mm. Here I will briefly summarize the characteristic features of nucleus along the path towards GPe: the exit from cortex was indicated by a marked reduction in the background; within the striatum, the background activity was slightly elevated but encounter of spiking units (medium-spiny neurons have low spontaneous firing rates) were sparse (Berke et al. 2004); the dorsal border of GPe was clearly marked by a drastic increase in the background and spiking activities; the ventral border of GPe was less clearly defined and was deduced by matching the border of the passing structures to their stereotaxic coordinates.

Because of the larger size of GPe, two $1 \mu\text{L}$ injections were performed at -6.0 mm and -5.5 mm DV. A synapsin promoter was used for pan-neuron expression, due to the lack of promoters specific for inhibitory neurons. Opsin expression in the entire GPe was achieved with this method, shown in the example fluorescence image from a hemi-Parkinsonian rat (Fig. 2.4). It was surprising, yet fortunate that the expression did not breach significantly into the striatum, despite the lack of cellular specificity. In fact, our results also confirmed that GPe had many efferent projection targets: including STN, GPi (Lindahl et al. 2013) and thalamic reticular nucleus (TRN) (Cornwall, Cooper, and Phillipson 1990; Gasca-Martinez et al. 2010; Oh et al. 2017).

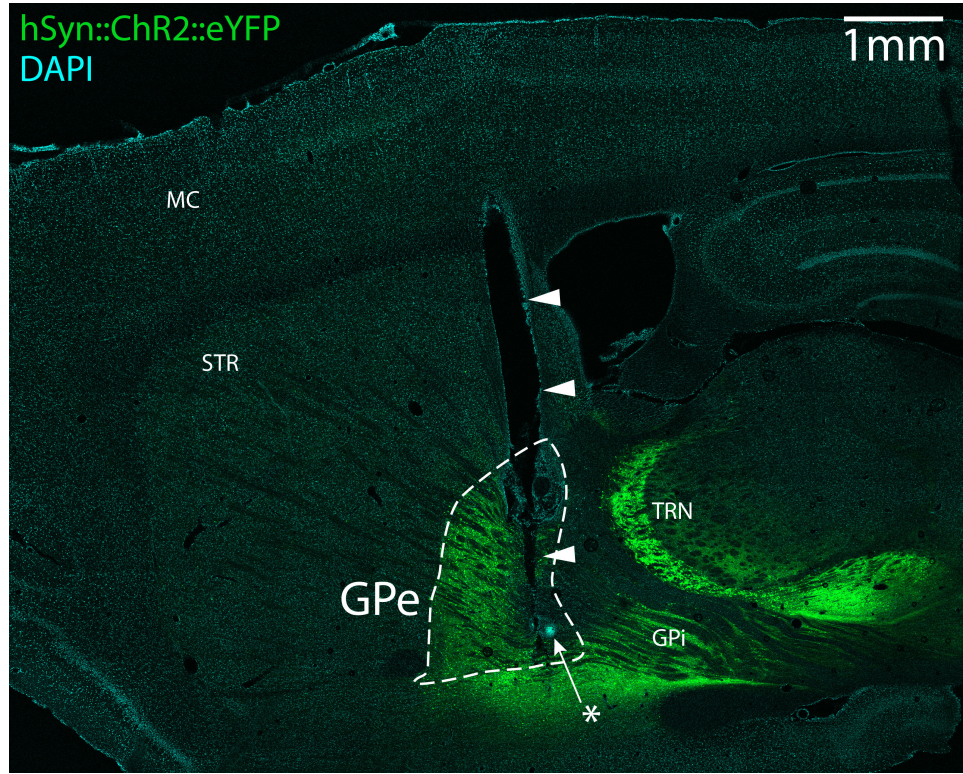


Figure 2.4: Trident probe was implanted within opsin-expressing GPe in a h-P rat. The dashed outline indicated the location of GPe. The Trident track (arrowheads) was observable. The location of the tip of the electrode was visualized through electrolytic lesion, which leads to the bright dot of DAPI fluorescence (*). The thalamic reticular nucleus (TRN, the shell-like structure on the right side of GPe) and GPi were also labeled as they are the projection targets of GPe.

2.3.2 Modulation of electrophysiology of GPe and the associated basal ganglia circuit by optogenetic excitation of GPe

First, in three healthy animals, we performed simultaneous extracellular recording in GPe and STN and confirmed that optogenetic activation of GPe lead to increased inhibitory actions on STN (Fig. 2.5).

Then, in two hemi-Parkinsonian animals, we implanted a Trident probe (Section 3.11.2) in the GPe and a 6x6 MEA in the ipsilateral motor cortex. Simultaneous extracellular recording showed that optogenetic stimulation at GPe lead to modulations in LFP and spiking activity in both GPe and the motor cortex. LFP responses in the GPe (Fig. 2.6 A,

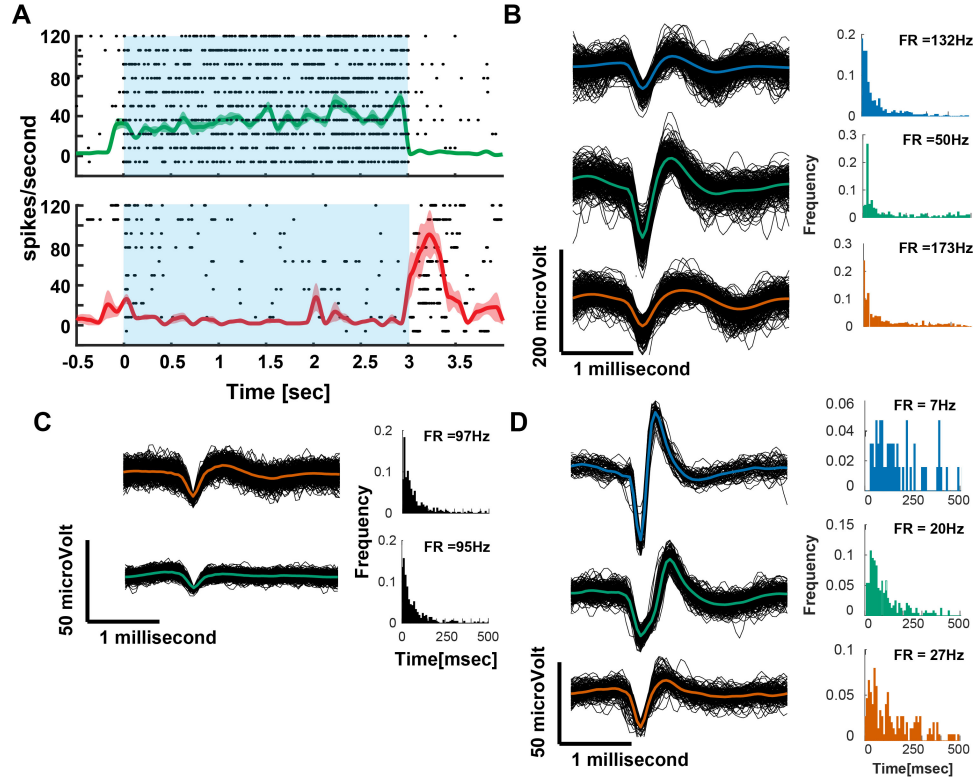


Figure 2.5: Electrophysiological modulations evoked by GPe-oStim in GPe and STN of healthy rats. **A.** Multi-unit PSTH for a pair of GPe (green) and STN (red) neurons peri-optical stimulation at GPe. The GPe channel showed clear excitation, in the form of ramping up of firing rate; while the STN firing rate showed sustained inhibition and a post-stimulus rebound effect. The firing rate trace was smoothed over a 100ms moving window for display. **B-D.** Examples of waveforms and interspike-interval (ISI) histograms from isolated GPe and STN units. **B.** Three units excited at a different GPe location. A mix of short and long duration spikes were observed. **C.** The GPe multiunit activity in A was sorted and two short duration, fast-spiking units were observed. **D.** Sorted STN units showed the stereotypical regularly spiking (RS), high-amplitude, wide, symmetrical morphology.

Fig. 2.7 A) showed negative deflection that corresponded to the excitation.

In the motor cortex, two peaks were found following a 10 msec pulse of GPe stimulation. The first peak, P1 (green arrow, Fig. 2.6 A), was found in all 32 channels, which had an onset latency of 5-9 msec (Fig. 2.6 B) and peaked at the termination of the stimulation. The presence of the onset delay distinguished this response from the direct photovoltaic phenomenon. A late and broader peak, P2 (blue arrow, Fig. 2.6 A), occurred after the

short stimuli in the medial part of the array (Fig. 2.6 C). When stimulation power was increased, while the amplitudes of P1 increased, amplitudes of P2 were not significantly changed (except that when P1 was large, the prominence of P2 was reduced, so did the reliability of P2 detection) (Fig. 2.6 D). This saturation effect also corroborated that LFP responses in the motor cortex were actual optogenetic responses.

While earlier studies showed electrical stimulation of the motor cortex evoked triphasic response (early excitation, short inhibition, and late excitation) in the GPe (MC→GPe) (A. Nambu et al. 2000), characterization of cortically-evoked responses by GPe stimulation (GPe→MC) was not available. Due to the inhibitory nature of GPe output, the short latency of P1 was unlikely a result of modulation through the canonical basal ganglia circuitry. A recently discovered GPe-MC direct GABAergic projection (Saunders et al. 2015) may shed light on the cause of this inhibition-like early response. While the time scale of P2 matched better with the dynamics of the basal ganglia, the suggested inhibitory effect was counterintuitive to the predictions of the canonical models.

When a longer stimulation pulse was applied, a negative deflection and a positive deflection spaced the duration of the stimulation in GPe and MC, respectively (Fig. 2.7, middle column). The motor cortex responses showed dose-dependent decrease in the peak latency and a plateau effect at high power levels, which was not clear in that of GPe. A negative deflection was found at the offset of the stimulus. During high-frequency stimulation (HFS) at 100Hz (50% duty cycle) of 0.5 sec, the high frequency component of the stimuli could be found in the GPe trace but was much weaker in the MC. The initial high amplitude responses did not last through the entire duration of stimulation, suggesting perhaps adaptation to optical stimulation occurred within 500 msec. The offset responses were similar to that of the 100 msec stimulation but were dissimilar to that of the 10 msec stimulation.

The modulation outcomes on the cortical spiking activities were diverse: early excitation, sustained excitation, early excitation-late inhibition, sustained inhibition, and lack of

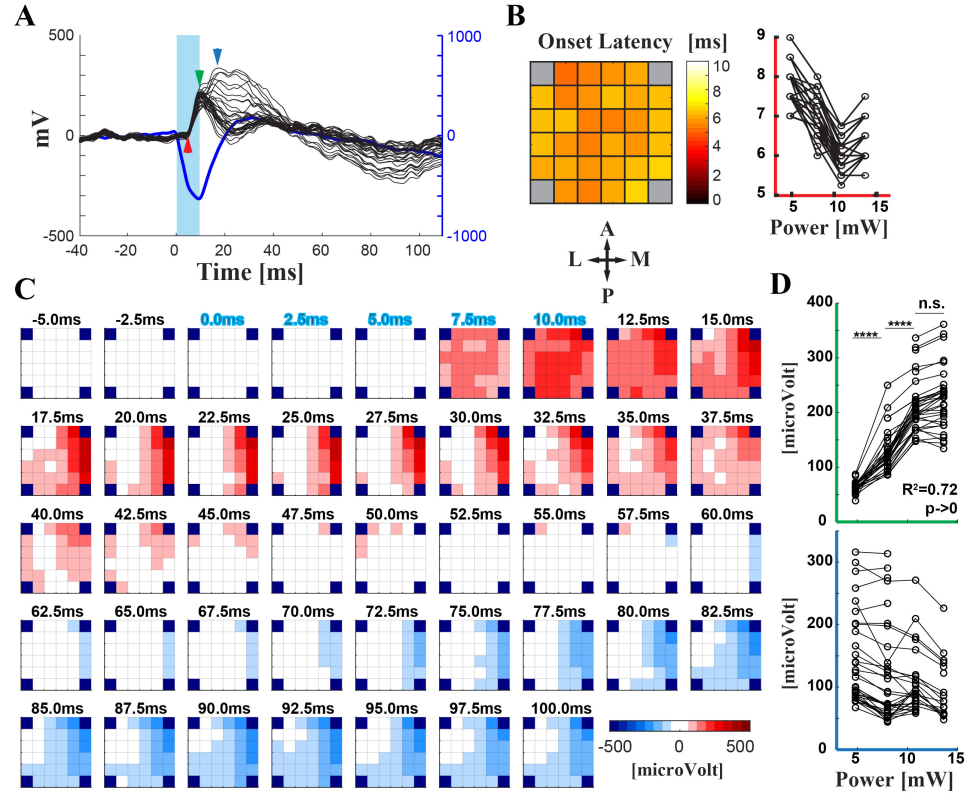


Figure 2.6: GPe-oStim evoked LFP responses in the motor cortex of a h-P rat. A. Overlaid traces of trial-average evoked LFP for each M1 channel (black, $n=32$) and one GPe channel (blue). The arrowheads marked the onset (red), early peak (green), and late peak (blue), respectively. The light shaded area indicated optical stimulation: 473nm, 10 msec pulses delivered at 2 seconds interval at 10 mW input power. **B.** Following the descriptions in A, the onset latency (red) across the array at 10 mW was illustrated in the heat map and then diagramed for different power levels. **C.** Temporal evolution of cortical evoked LFP responses depicted in the array map. Time of 0ms indicated the onset of the stimulation and the time of 10 msec indicated the offset. Continued increase in LFP amplitude was found in the medial portion of the array. A late long-duration depression was found in all channels and was pronounced in the medial channels. **D.** Dose response (power) of the amplitude of the early (green) and late (blue) positive deflections in the evoked response. A saturation effect was found for the early response (for the last pair $p < 0.5$). The slight decrease in the amplitude of the late response in high power cases was partially due to its reduced prominence as result of the increased amplitude in the early response ($p < 0.5$ for all pairs). For the arraymaps displayed in B and C, the orientation is defined as follows: top - bottom responds to rostral - caudal; left - right corresponds to lateral - medial. Paired t-test corrected for multiple comparisons were applied.

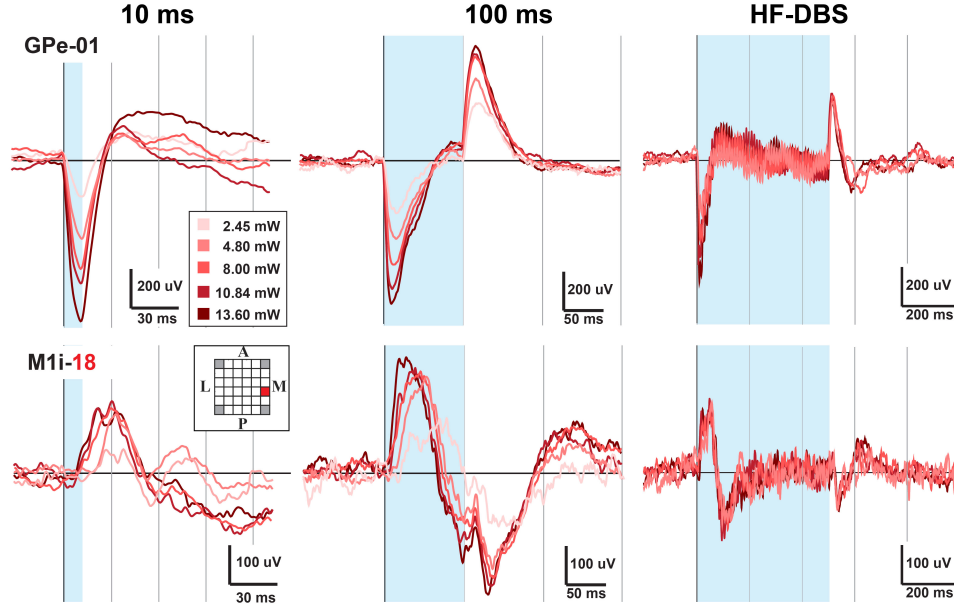


Figure 2.7: Examples of power-dose response of locally evoked LFP in GPe and cortically evoked LFP in the motor cortex by single-pulse or high-frequency (HF) GPe-oStim in awake h-P rat. Overlaid traces of trial-average evoked LFP for one GPe channel and one ipsilateral motor cortex channel. Three temporal stimulation protocols were used: single pulse of 10 msec pulse duration was repeated at 2 sec pulse interval; single pulse of 100 msec pulse duration was repeated at 2 sec pulse interval; and high-frequency (HF) stimulation at 100 Hz (80% duty cycle) of 0.5 sec burst duration was repeated at 2 sec burst interval. The blue shaded area indicated optical stimulation (473nm): in the HF case, the duration of the stimulation burst was shown. A least 30 trials were obtained for each condition.

clear modulation (Fig. 2.8). While the minimum power required for modulation of cortical spiking activity was below 0.62 mW, plateau effects also existed in sites showing increased firing rate by optical stimulation (Fig. 2.9, Fig. 2.10).

In summary, functional pan-neuron expression of excitatory opsins was achieved in GPe. Excitation of GPe lead to inhibition of STN and complex spatiotemporal responses in the motor cortex. While the former corroborated with the rate models, the motor cortex evoked responses exemplified the complexity of the network (convoluted responses through multiple pathways) beyond the (qualitative) model predictions.

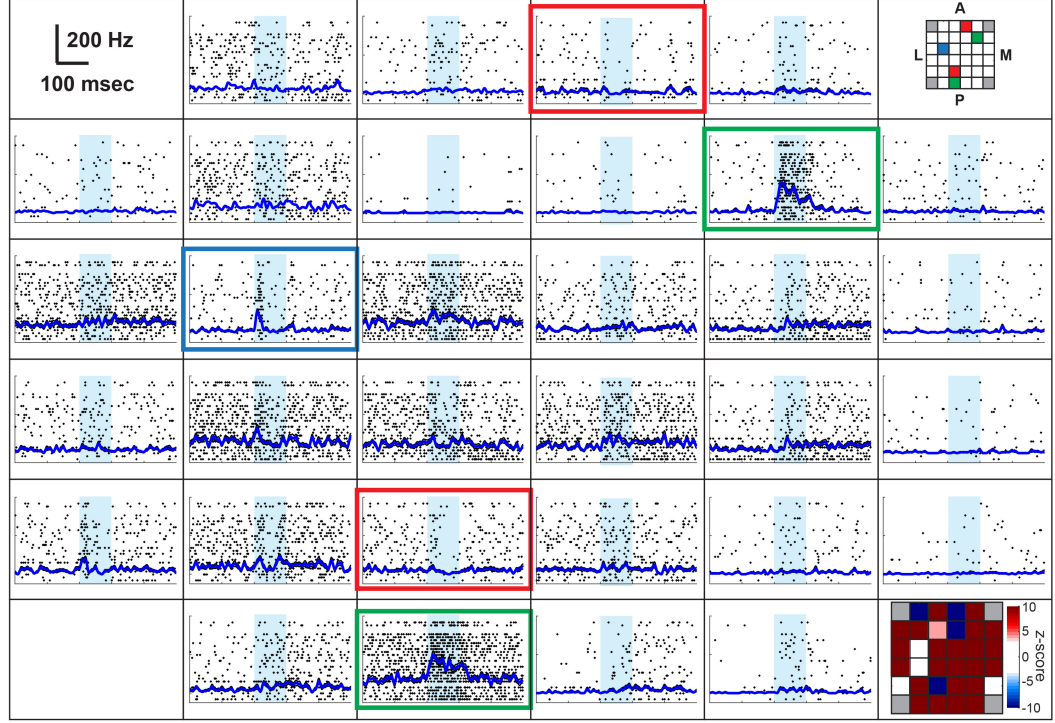


Figure 2.8: Trial-averaged response of spiking activity in the motor cortex to a 100 msec single-pulse GPe-oStim in awake h-P rat. A mixed outcome of early excitation (blue box), sustained excitation (green box), early excitation-late inhibition (red box), sustained inhibition, and lack of clear modulation were found across the motor cortex. The blue shaded area indicated the optical stimulation at 473nm. The raster plot was shown for each trail and each channel (some trials with movement artifacts were excluded in analysis but were still shown in the plot as empty rows). The array schematic on the top right corner showed orientation of the array as well as the locations of the three colored channels. The map on the bottom right corner summarized statistically significant effects: red colors for significant increase, blue for significant decrease, and white for unclear effects (z-test with respect to the average firing rate before stimulation). The size of the array was 2.0 mm x 2.0 mm.

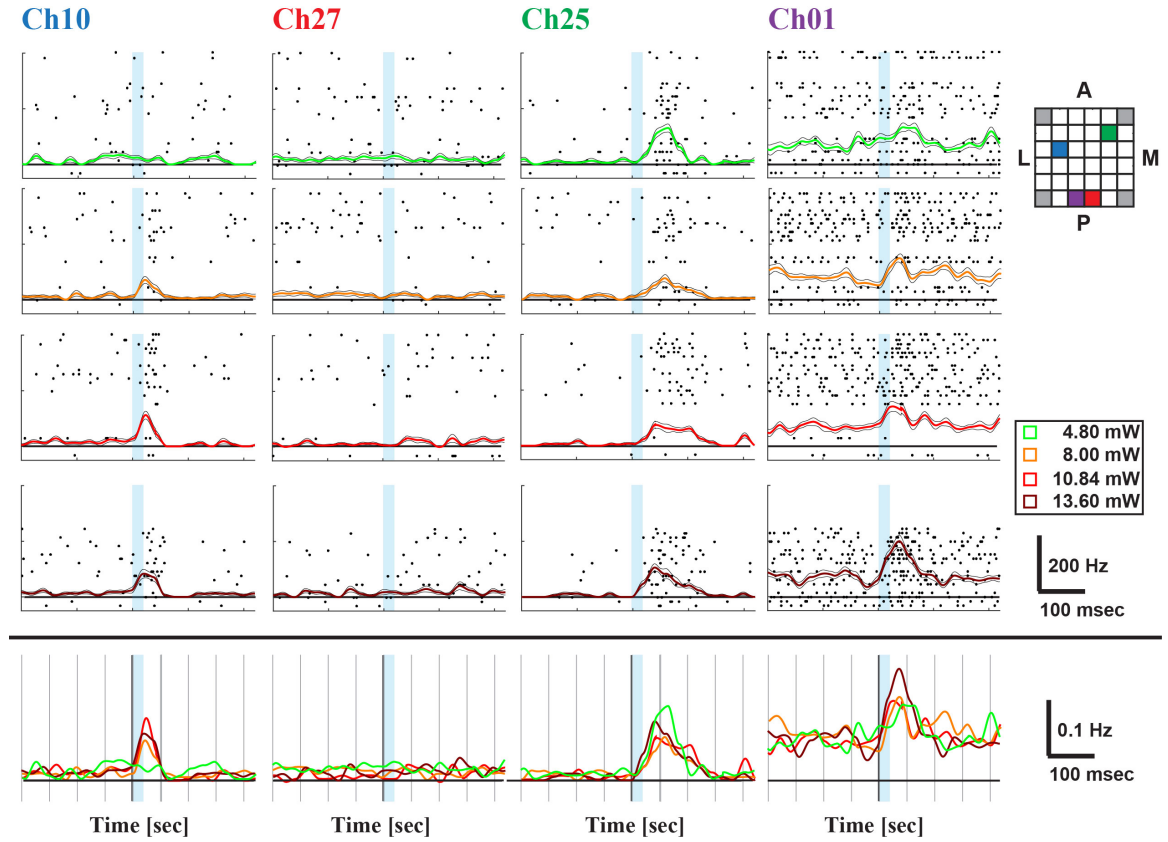


Figure 2.9: Dose-response characteristics of the cortically evoked spiking activity to a 10 msec single-pulse GPe-oStim in awake h-P rat transduced with ChR2. The 10 msec duration of the stimulation appeared to be shorter than the time required for modulation to reach the motor cortex. Excitatory responses were found in two varieties: short (Ch10) and long (Ch25 and Ch01) durations. Lack of clear modulation effects were also observed (Ch27). The threshold for modulation (Ch25) was below 4.80 mW (473 nm). Saturation in the responses were in general observed. The blue shaded area indicated the optical stimulation. The raster plot was shown for each trail and each channel (some trials with movement artifacts were excluded in analysis but were still shown in the plot as empty rows). The array schematic on the right side showed the orientation of the array as well as the locations of the colored channels.

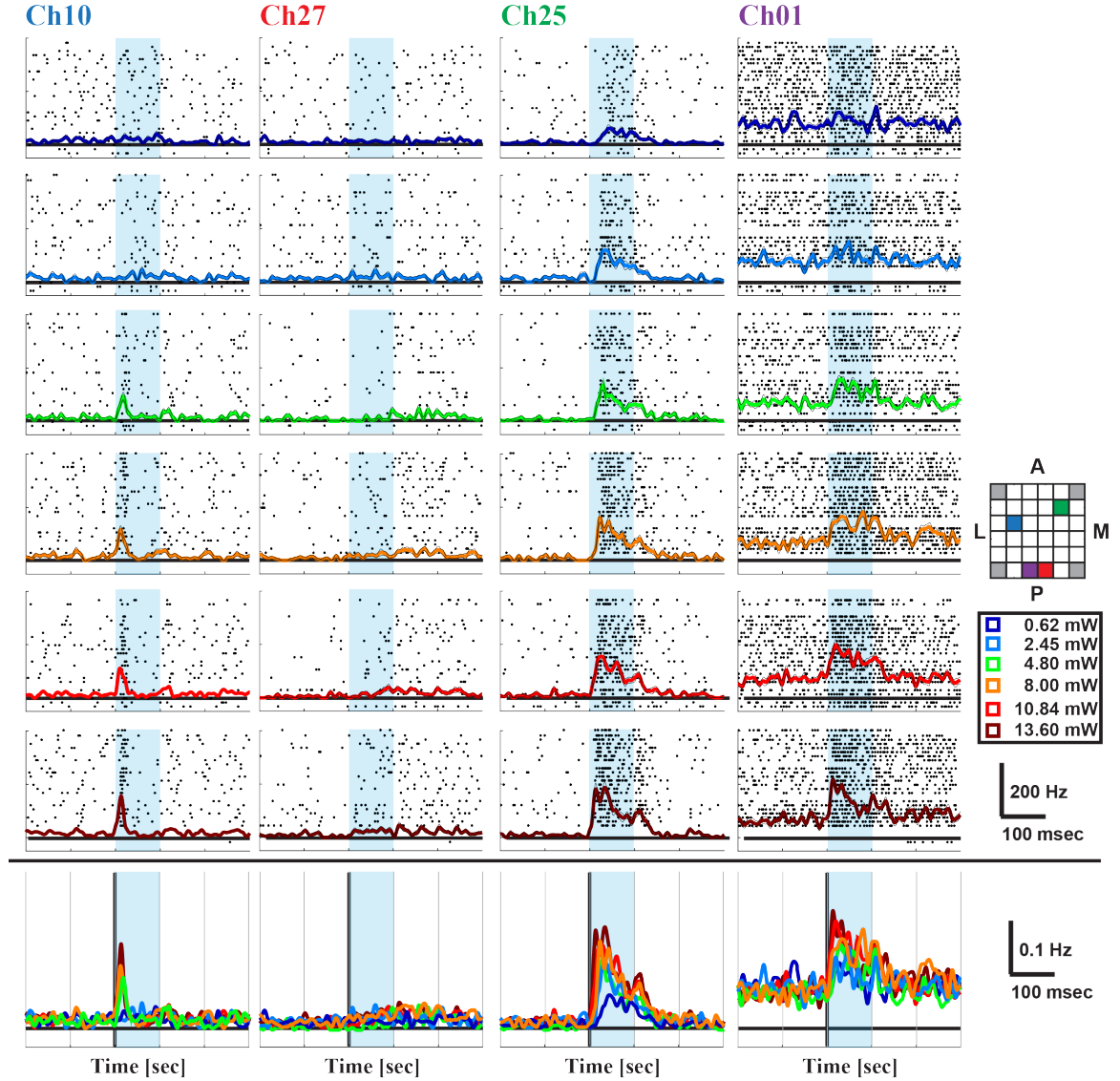


Figure 2.10: Dose-response characteristics of the cortically evoked spiking activity to a 100 msec single-pulse GPe-oStim in awake h-P rat transduced with ChR2. A variety types of responses were observed and four examples were illustrated: onset excitation (Ch10), offset rebound excitation (Ch27), delayed excitation combined with offset rebound excitation (Ch25), sustained excitation duration and after stimulation (Ch01). The threshold for modulation (green channel) was below 0.62 mW (473 nm). Saturation in the responses were in general observed.

2.4 Achieving Widespread Functional Opsin Expression in the Motor Cortex

Another crucial component of the basal ganglia circuitry is the motor cortex. Not only do both the striatum and STN receive excitatory glutamatergic projections from the motor cortex (Turner and Desmurget 2010), profound electrophysiological changes were observed in the motor cortex following dopamine depletion (Lindenbach and Bishop 2013a; Pasquereau, DeLong, and Turner 2016a; J. A. Obeso et al. 2017). Antidromic activation of motor cortex has been shown as a potential mechanism for STN-DBS through the hyperdirect pathway (Paz, Deniau, and Charpier 2005; S. Li et al. 2007; Q. Li et al. 2012; Kita and Kita 2012). Optogenetic activation of layer V neurons in the motor cortex and the hyperdirect pathway in transgenic mice both led to significant therapeutic improvement of motor functions (Gradinaru et al. 2009b). Its large size and accessibility by less-invasive stimulation methods poses motor cortex as a desirable locus for optogenetic modulation (see more detailed discussion in the following chapter). Despite the advantages, the large volume of the motor cortex present a challenge to achieve expression of necessary coverage, especially in rats for which transgenic methods for optogenetics are not yet well established or readily available.

In this section, I will show methods developed in this thesis to resolve this issue, which enabled optogenetic modulation of hemi-Parkinsonian motor symptoms by motor cortical stimulation discussed in the next chapter. Parts of this sections were excerpt and modified from one of my publications, with some sentences or paragraphs quoted verbatim (Zeyang Yu, Nurmikko, and Ozden 2017).

2.4.1 Limitations of the diffusion-based delivery method

Currently, opsin expression in the rat cortex is achieved through the local viral transduction technique (Fenno, Yizhar, and Deisseroth 2011; Tye and Deisseroth 2012). This is typically performed by inserting a needle or glass pipette into the region of interest in the brain, and

injecting a viral solution at a slow speed to generate a spatially localized virus mass in the brain tissue (Dai et al. 2015; Ozden et al. 2013a; J. Wang et al. 2012a). Consequently, the virus spreads by diffusion from the site of injection to the neighboring sites (diffusion-based delivery, DBD). While the slow injection rates utilized in the DBD method ensures a tolerable damage to the brain tissue, opsin expression ends up being limited to a volume of about 1 mm diameter (see for examples in Fig. 2.11, Fig. 2.12). In many applications where spatial specificity is crucial, such limited spread is ideal; however, for large spatial coverage, alternative methods are required. The most common method to compensate for the limited spatial coverage of DBD technique in cortical studies has been performing multiple DBD-based virus injections at multiple locations of about 1 mm apart in the cortex (Lu et al. 2015; Diester et al. 2011). However, this is a highly time-consuming process, considering that each injection can take up to 25 min.

In two hemi-Parkinsonian rats, we performed DBD-based single-site virus injections. Histology results (Fig. 2.12) confirmed that while local opsin expression was strong, the overall spatial extent was indeed limited.

One of the two rats also received bilateral MEAs implanted in the motor cortices (the array on the left hemisphere centered on the injection site). Modulation of spiking activities across the array on the modulated cortex showed high degree of spatial variability: robust modulations were only observed at several electrodes and electrodes at distance showed much less modulation (Fig. 2.13). Given the electrode-electrode pitch was 400 μm in this array, this result corroborated the limited volume of functional opsin expression. Another piece of evidence for limited opsin expression was indicated by the lack of modulation on the contralateral motor cortex as a result of interhemispheric projections.

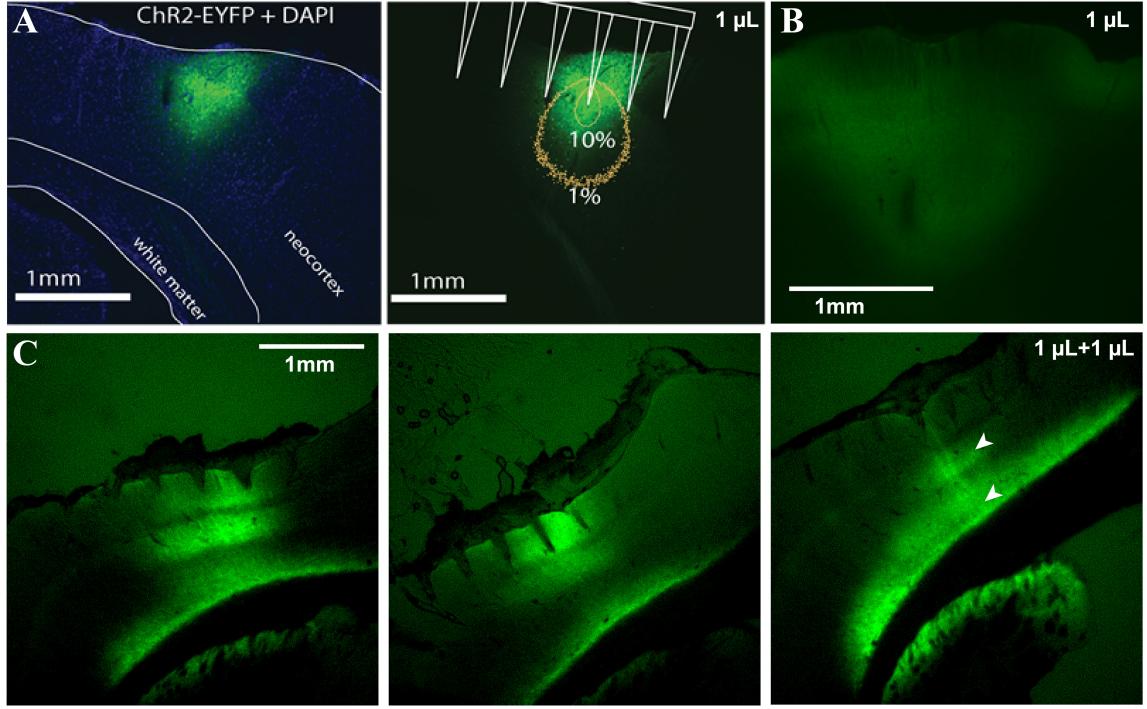


Figure 2.11: A summary of spatially limited expression in the motor cortex as an outcome of DBD-based virus injection observed in prior work of the lab. The green color indicated the fluorescence co-expressed with opsin. **A.** results of 1 μL injection by Wang et al. showed expression volume < 1 mm in diameter, adopted with permissions from Fig. 3 of (J. Wang et al. 2012). **B.** unpublished result by Ozden et al. showed expression volume ~ 1 mm in diameter 1 μL injection. **C.** results from Wagner et al. of 1 μL (-1.0 mm DV) + 1 μL (-2.0 mm DV) injections showed limited expression at superficial part of Layer V (~ 1 mm in diameter) and diffused long range expression in deep layers (deep Layer V and Layer VI) along the white matter. The two types of expression morphologies were indicated by the white arrowheads. The last image was adopted and modified with permission from Fig. 1F of (Wagner et al. 2015).

2.4.2 Development of the convection-enhanced delivery method

As a solution to this challenge, we adopted the convection-enhanced delivery (hereinafter CED) technique, a method which has been established for gene therapy and drug delivery applications in multiple species (Bobo et al. 1994; Yazdan-Shahmorad et al. 2016; Yin, Forsayeth, and Bankiewicz 2010; Fiandaca et al. 2008; Lam et al. 2014; Szerlip et al. 2007; M. Y. Chen et al. 1999). The technique is based on injecting a large amount of infusate at controlled high rates (high pressure and precision engineered fluid flow) so that

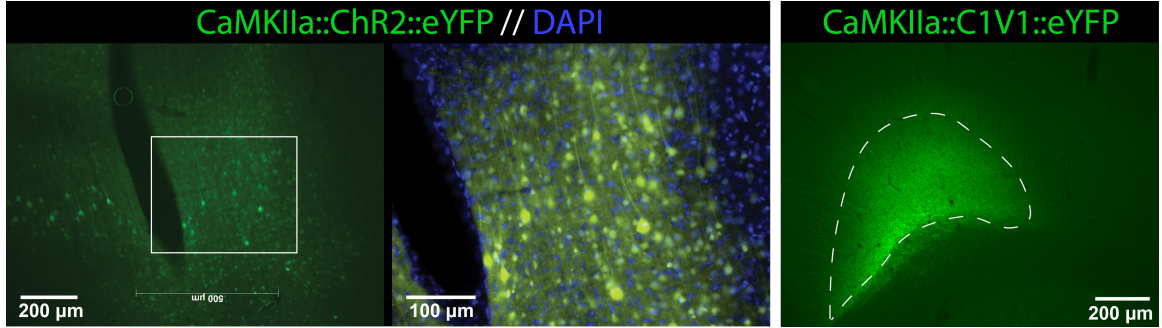


Figure 2.12: Strong, but spatially limited, expression in the pyramidal neurons of motor cortex was achieved with the DBD-based method in the preliminary studies. Fluorescence images showing coronal slices of motor cortex transduced with the DBD-based method, where 1 μL of virus was injected in two rats (left and right panels, respectively). **Left.** While pyramidal neurons were reliably labeled at the center of expression, the range of expression was limited to $< 1\text{mm}$ in diameter. The right subimage provided a zoom-in view of the area indicated by the white rectangle. **Right.** In a different animal, injected with 1 μL C1V1(t/t) via the DBD-based method, a similar limited spatial reach of the expression ($< 1\text{mm}$ in diameter) was observed.

the distribution of the infusate during injection is in the regime of advection and extends well beyond the site of injection (Fig. 2.14 A). Demonstration of (non-optogenetic) CED in rodents has been limited to large subcortical nuclei like the striatum (Yin, Forsayeth, and Bankiewicz 2010; Szerlip et al. 2007); and protocols for widespread virus delivery in a thin planar structure of rat brain, such as the cortex, have not been established. The outcomes of CED, in terms of the volume of distribution and reflux of the infusate, depend on the shape and size of the targeted area, hydraulic properties of the tissue, design of the cannula, and choices of injection parameters (Syková and Nicholson 2008; Yin, Forsayeth, and Bankiewicz 2010); hence CED protocols for the rat cortex cannot be directly extrapolated from those used for injection in other nucleus.

We first performed a series of validation experiments in the mechanically realistic brain phantom (0.6% agarose gel) with a virus-mimicking dye solution to test the design of our CED cannula and calibrate microfluidic injection parameters. In agarose gels, a successful CED (with minimal or lack of reflux) injection often resulted in infusate distributed

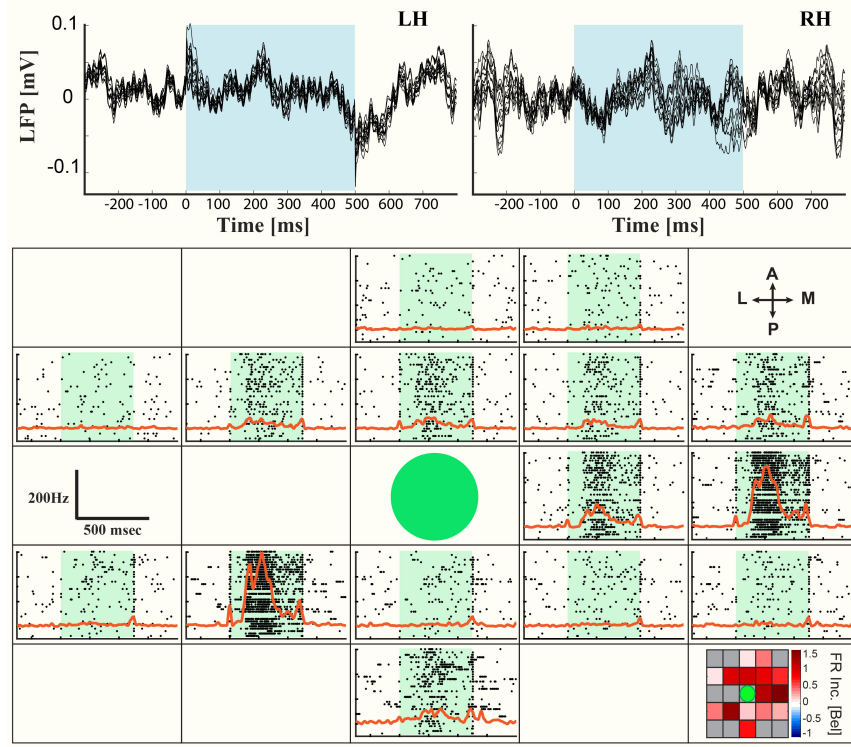


Figure 2.13: Example of cortically evoked responses to a 500 msec single-pulse ssM1-oStim in an awake h-P rat. Light was delivered through a single intracortical implanted fiber in the motor cortex on the left hemisphere (LH). Extracellular recording was performed with a 5x5 MEA on each hemisphere (RH, right hemisphere). A 561 nm laser was delivered at 5.5 mW. **Top.** Trial-averaged evoked LFP responses showed modulations on the stimulated hemisphere (LH), no clear interhemispheric inhibition was observed on the RH. **Bottom.** Modulation of spiking activity shown in the array map layout. While robust modulation was observed in several sites, not immediately adjacent to the fiber (green circle in the middle), distant electrodes saw much reduced change in activity. The orientation of the array was indicated on the top right corner, and the average firing rate increase at each site (period during stimulation compared to before stimulation) was shown on the lower right corner. The size of the array was 1.6 mm x 1.6 mm.

in the shape of an oblate spheroid (Morrison et al. 1999) (Fig. 2.14 C, right).

The design of our CED cannula was modified based on previous studies demonstrating the utility of a 1mm step at the tip of the cannula as a microfluidic backstop for reducing reflux and increasing the spread of infusate horizontally (Yin, Forsayeth, and Bankiewicz 2010; Krauze et al. 2005). The cannula was constructed from a gas-tight syringe connected to a 32-gauge hypodermic needle serving as the inner tube; then a larger

capillary tube was coaxially and tightly fixed over the hypodermic needle to form the reflux-preventing planar step (Fig. 2.14 B). Our results indicate that, for shallow injection targets such as the cortex, such a step-less injection needle might not be effective in CED regime, because of high probability of reflux even at relatively lower (compared to deep injection) injection speeds (Fig. 2.14 C, left).

A two-stage injection protocol was used to approximate a ramped regime (Yin, Forsayeth, and Bankiewicz 2010), which is thought to improve infusate distribution and reduce reflux (Sanftner et al. 2005; Barua et al. 2012). While the mechanism is not fully understood, the slower first stage injection may open passages in the tissue, reduce the hydraulic resistance, and thus facilitate active convection in the faster second stage. Also in the agarose gels, we experimented with different injection speeds for the two stages. The goal was to identify injection parameters that yielded largest horizontal spread (most pronounced diameter-to-depth ratio, Fig. 2.14 C, middle), yet minimal occurrence of reflux (backflow of the infusate along the track out of the tissue, Fig. 2.14 C, left). Ultimately, we chose 0.25 $\mu\text{L}/\text{min}$ and 0.6 $\mu\text{L}/\text{min}$ as the speed for the two stages, respectively, which produced outcome distributions like the example shown in Fig. 2.14 C, right.

With the injection parameters determined in the agarose gel, we tested virus transfection via CED in the cortex of healthy rats.

2.4.3 Widespread functional opsin expression achieved with CED-based opsin transduction

In thirteen healthy rat, we performed CED-based and DBD-based virus injections to compare the difference in the extent of spread of functional opsin expression. Fig. 2.15 summarized the procedures for the two methods. After allowing the expression to develop for six weeks, we conducted a series of electrophysiological and histological experiments to verify the spatial extent and functionality of the opsin expression.

