THE NEUROPROTECTIVE POTENTIAL OF STN DBS AND THE ROLE OF BDNF

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Neuroscience – Doctor of Philosophy

2015

ABSTRACT

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Parkinson's disease (PD) is a chronic, neurodegenerative disease that affects one percent of the population over the age of sixty. To our knowledge, there is no therapy that can slow the progression of the disease. Deep brain stimulation (DBS) of either the subthalamic nucleus (STN) or the globus pallidus interna (GPi) is well established to provide significant therapeutic efficacy in alleviating the motor symptoms of PD, yet our understanding of effects on disease progression remains limited. Previous studies have shown that long-term, high-frequency stimulation of the STN halts degeneration of the substantia nigra induced by intrastriatal 6-hydroxydopamine (6-OHDA) injections and significantly increases brain-derived neurotrophic factor (BDNF) in the nigrostriatal system, primary motor cortex and entopeduncular nucleus (EP). These results suggest that STN DBS can induce plasticity within basal ganglia circuitry and has the potential to provide neuroprotection in PD.

In order to examine the role of BDNF in the neuroprotective effects of STN DBS, I examined in our 6-OHDA rat model if blockade of the trophic receptor for BDNF, tropomyosin-related kinase type 2 (trkB), would alter the afforded protection. I also examined the effect of trkB antagonism on the recovery of motor function provided by STN DBS for unilaterally lesioned rats. Beyond elucidation of the mechanism for STN DBS-mediated neuroprotection, I evaluated if STN DBS would similarly facilitate nigral neuroprotection against α-synuclein overexpression-mediated toxicity. Lastly, I evaluated if DBS of the EP, the homologous structure of the GPi in the rat, would result

in similar results as STN stimulation, namely alleviation of a unilateral motor deficit, nigral neuroprotection and increased BDNF.

The data demonstrate that BDNF plays a critical role in the neuroprotective effects of STN DBS and in the alleviation of a unilateral motor deficit. The data also show that STN DBS is unable to provide neuroprotection against α-synuclein overexpression-mediated insult, but I call into question the usefulness of this model for the question of if STN DBS is disease modifying. I also present data that support abandoning EP DBS in the rat as an appropriate model of GPi DBS for PD. I conclude with remarks on the implications of this work for the clinic, including the use of a common variant in the gene for BDNF as a biomarker that may allow the development of 'precision medicine' approaches for the refinement of current medical practice guidelines for PD.

To my mother, who started it,
To my father, who showed me how,
To my partner, who helped me finish and
To my God, who blessed me so.

ACKNOWLEDGMENTS

It is my distinct honor and pleasure to acknowledge the generous contributions to this dissertation and to my training from the following individuals. First and foremost, I am most grateful for my advisor, Caryl Sortwell, who taught me that collectively we are smarter, better and more efficient scientists. Her pedagogic efforts were no less than Herculean, and her abilities as a mentor rival those of gray-eyed Athena. I also extend my thanks to the past and present members of our laboratory: Sara Gombash Lampe, Anne Spieles-Engemann, Christopher Kemp, Susan Wohlgenant, Nicole Polinski and Megan Duffy. Much of what I have learned has come from working with this fine group of people. A special thanks to my guidance committee members, Timothy Collier, Marc Breedlove, John Goudreau, Jack Lipton and Fredric Manfredsson, for their considerable service to my training. I must also acknowledge all past and present members of the Department of Translational Science & Molecular Medicine for making my time in graduate school most enjoyable and fulfilling and for helping me with my science; in this latter regard, Kathy Steece-Collier, Katrina Paumier, Allyson Cole-Strauss, Brian Daley and Nathan Kuhn deserve special mention. I am also fortunate to have had administrative support from Alisha Bergman, Michelle Gartland and Thuy Tran in our department, from Margo Smith and Cindy Arvidson in the MD/PhD Program and from James Galligan and Jim Stockmeyer in the Neuroscience Program. I would like to acknowledge Christine Shafer and Elahe Crockett for their telephone calls. Lastly, I wish to acknowledge my family and friends, especially my mother, Becki Telford, my

father, William Telford, my brother, Spencer Telford and my partner, Luke Ansorge, for their integral roles in carrying me to this point.

PREFACE

At the time of the writing of this dissertation, two of the following chapters are in the process of publication. Chapter 2 has been submitted and accepted for publication for inclusion in the text *Gene Therapy for Neurological Disorders*, edited by Dr. Fredric P. Manfredsson, as part of the Springer 'Methods and Protocols' series. Chapter 6 has been submitted to PLOS ONE and is under revisions. Chapters 3, 4 and 5 are in preparation for submission.

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KEY TO ABBREVIATIONS

6-OHDA	6-hydroxydopamine
α-syn	alpha-synuclein
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AADC	aromatic-L-amino acid decarboxylase
AAV	adeno-associated virus
ANA-12	*N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide
ANOVA	analysis of variance
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
CβA/CMV	chicken beta actin/cytomegalovirus
COMT	catechol-O-methyltransferase
CT	computed tomography
DA	dopamine
DAB	3,3’-diaminobenzidine
DAT	dopamine transporter
DBS	deep brain stimulation
DDC	3,4-dihydroxyphenylalanine decarboxylase
DMSO	dimethyl sulfoxide
DOPA	3,4-dihydroxyphenylalanine
DREADDs	Designer receptors exclusively activated by designer drugs

dtSNpc	dorsal tier of substantia nigra pars compacta
ELISA	enzyme-linked immunosorbent assay
Enk	enkephalin
EP	entopeduncular nucleus
FDA	Food and Drug Administration
GABA	γ-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GP	globus pallidus
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
HVA	homovanilic acid
IHC	immunohistochemistry
i.p.	intraperitoneal
ir	immunoreactive
JNK	c-Jun N-terminal kinase
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-3,4-dihydroxyphenylalanine-induced dyskinesias
LRRK-2	leucine-rich repeat kinase 2
LTD	long-term depression
LTP	long-term potentiation
LV	lentivirus
M1	primary motor

MAO	monoamine oxidase
MFB	medial forebrain bundle
MPP+	N-methyl-4-phenylpyridnium
MPTP	1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron
NGF	nerve growth factor
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl-D-aspartate
NR2B	N-methyl-D-aspartate receptor 2B subunit
NT-3	neurotrophin-3
NT-4	neurotrophin-4
ODT	optimized drug therapy
PD	Parkinson’s disease
PEG	polyethylene glycol
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PINK1	phosphatase and tensin homolog-induced putative kinase 1
PLC-γ	phospholipase C gamma
PPN	pedunculopontine (tegmental) nucleus
PTEN	phosphatase and tensin homolog
REM	rapid eye movement

RM-ANOVA	repeated measures analysis of variance
ROS	reactive oxygen species
SEM	standard error of the mean
shRNA	small hairpin ribonucleic acid
SN	substantia nigra
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNP	single nucleotide polymorphism
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SP	substance P
STN	subthalamic nucleus
TBS	tris-buffered saline
TH	tyrosine hydroxylase
tPA	tissue plasminogen activator
TrkB	tropomyosin-related kinase type 2
UPDRS	Unified Parkinson’s disease rating scale
VIM	ventral intermediate nucleus
VMAT-2	vesicular monoamine transporter 2
VTA	ventral tegmental area
vtSNpc	ventral tier of substantia nigra pars compacta
WT	wildtype

Chapter 1: Introduction

History of Parkinson's Disease

The Initial Descriptions of Parkinson's Disease

Parkinson's disease (PD) owes its initial description to the English surgeon and geologist James Parkinson and its name to the famed and influential French neurologist Jean-Martin Charcot who credited him. Parkinson's description of a single disease in "An Essay on the Shaking Palsy", published in 1817, linked together the symptoms of resting tremor, loss of movement and stooped posture into a triad within the context of relative sparing of cognitive and executive functions. Whereas previous descriptions of the hallmark tremor had existed for centuries through the works of Galen, Sylvius, Juncker and Cullen, Parkinson's monograph was the first to posit a single disease entity, which he called '*paralysis agitans*', for a triad of symptoms that is still present today as the core of the clinical depiction [1]. In addition, Parkinson also first characterized some of the non-motor features of PD, including sleep disturbances and constipation, and described the progressive course of PD from the mildest tremor to the most violet shakes over many years, a fact of paramount importance in our understanding and treatment of PD today.

James Parkinson's monograph, though foundational, was inaccurate in naming the disease '*paralysis agitans*', according to Jean-Martin Charcot at la Salpêtrière. Charcot maintained that tremor was a common association but not a requirement for the diagnosis; as such, he advocated that the name be changed to Parkinson's disease [2]. In addition, Charcot was struck by the slowness of movement, as separate from the

absence of movement, observed in PD patients, so it was his work that added bradykinesia as a defining symptom of PD, shifting the hallmark symptoms from a triad to a tetrad, despite the common practice of movement disorders neurologists today to use bradykinesia and akinesia interchangeably [3]. Beyond Charcot's christening of Parkinson's disease, he promulgated it as well, leading to a host of his students at la Salpêtrière to further our understanding of PD.

Connecting the Substantia Nigra to Parkinson's Disease

The substantia nigra (SN), or 'black substance', was first described in 1786 by Felix Vicq d'Azyr, using the name *locus niger crurum cerebri*, or the 'black place of the legs of the cerebrum', for its pigmented appearance on gross examination and its relative location to the large, easily identified, white matter tracts of the *crus cerebri* [2]. Despite his original contribution, his work was misappropriated by Jules-Bernard Luys in his original depictions of SN neurons to Samuel Thomas von Sœmmerring, resulting in what Martin Parent and André Parent eloquently refer to as, "an imbroglio regarding the paternity of a crucial finding" [2]. Despite this misattribution, Luys' work informed that of Giovanni Mingazzini, who described the stratified divisions within the SN, Domenico Mirto, who described SN neurons as projection neurons, and Torata Sano, who formally divided the SN into a pars compacta (SNpc) and a pars reticulata (SNpr) [2].

As knowledge of the substantia nigra grew, so did our knowledge of its relationship with Parkinson's disease, much in part from the influence of Jean-Martin Charcot as a mentor at la Salpêtrière. Gheorghe Marinescu, a neurologist, along with the pathologist

Paul Oscar Blocq first described the histology of a patient of Jean-Baptiste Charcot (the famed Charcot's son) with disseminated tuberculosis that resulted in a small, tuberculoma at the SN and parkinsonian tremor. The hypothesis of the SN as the critical site of pathology in PD was then put forth in a lecture by Edouard Brissaud. This pioneering work at la Salpêtrière culminated with Konstantin Nicolaevich Tretiakoff, who in the service of Pierre Marie and laboratory of Marinescu, compared brain tissue samples from patients of Constantin Alexander Economo Freiherr von San Serff ("Von Economo" of neuroscience fame). These patients had encephalitis lethargica and exhibited parkinsonian symptoms. As encephalitis lethargica had reached pandemic proportions in Europe, there was ample tissue. Tretiakoff correlated SN pathology seen in PD patients with that of post-encephalitic Parkinsonism, providing the needed demonstration of the connection between the SN and PD. (previous paragraph paraphrased from [2])

Connecting the Neurotransmitter Dopamine to Parkinson's Disease

After the connection between the substantia nigra and Parkinson's disease was made, it was only a matter of time before the neurotransmitter dopamine was implicated as well – about sixty years, in fact and as a side product of a different line of investigation. Following the discovery by Bernard Brodie that reserpine, a vesicular monoamine transporter antagonist, would deplete the brain of serotonin, Arvid Carlsson and colleagues initially sought to investigate the role of the catecholamine neurotransmitter norepinephrine due to its similarities in chemical structure to serotonin [4]. In an effort to reverse the physiological effects of reserpine in their model, they administered 3,4-

dihydroxyphenylalanine (DOPA), a precursor to norepinephrine, as catecholamines cannot cross the blood brain barrier. Much to their surprise, norepinephrine levels were not restored in the brain in conjunction with the relief of reserpine-induced physiological effects; hence, they looked to an earlier compound in the synthesis of norepinephrine, namely dopamine. At that time, dopamine could not be measured in the brain, so Carlsson and colleagues developed a method to do so, and showed that reserpine diminished dopamine levels and that DOPA replaced them. Additional work showed high levels of dopamine (DA) in the basal ganglia and a resultant parkinsonian state following reserpine administration, thereby laying the groundwork for dopamine's central involvement in PD. L-DOPA as a potential treatment for PD was first explored through studies employing intravenous administration and followed by an oral dosing regimen that widened L-DOPA use for PD [5] to its current use as standard of care for all PD patients. (previous paragraph paraphrased from [4])

The Initial Presentation and Diagnosis of Parkinson's Disease

Patients most often present to their primary care physician and then through a referral to a neurologist with the chief concern of tremor at rest, which may often be accompanied by rigidity, akinesia (and bradykinesia often combined with this) and postural instability; these four cardinal motor symptoms are often recalled by students through the acronym *TRAP* [6]. Motor symptoms are most often unilateral or at least asymmetric in onset, only expanding bilaterally later in the course of the disease; as such, asymmetry is included as a diagnostic criterion [7, 8].

Resting tremor is often described as a ‘pill-rolling’ movement [6, 9] between the thumb and index finger, though it may be present in any of the limbs or in the jaw or lips; however, tremor of the neck or of the voice is rare in PD and is far more consistent with a diagnosis of essential tremor [6]. Rigidity is described by a resistance to passive motion when applied to a muscle group, and in the diagnosis of PD, this is often present in the shoulder and was misdiagnosed as musculoskeletal in origin (e.g., arthritis, rotator cuff injury) [6]. Akinesia and bradykinesia, loss or slowness of movement, respectively, are two separate but related symptoms of PD. Onset of these symptoms is often subtle; some patients may have loss of fine motor skills (e.g., difficulty with buttoning a shirt) or drooling from decreased swallowing, whereas others may have a smaller arm swing when walking [6]. Bradykinesia results in poor reaction times in most cases; however, some environmental cues are capable of ‘restoring’ a patient’s response time to normal. For example, patients may be able to sprint out of a building if someone yelled, “fire!” This phenomenon is called ‘kinesia paradoxica.’ Postural instability is grouped with the cardinal features of PD, but it is not present at the time of diagnosis nor is it prominent over the first few years since motor symptom onset. Impaired postural reflexes along with orthostatic hypotension and a resistance to standard-of-care medications leads to an increased frequency in falls.

The definitive diagnosis of PD is made through the combination of the clinical and histopathological examinations made by the neurologist and pathologist, respectively. Since a definitive diagnosis is less useful to the patient post mortem, a working diagnosis from clinical data alone is used through the inclusion of hallmark symptoms of

PD and the exclusion of other probable causes. Presence of resting tremor, bradykinesia, rigidity and asymmetric onset, or any three of these in combination, suggests a probable diagnosis of PD in the absence of features suggesting an alternative diagnosis (e.g., dementia preceding motor symptoms or hallucinations unrelated to medications; see Table 1, Group B features for a complete list) [10].

Even though the motor symptoms provide the impetus for the clinical encounter and dominate the diagnostic criteria for PD, a careful history often reveals a much longer narrative that features non-motor symptoms as well. There are three cardinal symptoms associated with prodromal PD, viz. REM sleep behavior disorder, anosmia (loss of smell) and constipation [11]. It is estimated that prodromal PD may start as early as fifteen to twenty years before diagnosis [11]. An additional non-motor feature mentioned above, orthostatic hypotension, should be included as well [11].

The Pathophysiological and Pathological Hallmarks of Parkinson's Disease

Overview of Normal Neuroanatomy and Physiology

The caudate nucleus and the putamen (or collectively the striatum in rodents), the globus pallidus pars interna (GPi, or the entopeduncular nucleus, EP, in rodents) and pars externa (GPe, or simply globus pallidus, GP, in rodents), the subthalamic nucleus (STN) and the substantia nigra pars compacta (SNpc) and pars reticulata (SNpr) comprise the basal ganglia [12, 13]. Cortical, glutamatergic projections provide the primary input to the basal ganglia, and the GPi and SNpr are the two structures that provide the preponderance of the output from the basal ganglia. Information flow within

the basal ganglia has been classically described as two parallel, functionally antagonistic pathways, the direct pathway and the indirect pathway [14], from foundational work conducted by Mahlon DeLong, earning him the 2014 Lasker-Debakey Clinical Medical Research Award (along with Alim-Louis Benabid, whose work is discussed below) [15].

The Direct Pathway

The direct pathway derives its name from the fact that there is only one synapse between the first structure in basal ganglia processing, the putamen, and its primary output structures, the GPi and SNpr. Cortical input drives striatal neurons that express D1 dopamine (DA) receptors to release γ-aminobutyric acid (GABA) and substance P (SP) at the GPi and the SNpr [12]. Within this pathway, SP striatal neurons are typically at rest and the GPi and SNpr output neurons are tonically active [12, 14, 16, 17]. These output neurons transmit GABA to inhibit the thalamus and the pedunculopontine nucleus (PPN). Corticostriatal input suppresses these output neurons, thereby disinhibiting the thalamus and PPN and activating their target structures, including the cortex, to facilitate a motor program [12, 18]. SNpc neurons project to the striatum to release DA to activate SP striatal neurons through the D1 receptor [12, 18]; hence, the SNpc has a similar overall effect on basal ganglia output as corticostriatal input, viz. striatal DA facilitates movement.

The Indirect Pathway

The indirect pathway derives its name from the fact that there are three synapses—opposed to the one synapse of the direct pathway discussed above—between the putamen and the GPi and SNpr. Striatal neurons expressing D2 receptors project to the GPe to release GABA and enkephalin (Enk). The GPe in turn sends GABAergic input to the STN that releases glutamate to activate the GPi and SN (both the SNpc and SNpr) [12, 19]. Through nigrostriatal DA release, Enk striatal neurons are inhibited via the D2 receptor, thereby disinhibiting the GPe and consequently suppressing STN activity [18]. As the STN typically drives GPi and SNpr inhibitory output, suppression of the STN removes this effect and facilitates movement. Overall, striatal DA facilitates movement in the indirect pathway.

Pathophysiology of Parkinson's Disease

The defining pathophysiological characteristic of PD is the loss of dopamine (DA) in the caudate nucleus and the putamen. This loss of DA is a result of dysfunctional and subsequently degenerating nigrostriatal projections. Of importance, not all dopaminergic neurons of the midbrain are equally affected. SNpc neurons found in the ventral tier (vtSNpc), which primarily project to the putamen, are the most affected by PD and the first SN neurons to degenerate, resulting in at least 95% loss; SNpc neurons found in the dorsal tier (dtSNpc), which project to the caudate nucleus, are more resistant to degeneration in PD with usually an 80% loss observed [20-23]. In contrast, DA neurons of the nearby ventral tegmental area (VTA) that project to the nucleus accumbens, hypothalamus and cortex are relatively spared [21]. Furthermore, DA neurons of the periacqueductal grey are essentially unaffected by PD [21]. This

differential loss of dopaminergic neurons and their projections underlies the PD phenotype.

The PD motor phenotype is a result of DA loss within specific subregions of the basal ganglia. Loss of DA in the putamen is greatest within the dorsal and caudal portions, and loss of DA in the caudate is greatest within the dorsal and rostral portions [22, 24, 25]. Of note, while there is a net loss of DA in these regions, the remaining DA projections compensate by increasing DA turnover, evidenced by the increased levels of the DA metabolite homovanilic acid (HVA) measured in PD patients [22, 26]. In a similar vein, the postsynaptic system also compensates for loss of DA through increased expression of the D1 and D2 DA receptors [27, 28] and supersensitization of these same receptors, partly through reorganization of intracellular signaling pathways [29-31]. One should note, though that beyond the hallmark DA loss found in PD, other losses of neurons occur as well in the locus coeruleus, the raphe nucleus, the nucleus basalis of Meynert, the dorsal vagal nucleus and the sympathetic ganglia, resulting in alterations in adrenergic, serotonergic and cholinergic systems [21]. Non-DA neurotransmitter involvement in PD is exhaustively reviewed in [32].