First and foremost, utilizing epicortical fluorescence imaging and confocal imaging,

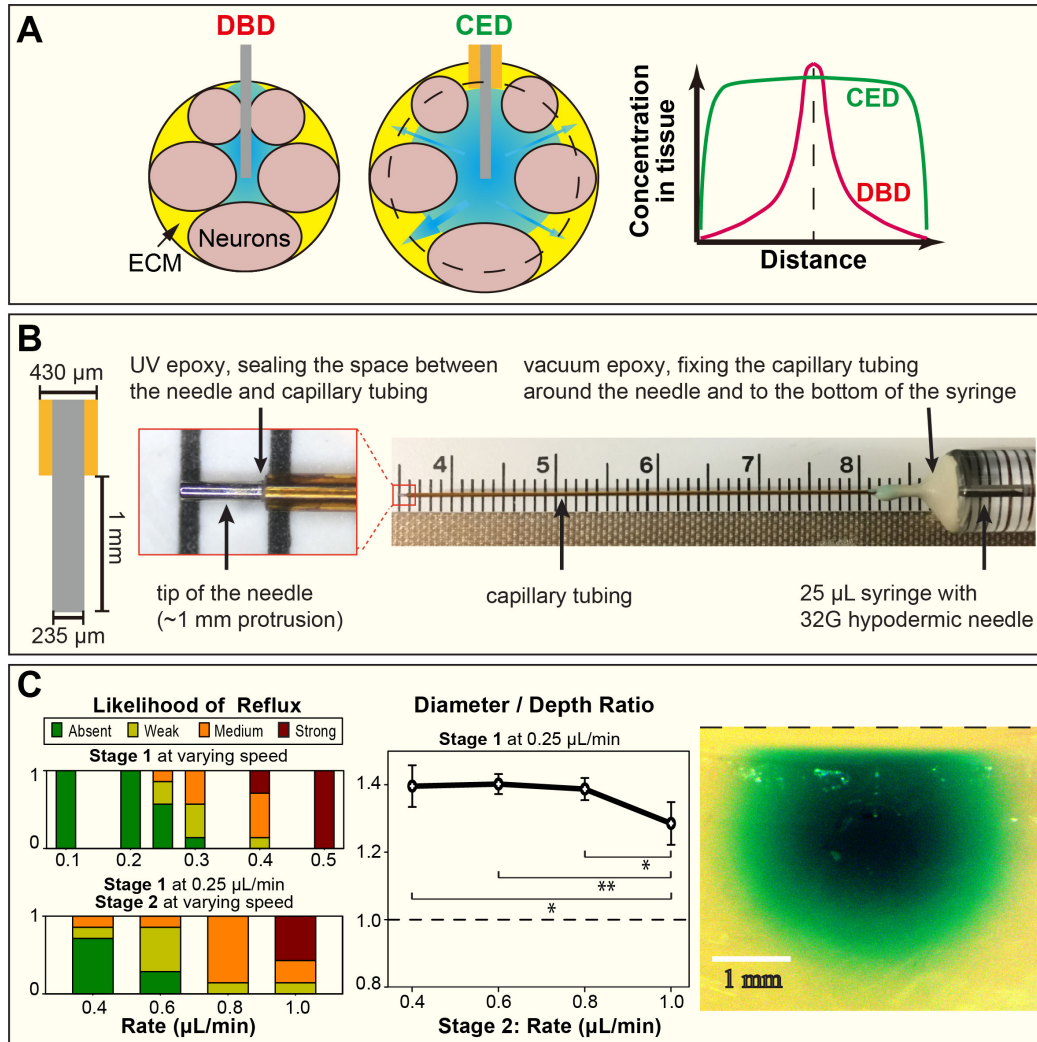


Figure 2.14: Illustration of the mechanisms, design of cannula, and selection of injection parameters for CED. **A.** Comparisons of the mechanism of DBD and CED. CED is suggested to open passages in the extracellular matrix under the high pressure and promote advection of virus solution further into the tissue (left and middle). Consequently a more uniform distribution of virus concentration could also be achieved (right). **B.** The CED cannula: dimensions and assembly process. **C.** Selected results from the agarose gel study for parameter validation: (left) one key consideration for the selections of injection speeds aimed to minimize the likelihood of reflux (backflow); (Middle) another key criteria aimed at the maximization of the diameter-to-depth ratio, i.e. to achieve more lateral spread than vertical spread; (right) an example outcome using the selected parameters showing minimum reflux and great horizontal spread.

we confirmed the drastic improvement in spatial coverage of opsin expressions by CED. As a first-order characterization, we performed epicortical fluorescence imaging in anesthetized rats. A large craniotomy ($\sim 4 \text{ mm} \times 7 \text{ mm}$) was made around the CED sites and, intraoperatively, the spread of opsin expression was qualitatively evaluated through epifluorescence imaging of the co-localized fluorescence proteins (Fig. 2.16 A). In all thirteen rats, fluorescence was reliably observed over the entire area of the rectangular craniotomy (Fig. 2.16 A, right), for all tested virus constructs (opsins and fluorescence tags). In addition, in two animals, after perfusion with PBS, we extracted the brain from the cranium without fixation, then carefully dissected out the entire cortex and flattened it. Epifluorescence imaging from the flattened cortex, free of possible confounds mentioned above, revealed an 8-shaped pattern of extra prominent expression for the dual-site CED (Fig. 2.16 B). Regression analysis showed that each of the two injections formed a circular expression field (with particularly elevated expression levels) of $3.07 \pm 0.10 \text{ mm}$ ($N=2$ rats, two sites per rat) in diameter. The area of interest (the motor forelimb area) was located in between the two expression fields. These expression fields were only conservative estimates of the actual spread sizes as expression clearly existed beyond these brighter areas.

Second, in the rest of the animals, brains were sliced either in coronal or sagittal direction after paraformaldehyde fixation and imaged under confocal microscope. Volumetric measure of expression was reconstructed through interpolation based on at least 8 slices through the expression volume. Although the cellular localization of fluorescence proteins varied for different virus constructs (mCherry showed mainly in cell bodies; eYFP filled many more processes), CED of all four types of virus constructs reliably resulted in widespread opsin expression spanning the entire cortical thickness (Fig. 2.17). The spatial extent of opsin expression ($7.4 \pm 1.0 \text{ mm}$ in the AP direction, $4.4 \pm 1.1 \text{ mm}$ in the ML direction, maximal cross-sectional area, combined $N = 7$ rats) matched the ones obtained by epicortical imaging (expression exceeded the area of the craniotomy), indicating that, with appropriate care, the epicortical fluorescence imaging data can indeed be used to identify

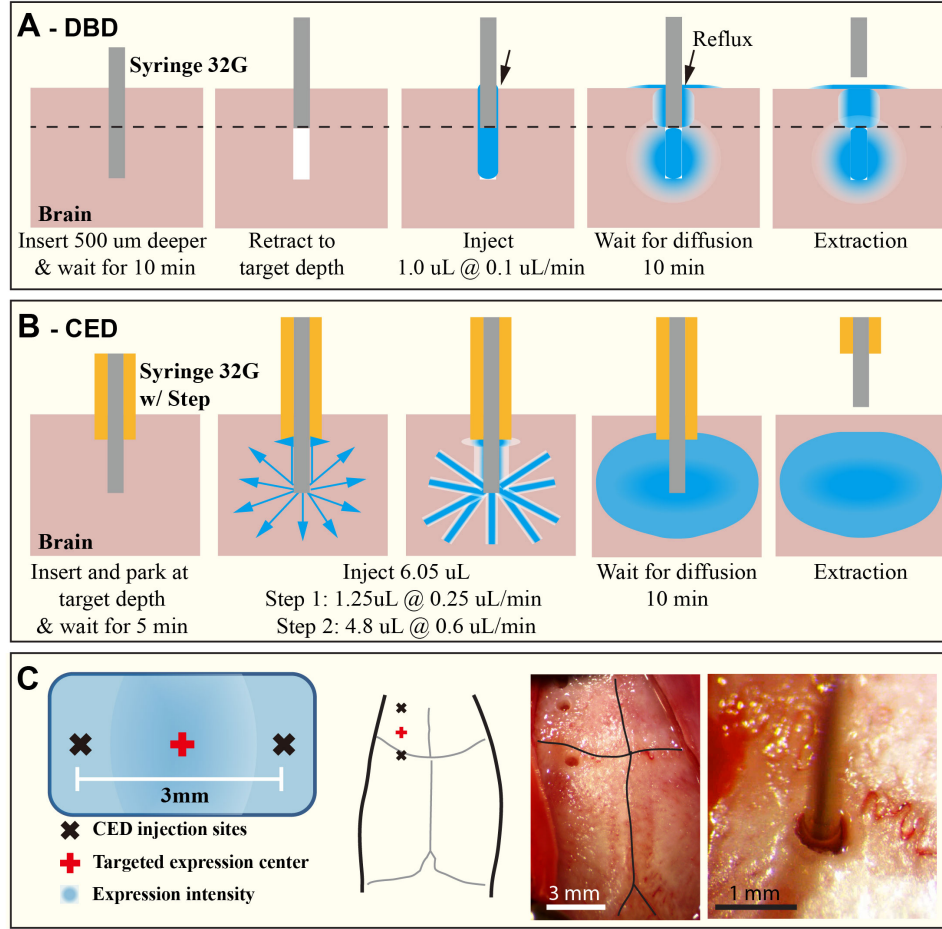


Figure 2.15: Injection procedures for DBD and CED, and layouts of dual-site injections in the rat cortex. **A.** Procedure for the typical DBD injection: the injection needle was typically lowered 500 μm beyond the target depth and retracted after 5 min wait to create a space allowing solution accumulation; then injection was performed at a slow speed 0.1 $\mu\text{L}/\text{min}$; before extraction, we waited another 10 min to allow diffusion of solution. **B.** Procedure for CED injection: the CED cannula was lowered to the target depth (no extra advancement) and 5 min was allowed to pass for tissue settling; then 6.05 μL of virus solution was injected in two steps at 0.25 $\mu\text{L}/\text{min}$ and 0.6 $\mu\text{L}/\text{min}$, respectively; before extraction, we waited another 10 min to allow diffusion of solution. **C.** Two injections were performed 3 mm apart centered on the target-of-interest. The middle two plots showed the locations of injection sites on the skull surface. The last image showed a CED cannula inserted into one of the injection site.

opsin expression in the deeper cortical layers in our case of CED.

Compared with the DBD method, CED not only achieved dramatically larger cortical coverage ($36.2 \pm 8.2 \text{ mm}^3$ with CED, $n = 7$ rats, and $1.50 \pm 0.31 \text{ mm}^3$ with DBD,

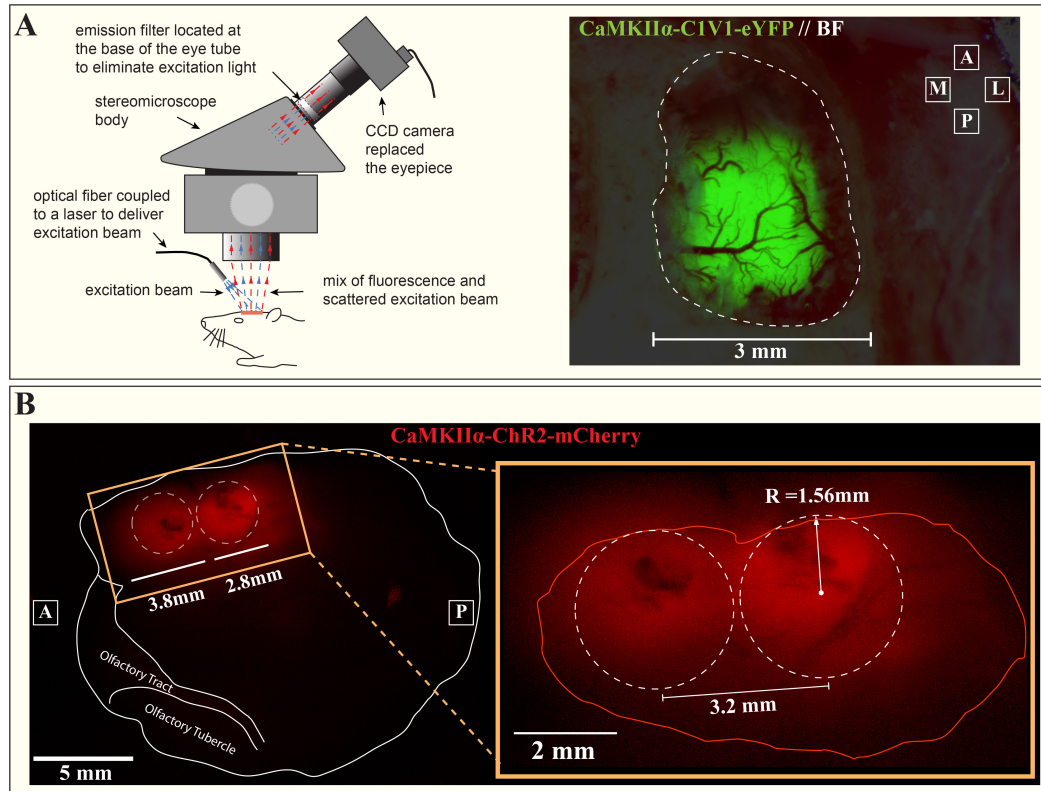


Figure 2.16: Intraoperative and post-mortem epifluorescence imaging of the CED transduced cortex. **A.** Intraoperative epifluorescence imaging through the craniotomy. (Left) A schematic of the epifluorescence setup. Scattered excitation light was filtered out, and fluorescence light was collected by the CCD camera. (Right) YFP epifluorescence image overlaid on the bright-field image (structure). Expression of opsin-colocalized YFP was found over the entire exposed cortical area (craniotomy outlined by the dashed line). **B.** Epifluorescence from a flattened cortex (Left): Strong expression of mCherry spanned a 9.5 mm $\times$ 4.4 mm area. (Right): A zoom-in view of the fluorescent area revealed the “8-shaped” pattern of prominent expression, as a result of the two CED injections 3 mm apart (3.2 mm estimated from imaging). Each injection led to a circular expression field of 3.1 ± 0.1 mm in diameter. These extra prominent expression fields were only identified as a conservative estimate for the spread from each CED injection; since opsin expression was also present beyond these fields, complete overlap of two fields was not required for coverage. The dark regions in the centers of the circles were due to blood accumulation on the surface at the injection sites which was caused by dura removal. Typically, the dura and pia fuse together at the injection locations. Orientations: A - anterior; P - posterior; M - medial; L - lateral.

$n = 3$ rats; $p < 0.0001$, one-tailed unequal-variance unpaired t-test, corrected for multiple comparison) (Fig. 2.17 B, right), the expression was also able to reach to more superficial layers (Fig. 2.17 A, right). From a more practical perspective, we compared the expression

to the volume of motor cortex in rats, which was estimated both from the atlas (volume of M1, $V_{M1} = 24.8 \text{ mm}^3$, (Paxinos and Watson 2007)) and from functional mapping studies (volume of functional motor cortex, $V_{Func MC} = 33.50 \text{ mm}^3$, (Fonoff et al. 2009)). The average expression volume achieved with CED was comparable with one of the estimates and significantly larger than the other was ($p \rightarrow 0$, one-tailed z-test, $n=7$) (Fig. 2.17 B, right). Hence a complete coverage of the targeted area (motor forelimb area) can be achieved with this procedure.

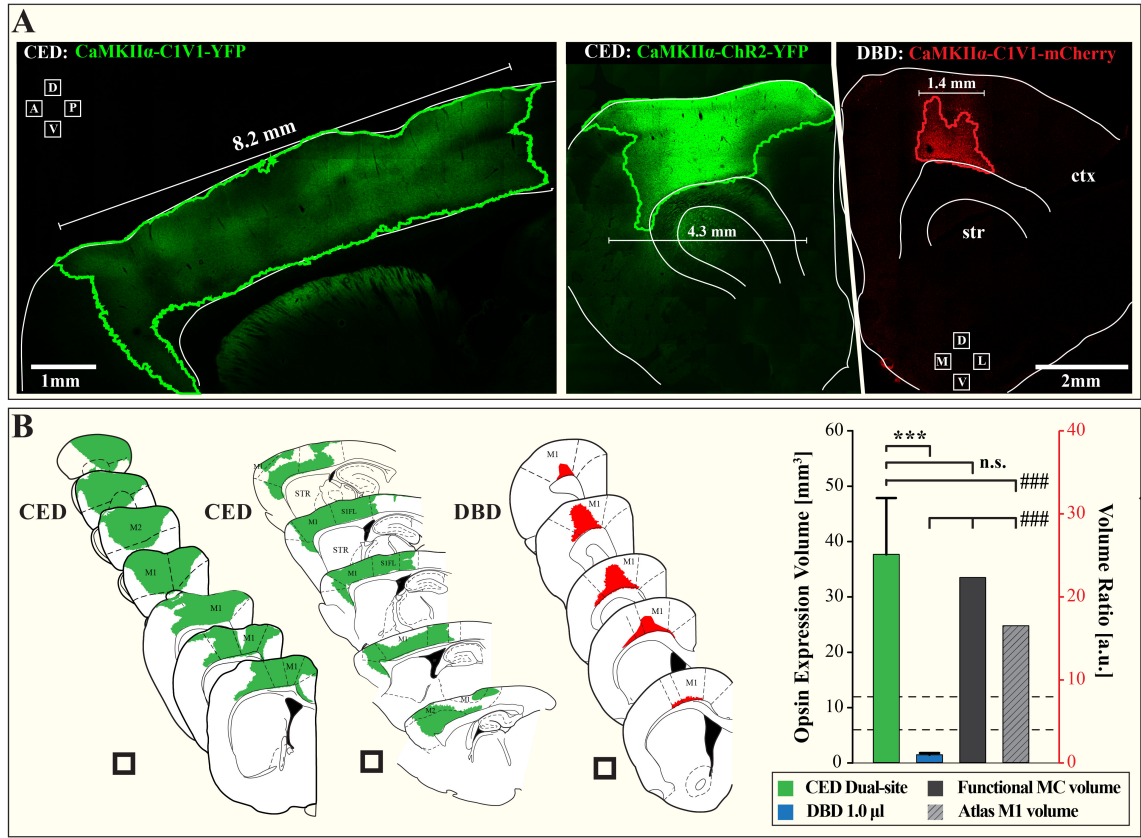


Figure 2.17: Comparisons of the expression patterns associated with CED and DBD-1 μ L. **A.** Confocal images, comparing expression patterns from DBD and CED rats. (Left) Widespread fluorescence in the cortex with an 8.2 mm span in the horizontal dimension (AP) was achieved by dual-site CED. (Right) Direct comparison of expression patterns from DBD (1 μ L) and CED methods in the two hemispheres of the same animal. DBD resulted in a 1.4 mm span of mCherry expression which was mainly confined in the deeper layers (left). CED achieved a 4.3 mm span of YFP expression throughout all cortical layers; the efferent projections from the labeled motor cortex were found in the white matter and striatum. **B.** Comparisons on the volume of expression by CED and DBD. (Left) Area of expression extracted from confocal images: two first two rats were injected with CED method (YFP tag) and the third rat was injected with DBD-1 μ L injection (mCherry). Note that the AP span was much smaller (+1.0 mm to +2.70 mm) in the last animal than that in the former two (-0.40 mm to +5.20 mm). Also note the injection track located just outside the medial border of the expression patterns in the third and fourth slices from the left. (Right) Summary of opsin expression volumes with CED and DBD in the cortex, compared with the volume of the motor cortex. The absolute volumes and the relative volume ratios with respect to the average volume of DBD (1 μ L) were shown on the two y-axes, respectively.

Figure 2.17 caption – continued. The two dashed lines indicated the volumes of 6 and 12 μL , respectively (amount of virus injected in CED). Asterisks (*) and palm signs (#) were used to represent statistically significant comparisons: *** $p < 0.001$ one-tailed unequal-variance unpaired t -test, corrected for multiple comparisons, $n=7$ trials; ### $p \rightarrow 0$, one-tailed z -test, corrected for multiple comparisons, $n=7$. Orientations: A-anterior; P-posterior; M-medial; L- lateral; D-dorsal; V-ventral.

2.4.4 Functional opsin expression enabled modulation of electrophysiology of the motor cortex and motor cortical projection targets

The viability and optogenetic functionality of the opsin-expressing neurons were examined through extracellular recording. For each animal, at least four recording sites were sampled over the area of the craniotomy. At the majority of the recording sites, where spontaneous spiking activity was detected, low power (lowest threshold < 0.1 mW) light stimulation reliably induced optogenetic responses (40 out of 42 sites, $n=2$ rats where we sampled the entire motor cortices). Among these sites, optically induced activity modulations were observed in the form of excitation, inhibition, or both excitation and inhibition in the multiunit spiking activities (Fig. 2.18).

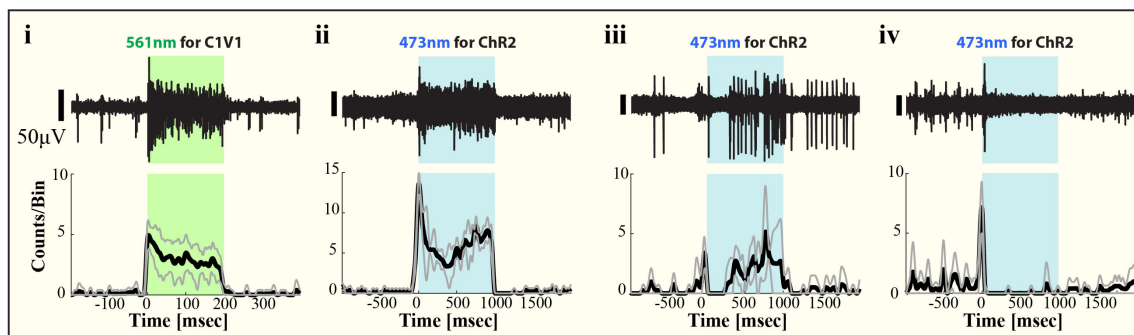


Figure 2.18: Optogenetic modulations of spiking activity sampled from various sites of the transduced (with C1V1 or ChR2) cortices. A variety of modulations of spiking activity were identified, including both excitation and inhibition. The top panels show representative traces for each of the four types of commonly observed modulations. The bottom panels show the peri-stimulus time histogram (PSTH, 10 msec time bin, stimulus-triggered average over 5-7 trials) of the corresponding case. i. C1V1-expressing neurons modulated by a 561 nm laser, with continuous-waveform (CW) light pulses of 200 msec duration. Excitation of spiking activity is observed. ii - iv: ChR2-expressing neurons modulated by a 473nm laser, with CW light pulses of 1 sec pulse duration. ii: excitation with fast onset. iii: excitation with delayed onset. iv: inhibition on spiking activity.

In terms of local field potentials (LFP), optical stimulation resulted in positive/negative deflections and induced oscillations (typically in the gamma band) as reported before (Lu et al. 2015). In two animals (one hemisphere injected with CED, the other hemisphere injected with DBD), mapping of optogenetically-evoked LFP responses was performed across the craniotomy, which further confirmed the spatial extent and robustness of the functional opsin expression with CED (Fig. 2.19). Not only was LFP modulation robust when recorded intracerebrally ($-500\text{ }\mu\text{m DV}$); it could also be measured reliably from the cortical surface in an ECoG-like mode (Fig. 2.19). However, surface illumination and surface recording on the DBD-hemisphere produced spatially much limited and much weaker responses (Fig. 2.19, B-C).

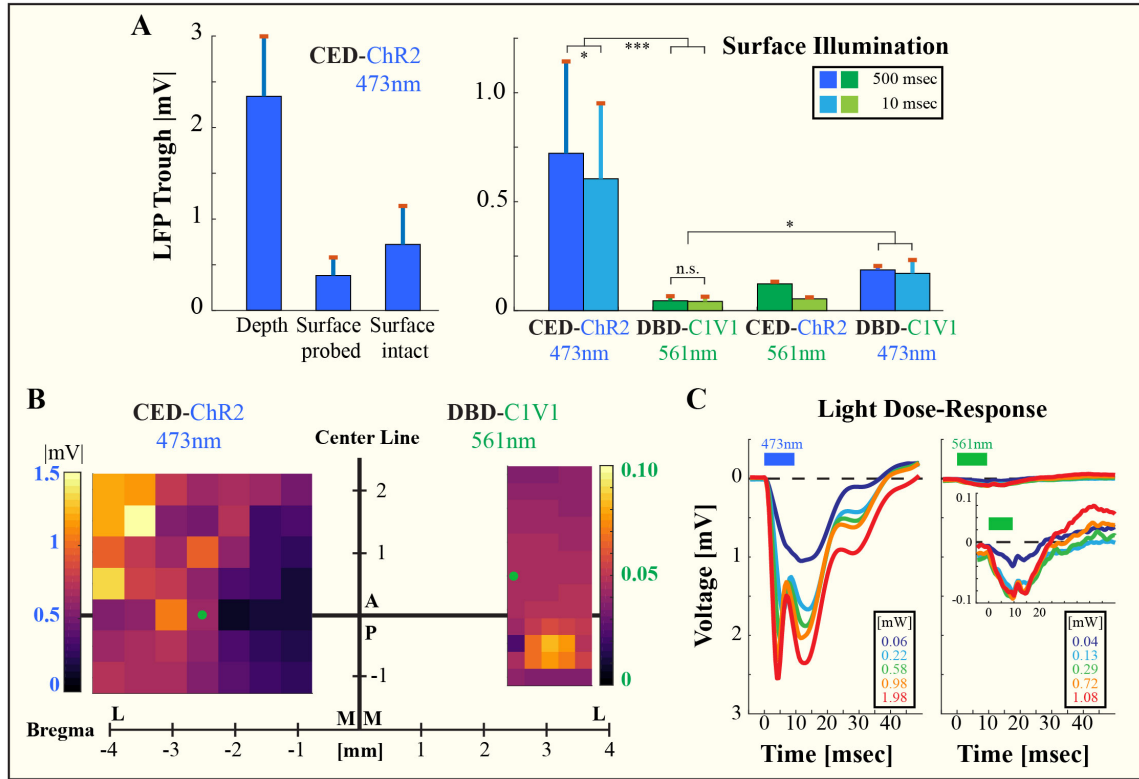


Figure 2.19: Optogenetic modulations of LFP in CED and DBD transduced cortices. Data were generated from two animals: one (Rat 1) with only CED-ChR2 on the left motor cortex, and the other (Rat 2) with CED-ChR2 and DBD-C1V1 (1 μ L) on the left and right hemisphere, respectively. An optrode delivered light pulses with durations of 10 msec or 500 msec at 4 sec intervals (473 nm laser for ChR2 and 561 nm laser for C1V1). Two schemes of light delivery/recording were tested: Surface - tip of the electrode touching the brain surface (ECoG-like) and tip of the fiber 500 μ m above the surface; Depth - optrode was lowered 1.1 mm into the brain. In all plots, the absolute amplitude of the first negative peak of the evoked LFP was taken to represent the strength of the response. All light power values (0.06–1.98 mW for 473 nm and 0.04–1.08 mW for 561 nm) were measured at the tip of the fiber, corresponding to intensities of 0.34–11.10 mW/mm² (473 nm) and 0.74–6.06 mW/mm² (561 nm) at the recording site. **A.** (Left) Evoked LFP amplitudes in the cortex and at the surface (before [intact] and after [probed] optrode insertion) across the CED-ChR2 cortex. Light power was 0.22 mW; pulse duration was 500ms. All pairs of comparisons were statistically significant ($p < 0.0001$, one-tailed unequal-variance unpaired t -test, $n = 30$ trials). (Right) Evoked LFP amplitudes at the surface were dramatically greater on the CED side ($p < 0.0001$, one-tailed unequal-variance unpaired t -test, $n = 30$ trials). In Rat 2, modulations of LFP on the DBD-C1V1 side with the 473nm laser (excites mainly ChR2 rather than C1V1) showed greater amplitudes than those with 561nm laser ($p < 0.01$, one-tailed unequal-variance unpaired t -test, $n = 30$ trials), which suggested the presence of strong interhemispheric projection from the CED-ChR2 hemisphere. Light power was 0.22 mW (473 nm) and 0.28 mW (561 nm).

Figure 2.19 caption – continued **B.** Heat maps of spatial distribution of the evoked LFP amplitudes recorded from the two hemispheres. Note the difference in the colorscales used for the two maps; the DBD side responses were much weaker. **C.** Light dose-response curves. Light stimuli (10 msec pulses) with various intensities were delivered to the brain surface at one site on each hemisphere, respectively. Left: Representative evoked LFP traces. Inset shows a zoom-in view of the DBD-C1V1 traces. Notice the slow onset of the first negative peaks in the DBD-C1V1 traces, which suggested dominance of network effect either from neighbouring area or from the activation of Chr2 terminals from the opposite hemisphere. Right: Plot of light power vs. amplitude of the first native peak in evoked LFP. Dotted lines represent the standard deviations.

In some experiments, we noticed that the activity of contralateral motor cortex was modulated by optical stimulation on the CED hemisphere, which was likely a result of interhemispheric conduction. Then an interesting experiment was conceived: in the DBD cortex of a rat, which had DBD-transduced cortical neurons labelled with C1V1 (561 nm) and interhemispheric afferents from the CED cortex labelled with Chr2 (473nm), different wavelength of optical stimulation can be used to preferentially activate each of the two neural elements. We found that LFP responses from the activation of interhemispheric afferents were actually stronger than that of DBD-transduced neurons (Fig. 2.19 A, right), providing an interesting perspective on the robustness of CED transduction. This was confirmed in histology showing strong labelling in the projection targets of the motor cortex by the efferent fibers: e.g. the striatum, the contralateral motor cortex, and the STN (Fig. 2.20). This turned out to support a particular paradigm with significance to the modulation of Parkinsonian motor symptoms (details will be discussed in the next chapter).

In two chronically implanted hemi-Parkinsonian rats, we recorded LFP activities from the STN and the contralateral motor cortex (ECoG-like signal obtained with screw electrodes) during cortical stimulation. STN is connected with the MC in two ways: through the striatum and the GPe (the indirect pathway) and through the cortico-subthalamic projections (the hyperdirect pathway). Not only did we find LFP modulation in the STN, evoked LFP waveforms were distinct when cortical stimulation was either delivered to Layer

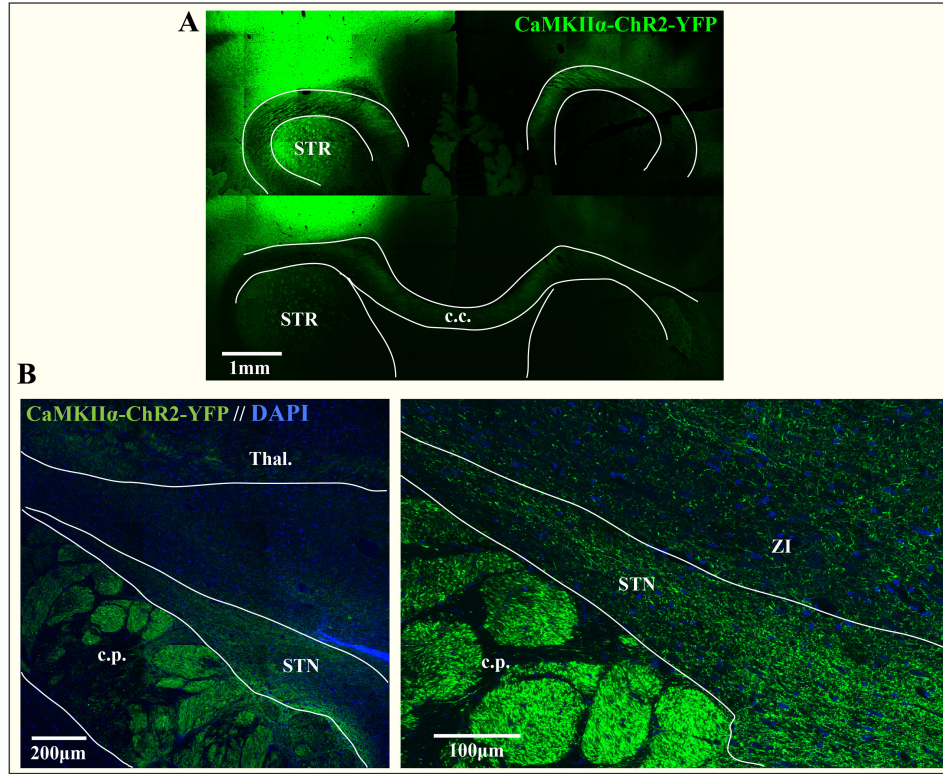


Figure 2.20: Opsin expression in the terminals of cortical projection targets in a healthy rat. **A.** Two coronal slices showed the inter-hemispheric callosal projections (contralateral terminals and fibers through the corpus callosum (bottom)), as well as corticostriatal projections. **B.** Left, a coronal view of cortical projections to the subthalamic nucleus (STN); right, a zoom-in view of the area. Efferent pyramidal tract fibers from the motor cortex and their organization was clearly observed in the cerebral peduncle. [Abbrev.: c.c., corpus callosum; STR, striatum; c.p.: cerebral peduncle (internal capsule); ZI: zona incerta]

V through an intracerebrally implanted optical fiber (ssM1-oStim) or delivered through surface illumination (seMCoS). Specifically, Layer V stimulation led to an early trough followed by a peak that was not observed during surface illumination (Fig. 2.21 middle). These short latency peaks were consistent with similar observations during electrical stimulation of the motor cortex (A. Nambu et al. 2000), and may reflect the activation of the hyperdirect pathway.

Evoked LFP activities were also observed in the contralateral motor cortex. However, the magnitude of response was much stronger during Layer V stimulation (Fig. 2.21 bottom). This was rather unexpected: as the penetrability of surface illumination was limited, and the cell bodies of callosal projections reside primarily in the shallow layers (I-IV) (Fame, MacDonald, and Macklis 2011), one might expect stronger response from surface illumination. After reviewing the literature, two possible factors may explain the appeared discrepancy: 1) the recording of ECoG-like field potential required synchronous activity of pyramidal neurons, but the homotopic nature of callosal projections (projecting to the same area contralaterally) would mean that spatially distributed stimulation would lead to spatially distributed contralateral modulation, hence resulting in the lack of synchrony; and 2) optical stimulation at Layer V may directly stimulate the white matter, where all callosal projections converge before crossing, which would likely lead to highly synchronous modulation.

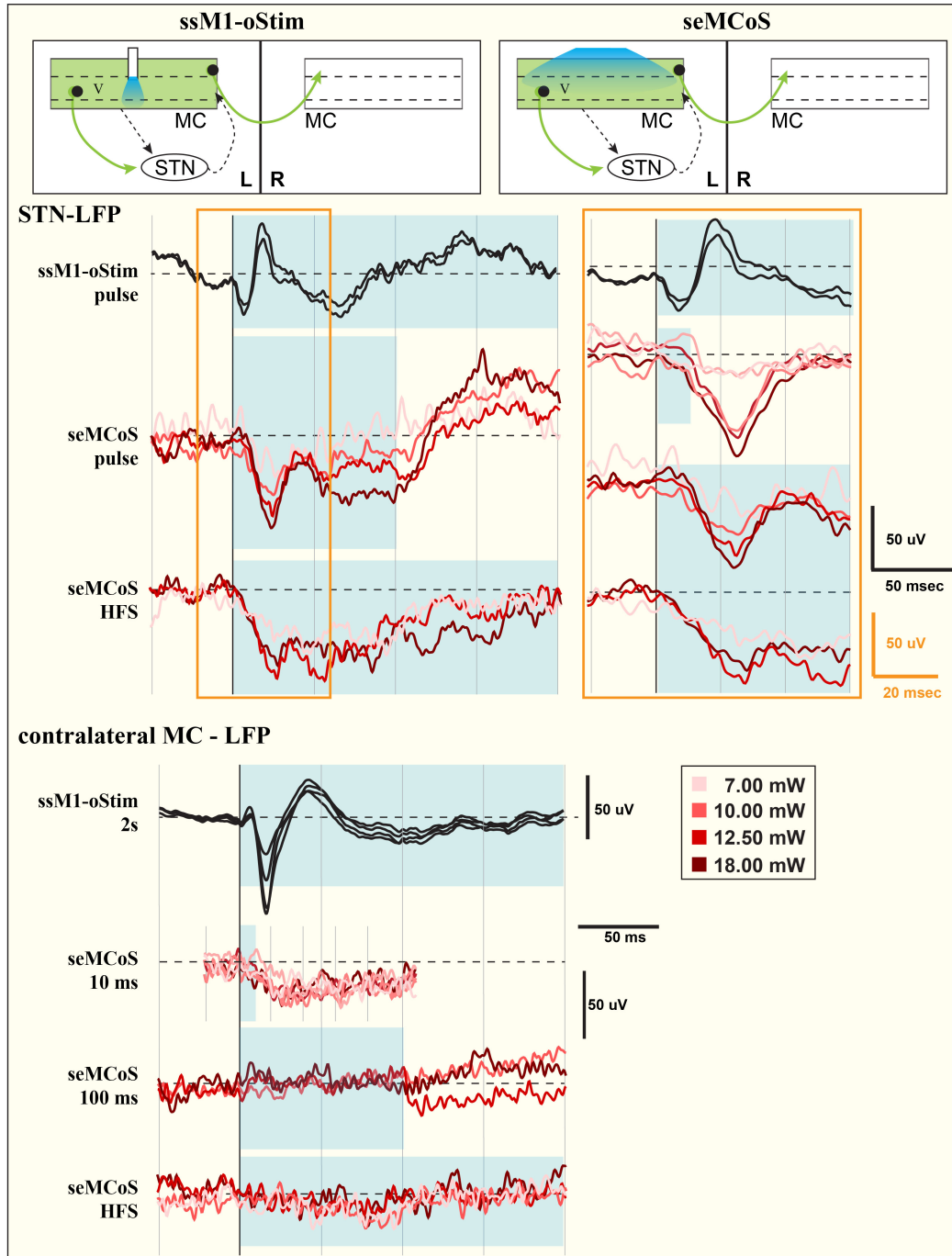


Figure 2.21: Modulation of LFP activities of cortical projection targets by motor cortical stimulation in two h-P rats. **Top.** Optical stimuli were delivered in two ways: to Layer V through an implanted fiber (left, ssM1-oStim) or as surface illumination (right, seMCoS). **Middle.** evoked LFP in the STN by different stimulation protocols. Note the absence of the short latency components in seMCoS evoked traces. A zoom-in view was provided for the region indicated by the orange rectangle.

Figure 2.21 caption – continued **Bottom.** evoked LFP in the contralateral motor cortex by different stimulation protocols. Evoked responses were much stronger in ssM1-oStim. ssM1-oStim was performed at the level of 2 mW and the traces represented recording from different electrodes; seMCoS was performed at several power levels from 7-18 mW and each trace represented averaged response from different electrodes. High-frequency stimulation (HFS) was composed of 5 msec duration pulses at 100 Hz repetition rate.

Utilizing the well-labeled cortico-subthalamic (MC-STN) projections, in the same animals, we also performed simultaneous recording from the STN and both motor cortices during optical stimulation of the MC-STN fibers (Fig. 2.22). Locally evoked LFP in STN had short latency; adaptation to repetitive and prolonged stimulation were observed (opsin properties)(Lorenz-Fonfria et al. 2014). Complex waveforms were found in the evoked LFPs in the ipsilateral MC, which perhaps reflected the combination of a fast antidromic effect and a slower orthodromic effect propagating through the basal ganglia. As for the contralateral motor cortex, it was unclear through what mechanisms this modulation was achieved. However, the longer latency (~ 15 msec) suggested the involvement of multiple intermediary steps.

Overall, our results confirmed that the widespread opsin expression in the motor cortex was indeed functional. Besides the robust modulation of the neural activities locally in the motor cortex, modulation of the activities in the projection targets of the motor cortex was also reliably achieved. This is an important premise to successful neuromodulation in the context of the Parkinson's disease in the next chapter.

2.4.5 Functional opsin expression enabled direct elicitation of motor response and optical mapping of the motor cortex

Optogenetics is commonly applied in the study of cortical networks and functions, thus we further evaluated the functionality of the transduced motor cortex, a commonly overlooked criterion for safety and effectiveness.

As for optical microstimulation with the traditional DBD method, eliciting visible

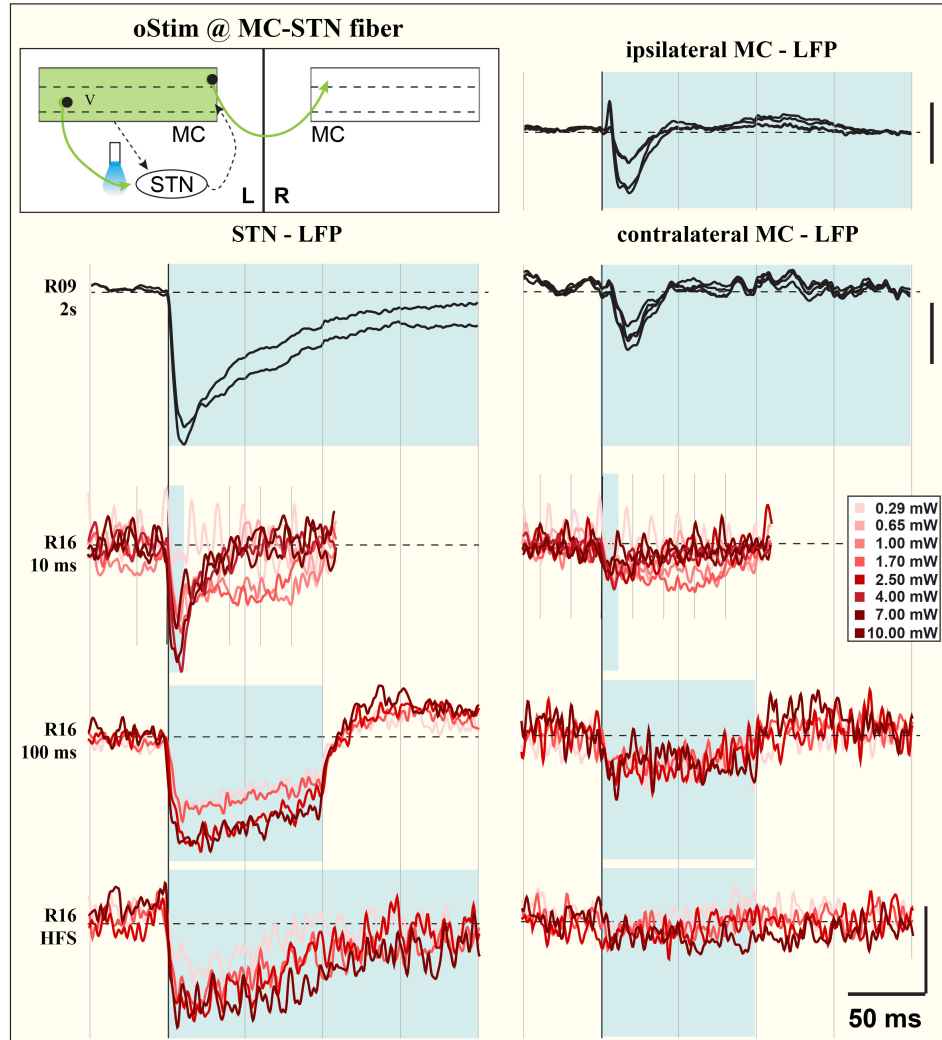


Figure 2.22: Modulation of LFP activities in the STN and bilateral motor cortices by optogenetic-stimulation of the cortico-subthalamic projections in two h-P rats. Upper-Left. Illustration of the stimulation paradigm. **Lower-Left.** Evoked LFP in the STN by different stimulation protocols. Note the short latency across all protocols and the adaptation effects presented in longer and repetitive stimulation. **Right.** Evoked LFP in the ipsilateral and contralateral motor cortices. Ipsilateral MC showed complex multi-phasic waveforms with a short positive peak, a longer negative trough, followed by a late and broad peak. Evoked responses in the contralateral motor cortex were weaker and lacked the short latency component. Stimulation for R09 was performed at 5 mW and the traces (black) represented recording from different electrodes; stimulation for R16 was performed at several power levels from 0.29-10 mW and each trace represented averaged response from different electrodes. High-frequency stimulation (HFS) was composed of 5 msec duration pulses at 100 Hz repetition rate.

motor responses (motion of body parts) has been difficult via optogenetics, partially due to the limited volume of opsin expression. In CED rats, we found that eliciting motor responses was relatively easy and the associated overt behavioral responses were robust, which allowed us to generate functional maps of the motor cortex by optical stimulation (Fig. 2.23). The functional maps obtained with optical stimulation resembled the ones with intracortical (electrical) microstimulation ($\sim 90\%$ overlap); yet they did differ at few locations as reported previously in Thy1-ChR2 transgenic mice (J. Lee et al. 2015), which perhaps was related to the spatial and cellular specificity of optogenetics.