Neuropathology of Parkinson's Disease

The Lewy Body – A Visual Hallmark

The neuropathological hallmark of PD is the Lewy body. The original histological description of the Lewy body was provided by Friedrich Heinrich Lewy in 1912 [33], and the name 'Lewy body' was given by Tretiakoff in his postmortem studies of PD patients

[34]. Gibb and Lees summarize Lewy's description in the following: "The Lewy body is a neuronal inclusion which is always present in areas of neuronal degeneration in Parkinson's disease. In its classical form in the substantia nigra it consists of a central core staining deeply with haematoxylin and eosin, surrounded by a body which stains less intensely, and then a peripheral halo which stains lightly or not at all. This appearance is relatively uncommon and more usually Lewy bodies have no core. Considerable variation in shape occurs, including elongated and serpiginous forms, and their appearance depends on some degree on their location within the nervous system. (The various forms were already described by Lewy in 1912)" ([35], quoted in [36]). Though classically haematoxylin and eosin histochemistry was used to reveal Lewy bodies, immunohistochemistry (IHC) for ubiquitin or α-synuclein is far more frequently employed today [33]. In fact, while immunohistochemical studies have revealed that Lewy bodies are primarily composed of these proteins, these studies have also shown that ubiquitin and α-synuclein are by no means a sufficient molecular description. Wakabayashi and colleagues [34] have compiled more than ninety molecules as constituents of Lewy bodies through IHC demonstrations, and they have organized these under thirteen groups; they also cite a proteomic study using laser capture microdissected tissue that included 300 proteins [37]. Using electron microscopy, Lewy bodies can be characterized into two types: a mixed granular-fibrillar type and a fibrillar type [33, 38]. The former, which is the more common type, has a primarily granular core with fibrils more dominant at the periphery and in a radial pattern, whereas the fibrillar type has curved fibrils in the shape of circles or ovals and is likely the homogeneous looking Lewy body "of uniform density with light microscopy" [33].

While Tretiakoff coined the term ‘Lewy body’ when examining the substantia nigra, Lewy originally observed the distribution of Lewy bodies throughout much of the neuraxis. As has been previously reviewed, Lewy bodies are found in “the olfactory bulb, hypothalamus, posterior pituitary, nucleus basalis of Meynert, substantia nigra, locus ceruleus, dorsal raphe nucleus, dorsal vagal nucleus, cerebellum and spinal cord [as well as] in the neurons of the amygdaloid nucleus and cerebral cortex, particularly in deep layers (V and VI) of the limbic system” [34]. Heiko Braak and colleagues have further characterized Lewy body location to six different stages of the disease process, starting with the olfactory bulb and the dorsal vagal nucleus, progressing “through the pontine tegmentum (stage 2), into the midbrain and neostriatum (stage 3), and then the basal procencephalon and mesocortex (stage 4) and finally through the neocortex (stages 5 and 6)” ([34], see also [39, 40]). The Braak staging has been highly influential in steering the PD research field to consider the hypothesis that pathologic α-synuclein spreads between cells [41], but its most important contribution is to provide a neuropathological explanation for the progressive nature of PD.

Slow, Relentless Progression – A Temporal Hallmark

A defining characteristic of PD is that it is progressive. Indeed, a slow onset of symptoms that develops over several years is one of the diagnostic criteria [10]. When patients are tracked using the Unified Parkinson’s Disease Rating Scale (UPDRS), they can expect an average two point change in the UPDRS total score every year starting at diagnosis [42, 43]. The premotor symptoms of PD occur many years prior to diagnosis,

and the motor symptoms develop many years after diagnosis [11], much of which correlates with Braak's staging hypothesis [40, 44]. PD, then, is a decades long process.

For putative neuroprotective therapies that focus on modifying the progression of the motor symptoms of PD, the key time courses are that of loss of striatal DA and SNpc DA neurons [45]. In a landmark study, Kordower and colleagues [46] conducted postmortem examinations on PD subjects at varying times after diagnosis where the subjects had deceased for reasons unrelated to their medical state. PD patients at diagnosis have lost 50-90% of their SNpc DA neurons and lose a marginal amount afterward, but they present at the clinic with modest losses of striatal DA that progresses to a near complete loss by four years post diagnosis [46]. This indicates that the window of opportunity for testing neurprotective therapies is within the first three to four years from diagnosis and that efforts should be made on pursuing therapies that protect nigrostriatal axons [45].

Etiology of Parkinson's Disease

Genetic Causes

With the advent and subsequent spread of sophisticated molecular/genetic laboratory techniques in the 1990s, the genetic basis for PD has developed considerably [47]. A common method for examining the genetic basis for a disease is through twin studies, i.e. the comparison of monozygotic twins versus dizygotic twins for a specified outcome, in this case, a diagnosis of PD. Although several twin studies for PD have resulted in

conflicting conclusions that are modest at best, the latest study suggests that PD is "moderately heritable" ([47], citing [48]). The gene that codes for α-synuclein, *SNCA*, was the first to be demonstrated as a single, definite genetic cause of PD when the A30P single nucleotide polymorphism is present [49]. Since then, other genetic mutations have been identified including duplications or triplications of *SNCA*, A53T and E46K single nucleotide mutations within *SNCA*, mutations in *LRRK2*, mutations in *PARK2*, which codes for parkin and mutations in *PINK1* [50-62]. The molecular biology of these mutations is further reviewed in Chapter 2. Other genetic mutations associated with PD that are not listed here are reviewed in [47]. In summary, the proportion of patients' diagnoses that can be fully or mostly explained by genetic causes is approximately ten percent [63].

Environmental Risk Factors

Whereas genetic factors are the primary cause in ten percent of cases [63], it is estimated that twenty percent of patients report a family history of PD, implying a role for environmental factors in its etiology [64]. Several toxicants have been linked to PD outbreaks, including manganese, lead, paraquat (a pesticide) and carbon disulfide; additionally, several occupations that include use and exposure of these toxicants have been linked to PD, including farmers, pesticide applicators, steel industry workers and welders [64]. Synthetic heroin users are also at risk in the event that its synthesis yielded the toxic impurity 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), the discovery of which by Dr. J. William Langston was a critical event in PD research [65]. Overall, while these exposures explain specific 'outbreaks' of parkinsonisms [64], they

cannot account for the remaining cases after genetic causes; hence, the field is still searching for risk factors for PD and a complete explanation for why many individuals develop PD.

Idiopathic Parkinson's Disease

According to William Haubrich, the term "idiopathic has come to be a word sometimes used to describe a condition of which one is uncertain or ignorant of the cause, yet to which one wishes to apply a high-sounding word intended to mask the fact. In this sense, 'idiopathic' (*idio-* + Greek *pathos*, 'disease') is equivalent to 'essential' or 'cryptogenic.' Originally, an idiopathic condition was thought to arise within the patient him[/her]self rather than occurring as a consequence of any recognized outside cause. Later, the sense shifted slightly to that of a condition peculiar to a given individual, in contrast to that being representative of a widely recognized disease" [66]. This description of the word 'idiopathic' is useful for the field of PD for two reasons: (a) in the former sense, it acknowledges that we cannot give a complete accounting for why most individuals develop PD and (b) in the latter sense, it underscores the tremendous heterogeneity of PD in that no two patients with PD have quite the same experience.

Aging – A Partial Accounting

Epidemiological studies have shown that of the many risk factors associated with development of PD, the greatest risk factor, from which no one is immune, is aging [67-70]. In an excellent review, Collier and colleagues argue that the processes of aging and the mechanisms behind development of PD are highly related at the cellular level

[70]. Specifically, they propose a 'stochastic acceleration hypothesis' for the development of PD. Multiple factors—genetic, environmental or otherwise—interact with aging to accelerate striatal dopamine loss to the degree that a threshold for development of PD is crossed during the individual's life [70].

Animal Models of Parkinson's Disease

There are three primary methods to generate animals models for PD: traditional transgenic approaches, neurotoxicant administration and the use of viral vectors. Genetic-based models and neurotoxicant-based models are covered below. Viral vector-based models of PD are covered in Chapter 2.

Genetic-based Models

Since a portion of PD cases result from a strong genetic basis (see above), these genes have been studied for what role they may play in the pathophysiology and if they may be used to model the disease (or components of it) in a model organism through transgenic approaches (viral vector approaches are discussed in Chapter 2). Several model organisms—*Drosophila melanogaster* [71], *Caenorhabditis elegans* [72], mice [73], rats [74]—have been employed to model *SNCA* mutations, but unlike the human condition, these classically-derived models do not lose DA neurons over a prolonged time course, do not form Lewy bodies and do not exhibit parkinsonian motor symptoms [75]. However, a recent study using a bacterial artificial chromosome (BAC)-transgenic rat expressing α-synuclein showed progressive nigral degeneration over time that reached significance at an aged time point [76]. *D. melanogaster* and *C. elegans* have

also been used to model *LRRK2* mutations, but as these organisms do not endogenously express α-synuclein, they are unable to recapitulate one of the key features of *LRRK2* mutations in patients, viz. α-synuclein aggregation [77, 78]. *LRRK2* mutations modeled in mice do not lead to the development of any SNpc degeneration, making this a poor model for testing neuroprotective therapies [79]. Using the *parkin*, *PINK1* or *DJ-1* mutations in *D. melanogaster* or mice offers little improvement – the mice do not even develop dysfunction of the nigrostriatal system, and the flies only develop DA neuron dysfunction and no other PD features [75]. However, recently developed *PINK1* and *DJ-1* models using these genes in rats have been able to generate a progressive, neurodegenerative process [80].

Neurotoxicant-based Models

The loss of SNpc DA neurons and development of a parkinsonian motor phenotype can be achieved through the administration of specific neurotoxicants, most commonly 6-hydroxydopamine (6-OHDA) in rats or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice or non-human primates (or unethically in humans, considering its origin detailed in [65]). In PD patients, STN glutamatergic activity is increased [81-85] leading to hyperactivity in the GPi and the SNpr and compensatory decreases in glutamate receptor [86-88]; 6-OHDA or MPTP administration results in similar results [82, 89-97]. Of importance, these models can be used to reliably generate motor impairments that model motor symptoms routinely observed in PD patients. Behavioral tests commonly employed in PD murine models are spontaneous or amphetamine-induced rotations and spontaneous forelimb use assessed in the cylinder task [98-101]. In addition, these

models can be used along with chronic administration of L-DOPA to generate the common side effect of L-DOPA-induced dyskinesias that are observed in PD patients after chronic L-DOPA use [102, 103]. The 6-OHDA rat model of PD is discussed more below.

Two other neurotoxicants that have been used to model PD are rotenone and paraquat. These pesticides (introduced above as environmental risk factors) are not routinely used due to a lower threshold for widespread toxicity in the animals and in non-specific toxicity to other, non-dopaminergic systems in the brain that are not relevant to idiopathic PD [104]. However, some groups are still advancing rotenone as a useful neurotoxicant for studying PD [105, 106].

Using 6-Hydroxydopamine to Model Parkinson's Disease in the Rat

Discovery

As an interesting parallel, the original use of 6-hydroxydopamine (6-OHDA) by Porter and colleagues [107] was in the research and depletion of norepinephrine in peripheral neurons, much as Carlsson first investigated norepinephrine in the brain. Ungerstedt then applied 6-OHDA to researching several monoaminergic areas of the brain including the caudate nucleus and SN, and he further noted that injections to the SN results in a loss of DA in the striatum, implying a process of anterograde degeneration [108]. Ungerstedt then joined by Arbuthnott demonstrated that animals unilaterally lesioned with 6-OHDA would rotate with the addition of amphetamine [109]. This unilateral model and amphetamine-induced rotations are still employed in PD research,

both for the power of an internal control of an intact side of the brain and because bilateral 6-OHDA lesions result in loss of eating and drinking and subsequent death [110, 111].

Mechanism of Toxicity

The primary mechanism of toxicity of 6-OHDA is induction of oxidative stress. The structure of 6-OHDA is similar enough to other catecholamines such that it is readily transported by the dopamine transporter (DAT) [112]. Accumulation of 6-OHDA in the cytosol creates a pool of potential oxidative species, either through spontaneous oxidation [111, 112] or through oxidation by monoamine oxidase (MAO). MAO processing of 6-OHDA generates hydrogen peroxide (H_2O_2), which is both a reactive oxygen species (ROS) itself and a precursor to other ROS [112]. There may be another role for 6-OHDA-mediated degeneration in the mitochondrion; specifically, 6-OHDA may enter this organelle and act as a complex I inhibitor, though this has only been demonstrated *in vitro* [112, 113].

A secondary mechanism of toxicity is through the activation of inflammatory processes. Activated microglia have been identified in association with 6-OHDA-lesioned nigrostriatal systems [114-123], although controlling for the role of the trauma induced by the injection/surgical process is not often done. The role of inflammation in the pathogenesis of PD has taken a leading role in the field within the last few years, bolstered by clinical data showing inflammation as a key mediator of the PD brain's

milieu [124-131], and its role in 6-OHDA and in other PD models (inflammation in viral vector models is reviewed in Chapter 2) is becoming more appreciated and studied.

Experimental Design Considerations

Like other catecholamines, 6-OHDA does not cross the blood-brain barrier, so it must be injected directly into the brain. Injections were first made into the SN directly or into the medial forebrain bundle (MFB), resulting in a rapid and nearly complete loss of SNpc DA neurons that is pathologically similar to late-stage PD [111, 132]. However, as the DAT is present in multiple locations on DA neurons and not exclusively on the soma, it is possible to induce degeneration via intrastriatal injections of 6-OHDA; this was first tried by Berger and colleagues in a demonstration of intrastriatal 6-OHDA being retrogradely transported to the SNpc and subsequent neuronal death [133]. Through modifying the injection paradigm, it is possible to generate DA terminal loss that precedes SNpc cell death [134], or to generate DA terminal loss without subsequent overt cell loss. Initiating the degenerative process with loss at the synapse and lengthening the timeline of degeneration (from days to weeks) are seen as superior features in using 6-OHDA to model the condition of the PD patient [134, 135]. Using 6-OHDA in this way is also advantageous for vetting experimental, neuroprotective therapies in that interventions can be initiated at a midpoint in the timeline of soma degeneration that is analogous to the level of degeneration present in PD patients who are first presenting to the clinic with symptoms (i.e., the earliest time at which a therapy can be employed) [20, 45, 46, 134, 135]. Another methodological consideration is the importance of a behavioral phenotype. Unilateral administration of 6-OHDA will trigger

a unilateral motor deficit on the contralateral side that is detectable by behavioral assays like the cylinder task when approximately 50% of SNpc neurons have degenerated [100, 134, 136]. Proprioceptive deficits can also be detected through a forelimb-placing task after vibrissae stimulation, and somatosensory deficits can be detected through ability and latency for removing adhesives placed on the rat's forepaws [99, 100]. Two major limitations of the 6-OHDA model are (a) degeneration of non-DA neurons observed in PD patients cannot be modeled [137, 138] and (b) Lewy body pathology cannot be produced [139]; ergo, the investigator may need to employ a different approach if either of these features is necessary for his or her question.

Current Dopamine-based Therapies for Parkinson's Disease Motor Symptoms

Pharmacotherapies

Essentially all medications used today to treat the motor symptoms of PD are designed to enhance dopamine (DA) signaling, referred to as the "Dopamine Axis" of PD therapy [140]. DA, a catecholamine, does not cross the blood-brain barrier, so it cannot be practically administered. There are several approaches to enhance DA signaling, the first of which is to increase its precursor, L-dihydroxyphenylalanine (L-DOPA, often referred to as levodopa), a compound that readily crosses the blood-brain barrier. Since L-DOPA is the product of the rate-limiting step in DA synthesis, i.e. the hydroxylation of tyrosine by tyrosine hydroxylase (TH), it is quickly converted to the end product. Oral administration of L-DOPA alone results in rapid conversion of the compound outside of nervous tissue by DOPA decarboxylase (DDC), so L-DOPA is combined with carbidopa, a drug that blocks peripheral DDC, in an effort to maximize L-

DOPA conversion by the DDC present in the brain [9]. L-DOPA is absorbed in the small intestine via amino acid transporters and crosses the blood-brain barrier similarly, so it should not be taken with a high protein meal in order to maximize absorption [141]. The plasma half life is one to three hours, but the effects are generally observed beyond this period, suggesting an ability to store/manage DA release [141]. A second approach to enhance DA signaling is to mimic DA via DA agonists, e.g. bromocriptine, pramipexole, ropinirole, apomorphine and cabergoline [9]; these have varying binding affinities for DA receptors and will not be contrasted here. A third approach is through decreasing DA metabolism, thereby achieving an increased effective concentration of synaptic DA. DA is metabolized via two enzymes, viz. catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), so inhibitors of these are used (in the latter case, specific MAO-B inhibitors are used since this is the primary isoenzyme found in the striatum) [141]. Two approaches that are not DA-centric are muscarinic receptor antagonists, which were used with modest success even before the discovery of L-DOPA, and amantadine, an antiviral drug that affects "DA release in the striatum, has anticholinergic properties and blocks [N-methyl-D-aspartate (NMDA)] glutamate receptors" and also has modest effects [141]. While the mainstay therapy for PD is L-DOPA, its use *per se*—i.e., apart from the effect of disease progression—has been linked to L-DOPA-induced dyskinesias (LIDs) [142] (though this is still hotly debated, see the 'Controversies in Movement Disorders' session during the 18th International Congress of Parkinson's Disease and Movement Disorders). Therefore, it is a common practice to delay the use of L-DOPA and to first employ MAO-B inhibitors and DA agonists until symptoms can no longer be adequately managed [143]. However, this

concern for delaying L-DOPA to delay LIDs is a common misconception. Age and disease duration more closely relate to development of LIDs, so the decision to choose L-DOPA or DA agonists for a patient's initial treatment should focus on other factors, as previously reviewed [144].

Surgical Therapies

L-DOPA provides adequate management of symptoms usually for a decade, at which point dyskinesias may become intolerable [145, 146] and new therapeutic, surgical options are considered [147]. Two main surgical approaches have been used to treat PD: lesioning and electrical stimulation [148]. Lesioning was used first with either the ventral intermediate nucleus (VIM) of the thalamus or the globus pallidus interna (GPi) targeted (thalamotomy and pallidotomy, respectively) [148]. Unilateral tremor can be reduced using thalamotomy, but bilateral surgery is fraught with complications, most commonly the introduction of swallowing and speech difficulties [149]; as most PD patients have progressed to considerable bilateral disease by the time surgery is discussed, complications from bilateral thalamotomy has limited its use. Pallidotomy, in contrast, can be used to reduce dyskinesias, tremor, rigidity and bradykinesia either unilaterally or bilaterally [148, 150, 151], leading to increased use through the 1990s. Pallidotomy then gave way in popularity to deep brain stimulation (DBS) at the beginning of the new millennium with United States Food and Drug Administration (FDA) approval of VIM DBS for essential tremor in 1997 and GPi or subthalamic nucleus (STN) DBS for PD in 2002 [152]. DBS for PD is discussed below.

Deep Brain Stimulation for Parkinson's Disease

A Brief History

The use of deep brain stimulation (DBS) for Parkinson's disease (PD) begins, like all matters academic, with the founder of the 'modern' philosophical era, René Descartes. His Cartesian coordinate system allowed for a precise, specific description of a point's location in space that several hundred years later, allowed for the ability to accurately target areas of the brain through mapping neuroanatomical structures onto a three-dimensional space. Targeting structures in this manner is achieved through a stereotactic apparatus, a device described by Sir Victory Horsley and Robert Clarke in 1908 [153] and subsequently used by Horsley in 1909 in an adolescent for treatment of hemiathetosis through removal of the portion of the precentral gyrus associated with the upperextremity [154], and the same procedure was used in two related cases in 1931 and 1937 by Paul Bucy and Theodore Case [155, 156]. A stereotactic device that could be used to insert a probe into the brain was first used in 1947 by Ernest Spiegel, Henry Wycis and colleagues [157], and it was subsequently employed for psychiatric and movement disorders indications, e.g. thalamotomy and pallidotomy for PD [158-168].