Therefore, our results demonstrated that CED generates “transgenic-like”, robust, functional opsin expression over large areas of the cortex, which is useful for studies targeting optical modulations across multiple cortical areas.

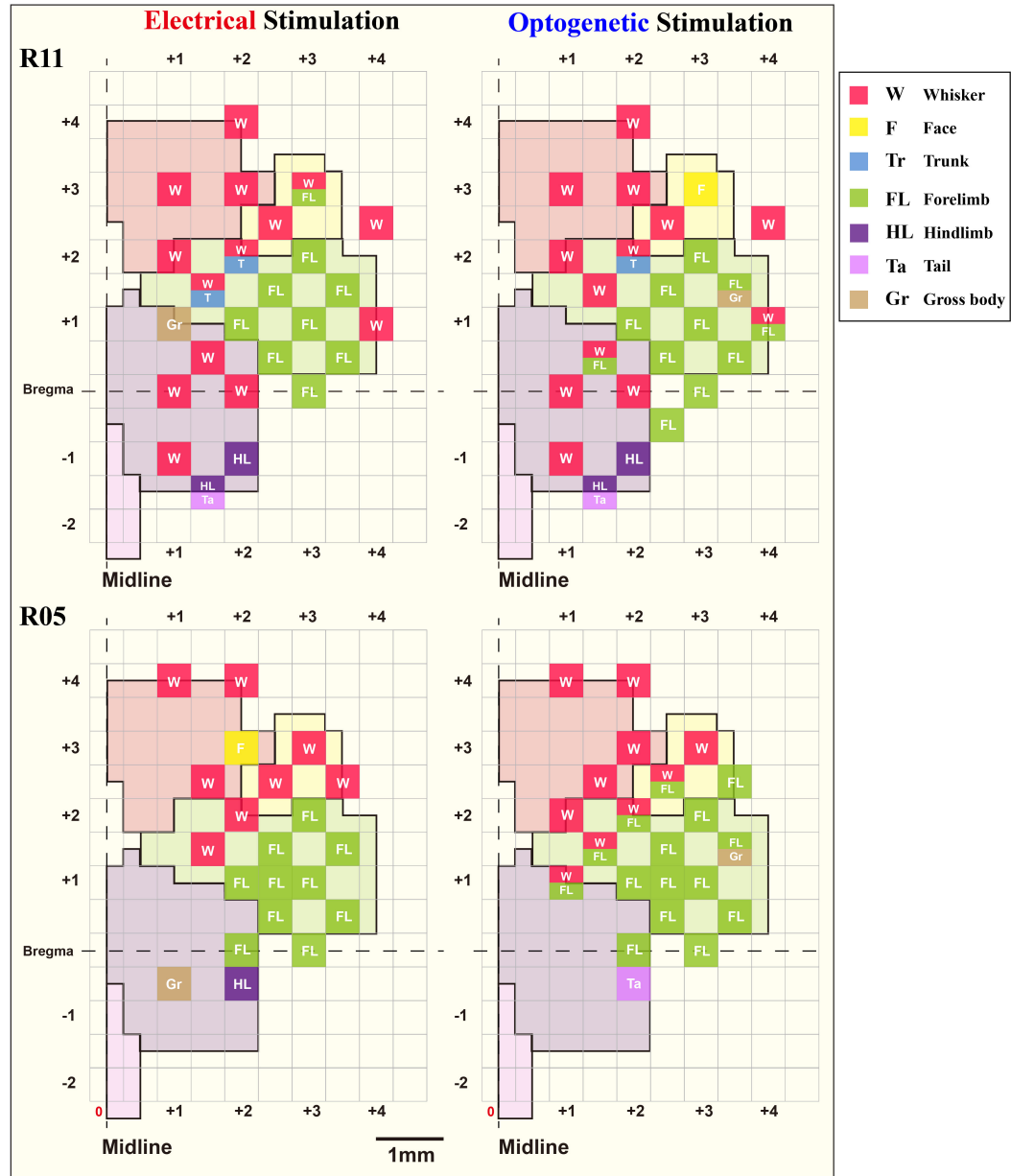


Figure 2.23: Functional motor maps acquired intraoperatively with electrical and optogenetic stimulations. CaMKII-ChR2-eYFP (for R11) / -mCherry (for R05) was injected via dual-site CED in the two rats, respectively. Both electrical and optogenetic stimulation reliably elicited overt behavioral response. The resulting functional mapping matched with the functional motor areas (transparent colored patches in the background: whisker, nose, forelimb, hindlimb, and tail) determined with epidural electrical stimulation by Fonoff et al. (adapted with permission (Fonoff et al., 2009)).

Figure 2.23 caption – continued Specifically, in our study, the forelimb areas were in high agreement with the results from Fonoff et al; the whisker areas spread into other areas; and the hindlimb modulation was seldom observed. Sites where no behavioral modulation was observed with stimulation larger than 200 μ A or 2.5 mW were considered as non-responsive, and excluded.

2.4.6 Section summary

By using the CED technique, we have successfully obtained robust and functional opsin expression over widespread areas of the rat cortex, overcoming the limitations of the diffusion-based delivery (DBD) methods. This method yielded large expression volumes exceeding that obtained by the conventional DBD method by about 24 fold in volume and by about 16 fold in the maximal cross-sectional areas (AP $\times$ ML horizontal plane). *in vivo* electrophysiology also showed that neurons within the identified fluorescent areas were indeed responsive to optical stimulation. The extent of the cortical opsin expression patterns in our rats resembled that of transgenic mice. For example, overt motor response could be elicited reliably by optical stimulation of the motor cortex at light power levels similar to those used to generate motor response in transgenic mice (Lee et al. 2015).

Overall, robust motor cortical modulation by optogenetics was made possible in animal models where transgenic methods are not currently available.

2.5 Conclusion

In this chapter, I described methods developed to achieve functional opsin expression in deep brain targets (STN and GPe) and in the motor cortex, as well as results from a series of validation experiments. Electrophysiology-assisted targeting allowed for improved accuracy in virus injection and probe implantation, especially in STN. Extracellular recording confirmed STN activities could be silenced by the photoactivation of the functionally expressed inhibitory opsin SwiChR. We also achieved pan-neuron expression of ChR2 confined to GPe and showed robust modulation by GPe-oStim not only locally in the GPe, but also

in STN and motor cortex. Lastly, convection-enhanced delivery was adopted to overcome the limited opsin expression in the motor cortex. As a result, not only were we able to achieve spatially widespread opsin expression and demonstrate optogenetic modulation of neural activities in the motor cortex and its projection targets, the strong and thorough expression even enabled us to elicit direct motor response with optogenetic stimulation and ultimately complete an optical motor map for the first time in non-transgenic rats.

The work of this chapter established a solid foundation for optogenetic-based modulations of hemi-Parkinsonian motor symptoms in the following chapter.

2.6 Detailed Methods for Chapter 02

2.6.1 Subjects

The animals used in this portion of the study were 37 male Long-Evans rats (Charles River Laboratories, Inc., Wilmington, MA; 250300 g at the start of the study), housed in the animal care facility at Brown University. The rats were kept on a 12-h light/dark cycle and housed individually with free access to food and water. All procedures were performed according to the National Institutes of Health guidelines and approved by the Brown University Institutional Animal Care and Use Committee (Protocol#: 1402000046, 1703000263).

2.6.2 Viral vectors

We used recombinant adeno-associated viral vectors, (serotype 2 pseudotyped with serotype 5, rAAV2/5) to transduce neurons to express excitatory or inhibitory opsins under the control of the Ca^{2+} /calmodulin-dependent protein kinase II- α (CaMKII α) promoter or the human synapsin 1 (hSyn) promoter.

For excitation, we used the channelrhodopsin (ChR2) ([rAAV5::CaMKII α -hChR2

(H134R)::EYFP-WPRE-pA], 6.2×10^{12} copies/mL) or its red-shifted variant, C1V1(T/T) ([rAAV5::CaMKII α -C1V1(E122T/E162T)::TS-EYFP], 2×10^{12} copies/ml, (or tagged with mCherry, same titer)). For GPe-oStim, vectors with hSyn promoters were used: AAV-hSyn-C1V1(E122T/E162T)-TS-p2A-eYFP-WPRE, 5.6×10^{12} copies/ml and AAV-hSyn-hChR2(H134R)-eYFP-WPRE-pA, 5.5×10^{12} copies/ml.

For inhibition, we used the third-generation halorhodopsin (eNpHR3.0) ([rAAV5::hSyn-eNpHR3.0::EYFP], 3.5×10^{12} copies/mL) or a potent inhibitory variant of channel-rhodopsin, SwiChR ([rAAV5::CaMKII α -SwiChRCA::TS-EYFP], 5.6×10^{12} copies/ml). Viral constructs were kindly shared by Dr. Karl Deisseroth at Stanford University and were packaged and distributed through the University of North Carolina Vector Core.

2.6.3 Design of CED cannula

The design of our CED cannula followed the overall description of Yin et al. (Yin, Forsayeth, and Bankiewicz 2010), yet we adapted the design to syringes of standard needle gauge. Therefore, the dimensions of our cannula were slightly different than described in Yin et al. Specifically, the cannula was made from a 25- μ L gas-tight syringe with a 32-gauge hypodermic needle (1702SN, flat tip; Hamilton) (Fig. 2.14, B). A fused silica capillary tubing (TSP320450, Molex) with an inner diameter of 320 μ m and an outer diameter of 435 μ m was fitted over the polyimide tube, leaving 1 mm of the tip of the hypodermic needle protruding. This 1mm “step” structure has been shown to effectively reduce reflux of infusate at high infusion rates (Yin, Forsayeth, and Bankiewicz 2010; Yazdan-Shahmorad et al. 2016). The capillary tubing was fixed at the base of the syringe with vacuum epoxy (Torr Seal; Kurt J. Lesker Company), and the hypodermic needle-polyimide tube-capillary tubing junction at the tip was sealed with UV epoxy (NOA-81, Norland). The air-tightness of the cannula and the integrity of the cannula orifice were checked before usage. Optionally a polyimide tube (#100, MicroLumen), with an inner diameter of 250 μ m and an outer

diameter of 300 μm , was inserted between the hypodermic needle and the capillary tube, to ensure that the hypodermic needle was properly centered with respect to the capillary tubing.

2.6.4 Stereotaxic injection of viral vectors

Animals underwent stereotactic surgery, head-fixed with a stereotaxic alignment system (David Kopf Instruments) under isoflurane anesthesia (1-3%). Ophthalmic ointment was applied to prevent eye drying.

(a) Diffusion-based delivery in the STN, GPe, and the motor cortex

The electrophysiology-assisted targeting method for STN and GPe was detailed in the results section due to its significance (Sec. 2.2.1 and 2.3.1).

A small hole (~ 1 mm in diameter) was drilled above the center of the area of interest (AOI): the STN (stereotaxic coordinates: -3.8 mm AP, -2.4 mm ML, ~ -7.5 to -7.8 mm DV from dura), the GPe (-1.0 mm AP, -3.2 mm ML, ~ -5.5 and -6.0 mm DV from dura) forelimb area of the motor cortex (+1.5 mm AP, 2.5 mm ML, -1.5 mm DV from dura). The dura was not removed, but a cut was made (the small size of the hypodermic needle alone allows for penetration through the cut). The tip of a hypodermic needle attached to a 25 μL syringe (1702SN, 18° or 60° bevel angle, Hamilton) mounted on a microinjection pump (UltraMicroPumps III; World Precision Instruments) was first inserted 500 μm deeper than the final target depth and was maintained at this depth for 5 min to allow the tissue to settle. The needle was then retracted to the target depth and 1 μL virus solution was injected at a speed of 0.1 $\mu\text{L}/\text{min}$ for 10 min. For GPe, two 1 μL injections were made at -5.5 mm and -6.0 mm, respectively (no waiting between two injections). After injection, we waited for 10 min before retracting the needle to allow relaxation of pressure. Craniotomies were sealed with silicone elastomer (Kwik-Cast; World Precision Instruments) and the skin

was sutured. We waited for transduction of virus and maturation of expression for at least 6 weeks.

(b) Convection-enhanced delivery in the motor cortex

Injections via the CED method were performed only for the motor cortex. Injections were made at two locations 3 mm apart (dual-site CED). The two injection sites (the anterior site: +3.0 mm AP, 2.5 mm ML and the posterior site: +0.0 mm AP, 2.5 mm ML) were chosen equidistant from the center of the forelimb area. At each injection location, first a small hole (~ 1 mm in diameter) was drilled, and the dura was carefully removed. The injection cannula mounted on a microinjection pump (UltraMicroPumps III; World Precision Instruments) was inserted 1.5 mm into the cortex. The tissue was allowed to settle for 5 min after insertion. Then, without retracting the cannula, a total volume of 6.05 μL of virus solution was infused in two steps: the first 1.25 μL was injected at a speed of 0.25 $\mu\text{L}/\text{min}$ as the slow stage (5 min), and the remaining 4.80 μL injected at a speed of 0.60 $\mu\text{L}/\text{min}$ as the fast stage (8 min) (Fig. 2.15 B). After injection was completed, the cannula was left at its place for 10 min to allow relaxation of intracortical pressure. Craniotomies were sealed with silicone elastomer (Kwik-Cast; World Precision Instruments), and the skin was sutured. We waited for transduction of virus and maturation of expression for at least 5 weeks.

2.6.5 Intraoperative electrophysiology of non-CED rats

After maturation of opsin expression (> 6 weeks after injections), the rats underwent a second stereotaxic surgery under either an initial dose of 100/10 mg/kg ketamine/xylazine (IP) anesthesia. The anesthesia was supplemented every one hour with additional ketamine (50 mg/kg). The cranial hole for the previous injection was reopened and enlarged. The dura was resected to prepare for the optode, comprising a tapered multimode optical fiber (105/125 μm core/cladding diameters) (AFS105/125Y, Thorlabs) glued next to a 100 kOhm

stainless steel electrode (EL30030.1A10, Microprobes, USA), to stimulate and record extracellular neuronal activities. In the optrode, the electrode tip was about 200 μm ahead of the tip of the fiber. For healthy animals with opsin expression in GPe, simultaneous recording from GPe and STN were obtained with the help of another 10/125 μm coaxial optrode (Ozden et al. 2013), due to the spatial proximity of GPe and STN. The implants of experiments involving h-P rats will be discussed in the next chapter. Recording and optical stimulation setup will be discussed in the next section.

(a) Electrolytic lesion for marking of electrode location

Stainless steel was chosen as the material for electrolytic marking of electrode location. At the end of recording, a series of weak 20 sec 20 μA anodal current (Model 2200, AM systems) was injected at several depths along the track, respectively. The trace amount of electrochemically deposited iron could later be visualized by Prussian blue staining (Green 1958; Fung, Burstein, and Born 1998)

2.6.6 Intraoperative electrophysiology of CED rats

(a) Verification of opsin expression

After maturation of opsin expression (~ 5 weeks after injections), the rats underwent a second stereotaxic surgery under similar schemes of anesthesia. A large craniotomy (>6 mm long in AP direction and 4 mm wide in the ML direction) was made over the motor cortex (centered on +1.5 mm AP, 2.5 mm ML). The dura was carefully removed. Two additional cranial holes were made above the cerebellum, and two stainless steel screws were screwed in to serve as reference and ground, respectively, in electrophysiological recordings.

(b) Extracellular recording and optogenetic modulation

An optode similar to the one used in Section 2.6.5 was prepared, except for using a 100 kOhm tungsten electrode (WE30030.1A3, Microprobes, USA).

In seven CED animals, at least four tracks were sampled over the craniotomy (areas with surface blood vessels were avoided). In two CED animals, the entire craniotomy area was sampled according to a square grid with a pitch of 500 μm .

A 473 nm laser was used for excitation of channelrhodopsins and a 561 nm laser for C1V1. A train of square pulses, 1 sec in duration with an inter-pulse interval (IPI) of 5 s, were generated from a pulse generator (Master-8, A.M.P.I. or Pulse Pal) to control the gating of the laser. The maximum power of light was 1 mW; sites where no clear modulation was observed below 1 mW power were considered unresponsive. Excitation or inhibition (the latter likely a network response) of spiking or background activities were all considered as acceptable optogenetic modulations. Neural activities were amplified 1000 times (Model 1800; AM Systems) and recorded with a data acquisition card (PCI-6221, National Instruments) and a custom-written LabVIEW (National Instruments) program.

(c) Local field potential mapping of the motor cortex

In two animals, we obtained amplitude mapping of optogenetically evoked LFP (1-100 Hz) activities over the entire craniotomy area, according to a 500 μm pitch square grid. The absolute amplitude of the first negative peak of the evoked LFP waveform was used as the metric. An optrode was used to deliver light pulses with durations of 10 msec or 500 msec at 4 sec intervals (lack of light artifact was verified in saline and in post-mortem tissue). In one of the two animals, we first performed depth recording at 1.1 mm below the brain surface; then probed the optically induced responses at the surface. In the other animal, in which the left hemisphere was injected with Chr2 via CED, and the right hemisphere was injected with C1V1 (1 μL) via DBD, we probed the surface response on both hemispheres.

Light power was held constant across sites at 0.22 mW (473 nm for ChR2) and 0.28 mW (561 nm for C1V1). A relatively low power was used to limit the volume of activation for the depth recording. At the area of maximal evoked response, we also obtained the light dose response relationship for the depth and surface recordings. The same pulse train was used, yet the light power was varied: 0.061.98 mW for 473 nm and 0.041.08 mW for 561 nm.

(d) Functional mapping of the motor cortex

In two CED animals, the functional motor map of the motor cortex was constructed with both electrical and optogenetic stimulation. An optrode (described above, now with a 100 kOhm stainless steel electrode (EL30030.1A10, Microprobes, USA)) was used to perform extracellular recording and deliver electrical or optical stimulation. The entire craniotomy area was sampled according to a square grid with 500 μm spacing. At each track, the construct was inserted to a depth of about 1.5 mm from the cortical surface. First, optogenetic modulation of neuronal activity was tested with input light power below 1 mW as described in the previous section. Then the functional cortical maps were obtained by electrical and optical stimulation, respectively.

For electrical stimulation, a 1 sec pulse train was delivered every 5 sec. Each pulse train was comprised of 10 bursts, at a frequency of 10 Hz and with burst duration of 30 msec. Each burst was further comprised of monophasic constant current square pulses that were 200 μsec in duration, 300 Hz in repetition rate. The amplitude of the current pulses was increased slowly at each probing site until a repetitive movement of a body part was visually observed (both cathodal and anodal currents were tested). The exact frequency of the elicited movement was not quantitatively measured. However, an artificial beep sound was played along with each stimulation pulse. Hence the repetitive movements can be reliably detected by the experimenters with the help of the stimulus-synchronized beeps.

If no repetitive movement was observed when 200 μ A amplitude was reached, the site was labeled as unresponsive.

For optical stimulation, a 1 sec pulse train was delivered every 5 sec. Each pulse train was comprised of a burst of square light pulses that were 20 msec in duration, 10 Hz in repetition rate. Similar to electrical stimulation, the pulse power was increased slowly until either repetitive movement of a body part was detected or 2.5 mW power was reached.

2.6.7 Intraoperative epifluorescence imaging

Intraoperative fluorescence imaging of the cortical surface, epi-(cortico)-fluorescence was performed through a stereomicroscope modified to operate as a fluorescence microscope (M80, Leica) (Fig. 2.16 A). For this modification, one of the eyepieces of the microscope was removed and a fluorescence filter was placed at the base of the body tube, and a CCD camera (A35140U, OMAX) was mounted with an adaptor to the location where the eyepiece was removed. The fluorescence filter was a bandpass filter: 542/20 nm (FF01-542/20-25, Semrock) for eYFP or 605/55 nm (D605/55m, Chroma) for mCherry. The excitation light was supplied through a multimode 105 μ m /125 μ m optical fiber (AFS105/125Y, Thorlabs) which was held about 5 mm above the cortical surface. The fiber was coupled to either a 473nm laser (MLL-III-473, OptoEngine) or a 561 nm laser (MLL-FN-561, OptoEngine) for eYFP or mCherry imaging, respectively.

2.6.8 Histology and image analysis

For histological analysis, rats were euthanized by a lethal injection of Beuthanasia-D (100 mg/kg, IP) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution. Brain was extracted and sequentially fixed in 4% paraformaldehyde in PB solution (for at least 1 day) and cryoprotected in 30% sucrose solution (until brain fully sunk in solution). The

brain was then frozen with dry ice and cut into 50 μm (for confocal imaging) or 100 μm (for fluorescence imaging) coronal slices with a microtome.

(a) Prussian blue, nuclear fast red, and Nissl staining

Prussian blue solution was prepared by mixing equal amount 4% potassium ferrocyanide (prepared from dissolving P3289-100G, Sigma Aldrich) with 4% hydrochloric acid. Slices suspected to contain the electrode track after visual inspection was immersed in the solution for 10-20 min, often immersion was terminated early as soon as the characteristic blue dot appeared). The slice was then counterstained in the nuclear fast red solution (N3020, Sigma Aldrich) very briefly for less than 2 min (prolonged staining may render overcoloration). The slices were then extensively washed in DI water for 10 min and then mounted. The adjacent slices will go through 0.5% Cresyl Violet staining for structural comparisons (see details in Paxinos and Watson, 1986).

(b) Immunohistology

An antibody for glial fibrillary acidic protein (GFAP) was used to label astrocytes and indicate lesions (1:100 AB206586 Anti-GFAP conjugated with Alexa Fluor 405, EMD Millipore). An antibody for Fox-3 protein (NeuN) was used to label neurons and verify the co-localization of opsin fluorescence (1:250 AB207279 Anti-NeuN conjugated with Alexa Fluor® 594, EMD Millipore). Slices were sequentially washed in phosphate buffer (PB; twice, 5 min each), PBS (three times, 5 min each), and then left in block solution for 1 h. The block solution was prepared with 0.1% Tween (SigmaAldrich, Missouri), 0.25% Triton-X (SigmaAldrich, Missouri), and 10% normal goat serum (EMD Millipore, MA) in PBS. Slices were then immersed in the mixture of antibodies (in block solution), covered with aluminum foil, and rotated for 2.5 days in a cold room (4°C). Afterward, slides were washed in PBS (three times, 5 min each) and PB (twice, 5 min each), and mounted on

microscope slides.

Slices were examined under Zeiss LSM 510 Meta and LSM 800 confocal laser scanning microscopes. For extraction of expression areas and volumes, a custom Matlab program was used to extract the areas with prominent fluorescence (after thresholding, small holes in the patterns were filled automatically). The identified areas were manually verified and corrected for exclusion of non-cortical structures and lesion tracks (dimming in opsin expression). The total volume was constructed through linear (2D) interpolation of the precisely stereotaxically registered brain slices. Similar methods were used for the quantification of spread patterns in the gel tests.

For characterization of fluorescence or immunoreactivities around injection sites, fluorescence images obtained in grayscale were normalized in intensity from 0 to 1. Square regions-of-interest (ROIs) of 200-250 μm size were manually selected within (avoiding the track itself) or outside the injection track (3-5 sites, respectively). The average normalized fluorescence intensities within these ROIs were calculated, which were used as surrogates for immunoreactivity and neuronal density measures. The relative differences (% change) of fluorescence within and outside the track region were then calculated. In some slides, manual cell counts with the ROIs were also performed (for NeuN).

2.6.9 Data analysis

(a) Inclusion criteria

Animals were included for in the final analysis of results if post-mortem histology confirmed desired opsin expression in the target area, and if intraoperative evaluation was performed, functional activation of opsins was verified either through modulation of spiking activity, LFP activity or motor response. For h-P rats, either their behavioral symptoms were verified or post-mortem TH-histology confirmed >80% DA depletion (see Chapter 3).

(b) Extraction of LFP, MUA and isolation of spikes

Raw signals were sampled at 20 kHz. LFPs were bandpass filtered between 1 and 300 Hz (forward-backward 3rd order Butterworth filter) and downsampled to 2 kHz. The MUA was bandpass filter between 300 Hz and 9 kHz (forward-backward 6th order Butterworth filter). Preprocessing procedures for the removal of artifacts related to breathing, chewing, scratching, and so on for both MUA and LFP was performed and will be detailed in the next chapter. Then an adaptive thresholding program determined the spiking threshold between 3.6 to 5 standard deviations. The isolation of units from detected spiking events was performed either with a PCA-based algorithm or with Offline Sorter (Plexon). Firing rate for each unit was computed over 20-50 msec time bins.

(c) Stimulus-triggered average (STA) of LFP and peri-stimulus time histograms (PSTH)

If optical stimulation was delivered as single pulses with long intervals ($\text{IPI} \geq 1 \text{ sec}$), the modulation effects from each pulses were extracted; otherwise for high-frequency stimulation each burst of pulses was considered as one event. First the onset and offset time points were extracted from the stimulation trigger signal. Any events with irregular durations or intervals (likely the first and last event) were excluded. A pre-stimulation period and a post-stimulation period was suggested by the program and confirmed by the experimenter. Finally, the corresponding periods of LFP and firing rate was excerpted for each event, and then averaged across events.

Chapter 3

Rescue of Pathological Rotational Behaviors by Optogenetic Neuromodulation in 6-OHDA hemi-Parkinsonian Rats

3.1 Introduction

Deep brain stimulation (DBS) of STN and globus pallidus internus (GPi) has proven to be efficacious for the alleviation of motor symptoms of PD (Alim Louis Benabid 2003; McIntyre, Savasta, et al. 2004; Weaver et al. 2009; Tan et al. 2016; Hickey and Stacy 2016; Fang and Tolleson 2017; Mansouri et al. 2018). Although the improvements are often drastic, a complete relief in motor symptoms cannot be achieved by DBS or at least by DBS alone.

After more than two decades of clinical application and continuous efforts in electrophysiological studies, the exact mechanisms of the therapeutic actions of DBS had remained unclear and largely controversial. This elusiveness comes mainly from the lack of specificity in the effects of electrical stimulation and the complexity of the basal ganglia circuitry.

On the former, electrical stimulation exerts its influence by directly interfering with the ongoing neural activity that is inherently sensitive to current inputs from endogenous and exogenous sources. What local neural element will be affected by electrical stimulation is a convoluted question, the answer to which depends many factors including, the size and geometry, local electrical properties, presence of generic and cell-specific ion channels on the membrane, the design of the DBS electrodes, and so on (Howell and Grill 2013; Chiken and Nambu 2015). A further complication comes from the varying stimulation outcome from the choices of stimulation parameters (pulse duration, frequency, amplitude, polarity, charge-balancing, and so on). While anodal stimulation is shown to be stimulatory and cathodal stimulation is inhibitory, high frequency anodal stimulation has been shown to cause inhibition (through depolarization block).

The overall anatomical complexity of the basal ganglia circuitry also adds to the mystery of DBS. Some evidence suggested that electrical stimulation may affect nearby axonal elements more strongly than neurons (McIntyre, Savasta, et al. 2004; Tehovnik and Slocum 2013). Both STN and GPi reside in a space closely packed by neighboring structures

and fibers of passage (f.o.b.), when potential excitatory and/or inhibitory stimulation effects are evoked on multiple neural elements, the overall pattern of modulation across the basal ganglia, as well as the ultimate behavior outcome, becomes prohibitively complex to predict. This non-specificity of stimulation effect may also contribute to side-effects of DBS, such as abnormal tingling sensations (paresthesias), speech problems (dysarthria), and impulse control problems (e.g., pathological gambling, compulsive eating) (Hälbig et al. 2009; Dodd et al. 2005; Broen et al. 2011; Merola et al. 2017).

Understanding of the mechanism of DBS and identification of new targets could benefit from pathway-specific and cell-type specific modulation methods. Also considering the potential necessity of high temporal precision for the stimulation, optogenetic modulation of specific elements of the basal ganglia circuitry has been put to test. A seminal work by Gradinaru et al. (Gradinaru et al. 2009a) demonstrated therapeutic effects by activation of afferent fibers of STN from the motor cortex in a transgenic mouse model, while inhibition of the principal neurons in STN of non-transgenic rats failed to improve motor functions. Subsequently, Yoon et al. (Yoon et al. 2014) reported conflicting observations that pan-neuron inhibition indeed improved certain aspects of motor function. Several other studies investigated the therapeutic effects of modulating neurons in other basal ganglia nuclei, including the striatal medium spiny neurons (MSNs) (Alexxai V. Kravitz et al. 2010)(Tecuapetla et al. 2014) (H. J. Lee et al. 2016), GABAergic neurons in globus pallidus externus (GPe) (Mastro et al. 2017), as well as the cortico-subthalamic hyperdirect pathway (Chu et al. 2017). However, there has not been a systematic study that compares the effects of modulations at multiple nuclei along specific pathways (e.g. the indirect pathway) of the cortico-basal-ganglia circuitry with the focus on a specific type of motor symptom (rotational behavior) in a non-transgenic animal model.

In this chapter, based on the developed methods for opsin expression in the STN, GPe, and motor cortex of non-transgenic rats, we investigated the therapeutic effects on normalization of rotational behaviors in a hemi-Parkinsonian rat model.

Just like the previous chapter, majority of the work in the chapter, expect hyper-direct pathway stimulation and GPe stimulation, was done in close collaboration with Dr. Ilker Ozden.

3.2 Verification of the 6-OHDA Model

3.2.1 Dopaminergic depletion and hemi-Parkinsonian motor symptoms

Near-complete dopaminergic depletion is necessary for the establishment of (hemi-)Parkinsonian motor symptoms (Smith et al. 2002; Deumens, Blokland, and Prickaerts 2002). As discussed in Section 1.3.1, we used the 6-OHDA lesion model, which mimics the pathology, pathophysiology and motor symptoms of human PD. Tyrosine hydroxylase (TH) immunostaining showed $> 90\%$ loss of dopaminergic neurons in the SNc and nucleus accumbens (NAc, data not shown), as well as loss of dopaminergic projection terminals in the striatum, in the lesion hemisphere following 6-OHDA injection to the medial forebrain bundle (MFB) (Fig. 3.1).

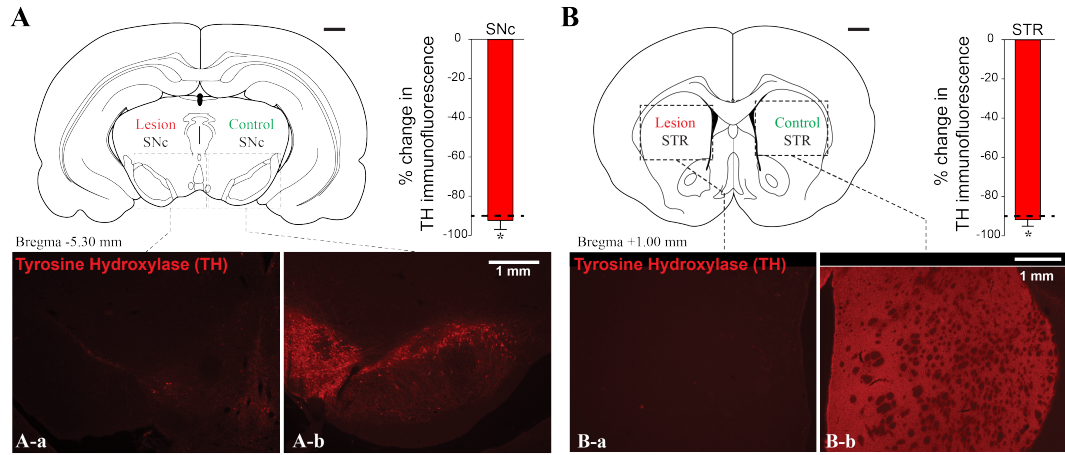


Figure 3.1: Tyrosine-hydroxylase immunohistology of dopamine depletion. Example confocal images showing TH-immunoreactivity in the substantia nigra pars compacta (SNc, **A.**) and striatum (STR, **B.**) and on the lesion (A-a, B-a) and control (A-b, B-b) hemisphere, respectively. Near complete ($> 90\%$) loss of dopaminergic neurons (in SNc) and their terminals (in STR) in five animals was summarized in the bar plot; one-tailed unequal-variance paired t -Test, $p \rightarrow 0$.

Stereotypical motor symptoms, such as akinesia/bradykinesia and motor asymmetry were observed in all lesion rats. During open-field free behavior, akinesia was clearly manifested as, after acclimation, animals remained still for the most of time. Bradykinesia was quantified in a cued lever pressing task, where the reaction time from cue to execution was increased by about 27% (4733 trials from two healthy rats and 855 trials from one h-P rat, $p < 1E-4$, Welch's t-test). Motor asymmetry (linked to akinesia and bradykinesia), due to the laterality of the lesion, manifested in several aspects: 1) limb-use asymmetry, meaning that animals had difficulty to or were reluctant to use the forelimb contralateral to the lesion hemisphere (Fig. 3.2); 2) lack of motor response to somatosensory stimulation (touch) on the lesion side body; 3) rotational bias: h-P rats predominately rotates towards the side ipsilateral to the dopamine depletion (CCW-direction in all of my rats due to the left hemispheric 6-ODHA lesion), during open-field free behavior (details in the next section).

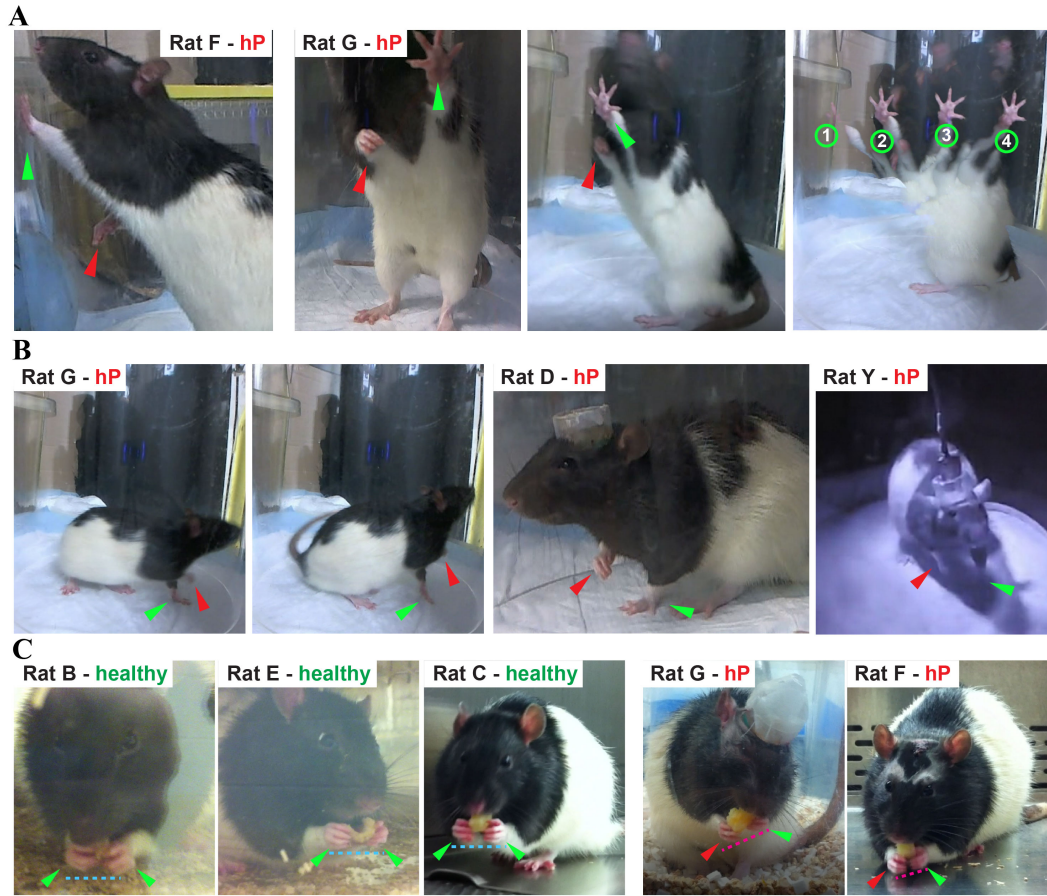


Figure 3.2: Example photos illustrating forelimb-use asymmetry in wall touching, standstill, and food manipulation. Multiple animals were included in this figure and their parkinsonian states (healthy or hP) were indicated after their ID. The position of their hands on the lesion hemibody (red arrowhead) and unlesion hemibody (green arrowhead) were also indicated. **A.** Wall-touch: during vertical exploration (rearing), animals often touched the wall with their hands. In h-P rats, animal predominantly use the good hand to make the wall touches. The image on the far right showed a maximum-intensity projection of four consecutive touches with the unlesion hand during the same rearing event (at the same time turning CCW). **B.** While standing still, h-P animals often displayed an abnormal posture where the good hand was on the floor but the lesion hand was lifted and unextended. **C.** Forelimb use during manipulation of food. In healthy rats, the two good hands were held at the same level (blue dashed lines); while in h-P rats the lesion hand was placed lower than the good hand (magenta dashed lines). When rats attempted rotate the food object, h-P rats only used the good hand to drive the rotation, while the lesion hand served merely as a support beneath the food.

3.3 Rotation Tests

3.3.1 Rotational behavior: stereotypes and mechanisms

Hemi-parkinsonian model utilizes the unilaterality of motor symptoms to create asymmetry in behavior as the indicator for the severity of Parkinsonian symptoms. An important aspect of motor asymmetry, the rotational bias, was characterized by three assessments: the spontaneous rotations (SRs), the amphetamine-induced rotations (Amph-IRs), as well as the apomorphine-induced rotations (Apom-IRs).

In open-field free behavior, hemi-Parkinsonian rats showed spontaneous rotational bias which manifested as predominant counter-clockwise (CCW, ipsilateral to the unilateral 6-OHDA injection on the left hemisphere) rotations. The exact mechanism that links unilateral dopamine depletion to asymmetrical rotation is not known. In fact, DA has profound influence in initiation of movement, action selection and motor flexibility across species via its role in the basal ganglia (Bestmann et al., 2015), all of which may contribute animal's reduced mobility on the lesion side of the body. It is noteworthy to stress that spontaneous rotations were not commonly observed consistently, as the bradykinetic aspect of the symptoms often led to complete lack of movement during a recording session, hampering the characterization of rotation. Specifically, after calming down from handling, animal usually remain completely still, sometimes even with the presence of aversive sensory stimuli; yet a postural asymmetry towards the ipsilateral (left, CCW) direction often persisted (Deumens, Blokland, and Prickaerts 2002). On the other hand, whereas animal was agitated, CCW SRs manifested reliably. Overall, while motor (rotational) asymmetry persists, it will only manifest when animal actually has initiated movement.

A common strategy to reveal the underlying rotation bias is to subject the animal to drug challenges that provokes constant movement. The rotational bias can be either exaggerated or compensated depending on the choice of drug.

Amphetamine readily crosses the membrane of dopaminergic terminals through passive diffusion or dopamine transporters (DATs) and triggers dopamine release (Fig. 3.3 A). A systemic injection of amphetamine leads to the successful release of dopamine only on the intact hemisphere, exaggerating the existing imbalance of activation in the striatum (Iancu et al. 2005; Tronci et al. 2012; Carey 1992; Gradinaru et al. 2009b; Carey 1986; Zeyang Yu et al. 2017; Da Cunha et al. 2008). In addition, persistent motion is illicitated due to the overall increased striatal activation; combined, animal is driven into a state of constant CCW (ipsiversive) rotations (peak >9 RPM) for about 30 - 60 min. This type of rotation manifests a stereotypical large turning-radius (Fig. 3.3 A). Note, contralateral (CW) rotations in Amph-IR have been reported at the beginning of the lesion (Carey 1992), which was also observed in our study; hence, animals were evaluated at least 1 week post-lesion.

Apomorphine, on the contrary, induces contralateral rotations. It functions as a direct agonist to the postsynaptic dopaminergic receptors. Systemic administration causes bilateral activation of the striatum in both hemispheres, bypassing the existing DA deficiency. In fact, apomorphine is used in dopamine-replacement therapy, leading to improvement in motor symptoms (Hutchinson et al. 1997; Pinter et al. 1999; Menon and Stacy 2007; Antonini 2009; Slotty et al. 2014; Boyle and Ondo 2015). In addition, dopaminergic receptors have been shown to supersensitize after DA depletion, perhaps as a compensatory mechanism (Ungerstedt 1971; Siever, Cohen, and Pert 1981; Marshall and Ungerstedt 1977). Hence, the denervated striatum is in fact more activated and animal turns away from this side of the brain (CW, contralateral). Stereotypically, animal displays small radius, tight rotations (Fig. 3.3 B, “chasing the tail”, “head-to-tail”). The peak speed of rotation (>16 RPM) is also higher compared to Amph-IR. One dose of apomorphine usually exerts effect over a period of 60 - 90 min. Although this vigorous CW rotational behavior would hardly warrant being considered as healthy, it does reflect a state of compensated or over-compensated Parkinsonism. In fact, dyskinesia (involuntary movement), which is a complication associated with prolonged dopamine-replacement therapy, bears much similarity

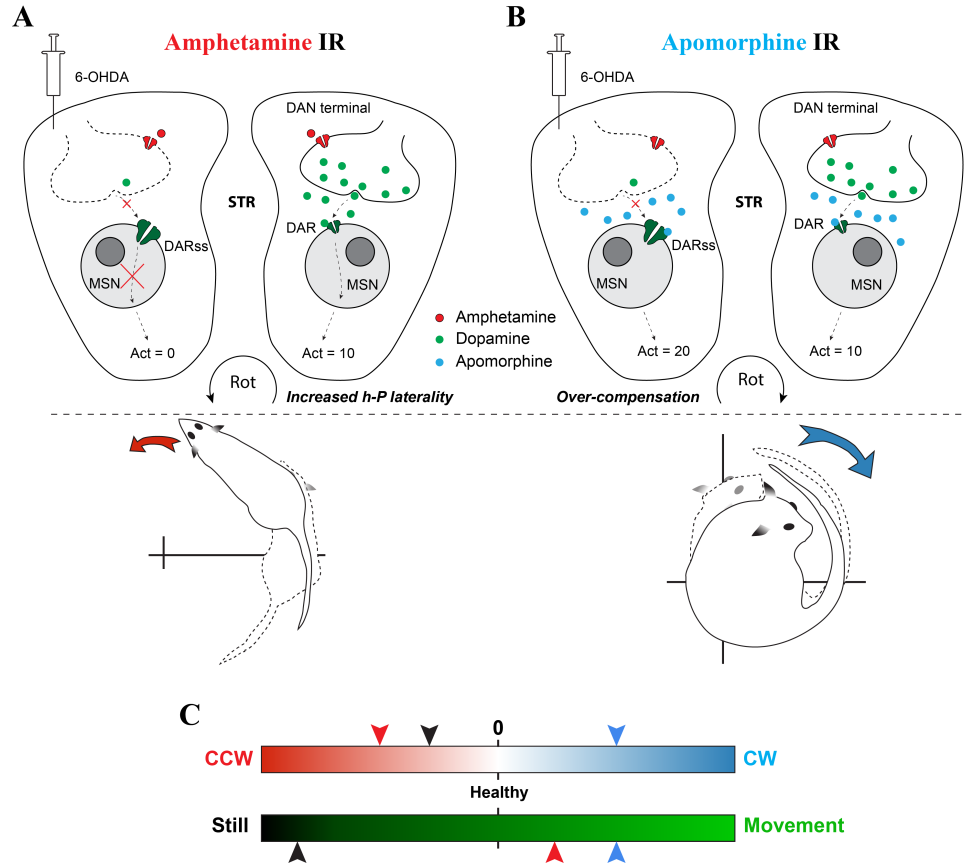


Figure 3.3: Mechanisms and behavioral consequences of drug challenges. A-B. Rotational biases can be either exaggerated or compensated by systemic administration of amphetamine or apomorphine. In both case, animal will be driven into a state of persistent movement, either increased counterclockwise rotations or clockwise rotations, respectively. Note the different sizes of the dopaminergic receptors, illustrating the super-sensitization (DARss). Also note the difference in stereotypical postures during rotation. C. Two movement parameters: rotation direction and total movement (number of rotations) summarized in illustration. Arrowheads represent spontaneous rotation (black), AmphIR (red), and ApomIR (blue). Part of the figure was adopted and modified from (Koshikawa 1994).

with behaviors during Apom-IR (Antonini 2009).

3.3.2 Implementation of a custom magnorotamer

Simultaneous neural recording during optical stimulation in freely behaving h-P animals cannot be reliably obtained over a long period of time, as the consistently unidirectional

rotations caused winding of wires and optical cable (Fig. 3.4 A). This was particularly an issue during high RPM rotations in Apom-IR and Amph-IR (peak rotations at > 10 RPM). As a result, electrical, optical, and even optic-electric hybrid commutators (also known as rotary joints or slip rings) were developed and employed to provide disentanglement. However there has not been a simple, robust, and cost-effective device developed for continuous monitoring of rotations for this experimental condition: 1) the bundle of electrical wires carrying neural signal and the optical patch cable together were rather bulky relative to the small arena required for rotation assessment, hence an image recognition based solution by video recording over the animal was not viable due to the blockage of the wiring; 2) video recording below the arena was also difficult as the surface often became dirty (discharge of urine and feces can be stimulated by amphetamine and apomorphine); 3) both the electrical and the optical aspects of the commutator required careful calibrations: on the electrical side: between low enough resistance for fluid rotation as well as the reduction of vibrational noise, and high enough contact force for good electrical connectivity; and on the optical side: the coupling efficiency at different angles needed to be uniform and high. Therefore modifications within the commutator was also ruled out.

The solution came to me while I was learning about car transmission (as a hobby), which used gear systems to transmit the rotations in the engine to the wheels. After several iterations, I was able to prototype a working solution, which was named magnorotamer (Fig. 3.4 B). The magnorotamer (hereinafter rotameter) used a 1:3 speed multiplier gear system to translate the rotation in the axis of the commutator (the rotation of the animal) to a parallel axis. At the top of the parallel axis was a diametrically magnetized disk magnet (N and S each occupied one hemidisk). The rotating magnetic vector was then measured using an angular Hall-effect sensor (AS5048 14-bit Magnetic Rotary Encoder, ams IC) and an analog signal was outputted with mapping to the absolute angular position. The increased speed in the parallel axis (1 rotation by animal translated to 3 rotations measured) increased the angular resolution of the measurement.

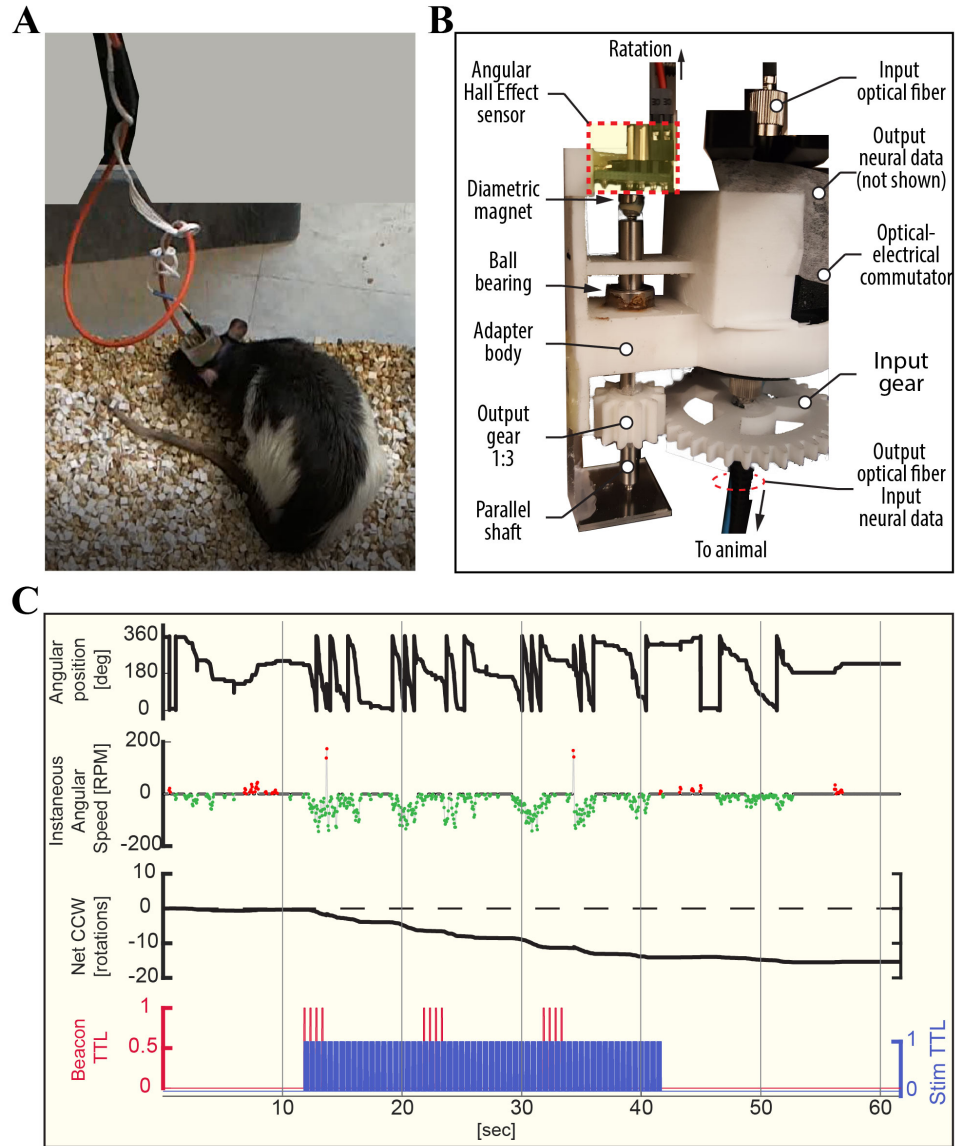


Figure 3.4: Custom magnorotameter for continuous rotation measurement. **A.** Example of entangled wiring in a rotating animal merely 1 min after the last unwinding, in absence of commutators. **B.** Schematic of the custom magnorotameter fitted to the Doric Lens optic-electric commutator. The rotation of the commutator was translated to the parallel axis through a speed multiplier gearing system (1:3 ratio). The rotation of the shaft leads to the rotation of a diametrically magnetized disk magnet and is thus measured by an angular Hall-effect Magnetic Rotary Encoder (AS5048, 14-bit, ams Integrated Circuits). **C.** Example rotation recording from a GPe-oStim subtrail during Apom-IR.

The analog signal was fed into an external input channel of our neural recording system and oversampled (just for data consistency). During analysis, the raw voltage of the rotameter was first converted into the actual instantaneous angular position of the animal (note that because only two gears were used in this prototype, the rotation direction was flipped in the recording). From there, we computed a series of parameters including an estimate for instantaneous angular velocity and other rotation parameters. An example of analyzed rotameter data was shown in Fig. 3.4 C.

I have also made improvements to modularize the device to fit for electrical-only and optical-only commutators. Moving forward, a few further improvements have been planned including improvement in the industrial design, incorporation of a universal joint so that non-parallel configurations can be allowed (also the tolerance for non-perfect gearing can be more lenient) and addition of torque arm to increase the sensitivity to minute head movement.

Overall, the magnorotamer enabled measurement of high speed unidirectional rotations during optical stimulation, electrical stimulation, as well as simultaneous neural recording in unprecedented precision and duration.

3.4 Summary of Hypotheses and Modalities of Modulation

We focused on three main target nuclei for optogenetic stimulation: the subthalamic nucleus (STN), the globus pallidus externus (GPe), and the motor cortex (MC). As discussed in Chapter 1, electrophysiological changes following dopamine depletion (especially in 6-OHDA rats) have been reported for all three nuclei (Hamani et al. 2004; Hegeman et al. 2016; Pasquereau, DeLong, and Turner 2016).

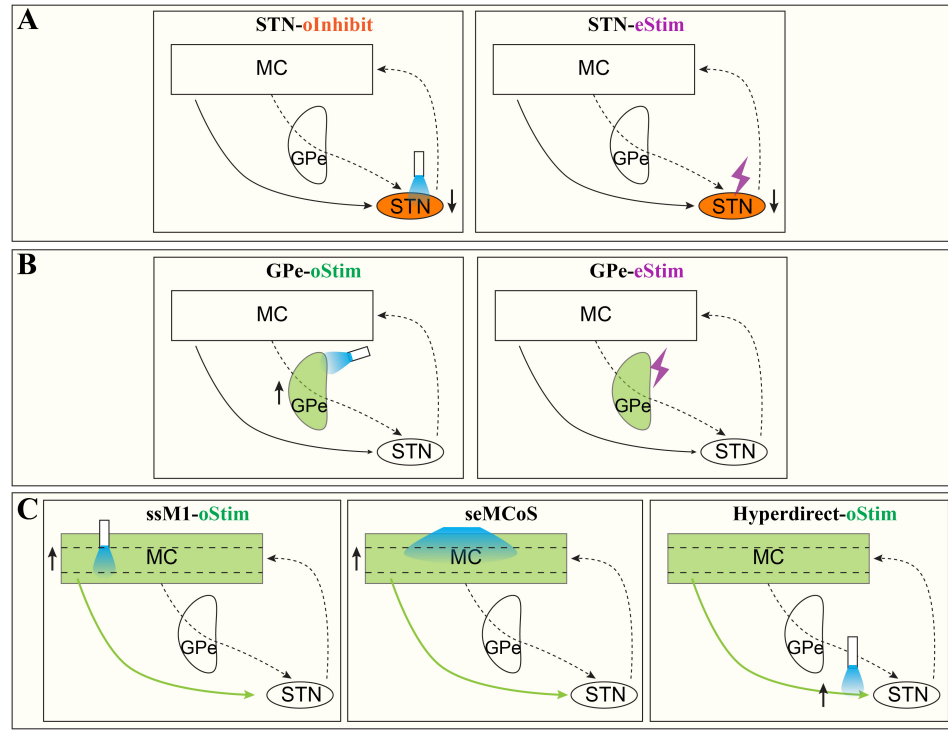


Figure 3.5: Schematic illustrations of modulation modalities investigated in this thesis. **A.** Modulation of the STN (left, optogenetic inhibition of principal neurons; right, electrical stimulation). **B.** Modulation of the GPe (left, pan-neuron optogenetic excitation; right, electrical stimulation). **C.** Modulation of the motor cortex: (left) intracortical single-site excitation of the layer V neurons (ssMC-oStim); (center) spatially-extended motor cortical optical stimulation (seM-CoS), which targets more superficial structures; (right) optogenetic stimulation of the hyperdirect pathway by light delivery at the terminals of cortical efferents at the STN.

3.4.1 Hypothesis 1: Inhibition of the principal neurons of STN is therapeutic for rescue of rotational bias

STN is a natural choice as the common target for deep brain stimulation. As part of the indirect pathway of basal ganglia circuit, dopamine depletion leads to overactivity of the indirect pathway and the STN (Miller and DeLong 1987; Fillion and Tremblay 1991). Inhibition of the principal neurons of STN was one of the most prevalent hypothesis for the mechanism of DBS (Pollak et al. 1993; A. L. Benabid et al. 1996; Deniau et al. 2010a; Sutton et al. 2013; Benazzouz and Hallett 2000). Global inhibition of STN (transient or reversible) by chemical modulation (Piallat, Benazzouz, and Benabid 1999; Petri et al. 2013) or electrolytic lesion (Carvalho and Nikkhah 2001; Hamani et al. 2004; Touchon et al. 2004) led to behavioral improvement in various mammalian models of PD; prior to DBS, subthalamotomy was a surgical treatment for PD (Bergman, Wichmann, and DeLong 1990; Su et al. 2002; Alvarez et al. 2005; J. A. Obeso et al. 2009; Martínez-Fernández et al. 2018; Volkmann 2018). However, two prior optogenetic studies (Gradinaru et al. 2009b; Yoon et al. 2014) reached conflicting results regarding the ability of STN-inhibition (halorhodopsin, NpHR, chloride pump) to modulate rotational behavior.

We set out to first investigate optogenetic inhibition of the excitatory neurons in the STN (Fig. 3.5 A, left) with a more potent inhibitory opsin (SwiChR_CA, chloride channel) by the Deisseroth group in Stanford University (Berndt et al. 2014). Electrical stimulation of STN will also be tested and serve as the gold-standard (Fig. 3.5 A, right).

3.4.2 Hypothesis 2: With the premise inhibition of STN is therapeutic, excitation of GPe, which sends inhibitory efferents to STN, shall also rescue rotational behavior.

GPe is a key component of the indirect pathway, which houses predominantly inhibitory GABAergic neurons that synapse strongly onto STN. A number of studies confirmed the prediction of the rate model that the activity of GPe and hence the inhibitory actions on

STN was reduced in a Parkinsonian state (Miller and DeLong 1987; Fillion and Tremblay 1991). It is worth pointing out that the changes in GPe electrophysiology is not completely indirect and through network effects. It has been shown that injection of 6-OHDA within GPe caused loss of DAergic neurons in SNc, as well as a reduction in the spontaneous firing rate of GPe neurons, hence displaying a direct effect from loss of dopamine (Bouali-Benazzouz et al. 2009).

Since I have demonstrated that additional inhibition at STN was therapeutic, our hypothesis is that activation of GPe, hence inhibiting STN, shall also be therapeutic for rescue of rotational bias. Furthermore, from a pragmatic perspective, the small size of the STN ($\sim 0.8mm^3$ (Hardman et al. 2002)) and the limited means of targeted genetic modifications in rats present challenges to further enhance the efficacy of optogenetics-based modulations. One technique to circumvent this problem is to utilize the existing anatomical connections and perform virus injection in a larger and more accessible brain region. Hence, GPe, a bigger nucleus ($\sim 10.3mm^3$ (Hardman et al. 2002)) immediately upstream to STN, is an attractive locus for optogenetic modulation.

In fact, a very recent report by Mastro et al. (Mastro et al. 2017) demonstrated rescue of bradykinesia by excitation or inhibition of subpopulations of GABAergic neurons in the GPe of mice with bilateral 6-OHDA lesion. However, the effect on rotational bias was not explored due to the bilateral lesion and transgenic mouse lines were used for targeted opsin expression.

Therefore, we investigated whether rotational bias can be rescued by optogenetic excitation of GPe (as well as by electrical stimulation) in rats with opsin expression achieved via viral transduction (Fig. 3.5 B, right).

3.4.3 Hypothesis 3: Excitation of motor cortex rescues rotational bias

One would not overlook the motor cortex in a study of neuromodulation for a movement disorder. Motor cortex plays important roles in the function of basal ganglia circuit, as both the striatum and STN receive excitatory glutamatergic projections from the motor cortex (Turner and Desmurget 2010). Profound electrophysiological changes were observed in the motor cortex following dopamine depletion (Lindenbach and Bishop 2013a; Pasquereau, DeLong, and Turner 2016a; J. A. Obeso et al. 2017). In addition, deep brain stimulation of the STN has been shown capable of modulating cortical activities (Dejean, Hyland, and Arbutnot 2009; Lehmkuhle, Bhangoo, and Kipke 2009; Pasquereau, DeLong, and Turner 2016). Most notably, direct activation of the fibers within the cortico-subthalamic hyperdirect pathway, which leads to antidromic activation of the cortex (Paz, Deniau, and Châprier 2005; S. Li et al. 2007; Q. Li et al. 2012; Kita and Kita 2012), may play an important role in the therapeutic mechanism of DBS. Indeed, in the seminal work by Gradinaru et al., optogenetic activation of layer V neurons in the motor cortex in transgenic mice led to more therapeutic actions than modulation of principal STN neurons (Gradinaru et al. 2009b); which was corroborated with following work by other groups (Q. Li et al. 2012; Sanders and Jaeger 2016a).

The motor cortex poses as a desirable locus for optogenetic modulation also because of its anatomy. First of all, the motor cortex consists of interconnected functional areas corresponding to detailed physiological (motor) mappings (Fonoff et al. 2009). In a practical point-of-view, not only does motor cortex has much larger volume (availability for spatially specific modulation), its accessibility also allows for easier targeting as well as possibility for less invasive methods of modulation (e.g. epidural stimulation ((Fregni et al. 2005; Elahi, Elahi, and Chen 2009; Gutierrez et al. 2009; Heumann et al. 2014; Chou et al. 2015; Flamez et al. 2016; Ferrucci et al. 2016)) and transcranial stimulation (Fregni et al. 2005; Elahi, Elahi, and Chen 2009; Gutierrez et al. 2009; Heumann et al. 2014; Chou et al. 2015; Flamez

et al. 2016; Ferrucci et al. 2016)).

Despite the advantages, the large volume of the motor cortex posed challenge for optogenetic studies in non-transgenic animal in the past. As an important indicator for the extent of opsin expression, prior to the application of convection-enhanced delivery of virus by this study (Zeyang Yu, Nurmikko, and Ozden 2017), directly evoked movement in body parts by optogenetic stimulation of the motor cortex had not been achieved. The functional and widespread opsin expression in the motor cortex allowed us to investigate the therapeutic potential of the motor cortex, as well as its involvement in the actions of DBS. In addition, the widespread expression also enabled modulation of the terminals of labelled MC-STN hyperdirect pathway at the STN, as this pathway received divergent input from all of the cortical areas (especially the motor cortex) (Monakow, Akert, and Knzle 1978; Atsushi Nambu, Tokuno, and Takada 2002; Kita and Kita 2012); thus a reliable basis for the optogenetic modulation of the hyperdirect pathway is established for non-transgenic rat models.

Specifically we investigated three paradigms of motor cortical stimulation (excitation by light, MC-oStim): the single-site intracortical stimulation (ssMC-oStim), the spatially-extended motor cortical optical stimulation (seMCoS), and the stimulation of the hyperdirect pathway (HD-oStim) (Fig. 3.5 C).

3.5 Electrical Stimulation of the Subthalamic Nucleus (STN-eStim), as the Gold-standard Modulation Paradigm, Rescued Rotational Behaviors in h-P Rats

As a ground-truth reference point, bipolar high-frequency (125Hz) electrical stimulation in the STN with charge-balanced biphasic pulses (60-240 μ sec, 100-200 μ A) was tested during spontaneous and drug induced rotations (also in other scenarios of behavior assessment).

Typically upon onset of a train of supra-threshold electrical stimuli, animal typically showed brief “nodding” (lowering of head and extension of neck), followed by increased whiskering, horizontal exploration, and rearing (vertical exploration). While animal's spontaneous movement was significantly increased and spontaneous rotational biases was reduced (data not shown), the asymmetry in posture, especially the lack of use of the lesion forelimb was not reversed. At very high stimulation amplitudes and long pulse duration, repetitive hand movements could be elicited; these parameters were avoided in rotation tests.

In amphetamine challenges, electrical stimulation of STN significantly reduced asymmetric Amph-IR by $37.4 \pm 4.9\%$ ($p < 1E-3$, $N = 6$ rats, 66 trials; $F_{1,4} = 63.6$, $p \rightarrow 0$) (Fig. 3.6 A). There was in general no long-lasting hysteric effects associated with electrical stimulation (as we avoided stimuli that produced direct movement). Another similar restriction was to limit current amplitude to avoid induction of persistent counter-clockwise (CW) rotations; as such CW rotations in Amph-IR represented over-compensation of hemiParkinsonism. As a result the therapeutic effects were obvious and consistent, in most animals residual CCW rotations were always observed, reflecting a more conservative estimate compared with other literature (Gradinaru et al. 2009b).

In terms of stimulation parameters, in most trials a 125 Hz pulse frequency was used, as its high efficacy were widely accepted in literature (Gradinaru et al. 2009b; Q. Li et al. 2012). For each animal, we first determined the threshold stimulation parameters:

starting out with short pulse durations (60 - 100 μsec), ramping up current amplitude from 50 to 200 μA ; if no clear modulation was found at 200 μA , a longer pulse duration was employed. Animals, not responding to pulses with duration greater than 300 μsec , were excluded in further testing for the high likelihood of misplacement of electrodes.

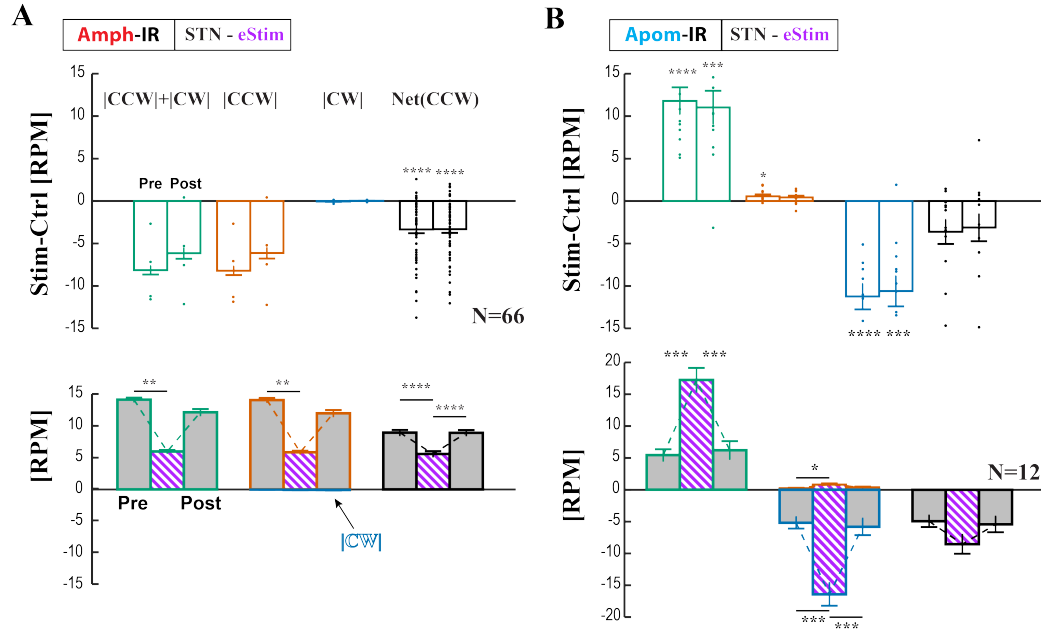


Figure 3.6: Rotational bias modulated by high-frequency electrical stimulation of STN. Four types of speeds were charted: |CW|, the total rotations in the CW direction (represented as negative values for consistency), |CCW|, the total rotations in the CCW direction, Net(CCW), the net rotations in the CCW direction (positive values meaning overall more CCW than CW, and vice versa), and |CCW|+|CW|, the total number of rotations in both directions (always positive). The top panels show the changes in speeds during stimulation subtrials from that of the pre-stim or post-stim control trials; and the bottom panels show the absolute speeds in the pre-stim, stim (stripped), and post-stim subtrials. Note that the Net CCW values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed taking account into the interburst intervals. The bars show average values and the error bars represent the s.e.m. The asterisks (*) indicated the level of significance (see methods for details). The interpretation of the graph also applies to the following figures in this chapter. **A.** Amph-IR results. STN-eStim significantly reduced biases in Amph-IR ($p < 1\text{E-}4$, paired t -test, $N = 6$ rats, 66 trials; $F_{1,4} = 63.6$, $p \rightarrow 0$). **B.** Apom-IR results. A significant increase in CW activities were observed ($p < 1\text{E-}4$, paired t -test, $n = 1$ rat, 12 trials; $F_{1,1} = 51.6$, $p \rightarrow 0$), however in behavior this increase manifested as a combination of increased rotation and increased back-and-forth head turns.

In apomorphine challenges, STN-eStim significantly increased the rotational motion

towards the CW direction by $199.1 \pm 30.0\%$ ($|CW|$, $n=1$ rat, 12 trials, $p<1E-4$ paired t-test; $F_{1,1} = 51.6$, $p \rightarrow 0$, ANOVA), on top of the existing CW driving force from the effect of the drug (Fig. 3.6 B). A positive correlation between current amplitude and CW rotation speed was also observed ($R^2 = 0.65$), although statistical significance cannot be asserted due to limited sample size for each amplitude condition. Upon closer inspection of animal's movement, we found the increase in CW activities composed of a combination of increased rotations and increased back-and-forth turning of the head (head-turns)(CW followed by CCW, but all individual CW and CCW actions were separately registered by —CW— and —CCW—; in the contrary, this effect would be canceled out in net(CCW) measurement). The headturns were time-locked with delay to the onset of bursts of stimuli. In fact, the effect of the delay in onset of motor response was manifested in the net CCW rotation (all ON-phase of stimulation bursts): it showed lower changes and higher variations, as in trials with short burst durations (0.5 sec), elicited movement occurred during OFF-phase due to delay. When net CCW rotations were measured taking in to account of both ON- and OFF-phase of the stimulation bursts, the outcome of modulation ($-201.5 \pm 30.0\%$, $p<1E-4$; $F_{1,1} = 51.8$, $p \rightarrow 0$) was much more consistent with total CW rotations.

The overall outcome of apomorphine challenge was not consistent with the previous reports of modest reduction of Apom-IR by DBS at STN in h-P rats (Q. Li et al. 2012; Badstuebner et al. 2017). While the previous studies employed continuous delivery of 125Hz pulses, stimuli was delivered in bursts (0.5 -1.0 sec intervals) in this study. We speculate some adaptation effect to electrical stimulation may contribute to the difference in the outcomes.

3.6 Optogenetic Inhibition of the Subthalamic Nucleus (STN-oInhibit)

Inhibition of the principal neurons of STN was one of the most prevalent hypothesis for the mechanism of DBS (Pollak et al. 1993; A. L. Benabid et al. 1996; Deniau et al. 2010a; Sutton et al. 2013; Benazzouz and Hallett 2000). Two prior studies (Gradinaru et al. 2009b; Yoon et al. 2014) reached conflicting results in ability of STN-inhibition (halorhodopsin, NpHR, chloride pump) to modulate rotational behavior. We set out to first investigate optogenetic inhibition of the excitatory neurons in the STN with a potent inhibitory opsin (SwiChR, chloride channel) by the Deisseroth group in Stanford University (Berndt et al. 2014).

Leading to the inhibition of STN principal neurons, we first tested two other stimulation regime that were also reported to be ineffective: excitation of STN excitatory neurons and pan-neuron inhibition of STN. In two rats, excitatory opsins (C1V1t/t) were expressed under Ca^{2+} /calmodulin-dependent protein kinase II- α (CaMKII α) promoter in the principal neurons of STN (Fig. 2.2). Optogenetic excitation of the excitatory population of neurons in the STN failed to produce any consistent prokinetic improvement (no modulation on rotational behavior or the overall mobility, data not shown), which agreed with the observations in previous literature (Gradinaru et al. 2009a). Subsequently, we confirmed that pan-neuron inhibition of STN (hSyn::eNpHR3.0) was not able to improve rotational behavior as previously reported (Gradinaru et al. 2009b; Yoon et al. 2014).

By notable contrast, optogenetic inhibition of the principal neurons in the STN by SwiChR (Berndt et al. 2014) led to marked reduction of Amph-IR by $16.3 \pm 4.5\%$ ($p < 0.001$, $n = 3$ rats; $F_{1,2} = 14.87$, $p < 0.001$) (Fig. 3.7), though not as potent compared with electrical stimulation. Although in general stimuli were delivered at high pulse frequency for consistency with electrical stimulation, we did not find significant difference in efficacy between stimuli delivered at 20-100 Hz. Apomorphine challenge was not conducted in this

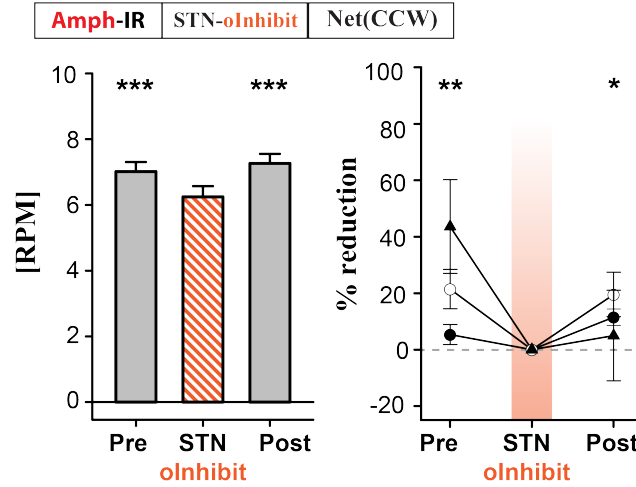


Figure 3.7: Rotational bias modulated by high-frequency electrical stimulation of STN. Both the speed of net CCW rotation (left column) and the percentage reduction in rotation (right column) was diagrammed. See methods for interpretations of * and #.

cohort of animals. In terms of spontaneous behavior (SR or bradykinesia), no noticeable improvement was found with STN inhibition.

The SwiChR opsin was designed with bi-stable (with ON and OFF states) functionality (Berndt et al. 2014) *in vitro*, but our *in vivo* verification study (Section 2.2.2) was not able to find any step-function kinetics in extracellular electrophysiology of STN. Interesting, long-lasting effects of STN-oInhibit were observed in some cases, which occurred on the scale of minutes, hours, or even days. In one animal (Rat G), CW rotations were elicited in spontaneous rotations in two trials of effective STN-oInhibit modulation; afterwards, even without stimulation, the animal manifested spontaneous CW rotations that lasted for seven days. A third stimulation trial 14 days after the first two again elicited CW rotations (Fig. 3.8 B). However, this observation was extremely rare, which we were unable to recreate in other animals. Another caveat was that these stimulation trials were all performed at very high light power (>15 mW) and heating became a likely confounding factor; however we confirmed in other animals that heating alone did not lead to long-lasting CW rotations.

Similar hysteresis effect was observed in another animal (Rat W) in Amph-IR: CW rotations was consistently observed in control trials following STN-oInhibit; no hysteresis was found in other animals during Amph-IR.

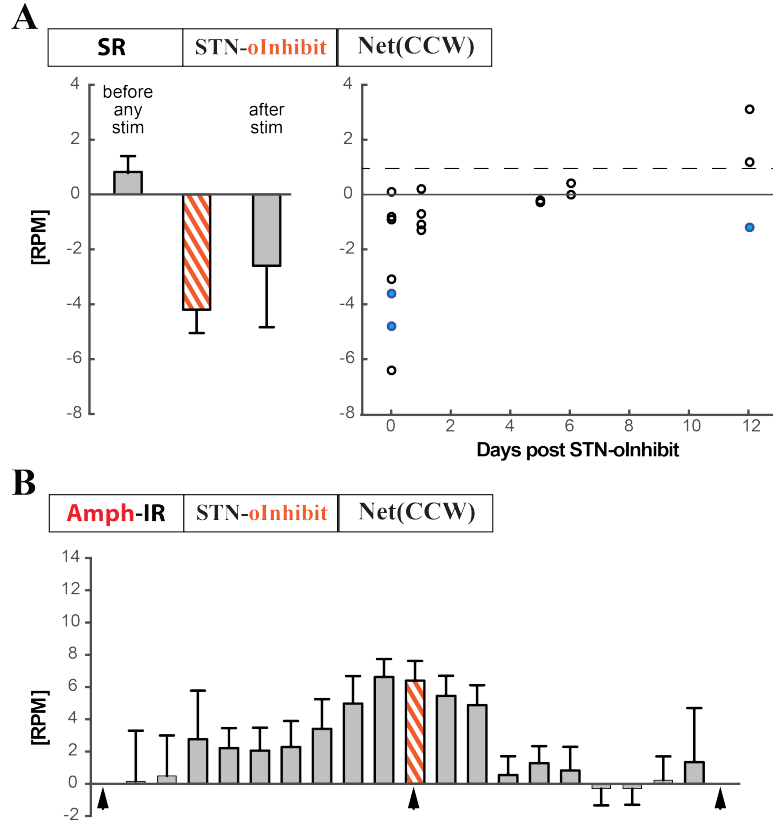


Figure 3.8: Example of hysteresis in the effect of STN-oInhibit. **A.** In Rat G, after two effective trials of STN-oInhibit, CW rotations (negative net rotations) occurred even without stimulation and persisted for days. **B.** In Rat W, hysteresis effect was found in the modulation of Amph-IR. The x-axis showed consecutive trials. The arrowheads indicated the previous, the current, and the next trial with optical modulation.

In summary, while inhibition of STN indeed provided anti-(hemi-)Parkinsonian actions and reduced severity of rotational bias, perhaps the small size of STN and the rather limited capability to optogenetically interrogate neuronal subtypes in non-transgenic rats constrained the parameter space one can explore for better stimulation outcome. In addition, the lesser efficacy compared with STN-eStim and the failure in improving overall

mobility by STN-oInhibit suggested possible parallel mechanisms of action of deep brain stimulation.

3.7 Optogenetic Excitation of the Globus Pallidus Externus (GPe-oStim)

GPe is a key component of the indirect pathway, which houses predominantly inhibitory neurons (subdivided into two major populations, see discussion later) that synapse strongly onto STN. A number of studies confirmed the prediction of the rate model that the activity of GPe and hence the inhibitory action on STN was reduced in a Parkinsonian state (Miller and DeLong 1987; Filion and Tremblay 1991). Since I have demonstrated that additional inhibition at STN was therapeutic, our hypothesis is that activation of GPe (hence inhibition of STN, as well as direct inhibition of basal ganglia output) is also therapeutic. From a pragmatism perspective, the small size of the STN and the limited means of targeted genetic modifications in rats present challenges to further enhance the efficacy of optogenetics-based modulations. One technique to circumvent this problem is to utilize the existing anatomical connections from GPe to STN and perform virus injection in the larger and more accessible GPe. Therefore, these factors present GPe as an attractive locus for optogenetic modulation. In this section, I show that pan-neuron optogenetic excitation of GPe was also and even more therapeutically effective.

3.7.1 Pan-neuron activation of GPe resulted in robust rescue of rotational bias

Excitatory opsins (ChR2 or C1V1(t/t)) were expressed non-specifically (human synapsin promoter, hSyn) in the region of GPe. Histology confirmed the spread of expression was relatively confined: unintentional expression was found posterior to GPe in thalamic reticular nucleus but not in the striatum (Fig. 2.4).

For amphetamine challenges, activation of GPe produced pronounced reduction of Amph-IR by $-54.3 \pm 4.5\%$ ($p < 1E-4$, $n = 2$ rats, 33 trials) (Fig. 3.9 A), which was as potent as STN-eStim: in many trials complete abolishment of CCW rotations were achieved. Exploration of the parameter space was performed while holding a constant overall duty cycle. A positive relationship was found between pulse frequency and CCW reduction ($R^2=0.46$, $p<0.001$, Fig. 3.9): low frequency stimulation tended to be ineffective. In addition, a rather non-linear power-dependency was noted: reduction of rotations was consistent above threshold power (5 mW), which matched the saturation effects in modulation of electrophysiology (Fig. 2.6 D).

As stimulation were delivered in bursts (0.5 - 10 sec) of high frequency pulses (100 Hz), we observed two interesting phenomenons, namely the “burst-onset” effect and the “train-onset” effect. “Burst-onset” effect refers to the reduction of CCW rotation speed time-locked to the onset of each burst (short delay). In some cases, back-and-forth (CW followed by CCW returning to the start position) turning of the head (“head-turns”) was also noticed during each burst. However, not all bursts in the same stimulation train were equally effective. Stimulation effect was most pronounced at the beginning of each stimulation train (“train-onset” effect) and a period of complete stop was reliably observed, the duration of which was variable and ranged from 5 to 30 sec (Fig. 3.9 Ea). Regardless of stimulation parameter (frequency, power, and duty cycle), the animal resumed CCW rotation in most trials at the latter part of a stimulation train, though the rotation speed was lowered. Another clear evidence for the “stopping power” of GPe-oStim was indicated by the sudden increase in CCW-rotation immediately after the offset of the stimulation train (“rebound effect”), which lasted less than 10 sec. While we did not start stimulation immediately after the end of the recoil, the effectiveness of stimulation was restored after 30 sec of rest. Combined, our result seemed to suggest competition between amphetamine-induced CCW driving force and the stimulation-evoked CW correction force.

The rhythmic head-turns occurred during stimulation may suggest evoked direct

motor response. Subsequent experiments looked at stimulation effect on spontaneous behaviors and found that the stereotypical CCW rotations were indeed overridden with CW rotations during GPe-oStim. While the head posture changed from pointing towards left-side (ipsilateral) to towards right-side (contralateral), CW rotations were not found in trials without CCW rotations before stimulation onset. Hence, I next studied whether animal's intent to move affects the stimulation outcome. When the animals were initially exposed to slight tactile agitation (by a cotton Q-tip), exploration was increased and CW-rotations were reliably evoked by stimulation (Fig. 3.9 C). After acclimation to the agitation, animals no longer responded to touches and stimulation failed to induce rotations in either CCW or CW directions.

When animal was in the over-compensated state induced by apomorphine, a drastic further increase in rotation was evoked by GPe-oStim ($185.5 \pm 16.6\%$, $p < 1E-4$, $n = 2$ rats, 81 trials, paired *t*-test, Fig. 3.9 D). The stereotypical small radius chasing-the-tail posture was maintained during stimulation. Higher stimulation effect weakly correlated with light power and strongly correlated with higher pulse duration and duty cycle.

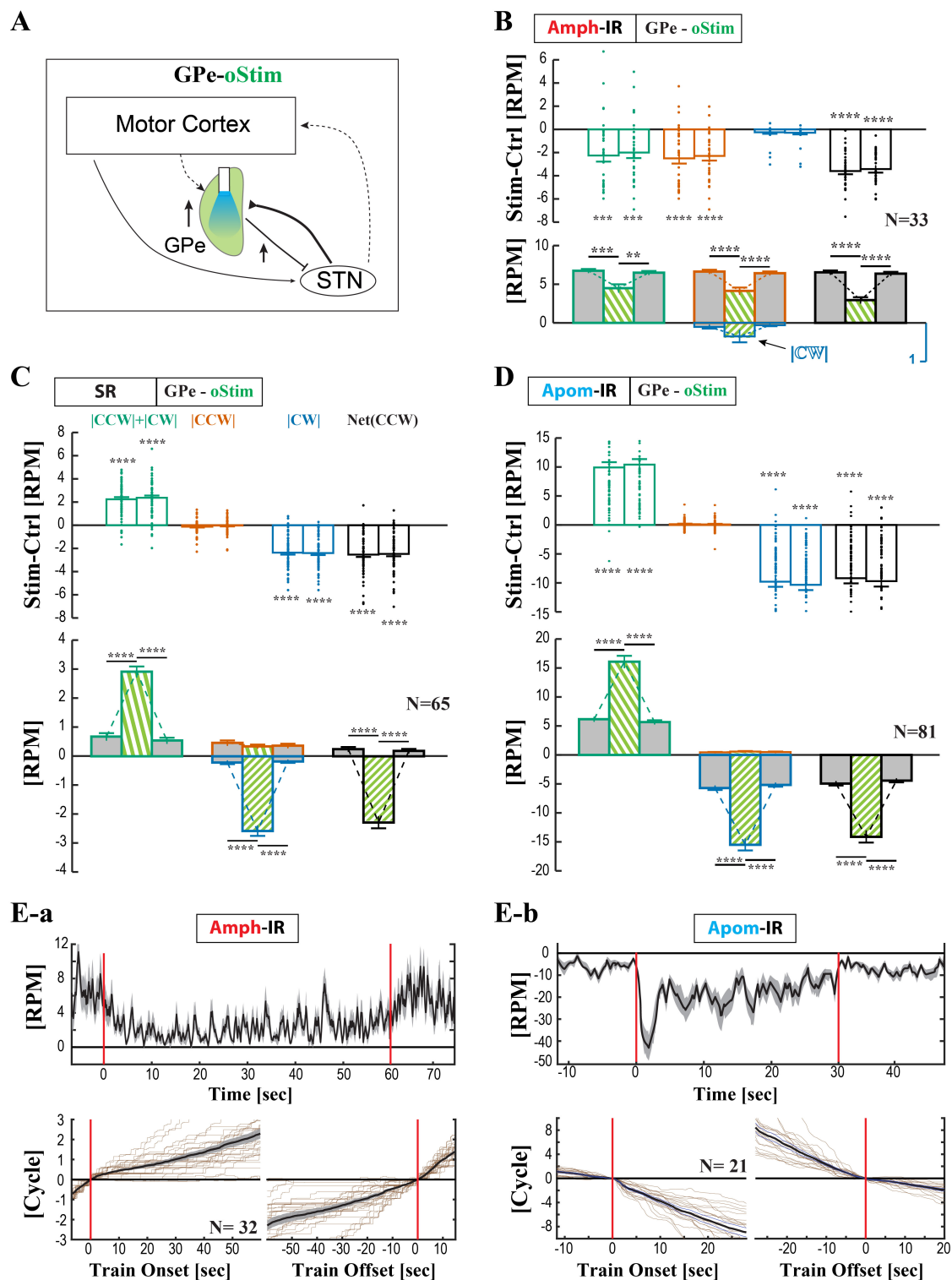


Figure 3.9: Rotational bias alleviated by optogenetic stimulation of GPe (GPe-oStim).

Figure 3.9 caption – continued **A.** Illustration of the stimulation paradigm. GPe-oStim theoretically would increase the activity locally in the GPe and also increase the inhibitory action onto STN (flat-terminus line). The excitatory STN->GPe (triangle-terminus line) and the cortico-subthalamic (the line with small arrowhead) fibers were also depicted. The final dashed arrow line showed the ultimate feedback output from basal ganglia to cortex through thalamus. **B.** Amph-IR results. GPe-oStim significantly reduced biases in Amph-IR ($p < 1E-4$). **C.** Spontaneous rotation. CW rotations were elicited during stimulation. The extent of CW rotations depended on the intent to move of the animal at the instance. **D.** Apom-IR results. A drastic increase in CW rotations were observed ($p < 1E-4$). **E.** Examples of trial-averaged rotation traces obtained during AmphIR (E-a) and ApomIR (E-b) in Rat G03. Traces of the instantaneous rotation speed (with s.e.m., gray area) were shown in top panels (the two red bars indicated the onset and offset of a stimulus train); and the bottom panels showed the progression of rotation in separate trials (brown thin traces) and in trial-average (black thick trace, with s.e.m., gray area). E-a: Many trials showed train onset abolishment of rotation but the rotation would ultimately resumed despite the ongoing stimulation (see the change in slope in the bottom left plot around 20 sec). At the offset, a clear increase in rotation speed was found. E-b: Note the astonishing peak rotation speed of >40 RPM evoked by stimulation. Both the absolute speed (top panels) and changes in the speed (bottom panels) were shown. Note that the Net CCW values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed from both ON- and OFF-cycle of the bursts. The asterisks (*) indicated the level of significance (see methods for details).

Other direct and indirect evidence also existed for the therapeutic effect of modulation of the striatopallidal pathway. In a seminal study aiming to understand pathway specific function in PD, Kravitz et al. showed increased spontaneous contraversive rotation could be induced by activation of D1-MSN and reduced overall movement by activation of D2-MSN (Alexxai V. Kravitz et al. 2010). A later study showed activation of D2-MSN in fact led to increased ipsiversive rotation and reduced contraversive rotations in healthy mice (H. J. Lee et al. 2016). However, no consensus was reached regarding the effect on rotations by inhibition of D2-MSN. Another study in healthy mice revealed increased ipsiversive rotation by inhibition of either D1- or D2-MSNs; and contraversive rotation required activation of both types of MSNs (Tecuapetla et al. 2014). In this regard, our results that pan-neuron activation of GPe, as a means of increasing inhibition on D2-pathway, could induce contraversive rotations conditioned on the existing intention to move, filled the gap in literature and provided new perspective on the functional significance of the indirect-pathway.