The birth of DBS came from the marriage of stereotactic neurosurgery with chronic, electrical stimulation. This was first employed by Hassler with the experimental finding that high-frequency stimulation of the pallidum would ameliorate tremor [169, 170], and this was employed as a treatment in 1967 by Natalia Bekhtereva and colleagues [171]. However, after the widespread and very successful treatment of PD with L-DOPA in the 1960s, the use of surgery in general for movement disorders was primarily confined to

medically intractable tremor [172]. A resurgence in stereotactic neurosurgery for movement disorders in the 1990s occurred with the convergence of Louis Benabid's use of chronic, bilateral DBS of the VIM of the thalamus for the treatment of tremor (and subsequent FDA approval in 1997) [172-174] and the revival in pallidotomy for PD as reinitiated by Lauri Laitinen [175]. Combining these movements, DBS of the globus pallidus interna (GPi) for treatment of PD was explored by Jean Siegfried and Bodo Lippitz and resulted in FDA approval in 2002 [176]. However, the use of DBS for PD took a turn with the hypothesis by Mahlon DeLong that the subthalamic nucleus (STN) could be targeted for the treatment of PD [172, 177]. Benabid and his team implanted a DBS electrode into a PD patient in 1994 [178], and subsequent studies showed that stimulation of the STN provided superior results than targeting the GPi [179, 180], resulting in FDA approval in 2002 [172] and the replacement of the GPi with the STN as the traditional and gold-standard surgical therapy for PD. However, the tide has begun to turn with the demonstration of equal benefit for motor symptoms between the GPi and STN [181]. The current use of GPi and STN DBS is discussed more below. (This section was drawn heavily from historical research presented in [182].)

DBS Surgery

As is the case with all non-emergent surgeries, the procedure begins with planning and any necessary preoperative imaging. Targeting may be accomplished through indirect or direct methods. Indirect targeting uses a reference standard/atlas for determining coordinates, whereas direct targeting uses a combination of imaging modalities of the individual patient. Direct targeting usually requires magnetic resonance imaging (MRI)

for identifying the anterior and posterior commissures, which are commonly used neurosurgical landmarks, and the STN; their MRI images may then be overlaid on a computed tomography (CT) images acquired with discrete stereotactic references, allowing for precise coordinates to be used for the target structure.

The surgical procedure is dependent on a stereotactic head frame that allows for precise movement within the coordinate system. Setting the stereotactic coordinates allows for location of the point of entry, a bur hole is made and the dura mater is opened and the pia mater coagulated [182]. Once the electrode lead is inserted within the region of the target, microelectrode recording is used to confirm lead location. Of note, sedation must be stopped prior to microelectrode recording since it may affect the electrophysiological measurements. Upon confirmation of the target structure, the electrode lead is implanted and affixed to the skull via an anchoring device or sometimes with bone cement or plates [182]. The impulse generator, which resides beneath the ipsilateral clavicle, may be implanted at the same time or staged at a later date. The wire is fed subcutaneously from the bur hole to the pulse generator. The pulse generator then is programmed after recovery from the surgical procedure.

Complications from DBS surgery are relatively minor compared to many other neurosurgical procedures. Intracranial hemorrhage is the worst possible complication since it results in severe neurological deficits or death; however, it only occurs 0.2% to 12.5% of the time—likely partially due to a reflection of a center's experience with the operation—and is likely due to injury of an arterial blood vessel [170, 182, 183]. Of

note, the risk of hemorrhage may be related to the chosen target with the GPi being more likely associated with hemorrhage than the STN by an approximate threefold difference [184, 185]. Transient, postoperative confusion is possible in elderly patients, and it may be reduced with staging bilateral implantations across separate surgical sessions [182]. There is also the risk of hardware-associated infection, which is most common at the site of the pulse generator, and may be treated with antibiotics or sometimes requiring removal of the subcutaneous wire or in some cases, the electrode lead itself. Hardware may also fail; leads may fracture, migrate or erode [170, 182]. Lastly, complications from stimulation are often reported, though are most often transient and manageable through changes in programming parameters. Such complications include dysarthria, weight gain, depression, eyelid-opening apraxia, stimulation-induced dyskinesias, muscle contractions, diplopia, worsening of postural instability or gait, hypersexuality, mood disorders, psychosis or hallucinations [186]. The most common complications from electrode lead implantation, which may or may not be stimulation-dependent, is a loss in verbal fluency [187-191] and cognitive decline when already present before surgery [192, 193]. (This section was drawn heavily from research presented in [182].)

Efficacy and Side Effects of DBS

STN DBS

Considered the gold-standard surgical therapy for PD, STN DBS has shown improvements in UPDRS motor scores of approximately 50% [181, 193-198]. STN DBS has also shown improvements on non-motor features, including quality-of-life measures

and reductions in L-DOPA dosages [181, 199]. STN DBS may also be superior to optimized medical therapy in reduction of PD motor symptoms [200]. Lastly, STN DBS has shown superiority in economic cost-benefit analyses [201, 202]. In contrast to these benefits, STN DBS may exacerbate preexisting depressive symptoms [181] or even increase impulsivity [203].

GPi DBS

Though fewer PD patients have received GPi DBS than STN DBS, its use is on the rise with the results from a rigorous study completed by Kenneth Follett and colleagues showing that GPi and STN DBS were equivalent insofar as relief of PD motor symptoms were concerned [181]. GPi DBS is less likely associated with a reduction in L-DOPA dosages, but it is less likely to exacerbate preexisting comorbidities such as cognitive dysfunction or depression [181, 204-206].

Patient Selection for DBS

When to consider?

Since its inception in the clinic, DBS has been used as a treatment of last resort for PD. This practice was reasonable at the time because of the low familiarity/comfort of neurosurgeons with the procedure, less-refined practices in programming and the overall risks of functional neurosurgery, which are not trivial, and were relatively higher than they are today due to an increase in case volume at what are now experienced centers. Compounded onto these is the fact that even neurologists specializing in movement disorders are unable to diagnose PD better than 26-53% of the time within

the first five years, depending on presentation [207], meaning DBS within that initial interval may be unreasonable. These factors led to the original consideration of (and FDA approval for) candidates for DBS only when they were at least five years post diagnosis and a Hoehn-Yahr scale stage three or greater (i.e., mild-moderate disability, impaired righting reflexes but still physically capable of independence) [198, 208-210]. Clinical practice under these guidelines resulted in patients receiving DBS surgery twelve to fourteen years post diagnosis (Volkmann 2004).

Patients are considered optimal candidates for DBS if they follow additional, specific inclusion and exclusion criteria. Patients are generally excellent responders to stimulation if they are also excellent responders to L-DOPA, usually measured by if they have a greater than 33% reduction in UPDRS motor score [182, 198, 209]. Patients are considered excellent DBS candidates, then, if their L-DOPA response is robust but short lived (i.e., low 'on' versus 'off' time) and their L-DOPA-induced dyskinesias are unmanageable, as L-DOPA dosage can be lowered with stimulation; however, tremor specifically does not have to respond as robustly to L-DOPA as other symptoms for consideration of DBS [182]. As a possible exclusion criterion, patients hoping to benefit specifically in regard to axial symptoms, like postural instability, speech issues and gait abnormalities, should not expect this from DBS, as it primarily affects symptoms that affect the limbs [170, 182]. Patients with atypical parkinsonism are not good candidates for DBS since their symptoms will not improve and may worsen [182, 211, 212]. Patients without dedicated caregivers are also poor candidates for DBS [212].

Which to consider?

In the last five years, the practice of DBS for PD has shifted from the question, “Which target is better?” to, “Which target is more appropriate for this patient?” In this light, it is highly recommended that DBS only be practiced at experienced centers and under the evaluation of a multidisciplinary team [213, 214]. A thorough comparison between the GPi and the STN as targets has been completed by Nolan Williams, Kelly Foote and Michael Okun [214]. In brief, patients can expect an advantage for bilateral GPi stimulation for “L-DOPA-responsive gait and balance issues,” for “dyskinesia suppression,” preservation of cognition, “flexibility in long-term medication adjustments,” “ease of programming and east of long-term management,” for using a unilateral approach and (in unilateral cases) for overall quality of life [214]. In contrast, patients can expect an advantage for bilateral STN stimulation for bradykinesia and are likely to have a 50% reduction in L-DOPA dosage [179, 181, 182, 185, 214-217]. In addition, payers may expect an economic advantage from STN stimulation [202, 214].

The Mechanism of STN DBS

Since the advent of STN DBS, several hypotheses have been proposed for its mechanism. As an extension of the origins of STN DBS from pallidotomy or subthalamotomy for PD, the first explanation was that of a ‘depolarization block.’ The argument was by analogy (notably, a weak form of logic): lesioning the STN and electrically stimulating it produced similar clinical effects, so they must work by a similar mechanism and STN DBS is a ‘functional ablation’ [218-221]. However, if this hypothesis were true, it would stand to reason that reduced STN activity would result in

reduced glutamate release and a subsequent reduction in measured glutamate at postsynaptic targets; however, this was not shown. DBS actually promotes STN activity [222, 223], and glutamate levels as measured by microdialysis are increased in the substantia nigra pars reticulata (SNpr) and GPi, two targets of the STN, in association with STN DBS [224, 225]. To add a nuance to this hypothesis, STN DBS has shown both excitatory and inhibitory effects that result in a replacement of pathologic, intrinsic STN activity with a new pattern, thereby allowing STN neurons to become stable oscillators, as first shown *in vitro* [226] and later *in vivo* [227]. In a groundbreaking study in parkinsonian rats, Viviana Gradinaru, Anatol Kreitzer and colleagues used optogenetic approaches to demonstrate the activation of STN afferents and the hyperdirect pathway from the primary motor (M1) cortex to the STN may explain the therapeutic effects of STN DBS [228]; this hypothesis was further tested in non-human primates and seems to hold true given that stimulation intensities are above a threshold [229]. In contrast, one study showed that STN DBS may isolate the STN from pathology in other regions by decoupling it from upstream and downstream areas through axonal failure, allowing the STN to resume normal firing patterns [230]. Using circuit activity/patterns as a way to explain STN DBS has garnered much attention. Many studies have shown that PD is associated with increased beta oscillations within the basal ganglia and that STN DBS decreases these by altering STN firing patterns [231-236].

Is STN DBS Disease-Modifying?

Beyond therapeutic efficacy, several clinical studies have investigated if STN DBS may be used to slow or halt the progression of PD. This proposition first came from anecdotal reports from neurologists who believed their PD patients receiving STN DBS did better than those who did not elect surgery. This clinical observation resulted in retrospective analyses that showed STN DBS could maintain subjects' off-medication motor symptoms several years after electrode implantation [193, 195, 215, 237]. In contrast, a prospective study showed equivalent disease progression as measured by striatal fluorodopa uptake in subjects either receiving STN DBS or not [238]. Of note, these studies examined disease modification in subjects who were in late-stage PD since the average age of patients at electrode implantation is about twelve to fourteen years post diagnosis [239]. Since the completion of these clinical studies, the PD field has advanced its knowledge of the state of striatal DA loss relative to the time of diagnosis. Since the majority of terminal loss occurs by four years post diagnosis [45, 46], it is unreasonable to assess the question of disease modification in the context of a PD subject who has already lost (and subsequently compensated for) the very thing the investigator is trying to save. A more recent clinical trial has employed STN DBS at an earlier time in the disease course (i.e., about seven to eight years post diagnosis) [240], but not early enough to overcome this experimental design hurdle. The only clinical cohort that may be able to address the question of disease modification is at Vanderbilt University, though current follow-up is too short and the cohort too small [241]. As such, from a clinical perspective, 'the jury is still out' on whether STN DBS is a disease-modifying therapy for PD because an appropriately designed clinical trial has yet to be conducted.

Even though the definitive clinical trial has yet to establish if STN DBS is disease-modifying, several groups in parallel with the clinical studies discussed above have shown a neuroprotective effect of STN DBS. In rats, STN DBS used immediately after 6-OHDA administration results in a doubling of the remaining tyrosine hydroxylase immunoreactive (THir) neurons in the SNpc compared to rats without activated electrodes [242], and when STN DBS is activated one week or two weeks after 6-OHDA, the SNpc neurons that remain are protected [134, 243, 244]. Similar results have been found in non-human primate models of PD using MPTP with either pretreatment with STN DBS or waiting 6 days after MPTP [245]. STN DBS-mediated neuroprotection in a 6-OHDA rat model of PD showed a stimulation-specific increase in brain-derived neurotrophic factor (BDNF) in the SNpc in lesioned rats or the striatum in intact (i.e., no 6-OHDA) rats, providing a possible explanation for the observed neuroprotection [136]. Indeed, a role for BDNF in stimulation-mediated effects in other contexts has been shown by other laboratories [246-248].

Neurotrophins

Meet the Family

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) comprise the neurotrophin family. NGF was discovered first through the work of Viktor Hamburger, Rita Levi-Montalcini and Stanley Cohen [249-254] for which the 1986 Nobel Prize in Medicine was awarded though not entirely shared. The other neurotrophins are structurally similar to NGF, including by a

shared ‘cysteine knot’ tertiary structure, and are approximately 12 kD [255-258]. All neurotrophins are secreted proteins and bind tropomyosin-related kinase (trk) receptors, leading to dimerization and receptor activation [259, 260]. For the present work, BDNF plays a central role and is discussed in greater detail below.

Brain-Derived Neurotrophic Factor

Structure & Function

Yves-Alain Barde, Hans Thoenen and colleagues observed that “glioma-conditioned medium […], the source of glial factor, can support both survival and fibre formation of isolated chick sensory neurons and that neither NGF nor glial factor are responsible for this effect” [261] and they were able to replicate these result using rat brain extracts [262]. Identification of the responsible, new factor was conducted by the same laboratory group [263] and later became known as brain-derived neurotrophic factor (BDNF). BDNF shares 50% of its primary structure with NGF, including the key six cystine residues, and has similar secondary structure to NGF from its disulfide bridges, so BDNF was classified as the second neurotrophin after the category-defining NGF [264].

Extending the work of Barde and colleagues, Johnson and colleagues showed that BDNF enhances survival of cultured fetal retinal ganglion cells [265]. One should note, though, that the role of BDNF is distinctly different than that of NGF. NGF may be characterized as ‘anti-death’ since its presence appears necessary for developing sensory neurons [266, 267]; however, BDNF is ‘pro-growth/survival’ since the addition

will rescue motor neurons during a period of cell death during development, but genetic knockout of BDNF has no effect on the same population of neurons [268-270].

The effects of BDNF are myriad and extend beyond just the presence or absence of perikarya during development. BDNF has been found in the hippocampus, cortex, claustrum, cerebellum, olfactory bulb, thalamus, preoptic area, hypothalamus and pontine nuclei as well as in the heart, lungs, platelets and Schwann cells [271-274]. Of importance to the present work, BDNF mRNA was found in the entopeduncular nucleus (EP), the ventral tegmental area (VTA) and in dopaminergic (DA) neurons of the substantia nigra (SN) [275], and BDNF enhances the survival of cultured DA neurons [276].

Synthesis, Release and Signaling

Production of BDNF proceeds even in excess, but trafficking and degradation of BDNF is modulated in response to neuronal activity [277]. Glutamate as well as acetylcholine have been shown to increase BDNF production by postsynaptic targets [278, 279]. Glutamate-mediated increases in BDNF require involvement of both NMDA and non-NMDA postsynaptic receptors; cholinergic-mediated increases require muscarinic but not nicotinic acetylcholine receptors [280-286]. BDNF is first produced in a ‘pro’ form, proBDNF. proBDNF contains an “amino-terminal pro-domain that assists in their proper folding and dimerization” [287]. proBDNF is constitutively released to interact with its low-affinity receptor p75 [287]. Ligand-bound p75 in association with the protein Sortilin acts through c-Jun N-terminal kinase (JNK) to ultimately result in apoptosis [287].

Activity-dependent release of BDNF opposes the constitutive-release pathway and offers the ability for the target neuron to be ‘fine-tuned’, called the “Yin-Yang Hypothesis” [287]. Upon the firing of an action potential, the presynaptic neuron releases neurotransmitter and co-releases vesicular proBDNF into the synaptic cleft [287, 288]. The pro-domain is proteolytically cleaved either intracellularly by endoproteases or proprotein convertases or extracellularly by tissue plasminogen activator (tPA), thereby converting the proBDNF form into the mature form that is referred to simply as BDNF [289, 290]. Then, the mature form of BDNF can act via two signaling pathways on the postsynaptic neuron: the canonical and the non-canonical pathways.

In the canonical pathway, BDNF binds to its high-affinity receptor tropomyosin-related kinase type 2 (TrkB). TrkB signaling activates PLC-γ, PI3K-Akt and Erk, resulting in neuronal survival, growth/arborization and regulation of synaptic plasticity through mediating long-term potentiation (LTP) [287, 291, 292]. Of note, the affinity of BDNF for TrkB actually is increased when TrkB is complexed with p75Ntr [293, 294]. While the time course for the signaling events is on the course of many minutes, the measureable effects take much longer on the order of hours, as they require alterations in transcription and translation of specific genes and the production of a complement of new proteins [287].

In the non-canonical pathway, the effects of BDNF are still mediated through TrkB, but the intracellular signaling takes a tangential path. Through PI3K-Akt signaling, an intermediate called Girdin is phosphorylated and combines with Src to phosphorylate the NMDA receptor 2B subunit (NR2B) [295, 296]. NR2B phosphorylation results in a potentiated response by NMDA receptor-mediated currents [295, 297]. It is also important to note that the non-canonical pathway is much faster than the canonical one due to it only being a series of phosphorylation events. Lastly, it should be noted that TrkB is also present in a truncated form that was once thought to serve as a 'sink' for extracellular BDNF, but an intracellular signaling pathway has recently been elucidated [287]; knowledge of the truncated form of TrkB is still sparse, though it may very well play a role in PD research with the recent finding that both full-length and truncated TrkB have differential distributions between the dendritic, somatic and axonal compartments of striatal and nigral neurons in postmortem PD tissue [298]. BDNF-trkB signaling also has effects on DA signaling. In a BDNF heterozygous knockout mouse (i.e., decreased BDNF levels), there is decreased DA release and DA transporter (DAT) function, and these DA handling deficits were partially reversed with acute BDNF treatment [299]. In addition, selective removal of trkB signaling on striatal enkephalinergic neurons results in hyperlocomotion [300].

Of great relevance to the present research, BDNF release can be driven through electrical stimulation. In neuronal cultures, high-frequency stimulation leads to increased (mature) BDNF release and release of tPA, which converts proBDNF to the mature form, and low-frequency stimulation leads to increased proBDNF release [290].

In addition, long-term, high-frequency STN DBS increases BDNF levels in the nigrostriatal system [136]. Since high-frequency STN DBS is therapeutic for PD and low-frequency STN DBS worsens PD symptoms and promotes pathologic synchrony [236, 301-303], this offers a potential mechanism for this observed difference.

BDNF for Parkinson's Disease

With the discovery that DA neurons of the SN would fare better with the addition of BDNF [276], the field of PD research extended that finding into a possible therapeutic avenue. Of importance, there was a sound basis for this research: BDNF levels are reduced in the brains of PD subjects [304, 305]. *In vitro* studies led the way in showing a neuroprotective effect of BDNF application to mesencephalic DA neurons from N-methyl-4-phenylpyridnium (MPP+)- or 6-OHDA-induced cell death [306]. *In vivo* studies then provided more of a conceptual basis for using BDNF in the basal ganglia by demonstrating BDNF application to the striatum or the SN would enhance DA turnover [307]. This was followed by BDNF being used as a neuroprotective agent against MPP+ in rodents, resulting in fewer SN neurons lost and a reduction in pathologic turning as assessed by amphetamine-induced rotations [308, 309], and the overall findings were replicated in non-human primates [310]. Given that BDNF is particularly trophic for primary mesencephalic cultures, Yurek and colleagues used it as a supportive therapy to transplanted fetal DA neurons and showed increased innervation of the striatum by the transplanted neurons in a 6-OHDA rodent model [311]; DA neuron grafts are still under investigation. However, BDNF has not been tested in human subjects as a monotherapy for PD.

Chapter 2: Viral Vector-Based Modeling of Neurodegenerative Disorders: Parkinson's Disease

Abstract

Gene therapy methods are increasingly used to model Parkinson's disease (PD) in animals in an effort to test experimental therapeutics within a more relevant context to disease pathophysiology and neuropathology. We have detailed several criteria that are critical or advantageous to accurately modeling PD in a murine model or in a non-human primate. Using these criteria, we then evaluate approaches made to model PD using viral vectors to date, including both adeno-associated viruses and lentiviruses. Lastly, we comment on the consideration of aging as a critical factor for modeling PD.