One caveat of our optogenetic method had to do with the lack of specificity to the

cellular subtypes in GPe. There are multiple subgroups of output neurons in GPe that project to STN, differentiated by variable expression of cellular markers, Lim homeobox 6 (Lhx6) and parvalbumin (PV) (Mastro et al. 2014). In a recent seminal discovery, using transgenic mice, Mastro et al. was able to achieve rescue of bradykinesia with selective modulation of subpopulations in GPe, while pan-neuron activation of GPe did not result in increased mobility (Mastro et al. 2017); this is in high accordance with our finding that overall mobility and the intent to move was not increased by GPe-oStim.

In conclusion, our results pointed to an overall correction of rotational bias, but not an increase in the intention to move, as a result of pan-neuron activation of GPe.

3.7.2 Electrical stimulation of the globus pallidus externus (GPe-eStim)

The Trident probes allowed electrical stimulation in the same region where optical stimulation was delivered. In amphetamine challenges, the speed of rotation was consistently suppressed by GPe stimulation and the extent of reduction ($58.3 \pm 5.0\%$, $p < 1E-4$, $n = 2$ rats, 34 trials, paired t -test) was comparable with that caused by GPe-oStim. Also similar to GPe-oStim, an initial stop in rotation did not last through the entire length of a stimulus train (30s); and the animal ultimately resumed CCW rotation at a reduced speed (Fig. 3.10 E). A modest, yet consistent increase in the occurrence of CW rotations were also found. A key difference in the behavior, however, was the lack of obvious repetitive head-turns in the CW-direction. Immediately after the offset of stimulation train, rotation speed increased to higher than the pre-stimulus values (Fig. 3.10 E, right).

In apomorphine challenges, both increase and decrease in the heightened CW rotation were observed, and no consistent additional motor activation on the lesion hemisphere was realized ($p=0.25$, paired t -test). In addition, while not formally characterized, the spontaneous rotation of the animal without agitation was not increased, suggesting the lack of effect on improving bradykinesia or akinesia.

While electrical stimulation of GPe is not a common target for deep brain stimulation, improvement in parkinsonian motor signs was observed in the acute clinical study (Vitek et al. 2004). Its functional importance to the function of basal ganglia has long been investigated. Early *in vivo* studies in numerous models consistently demonstrated the elicitation of contraversive rotation with unilateral stimulation of the GPe (and homologous nucleus) (Crossman, Lee, and Slater 1977, 1978; L. A. Lee and Slater 1981). However, the pallidal area, just like STN, is infiltrated with corticofugal fibers and fibers from the striato-nigral (direct) pathway (this is exemplified by the D1-Cre fluorescence occurred in the GPe region in Figure 1 of the work by Kravitz et al. (Alexxai V. Kravitz et al. 2010)); thus electrical stimulation could affect activities of the local GPe neurons and the fiber of passage (Asdourian, Lentz, and Kelland 1991). Other reported afferents, efferents, and fibers of passage were summarized in Fig. 3.10 B.

In fact, the therapeutic effects found during GPe-eStim would appear to contradict the predicted inhibitory effect of high-frequency stimulation, as inhibition on GPe neurons would lead to deterioration in motor symptoms according to the canonical model. Indeed, a wealth of evidence has shown that high frequency electrical stimulation could preferentially activate fibers (McIntyre, Savasta, et al. 2004; McIntyre, Grill, et al. 2004; Arle et al. 2018; Feng et al. 2017) and increase the output from the stimulated structure (Hashimoto et al. 2003). Providing additional support, albeit indirect, Asdourian et al. reported abolishment of GPe-eStim-evoked contralateral muscle activity with chemical lesion to the fibers, but not neurons of GPe (Asdourian, Lentz, and Kelland 1991). If GPe-eStim were to exert its effect through preferential modulation of fiber, the effect of increased GPe output by GPe-eStim might be regarded functionally equivalent to the optogenetic activation of GPe neurons. In addition, GPe-eStim may also affect fiber of passages (Fig. 3.10 B) and the activation of direct pathway fibers would also lead to enhanced motor activation. A perhaps more indirect evidence, showing the pro-kinetic effect of GPe-eStim, comes from study of other hyperactivity-related motor disorders: hyperactivity of GPe was reported

in patients with Tourette Syndrome and electrical stimulation of GPe has been considered as a potential treatment (Andrade and Visser-Vandewalle 2016). Hence, the activation hypothesis of electrical stimulation in GPe (or its dominance over the inhibitory effect) could address the therapeutic effect during Amph-IR.

The lack of effect on Apom-IR (sharing the similar cellular mechanism to that of dyskinesia) by electrical stimulation was in contrast to the observation of increased likelihood of dyskinetic symptoms in human patients receiving GPe electrical stimulation (Vitek et al. 2004); and our previous finding of drastically increased Apom-IR by GPe-oStim. To some extent, this discrepancy suggested the presence of non-excitatory effect of electrical stimulation on the GPe neurons (premising on the anatomical complexity of the GPe region). Perhaps the inhibitory stimulation became evident at a state of heightened GPe activity (by apomorphine); the activity within the GPe is increased during successful dopamine-replacement treatment (e.g. L-DOPA) compared to the lowered level in the Parkinsonian state (Boraud et al. 1998).

Overall, while producing rescuing effect on rotational bias consistent with observations in GPe-oStim, electrical stimulation led to several key discrepancies. In fact, the mixed effects added to the significance of optogenetics in the dissection of pathway-specific functions.

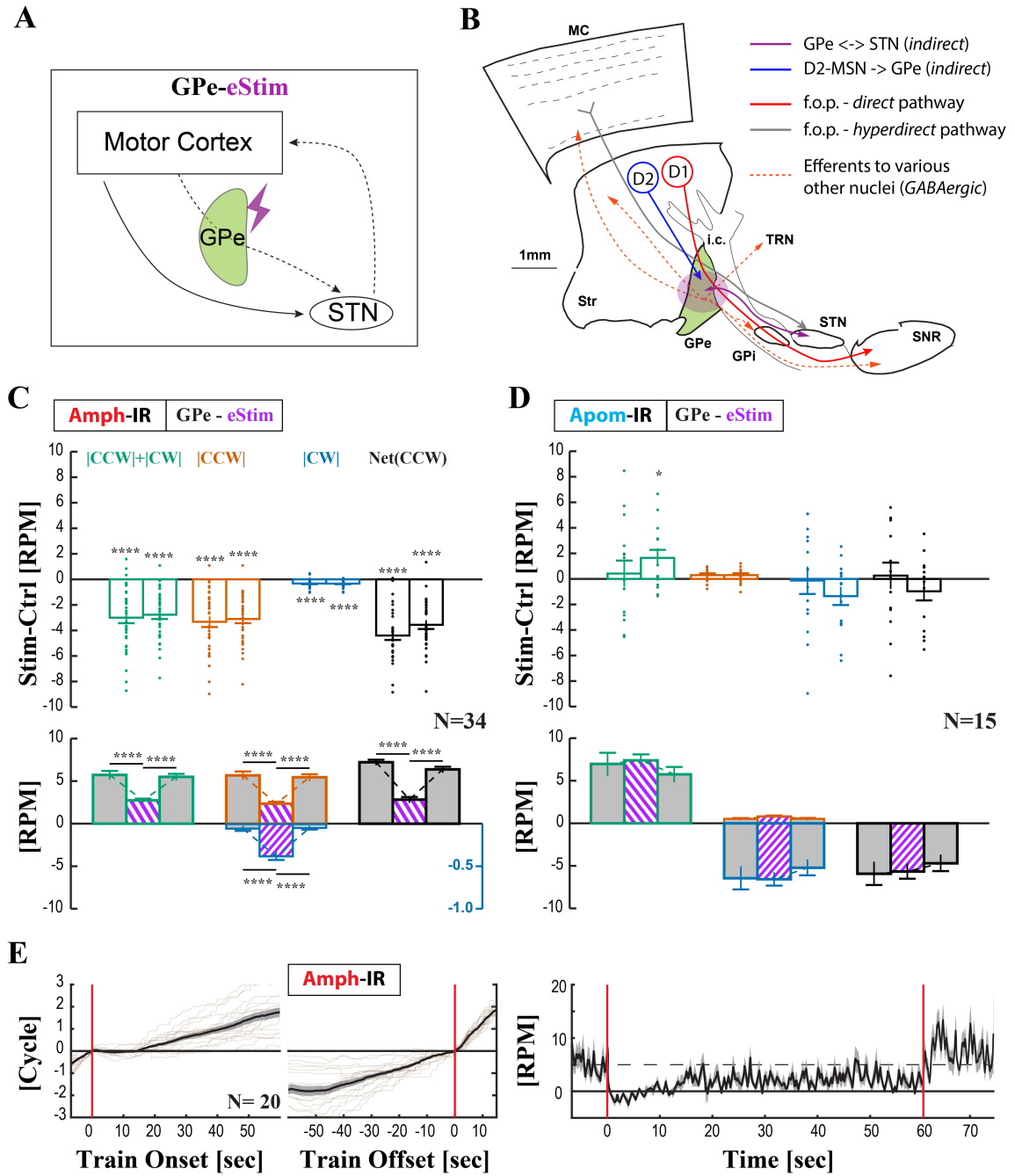


Figure 3.10: Rotational bias modulated by electrical stimulation of GPe (GPe-eStim).

Figure 3.10 caption – continued **A.** Illustration of the stimulation paradigm. **B.** A more realistic depiction of anatomical connections potentially affected by GPe-eStim. Besides the well known inputs from the D2-MSN, as well as the directional connections with STN, GPe also sends efferent projection to the striatum (Alexxai V. Kravitz et al. 2010; Mallet et al. 2012; Abdi et al. 2015; Saunders, Huang, and Sabatini 2016), to the GPi and SNr (Lindahl et al. 2013), to thalamus (e.g. TRN (Cornwall, Cooper, and Phillipson 1990; Gasca-Martinez et al. 2010; Oh et al. 2017), and to the frontal cortex (Saunders et al. 2015). This region is also pact with fiber of passages including the fibers of the direct pathway (Alexxai V. Kravitz et al. 2010; Mallet et al. 2012; Abdi et al. 2015). The region of GPe is colored in light green. The span (for illustration only, not to scale) of electrical stimulation is colored in transparent purple. (abbrev. f.o.p: fiber of passage; TRN: thalamic reticular nucleus. **C.** Amph-IR, the exaggerated rotational bias was significantly reduced by GPe-eStim ($p < 1E-4$). A modest, yet consistent increase in the occurrence of CW rotations were also found. **D.** No consistent increase in CW rotations was noted during Apom-IR. A slight non-significant increase in the overall movement (total CW and CCW rotations) was found. **E.** Examples of trial-averaged rotation traces obtained during AmphIR in Rat G03. (Left) The progression of rotations in separate trials (brown thin traces) and in trial-average (black thick trace, with s.e.m., gray area). (Right) traces of the instantaneous rotation speed (with s.e.m., gray area); the two red bars indicated the onset and offset of a stimulus train; and the dashed line indicated the average speed pre-stim. A clear manifestation of the “train-onset” rotation abolishment was shown as the lack of change in the number of rotations and the null values in rotation speed. The rotation re-initiated ultimately on average after 15 sec. At the offset, a clear increase in rotation speed was found (higher than the pre-stim value). Note that the “Net CCW” values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed from both ON- and OFF-cycle of the bursts. The asterisks (*) indicated the level of significance (see methods for details).

3.8 Optogenetic Excitation of the Motor Cortex (MC-oStim)

As discussed in the previous sections, motor cortex is crucial in the function of basal ganglia circuit; physiological changes in the motor cortex were shown to correlate with motor symptoms of Parkinson's disease. Antidromic activation of motor cortex has been shown as a potential mechanism for STN-DBS through the hyperdirect pathway (Paz, Deniau, and Charpier 2005; S. Li et al. 2007; Q. Li et al. 2012; Kita and Kita 2012). Optogenetic activation of layer V neurons in the motor cortex and the hyperdirect pathway in transgenic mice both led to more therapeutic improvement of motor functions (Gradinaru et al. 2009b). Its large size and accessibility by less-invasive stimulation methods attracted me to further investigate the therapeutic potentials of motor cortical optogenetic stimulation in non-transgenic rats.

In this section, focusing on the rescue of rotational behavior, we investigated three

paradigms of motor cortical stimulation (excitation by light, MC-oStim): the single-site intracortical stimulation (ssMC-oStim), the spatially-extended motor cortical optical stimulation (seMCoS), and the stimulation of the hyperdirect pathway (HD-oStim).

3.8.1 Single-site motor cortical stimulation (ssMC-oStim)

In an early preliminary study, we found single-site stimulation in the motor cortex without widespread opsin express, though elicited clear and spatially limited electrophysiological response (see 2.4.1), was not able to evoke rescue of rotational behavior or induce any visible motor response. However, evidence from healthy animal with widespread excitatory opsin expression in the motor cortex confirmed the evoked direct motor movement by intracortical optical stimulation (Zeyang Yu, Nurmikko, and Ozden 2017). Here I will discuss results from intracortical single-site excitation by optical stimulation.

Within a well labeled motor cortex, spatially-limited intracortical light illumination (volume of opsin expression much greater than volume of suprathreshold light distribution) was able to evoke strong modulation of local neurons, neurons in the contralateral cortical areas through interhemispheric connections, as well as field potentials in the STN (2.4.3 and 2.4.4).

In terms of behavioral modulation in awake and freely moving animals, ssMC-oStim of the motor forelimb area led to modest ($-13.5 \pm 4.1\%$, $n = 2$ rats, 34 trials), yet significant ($p < 0.01$, paired t -test) reduction of Amph-IR (Fig. 3.11 B), which was comparable to STN-oInhibit. In apomorphine challenges, in great contrast to the previous observations of both the increased CW rotations by GPe-oStim and STN-eStim and the lack of change by GPe-eStim, a pronounced decrease in rotation was observed (Fig. 3.11 C). On the first glance, this observation would appear to be contradicting the assumed function and pathophysiology of the motor cortex, while further analysis of literature revealed possible explanations (which will be discussed in later part of this section).

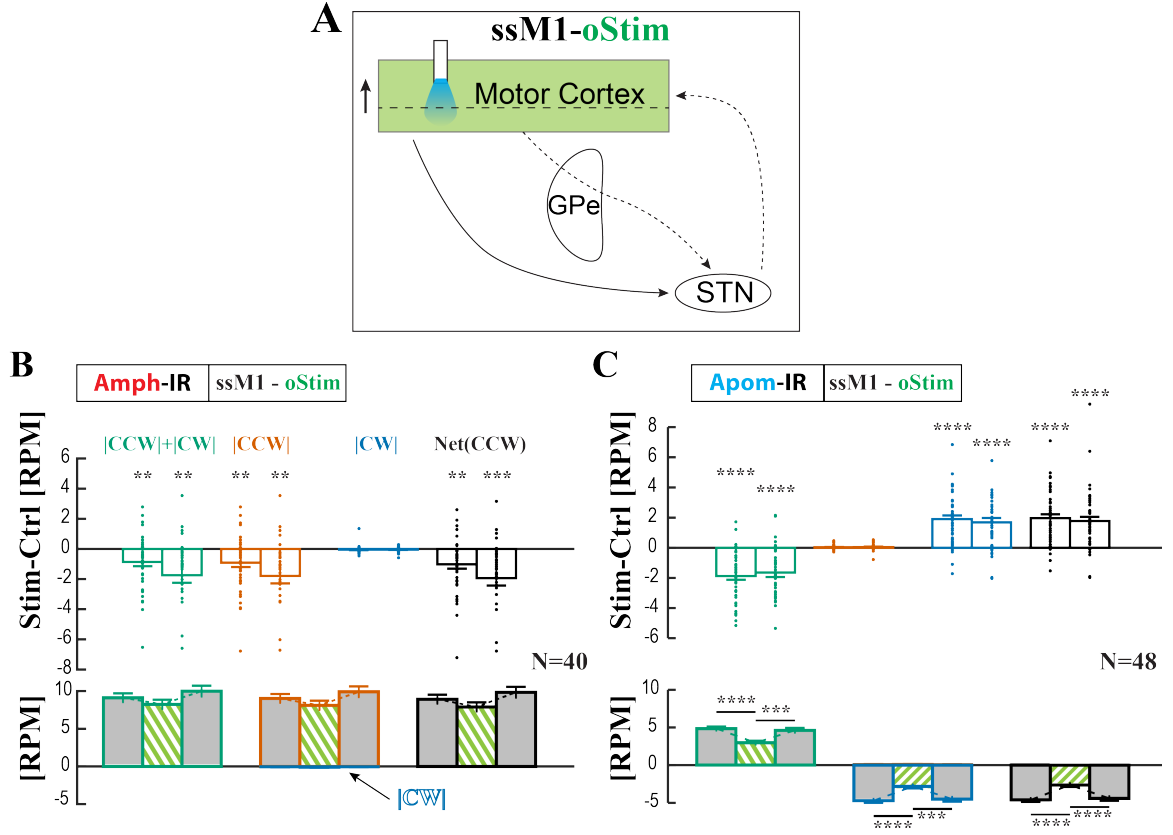


Figure 3.11: Rotational bias reduced by intracortical single-site stimulation of the motor cortex (ssMC-oStim). **A.** Illustration of the stimulation paradigm. The sizes of nuclei, the diameter of the optical fiber, and the lateral spread of the light were not drawn in scale. Yet, we did intend to show that delivery of light was targeted to the Layer V (dashed horizontal line) neurons. Theoretically cortical neuronal activity would be increased during the stimulation (the upward arrow). **B.** Amph-IR. The exaggerated rotational bias was modestly, yet significantly reduced ($-13.5 \pm 4.1\%$, $n = 2$ rats, 34 trials, $p < 0.01$, paired t -test). Neither consistent occurrence of CW rotations, nor any obvious contraversive evoked movement of body parts was observed. **C.** Apom-IR. In contrary to the enhancement observed in GPe-oStim and STN-eStim, as well as the lack of change observed in GPe-eStim, a marked reduction in the over-compensated CW rotations were noted ($-37.1 \pm 5.8\%$, $p < 1E-4$, $n = 2$ rats, 48 trials, paired t -test). Note that the Net CCW values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed from both ON- and OFF-cycle of the bursts. The asterisks (*) indicated the level of significance (see methods for details).

Upon closer inspection, the stimulation effects, similar to that of the previous stimulus paradigm, showed dependence on the time course of the stimulus. For example, the

reduction of rotations was more pronounced at the beginning of each stimulus burst (burst-onset effect). Also, the effect became less noticeable near the end of each stimulus train (train-onset effect).

Overall, ssMC-oStim produced therapeutic effect towards rescue of rotational behavior. While rather accurate in the targeting of Layer V neurons, the limited stimulation volume (< 1 mm in radius) cast questions in whether the efficacy could be improved by engaging more expressed cortical areas. In this paradigm, further increase of stimulation volume would not be trivial, as local optico-thermal effect may become hazardous (see discussion in 3.9.1). In fact, another unexpected (yet not surprising) complication to this paradigm was the epileptogenesis associated with focal activation of cortex, which was exacerbated also by the implantation related injuries (SecRef Appendix A).

3.8.2 Spatially-extended motor cortical optical stimulation (seMCoS)

Rising up to the challenges and limitations of the single-site motor cortical stimulation (ssMC-oStim), I will show results from a less invasive method of light delivery, the spatially-extended motor cortical optical stimulation (seMCoS), which is achieved essentially through epicortical illumination to a large cortical area.

The choice of this stimulation modality is motivated from several aspects. First, during in vivo evaluation of the CED expression, we found reliable evoked cortical ECoG-like potential by epicortical illumination (Zeyang Yu, Nurmikko, and Ozden 2017). Secondly, earlier work by Huber et al. demonstrated the use of LED as light source for epicortical stimulation to evoke changes in sensorimotor behavior, albeit in mice (Huber et al. 2008). Finally, due to the limited penetration of light, surface illumination more likely engages the superficial layers (Layer II/III and superficial Layer V), from which corticostriatal projections are more likely to innervate with D1-MSNs in the striatum, leading to preferential activation of the pro-kinetic direct pathway (Reiner et al. 2003; Lei et al. 2004; Mathai

and Smith 2011; Liu et al. 2011; Lindenbach and Bishop 2013; Wall et al. 2013).

(a) Cranial windows for optical access

A cranial window is required in seMCoS for chronic stable optical access. Two materials, PDMS and glass, were contested to fabricate the cranial window.

Besides transparency, an ideal optical window would allow also access to the brain for recording. Thus, PDMS was initially selected, because after implantation, electrodes can be inserted through the PDMS; and the PDMS can be resealed after electrode extraction with clear cyanoacrylate adhesive (Heo et al. 2016). I also designed and made custom molds for fast production of PDMS windows with specific size and thickness ($\sim 400 - 500 \mu\text{m}$). One major disadvantage of PDMS was that its surface became dirty quite easily. In addition, while the debris did not tend to adhere to PDMS, cleaning of the debris often required applying pressure over the window; since the window was flexible, such pressure could be transferred to the brain and cause irritation.

A glass cranial window (5 mm diameter, 0.7 mm thickness, #502040 WPI), on the other hand, could be cleaned with much ease. Optical clarity was maintained over the entire period of implantation (Fig. 3.12); yet granulation tissue and bone regrowth was often observed beneath the cranial window around the craniotomy, which affected the optical access. However, this is at the cost that electrophysiology below the window will not be accessible (at least not with electrical recording in freely behaving animals).

We also studied whether filling material in between the cortical surface and the cranial window was necessary. In 12-well cell culture plates, PDMS films of the same thickness ($400 \mu\text{m}$) were formed at the bottom of each well. On top of the PDMS, a 3 mm thick of saline, surgical lube (water-soluble thickened glycerin), or quick-curing PDMS (Kwik-Sil) was layered. Then the relative transmittance of a 473nm laser was measured through this structure, using the values in air (through the culture plate) as reference. The

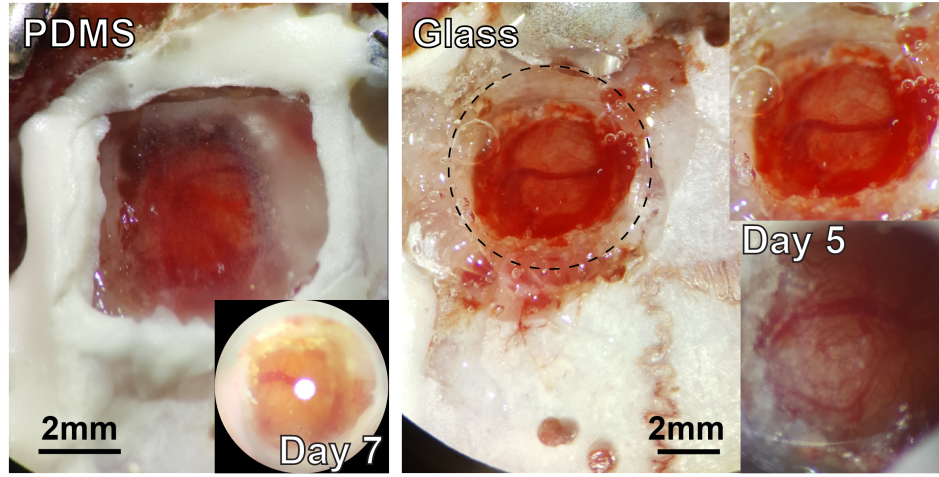


Figure 3.12: PDMS and glass cranial windows. Images of two types of cranial windows taken during implantation and during inspection after implantation (insets with date labeled). **Left.** PDMS window during the implantation showed diffusive properties, which was improved after slight wetting of the surface. Day 7 post-op inspection revealed good optical access, albeit yellowish granulation tissue growth (bright dot was the glare from light source). **Right.** Glass window during implantation and post-op (day 5) remained clean on the surface. Fine vasculature could be discerned, showing good optical transmittance. Granulation tissue growth also presented around the craniotomy in this case (the difference in brightness was caused by an angle between the incidence light and the axis of the microscope).

quick-curing PDMS was quickly eliminated for entrapment of air bubbles (not feasible to perform degassing given the fast rate of curing) and hence the lowest relative transmittance ($\sim 93.1\%$). Bubble entrapment ($\sim 95.4\%$ transmittance) was also found in the cells with surgical lube; despite of clearing of the bubbles, the transmittance of the filling material decreased significantly (visual inspection observed translucent substances) *in vivo*. In the end, we chose to fill the window with saline, as not only did it show the highest transmittance ($\sim 98.2\%$), its low viscosity also facilitates bubble elimination during implantation.

Using cranial windows, we were able to maintain a sealed environment for the brain, physically isolate the apparatus for light delivery from biological tissues, and monitor the brain surface over time.

(b) Findings from rotation tests

In three CED rats, we implanted suspended fibers over the motor cortex (isolated from the brain with a cranial window, see Fig. 3.19 A for illustration). As discussed in Section 2.4.4, modulation of neuronal activity was confirmed in the motor cortex on the contralateral hemisphere, as well as in the STN. Due to the physical hindrance of the cranial window and the large volume of illumination, hence light-induced artifact, simultaneous electrical recording in the stimulated area could not be obtained during seMCoS.

Rotational bias was also reliably modulated by seMCoS. Animals with amphetamine-induced enhanced h-P rotational bias showed significant decrease in the exaggerated CCW rotations (Fig. 3.13 B). This reduction ($-52.1 \pm 4.9\%$, $n = 3$ rats, 46 trials, $p < 1E-4$, paired t -test) was stronger compared with that of ssMC-oStim ($\sim -16\%$, $p < 1E-4$, unpaired, unequal variance, t -test), demonstrating an increased therapeutic effect by engagement of larger cortical area; it was also comparable to effect of STN-eStim ($\sim -37\%$), GPe-eStim ($\sim -58\%$), and GPe-oStim ($\sim -54\%$). Closed-up inspection revealed burst-onset locked head rotations, as well as a similar desensitization near the end of the stimulus train (Fig. 3.13 E).

In spontaneous behavior, seMCoS evoked CW-direction activities ($287.3 \pm 131.8\%$, $p < 0.05$, $n = 3$ rats, 33 trials, paired t -test) in small amplitudes; these activities did not translate into rotations (the low absolute amount of rotations) and bore high variability, despite of the somatosensory agitation. Failed to alleviate akinetic symptom or promote consistent rotations, we were unable to determine whether the simultaneous increase in CCW and CW rotations actually reflected correction of spontaneous rotational bias (Fig. 3.13 C).

For apomorphine challenges, seMCoS led to overall decrease in the overcompensation-related CW rotations (Fig. 3.13 D, $-34.9 \pm 4.9\%$, $n = 1$ rat, 18 trials, $p < 1E-3$, paired t -test), which was comparable to that of ssMC-oStim (-37%), but in contrary to the observations of additional increase in CW rotations by GPe-oStim and STN-eStim.

The differences in the direction of actions of seMCoS and ssMC-oStim to Amph-IR and Ampo-IR may imply differential abilities of corticostriatal inputs to modulate MSN output in response to the levels of DA-ergic inputs. During Amph-IR, the lack of DA spared the DA-ergic modulation in the lesion striatum; hence therapeutic reduction of rotational bias may also come from the activation of the direct pathway. During Apom-IR, as D1-MSN activity is increased and D2-MSN activity is suppressed in response to the DAR agonist, cortical excitatory inputs may appear more effective in driving anti-kinetic outcome from the re-activation of D2-MSN. As apomorphine induced involuntary movement has been considered as equivalent signs of dyskinesia, the successful suppression by seMCoS added to the advantage of motor cortex as a potential target for therapeutic modulation. Another benefit of seMCoS was the lack of epileptogenesis (absence seizure-like discharge) even at high input powers (~ 18 mW). Overall, seMCoS enabled pronounced therapeutic effects towards the rescue of rotational behavior.

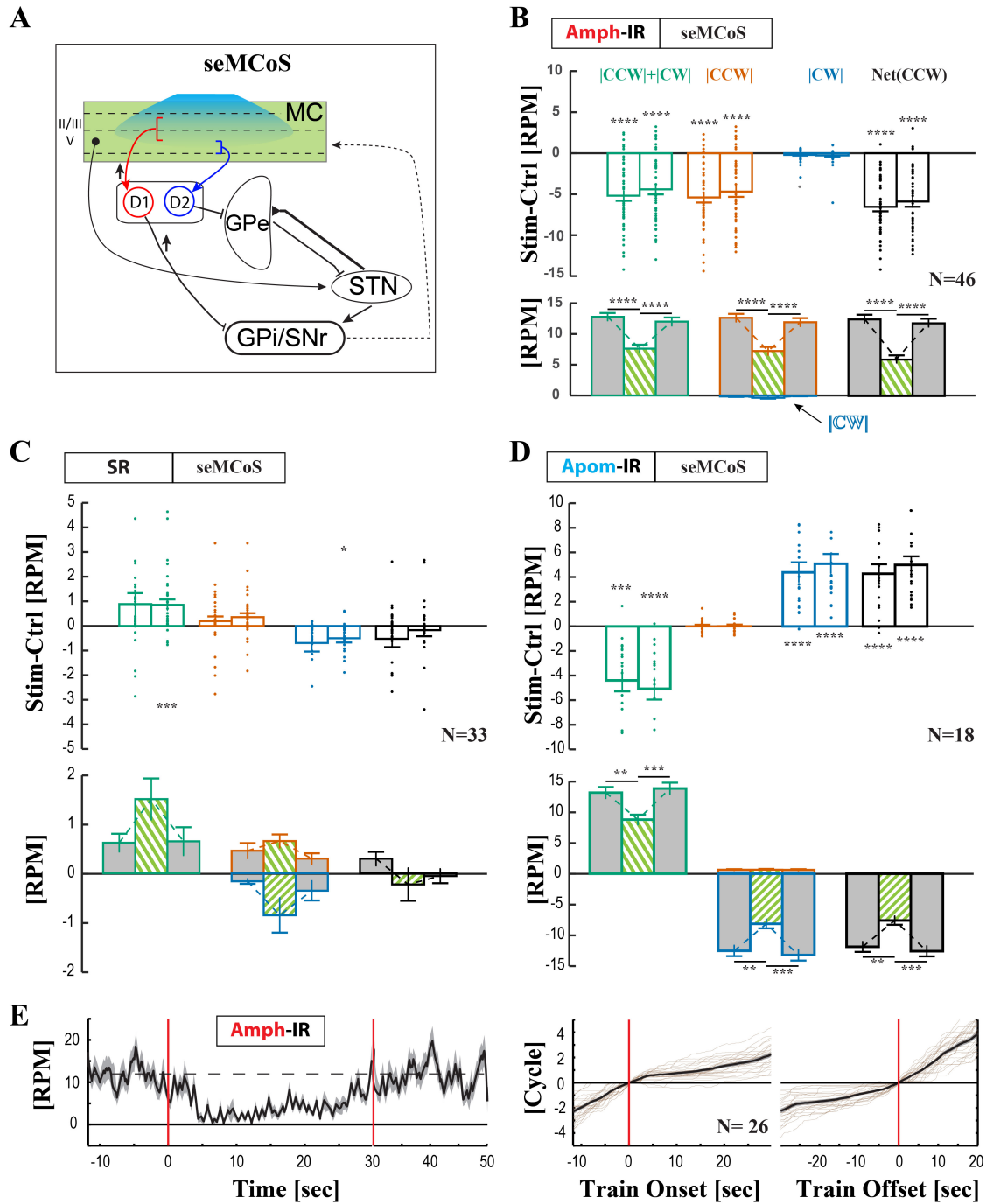


Figure 3.13: Rotational bias reduced by spatially-extended stimulation of the motor cortex (seMCoS). **A.** Illustration of the stimulation paradigm (not in scale). The direct excitatory effect of epicortical stimulation is likely to reach only the upper part of Layer V of the motor cortex, which may lead to preferential stimulation of corticostriatal efferents of the direct pathway.

Figure 3.13 caption – continued **B.** Amph-IR. The exaggerated rotational bias was significantly reduced ($-52.1 \pm 4.9\%$, $n = 3$ rats, 46 trials, $p < 1E-4$, paired t -test). **C.** Spontaneous rotations. An increase in CW rotations, though only marginally significant ($287.3 \pm 131.8\%$, $p < 0.05$, $n=3$ rats, 33 trials, paired t -test), was induced by seMCoS, accompanied by the simultaneous, more modest, increase (not significant, $p = 0.22$, paired t -test) in CCW rotations. **D.** Apom-IR. In agreement with results from ssMC-oStim, yet more pronounced, a reduction in the CW rotations were measured ($-34.9 \pm 4.9\%$, $n = 1$ rat, 18 trials, $p < 1E-3$, paired t -test). **E.** Examples of trial-averaged rotation traces obtained during AmphIR in Rat C16. (Left) Traces showing the instantaneous rotation speed (with s.e.m., gray area). The two red bars indicated the onset and offset of a stimulus train and the dashed line indicated the average speed pre-stim. (Right) The progression of rotations in separate trials (brown thin traces) and in trial-average (black thick trace, with s.e.m., gray area); A slight delay of above 5 sec was observed before reduction in speed took place. Also the stimulation effect gradually weakened near the end of the 30s stimulus train. Note that the Net CCW values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed from both ON- and OFF-cycle of the bursts. The asterisks (*) indicated the level of significance (see methods for details).

(c) Developing an LED-based seMCoS

In addition to the suspended fiber approach, I also developed an LED-based system for seMCoS. This was motivated by a preliminary experiment in a Thy1 transgenic mouse (Chr2 expression in Layer V), in which we intraoperatively confirmed directly evoked forelimb motor response by epicortical illumination with a blue LED (data not shown). In our concept, an LED would be implanted on top of the cranial window similar to that of the fiber (Fig. 3.14 A). It is advantageous for light source to be located on the animal, so that only electrical connections are required as part of the tether to the animal. This is particularly important for simultaneous recording and stimulation from freely behavior animals: 1) the robustness of the tether is greatly improved and 2) the use of a separate optical rotary joint can be eliminated (an electrical commutator is already in use for neural recording). In addition, LEDs have sufficiently narrow spectral bandwidth (FWHM $\sim 10\text{nm}$), short onset latency, high power, a range of angular output profiles, as well as being cost-effective.

Figure 3.14 illustrates some preliminary characterization of light output. In addition, thermal safety is a big concern for implants containing active electronics. Indeed, in agarose gel, we found that heating from LED comes from two sources: light absorption by tissue

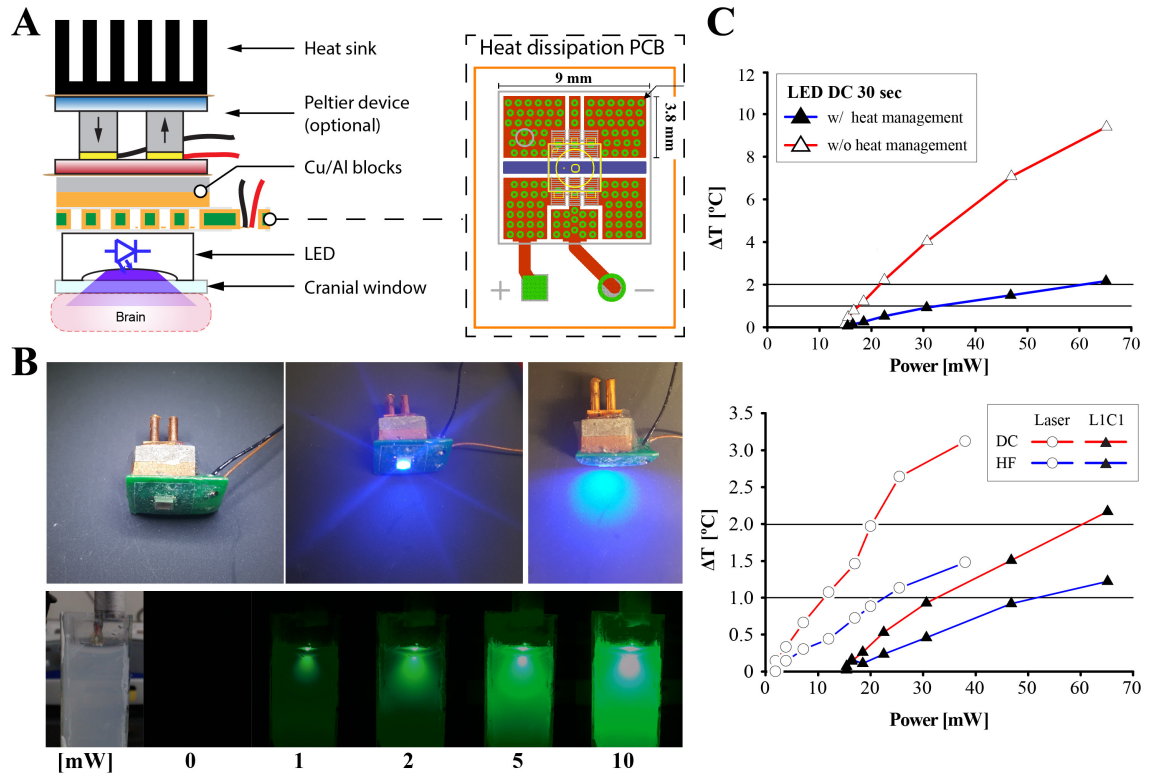


Figure 3.14: The LED approach for epicortical illumination: light output profile and thermal management. **A.** Illustration of concept: LED together with passive (heat dissipation PCB, heat sink) and active (optional, thermoelectric cooler, Peltier device) heat management units. **B.** One of two prototypes (one green and one blue) without the Peltier device. Shown in the bottom row was demonstration of light distribution in air and in agarose gel. **C.** Temperature changes by illumination of LED (green “L1C1”) or 561 nm laser was measured by a thermistor 2 mm the surface of agarose gel. (Top) With passive heat management heating from LED can be effectively controlled, even when an impractical DC stimulation (30 sec) was tested. (Bottom) We then compared the thermal effects of the LED (with passive heat sinks) with that of a 561 nm laser. The focality of the laser led to faster temperature increase. When high-frequency (100Hz, 50% duty cycle, 30 sec) stimulation was delivered, the heating effect was reduced almost by one half as predicted from the lowered average power.

and resistive effects in the LED. While heating from light absorption has been validated by simulation (3.9.2), the heating of the LED itself needs to be minimized or redirected away from the brain. As a solution, I designed a heat management system for the LED implant, comprising of both a passive and an active component. The passive component included a custom heating dissipation PCB, multiple aluminum and bronze blocks (balanced thermal

conductivity and capacity), and a pin-fin heat sink. Optionally an active peltier device can be added in between pin-fin heat sink and the blocks: peltier device can accelerate the removal of heat from one surface to the other surface (Fig. 3.14 A).

Two prototypes were assembled, with one green (Luxeon L1C1, digiKey) and one blue LED (LB-GS6P, digiKey), respectively. Both LEDs were able to achieve necessary output power for seMCoS (x-axis in Fig. 3.14 C). In agarose gel, I evaluated the effectiveness of the heat management of the assembly. We found that with the passive heat management, temperature increase can be controlled within the safety range with typical seMCoS parameters. When contrasted with the heating effect from a surface illuminating laser, the LED led to much lower temperature increase. Although, using typical seMCoS parameters (high frequency, 100 Hz, 50% duty cycle), both approaches generated heating within safety limit. Agarose gel model did not provide active cool due to blood circulation, thus even less heating may be observed *in vivo*.

Unfortunately and fortunately, because of the satisfactory modulatory effects by the suspended fiber approach, we did not further pursue LED in *in vivo* testing or implantations. However, I believe this approach has great potential moving forward, especially when high input powers and stable optical coupling is required.

3.8.3 Optogenetic excitation of the hyperdirect pathway (HD-oStim)

As discussed previously, CED-based method allowed robust labeling and electrophysiological modulation of projection terminals in output targets of motor cortex (Fig. 2.20 B). In two CED rats, we evaluated direct modulation of the hyperdirect pathway on rotational behavior (Fig. 3.15 A).

HD-oStim also led to marked reduction in rotational bias during Amph-IR ($-27.4 \pm 5.2\%$, $n = 2$ rats, 32 trials, $p < 1E-3$, paired *t*-test), although bearing trial-to-trial variabilities (Fig. 3.15 B). In apomorphine challenges, either reduction or enhancement (some in great

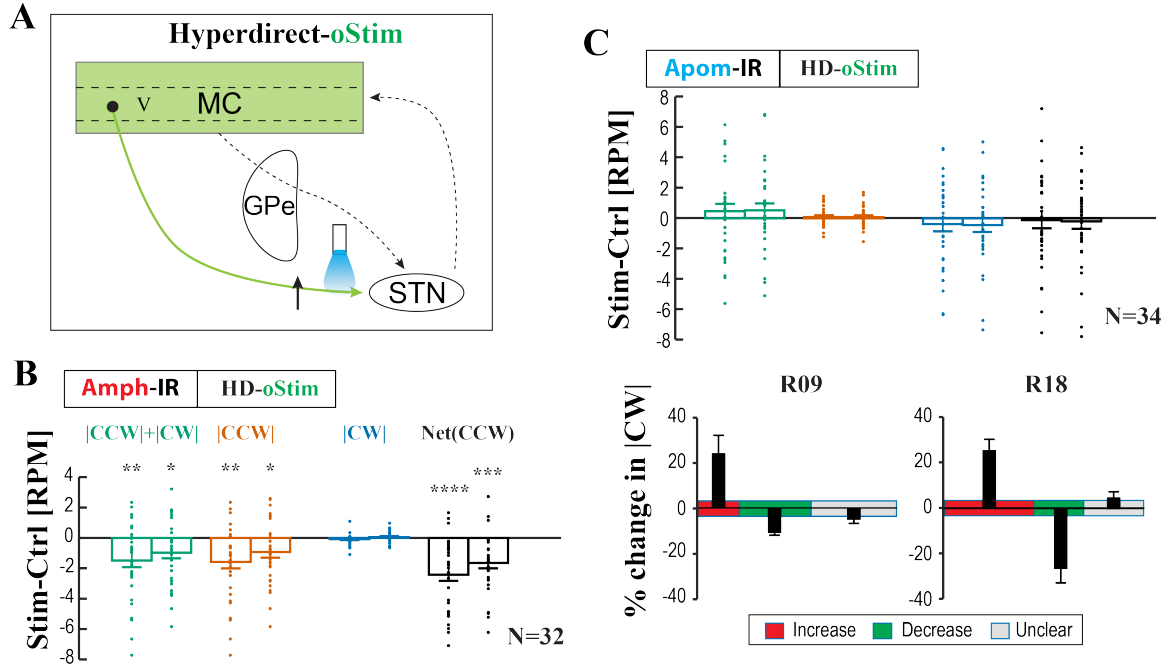


Figure 3.15: Rotational bias reduced by stimulation of the hyperdirect pathway (HD-oStim). **A.** Illustration for the stimulation of hyperdirect pathway (not drawn in scale). Cortico-subthalamic projections originate from Layer V pyramidal neurons. **B.** Amph-IR. The exaggerated rotational bias was significantly reduced ($-27.4 \pm 5.2\%$, $n = 2$ rats, 32 trials, $p < 1E-3$, paired t -test). Neither consistent occurrence of CW rotations, nor any obvious contraversive evoked movement of body parts was observed. **C.** Apom-IR. While changes in both directions were observed, no consistent reduction or enhancement in the over-compensated CW rotations were noted ($3.8 \pm 4.3\%$, $p = 0.29$, $n = 2$ rats, 34 trials, paired t -test). The bottom panel depicted the distribution of incidences of increase (stim higher than both controls), decrease (stim lower than both controls), and unclear (stim was lower than one control and higher than the other) trials. The horizontal colored bar indicated the frequency of occurrence for each type of trials. The difference between the two animals was not significant ($p = 0.76$, $F_{1,2} = 0.10$, nested ANOVA). Note that the “Net CCW” values were extracted from the ON-cycle of all stimulation bursts; while the other parameters were computed from both ON- and OFF-cycle of the bursts. The asterisks (*) indicated the level of significance (see methods for details).

extent) occurred for the over-compensated CW rotations; however overall there was no consistent effects ($3.8 \pm 4.3\%$, $p = 0.29$, $n = 2$ rats, 34 trials, paired t -test) (Fig. 3.15 C). Although the number of animals was limited, the inconsistency in the stimulation effect was observed in both animals. While the each type of trials were statistically different from each other ($p < 1E-4$, $F_{2,1} = 40.62$, nested ANOVA) within each animal, there was no

significant difference in the distribution of trials across the two animals ($p=0.69$, $F_{1,2}=0.16$, nested ANOVA).

A recent study by Chu et al. revealed decreased strength of corticosubthalamic transmission, accompanied by actual reduction in the number of corticosubthalamic axon terminals, following chronic loss of dopamine (Chu et al. 2017). They further demonstrated that the downregulation of hyperdirect input was a plastic change in response to the disinhibition of afferents from GPe to STN. These findings not only provide support for the relatively weaker therapeutic effect of HD-oStim in hP rats, they further help established a connection GPe-oStim, STN-oInhibit, and HD-oStim for comprehensive mechanisms of action beyond isolated rate modulation.

3.9 Simulation Verification of Stimulation Paradigms

The activation of opsin (achieving a significant probability of channels opening) is highly correlated with the intensity of light power, i.e. optical irradiance. While the threshold levels of optical irradiance to illicitate action potentials also depend on the properties of the opsin and the extent of expression, commonly assumed values are between 1 and 5mW/mm² (Klapoetke et al. 2014), (F. Zhang et al. 2006). As multiple paradigms of light delivery were employed in this study, the desired spatial profiles differ drastically for each application. For example in the case of STN stimulation, an optical fiber was implanted at the dorsal border of this disk-shaped structure and vertical spread of light was not required. Yet, in motor cortex stimulation, a single implanted optical fiber may not be able to provide the desired lateral and vertical light coverage.

On the other hand, a potential confounding factor to the actual optogenetic modulations is the changes of neural activity caused by thermal challenge from the direct absorption of light energy by the brain tissue (Deng et al. 2014; Arias-Gil et al. 2016). Outside the realm of optogenetics, this effect has mostly been studied on the perspective

of thermal safety, i.e. avoiding thermal lesion to tissues (Nourhashemi, Mahmoudzadeh, and Wallois 2016) by radio frequency electromagnetic wave (or ultrasound) exposure or active bio-implants. Extreme temperature changes could lead to various irreversible adverse effects, including protein denature and cell death (Dewhirst et al. 2003). While a temperature increase within 6-8 °C was reported to result in reversible thermal effects (Thomsen 1991; Deng et al. 2014; Arias-Gil et al. 2016), physiological changes from heating could be observed at smaller temperature increase of 1.5-3 °C (Deng et al. 2014; LaManna et al. 1989). The requirement on thermal safety for implantable devices based on ISO standard and regulations by FDA (United States Food and Drug Administration) stated that the change in temperature of the outside surface of an implantable device should be within 2 °C of the surrounding (International Organization for Standardization 2017).

In optogenetics-based studies, especially for elicitation of behavioral modulation, often higher light power was required. It is particularly a concern for optical fiber based delivery, because the scattering and absorbing properties of the tissue led to localized light distribution and heat generation. In fact, in acute experiments where the tip of the fiber was accidentally covered with coagulated blood (light cannot escape from the fiber and hence got concentrated at the tip), we had observed signs of charring (on the fiber) after extraction. More modest localized temperature changes had also been shown to affect the electrochemistry of the neuronal membrane and lead to non-optogenetic (and non-specific) modulations of neural activity, (Yizhar et al. 2011; Kim and Connors 2012; Han 2012), e.g. hyperthermia leads to increased excitability in excitatory and inhibitory hippocampal neurons.

Accordingly, I will discuss results from simulation studies that characterize the spatial and temporal profiles of light distribution (adopted and modified from the algorithms reported by (Ozden et al. 2013)) and the associated photothermal effects (adopted and modified from the algorithms reported by (Ozden et al. 2013; Stujenske, Spellman, and Gordon 2015)) in the various light delivery scenarios employed in this thesis.

3.9.1 Sufficient light coverage was achieved for deep brain light delivery without significant risk for photothermal effects

We first studied the light delivery from intracerebrally implanted optical fibers. In most of the prior simulation studies, a flat-terminal optical fiber was modeled and the lateral spread of light was limited and was primarily due to scattering (Ozden et al. 2013; Wagner et al. 2015; J. Lee et al. 2015a). However, in practice such configuration is almost never adopted due to the poor tissue penetrability. Instead, optical fibers were commonly polished (more controlled angle of the taper) or thermally pulled (higher variability but smoother surface for better light exit) to form tapered terminals. In my implantations, the 60° polished tapers were most commonly used; in more recent implants, laser-pulled, much sharper tapers (5° at the very tip) were made. These sharp-tapered fiber were adopted for the engineered increase in lateral spread of light, the superior mechanical penetrability, and lowered light intensity at the surface of the probe (larger surface area).

At two power levels where therapeutic effects were typically elicited (5, 10 mW), the volume of tissue with suprathreshold light intensity ($1\text{ mW}/\text{mm}^2$, Fig. 3.16 A) spanned about 1.3 mm^3 (size of STN is about 0.8 mm^3 (Hardman et al. 2002)) and 3.2 mm^3 ($\sim 31\%$ volume of GPe (Hardman et al. 2002)), respectively. For STN, the disk-shape of the structure resulted in considerable, yet not complete coverage in the lateral dimension (100% with $1\text{ mW}/\text{mm}^2$, $>55\%$ at $5\text{ mW}/\text{mm}^2$, and $>39\%$ with $10\text{ mW}/\text{mm}^2$) (Fig. 3.16 B). For GPe, the vertically elongated shape matched well with the ellipsoidal shape of light output (Fig. 3.16 B); despite of its larger size (10.3 mm^3 (Hardman et al. 2002)), considerable coverage in the lateral (73% with $1\text{ mW}/\text{mm}^2$, $>27\%$ at $5\text{ mW}/\text{mm}^2$, and $>20\%$ with $10\text{ mW}/\text{mm}^2$) and vertical (100% with $1\text{ mW}/\text{mm}^2$, $>96\%$ at $5\text{ mW}/\text{mm}^2$, and $>89\%$ with $10\text{ mW}/\text{mm}^2$) dimensions could be achieved (Fig. 3.16 C).

In terms of heating, besides the commonly used power levels, a 20 mW (pulsed) input power was particularly analyzed as a worst-case scenario. Temperature change reached steady-states in all simulated scenarios, within the first 5-10 sec of stimulation (the extreme

case of 20 mW shown in Fig. 3.17 A), indicating that heating was within the capacity of active hemodynamic management; therefore these simulation results should be applicable to longer stimulation protocols (typical stimulation protocols lasted from 30 to 180 sec). Commonly used power levels (≤ 10 mW) all lead to temperature increases below 1.0°C ($<0.08^{\circ}\text{C/mW}$) (Fig. 3.17 B). At 20 mW, the peak temperature change for even the flap tip fiber was lower than 3°C ($<0.15^{\circ}\text{C/mW}$), while for the two actually implanted fibers the temperature increases were kept below 1.5°C (Fig. 3.17 C). Moreover, the results also suggested that significant thermal effect would be well contained within a range of 500 - 1000 μm .

Therefore, we conclude desirable light coverage can be achieved in deep brain optical stimulation with thermal safety.

3.9.2 Suspended fiber illumination increased lateral coverage of light stimulation

For ssMC-oStim, a larger diameter 200/230 μm fiber was inserted into motor cortex to a depth of -1.0 mm DV (targeting Layer V neurons). Lower power levels (< 7 mW) were usually used, compared with deep brain stimulation; this was partially due to the exceptional opsin expression by CED and also to avoid epileptogenesis (lower threshold in cortex). In fact therapeutic effects were observed at rather low power level (2 mW). Lateral spread of low power stimulation was indeed spatially limited (Fig. 3.18B: 1 mW: radius < 0.30 mm; 2mW: radius < 0.47 mm). Heating was further reduced by the larger diameter fiber, hence thermal safety did not pose a significant risk (Fig. 3.18 C).

When a suspended fiber was used for surface illumination, lateral coverage was drastically improved. Light was able to reach to a depth of 1mm with around 10% of the maximum irradiance. Although, the large surface area at the input resulted in rather weak irradiance even at the brain surface. In experiments, commonly 10-20 mW of power was used for behavior modulation. However, in our experiments, LFP modulation can

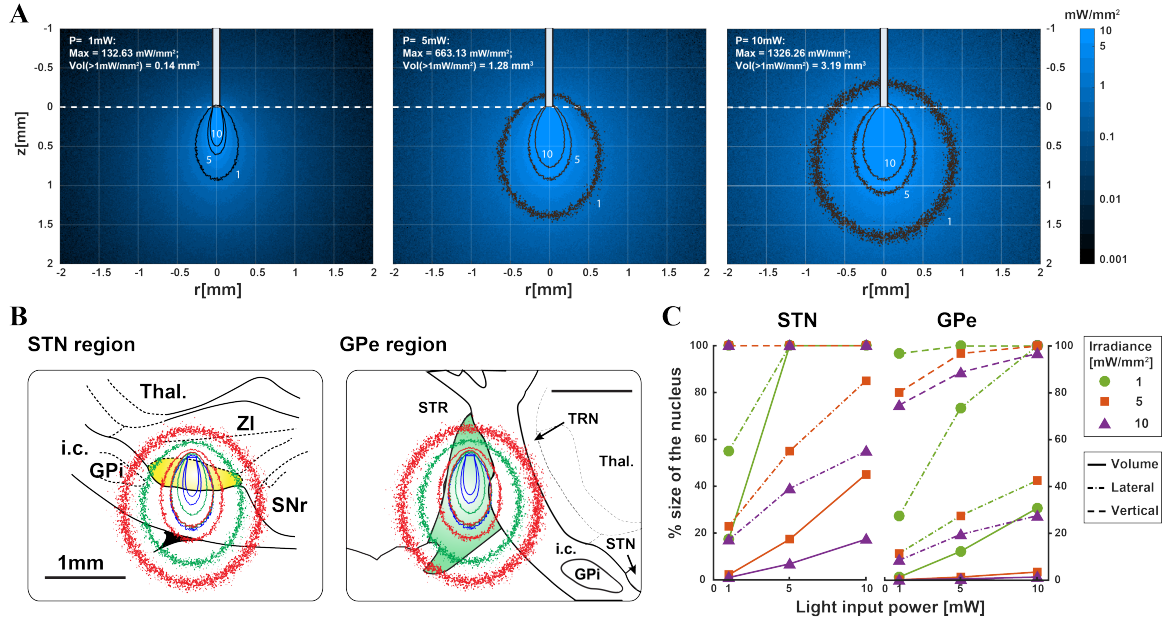


Figure 3.16: Light distribution from implanted optical fiber in relation to the geometry of the STN and GPe. **A.** Heatmaps showing the light intensity distribution from a flat termination fiber at 1 (left), 5 (middle), and 10 (right) mW input power (473 nm). The black scattered dots formed iso-intensity contours at 1, 5, and 10 mW/mm², with the first being the lowest threshold for opsin activation reported in literature. **B.** Iso-intensity contours of the three scenarios in A (blue for 1 mW, green for 5 mW and red for 10 mW) overlaid on the anatomical illustrations of the STN and GPe. **C.** The ratios of the volume, lateral dimension, and vertical dimension of the STN and GPe covered by the stimulation volume at each input power level.

be observed at input power of 7 mW and behavioral modulation was found above 10 mW (Figure. 3.19), corresponding to at most 0.5 and 0.72 mW/mm² at the brain surface. Therefore, we conclude that the direct optogenetic modulations were most likely subthreshold; yet subthreshold or suprathreshold secondary modulations might have been achieved through network.

In conclusion, simulation results confirmed that the seMCoS through a suspended fiber indeed led to drastic increase in the lateral spread of the stimulus light within the motor cortex.

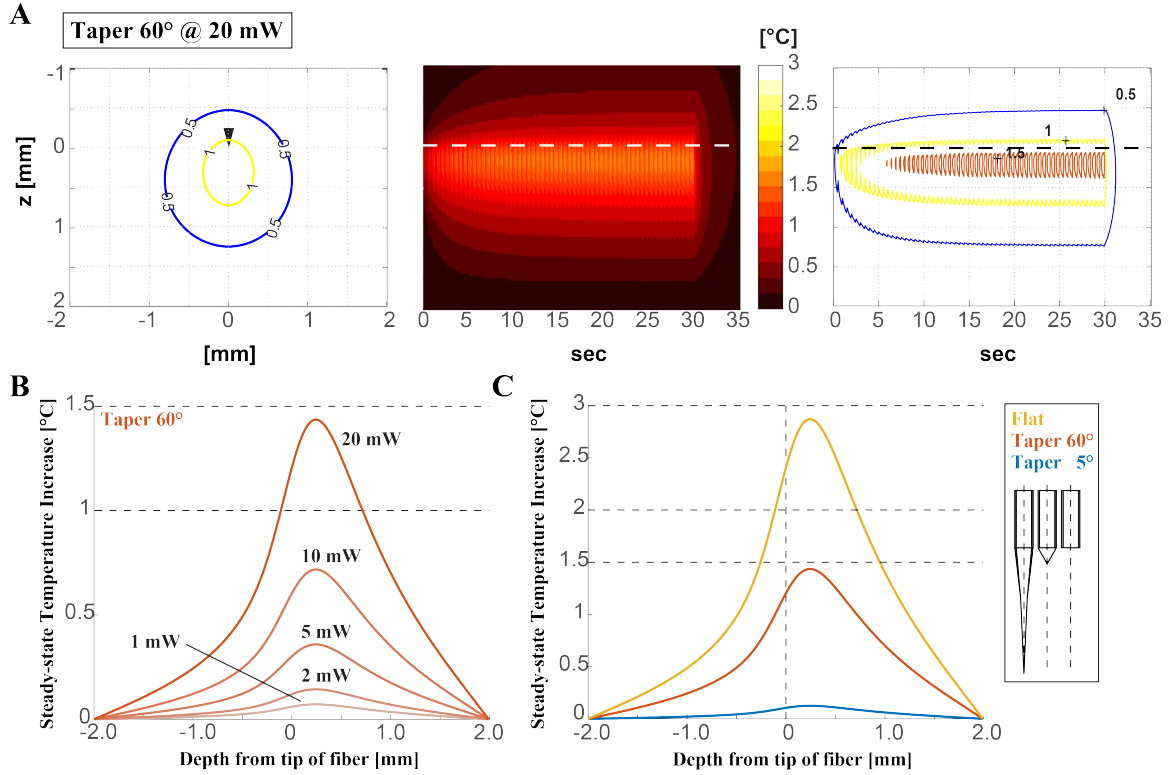


Figure 3.17: Spatial temporal temperature increase profile in relation to taper geometry and input power. **A.** Temperature increase resulted from 60° fiber at 20 mW. (Left) Coronal-section spatial profiles of the temperature increase in steady state. The black arrowhead indicates the location of the tip of the fiber. The next two plots diagrammed the temporal profile of temperature increase along the axis, averaged within a radius of 250 μm : displayed in heat map (Center) and isocontour plot (Right). The dashed lines mark the level of the fiber tip. **B-C.** Steady-state temperature increase measured along the axes of the fibers: varying power for the 60° taper. **B.** and varying types of taper at fixed power of 20 mW. **C.** Positive depths indicate deeper than the tip of the fiber. The three horizontal dash lines marked the lower bound temperature increase for reversible physiological change, the FDA safety limit, and the upper bound reversible changes. The three types of taper geometry were drawn in the same scale for comparison.

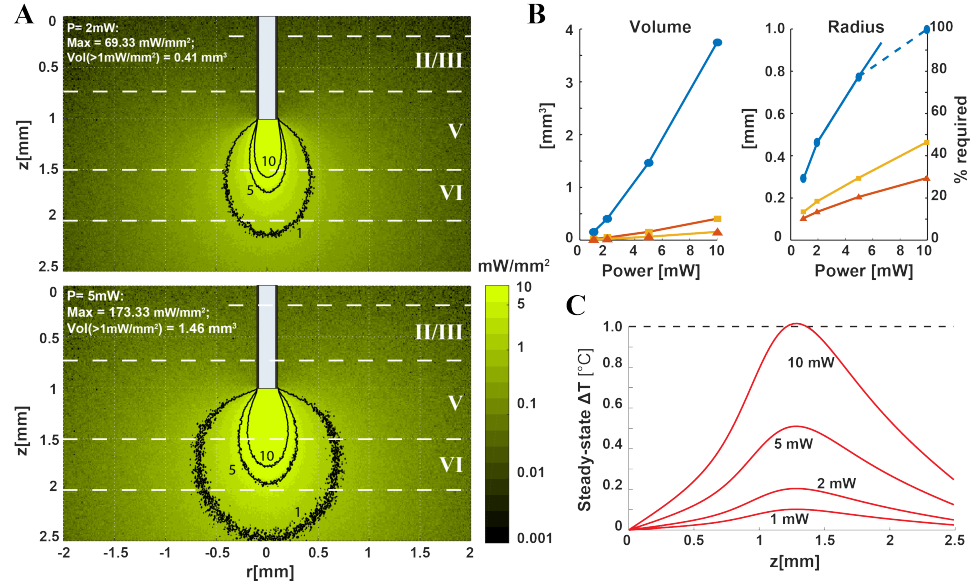


Figure 3.18: Simulated light output and effects of heating from an intracortical implanted optical fiber. This configuration models the implantation (200/230 μm , multimode, 0.22NA) for ssMC-oStim. For simplicity, simulation was performed for a blunt taper. **A.** Spatial profile of the optical irradiance (transmitted light intensity) was shown in logarithmic-scale heatmap at input power levels of 2 mW (top) and 5 mW (bottom). The black scattered dots formed iso-intensity contours at 1, 5, and 10 mW/mm², with the first being the lowest threshold for opsin activation reported in literature. **B.** The enclosure volumes and lateral dimensions of the iso-intensity contours at 1 (blue), 5 (yellow), and 10 (red) mW/mm². **C.** Steady-state heating effect from a typical 30s HFS (125 Hz, 40% duty cycle) light stimulation. A 561 nm laser was used as source. Temperature changes were within safety limit.

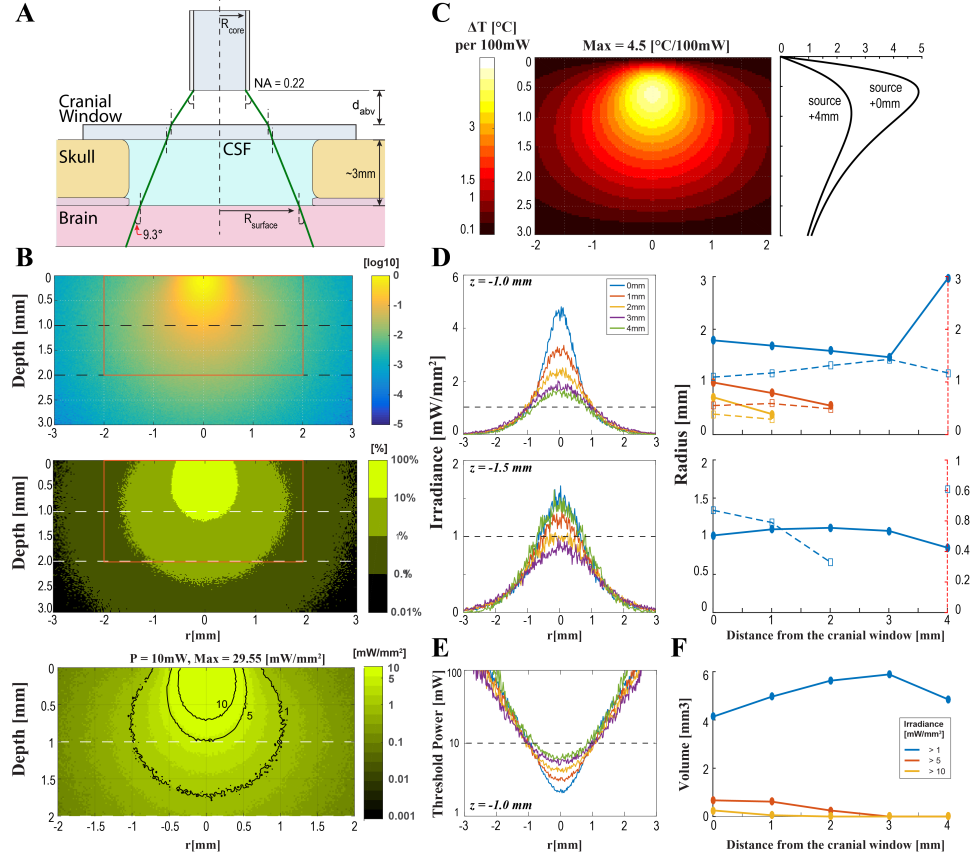


Figure 3.19: Improved lateral spread of light using a suspended fiber for seMCoS. **A.** Schematic of optical paths in seMCoS with cranial window and suspended fiber. No filling material, except saline was used in between the cranial window and the brain surface. **B.** Spatial profile of the relative intensity in logarithmic-scale (top), isointensity contours in relative intensity (middle), and optical irradiance (transmitted light intensity, bottom) were plotted at input power of 10 mW. The fiber was suspended 3 mm above the window. The black scattered dots formed iso-intensity contours at 1, 5, and 10 mW/mm^2 , with the first being the lowest threshold for opsin activation reported in literature. **C.** The enclosure volumes and lateral dimensions of the iso-intensity contours at 1 (blue), 5 (yellow), and 10 (red) mW/mm^2 . **C.** Steady-state heating effect from a typical 30s HFS (125 Hz, 40% duty cycle) light stimulation. The maximum temperature change was found to be $4.5^\circ\text{C}/100 \text{ mW}$, when the fiber was in contact with the cranial window. Illuminating at a distance, this value reduced further. A 561 nm laser was used as source. **D-F.** Summary of spatial profiles obtained by varying the height of the fiber (0-4mm): cross-sectional irradiance profile at 1.0 and 1.5 mm, as well as the width of profile with irradiance greater than 1 mW/mm^2 (D); the input power required to achieve 1 mW/mm^2 for each horizontal location at 1mm depth (E); enclosure volumes of the isocontours at 1, 5, and 10 mW/mm^2 .

3.10 Conclusions

3.10.1 Summary

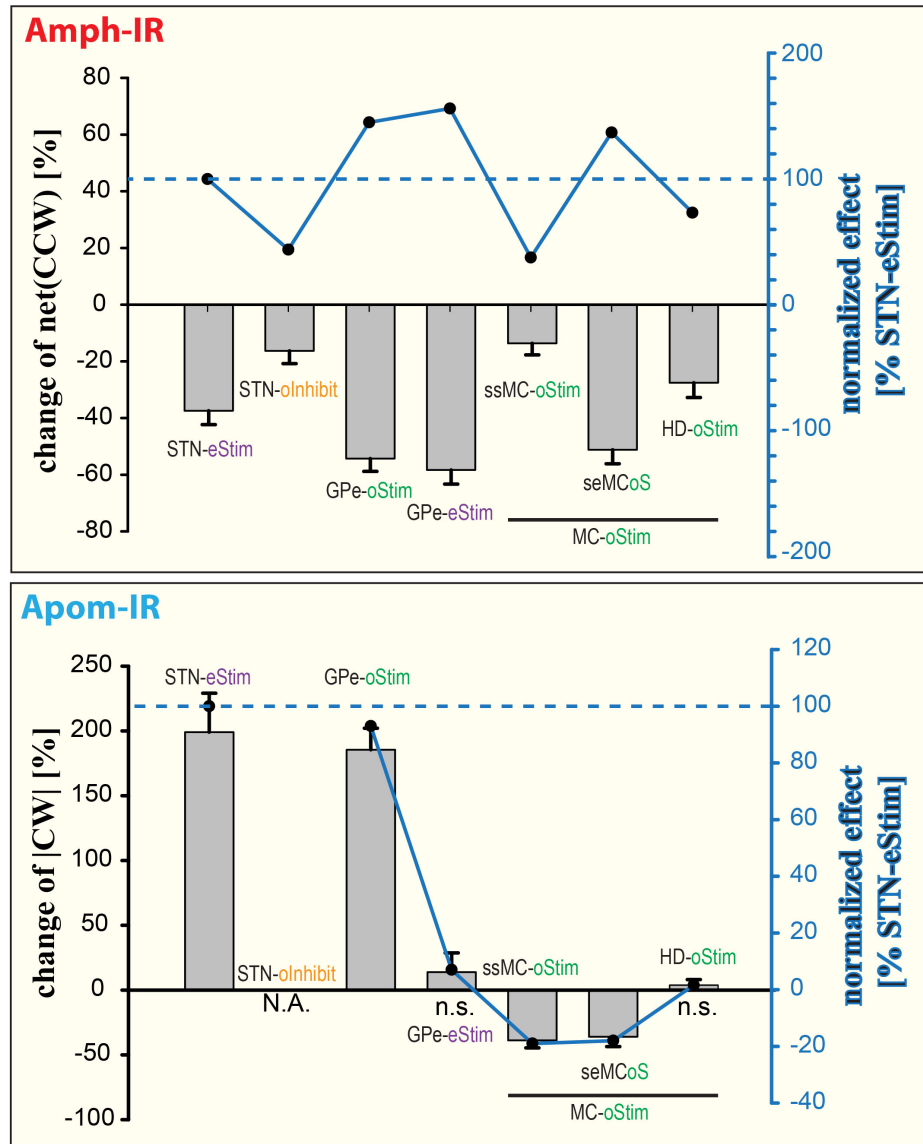


Figure 3.20: Bar graph summary of the rescue of rotational bias. The second y-axis on the right showed the comparisons with reference to the effect of that of STN-eStim. Unless otherwise specified (labeled with n.s.), all changes were significant at least at $p < 0.01$. Note that STN-oInhibit in Apom-IRs was not systematically characterized, thus labelled as not available (N.A.).

Our results demonstrated that rotational biases in hemi-Parkinsonian rats can be alleviated to different extents by optogenetic modulations of STN, GPe and the motor cortex. First we established a reference point by characterizing the therapeutic effects of electrical stimulation of STN (representing the conventional DBS paradigm). We found STN-eStim effectively reduced the Amph-IRs which represented an exaggerated Parkinsonian state and further increased the Apom-IRs, providing additional anti-Parkinsonian benefits. Second, while previous studies did not find alleviation of rotational bias by optogenetic STN-inhibition, leveraging on a new generation potent inhibitory opsin, consistent although modest reduction of Amph-IRs were indeed discovered in our results.

Furthermore, the inhibition of STN could theoretically be achieved also by the excitation of GPe, which sends GABAergic projections to STN. Indeed, pan-neuron GPe activation led to drastic reduction in the rotational bias, which was even more potent than STN-eStim. GPe-oStim also provided potent extra anti-Parkinsonian drive concurrent to the effect of the pharmacologically anti-Parkinsonian agents (apomorphine). While GPe was not commonly chosen as a DBS target in human patients, we found electrical stimulation at this nucleus also provided strong alleviation of rotational bias; however, no additional anti-Parkinsonian effects were found. In this regard, optogenetic stimulation was superior to electrical stimulation at GPe.

Finally, direct motor cortical excitation by optogenetic stimulation was performed in three manners: single-site intracortical illumination (ssMC-oStim), spatial extended epicortical illumination (seMCoS), and optical stimulation of the hyperdirect pathway (HD-oStim). All three paradigms resulted in significant improvement in rotational bias: in particular, seMCoS provided the most alleviation and the effects of ssMC-oStim were limited as a result of spatially limited illumination. We further noted that while the extra anti-Parkinsonian effect presented in cases of motor cortical illumination, stimulation of the hyperdirect pathway did not lead to consistent effects.

To our knowledge, demonstration of therapeutic effects of optogenetic modulation

of the GPe, the motor cortex, and the hyperdirect pathway in the non-transgenic rats had never being done before this study.

3.10.2 Parallel mechanisms of actions of DBS

(a) **Therapeutic effects achieved through upstream and downstream modulations confirmed the important role the indirect pathway to motor symptoms.**

High frequency DBS has long been suggested to provide inhibitory actions on local neurons (Pollak et al. 1993; A. L. Benabid et al. 1996; Deniau et al. 2010a; Sutton et al. 2013; Benazzouz and Hallett 2000), hence according the rate model result in the overall disinhibition of the output of the indirect pathway. Prior to this thesis, this perspective has been supported by pharmacologically (Piallat, Benazzouz, and Benabid 1999; Petri et al. 2013) and electrolytically induced inhibition (Carvalho and Nikkhah 2001; Hamani et al. 2004; Touchon et al. 2004). However optogenetic inhibition has provided mixed outcomes (Gradinaru et al. 2009b; Yoon et al. 2014). With the consistent alleviatory effects by both the upstream GPe-oStim and the downstream STN-oInhibit, our results further supported the therapeutic effects of the disinhibition of the hyperdirect pathway.

One limitation in the interpretation of our results is the lack of cellular specificity in GPe opsin expression, as there are multiple subgroups of output neurons within GPe (forming a local negative feedback network) (Mastro et al. 2014). It is not clear whether opsin expression was uniform across the subgroups of neurons in our study. Recently, in transgenic mice, Mastro et al. was able to achieve rescue of bradykinesia with selective modulation of subpopulations in GPe, while pan-neuron activation of GPe did not result in increased mobility (Mastro et al. 2017). On one hand, this result is in high accordance with our finding that overall mobility and the intent to move was not increased by GPe-oStim; on the other hand, since rotational bias was not investigated by Mastro et al., how improvement in rotational bias attributes to the modulation of each subpopulation remained unclear.

(b) Therapeutic effects of HD-oStim: orthodromic excitation of the STN or antidromic activation of the motor cortex?

DBS of the STN has been shown capable of modulating cortical activities (Dejean, Hyland, and Arbuthnott 2009; Lehmkuhle, Bhangoo, and Kipke 2009; Pasquereau, DeLong, and Turner 2016). Most notably is the direct activation of the fibers within the cortico-subthalamic hyperdirect pathway, which leads to antidromic activation of the cortex (Paz, Deniau, and Charpier 2005; S. Li et al. 2007; Q. Li et al. 2012; Kita and Kita 2012). Our results were consistent with this perspective in three ways: 1) direct optical stimulation of the hyperdirect pathway produced therapeutic effects; 2) direct stimulation of the deep layers of motor cortex (where the soma of corticosubthalamic projection neurons reside) also produced therapeutic effects; and 3) optogenetic inhibition of STN was also effective.

Should STN-excitation exacerbate rotational bias? Exacerbation of rotational bias by optogenetic stimulation of STN was not found or reported: in the very early phase of the study, we attempted optogenetic excitation of STN in two h-P rats and found lack of clear behavioral modulations (not included in this report due to the small sample size), while matching other reports in literature (Gradinaru et al. 2009a). Earlier studies with pharmacologically induced STN excitation did report abnormal behavior but only at high dosage and was related to generation of seizure (Dybdal and Gale 2000).

While the volume and the number of neurons in the STN was not reported to change in Parkinsonism (Hamani et al. 2017). A recent study by Chu et al. revealed decreased strength of corticosubthalamic transmission, accompanied by actual reduction in the number of corticosubthalamic axon terminals in the PD animals (Chu et al. 2017). This finding and other reports in nonhuman primates (Mathai et al. 2015) may support the relatively weaker therapeutic effect of HD-oStim in hP rats.

(c) Therapeutic effects from motor cortical stimulation: engagement of deeper layers versus superficial layers

In our study, motor cortical stimulation targeting deeper (mostly Layer V by ssMC-oStim) and shallower (mostly Layer II/III, but also limited Layer V by seMCoS) layers both produced therapeutic effects, but with differences. Indeed, the laminar structure of motor cortex may play a role in their mechanisms of actions.

First of all, the Layer V neurons of motor cortex are part of the nominal motor output (corticospinal and corticopontine) pathways, the hyperdirect pathway (Degos et al. 2008b), and the corticostriatal pathways. Second, topographical difference in corticostriatal inputs to the direct and indirect pathway has also been shown in literature (Reiner et al. 2003; Lei et al. 2004; Mathai and Smith 2011; Liu et al. 2011; Lindenbach and Bishop 2013; Wall et al. 2013). In brief, two morphologically distinct types of striatal-projecting pyramidal cells exist in the motor cortex (also in other cortical areas). The Intratelencephalic-type (IT) neurons, residing in Layer II/III, have projects to striata in both hemispheres; some evidence suggested that IT neurons may preferentially innervate with the direct pathway (Reiner et al. 2003; Lei et al. 2004). A second type of corticostriatal projections, the pyramidal-tract-type (PT) projections are formed by the collateral branches of Layer V pyramidal tract neurons as their fibers travel downstream through the striatum. While this type of projections target both D1- and D2- MSNs, in motor cortex, larger proportion of PT projections were shown to innervate with the indirect pathway (Wall et al. 2013).

As a consequence, 1) for the hyperdirect pathway, although theoretically it could be activated by both modalities, its engagement by ssMC-oStim would be more prominent; and 2) for the corticostriatal pathways, both modalities could elicit increased cortical input to the direct and indirect pathways, however ssMC-oStim may affect more on the indirect pathway and seMCoS may affect more on the direct pathway.

3.10.3 Therapeutic effects of seMCoS suggest opportunities for non-invasive neuromodulation paradigms

The larger size and ease of accessibility of motor cortex allow for easier targeting as well as possibility for less invasive methods of modulation (e.g. epidural stimulation ((Fregni et al. 2005; Elahi, Elahi, and Chen 2009; Gutierrez et al. 2009; Heumann et al. 2014; Chou et al. 2015; Flamez et al. 2016; Ferrucci et al. 2016)) and transcranial stimulation (Fregni et al. 2005; Elahi, Elahi, and Chen 2009; Gutierrez et al. 2009; Heumann et al. 2014; Chou et al. 2015; Flamez et al. 2016; Ferrucci et al. 2016)). The demonstration of therapeutic effects of seMCoS further promotes this idea and even suggests a potential benefit over deeper stimulation due to the preferential engagement of the direct pathway.

(a) An outlook for further improving optogenetic motor cortical stimulation

Despite the therapeutic efficacy achieved through the global activation of the motor cortex by seMCoS, an implementation of spatially selective light delivery would be more preferable. Moving forward, two possible paths of improvement have been proposed. First, our lab has developed a fully transparent multi-shank ZnO optoelectrode array for simultaneous light delivery and electrophysiological recording (J. Lee et al. 2015a). ZnO array would not only help overcome the lack of simultaneous recording capability in the area being stimulated, but also enable spatially patterned optical stimulation. In fact, unlike the current paradigm where light stimulation scatters through the cortex, spatially extended stimulation can be achieved at the desired depth (e.g. Layer V). But the array approach also bears many challenges. Most notably, precise and robust light coupling to each optoelectrode is required, which is a non-trivial task, especially aiming for operation in a freely-behaving animal. In addition, the ZnO array, similar to the standard Utah MEA has a pitch between each shaft of 400-500 μm , leading to unsatisfactory spatial resolution especially for study of rodent cortex.

An all-optical approach for simultaneous recording and stimulation is another option, which has superior spatial resolution (subcellular). For a complete review of in vivo neuroimaging (intrinsic signals, voltage sensitive dyes, or calcium indicator dyes), readers are referred to the review by Yang et al. (W. Yang and Yuste 2017). Complementing the advancement in imaging of neural activity, the compatibility with simultaneous optogenetic stimulation has been demonstrated and is under continuous development (Bègue et al. 2013; Akerboom et al. 2013; Rickgauer, Deisseroth, and Tank 2014; Hernandez et al. 2016; W. Yang et al. 2016; Carrillo-Reid et al. 2017; W. Yang et al. 2018). Nevertheless, the challenge to implement on freely-behaving animal remains; and most of the state-of-art methods still require head-fixation (efforts to mimic free behavior include integration of spherical treadmills and virtual reality) (Leinweber et al. 2014; Voigts and Harnett 2018). Compromising spatial resolution, more miniaturized solutions that allow for wide enough field of view (~ 1 mm x 1mm) are also being developed (Szabo et al. 2014).

3.10.4 Apomorphine-induced rotations: therapeutic over-compensation or dyskinesia

PD patients may develop dyskinesia, the abnormal, uncontrolled, involuntary movement, over chronic treatment with L-DOPA. DBS has been shown to reduce the severity of dyskinesia (Fraix et al. 2000; Toda, Hamani, and Lozano 2004; Aristieta et al. 2012; Toda et al. 2016), however, this effect is perhaps largely a result of the reduced dosing frequency and dosage of L-DOPA during effective DBS (Toda, Hamani, and Lozano 2004); there is a lack of definitive neuromodulatory explanations (Dostrovsky and Lozano 2002; Herrington, Cheng, and Eskandar 2016). Quite on the contrary, dyskinetic movement has been reported in some cases when small lesion to STN was caused by insertion of electrode or during the initial parameter adjustment of DBS (A. L. Benabid et al. 2000; Hamani et al. 2004).

In this regard, it is arguable whether the CW Apom-IRs should be regarded as

dyskinesia or just an over-compensated state (the “ON” state). On one hand, apomorphine in 6-OHDA animals has been used by several groups to induce dyskinetic movements ((Fraix et al. 2000; Toda, Hamani, and Lozano 2004; Aristieta et al. 2012; Toda et al. 2016). Yet, in our experiments, the various therapeutic modulations (to Amph-IRs) did not affect Apom-IRs ubiquitously. While STN-eStim drastically increased Apom-IRs (despite the common belief that DBS does not exacerbate dyskinesia), GPe-eStim did not do so (despite, increased dyskinetic symptoms in human patients receiving GPe electrical stimulation was demonstrated (Vitek et al. 2004)). It was further complicated by the increase of Apom-IRs by GPe-oStim (Section 3.7.1), the reduction of Apom-IRs by ssMC-oStim and seMCoS (despite the common beliefs that suggest otherwise, results and reasoning detailed in Section 3.8.1 and 3.8.2), and the lack of consistent modulation by HD-oStim (results and speculations described in Section 3.8.3).

Nonetheless, it is worth pointing out that high dosage of DA-agonist would not have been administered in patients with DBS. For the aforementioned inconsistencies and for the lack of dependence on repetitive, chronic DA-agonist dosing, I suggest regarding Apom-IR not as a dyskinetic symptom, but as a behavioral sign to an over-compensated parkinsonian state; which by no means reflects normal brain function, but is certainly “less” parkinsonian. In this sense, our results could shed light on the possible tradeoffs between the anti-parkinsonian effects and dyskinetic movement.

3.11 Detailed Methods for Chapter 03

3.11.1 Subjects

The animals used in this portion of the study were 12 male Long-Evans rats (Charles River Laboratories, Inc., Wilmington, MA; 250300 g at the start of the study), housed in the animal care facility at Brown University. The rats were kept on a 12-h light/dark

cycle and housed individually with free access to food and water. All procedures were performed according to the National Institutes of Health guidelines and approved by the Brown University Institutional Animal Care and Use Committee (Protocol#: 1402000046, 1703000263).

3.11.2 Probe fabrication and assembly

(a) Trident construct

Two 100 kOhm stainless steel electrodes (EL30030.1A10, Microprobes) were glued (UV-epoxy, NOA-81, Norland) to a tapered glass optical fiber (0.22 NA, 105/125 μm core/cladding diameters, AFS105/125Y, Thorlabs) previously connectorized to a LC ferrule with metal flange (FAZI-LC-126, Kientec). The adhesion was reinforced at the base of the fiber with vacuum epoxy (Torr Seal; Kurt J. Lesker Company). The electrode tips were about 200 μm ahead of the tip of the fiber. Connectors with (34G, CZ1324, Conner wire) gauge wires were attached to electrodes with silver epoxy and the electrical connection was protected also with vacuum epoxy. Care was taken to minimize the cross sectional area of the construct (Fig. 3.21 A). The preservation of tips of electrodes and optical fiber, the impedance of electrode through the attached connectors, as well as the optical coupling efficiency were measured or confirmed after fabrication.

(b) Fixed array

Fixed array refers to a custom designed microwire electrode based structure for recording and stimulation. A 3D printed housing structure had two towers, intended for symmetrical implantation of two sets of 2x2 electrodes and two optical fibers in motor cortices of two hemispheres (Fig. 3.21 B). The 100 kOhm, 81 μm diameter, tungsten electrodes (WE30030.1A3 as spaced at 1 mm pitch and the optical fiber located in the center of the 2x2 pattern. The electrodes were about 5 mm long outside the housing ($\sim 4.5\text{mm}$ for the

optical fiber). The smaller diameter and longer electrode length allowed reduced trauma and increased visibility during implantation to avoid surface blood vessels or at least easier cleaning of blood.

(c) Screw ECoG construct

While in most cases, ECoG screws were individually placed into cranial holes during the surgery. The wire to each ECoG would wind up and cause difficulty to achieve a small pitch. In response, I fabricated a screw ECoG construct where screws were held together by a teflon piece with their tips just protruding out. An optical fiber was placed in the middle a 2x2 matrix of screws (Fig. 3.21 C). Alternatively, electrical connection was made to small washers, hence only indirectly screws. This way the action of the screws were not hindered during insertion (Fig. 3.21 C). We verified that screws can also reliably record optogenetically evoked potentials in the cortex, as the photovoltaic artifacts were minimal compared with the actual evoked activities.

(d) MEAs

Utah MEAs were purchased from Blackrock Microsystems in configurations of 6x6 arrays or dual 5x5 arrays (Fig. 3.21 D). A customized version of the former had a rod on the backside for holding. The latter had one hole in the middle of each array, and we fixed optical fibers through these holes for optogenetics in the motor cortex.

3.11.3 Surgical procedures

(a) General surgical procedures

All survival procedures were performed under aseptic conditions. Surgery area was decontaminated with chlorhexidine. Surgical tools were sterilized by autoclave beforehand, and

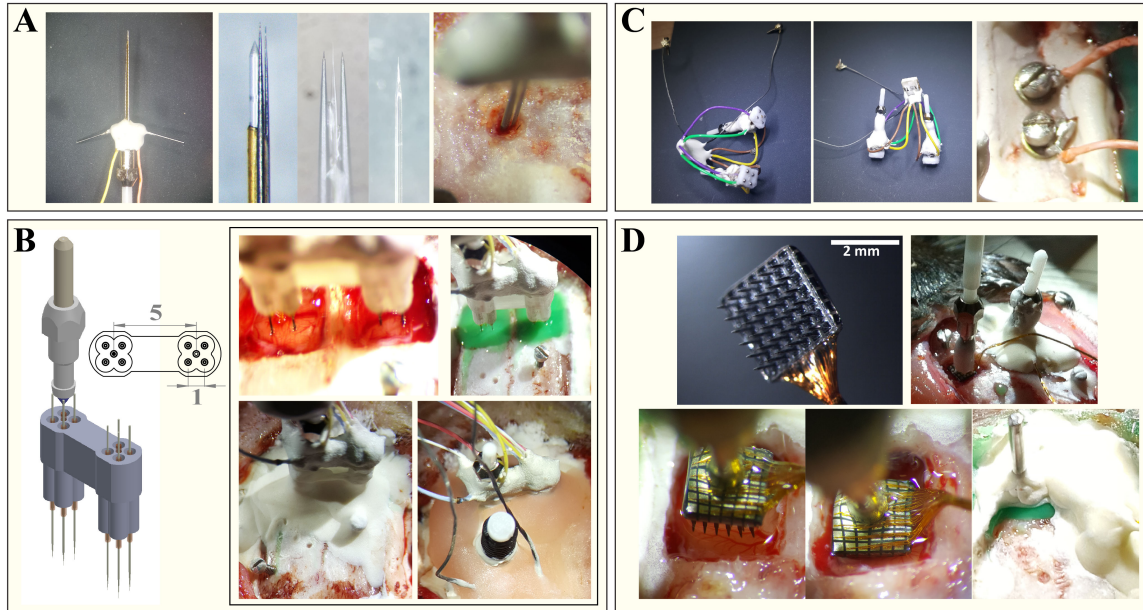


Figure 3.21: Probes and their implantation. **A.** Trident probes: in middle three plots showed from left to right, a Trident with 60 deg taper, a Trident with laser pulled taper, and a laser pulled optical fiber. **B.** Fixed Array: (left) CAD model, (right) implantation; **C.** Screw ECoG: (left & middle) a version that was pre-assembled before surgery; (right) otherwise ECoG screws were placed individually during surgery together with a washer which had premade electrical connections. **D.** MEAs. (top-left) a 6x6 MEA; (top-right) implantation of dual 5x5 optical-fiber integrated arrays, one on each hemisphere; (bottom) implantation of a customized 6x6 MEA with a rod on the back for holding.

using a hot-bead sterilizer during the surgery. Implanted materials, unable to withstand autoclaving, were sterilized with ethylene oxide (EtO) gas sterilization at least one week before implantation. Consequently, the devices were allowed to “off gas” an appropriate length of time and then rinsed two times with warm sterile saline prior to implantation.