Introduction to Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder with approximately "100-300 per 100,000 persons" affected (see chapter 2 in [312]). The primary risk factor for PD is aging with PD affecting one-percent of the population over sixty-five years of age [313]. Akinesia, bradykinesia, rigidity and resting tremor are the most common symptoms. While motor dysfunction is the primary basis for diagnosis, patients may also experience depression, cognitive dysfunction, agnosia and other symptoms at clinical presentation [6]. The progressive degeneration of the dopaminergic cells of the substantia nigra pars compacta (SNpc) and their projections to the striatum results in the loss of dopamine to the caudate nucleus and the putamen and the motor symptoms at clinical presentation. As a result, current pharmacotherapies (e.g., L-DOPA) attempt to bolster nigrostriatal dopaminergic

transmission. However, as disease progression continues, these pharmacotherapies lose symptomatic efficacy and can yield troubling dyskinesias [314], making the development of neuroprotective therapies critical.

While a clinical diagnosis of PD can be made based on symptoms and signs alone, the definitive diagnosis is made *post mortem* by a neuropathologist. The pathological hallmark of PD is the Lewy body, a "proteinaceous neuronal cytoplasmic inclusion" that is often immunoreactive for ubiquitin and most specifically for α-synuclein (α-syn) (see chapter 12 of [312] as well as [33, 34, 315]). α-syn is a natively unfolded protein of 140 amino acids that binds and bends membranes, though its biological function is not completely understood [316]. (The relationship between α-syn and PD is discussed more below.)

Criteria for Evaluating Animal Models of Parkinson's Disease

Our ability to study the mechanisms driving the pathophysiology in PD and to test experimental therapeutics is only as strong as our capacity to accurately model the human condition in a laboratory animal. Neurotoxicant-based and transgenic models of PD have proven valuable in advancing our understanding of the consequences of dopamine denervation and the biological function of particular genes within the context of the whole brain environment; however, PD is still without a disease-modifying agent. Through targeted induction of oxidative stress, neurotoxicant-based models of PD can be used to produce progressive degeneration of the SNpc, but only of the perikarya since the terminals degenerate almost immediately (e.g., [134]). Limitations of the

neurotoxicant-based PD models (e.g., 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) include inadequate construct validity, as oxidative stress is only one of many contributors to the disease process, and an absence of the pathological hallmark of PD, Lewy bodies. The overwhelming majority of germ line transgenic models that overexpress normal or mutated forms of genes linked to PD have failed to recapitulate the magnitude of nigrostriatal degeneration observed in the parkinsonian brain. In this chapter, we review viral vector-based models of PD to determine whether this approach more appropriately recapitulates the human condition. In the following sections, we will review the progress that has been made in several different viral vector-based models of PD and evaluate them based on the following criteria that we propose are critical or advantageous to studying the disease and exploring therapeutics.

Does the model have construct validity?

Construct validity requires that the causes and pathophysiological changes that occur in PD patients are comparable to what drives and occurs in the animal model. The causes of PD are for the most part unknown because the majority of cases are idiopathic. A combination of genetic and environmental factors has been posited to increase the risk of developing the disease. Nonetheless, a small percentage of PD cases can be entirely explained by our current understanding of genetics (see chapter 15 of [312], cf. chapter 16, ib.). With this in mind, viral vector-based animal models of PD have targeted overexpression of normal or mutated forms of some of the causative PD genes to the nigrostriatal system. These include overexpression of normal or mutated forms of α-syn, leucine-rich repeat kinase 2 (LRRK-2) and parkin substrates as well as

knockdown of PTEN-induced putative kinase 1 (PINK1). Further, with the concept in mind that PD motor symptoms are primarily driven by loss of nigrostriatal dopamine, some research has explored silencing tyrosine hydroxylase (TH) using shRNA to effectively deplete dopamine in the nigrostriatal system.

Is the model consistently reproducible?

An important criterion for any model is the level of variability and the ease of reproducibility. We will examine the variability of each model and use the literature to confirm reproducibility both within the same laboratory and between laboratories employing the same model. It should be noted that although viral vector constructs may express the same PD-related gene, these vector constructs may differ in other important aspects such as the promoter used, titer or injection parameters. Therefore, results may vary between specific vector constructs, so some leniency must be afforded when comparing studies. Of note, this lack of standardization has led to some confusion, and perhaps trepidation, by other groups in using certain models. Laboratories employing viral vector-based animal models of PD should take this into account when developing models, comparing their models to those from other groups and communicating their results.

Is the model appropriately progressive?

Nigrostriatal degeneration occurs over many years both before and after symptom onset and diagnosis. Of great importance, this process proceeds in stages, the very first of which is dysfunction/loss of dopamine (DA) terminals in the caudate nucleus and the

putamen (collectively termed the striatum in rodent species). A recent, detailed, post-mortem analysis of PD brains confirms that loss of striatal DA innervation is a critical early event that precedes and exceeds loss of DA neuron cell bodies [46]. Therefore, an ideal model of PD should display substantial striatal terminal dysfunction/loss prior to overt nigral DA neuron loss. In accordance with the progressive nature of PD, degeneration of DA neurons of the SNpc should occur over months; for the purposes of discussion, we set our minimum at eight weeks (i.e., degeneration should be complete no sooner than eight weeks post insult). Finally, it is advantageous for the model to progress to at least 60% loss of nigral DA neurons in order to mimic late-stage PD, allow for functional evaluations of motor performance and to give the investigator a large window to observe a disease-modifying effect.

Does the model recapitulate the neuropathological hallmarks of PD?

Since the definitive diagnosis of PD is made *post mortem* upon confirmation of specific neuropathological hallmarks, a model of PD should also include the relevant neuropathology. Nigral DA neurons should possess Lewy body-like inclusions that are immunoreactive for ubiquitin and α-syn as well as dystrophic (or Lewy) neurites in the striatum. Further, as the nigrostriatal system in the PD brain is associated with neuroinflammation [124-131], markers indicative of reactive microgliosis should be observed. In addition, overt pathology that is not commonly associated with PD should not be observed, thereby demonstrating the model is somewhat specific in its effects.

Does the model result in quantifiable parkinsonian symptoms?

The most important outcome for PD patients is in a therapy's amelioration of symptoms. In this light, PD models should result in quantifiable symptoms analogous to those that observed in PD patients to allow for the study of how therapeutic intervention can slow, halt or reverse functional deficits. Therefore, motor symptoms, such as akinesia, bradykinesia, postural instability or tremor, are desirable qualities in a PD model, and their measurement must possess enough sensitivity to observe a therapeutic effect. Furthermore, motor symptoms should ideally present at a time when approximately 50% of dopaminergic input to the striatum has been lost, thereby mirroring clinical presentation [45, 46].

Viral Vector-Based Models of Parkinson's Disease

Using genetic insights into PD pathology, several gene therapy-based models have been developed with varying degrees of success at recapitulating the human condition. These models include overexpression of α-syn, leucine-rich repeat kinase 2 (LRRK-2) and parkin substrates as well as knockdown of tyrosine hydroxylase (TH) and PTEN-induced putative kinase 1 (PINK1). Many of these models have been comprehensively reviewed before [317-323], but we will evaluate the currently employed models according to the above criteria in a more prescriptive fashion. A summary is presented in Table 1. Please note, while the following gene therapy-based models have their respective roles in modeling genetic forms of PD and in studying some important cell and molecular biology, we are limiting our discussion to how well they recapitulate the idiopathic form of PD *in vivo* in the mammalian brain.

Table 1. Overview of Viral Vector-Based Models of Parkinson's Disease.

Model	Construct Validity?	Reproducible?		Progressive Nigrostriatal Degeneration?				Parkinsonian Pathology?				Parkinsonian Symptoms?	References		
	Construct validity for idiopathic PD	Within Group	Across Groups	Striatal DA Terminal Dysfunction/Loss (over weeks)	Terminal Loss Precedes Overt Cell Loss	Nigral DA Neuron Loss (≥8 weeks)	Endstage Nigral DA Neuron Loss >60%	Lewy-body-like Inclusions	Dystrophic Neurites	No Overt, non-PD Pathology	Neuroinflammation	Motor impairment	Rats	Mice	Non-Human Primates
α-Synuclein Overexpression:															
Wildtype	*Yes*	Yes	Yes	Yes	Yes	Yes	With high expression	Yes	Yes	Yes	Yes	Yes	[74, 325, 327-339, 356]	[340-343]	[344-346, 354]
A30P	*Yes*	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No data	No	[74, 325, 328, 330, 347-349]	[350]	
A53T	*Yes*	Yes	Yes	Yes (on limited data)	No (on limited data)	Yes	No	Yes	Yes	Yes	Yes	Yes	[74, 325, 330, 351, 352, 353]	[342]	[344, 345, 354]
E35K, E46K, E57K	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	No data	No data	Yes	No data	No data	[330]		
S129A	*Yes*	Not demonstrated	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No data	No data	[327-329, 355]		
LRRK-2 Overexpression															
Wildtype	*Yes*	Not demonstrated	Yes	No	No	No	No	No	Yes?	No	No	No	[360]	[361]	
G2019S	*Yes*	Not demonstrated	Yes	Yes	No (on limited data)	Yes	No	No	Yes?	No	Yes (on limited data)	No	[360]	[361]	
D1994A	*Yes*	Not demonstrated	Not demonstrated	No	No	No	No	No data	No data	No	No data	No		[361]	
G2019S + D1994A	*Yes*	Not demonstrated	Not demonstrated	No	No	No	No	No data	No data	No	No data	No		[361]	
TH shRNA	*No*	Not demonstrated	Yes	Yes, dysfunction only	No	No	No	No	No	No	No data	Yes	[370]	[369]	
Parkin Subtrates:															
CD-Crel1	*Yes*	Not demonstrated	Not demonstrated	Yes	Yes	Yes	No	No data	No data	No	No	No data	[364]		
Pael-R	*Yes*	Not demonstrated	Yes	Yes	No	Yes	Not quite	No	No	No	No	Yes (stepping test only)	[366]	[365]	
hu p38/JTV	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	No	No	No	No data	No data		[367]	
hu wt synphilin-1	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	No	No data	No	No data	No data		[368]	
hu wt synphilin 1 on A30P α-syn background	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	Yes	No data	No	No data	No data		[368]	
hu R621C synphilin-1	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	No	No data	No	No data	No data		[368]	
hu R621C synphilin-1 on A30P α-syn background	*Yes*	Not demonstrated	Not demonstrated	No data	No data	No	No	Yes	No data	No	No data	No data		[368]	
PINK1 shRNA	*Yes*	Not demonstrated	Not demonstrated	No	No	No	No	No	No	No	No data	No data		[371]	

α-Synuclein Overexpression

Mutations in the gene encoding α-syn, *SNCA*, have been definitively linked to familial PD [49]. Increases in overall α-syn expression by duplications or triplications of the α-syn gene *SNCA* and single nucleotide mutations (viz., A30P, A53T and E46K) within *SNCA* are associated with development of PD and an earlier onset of disease symptoms [49-54]. In addition, the primary component of Lewy bodies is aggregated α-syn [324]; hence, targeted overexpression of wildtype or mutated α-syn to the adult, non-human nigrostriatal system was the first viral vector-based approach to model PD [74, 325, 326]. Since that time, gene transfer of α-syn to the SN has been the most extensively studied with numerous reports in mice, rats and non-human primates and using both adeno-associated viruses (AAV) and lentiviral (LV) vector constructs [74, 325-355]. Over time, as technological advances allow for higher vector titers to be achieved and the identification of more efficient promoters, viral vector-mediated overexpression of α-syn has become more consistent and yielded a greater magnitude of effects. Present use of AAV or LV to overexpress α-syn recapitulates several components of PD neuropathology, including: a) early striatal terminal dysfunction [352, 356], b) progressive loss of striatal, dopaminergic terminals, c) progressive loss of dopaminergic neurons of the SNpc following loss of terminals, d) Lewy body-like inclusions containing α-syn, e) dystrophic neurites resembling Lewy neurites [325, 331, 336, 357] and f) neuroinflammation [123, 334, 341, 352]. In addition, viral vector-mediated α-syn overexpression results in PD-like motor symptoms that correlate with an approximate 50% loss of striatal DA as observed in PD patients [337, 339].

In our own laboratory, we have characterized the degeneration, pathology and behavioral phenotype induced by intranigral injections of recombinant AAV serotype 2/5 (AAV2/5) in which expression of the human wildtype α-syn transgene is driven by the chicken beta actin/cytomegalovirus (CβA/CMV) enhancer-promoter hybrid that results in efficient gene expression in neurons [339] (see Figure 1). This model results in: a) transduction of the nigrostriatal system with human wildtype α-syn, b) 60% nigral DA neuron loss and 40% reduction in striatal TH immunoreactivity eight weeks post injection, c) marked microgliosis in the SN associated with α-syn overexpression and d) significant impairment in contralateral forelimb use eight weeks post injection. Lastly, nigral neuron degeneration correlates with α-syn expression levels and can be adjusted by the investigator through altering the vector titer or construct [339], offering a methodological advantage.

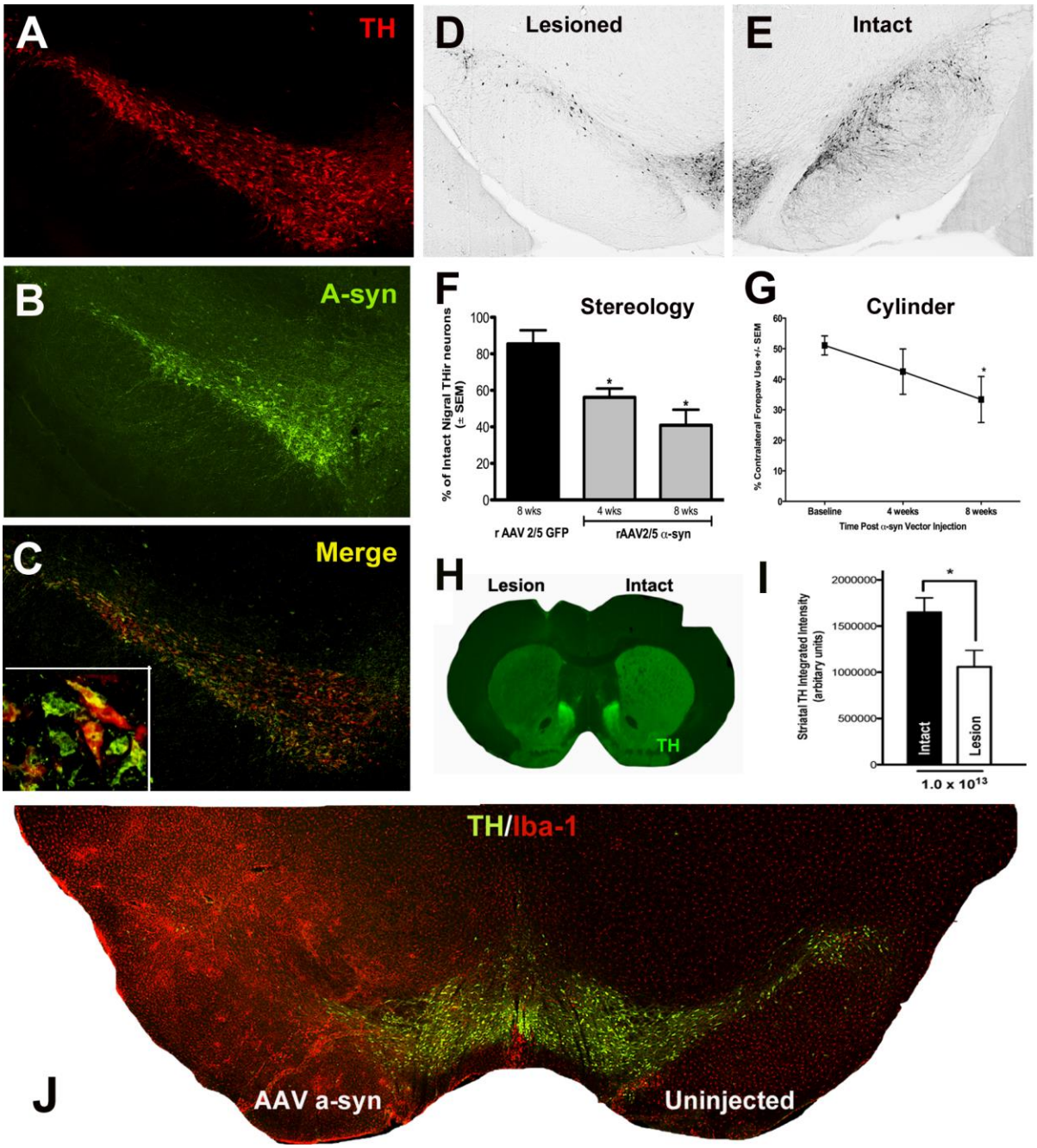

Figure 1. Overexpression of α-Synuclein in the Rat Nigrostriatal System Via AAV2/5 Results in Nigral Degeneration, Contralateral Forelimb Impairment and Microgliosis. Intranigral injections of AAV2/5 α-syn (2 x 2 µls of 1 x 10^{13} vg/ml, AP -5.3, ML +2.0 mm, DV -7.2 mm; AP -6.0 mm, ML +2.0 mm, DV -7.2 mm) were made to young-adult, male, Sprague Dawley rats as described previously [339].

Figure 1 (continued)

A-C. Coexpression of human, wildtype α-syn (green) in TH immunoreactive (THir, red) neurons within the SNpc at two weeks following injections, prior to degeneration. **D.** Degeneration of THir neurons of the SNpc at eight weeks after AAV2/5 α-syn injections compared to the uninjected, contralateral SN **(E)**. **F.** Stereological assessment revealed that after four weeks post α-syn vector injections, there is an approximate 40% decrease in THir neurons in the SNpc that progresses to about 60% at eight weeks (*, $p < 0.05$ compared to green fluorescent protein (GFP) control). **G.** Significant deficits in contralateral forelimb use are observed eight weeks after AAV2/5 α-syn injections (*, $p < 0.05$ compared to baseline). **H.** Partial dopaminergic striatal denervation ipsilateral to α-syn overexpression at eight weeks visualized using near infrared immunofluorescence and quantified in **I** (*, $p < 0.05$ compared to baseline). **J.** Microgliosis is associated with α-syn overexpression and nigral degeneration. THir SNpc neurons (green) and ionized calcium binding adaptor molecule 1 (Iba-1, microglia-specific, red) immunofluorescence reveals marked microgliosis eight weeks after AAV2/5 α-syn injections. Adapted from [339].

LRRK-2 Overexpression

LRRK-2, a protein without a definitively known function or substrate, is 2527 amino acids long and located in the cytoplasm (reviewed in [358]). Several different mutations in the *LRKK2* gene have been linked to the development of PD in an autosomal dominant inheritance pattern [55-58]. These cases generally include the formation of α-syn immunoreactive Lewy bodies [359]. They also result in the hallmark loss of striatal

DA and nigral neuron degeneration found in idiopathic PD. Viral vector-mediated overexpression of LRRK-2 has been used far less extensively (i.e., in only two laboratories) than α-syn-based models [360, 361]. This is primarily due to the prohibitively large size of the LRRK-2 coding sequence, precluding its use in standard vectors such as AAV or lentivirus. Loss of striatal fibers and nigral neurons is of too low a magnitude to model anything more than very early PD. Furthermore, these models produce LRRK-2 complexes that are α-syn deficient and do not resemble Lewy bodies. On the other hand, dystrophic neurites are observed in the striatum with immunoreactivity for the pathological phospho-tau epitope (pSer202/pThr205), but α-syn immunoreactivity is not reported. No data exist on whether motor impairments are present; however, the low magnitude of striatal DA loss suggests motor deficits are unlikely to exist.

Parkin Substrates

Mutations in the gene for parkin have been linked to an autosomal recessive inheritance pattern of a very early-onset (i.e., juvenile) form of PD [59, 60]. These forms of PD may be better described as parkinsonisms in that they do not show the formation of Lewy bodies, but they do still exhibit loss of nigral DA neurons [362, 363]. Manipulating parkin for modeling PD has proven more difficult for gene therapy approaches since the development of the model requires a complete knockdown of the protein in order to mimic both copies of the gene being mutated in the human condition and to result in pathology. However, since patients with parkin mutations have increased levels of parkin substrates (i.e., parkin is unable to process the increasing supply of substrate),

viral vector-mediated overexpression of parkin substrates instead has been used to create a ‘loss-of-function’ paradigm—rather, loss-of-function paradigms, as four parkin substrates have been used with variable results.