Animals were anesthetized with isoflurane (1-3%). During the surgery, isoflurane level was to maintain a steady level of anesthesia, as assessed by the paw withdrawal reflex, as well as the regularity and depth of breathing. After induction, 0.2 mg/kg dexamethasone was administered (i.p.) to reduce brain swelling during the surgery. Hair over the surgical site was trimmed and the head of the animal was fixed in a Kopf stereotaxic apparatus. The surgical site was cleaned with betadine and alcohol wipes (3 times). Sterile drapes

were used to cover the body of the animal and the stereotaxic frame to expose only the surgery site. Before the incision, a mixture of lidocaine and epinephrine (4 mg/kg) maybe administered (intradermal) for additional local anesthesia.

An approximately 1-1.5 cm-long skin incision was made along the midline. Fascia and other periosteum connective tissue were removed from the skull surface. Stereotaxic measurement was taken from the suture landmarks and the head of the animal was leveled accordingly. In many cases, the temporalis muscle was detached from the temporal bone and dental acrylic was applied on the temporal bone for additional fixation. After cleaning of the skull and alignment of the head, anchor screws were placed over the multiple points on the skull. A thin layer of dental acrylic was applied over the edge of the exposed skull surface, as well as at the base of the screws, which acted as foundation for further application of adhesives. I have noticed significant improvement in the integrity (strength of adhesion, as well as lack of cavity) of the final implant with the practice of dental acrylic foundation.

(b) 6-OHDA lesion procedure

On the day of injection, 3.4 mg/ml (free-base) 6-hydroxydopamine-Br solution (with ascorbic acid, H116, Sigma Aldrich, MO) was prepared with sterile injectable saline. For protection of noradrenergic and serotonergic neurons (Thiele, Warre, and Nash 2012), 25 mg/kg desipramine hydrochloride (D3900, Sigma Aldrich, MO) solution was injected i.p., 30 min prior to 6-OHDA injections. To achieve complete DA depletion, 3.0 μ L ($\sim 10 \mu$ g free-base) of 6-OHDA was injected into the medial forebrain bundle (MFB) of the left hemisphere (-4.4 mm AP, -1.2 mm ML, -8.0 mm DV) with a 32-gauge hypodermic needle at a speed of 0.1 μ L/min.

(c) General implantation procedure

After implantation, dental acrylic was carefully applied to provide physical support. In some cases, fast-curing biocompatible PDMS (Kwik-Cast; World Precision Instruments) was applied around wound opening before the application of dental acrylic; this prevented adhesion of soft tissue to dental acrylic and reduced irritation to the animal during recovery. A polypropylene implant protector taking either cylindrical or conical shape was fixed over the implants to prevent animal from scratching the devices. The protecting PDMS was removed after dental acrylic had cured. Finally, a purse-string suture was performed to close the skin loosely around the body of the dental acrylic.

(d) Implantation and management of cranial windows

A craniotomy was made with a dental drill at a size large enough ($\sim 3 \times 3$ mm) to expose the majority of the motor cortex (AP: 1.5mm, ML: 2.5 mm). A duratomy was performed for optical access. Epifluorescence imaging was performed to confirm the extent of opsin expression; note since epifluorescence was the combined process of light delivery and backscattering, confirmation of epifluorescence over the region-of-interest gave me confidence in achieving spatially-extended stimulation (seMCoS). During the measurement, the brain surface was kept hydrated with sterile saline. Afterwards, the PDMS (measured and cut) or glass cranial window was placed over the brain surface (window material was slightly larger than the craniotomy) and then fixed to the skull with biocompatible adhesives and dental acrylic (MetaBond). Sterile saline solution was used to fill the space between the exposed brain surface and the cranial window within the craniotomy. Another epifluorescence measurement was taken to confirm optical transparency of the window.

After the implantation surgery, the cranial window was inspected daily in the first week and afterwards at least once a week. Before seMCoS sessions, the surface of the cranial window was debrided and washed with sterile saline and low-concentration of chlorhexidine.

Fibrotic growth beneath the cranial window on the brain surface was also monitored. After explant, the transmittance of the cranial window was measured.

(e) Implantation of recording and stimulation devices

For implantation of Trident constructs and optical fibers, a cranial burr hole (~ 1 mm) was made according to the stereotaxic coordinates. A small cut on the dura was carefully made. The point of location was slightly adjusted to avoid obvious surface vasculature (Fig. 3.21 A). For STN (-3.8 mm AP, -2.4 mm ML, -7.8 mm DV) and GPe (-1.0 mm AP, -3.0 mm ML, -5.5 mm DV), the exact depths of the nuclei were confirmed by extracellular recording with a single microwire electrode (or determined during virus injection). Implantation in motor cortex targeted the center of the motor forelimb area (+1.5 mm AP, -2.5 mm ML, -1.5 mm DV). The rest of the cranial burr hole was filled with PDMS; and then the implants were secured to stainless steel skull anchoring screws with dental acrylic.

For fixed array and MEA implantations in the motor cortex, a larger craniotomy (> 4 mm x 4 mm) were made over the forelimb area. The dura was removed. Often the dura might have adhered to pia mater at the cranial holes previously made for virus injection, and rupture of surface blood vessels would likely occur. If so, the blood was carefully cleaned away before implantation.

Two screws were placed over the cerebellar surface as reference and ground. In animals recorded with screw ECoG, screws were placed over the bilateral forelimb areas in a 2x2 layout with a pitch of 1 mm. However in seMCoS animals, to ensure coverage of light, screws for ECoG recording were only placed on the contralateral motor cortex.

3.11.4 Assessment of spontaneous and drug-induced rotations

Hemi-parkinsonian motor symptoms including akinesia/bradykinesia and motor asymmetry were assessed with a video camera during animals free behavior in a 30.5 cm (12 in)

diameter acrylic cylinder with a video camera. An important aspect of motor asymmetry, rotational bias, was characterized by three assessments: the spontaneous rotations (SRs), the amphetamine-induced rotations (Amph-IRs), as well as the apomorphine-induced rotations (Apom-IRs).

In open-field free behavior, hemi-Parkinsonian rats showed spontaneous rotational bias which manifested as predominant counter-clockwise (CCW, ipsilateral to the unilateral 6-OHDA injection on the left hemisphere) rotations. However, the bradykinetic aspect of the symptoms often led to complete lack of movement during a recording session, hampering the characterization of rotation.

In Amph-IR experiment, D-amphetamine (2.2 mg/kg, free-base, i.p.) blocks dopamine reuptake and stimulates dopamine release on the healthy hemisphere (Carey 1992), hence further increases the rotational biases and drives rats into a state of continuous locomotion (rotation) for 3060 min. On the other hand, apomorphine (0.5 mg/kg, free-base, s.c. around the neck) is a non-selective agonist to dopamine receptors, which directly activates the dopaminergic pathway. While the action of the apomorphine compensates the imbalanced dopamine, it has been suggested that dopamine receptors after long denervation dopamine inputs develop hypersensitivity to dopamine, leading to overactivation of striatum on the lesion hemisphere; this is also believed to cause the overcompensation in rotational bias, which drives animal into persistent clockwise (CW) rotations (Ungerstedt 1971; Siever, Cohen, and Pert 1981; Marshall and Ungerstedt 1977). Typically the effect of Apom-IR lasts around 6090 min.

After injection, assessment started after 10 min wait for rotational behavior to establish and stabilize. Before implantation, rotation data was extracted based on video recording. After implantation, the instantaneous heading (angular position) of the animal was recorded with a custom magnorotameter (Fig. 3.4 B). Angular velocity, net CCW rotations, total CCW rotations, total CW rotations, total rotations were extracted and computed offline in MATLAB. Depending on the specific experiment, each recording file

was 30 sec to 3 min in length, which is defined as a “subtrial”; it is the basic unit of the semi-automated experiment.

When studying the effects of neuromodulation on rotational behaviors, subtrials with (“treatment”) or without (“control”) stimulation were organized, commonly interdigitated, into groups as “trials”. Commonly a trial takes a three-subtrial [Control (1/2 length), Treatment, Control (1/2 length)] or four-subtrial [Control, Treatment, Treatment, Control] format.

1–2 weeks after 6-OHDA lesion, rats were first tested with amphetamine challenge; only the animals showing peak Amph-IR of more than 6 RPM were included in this study. Immunohistology results confirmed near complete dopamine depletion in qualifying animals.

3.11.5 Electrophysiological recording and stimulation in freely-behaving animal

In optrode implanted animals, subset of rotation tests (SR, Amph-IR, or Apom-IR) were carried out by cabling optical/electrical signals aided by a joint electrical and optical commutator (HRJ-OE, Doric Lenses, Canada). A custom-made gear-based magnetic rotameter was used accurately record the rotational position of the animal, which was synchronized with electrophysiology and the trigger signals of stimuli. To account for the transient variations in the baseline, each stimulation trial was performed together with two no-stimulation trials (pre-stim and post-stim, forming c-s-c structure). In some sessions of STN-eStim, GPe-oStim, GPe-eStim, and seMCoS, the two consecutive stimulation trials were performed (forming c-s-s-c structure). The recording was performed with the Open Ephys system (Headstage: RHD2132 32-inputs amplifier with 3-axis accelerometer, C3324, Intan; Open Ephys acquisition board) or the Plexon system (32-channel VLSI head stage, 20 $\times$ gain, model HST/32V-G20; 32-channel amplifiers, 0.7 Hz-8 kHz, 50 $\times$ gain, model PBX3/32wb-G50), multifunction DAQ from National Instruments (NI PCI-6259 or PCIe-6259, M Series DAQ, 32 AI, 48 DIO, 4 AO); custom software in LabVIEW; National Instruments). Good

optical coupling and electrical contacts were confirmed for each stimulation trial based on the modulations on electrophysiology; trials without consistent stimulus-related artifact or LFP modulations were discarded.

The temporal structure of stimuli is defined by the phase, pulse, burst, and train (Fig. 3.22). Pulses can be monophasic (optical stimulation) or biphasic (charge-balanced for electrical stimulation). Then periodically occurring pulses at the same pulse intervals are grouped as a burst. Lastly, a train contains burst of the same burst intervals (Fig. 3.22).

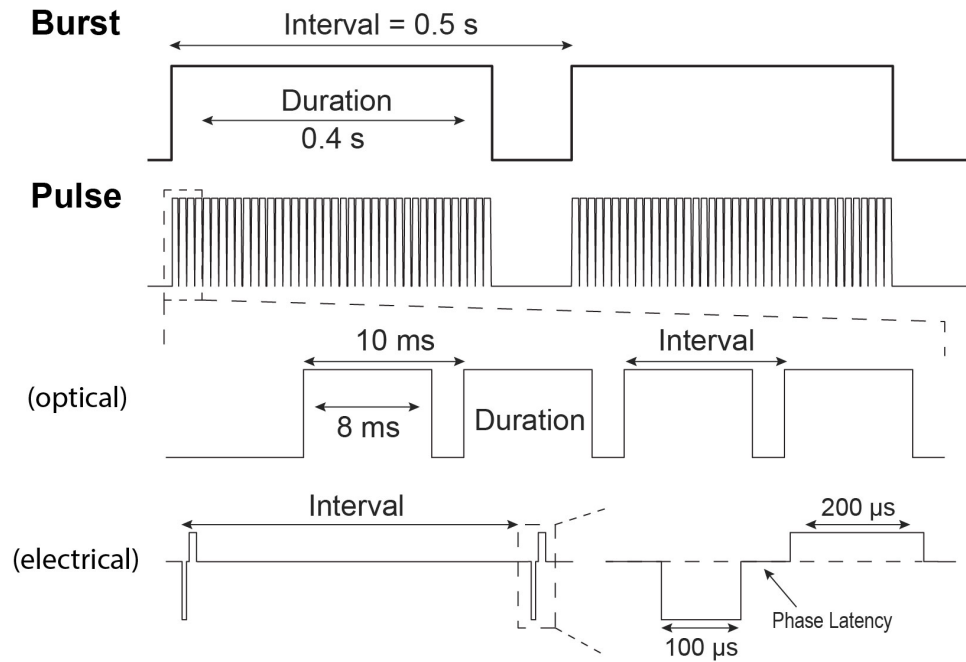


Figure 3.22: Temporal structures of optical and electrical stimulation protocols.

(a) Optical stimulation

For optical stimulation, either a 473 nm laser (for ChR2 and SwiChR, MLL-III-473, Opto-Engine) or a 561 nm laser (for C1V1 and eNpHR, MLL-FN-561, OptoEngine) was coupled to a patch cable, which was further connected to the optical fiber on the animal's head

through an optical commutator. In each animal, we first confirmed the presence of optically induced effects and determined the threshold light power. In terms of the temporal protocol of the stimulation, a train of square pulses, 4 msec in duration and 100 Hz in frequency (50% duty cycle), was generated from the pulse generator to control the gating of the laser. In some experiments, a train of light pulses was further organized into several identical bursts, 1.59.0 sec burst duration and 0.51.0 sec interburst interval. The power of light delivered to the brain was ranged from 3 to 12 mW.

(b) Electrical stimulation

For electrical stimulation, bipolar high-frequency (125Hz) electrical stimulation was delivered to the STN through the two stainless steel electrodes. Biphasic charge-balanced pulses (60240 μ sec) were profiled with a pulse generator (Master-8, AMPI, Israel or PulsePal, USA), applied as isolated constant current stimuli (Model 2200, AM systems), and delivered either continuously or a defined burst duration and repetition rate. The duration and duty cycle of the bursts are similar the ones used in optical stimulation. The current intensities (100200 μ A) used did not trigger direct motor responses.

3.11.6 Experimental session control

A MATLAB script was developed for the semi-automatic control of data acquisition during rotation tests. The program controlled the recording of the video camera, recording of the rotameter data. It also controlled the randomly selection of stimulation parameters from the set of parameters pre-specified by the experiment prior to the session. After selection, the stimulation protocol was formulated, downloaded to the PulsePal (based on Arduino) for TTL pulse generation. For optical stimulation, this trigger TTL signal was passed directly to the laser current driver; and for electrical stimulation, trigger signal was directed to the battery operated optically insulated stimulator. Typically, an additional TTL signal was

generated to drive an infrared (IR) LED (cannot be seen by the rat) placed within the field-of-view of the video camera. This IR-beacon signal was recorded together with the rotameter data and the stimulation trace for post-hoc synchronization.

The vigorous rotations pose tremendous challenges for the maintenance of stable optical coupling and electrical contact over a 1-2 hr session. In fact, the reason for simultaneous electrophysiology during optical recording is in part to provide continued monitoring on the establishment of desired coupling based on the evoked response. In addition, although it has been a considerable burden on the experimenter, I found it worthwhile to perform mandatory checks on the status of the coupling. Therefore the program was instructed to pause after specified number of trials and wait for user confirmation of coupling check.

A limitation of this setup was the requirement of analog control the power of laser output. In sessions where varying of the stimulation power was required, automation cannot be achieved. As a work around, the experimenter was instructed by the program with the randomly selected power level, and a dial on the laser driver was manually set.

3.11.7 Simulation of spatial light distribution and photothermal effects

Light, when traveling through the brain tissue, behaves not unlike how it travels through other turbid medium, such as the cloud. Photons can be absorbed or their propagation can be redirected (scattering), the strength of which are dependent of wavelength and tissue properties. Monte Carlo simulation of photon transport in a scattering medium has been a widely-used algorithm for this purpose (L. Wang, Jacques, and Zheng 1995). In addition, when milliWatt of light being delivered through an $\sim 100\mu\text{m}$ diameter fiber, heating at very local range is expected from a simple energy perspective. Yet the “aqueous” environment of the brain allows for dissipation of local heating through passive thermal diffusion and active heat transport through blood flows (see (Aronov and Fee 2011; Stujenske, Spellman,

and Gordon 2015) for details).

The algorithm used for Monte-Carlo simulation for photon transport in turbid medium was adopted and modified from the work by Ozden et al. (Ozden et al. 2013)) and associated photothermal effects was simulated based on the work described by both Ozden et al. and Stujenske et al. (Ozden et al. 2013; Stujenske, Spellman, and Gordon 2015)).

Specifically, while numerous advancement has been implemented to incorporate inhomogeneity of optical properties and complex geometry of the medium (Shin and Kwon 2016), in our implementation (algorithms modified from the work by Ozden et al. (Ozden et al. 2013)), the medium was assumed to have homogeneous properties (irrespective of targeted nucleus) and take simple, cylindrical geometry for the simplicity of computation. This choice is further justified by the relatively regular structure of ROI in some of our paradigms (e.g. motor cortex). Many prior studies have characterized optical properties of brain tissue in ex vivo and in vivo conditions (Yaroslavsky et al. 2002; Johansson 2010; Mesradi et al. 2013; Azimipour et al. 2014; Gysbrechts et al. 2016); suitable parameters were extracted from these literature after careful review (summarized in Table).

The brain volume being simulated was simplified as as cylinder of 6 mm diameter and 4 mm thickness (smaller than used by Stujenske et al.). Bigger simulation volume did not produce significantly different predictions, except at the boundaries; this is because 1) the simulated volume was at least 40 times of the photon mean-free-path (mfp, a measure of average distance traveled by photon before absorption or scattering occurred), such that most photons would not exit this volume; 2) both heat accumulation and dissipation in this model were normalized to unit volume; and 3) a constant temperature boundary condition was specified.

Optical fibers being modeled are glass, multimode, 0.22NA fibers of various core/cladding diameters (105/125 μm , ASF105/125Y; and 200/230 μm (FT200UMT, Thorlabs). Three

types of tapers (tip full angle, symmetrical), 180° (flat), 60° (polished, BV-10, Sutter Instrument), and 5° (laser pulled, P-2000) were modeled. The 60° polished fibers were incorporated in many of my Trident probes: the shorter taper promises better confidence in the parallel placement of recording electrodes within the light coverage (usually electrodes were $\sim 200\text{-}500\text{ }\mu\text{m}$) ahead of the fiber. More recently, the 5° laser pulled (upper bound of taper angle) fibers were adopted: they provided improved overall output efficiency, showed the superior mechanical penetrability, and assumed lowered light intensity at the surface of the probe.

Both 473 nm and 561 nm wavelength light was used in the simulation. For majority of photothermal simulation, 473nm light was used for two reasons: 1) it is the peak excitation wavelength for both ChR2 and SwiChR_{CA}, the two major opsins used in this thesis; and 2) shorter wavelengths are more likely to be absorbed, hence for the same input power, more localized heating may occur (Stujenske, Spellman, and Gordon 2015). A 30 sec train of high frequency stimulation, [Pulses: 100 Hz, 8 msec duration; Bursts: 0.4/0.5 sec], was used for heat simulation (Table. 3.1).

The heat capacity of the fiber was not considered in this simulation. The realistic heating effect may be further reduced with the implant as a heat capacitor. In this thesis, other glass fibers with greater NA (e.g. 0.39) had been used; however larger NA would entail better light spread, lower intensity at the emission surface, and hence even better performance in terms of thermal safety.

For seMCoS with suspended fibers, we also performed analysis of photothermal effects, following similar procedures. Fiber was simulated to be 0, 1, 2, 3, and 4 mm above the cranial window, respectively (Table. 3.1).

Key Parameters (for brain unless otherwise stated)	Value	Units
Refractive index - brain	1.36	
Refractive index - CSF	1.33	
Refractive index - cranial window	1.52	
Absorption coefficient (<i>wavelength dependent</i>)	370 - 260	m^{-1}
Scattering coefficient	10600	m^{-1}
Anisotropy factor	0.887	
Wavelength	473 or 561	nm
Density	1.04×10^{-6}	kg/mm ⁻³
Specific heat	3.65×10^3	J/(kg·°C)
Thermal diffusivity constant	0.527	mW/(m·°C)
Blood perfusion rate (normalized)	8.50×10^{-3}	s^{-1}
Initial temperature	37	°C

Table 3.1: (Yaroslavsky et al. 2002; Johansson 2010; Stujenske, Spellman, and Gordon 2015).

3.11.8 Histology and image analysis

For histological analysis, rats were euthanized by a lethal injection of Beuthanasia-D (100 mg/kg, IP) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) solution. Brain was extracted and sequentially fixed in 4% paraformaldehyde in PB solution (for at least 1 day) and cryoprotected in 30% sucrose solution (until brain fully sunk in solution). The brain was then frozen with dry ice and cut into 50 μm (for confocal imaging) or 100 μm (for fluorescence imaging) coronal slices with a microtome. Slices were examined under a Zeiss LSM 510 Meta confocal laser scanning microscope or a Zeiss Axiovert 200M fluorescence microscope. In a subset of rats ($N = 5$), the extent of dopaminergic lesion was evaluated with tyrosine hydroxylase (TH) immunostaining. Slices were sequentially washed in PB (twice, 5 min each) and PBS (three times, 5 min each), and then incubated in block solution for 1 h. The block solution consisted of 0.1% Tween (SigmaAldrich, MO), 0.25% Triton-X (SigmaAldrich, MO), and 10% normal goat serum (EMD Millipore, MA) in PBS. Slices were then immersed in the primary antibody solution (1:1000 AB152 Anti-TH in block solution EMD Millipore), covered with aluminum foil, and shaken for 2.5 days in a cold room

(4°C). Afterward, slides were thoroughly washed in PBS (five times, 5 min each), again immersed in block solution for 1hr, and incubated in secondary antibody solution (1:500, Alexa Fluor ® 594 donkey anti-rabbit IgG in block solution, Life technology, CA) for 2 hr. After the final washing steps with PBS (three times, 5 min each) and PB (twice, 5 min each), slides were mounted on microscope slides with DAPI mounting medium (H-1200, Vector Laboratories, CA).

For characterization of TH immunoreactivity, fluorescence images of slices (containing striatum (STR) or substantia nigra compacta (SNc)) obtained in grayscale were normalized in intensity from 0 to 1. Square regions-of-interest (ROIs) of 200-250 μm size were manually selected from the nucleus at question on each hemisphere. The average normalized fluorescence intensities within these ROIs were calculated (background intensity subtracted), which were used as surrogates for immunoreactivity. The relative difference (% change) of fluorescence on the lesion and control hemispheres was calculated.

3.11.9 Statistics

Rotation results were pooled across all animals receiving each modulation paradigm. Rotation results are presented as mean standard error of mean (s.e.m.). All statistical analyses were performed with MATLAB (MathWorks, MA). Pairwise *t*-test was performed for comparisons of the means between two groups. Nested analysis of variance (ANOVA) was used to separate effects of multiple groups of variations. Statistical significance was granted for values of $p < 0.01$. For statistically significant comparisons, the effect size of the difference was measured with the Cohen's *d* parameter. In figures, unless otherwise specified, asterisks (*) indicate the level of significance (* $p < 0.01$, ** $p < 1\text{E-}3$, *** $p < 1\text{E-}4$) and palm signs (#) indicate the Cohen's *d* effect size (# $d > 0.8$, ## $d > 1.2$, ### $d > 2.0$).

Chapter 4

Power Inhomogeneity and Phase Coherence of the Pathological Beta Oscillations in the Motor Cortex

4.1 Introduction

Excess beta-band (13-30Hz in humans and 13-45Hz in rats) power in the local field potentials (LFPs) in the motor cortex and in several basal ganglia nuclei is the most prominent feature of Parkinsonian pathophysiology (Brown 2003; Sharott et al. 2005; Brittain and Brown 2014; Delaville et al. 2014; Sharott et al. 2018).

However, it is not known how and to what extent pathological beta activity might override the functional activity in the motor cortex. Specifically, little information has been revealed regarding the spatial distributions and temporal dynamics of the pathological oscillatory activities within the Parkinsonian motor cortex, characterized at suitable spatial resolution.

To address this question, we utilized microelectrode arrays (MEAs, 400 μ m electrode pitch) unilaterally (6x6 MEA) or bilaterally (two 5x5 MEAs) implanted into the anterior forelimb area of motor cortices in nine 6-OHDA-induced hemi-Parkinsonian rats. Recordings were obtained during free behavior. LFPs were extracted from MEA recordings and their spatiotemporal dynamics were examined with spectral and coherence analyses.

In this chapter, I will introduce our preliminary findings regarding the spatiotemporal inhomogeneity in the beta activities within the hemi-Parkinsonian (hP) motor cortex. In brief, we found excess beta power presented on the lesion motor cortex. However, beta activities with elevated power also showed inhomogeneity across multiple dimensions: they did not manifest as 1) uniform distributions in space, 2) stable events in duration, or 3) constant bands in frequency. Examination of instantaneous beta power (beta events) revealed potential basis for a point-process perspective. Furthermore, we identified phase coherence of beta oscillations measured with the phase locking index (PLI) as a more conserved measure among sites within the lesion motor cortex: for which 1) sites with higher beta power in general also showed higher phase coherence between them; and 2) lesion sites without high beta power still show higher phase coherence.

4.2 Findings

4.2.1 Spectral power of beta oscillations was elevated on the lesion motor cortex

First of all, in two rats, MEAs were implanted bilaterally to both motor cortices. During spontaneous behavior, we evaluated the levels of beta activities on the healthy and lesion hemisphere. Significant increase ([Rat D: $1.91 \pm 0.06\%$ on control hemisphere to $2.23 \pm 0.06\%$ on lesion hemisphere, 17% increase, 2531 sec; $p \rightarrow 0$, Welch's t -test; $F_{1,40}=614$, $p \rightarrow 0$, Nested ANOVA] and [Rat G: $2.40 \pm 0.12\%$ on control hemisphere to $2.83 \pm 0.14\%$ on lesion hemisphere, 20% increase, 622 sec, $p \rightarrow 0$, Welch's t -test; $F_{1,10}=176$, $p \rightarrow 0$, Nested ANOVA]) was found in the normalized power at frequencies between 15 - 45 Hz on the lesion hemisphere (Fig. 4.1 A-B). When visualized in reference to the physical layout of the arrays (“arraymaps”), this difference could be easily identified in terms of both the average beta power and the maximum beta power, which were measured within the 60-120 sec long recording segments (Fig. 4.1 C).

4.2.2 Frequency variations of beta oscillations

In several other animals implanted with single MEA on the lesion motor cortex, we also found prominent bands in the beta range (Fig. 4.2 A). However, the center frequencies of the beta band tended to vary from animal to animal (from 15-30 Hz to 25-40 Hz), while being stable within each animal over weeks of recording. We hence first quantified the distribution of the center frequencies of peaks identified in the mean LFP spectra (across all channels) (Fig. 4.2 B) and found that the overall broad beta bandwidth in each animal was a result of narrower (in frequency domain) beta peaks occurring at varied center frequencies. In the two dual-array animals, these maximum power frequency distributions displayed higher mean values on the control hemispheres than on the lesion hemispheres (“Rat D”: 32.3 ± 4.0 Hz , lesion, versus 34.3 ± 7.1 Hz, control, $p \rightarrow 0$, Kolmogorov-Smirnov similarity test, KS

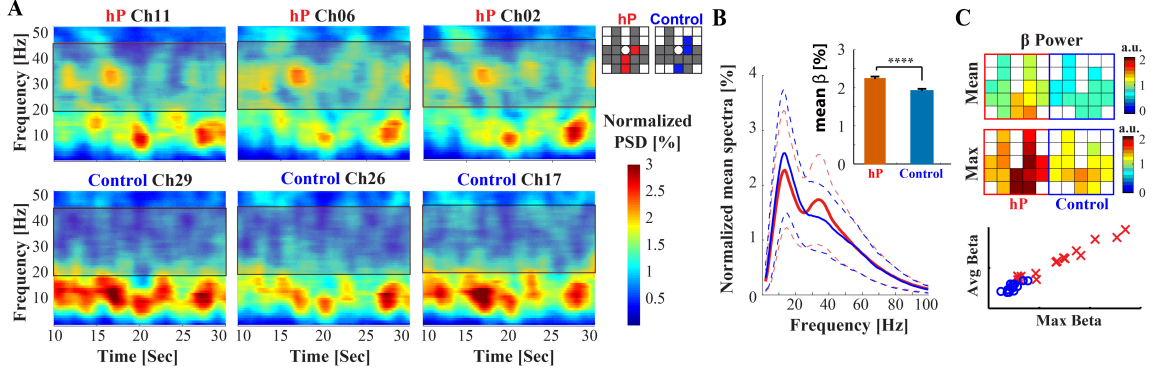


Figure 4.1: Elevated beta power on the lesion motor cortex. Example data was recorded from Rat D. **A.** Excerpts from the LFP time-frequency representations (TFRs in the form of normalized power spectral density) during spontaneous behaviors. Activities in the 20-45 Hz band were absent in control channels. The locations of the channels were shown in the array layout on the top right corner. PSDs were normalized (percentage of total power at each time point), and smoothed over time and frequency for illustration. **B.** Average spectra over time and across channels. The dashed lines show the standard deviations. The inset compared the average beta power on the lesion versus control hemisphere ($p < 10^{-4}$, unpaired t -test). **C.** Time averaged power and maximum power of beta activities in each channel displayed in the array layout. In the bottom panel. Segregation of lesion versus control channels were found in terms of beta power (bottom).

= 240; “Rat G”: 27.9 ± 6.0 Hz, lesion, versus 33.5 ± 8.6 Hz, control, $p \rightarrow 0$, KS similarity test, KS = 107; Fig. 4.2 B). The bandwidths for individual peaks were also narrow, e.g. 5.6 ± 1.9 Hz (lesion) versus 4.5 ± 2.0 Hz (control) for “Rat D” and 4.7 ± 1.8 Hz (lesion) versus 5.2 ± 1.7 Hz (control) for “Rat G”. Scrutinizing this variation in frequency at finer temporal scale (0.2 sec), we computed the distributions from peak frequencies extracted at each time point of the TFRs (rather than from the entire recording), and found that instantaneous peaks could in fact occur over an even greater frequency range, albeit that they were more likely to occur in the manifested beta frequencies (Fig. 4.2 C, also see Fig. 4.1 A). Similar observations of varied individual beta frequency were previously reported in ECoG recording of motor cortex in rats (Lehmkuhle et al. 2009) and were shown related to the underlying behavioral state, i.e. higher frequencies tended to appear during active movement.

Hence, beta activity was not broad-band in frequency but was rather an epiphenomenon of the aggregation of narrow beta peaks at varying frequencies.

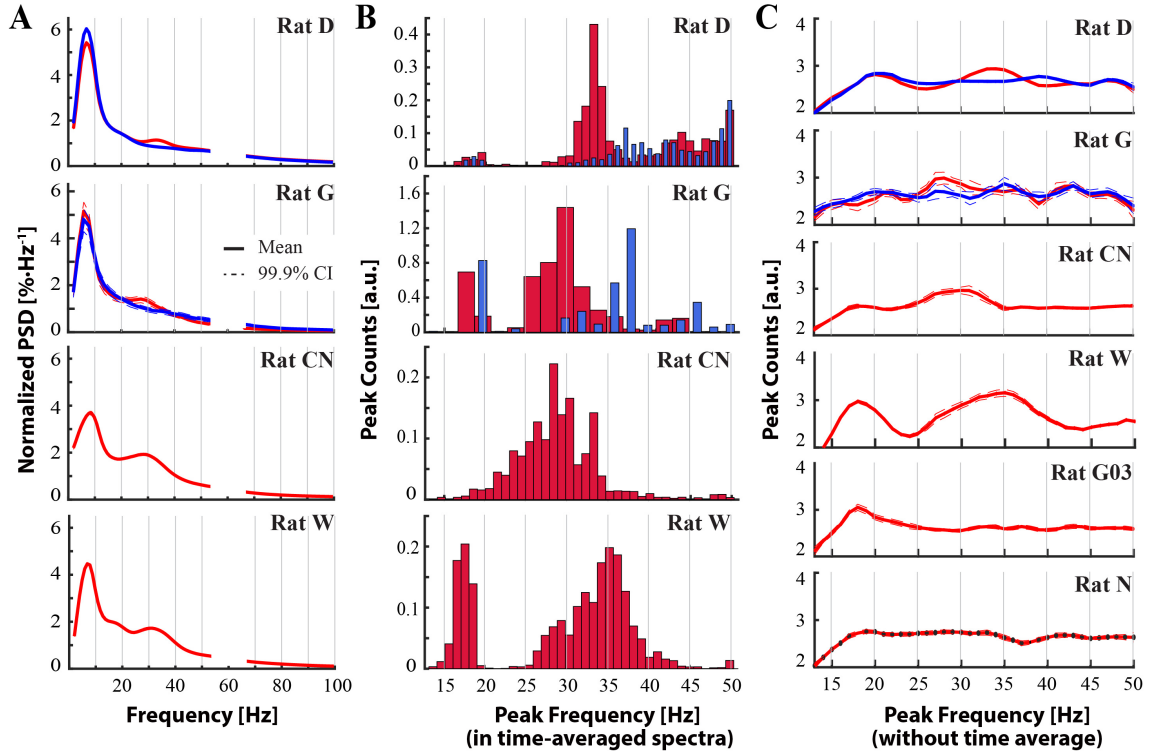


Figure 4.2: Variations in the peak center frequencies of beta activities. **A.** Example time-averaged channel-averaged LFP spectra (normalized) from four rats shown in mean and 99.9% confidence interval. The beta band frequencies varied across animals, the center frequencies of which were indicated by the black arrowheads. **B.** Maximum-power frequency distributions extracted from the time-averaged spectra. Top two panels showed distributions of peak frequencies from both lesion and control hemispheres in two dual-array animals. **C.** Maximum-power frequency distributions computed at each time point of the TFRs. The mean distributions were shown together with 99.9% CI. Peaks occurred over wide range of frequencies.

4.2.3 Temporal dynamics of beta oscillations as individual events

Over time, beta oscillations did not appear in a step-function-like “ON/OFF” fashion; the example TFRs in Fig. 4.1 A also demonstrated this characteristics. While evidence in the phasic nature of beta activities had been shown for STN (Tinkhauser et al. 2017), it has not been well characterized in the motor cortex. Therefore we further study the temporal dynamics of beta oscillations by using a wavelet-based method (Loughlin and Cohen 2010) to obtain beta power with high temporal resolutions. Then we identified temporal beta events

(peaks) at various amplitude thresholds (measured by percentiles of mean beta power) and characterized their amplitudes (power), durations (full-width half-prominence), and occurrence rates.

In terms of amplitudes (demonstrated by data from Rat D in Fig. 4.3), the control hemisphere had higher number of low amplitude beta events, and *vice versa* for the lesion side. Upon closer inspection of the amplitude distribution at various threshold levels, we found that as the threshold amplitude increased, the control and lesion beta distributions became more and more similar, despite more beta events continued to occur on the lesion side. In other words, high amplitude beta events were not exclusive to the lesion motor cortex, rather they were just produced more frequently. Similar relations were also observed in other (dual- and single- array) animals.

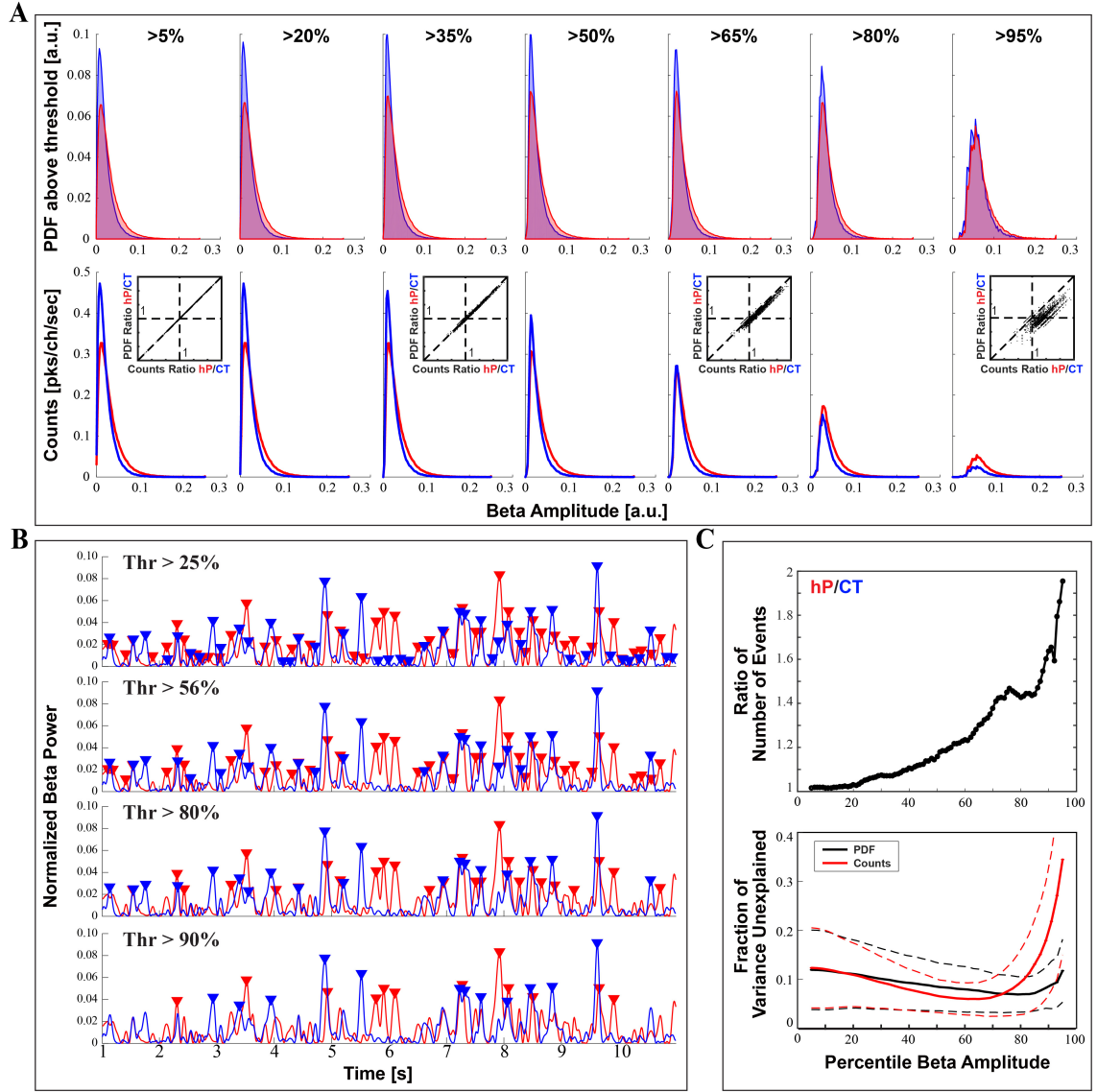


Figure 4.3: Amplitudes of beta events. **A.** Detected beta events with amplitudes greater than a certain amplitude threshold (measured by a percentile of the beta power) were extracted from all recordings of Rat D. **A.** Normalized (PDF; top, shaded) and unnormalized (counts; bottom) distributions of beta amplitudes above increasing amplitude thresholds. Control hemisphere (CT) showed more frequent low amplitude beta events. Among high amplitude events, although the CT and hP distributions became more similar, hP events occurred more often. Inset showed the ratio of normalized distributions plotted against that of the unnormalized distributions.

Figure 4.3 caption – continued **B.** Example beta power traces from one lesion (red) and one control (blue) channel. Suprathreshold beta events were indicated by the inverted triangles. High amplitude events were also found in control channel. **C.** To quantify the evolution of relations between the normalized and unnormalized distributions, we computed the ratio of the total numbers of suprathreshold beta events (hP/CT), which increased over threshold (top), and the similarity metric that decreased at high threshold between unnormalized distributions, but remained high between normalized ones (bottom). The median FVU were plotted together with values at 25th and 75th percentile (dashed lines).

The durations of beta events varied from 2 to 4 beta cycles (depended on the varying center frequencies) and were distributed rather similarly overall in lesion and control channels. For beta events with higher amplitudes, their durations were also longer and they occurred more often on the lesion side; however, the difference between high amplitude distributions on the lesion and control hemispheres was not significant (Fig. 4.4). Our results further disputed the hypothesis of stably elevated beta band on the hP motor cortex.

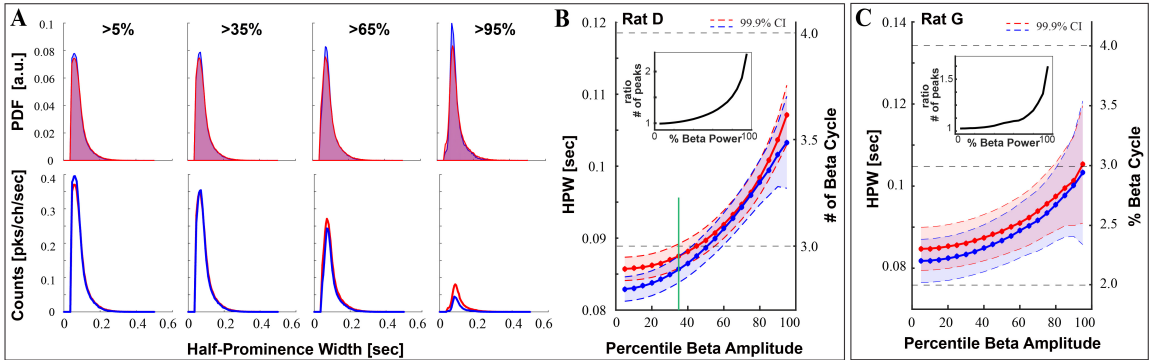


Figure 4.4: Durations of beta events. Durations of suprathreshold beta events, measured by the half-prominent full-width (HPW), were extracted from all recordings of Rat D (A-B) and Rat G (C). **A.** Normalized (PDF; top, shaded) and unnormalized (counts; bottom) distributions of beta durations above increasing amplitude threshold. Lesion hemisphere showed longer duration beta events that also had higher amplitude; otherwise the distributions were rather alike. **B.** Mean durations estimated from the lesion and control distributions in part A (based on Poisson distributions) were shown with the 99.9% confidence intervals. The green vertical line indicated the level below which the means were outside the confidence intervals of each other. Inset showed the ratio (hP/CT) of numbers of events at each beta power level. **C.** Data from Rat G. Beta durations were also higher on lesion side but not significant w.r.t. the 99.9% CI.