In two models overexpressing parkin substrates, CDCrel-1 or Pael-R, a partial loss of striatal DA terminals and concomitant loss of DA is observed, and this loss is progressive on the order of weeks [364-366]. For models overexpressing other parkin subtrates, p38/JTV or synphilin, no data on terminal status are available [367, 368]. It is unfortunate that the magnitude of terminal loss matches the loss of nigral DA neurons, suggesting that overt terminal loss does not precede overt loss of perikarya, although this has not been directly examined. Using CDCrel-1 or Pael-R has the advantage over p38/JTV or synphilin in that nigral neuron loss occurs over many weeks proceeding to an eventual loss that corresponds to late-stage disease. Achieving half of the cell loss of these models over a similar timespan with p38/JTV or synphilin requires the addition of A30P α-syn expression to create a pro-pathology environment. In these models, A30P α-syn expression lends the advantage of producing some neuropathology, including thioflavin-S positive inclusions. Lastly, the data available do not include behavioral assays to assess motor symptoms, with one exception: Pael-R overexpression will result in contralateral forelimb akinesia in the stepping test, but deficits were not observed in amphetamine- or apomorphine-induced rotations nor in the cylinder task [366].

TH Knockdown

The rate-limiting step in DA synthesis requires the enzyme TH. Virally-delivered shRNA-mediated knockdown of TH has been used by two laboratories to deplete striatal DA [369, 370]. This approach results in a phenotype that neurochemically resembles PD but not morphologically, so long as the titer is low enough to avoid non-specific degeneration. While not directly examined, there is no reason to believe that striatal terminals underwent degeneration, nor is there loss of SNpc neurons. Other aspects of neuropathology are also absent, and no data exist on the role of neuroinflammation. Lastly, behavioral deficits do exist in this model. It is unfortunate that the vector construct and injection parameters lead to transduced dopaminergic neurons outside the nigrostriatal system, namely those found in the adjacent ventral tegmental area (VTA). As these neurons are actually found to be resilient to degeneration in PD, an impact on their function is not desirable for accurately modeling the disease.

PINK1 Knockdown

Autosomal recessive loss-of-function mutations in PINK1 have been identified in familial PD [61, 62]. PINK1 plays an important role in mitochondrial homeostasis and is critical for parkin recruitment into mitochondria; therefore, loss-of-function PINK1 mutations lead to deficient mitochondrial homeostasis [62]. Only one laboratory to date has used virally-delivered shRNA to knockdown PINK1 [371]. Direct injection of adeno-associated virus expressing PINK1 shRNA into the striatum of mice did not affect nigral dopamine neuron survival during the short interval studied, but it did exacerbate the

degeneration induced by MPTP. This study did not report effects of PINK1 knockdown on striatal dopamine levels, dopaminergic terminals or neuroinflammation.

Consideration of Aging in Vector-Based Models of PD

Aging is known to be the primary risk factor for PD since the vast majority of idiopathic cases occur in patients over the age of sixty-five [70]. All reports to date in which vector-based modeling of PD has occurred have used exclusively young-adult animals. In an effort to examine the impact of nigrostriatal α-syn overexpression in the aged brain environment, we conducted studies using AAV2/5 human wildtype α-syn vector injected into the SN of three month-old, young-adult rats and twenty month-old, aged rats (Figure 2). We found that the transduction efficiency of AAV2/5 α-syn is significantly compromised in the aged brain, resulting in significantly fewer transduced cells in the mesencephalon and significantly less α-syn expression in the striatum, regardless of the titer used or duration of expression. Of interest, despite decreased transduction and lower levels of α-syn expression, the aged brain displayed an equivalent magnitude of α-syn-mediated nigral degeneration (Figure 2). We continued these experiments using various pseudotypes of AAV or LV expressing the reporter green fluorescent protein (GFP) in young adult and aged rats to determine whether age-related transduction deficiencies were specific to the AAV serotype or transgene expressed. We found that AAV2/5, AAV2/2 and LV were all deficient in facilitating gene transfer to the aged nigrostriatal system, whereas AAV2/9 yielded equivalent levels of transduction between young-adult and aged rats [372] (unpublished data), confirming previous results [373, 374]. Future studies using vector-based models of PD that include aging as a covariate

should consider aging-related deficits in viral vector transduction. With careful control over this potential confound, studies using viral vector-based PD modeling in aged animals will be poised to yield important insights on the interaction of aging and causative PD genes.

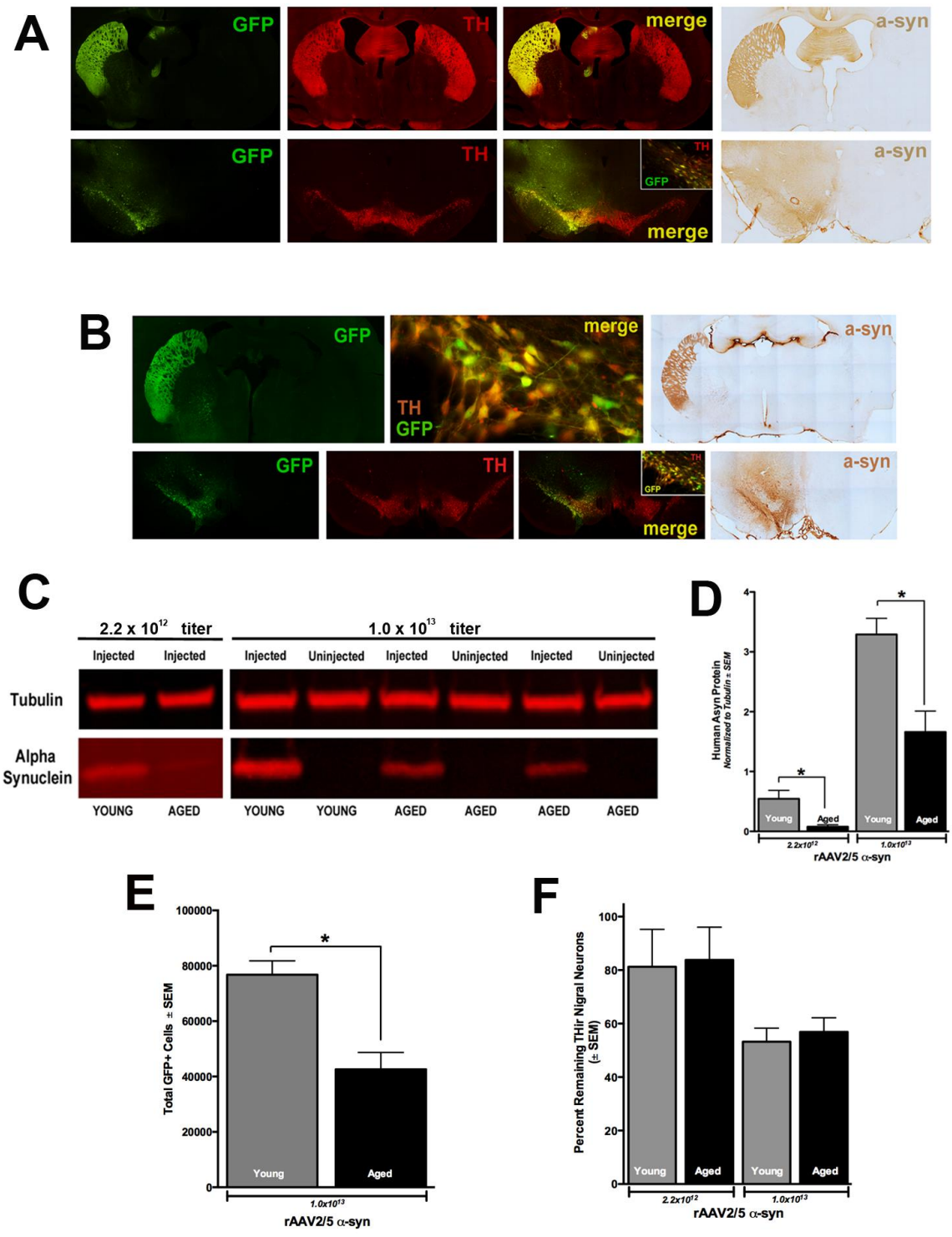

Figure 2. AAV2/5-mediated α-Synuclein transduction is significantly reduced in the aged nigrostriatal system. Intranigral injections of AAV2/5 α-syn/GFP were made to young-adult and aged (20-month), male, Sprague Dawley rats as described

Figure 2 (continued)

previously [339]. **A-B.** Immunofluorescent and immunohistochemical labeling of transduction of the nigrostriatal system demonstrating GFP (green), TH (red) and human wildtype α-syn (brown) at one month following injections into young-adult (**A**) and aged (**B**) rats. Despite the appearance of efficient transduction in both young-adult and aged rats, western blot revealed reduced human α-syn in striatal samples of aged rats compared to young-adult rats. **C.** Representative western blot of α-syn immunodetection in striatal samples of young-adult and aged rats injected with two different AAV2/5 α-syn titers (2.2×10^{12} vg/ml three months after injection or 1.0×10^{13} vg/ml one month after injection). The striatum ipsilateral (Injected) and contralateral (Uninjected) to AAV2/5 α-syn injections is depicted. **D.** Quantification of striatal human α-syn revealed significant deficits in human α-syn expression in aged rats for both vector titers examined (*, $p < 0.05$). Values are expressed as the mean optical density scores, normalized to tubulin controls ± SEM for each group. **E.** Stereological assessment of total α-syn immunoreactive cells in young-adult and aged rats twelve days following AAV2/5 α-syn injections (1.0×10^{13} vg/ml), prior to onset of degeneration. Significantly fewer transduced cells are evident in the aged brain (*, $p < 0.05$). Values represent mean α-syn cell counts per injected mesencephalon ± SEM for each group. **F.** Despite transduction of significantly fewer cells and significantly less α-syn expression, aged rats demonstrate equivalent degeneration of the SNpc, suggesting that α-syn neurotoxicity is exacerbated in the aged nigrostriatal system. Stereological assessment of THir neurons revealed that young-adult and aged rats injected with 2.2×10^{12} vg/ml AAV2/5 α-syn displayed equivalent loss (≈20%) of THir SNpc neurons three

months after injections. Young-adult and aged rats injected with 1.0 x 10^{13} vg/ml displayed equivalent loss (≈35%) of THir SNpc neurons one month after injections. Values are expressed as the percent remaining THir SNpc neurons as compared to the contralateral hemisphere ± SEM for each group.

Conclusions

Gene therapy approaches allow investigators to model PD through the overexpression or knockdown of genes of interest specifically in the nigrostriatal system. Substantial improvements in the last decade have led to more consistent and relevant models of PD than their first generation predecessors. We argue that the most important features of a model for testing experimental therapeutics are: a) to model progressive striatal terminal dysfunction and DA loss prior to overt nigral neuron degeneration and b) that this dysfunction and subsequent loss results in a demonstrable behavioral deficit. Based on our criteria, the α-syn overexpression model most closely recapitulates a comprehensive model of idiopathic PD. Even so, several models have come close on several measures. Overexpression of LRRK-2 with the G2019S mutation shows promise on measures of progressive nigrostriatal degeneration and neuropathology, and overexpression of the parkin substrates CD-Crel1 and Pael-R also show potential, though these require further work to more closely mimic the development of neuropathology and emergence of motor symptoms seen in PD patients. Since only the α-syn overexpression models have been validated in non-human primates, all of the other models discussed deserve consideration for further study in the non-human primate, as there may be significant interactions between the model organism and the

chosen gene or vector construct that may affect the translational value of the model. After all, the α-syn overexpression model is a result of several iterations by many laboratories around the globe using several model organisms over the last twelve or more years. The other models reviewed in this chapter have received less attention. Lastly, incorporation of aging as a covariate will more closely recapitulate the parkinsonian brain. The fields of PD research and experimental therapeutics stand much to gain in focusing our efforts to refine our use of all of the models reviewed here with respect to our proposed criteria for evaluating a comprehensive model of idiopathic PD.

Chapter 3: TrkB Signaling Is Necessary for the Neuroprotection Provided by Subthalamic Nucleus Deep Brain Stimulation in the Rat 6-Hydroxydopamine Model of Parkinson's Disease

Abstract

High-frequency deep brain stimulation (DBS) of the subthalamic nucleus (STN) is the most common neurosurgical treatment for the alleviation of Parkinson's disease (PD) motor symptoms. Beyond symptomatic efficacy, our laboratory and others have demonstrated that STN DBS provides neuroprotection for dopaminergic neurons of the substantia nigra pars compacta (SNpc) in preclinical models. Further, we have previously demonstrated that STN DBS increases brain-derived neurotrophic factor (BDNF) in the nigrostriatal system and primary motor cortex. However, whether BDNF signaling through its receptor, trkB, participates in the neuroprotective effects of DBS remains unknown. In the present study we investigated the impact of *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (ANA-12), a trkB antagonist, using our STN DBS rat model. We conducted long-term, STN DBS in male, Sprague-Dawley rats that received unilateral, intrastriatal 6-OHDA. Stimulation and ANA-12 treatment were initiated ten days following 6-OHDA. Rats were randomly assigned to receive either continuous Active or Inactive stimulation during the ongoing nigrostriatal degeneration induced by 6-OHDA. Within each stimulation cadre, rats were randomized to receive ANA-12 (0.5 mg/kg) or vehicle twice per day. Rats were sacrificed four weeks after 6-OHDA, an interval that is normally associated with 70-75% SNpc neuron loss. Tyrosine hydroxylase immunoreactive neurons of the SNpc were quantified in rats with verified STN electrode

placement using unbiased stereology. Our data show that chronic ANA-12 administration abolishes the neuroprotective effect of long-term stimulation, highlighting the importance of BDNF-trkB signaling in the neuroprotective effects of STN DBS.

Introduction

Deep brain stimulation (DBS) is currently the gold-standard neurosurgical therapy for Parkinson's disease (PD) with the subthalamic nucleus (STN) as the most commonly targeted site as well as the best studied. Since Food and Drug Administration (FDA) approval in 2002, over 100,000 patients have received STN DBS [375]. Since dopaminergic pharmacotherapy is effective for many years following diagnosis, DBS is only considered once medical management no longer provides adequate control of symptoms; hence, the average patient undergoing DBS surgery is 12-14 years post diagnosis [239].

Apart from its symptomatic benefit, STN DBS has been investigated as a disease-modifying therapy. Conflicting clinical studies have been unable to definitively determine if STN DBS is disease-modifying ([193, 195, 215, 237], cf. [376]). A skeptical interpretation of these studies' conclusions is warranted, however, as they included patients with late-stage PD undergoing electrode implantation. Since striatal terminal loss occurs by four years after diagnosis [45, 46], the window of opportunity for disease modification in PD is a decade earlier than the average time point used in these studies.

In parallel to the conflicting clinical work on the potential disease modification from STN DBS were a series of preclinical studies in parkinsonian animal models. STN DBS was shown to be neuroprotective in the 6-OHDA rat model of PD and in a MPTP-based non-human primate model of PD [134, 242-245].

The mechanism for STN DBS-mediated neuroprotection in preclinical models of PD is currently unknown, yet evidence suggests a role for brain-derived neurotrophic factor (BDNF). Our laboratory was the first to show that STN DBS increases BDNF in the nigrostriatal system and the primary motor cortex in a stimulation-dependent manner [136, 377], and a role for BDNF in DBS has been the subject of other studies as well [246-248]. BDNF promotes survival, growth and synaptic plasticity via activation of its high-affinity receptor tropomyosin-related kinase type B (trkB) and resultant signaling through PLC-γ, PI3K-Akt and Erk [287, 291, 292]. However, the question remains: is BDNF necessary for STN DBS-mediated neuroprotection to occur, or is increased BDNF an epiphenomenon from stimulation? We hypothesize that BDNF-trkB signaling is necessary for our observed, STN DBS-mediated neuroprotection in our progressive, 6-OHDA, rat model of PD.

Methods

Animals

A total of twenty-six, male, Sprague-Dawley rats (Harlan, ≈250 g) were used in this study. Rats were only included in the final analysis if they successfully completed the study and electrode placement adequately targeted the STN as previously described

[135]. Animals were allowed food and water *ad libitum* and were housed in reverse dark-light cycle conditions in an AAALAC approved facility. An Institutional Animal Care and Use Committee specifically approved this study.

Intrastriatal 6-OHDA Injections

Intrastriatal 6-OHDA injections were conducted as described previously [134]. Rats were anesthetized prior to surgery with Equithesin (0.3 ml / 100 g body weight i.p.; chloral hydrate 42.5 mg/ml + sodium pentobarbital 9.72 mg/ml). They received two unilateral, intrastriatial injections (AP +1.6 mm, ML +2.4 mm, DV −4.2 mm and AP +0.2 mm, ML +2.6 mm, DV −7.0 mm relative to bregma) of 6-OHDA (MP Biomedicals, Solon, OH; 5.0 µg/µl 6-OHDA in 0.02% ascorbic acid, 0.9% saline solution, injection rate 0.5 µl/minute, 2.0 µl per site). Drill holes were filled with gel foam or bone wax to prevent entry of cement from electrode placement. These 6-OHDA lesion parameters result in ≈50% loss of substantia nigra pars compacta (SNpc) tyrosine hydroxylase immunoreactive (THir) neurons after two weeks that progresses to ≈75% loss after four weeks and is stable at six weeks [134].

Electrode Implantation

Rats were implanted with electrodes immediately following intrastriatal 6-OHDA injections. Rats were unilaterally implanted (ipsilateral to 6-OHDA injections) with a bipolar, concentric microelectrode (inner electrode projection 1.0 mm, inner insulated electrode diameter 0.15 mm, outer electrode gauge 26, Plastics One, Roanoke, VA) targeted to the dorsal border of the STN (AP −3.4 mm, ML +2.5 mm, relative to bregma

and DV −7.7 mm, relative to the dura mater). Burr holes were drilled in the skull; the electrode was fixed in place using bone screws and dental acrylic. Electrodes were lowered to coordinates corresponding to the dorsal border of the STN in order to minimize damage to the nucleus.

Long-Term, Continuous Stimulation Platform

Rats were randomly assigned to receive stimulation (Active) or not (Inactive). For rats in the Active group, stimulation was continuously delivered in a freely moving setup as previously described [134]. Stimulation was generated by an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments, Sarasota, FL). Stimulation parameters consisted of a frequency of 130 Hz, a pulse width of 60 μs and an intensity of 30-50 μA. At the onset of stimulation, intensity settings were increased until orofacial or contralateral forepaw dyskinesias were observed in order to confirm stimulation delivery, and immediately following a positive dyskinetic response, the intensity was set below the lower limit of dyskinesias, such that no rat was functionally impaired by stimulation. When rats were not being stimulated, they were still physically connected within their stimulator bowls to a commutator for the duration of the behavioral task.

ANA-12 and Vehicle Manufacture and Injection Schedule

N-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide, or ANA-12 (Catalog #4781, Tocris Bioscience, Bristol, United Kingdom),

was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific BP231-1, Lot #072871) and added to a solution of polyethylene glycol 400 (PEG-400, Fluka 81172, Lot #BCBK4383V) and normal saline (Teknova, S5812, Lot #S581204D1301) for a final solution of 5% DMSO, 40% normal saline and 55% PEG 400. For example, for a 10 mL final solution, 5 mg ANA-12 would be dissolved in 500 µL DMSO followed by 4.0 mL normal saline and 5.5 mL PEG 400 mixed by vortex. For an injection of vehicle, all components remain except the ANA-12. For each injection of ANA-12, the compound was dissolved and a new solution made. Within the Active and Inactive arms, rats were randomly assigned to receive ANA-12 or vehicle twice per day, i.p., 0.5 mg/kg from day 10 to day 28 post surgery, resulting in four groups total.

Sacrifice

At four weeks post surgery, rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized normal saline at 37°C followed by ice-cold normal saline. Care was taken to minimize the tissue damage resulting from removing the skull with the electrode still intact. All brains were placed in ice cold normal saline for one minute and then hemisected on the coronal plane at the optic chiasm. The caudal half was fixed in 4% paraformaldehyde for one week and transferred to 30% sucrose in 0.1 M phosphate buffer. The rostral half was immediately flash-frozen in 3-methyl butane and stored at -80°C.