In summary, we found beta activities occurred more frequently on the hP motor

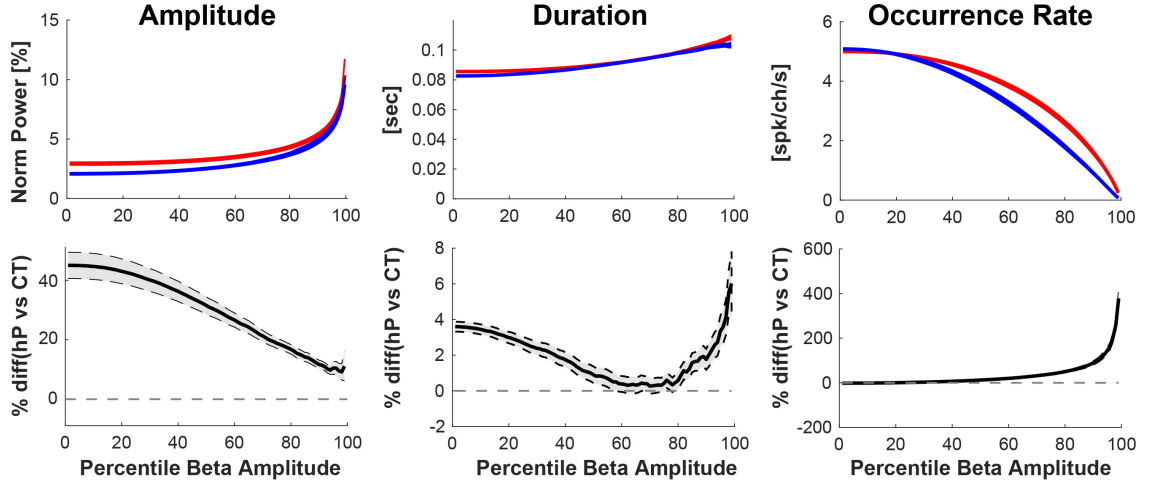


Figure 4.5: Comparisons of amplitudes, durations, and occurrence rates of beta events. (*Top*) Mean values of beta events shown with 99.9% confidence interval. (*Bottom*) Mean percentage difference between hP (red in part A) and CT (blue in part A) for each variable. The gray shaded area illustrated the s.t.d. over time around the means.

cortex (Fig. 4.5 right), which was particularly the case for events with high amplitudes. At the same time, the control hemisphere was not void of events of comparable amplitudes. Despite differences in event amplitudes and frequency, durations of beta events were rather similar and more importantly short, spanning only a few beta cycles.

4.2.4 Spatial variations of beta oscillations

In previous sections, we showed that beta activities were elevated on the lesion motor cortex and had variations in the fine temporal structure. Does beta power also vary across different cortical sites? Would sites with high beta power always show higher beta power? To address these questions, we studied the spatial (and temporal) variations of beta activities.

First, a power based metric was quantified conjecturing that beta power greater than the grand average level across lesion sites was “hP-like” and the ones smaller than the grand average value across control sites were “CT-like”. Each channel at each time was assigned a numeric label of 1 for being “hP-like”, -1 for being “CT-like”, and 0 otherwise.

Then the labels were averaged over time and compiled into a variable termed, the beta-state likelihood (BSL). Fig. 4.6 A showed the BSL in two animals: while it was clear that the hP hemisphere had greater BSL values, not all hP sites had BSL greater than 0. In addition, within the hP motor cortex, sites manifesting high beta power varied over time (Fig. 4.6 A-b). Subsequently, in order to improve the robustness against transient variations in power, an alternative measure, the average beta rank (normalized) was computed to measure the spatial and temporal stability of beta distributions. In brief, at each time point, each channel was designated a rank based on its instantaneous beta power; the ranks were then averaged across all time and normalized between 0 (always the lowest power) and 1 (always the highest power) (hereinafter, it will be referred to as the average beta rank). Despite the increased robustness, we still found several CT sites showing higher average beta ranks than some lesion sites (Fig. 4.6 B).

Then, we asked whether we can correctly classify the identity of motor cortical sites (hP or CT) based on spectral power. First, using a naive Bayesian classifier, as we varied the threshold on beta power (a common practice in adaptive DBS), a reasonable performance was achieved using average values from long segments of recording (AUC 74.2%, Jmax = 41.1%, Fig 4.6 C-left); and the performance was enhanced by using beta ranks instead (AUC 79.8%, Jmax = 48.6%). While these analyses required long period of recordings, subsequently we also attempted classifications on short time windows (1 sec), by taking multiple frequency bands into consideration. In brief, the frequency spectrum was divided into five frequency bands of equal bandwidth (5 - 20, 25-40, 45-60, 65-80, and 84-99 Hz) and their temporal means and standard deviations of power were computed and stitched into a 10-element feature vector for each time window (non-overlapping). Afterwards, we performed dimension reduction based on principal component analysis (PCA), and the reduced feature vectors across channels and over time (each channel at each time was treated as independent) were classified with supervised quadratic discriminant analyses (10-fold cross-validation). The outcome of the classifier was significantly above chance level,

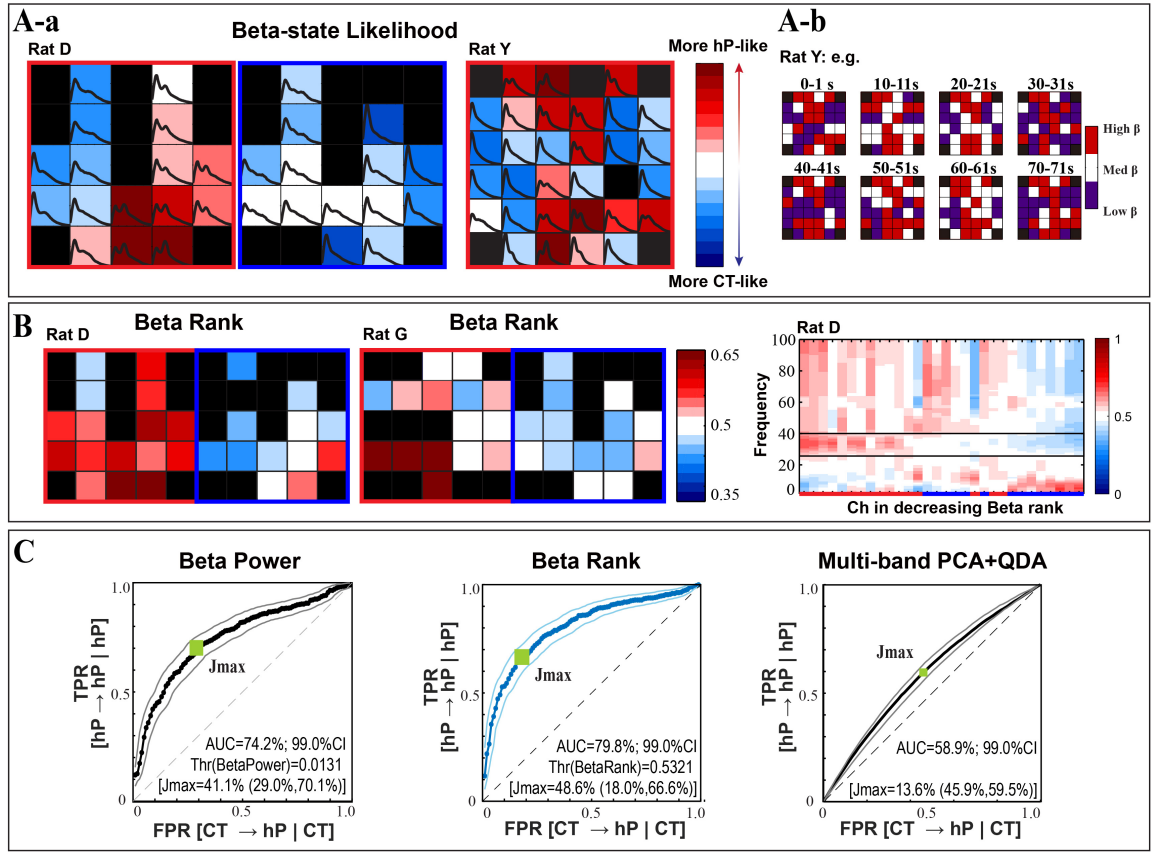


Figure 4.6: Spatiotemporal variations of beta power. **A.** (a) Spatially-resolved power spectral density (PSD) in the motor cortex. Red and blue colors in the background showed the beta-state likelihood. The traces are PSD plots between 0-100 Hz. Black squares are disconnected channels. (b) Similar plots showing that beta-band activity fluctuated between high and low power levels at each recording site. However, the sites, which were classified as hP in part (a), switched more frequently to high beta-band level. **B.** Time-average power ranks corroborated the fluctuations in beta activities. Red color represented higher ranks. (Right) Average ranks of all frequencies of each channel ordered based on decreasing average beta rank. **C.** ROC curves for naive Bayesian classification by varying power threshold (left) or rank threshold (middle) based on long recording segments, and for quadratic discriminant analysis of power of multiple frequency bands obtained at finer time resolution (1 sec) (right). True positive rates (TPR) are the fraction of hP sites being correctly classified as hP; and false positive rates are the fraction of CT sites being falsely classified as hP.

yet the performance was much inferior to the previous cases (Fig. 4.6 C). This reflected the strong overlap in beta band power among lesion and control sites, which was exacerbated when shorter time bin was used due to the temporal inhomogeneity.

4.2.5 Phase locking was elevated among the lesion motor cortex

Excessive coupling between basal ganglia nuclei at the beta band had been widely reported in Parkinsonism (Silberstein et al. 2005; Singh 2018). Its importance has been further emphasized by recent studies that showed in human patients the phase of cortical beta oscillations precedes those in the basal ganglia (e.g. STN), implying a role of motor cortex in shaping overall oscillatory pathophysiology in the basal ganglia (Ahn et al. 2015; Singh 2018). The use of MEAs provided us the unique opportunity to investigate the synchrony of these beta activities over fine spatial resolutions within and across the motor cortices. Specifically, we focused on the temporal dynamics in the synchrony of beta phase and computed the phaselocking index (PLI), a metric borrowed from EEG studies. PLI disregards the absolute phase differences and instead, keeps track of the consistency of the leading/lagging relation over time between phases of a pair of recording sites, which yields the advantage of being less sensitive to the effect of volume conduction (physiologically unrealistic perfect zero-phase lags, the reader is referred to (Stam et al. 2007; Vinck et al. 2011) for review). In this study, PLIs were calculated more computationally efficiently based on cross-spectrogram, rather than from instantaneous phases extracted with Hilbert transformation, while the two methods yielded similar outcomes. In this section, we define “marginal PLI” of a channel as the average PLI across all pairs involving this channel (including inter-hemispheric pairs); and “areal PLI” of a channel was computed based on the channel identity (hP or CT) and was the average across all pairs involving this channel and other channels on the same motor cortex (only intra-hemispheric pairs).

Firstly We found that marginal PLIs were not uniform among all sites ($p \rightarrow 0$, $F_{2,9} = 532$, ANOVA). Moreover, the phase-locking effects were stronger among sites on the lesion hemisphere (0.091 ± 0.002 for hP and 0.037 ± 0.001 for CT, $p \rightarrow 0$, $F_{1,40} = 782.5$, nested ANOVA). To breakdown this effect, we found higher power sites also tended to have higher areal and marginal PLI values, which was corroborated by similar relations found

for beta ranks (Fig. 4.7 A-B). More interestingly, in both cases, several lesion sites showed lower average phase synchrony, and sites with lower power could also manifest high phase synchrony. After reordering channels based on the instantaneous power, a baseline coupling between beta power and phase synchrony was revealed (Fig. 4.7 C) but the magnitude was much weaker. Therefore, increased phase synchrony was not solely dependent on the elevated power.

As discussed previously, beta power and beta rank can be used to classify identity of recording sites, to some degree (recapped in Fig. 4.7 A-B (right)). Here we show that phase synchrony, both marginal PLI and areal PLI, yielded better classification performance (AUC 90.4%, $J_{\max} = 41.1\%$ for aPLI and AUC 81.9%, $J_{\max} = 51.4\%$ for mPLI, Fig. 4-7 D-E). On one hand, this results implied that synchrony may be more essential to the parkinsonism compared to the elevated power. Indeed in other animals with dual arrays or single arrays, we have found more exaggerated discrepancies between phase synchrony and power of beta oscillations (Fig. 4.8). On the other hand, the high performance of marginal PLI based classification was rather surprising to us, as the metric was diluted by the contributions of lesion-control pairs. Therefore, phase synchrony may also have diagnostic implications as the computation of the metric did not require prior knowledge of the underlying parkinsonism.

During this thesis, I have also developed analytical methodologies for preliminary analysis of the spatial variations in phases, as well as the variations in the time-derivative (changes over time) and spatial gradient (changes over space) of phases, based on circular statistics. Preliminary analysis in dual array animals found separable differences in these metrics between lesion and control motor cortices. More detailed analyses would be required to fully reveal the spatiotemporal dynamics in the synchrony of activities in the parkinsonian motor cortex.

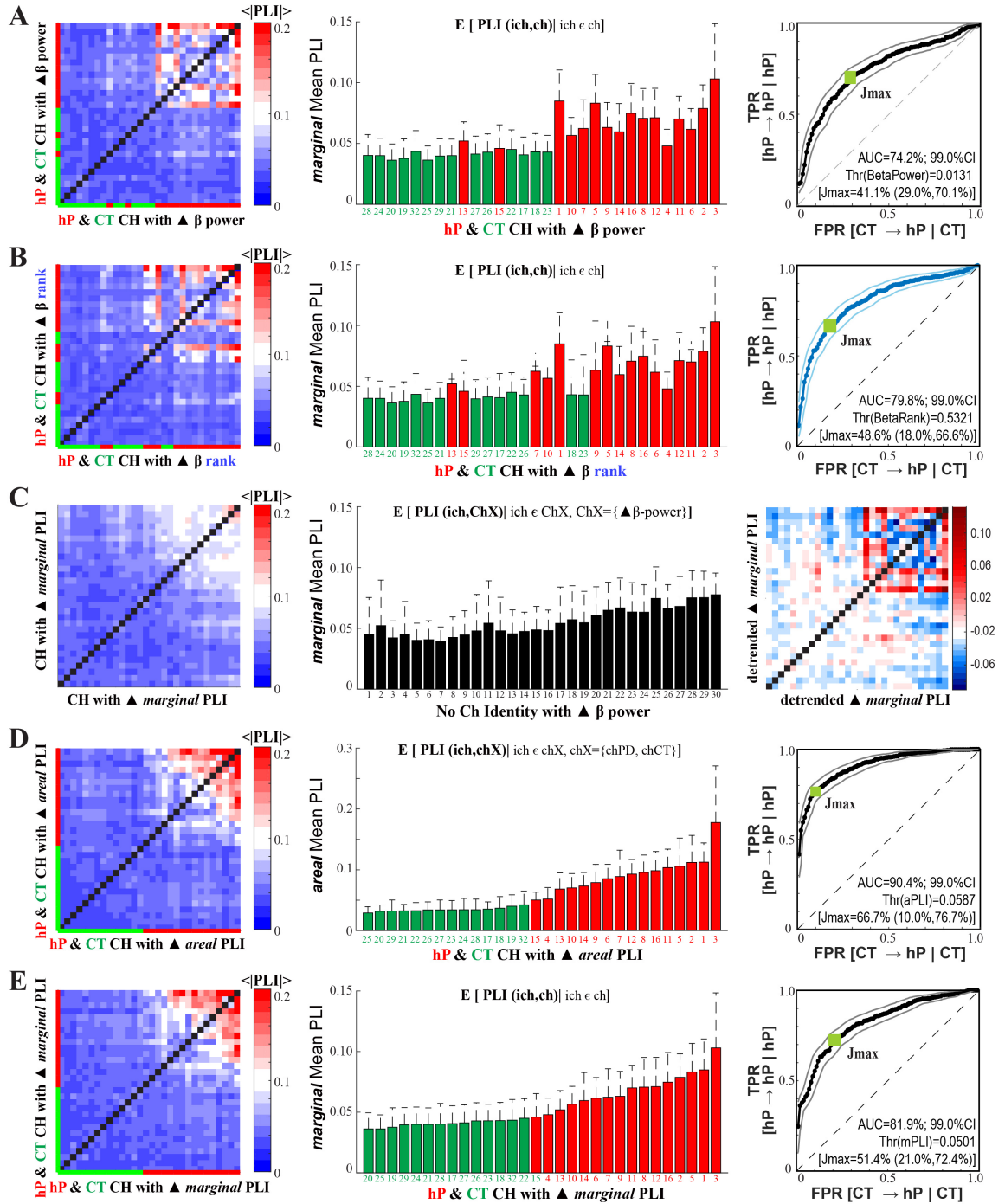


Figure 4.7: Increased phase synchrony over time within the lesion motor cortex. Data was extracted from all recording of Rat D. The left column showed the PLI values of all pairs of sites with ordering based on increasing values of beta power, beta rank, marginal mean PLI and areal mean PLI.

Figure 4.7 caption – continued The center column depicted the mean-PLI values with respect to the ordering in the left column. The right column (except for C) showed the ROC curves obtained by varying the threshold four metrics above. **A.** Power-Power-PLI: high beta power sites appeared in cluster and showed higher PLI values. **B.** Rank-Rank-PLI: ordering of PLI by beta rank also showed similar clustering of lesion sites with higher PLI values. **C.** During calculation of PLI, at each time point, the phase was reordered based on the instantaneous beta power, regardless of channel identity. This yielded a control distribution to exam the relation between beta power and beta PLI. **D.** Ordering based on areal PLI correctly separated all lesion and control sites and showed highest ROC performance. **E.** Ordering based on marginal PLI showed yielded similar results.

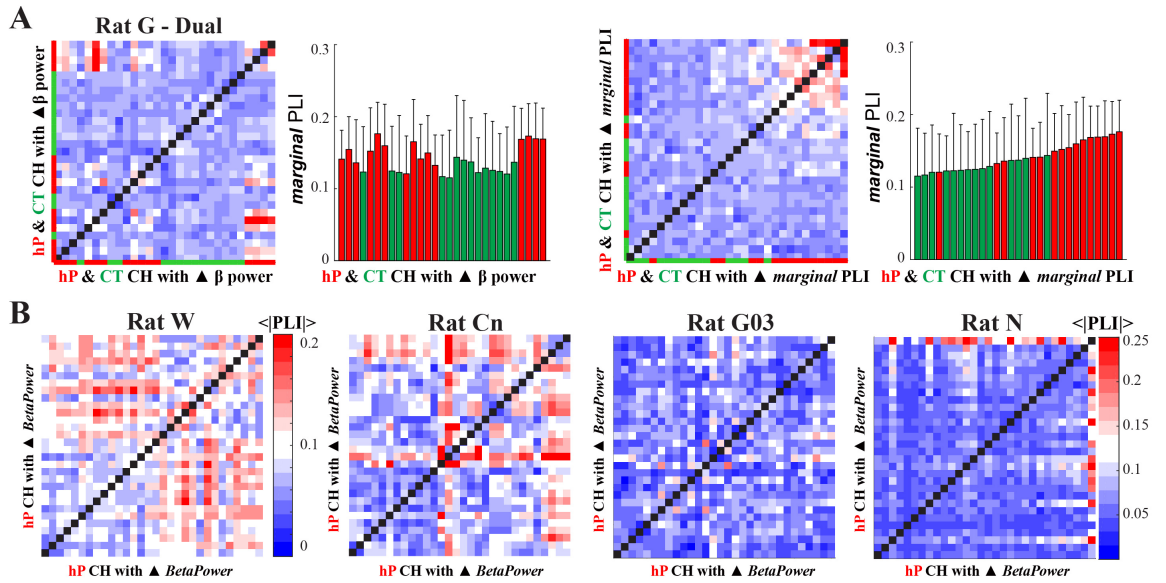


Figure 4.8: Phase-locking analyses in other animals. **A.** In another dual-array animal, the high beta powers did not correlate well with the phase synchrony (left), and the marginal PLI well segregated lesion and control sites. **B.** In single array animals, discrepancies between beta power and beta synchrony were also found.

In summary, the hP motor cortex showed elevated phase synchrony besides the exaggerated power in beta frequencies. The distributions of the phase synchrony were also not uniform spatially and the magnitude of synchrony was not solely explained by the increase in power. Collectively the beta phase-locking index better captured the presence of parkinsonism on the lesion motor cortex.

4.3 Conclusions

4.3.1 Summary of results

In summary, we characterized that excess beta power within the hemi-Parkinsonian motor cortex, the distributions of which bore inhomogeneity across multiple dimensions. First we found evidence that the broad band beta activities were formed collectively by narrow band activities with peak frequencies distributed in this band. We also found that beta power did not appear to be spatially uniform across motor cortex sites or stable over long period of time.

Furthermore, characterization of the properties of beta events had not been well established in literature and only very recently have several studies shown increased durations of beta events in the STN of human patients (Tinkhauser et al. 2017; Tinkhauser et al. 2017). Upon inspection of individual beta events in the motor cortex, we discovered that 1) beta events were longer on lesion sites, but the differences were small and the events typically did not last more than a few beta cycles; 2) although higher beta power events occurred more often on lesion sites, the control sites were also capable of producing large amplitude beta events. Overall, our results implicated a point-process perspective for the observed elevated beta power (Sherman et al. 2016), in other words, each beta event may be the basic unit of certain underlying neural mechanisms and the generation of which were affected by the parkinsonism.

Finally, we showed that a phase-lock index based algorithm showed higher predictive power in identifying lesion sites and hence the presence of parkinsonism than beta power. Specifically, beta synchrony was also not spatially uniformly and was more pronounced between sites with higher beta power. While the magnitude of PLI was not independent of the spectral power, i.e., sites showing high average beta power were more likely to have higher PLI values, this correlation alone was not able to explain the observed trends in PLI of lesion and control sites.

Overall, our results showed that sites in the lesion motor cortex may have varying levels of parkinsonism that fluctuate also over time. Besides the commonly used spectral power, phase synchrony at the beta band within the motor cortex may serve as a superior biomarker. The current study also had several limitations. Most notably, mechanistic explanations would need to be developed to link the observed inhomogeneity in spectral power to the elevated phase synchrony, especially how phase synchrony better captured the underlying parkinsonism. In addition, it has not been characterized how the inhomogeneities in beta oscillation change in relation to the behavioral state of the animal, and whether phase synchrony would be affected by pharmacological and neuromodulatory treatments of PD. However, we have also collected ample amount of motor cortical recording during the investigation of optogenetic modulations in the previous chapters; analysis of these data may provide basis to address these questions for future studies.

4.3.2 An outlook for optogenetic motor cortical stimulation with spatial and temporal specificity

Manipulating cortical oscillations with optogenetics has been demonstrated in rodent (Cardin et al. 2009; Sohal et al. 2009; Adesnik and Scanziani 2010) and in non-human primates (Lu et al. 2015). Yet it remained unclear how to achieve suppression of beta oscillation. One ongoing hypothesis is to enhance the gamma range activities, which are known to be prokinetic (Brown 2003). Admittedly, increase in gamma power may not lead to reduction of beta oscillations, as the circuit mechanisms objecting the capability of cortex to simultaneously support these two oscillations had not been demonstrated. On the other hand, increase in gamma activities were associated with the reduction of beta power after L-DOPA administration (Cassim et al. 2002; Silberstein et al. 2005; Avila et al. 2010; Dupre et al. 2016). In addition, cortical oscillations shift from low beta band during resting to high beta/gamma band during mobility in h-P rats (Avila et al. 2010) which might indicate the difficulty in switching from akinetic beta state to the prokinetic gamma state.

Furthermore, in Chapter 3, motor cortical optogenetic stimulations were delivered at high frequency (100 Hz) and led to symptomatic reduction, corroborating the potential importance of gamma range activities. Despite the success, the optical stimuli were not delivered in a spatially specific manner. I have also discussed the importance of further developing all-optical approaches to allow simultaneous recording and stimulation of the motor cortex. In corroboration, the observed variations in beta-band power and phase synchrony across the motor cortex confirmed the necessity for developing spatially-and-temporally selective motor cortical stimulation methods. It might offer a route to potential therapeutic benefits via differential neuromodulation at different motor cortical sites, allowing more specific control of motor cortical activity, and thereby motor symptoms, while reducing interference of stimulation on eloquent functional motor activity, hence reducing side effects.

4.4 Detailed Methods for Ch04

4.4.1 Subjects

The animals used in this portion of the study were 9 male Long-Evans rats (Charles River Laboratories, Inc., Wilmington, MA; 250300 g at the start of the study), housed in the animal care facility at Brown University. The rats were kept on a 12-h light/dark cycle and housed individually with free access to food and water. All procedures were performed according to the National Institutes of Health guidelines and approved by the Brown University Institutional Animal Care and Use Committee (Protocol#: 1402000046, 1703000263).

4.4.2 Data analysis

(a) Recording and signal processing

Electrophysiological recording from the MEA was performed during animals free behaviors in an open field. A hybrid electrical and optical commutator (HRJ-OE, Doric Lenses, Canada) was used to release the torsion in the cabling. Raw signals were sampled at 20 kHz and recorded with the Open Ephys system (Headstage: RHD2132 32-inputs amplifier with 3-axis accelerometer, C3324, Intan; Open Ephys acquisition board) or the Plexon system (32-channel VLSI head stage, 20 $\times$ gain, model HST/32V-G20; 32-channel amplifiers, 0.7 Hz-8 kHz, 50 $\times$ gain, model PBX3/32wb-G50).

Some of the recordings were obtained together with signals from BG nuclei in two out of the 32 channels; these channels were excluded from analysis in this chapter, leaving 30 channels of raw data available. LFPs were bandpass filtered between 1 and 300 Hz (forward-backward 3rd order Butterworth filter) and downsampled to 2 kHz. MUAs were bandpass filter between 300 Hz and 9 kHz (forward-backward 6th order Butterworth filter). Preprocessing procedures was performed to identify time segments containing artifacts related to breathing, chewing, scratching, and so on based on both MUA and LFP features;

these time segments were later excluded from processing.

(b) Spectral analysis

Spectral analysis was performed using customized functions based on the Chronux toolbox (<http://chronux.org/>; Mitra and Bokil, 2007). For full bandwidth TFRs, we used the multi-taper method with a sliding time window of 0.2 or 0.5 sec, stepped at 0.04 or 0.10 sec, a time half bandwidth product $TW = 1$ or 2 , and number of tapers $K = 1$ or 3 . Parameters were chosen to obtain a reasonable degree of smoothness while maintaining enough band separation. The obtained TFRs were initially computed as power spectral density and were then normalized across all frequencies (ignoring power within 55-65 Hz for line frequencies) at each time point (normalized power, with the unit of $[Hz^{-1}]$). The beta band for each animal was determined for each animal as a 15 Hz wide band around the center frequencies in the average power spectral densities. Power of the beta band was computed from the normalized TFRs using linear interpolation in frequency and was further represented as an average power within the beta band to eliminate biases in later analysis due to variations in bandwidth.

Beta state likelihood (BSL) was computed by firstly assigning each channel at each time a value of 1, if its power was greater than the lesion side average, a value of -1, if its power was smaller than the control side average, and otherwise a value of 0; then labels of each channel were averaged over time. BSL for each site varies from -1 to 1, with a lower value indicated a state more similarity to control sites and a higher value indicated more similarity to lesion sites. The channel-wise rank of the beta power at each time point of the TFRs was also extracted and normalized (1/30 for the lowest rank and 1 for the highest rank), which was referred to as the beta rank.

(c) Extraction of beta events

A Morlet-wavelet based TFR method was performed on z-scored LFPs (equivalent to the normalization in TFR) to extract the temporal evolution of beta activities, within the beta band identified in the multitaper TFRs, which was used in the extraction of beta events. Beta events were detected as local peaks (maxima) in the time resolved beta power with a fixed criterion on the minimum half-prominence duration of one beta cycle (~ 40 msec) and a varying threshold on the amplitude of the peaks. The amplitude threshold was varied according to the percentile of the average beta power in each channel. The duration (full-width half-prominence) and amplitude of each beta event was logged together with the average occurrence rate. For the ease of interpretation, durations were often displayed with reference to the length of a beta cycle (different for each animal).

We further computed the histograms of these parameters for each channel across all time in two flavors: normalized (empirical probability density function) or unnormalized (counts). The histograms of channels from lesion and control motor cortices were separately compiled. The agreement between the lesion and control histograms were quantified by Kolmogorov-Smirnov similarity tests and by the fraction of variance unexplained (FVU). The KS similarity test characterized the Euclidean distance between two empirical cumulative density functions and some of its limitations were discussed in (Filion 2015). The FVU measured the total error (sum of squared errors) normalized by the sum of squared values. A larger FVU indicated larger discrepancy.

(d) Cross-spectrogram

Given time domain signals $x(t)$ and $y(t)$ in a moving window, while the complex spectra ($S_{x(w)}$ and $S_{y(w)}$) are the Fourier-transformed autocorrelation functions ($r_{xx}(t)$, $r_{yy}(t)$), the Fourier transformed cross-correlation functions ($r_{xy}(t)$), the cross-spectra ($S_{xy}(\omega)$) can

also be obtained from TFR calculations. Cross-spectra obtained through the moving window form the complex cross-spectrogram ($S_{xy}(\omega)$). Likewise, a similar procedure can be performed for wavelet-based TFR methods. The instantaneous phase difference ($\theta_{xy}(t) = \phi_x(t) - \phi_y(t)$) between the signals were directly obtained from the imaginary component of the complex cross-spectrogram ($\Im\{S_{xy}\}$).

(e) Phase-locking index

Phase-locking index, or phase-lag index, is a measure of the consistency in phase relations between these two signals. It was calculated:

$$\begin{aligned} PLI_{xy}(\beta) &\triangleq \|E_t[\sin(\theta_{xy}(t|\beta))]\| \\ &\simeq \|E_t[\text{sgn}(\Im\{S_{xy}(\beta|t)\})]\| \end{aligned} \tag{4.1}$$

where $E_t[\cdot]$ denotes the expectation over time and $S_{xy}(\beta|t)$ refers to the beta frequency components of the cross-spectrograms computed with a moving time window. The values of PLI vary from 0 (no phase locking, leading and lagging was equally likely over time) to 1 (perfect phase locking, one signal is always leading or lagging another). PLI does not track the absolute phase differences and has the advantage of being insensitive to the effect of volume conduction (non-physiological perfect zero-phase lags, e.g. effects from common referencing). This kind of phase synchrony index and its variants have been used to study neural oscillatory synchronization of widely varying strength and spatial resolutions (Stam et al. 2007; Vinck et al. 2011).

PLIs were computed for each pair of channels, forming a 30×30 matrix. For a given channel, average of PLIs across the involving pairs can be obtained in two ways: the areal (average) PLI, which was averaged across its pairs with channels of the same identity (hP or CT), and the marginal (average) PLI, which was averaged across all of its involving pairs. The matrix of PLI pairs can thus be reorganized based on increasing values of average beta

power, average beta rank, areal PLI, or marginal PLI. A pseudo-permutation baseline was also computed: total recording time was subdivided into 60 sec segments and a PLI matrix was computed for each segment, which was reorganized based on the average power power; these PLI matrices were averaged element-wise across segment to obtain a final matrix that ignored the exact channel identity. This baseline matrix would be identical to the beta-power organized PLI matrix, if the ordering of beta power in channels remained exactly the same during all segments. A more permuted baseline situation, where permutation takes place within calculation of PLIs, would have much less practical implications and was hence excluded from analysis.

(f) Classifications

Naive Bayesian classifications on the channel identity (hP versus CT) were performed using the average power, average rank, areal or marginal PLI of beta activities as features (for the aforementioned 60 sec long segments), respectively.

A linear/quadratic discriminant analysis-based approach was also employed for classification at finer temporal scale (~ 1 sec). The frequency spectrum was divided into five frequency bands of equal bandwidth (5 - 20, 25-40, 45-60, 65-80, and 84-99 Hz) and the temporal mean and standard deviation of power were computed for each frequency band, forming a 10-element feature vector for each time window (non-overlapping). Afterwards, we performed dimension reduction based on principal component analysis (PCA), and the reduced feature vectors across channels and over time (each channel at each time was treated as independent) were subjected to supervised linear/quadratic discriminant analyses (10-fold cross-validation). Hyperparameters were varied to trade-off false detection with false rejection.

For both methods, the scores were obtained at various threshold levels (of each parameter or hyperparameter) and were compiled receiver operating characteristic curve

(ROC). As a performance metric, we used the area under the ROC curve (AUC) and reported the parameter that gives rise to the highest Youden's J statistics (sensitivity - false positive rate).

(g) Statistics

All comparisons were first subject to one-way ANOVA test. Then the means of the groups were compared with Welch's t -test (unpaired, unequal variance). Nested ANOVA tests were used to separate effects of multiple groups of variations (hP/CT, individual channels, and file segments). The comparisons of beta ranks were further verified with Mann-Whitney rank sum tests. Confidence intervals for average power spectral density and mean beta event statistics were computed with permutation tests by bootstrap (10,000 estimates) (May et al. 2014; Wagner et al. 2015).

Chapter 5

Discussions and Conclusions

5.1 Summary

In this thesis, I have introduced methodological approaches to achieve robust optogenetic modulations in three different nuclei, with drastically different requirements and restrictions, in the cortico-basal-ganglia circuitry of non-transgenic rats. Subsequently, I have investigated the rescue of the symptomatic rotational behaviors in the 6-OHDA-induced hemi-Parkinsonian rat model by modulation paradigms that were based on three hypotheses: inhibition of STN, excitation of GPe, and excitation of the motor cortex. Here I will summarize our findings by the modalities of hypotheses.

First and foremost, we successfully recreated the unilateral 6-OHDA hemi-Parkinsonian (h-P) rat model achieving near completely $> 90\%$ dopaminergic depletion in the SNc and in the striatum. h-P animals showed clear signs of akinesia, forelimb-use asymmetry and stereotypical rotational behaviors (spontaneous rotations, amphetamine-induced rotations - Amph-IRs, and apomorphine-induced rotations - Apom-IRs). I have also developed a custom device (magnorotameter) for high temporal resolution, continuous recording of the fast rotational behavior during simultaneous stimulation and electrophysiology in a confined space.

Through a series of electrolytic lesion studies, I was able to fine tune the stereotaxic targeting methods for the small STN nucleus with reference to the characteristic neural activities along the STN track. We mastered the art of identifying STN by listening to the audiotaped extracellular recording. Subsequently, we confirmed functional expression of inhibitory opsin SwiChRCA in the principal neurons of STN. Building upon this result, we showed that inhibition of principal neurons of STN led to modest (compared with electrical stimulation of STN) yet significant reduction in the rotational bias (Hypothesis #1).

We asked whether we could use existing anatomical (inhibitory) connections from GPe to STN to achieve the equivalent STN-inhibition through GPe-excitation (Hypothesis #2). Indeed, we found drastic improvement in the rotational bias when GPe was stimulated,

which was comparable to that of STN-eStim (equivalent of DBS, used as a gold standard). In addition, when the hemi-Parkinsonian state was already compensated by apomorphine, mimicking the “ON” state of patients with PD, both GPe-oStim and STN-eStim were able to produced additional anti-parkinsonian effects. On the contrary to the optogenetic modulation, while GPe-eStim also ameliorated rotational bias, it was not able to produce extra therapeutic effects when the BG was primed with dopaminergic inputs, hence demonstrating the power of specificity in stimulation by optogenetics. Besides the main findings, my results also confirmed the modulation of unit activities and LFP activities within the GPe, in the STN (inhibited), and in the motor cortex (complex responses across the MEA). The simultaneous MEA recording during rotational behavior and effective optical modulation also set the ground for future projects and analysis to investigate for causal relationship between motor cortical features of pathophysiology and improvement of behaviors.

Furthermore, motor cortex stimulation (Hypothesis #3) was also found to be therapeutic in the rescue of rotational behavior. However, this modulation effect cannot be achieved with the traditional diffusion-based virus injection methods. In response, we adopted the principles of the convection-enhanced delivery (CED) method and achieved widespread function opsin across the motor cortex. Of particular significance was that the high extent of robust opsin expression allowed for the first time reliable elicitation of motor movement and mapping of the motor cortex with optogenetics in non-transgenic rats. This breakthrough had further widened the application of motor cortical optogenetics in the study and manipulation of motor behaviors.

Additionally, we contrasted therapeutic effects from single-site intracortical stimulation (ssMC-oStim), to a method that engaged much larger areas of the motor cortex (seMCoS), and the stimulation of the hyperdirect pathway (HD-oStim, a highly regarded potential mechanism of action by DBS). A greatest correctional effect on rotational bias was achieved by seMCoS and was second by HD-oStim, which posits motor cortex as a

favorable potential therapeutic target. It remained unclear, however, why MC-oStim counteracted the effect of Apom-IR and why HD-oStim did not show consistent effect; perhaps their proper explanations are beyond the rate model.

Finally, I discussed preliminary findings on the characterization of motor cortical beta band oscillations in hP rats. We showed that the elevated beta band oscillations contained variations in spatial distribution, fluctuations in temporal dynamics, and instability in the center frequencies. Particularly, individual beta events lasted only a few cycles and was produced more frequently, but not larger in amplitude on the lesion hemisphere. We also found elevated phase synchrony in the beta frequencies between lesion motor cortical sites, which had more predictive power to the underlying parkinsonism than beta power. Our results implicated 1) the variability of cortical parkinsonism, 2) the potential of motor cortex oscillations as biomarkers for PD, and 3) the need for spatially and temporally resolved treatment options to address cortical pathophysiology.

5.2 What We Learned about the Basal Ganglia, the Parkinsonism, and the Deep Brain Stimulation

First of all, the consistent therapeutic effects by STN-oInhibit and GPe-oStim confirmed the rate model perspective of an increased tonic inhibition on motor output through the indirect pathway in PD. Secondly the discrepancy of outcome by GPe-oStim and GPe-eStim was of particular interest. Through the widespread projection targets found in histology and electrophysiological modulation effects, we confirmed the anatomical complexity of the GPe, a structure that essentially connected with the entire basal ganglia. Predictions the rate model may be complemented by the addition of these local networks and projections, and any interpretation of symptomatic cause and therapeutic mechanism with monotonic modulatory effect shall be avoided. We also identified opportunities to use optogenetics in GPe to causally interrogate neural correlates of behavior symptoms in other basal ganglia

nucleus.

In terms of the motor cortex and the hyperdirect pathway, firstly our motor cortical CED virus transduction approach fitted well with the divergent cortical inputs of the hyperdirect pathway and enabled for the first time reliable opsin expression of the hyperdirect pathway in rats; furthermore the therapeutic effects of HD-oStim and MC-oStim corroborated the predicted hypoactivity in the motor cortex; the efficacy of seMCoS further indicated the potential functional significance of layer-specific distribution of corticostriatal projections. Yet, it was perplexing how MC-oStim lead to the reduction of Apom-IR. Would it be attributable to the differential sensitivity of D1- and D2-MSNs to cortical inputs at the presence or absence of DA-agonist?

Last but not least, the overall lack of improvement in symptoms like akinesia and limb-use asymmetry suggested multiple parallel mechanisms of symptomatic generation. In terms of DBS, as discussed in the end of Chapter 3, our results confirmed the multitude of potential mechanisms through which DBS could exert its efficacy, including inhibition of STN, activation of GPe-STN GABAergic projections (or through antidromic activation of GPe rather than activation of these projections), as well as the antidromic activation of the motor cortex through the hyperdirect pathway. On the other hand, our results perhaps introduced further questions. For example, given the complexity of the GPe anatomy, was it just serendipity that we achieved therapeutic effects by GPe-eStim? Perhaps more systematic interrogation in the frequency-dependence of the stimulation effects shall shed light on these mysteries.

5.3 Remarks on Optogenetic Modulations for Potential Symptomatic Management

Our results demonstrated the power of using optogenetic modulations of multiple locations of the cortico-basal-ganglia circuitry to alleviate parkinsonian motor symptoms. Therefore,

theoretically these paradigms shall have potentials in translation into human therapy. In this regard, I would particularly advocate for the motor cortical stimulation due to its accessibility and availability to non-invasive neuromodulation techniques. This is further motivated by the rich variety of cortical activity states (Ch04) in the motor cortex and the fact that these states can be manipulated by optogenetics and other neuromodulation methods.

The requirement of genetic modifications (especially in the brain) is first and foremost a significant hurdle. However, there are in fact past and ongoing clinical trials involving gene therapy for PD in the following three categories (Blits and Petry 2016; Werner Poewe et al. 2017): 1) AAV-mediated expression of glia-derived neurotrophic factor (GDNF) for protection of nigral DA neurons (Bartus and Johnson 2017b, [a] 2017); 2) lentivirus or AAV-mediated expression of tyrosine-hydroxylase and cofactors in non-DA neurons in the striatum and allow them to produce dopamine (from tyrosine or from L-DOPA) (Mittermeyer et al. 2012; Palfi et al. 2014); 3) AAV-mediated expression of glutamic acid decarboxylase (GAD) in STN neurons, so that transduced STN neurons can produce GABA from glutamate (During et al. 2001; Carlsson et al. 2005; LeWitt et al. 2011). Therefore, this hurdle of genetic modifications may eventually be overcome, as long as superior therapeutic effects or mitigation of certain side-effects can be demonstrated for optogenetic modulation. A second hurdle is the requirement for the light delivery mechanism, especially for deep brain nuclei. There are always going to be difficult tradeoffs between invasiveness, spatial specificity, power density (to ensure opsin activation), and overall illumination volume. If gene therapy or stem cell therapy is permitted, besides the aforementioned approaches currently in clinical trials, many potential treatment options can be explored. Perhaps more so, by building upon the mechanisms discovered through therapeutic optogenetic modulations (Chu et al. 2017; Mastro et al. 2017).

The true advantage of optogenetic modulation over the other pharmacological (including chemogenetics) or gene therapy is evident if the temporal precision is required for

stimulation; for example the frequency dependence of stimulation outcome was reported for the hyperdirect pathway stimulation (Gradinaru et al. 2009a). For another application, an integration of optogenetics with stem cell (DA) therapy was demonstrated in PD rodents, so that DA production can be switched on or off on demand (Steinbeck et al. 2015). It is truly a regret that I was not able to more extensively test the frequency dependence of the modulation paradigms in the thesis. However, we also note that the possible parameter space (especially with the addition of stimulation bursts, as an intermediate level between pulses and trains in our study) is prohibitively large. This was further complicated by the limited duration of drug-induced rotations and the necessary wait before consecutive evaluations. Therefore only few frequencies were tested. Based on the limited data acquired for each stimulation paradigm (especially the stimulation/excitation modalities), we were not able to observe the frequency dependence reported in literature. A speculation was developed questioning the effective delineation of frequency (i.e. the pulse repetition rate or the inverse of which, the interpulse interval), the pulse duration (bounded by the interpulse interval), and the overall duty-cycle.

Overall, despite the unclear path of clinical translation for optogenetic modulation, it is clear that in the foreseeable future, optogenetic modulations will continue to shine due to its irreplaceable role in the dissection of Parkinsonism and in the exploration of symptomatic treatment.

5.4 An Outlook Towards the Closed-loop, Adaptive Neuro-modulation

The ultimate goal of my thesis was to develop a closed-loop system using cortical pathophysiology features to inform the on-demand delivery of effective symptomatic treatment. In the first part of this dissertation, I showed multiple optogenetic modulation paradigms targeting the cortico-basal-ganglia circuitry that yielded therapeutic amelioration of motor

symptoms, eluding to the use of optogenetic modulations as the write-in approach for the closed-loop system in dream.

Then we asked which neural biomarker can be used for "read-out" that will need to capture the ongoing severity of the disease, i.e. the necessity of stimulation. First attempts of closed-loop systems often set particular thresholds on the power, and recently the duration (Tinkhauser et al. 2017), of beta activity in the STN. However, the determination of this threshold has mostly been arbitrary (Little et al. 2013; Rosa et al. 2015; Piña-Fuentes et al. 2017; Tinkhauser et al. 2017). Based on the results from Chapter 4, I believe the motor cortical beta oscillations may serve as a better biomarker. First, we demonstrated the variability of the underlying parkinsonism in the motor cortex through the inhomogeneity in beta oscillations across multiple dimensions. However, beta power in the cortex alone may suffer from low separability at times due to the strong overlap of its distribution on the lesion and control areas, which could lead to missed stimulation or overstimulation. We subsequently found that phase synchrony of the beta activities within the motor cortex was a more conserved measure, yielding more consistent classification of control versus lesion cortical sites.

Therefore, combining power and coherence measures of beta activities obtained from multiple cortical sites could potential enhance the tracking of on-going parkinsonism. In addition, based on the observed spatial inhomogeneity in the beta activity, beyond the timing of stimulation, a multi-site cortical stimulation paradigm may also bring about increased stimulation specificity and reduced interference on the eloquent neural activities. Overall, our results provided new considerations for the design of an adaptive neuromodulation paradigm.

Appendix A

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