Tyrosine Hydroxylase Immunohistochemistry for SNpc Neurons

Saline-perfused and paraformaldehyde-postfixed brains (caudal half after hemisection) were frozen on dry ice and sectioned at 40 μm thickness using a sliding microtome in six series. One series (i.e., every sixth section) was stained with antisera for tyrosine hydroxylase (TH) using the free-floating method. Tissue was blocked in serum and incubated overnight in primary antisera directed against TH (Chemicon MAB318, mouse anti-TH 1:4000). Cell membranes were permeabilized with the addition of Triton-X (0.3%) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). TH immunoreactive (THir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in Tris buffer. Sections were mounted on subbed slides, dehydrated with ethanol and then xylenes and coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Kluver-Barrera Histology

Every sixth section of the subthalamic nucleus (STN) was stained using Kluver-Barrera histochemistry [378] to evaluate for appropriate targeting of the electrode to the STN. Only rats with correctly positioned electrodes were included in the data analysis. Electrode location was considered to be appropriate if the tip of the electrode was observed within 250 μm of the border of the STN within any of the sections based on previous studies in which current spread was determined [135].

Unbiased Stereology of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to 6-OHDA injection was quantified using unbiased stereology with the optical fractionator principle. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X, and THir neurons were counted according to stereological principles at 60X (NA 1.4). Percent remaining THir neurons of the ipsilateral, lesioned SNpc relative to the contralateral, intact SNpc were calculated. The Schmitz-Hof Coefficients of Error were less than or equal to 0.15 for all analyses.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). The effect of 6-OHDA on THir neuron survival was determined by a two-tailed Student-s t-test by comparing the intact hemisphere to the lesioned one. The impact of chronic ANA-12 administration on the extent of the 6-OHDA lesion was determined by a two-tailed Student's t-test comparing the percent lesion between the two Inactive groups. A one-way ANOVA followed by a least significant difference *post hoc* analysis was conducted to assess differences in THir neuron survival due to ANA-12 administration in rats receiving stimulation or not. Statistical significance was set at $p < 0.05$.

Results

Chronic ANA-12 Administration Does Not Exacerbate Nigral Degeneration

Rats receiving intrastriatal 6-OHDA and Inactive stimulation, regardless of Vehicle or ANA-12 treatment, possessed significantly fewer SNpc THir neurons ipsilateral to the injections/electrode compared to the contralateral SNpc ($t_{(16.29)} = 5.592$, $p < 0.001$). The intact SNpc in Inactive rats possessed 10,518 ± 1,162 THir neurons, whereas the lesioned SNpc contained 3,443 ± 500, or ≈67% fewer THir neurons than the unlesioned SNpc, as expected from this lesion paradigm [134, 379]. No significant difference was observed in the magnitude of degeneration measured in Inactive+Veh vs. Inactive+ANA-12 rats ($t_{(11)} = -1.086$, $p = 0.301$). These results are illustrated in Figure 3. Since there was no significant difference, these two groups were combined for the subsequent analysis.

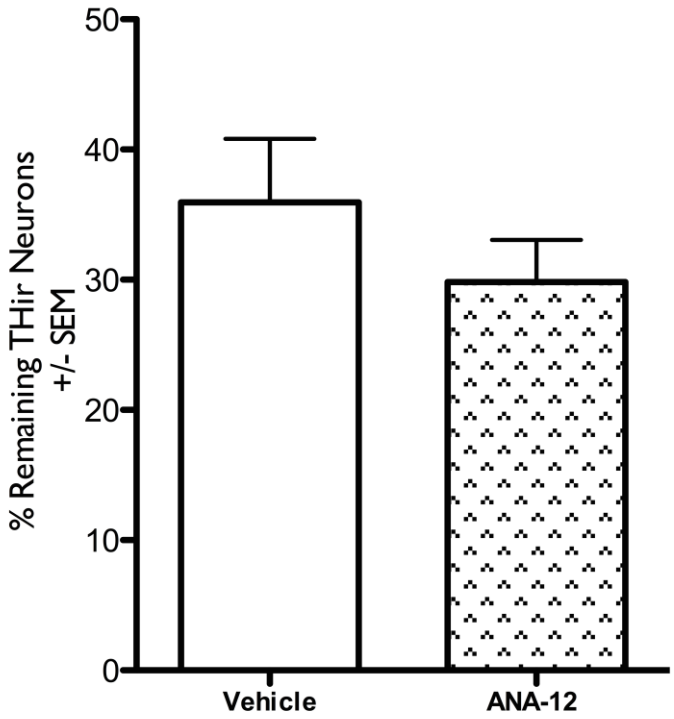

Figure 3. Impact of ANA-12 on 6-OHDA-Induced Degeneration. The percent remaining THir SNpc neurons as measured by unbiased stereology are shown for 6-OHDA-injected rats with implanted but not activated electrodes receiving Vehicle or ANA-12 injections from day 10 to day 28. There is no significant difference between the two groups (p = 0.307).

ANA-12 Abolishes STN DBS-Mediated Neuroprotection

A one-way ANOVA was used to compare three groups having received intrastriatal 6-OHDA: Active stimulation with ANA-12, Active stimulation with Vehicle and Inactive stimulation (combined ANA-12 and Vehicle groups from the previous analysis). Specifically, the Active+ANA-12 group had 23.4 ± 5.1%, the Active+Veh group 43.4 ± 6.0% and the Inactive group 32.2 ± 2.8% remaining THir SNpc neurons, comparing the lesioned side to the intact one. A statistically significant difference was observed

between the groups ($F_{(2,23)} = 4.319$, $p < 0.05$). *Post hoc* comparisons showed a significant difference between the Active+Veh and Active+ANA-12 groups ($p < 0.05$) and a trend toward a difference between the Active+Veh and Inactive groups ($p = 0.066$).

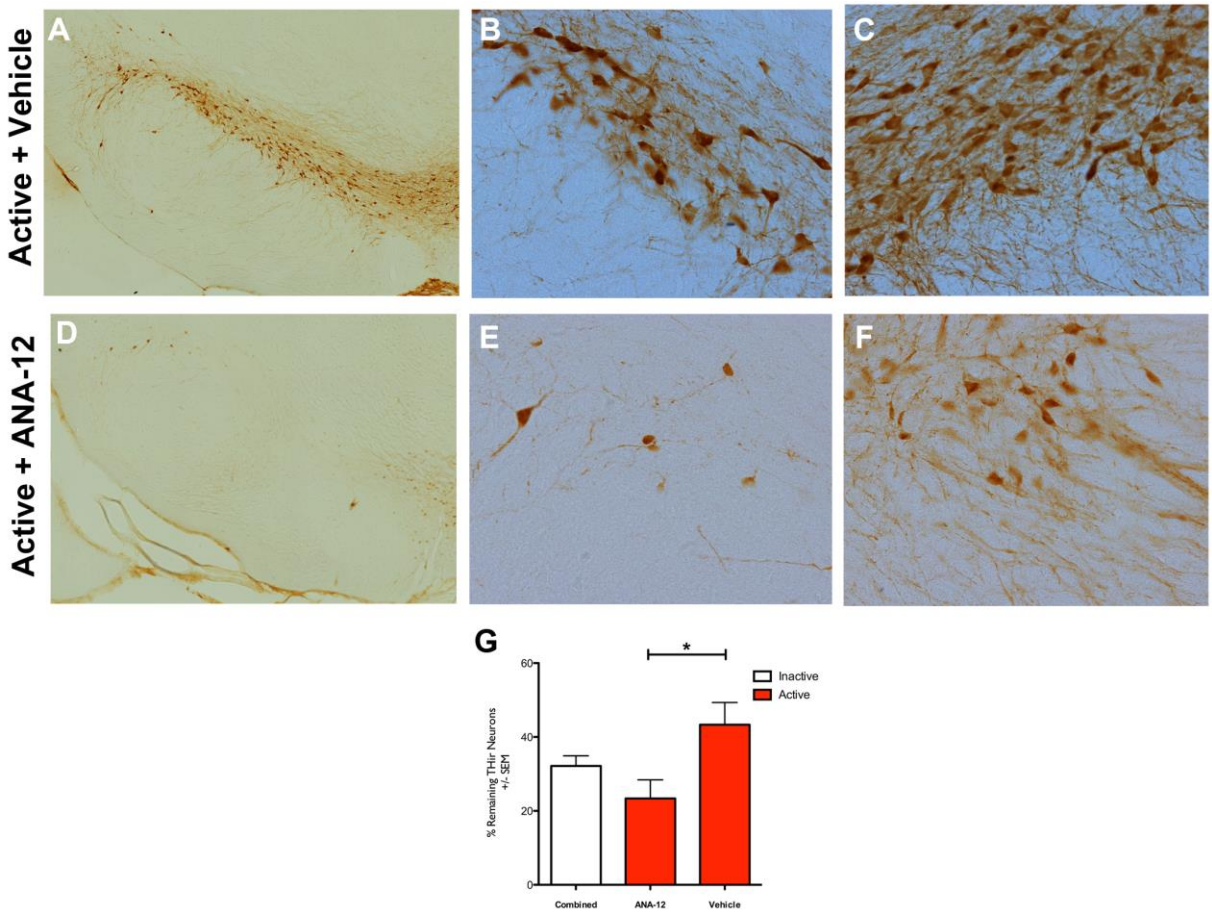

Figure 4. Impact of ANA-12 on STN DBS-Mediated Neuroprotection from 6-OHDA. The lesioned hemisphere for 6-OHDA-injected rats with activated electrodes receiving Vehicle or ANA-12 injections, respectively, from day 10 to day 28 at low magnification (**A, D**) and high magnification (**B, E**) as compared to the intact hemisphere shown at high magnification (**C, F**). **G.** The percent remaining THir SNpc neurons as measured by unbiased stereology are shown for rats with Active electrodes (red bars) or Inactive electrodes (white bar). Active groups received either Vehicle or ANA-12; Vehicle- or ANA-12-injected rats comprise the Inactive group, as per the previous analysis showing no significant difference due to treatment. There is a significant difference between stimulated rats receiving ANA-12 vs. Vehicle ($p < 0.05$).

Discussion

DBS-Mediated BDNF-trkB Signaling as Neurotrophic

STN DBS likely increases BDNF release in an activity-dependent manner. High-frequency stimulation of hippocampal neurons *in vitro* results in release of BDNF [285, 286]. STN DBS *in vivo*, as shown by our laboratory, results in increased measured BDNF in the nigrostriatal system—specifically in the SN of lesioned animals and the striatum of intact ones—and in the primary motor cortex (M1), regardless of lesion status [136]. In addition, we showed a stimulation-dependent increase in BDNF transcript in the SN [136].

BDNF is produced in excess with a pro-domain at the amino terminus and sequestered in synaptic vesicles by sortilin [277]. Activity-dependent release of proBDNF is accompanied by a co-release of tissue plasminogen activator (tPA) that cleaves the pro-domain, thereby producing the 'mature' form of the protein [287-290]. It is the mature form of BDNF that binds to its high-affinity receptor trkB and results in signaling through the PLC-γ, PI3K-Akt and Erk pathways [287, 291, 292]. In contrast, proBDNF released in a non-activity-dependent manner (i.e., constitutive release) is not accompanied by tPA, so it retains its pro-domain. proBDNF instead binds the low-affinity receptor p75 and initiates a different set of signaling pathways that are generally pro-apoptotic or facilitate long-term depression (LTD) [287].

ANA-12 is a low-molecular weight ligand for trkB that readily crosses the blood-brain barrier. When ANA-12 binds trkB, it blocks BDNF from binding with a high potency and

shows decreased downstream signaling for trkB but without changing trkA or trkC functions [380]. Intraperitoneal administration of ANA-12 results in peak plasma and brain levels within an hour, but attenuation of trkB phosphorylation is observed at least four hours post injection [380]. Since antagonism of trkB via ANA-12 in the present experiment results in a loss of neuroprotection, it stands to reason that STN DBS leads to the activity-dependent release of BDNF, which includes tPA for cleavage of the pro-domain, the subsequent binding of trkB and the activation of its canonical signaling pathways that in the parkinsonian rat are neuroprotective.

BDNF and trkB Are the Players, But On Which Playing Field?

The use of ANA-12 for examining the role of BDNF in STN DBS-mediated neuroprotection was a double-edged sword. Systemic administration of ANA-12 allows for targeting of all trkB-expressing cells, and as trkB is expressed ubiquitously [381-386], essentially the entire brain is implicated. If ANA-12 had no effect on STN DBS-mediated neuroprotection, then acceptance of the null hypothesis would rule out any further investigation of trkB signaling pathways. However, rejection of the null hypothesis leaves the issues of identifying within which system(s) trkB antagonism blocked the neuroprotective effects and the source(s) of BDNF.

trkB signaling is firstly an intracellular process that exerts pro-survival effects on the same cell. Disruption of trkB signaling on DA neurons of the SNpc is the most likely location for the disruption of the neuroprotective effects of STN DBS by ANA-12; however, trkB is present at both the soma and the synapse [387]. In our intrastriatal 6-

OHDA model, a near complete loss of THir terminals in the striatum is observed within twenty-four hours of 6-OHDA administration [134], so it is likely that the neuroprotective trkB signaling occurs at the soma. As decreased trkB expression is observed at the somata and dendrites of the DA SNpc neurons in postmortem PD patients [298], it may be that decreased somatic trkB expression and signaling is a component of PD pathophysiology.

The source(s) of BDNF for STN DBS-mediated neuroprotection is/are also unknown, though our laboratory's previous work provides clues. In rats that received stimulation but were not injected with 6-OHDA, stimulation-dependent increases in BDNF were observed in the ipsilateral striatum; however, lesioned animals showed BDNF increases in the ipsilateral SN but not in the striatum [136]. BDNF transcript was increased in the SN regardless of lesion status [136]. Since increased glutamate, as is present in STN projections to the SN and is measurably increased in the SN with STN DBS (Windels 2000; Boulet 2006), can increase postsynaptic BDNF production [278, 279], we hypothesize that STN DBS increases in glutamate lead to increases in SNpc BDNF production, and in the case of intact/unlesioned rats, is transported to the terminals for activity-dependent release. BDNF release then acts in an autocrine/paracrine fashion to provide neuroprotection to the SNpc (Figure 5, panel A). As an alternative, the neuroprotective BDNF may be released from subthalamic, glutamateric projections to the SN (Figure 5, panel B).

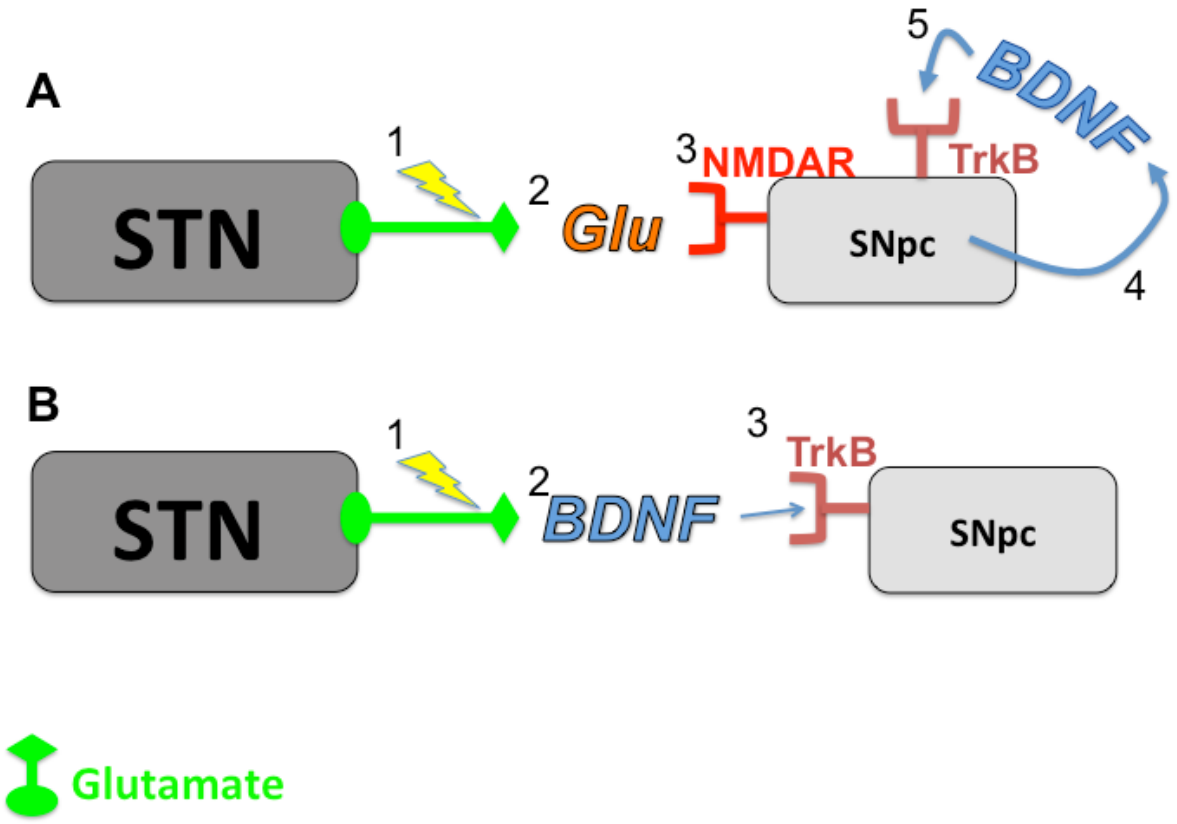

Figure 5. Proposed Mechanisms for BDNF-Mediated Neuroprotection. A. STN stimulation (1) increases activity, (2) increases glutamate (Glu) release at the SNpc and (3) binding to NMDA receptors (NMDAR). SNpc activation results in production of BDNF transcript, translation and (4) activity-dependent release of BDNF that (5) binds to trkB for an autocrine/paracrine trophic effect. **B.** STN stimulation (1) increases activity, (2) increases activity dependent release of BDNF at the SNpc and (3) binding to trkB for a trophic effect.

Future Directions – Novel, Experimental Therapies?

With the added knowledge of trkB involvement in STN DBS-mediated neuroprotection, an avenue is opened for evaluating new experimental therapies. STN DBS is the neurosurgical gold-standard for PD, but as an invasive procedure, it carries with it a set of risks that are inherent to functional neurosurgery. As such, despite increasing demand and use by PD patients, the PD research community has been reluctant to expand its scope with only recent efforts at examining its use in early- to mid-stage disease states [240, 241], let alone the issue of examining its disease-modifying potential. However, the hurdle for examining a new pharmacotherapy is lower.

Further investigation into harnessing trkB signaling as a neuroprotective strategy is warranted. At least two medications should be considered. First, a selective trkB agonist that is orally bioavailable and crosses the blood-brain barrier called 7,8-dihydroxyflavone should be further tested in preclinical model of PD [388, 389]. Second, an FDA-approved drug for multiple sclerosis, fingolimod [390], should be vetted in PD animal models, as it has shown to increase the activity-dependent release of BDNF in a mouse model of Rett syndrome [391] and in other contexts [392, 393]. Querying a compound library may also yield potential drug candidates.

Pharmacotherapies, however, lack neuroanatomical specificity, resulting in off-target effects that may limit the clinician's ability to titrate the dose to a high-enough level for the therapeutic effect. Gene therapy approaches offer specificity in both phenotype and neuroanatomy. BDNF-trkB signaling could be modulated via viral vectors through increasing either BDNF or trkB (or both). DREADDs (Designer Receptors Exclusively

Activated by Designer Drugs) could also be used to harness specific downstream signaling pathways of trkB. However, the source nor the target of STN DBS-mediated BDNF-trkB signaling is definitely known, so these therapeutic approaches require additional exploration of our phenomenon. (Proposed experiments for continued investigation into this mechanism are discussed in Chapter 7.)

Chapter 4: TrkB Signaling Contributes to the Relief of Symptoms Provided by Subthalamic Nucleus Deep Brain Stimulation in the Rat 6-Hydroxydopamine Model of Parkinson's Disease

Abstract

High-frequency deep brain stimulation (DBS) of the subthalamic nucleus (STN) is the most common neurosurgical treatment for the alleviation of Parkinson's disease (PD) motor symptoms. Beyond symptomatic efficacy, our laboratory and others have demonstrated that STN DBS provides neuroprotection for dopaminergic neurons of the substantia nigra pars compacta (SNpc) in preclinical models. Further, we have previously demonstrated that STN DBS increases brain-derived neurotrophic factor (BDNF) in the nigrostriatal system and primary motor cortex. However, whether BDNF signaling through its receptor, trkB, participates in the behavioral effects of DBS remains unknown. In the present study we investigated the impact of ANA-12, a trkB antagonist, using our STN DBS rat model. We conducted STN DBS in a cohort of rats with stable, near-complete unilateral nigrostriatal degeneration six weeks following intrastriatal 6-OHDA. Contralateral forelimb use was then assessed in rats receiving ANA-12 (0.5 mg/kg) or vehicle during acute STN DBS. Our results show that ANA-12 attenuates the behavioral effects of STN DBS, highlighting the importance of BDNF-trkB signaling in the alleviation of motor symptoms by STN DBS.

Introduction

High-frequency deep brain stimulation (DBS) is the gold-standard neurosurgical therapy for Parkinson's disease (PD) with over 100,000 patients having undergone surgery

[375]. Whereas a recent study showed equal efficacy in alleviation of motor symptoms between the stimulation sites of the globus pallidus interna (GPi) and the subthalamic nucleus (STN) [181], the STN was the preferred target for years and has received far more attention in the clinic and the laboratory. However, even with advances in patient selection for neurosurgery and the increasing experience of movement disorders clinics with targeting and programming, the therapeutic response to stimulation is still exceedingly wide – not all patients enjoy the same benefit for which DBS is known. Indeed, the heterogeneity in responses to anti-parkinsonian therapies is currently a common theme in the field. The National Institute of Neurological Disorders and Stroke (NINDS) PD 2014 Recommendations stated: “Recent research has begun to reveal the molecular and clinical heterogeneity of PD, a complex neurodegenerative process that likely derives from multiple molecular drivers that vary among individuals, act over years prior to clinical expression, underlie an individual’s ensemble of motor and non-motor symptoms, and likely dictate response to treatment and its complications” [394].

Preclinical studies have shown that the STN DBS clinical paradigm can be modeled in the rat and non-human primate [135, 136, 227, 242, 395-403]. Unilateral depletion of striatal dopamine (DA) via intrastriatal 6-hydroxydopamine (6-OHDA) injections results in motor impairments on the contralateral side, as measured by spontaneous forelimb akinesia in the cylinder task, amphetamine-induced rotations etc. [98-100, 134, 135]. Alleviation of these motor symptoms can be achieved through STN DBS, even over a very short time period as is observed in the clinic [135, 136, 227, 242, 395-400]. As

such, questions regarding the therapeutic efficacy STN DBS for PD can be asked in the parkinsonian rat.

In regard to the mechanism of DBS, the role of glutamate must be underscored. STN DBS *in vivo* alters glutamate transmission by increasing STN activity [222, 223] and increasing glutamate release in the substantia nigra pars reticulata (SNpr) and GPi [224, 225]. In addition, activation of STN afferents and the hyperdirect pathway from the primary motor cortex to the STN may explain the therapeutic effects of STN DBS [228, 229].

Several groups have investigated the neuroprotective potential of STN DBS in preclinical models [134, 242-245]. In these studies, a role for glutamate has emerged in its connection to activity-dependent release of brain-derived neurotrophic factor (BDNF). High-frequency stimulation of cultured hippocampal neurons results in released dendritic and later axonal BDNF [285, 286]. In addition, glutamate release in the basal ganglia increases BDNF transcript in the SN [136]. Our laboratory was the first to observe a stimulation-dependent increase in BDNF in the nigrostriatal system [136]. The canonical signaling pathway for BDNF through its high-affinity receptor tropomyosin-related kinase type 2 (TrkB) results in activation of PLC-γ, PI3K-Akt and Erk. These pathways instigate the production of proteins and a cellular response that ultimately promote survival and growth as well as remodeling at the synapse for long-term potentiation (LTP) related changes [287, 291, 292]. Given these data, BDNF is an important player in the neurobiological response to stimulation; however, its role

heretofore has only related to the long-term, neuroprotective effects of STN DBS in PD models.

BDNF may exert effects within the paradigm of STN DBS for PD beyond its canonical 'pro-growth' role. For instance, BDNF affects DA transmission. In a heterozygous BDNF knockout mouse, which exhibits decreased BDNF levels, there is decreased DA release and DA transporter (DAT) function, and these effects are reversed with replacement of BDNF [299]. BDNF can also affect glutamatergic transmission in a more acute timescale. TrkB signaling through the PI3K-Akt pathway phosphorylates the 2B subunit of the NMDA receptor for glutamate well within an hour [295]. Since the symptomatic benefit of STN DBS is intimately connected to alterations in STN activity and glutamate transmission in the basal ganglia [222-225, 227-236], and long-term STN DBS results in increases in BDNF [136, 404], is there a role for BDNF-trkB signaling in the therapeutic effect of stimulation? We sought to answer this question in the present work using our long-term stimulation platform and our well-characterized 6-OHDA rat model of PD. We chose to use the compound *N*-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide (ANA-12) since it crosses the blood-brain barrier and specifically and potently blocks trkB signaling about four hours after intraperitoneal administration [380]. In this experiment, ANA-12 was given at least four hours before the animal was subjected to behavioral assessment in order to coordinate the timing of the drug's effect and the primary outcome measure.

Methods

Animals

A total of ten, male, Sprague-Dawley rats (Harlan, ≈250 g) were used in this study. Rats were only included in the final analysis if they successfully completed the study and electrode placement adequately targeted the STN as previously described [135]. Animals were allowed food and water *ad libitum* and were housed in reverse dark-light cycle conditions in an AAALAC approved facility. An Institutional Animal Care and Use Committee specifically approved this study.

Intrastriatal 6-OHDA Injections

Intrastriatal 6-OHDA injections were conducted as described previously [134]. Rats were anesthetized prior to surgery with Equithesin (0.3 ml / 100 g body weight i.p.; chloral hydrate 42.5 mg/ml + sodium pentobarbital 9.72 mg/ml). They received two unilateral, intrastriatial injections (AP +1.6 mm, ML +2.4 mm, DV −4.2 mm and AP +0.2 mm, ML +2.6 mm, DV −7.0 mm relative to bregma) of 6-OHDA (MP Biomedicals, Solon, OH; 5.0 µg/µl 6-OHDA in 0.02% ascorbic acid, 0.9% saline solution, injection rate 0.5 µl/minute, 2.0 µl per site). Drill holes were filled with gel foam or bone wax to prevent entry of cement from electrode placement. These 6-OHDA lesion parameters result in ≈50% loss of substantia nigra pars compacta (SNpc) tyrosine hydroxylase immunoreactive (THir) neurons after two weeks that progresses to ≈75% loss after four weeks and is stable at six weeks [134].

Electrode Implantation

Rats were implanted with electrodes immediately following intrastriatal 6-OHDA injections. Rats were unilaterally implanted (ipsilateral to 6-OHDA injections) with a bipolar, concentric microelectrode (inner electrode projection 1.0 mm, inner insulated electrode diameter 0.15 mm, outer electrode gauge 26, Plastics One, Roanoke, VA) targeted to the dorsal border of the STN (AP −3.4 mm, ML +2.5 mm, relative to bregma and DV −7.7 mm, relative to the dura mater). Burr holes were drilled in the skull; the electrode was fixed in place using bone screws and dental acrylic. Electrodes were lowered to coordinates corresponding to the dorsal border of the STN in order to minimize damage to the nucleus.

ANA-12 and Vehicle Manufacture

N-[2-[[(Hexahydro-2-oxo-1*H*-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide, or ANA-12 (Catalog #4781, Tocris Bioscience, Bristol, United Kingdom), was dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, BP231-1, Lot #072871) and added to a solution of polyethylene glycol 400 (PEG-400, Fluka, Catalog #81172, Lot #BCBK4383V) and normal saline (Teknova, Catalog #S5812, Lot #S581204D1301) for a final solution of 5% DMSO, 40% normal saline and 55% PEG 400. For example, for a 10 mL final solution, 5 mg ANA-12 would be dissolved in 500 µL DMSO followed by 4.0 mL normal saline and 5.5 mL PEG 400 mixed by vortex. For an injection of vehicle, all components remain except the ANA-12. For each injection of ANA-12, the compound was dissolved and a new solution made.

Behavioral Testing

Spontaneous forelimb use was assessed using the cylinder task as previously described [99, 136, 405]. Other behavioral measures were not employed due to their incompatibility with the external hardware required for continuous stimulation in an awake animal. Before each behavioral assay, rats received injections of either ANA-12 or vehicle (0.5 mg/kg, i.p.) roughly within two hours of the start of the dark cycle, stimulation was turned on 3.5 to 4.0 hours after injection and behavior was assessed at least four hours post injection (i.e., rats were assayed having adapted to stimulation parameters at least 30 minutes). The cylinder task was employed at the following timepoints under the conditions listed: (a) prior to surgery, vehicle injected (b) six weeks following surgery, off stimulation, vehicle injected (c) 6.5 weeks following surgery, on stimulation, vehicle injected and (d) 7.5 weeks following surgery, on stimulation, ANA-12 injected. For six of the ten rats, they were assayed as well at: (e) 9.5 weeks following surgery, on stimulation, vehicle injected and (f) 10 weeks following surgery, off stimulation, vehicle injected.

During the dark cycle, rats were videotaped and placed in a clear plexiglass cylinder until twenty, weight-bearing forelimb placements on the side of the cylinder occurred, or until a maximum trial time of five minutes had elapsed. To determine if forelimb preference was present, the number of contralateral, ipsilateral, and simultaneous paw placements was quantified. Data are reported as the percentage of contralateral (to 6-OHDA and electrode) forelimb use: [(contralateral + ½ both)/(ipsilateral + contralateral + both)] x 100%. Rats with a unilateral nigrostriatal lesion will show a bias toward using the ipsilateral limb. A forelimb deficit was defined as possessing a minimum of a 20%

relative reduction in contralateral forepaw use compared to baseline; inclusion in this study required a forelimb deficit at the second behavioral task six weeks post surgery.

Continuous Stimulation Paradigm

When rats were to receive stimulation at a given time, stimulation was continuously delivered in a freely moving setup as previously described [134]. Stimulation was generated by an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments, Sarasota, FL). Stimulation parameters consisted of a frequency of 130 Hz, a pulse width of 60 μs and an intensity of 30-50 μA. At the onset of stimulation, intensity settings were increased until orofacial or contralateral forepaw dyskinesias were observed in order to confirm stimulation delivery, and immediately following a positive dyskinetic response, the intensity was set below the lower limit of dyskinesias, such that no rat was functionally impaired by stimulation. When rats were not being stimulated, they were still physically connected within their stimulator bowls to a commutator for the duration of the behavioral task.

Sacrifice

At eleven weeks post surgery, rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized normal saline at 37°C followed by ice-cold normal saline. Care was taken to minimize the tissue damage resulting from removing the skull with the electrode still intact. All brains were placed in ice cold normal saline for one minute and then hemisected on the coronal plane at the optic

chiasm. The caudal half was fixed in 4% paraformaldehyde for one week and transferred to 30% sucrose in 0.1 M phosphate buffer. The rostral half was immediately flash-frozen in 3-methyl butane and stored at -80°C.

Tyrosine Hydroxylase Immunohistochemistry for SNpc Neurons

Saline-perfused and paraformaldehyde-postfixed brains (caudal half after hemisection) were frozen on dry ice and sectioned at 40 µm thickness using a sliding microtome in six series. One series (i.e., every sixth section) was stained with antisera for tyrosine hydroxylase (TH) using the free-floating method. Tissue was blocked in serum and incubated overnight in primary antisera directed against TH (Chemicon MAB318, mouse anti-TH 1:4000). Cell membranes were permeabilized with the addition of Triton-X (0.3%) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). TH immunoreactive (THir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in Tris buffer. Sections were mounted on subbed slides, dehydrated with ethanol and then xylenes and coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Kluver-Barrera Histology

Every sixth section of the subthalamic nucleus (STN) was stained using Kluver-Barrera histochemistry [378] to evaluate for appropriate targeting of the electrode to the STN. Only rats with correctly positioned electrodes were included in the data analysis.

Electrode location was considered to be appropriate if the tip of the electrode was observed within 250 μm of the border of the STN within any of the sections based on previous studies in which current spread was determined [135].

Unbiased Stereology of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to 6-OHDA injection was quantified using unbiased stereology with the optical fractionator principle. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X, and THir neurons were counted according to stereological principles at 60X (NA 1.4). Percent remaining THir neurons of the ipsilateral, lesioned SNpc relative to the contralateral, intact SNpc were calculated. The Schmitz-Hof Coefficients of Error were less than or equal to 0.15 for all analyses.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). A two-way RM-ANOVA followed by a least significant difference *post hoc* analysis was conducted to confirm the presence of functional deficits, the behavioral response to DBS and the effect of ANA-12 on spontaneous forelimb use. Differences in THir neuron survival were determined by a two-tailed Student's t-test and comparing the unlesioned hemisphere to the lesioned hemisphere. Statistical significance was set at $p < 0.05$.

Results

ANA-12 Attenuates Functional Efficacy of STN DBS

A RM-ANOVA was used to compare spontaneous forelimb use across four time points at 0 (just before surgery), 6, 6.5 and 7.5 weeks post surgery. Values for spontaneous forelimb use are expressed as a percent of contralateral (to the lesion) forepaw use of the total. Averages for each time point are as follows: 52.0 ± 2.1% on week 0 ("Baseline"); 24.5 ± 3.4% on week 6 ("Lesion"); 47.0 ± 3.7% on week 6.5 ("Stim-On"); and 33.8 ± 5.5% on week 7.5 ("ANA-12"). A statistically significant difference was observed between the time points ($F_{(3,27)} = 13.465$, $p < 0.001$). A *post hoc* analysis showed a significant difference between: Baseline vs. Lesion ($p < 0.001$), Lesion vs. Stim-On ($p < 0.001$) and Stim-On vs. ANA-12 ($p < 0.05$). There was a trend toward a significant difference for the Lesion vs. ANA-12 comparison ($p = 0.108$). Results are depicted in Figure 6.

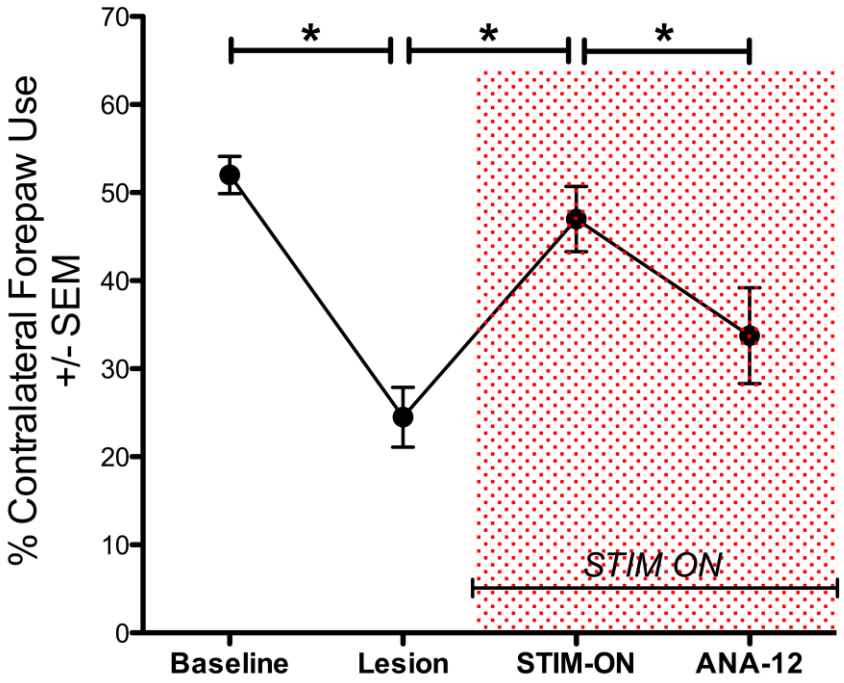

Figure 6. TrkB Signaling Contributes to Functional Efficacy of STN DBS. Rats were assessed for forelimb asymmetry using the cylinder task before surgery with vehicle ("Baseline"), six weeks after intrastriatal 6-OHDA with vehicle ("Lesion"), receiving acute STN stimulation with vehicle ("STIM-ON") and receiving stimulation with ANA-12 ("ANA-12"). Significant differences were observed for Baseline vs. Lesion ($p < 0.001$), Lesion vs. STIM-ON ($p < 0.001$) and STIM-ON vs. ANA-12 ($p < 0.05$).

A subset of rats were further assessed by the cylinder task, after the above data were collected, on two more occasions: 9.5 weeks following surgery, on stimulation, vehicle injected and 10 weeks following surgery, off stimulation, vehicle injected. The former measurement showed an average contralateral forepaw use of 27.4 ± 9.2%, and for the latter measurement, 26.3 ± 9.1%.

Unilateral 6-OHDA Effectively Lesions the SNpc

All rats included in this study had significantly fewer THir SNpc neurons on the side ipsilateral to the injections of 6-OHDA and the indwelling electrode lead compared to the contralateral SNpc ($t_{(18)} = 4.143$, $p < 0.001$). There was an approximate 42% loss with the intact and lesioned sides having 10370 ± 1137 and 4001 ± 1035 THir SNpc neurons, respectively. These data are depicted in Figure 7.

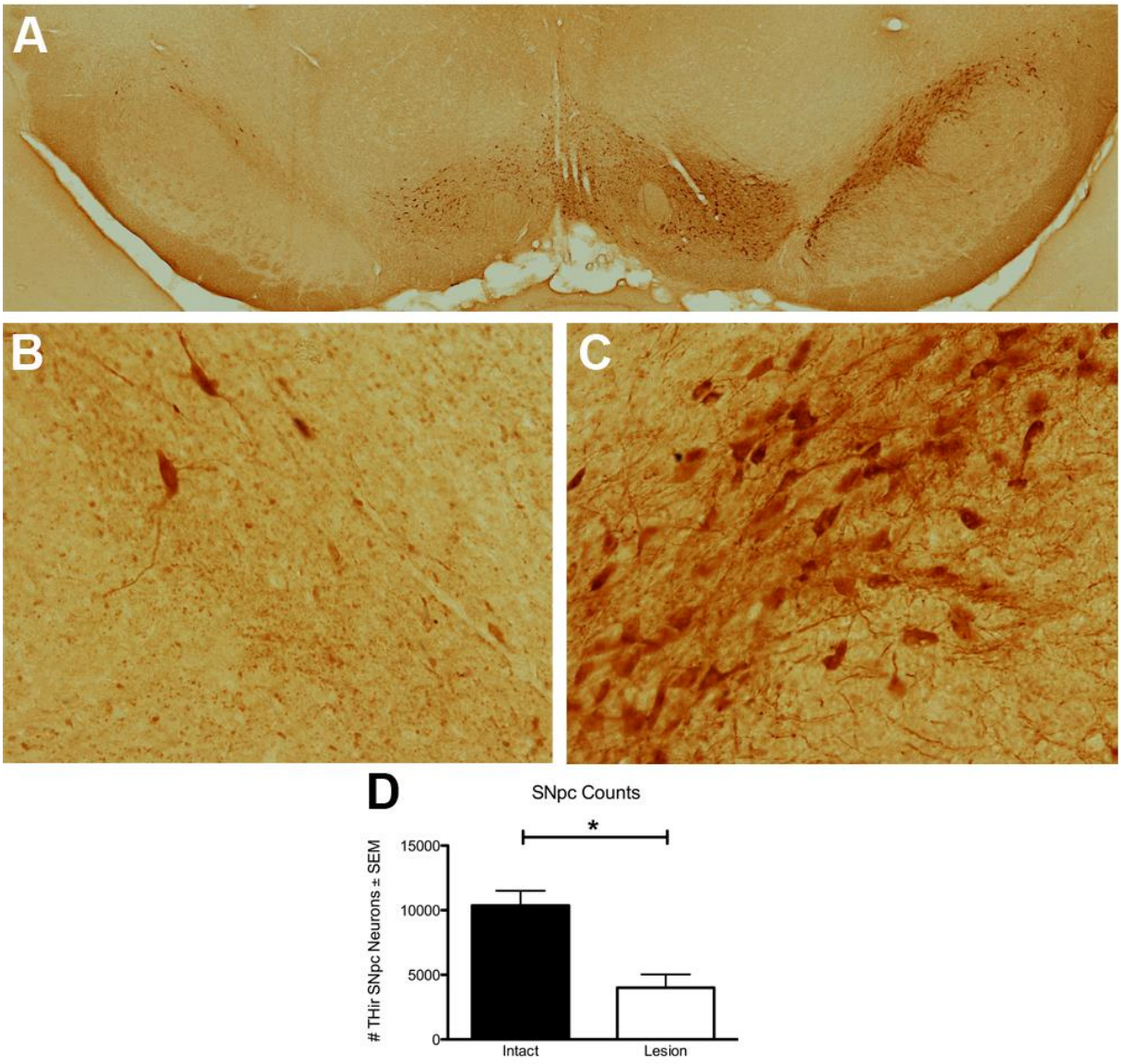

Figure 7. 6-OHDA Effectively Lesioned the SNpc. **A.** A low magnification view of a representative section showing the lesioned SNpc on the left side. Higher magnification views of the lesioned and intact SNpc are shown in **B** and **C**, respectively. **D.** The average number of THir SNpc neurons on the intact or lesioned sides is shown, and there was a statistically significant difference between the sides ($p < 0.001$).

Discussion

Spontaneous forelimb use as assessed by the cylinder task was used to measure unilateral akinesia, as we have demonstrated in this model before [136]. In contrast to our previous work [136], we chose for the behavioral deficit, not the stereological counts of THir SNpc neurons, to serve as the primary criterion for whether an animal's data were included for having a sufficient lesion. This decision was based on the fact that our primary question relied on behavioral data, so a robust behavioral deficit from 6-OHDA seemed the best choice. After all, it is not the loss of SNpc neurons *per se* but the loss of striatal DA that results in parkinsonian motor symptoms [20-25, 45, 46].

The timing of drug administration and stimulation relative to the behavioral assays is critically important for interpretation of this experiment's data. ANA-12 has a relatively short plasma half-life, but its antagonistic effects on trkB phosphorylation, which is the activated form, are significantly decreased four hours after a single, intraperitoneal dose [380]. In this experiment, the cylinder task was used at least four hours after vehicle or ANA-12 administration. Electrode leads were activated to deliver stimulation at least thirty minutes prior to the cylinder task, thereby allowing the animals enough time to adjust to the stimulation. The benefits of stimulation are in place almost immediately after it begins, both in the clinic and in our platform. The cylinder task, then, is assaying the rat's functional ability in the context of dampened trkB signaling and acute stimulation, where 'acute' stimulation is on the order of minutes, not hours. Of note, ANA-12 had lingering effects on the system since stimulation even weeks after a single ANA-12 injection was unable to fully recover to provide the same, robust response to stimulation as before in the Stim-ON condition.

The canonical description for trkB signaling involves long-term changes. Ligand binding followed by autophosphorylation leads to activation of PLC-γ, PI3K-Akt and Erk that in turn promote cell survival and growth through changes in gene transcription and production of a new cadre of proteins [287, 291, 292]. Effects of canonical trkB signaling, then, generally occur on the order of hours. The phenomenon described in this experiment though seems to fit a different time course. Antagonism of trkB in a single dose attenuates the robust, functional response to stimulation that our laboratory normally observes. Ergo, another trkB-mediated effect must explain our data.

In recent years, a non-canonical trkB signaling mechanism has emerged. Through the canonical activation of PI3K-Akt, a tangential pathway leads to Girdin phosphorylation and subsequent combination and activation of Src [295, 296]. This activated unit then phosphorylates the neuron's NMDA receptor 2B subunit (NR2B) [295, 296]. NR2B phosphorylation potentiates the response by NMDA receptor-mediated currents [295, 297]. This non-canonical pathway, which takes place over a shorter time course than the canonical trkB signaling pathways, has been used to explain other phenomena with behavioral measures [296, 406]. Of importance for our experiment, decreased BDNF levels in a heterozygous knockout mouse result in a decreased release of DA as well as decreased DAT function that can be partially reversed through BDNF administration [299].

Using ANA-12, a systemic drug with no specificity in its neuroanatomical target, in this experiment allowed for all of the trkB receptors to be queried as to whether they are involved in STN DBS-mediated functional/behavioral effects. Since ANA-12 did have an effect, then all of the trkB receptors are implicated. Hence, as was discussed in the previous chapter, the source of BDNF and the location of the trkB receptors that specifically modulate this behavioral effect cannot be known from this experiment; however, there are some likely possibilities.

In our previous work, our laboratory showed that STN DBS increases BDNF in the primary motor (M1) cortex. Gradinaru et al. [228] demonstrated using optogenetics that the functional efficacy of STN DBS can be explained through activation of the hyperdirect pathway, a set of fibers from Layer V neurons in the M1 cortex that project to the STN. As these projections are glutamatergic, they may also release BDNF to act on trkB receptors found on STN neurons. As an alternative, the rat has a proportion of STN fibers, which are also glutamatergic, that innervate the M1 cortex [407]. BDNF may then be released by STN neurons at the M1 cortex and act on trkB receptors found there. However, these reciprocal, subthalamocortical fibers are not present in primates [408], so it is unlikely that the clinical phenomenon can be explained by these fibers alone.

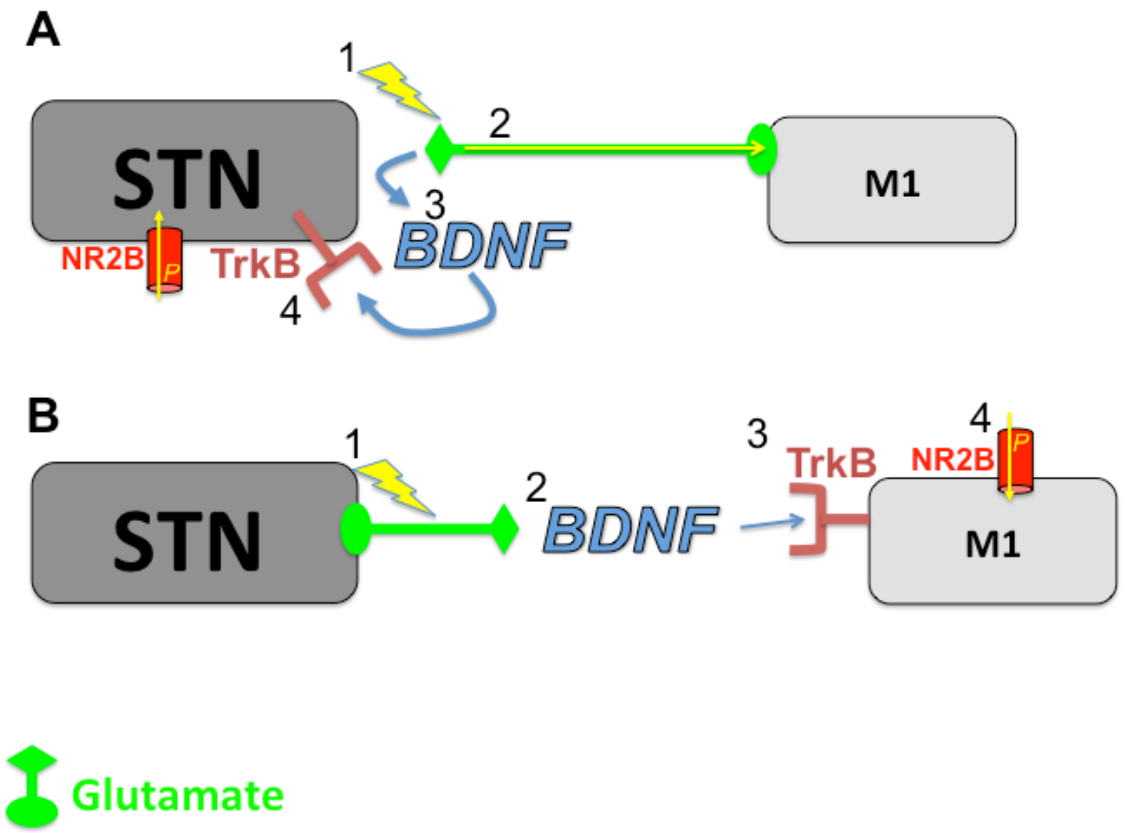

Figure 8. Proposed Mechanisms for BDNF-Modulated Functional Efficacy of STN DBS. **A.** STN stimulation (1) results in (2) antidromic activation of glutamatergic M1 fibers. Increased M1 activity leads to (3) activity-dependent release of BDNF and subsequent (4) activation of trkB and non-canonical signaling for NR2B phosphorylation, increased calcium influx and increased STN activity. **B.** STN stimulation (1) results in (2) activity-dependent release of BDNF from subthalamocortical, glutamatergic projections that are found in the rat. Activation of TrkB on M1 neurons results in non-canonical signaling, phosphorylation of NR2B and increased calcium influx.

The knowledge that trkB signaling contributes to the functional efficacy of STN DBS is the opening of a new avenue for anti-parkinsonian therapies. Dopaminergic pharmacotherapies comprise the vast majority of PD therapies to date. Harnessing the non-canonical pathway of trkB signaling is a potential option for new medications. One such medication is 7,8-dihydroxyflavone, a selective trkB agonist that is orally bioavailable and readily crosses the blood-brain barrier [388, 389]. Encouraging the system to release more BDNF in order to increase trkB signaling is another option through the use of fingolimod, an FDA-approved drug for multiple sclerosis that has seen positive results in several disease states including PD [390-393].

Should pharmacotherapies not prove successful in taking advantage of non-canonical trkB signaling, some neurosurgical approaches are available. The issue in patients may be deficient trkB expression in the appropriate neuronal compartment, as in at least one study [298], so a viral vector-mediated overexpression approach could be employed. Another approach would be viral vector delivery of an engineered DREADD (Designer Receptor Exclusively Activated by Designer Drugs) in order to specifically activate the non-canonical trkB signaling pathway. These approaches, though, would require additional characterization of our phenomenon, specifically in identifying the source of BDNF and the location of the trkB receptors that are mediating this effect.

Chapter 5: The Neuroprotective Potential of Subthalamic Nucleus Deep Brain Stimulation in a Viral Vector-Mediated Nigrostriatal α-Synuclein Overexpression Model of Parkinson's Disease

Abstract

Deep brain stimulation (DBS) is the most common neurosurgical treatment for the alleviation of Parkinson's disease (PD) motor symptoms. However, the current practice of employing subthalamic nucleus (STN) DBS as a treatment of late-stage disease, after the majority of nigral dopamine (DA) neurons and DAergic innervation in the putamen has degenerated, has prevented our ability to evaluate its disease-modifying potential. Our laboratory and others have demonstrated that STN DBS provides neuroprotection for DA neurons of the substantia nigra (SN) in neurotoxicant models of PD. One major limitation of these previous studies is that the predictive validity of the PD neurotoxicant models is low. In the present study, we sought to determine whether STN DBS applied in a model of alpha-synuclein (α-syn) nigrostriatal toxicity is neuroprotective. A large body of evidence points to α-syn involvement in PD. Further, α-syn overexpression targeted to the nigrostriatal system via direct, intranigral injections of viral vector overexpressing α-syn results in a neuropathological and behavioral phenotype that recapitulates key features of PD. Young-adult, male rats received two, 2.0 µl, unilateral, intranigral injections of recombinant adeno-associated virus pseudotype 2/5 (rAAV2/5) expressing human wildtype α-synuclein (α-syn, 1.2×10^{13} genome copies per ml). Rats were implanted ipsilaterally with a DBS electrode in the STN 18 days following vector injections and assigned to either Active stimulation (n = 6) or no stimulation (Inactive, n = 8) treatment groups. An additional group of rats received

α-syn vector injections with no electrode implantation (n = 5). Active rats received continuous STN stimulation for four weeks starting week five after vector surgery (130 Hz, 60 μs, amperage adjusted below the level of dyskinesia). Rats in the Inactive group received no stimulation during the same four-week interval and served as a critical control for the effects of electrode implantation. Cylinder task was used to track functional effects over the lesion time course and verify electrode patency. A significant forepaw use asymmetry developed over time in all groups, and acute stimulation exacerbated this effect. Nigrostriatal α-syn transduction and STN electrode placement were verified using immunohistochemical and histochemical methods, respectively. Stereological quantification of tyrosine hydroxylase immunoreactive (THir) SN neurons and THir striatal terminal density showed little to no loss of nigral DA neurons with modest alterations in striatal terminal density across groups, though all groups showed equivalent striatal α-syn expression. We conclude that STN DBS does not offer a disease modifying effect in the viral vector-mediated α-syn overexpression model of PD.

Introduction

The current gold-standard and *de facto* neurosurgical therapy for Parkinson's disease (PD) is deep brain stimulation of the subthalamic nucleus (STN DBS) with over 100,000 patients having received surgery since approval in the United States in 2002 [375]. Current practice is to consider DBS after adequate control of symptoms can no longer be achieved through medical management. Since the available pharmacotherapies are quite effective in early to mid-stage PD, the average patient undergoing DBS surgery is 12-14 years post diagnosis.

Only a minority of clinicians and researchers has supported the investigation of STN DBS as a disease-modifying therapy. Early clinical studies yielded conflicting conclusions ([193, 195, 215], cf. [376]), though they all shared the same average disease duration at time of implantation, namely over ten years from diagnosis. The window of opportunity for disease modification in PD, however, is within four years after diagnosis since striatal terminal loss is complete by that time [45, 46].

As clinical research has yet to provide a definitive conclusion, preclinical studies in parkinsonian animal models have attempted to do so. STN DBS was shown to be neuroprotective in the 6-OHDA rat model of PD and in a MPTP-based non-human primate model of PD [134, 242-245]. Despite these optimistic findings, the PD research community has been slow to commit to conducting a clinical trial of STN DBS in early-stage PD (about 0 – 4 years post diagnosis)—only now is one in the planning stages by Dr. P. David Charles at Vanderbilt University, having completed a successful Phase I (pilot/safety) clinical trial [241, 409-412]. Part of this hesitation is due to criticisms of the 6-OHDA and MPTP animal models of PD, as these models suffer from poor predictive validity. Numerous antiparkinsonian therapies have been tested in these animal models, yet there is still no known disease-modifying therapy.

Our laboratory (and others) employs a rat model of PD through viral vector-mediated overexpression of α-synuclein (α-syn) by dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc). The protein α-syn has developed into a key player in PD

research over the last fifteen years [41, 49-54], and nigrostriatal overexpression via a viral vector has been characterized as a powerful model of progressive PD with good construct validity and is considered to have better predictive validity. In the present experiment, we explored the neuroprotective potential of STN DBS in a viral vector-mediated nigrostriatal α-syn overexpression model of PD.

Methods

Animals

A total of 19, male, Sprague-Dawley rats (Harlan, ≈250 g) were used in this study. Rats were only included in the final analysis if they successfully completed the full stimulation interval and electrode placement adequately targeted the STN as previously described [135]. Animals were allowed food and water *ad libitum* and were housed in reverse dark-light cycle conditions in an AAALAC approved facility. An Institutional Animal Care and Use Committee specifically approved this study.

Production of Recombinant Adeno-Associated Viral Vectors

The production of the α-syn-expressing, recombinant adeno-associated viral vector pseudotype 2/5 (rAAV2/5-α-syn) was conducted as previously described [339, 413]. Briefly, human cDNA was used to produce a clone of the wildtype *SNCA* gene that was then inserted into the AAV plasmid backbone. The chicken beta actin/cytomegalovirus enhancer-promoter hybrid was used to drive the expression of the *SNCA* gene. The vectors contained AAV2 inverted terminal repeats and co-transfection was accomplished through rAAV *rep* and *cap* genes and adenovirus helper functions,

thereby packaging the vector into AAV5 capsids. Iodixanol gradients and q-sepharose chromatography were used to purify vector particles and dot blot to measure the vector titer [414]. The viral vectors were stored at 4° C, never frozen. Surfaces that were in contact with virus were coated beforehand with Sigmacote (Sigma-Aldrich, St. Louis, MO). The rAAV2/5-α-syn titer used in this study was 1.2×10^{13} genome copies per ml.

Intranigral Vector Injections

Intranigral vector injections were conducted as described previously [339]. Prior to surgery, anesthesia was induced with 5% isofluorane in O_2, and rats were maintained under anesthesia with 2% isofluorane in O_2. Rats received two unilateral, intranigral injections (AP -5.3 mm, ML +2.0 mm, DV −7.2 mm and AP -6.0 mm, ML +2.0 mm, DV −7.2 mm relative to *dura mater*) of rAAV2/5-α-syn (injection rate 0.5 µl/minute, 2.0 µl per site).

Electrode Implantation

Rats assigned to the Inactive and Active groups were implanted with electrodes seventeen days following vector injections. Rats were anesthetized prior to surgery with Equithesin (0.3 ml / 100 g body weight i.p.; chloral hydrate 42.5 mg/ml + sodium pentobarbital 9.72 mg/ml); they were subsequently, unilaterally implanted (ipsilateral to vector injections) with a bipolar, concentric microelectrode (inner electrode projection 1.0 mm, inner insulated electrode diameter 0.15 mm, outer electrode gauge 26, Plastics One, Roanoke, VA) targeted to the dorsal border of the STN (AP −3.4 mm, ML +2.5 mm, relative to bregma and DV −7.7 mm, relative to the *dura mater*). Burr holes were

drilled in the skull; the electrode was fixed in place using bone screws and dental acrylic. Electrodes were lowered to coordinates corresponding to the dorsal border of the STN in order to minimize damage to the nucleus.

Behavioral Testing

Spontaneous forelimb use was assessed using the cylinder task as previously described [99, 136, 405]. Other behavioral measures were not employed due to their incompatibility with the external hardware required for continuous stimulation in an awake animal. The cylinder task was employed at the following times: (a) prior to vector surgery, (b) two weeks following vector surgery and before electrode implantation, (c) 54 days following vector surgery, on stimulation and (d) 55 days following surgery, off stimulation.

During the dark cycle, rats were videotaped and placed in a clear plexiglass cylinder until twenty, weight-bearing forelimb placements on the side of the cylinder occurred, or until a maximum trial time of five minutes had elapsed. To determine if forelimb preference was present, the number of contralateral, ipsilateral, and simultaneous paw placements was quantified. Data are reported as the percentage of contralateral (to vector and electrode) forelimb use: [(contralateral + ½ both)/(ipsilateral + contralateral + both)] x 100%. Rats with a unilateral, nigrostriatal lesion will show a bias toward using the ipsilateral limb. A forelimb deficit was defined as possessing a minimum of a 20% relative reduction in contralateral forepaw use compared to baseline; inclusion in this study required a forelimb deficit at the second behavioral task six weeks post surgery.

Continuous Stimulation Paradigm

When rats were assigned to receive stimulation (viz., the 'Active' group), stimulation was continuously delivered in a freely moving setup as previously described [134]. Stimulation was generated by an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments, Sarasota, FL). Stimulation parameters consisted of a frequency of 130 Hz, a pulse width of 60 μs and an intensity of 30-50 μA. At the onset of stimulation, intensity settings were increased until orofacial or contralateral forepaw dyskinesias were observed in order to confirm stimulation delivery, and immediately following a positive dyskinetic response, the intensity was set below the lower limit of dyskinesias, such that no rat was functionally impaired by stimulation. When rats were not being stimulated, they were still physically connected within their stimulator bowls to a commutator for the duration of the behavioral task.

Tissue Processing, Histology and Quantification

Sacrifice and Sectioning

At eight weeks (or 56 days) post vector surgery, rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized normal saline at 37°C followed by ice-cold paraformaldehyde (PFA). Care was taken to minimize the tissue damage resulting from removing the electrode from the skull. All brains were postfixed in 4% paraformaldehyde for twenty-four hours and transferred to 30% sucrose

in 0.1 M phosphate buffer. PFA-perfused and postfixed brains were frozen on dry ice and sectioned at 40 μm thickness using a sliding microtome in six series.

Tyrosine Hydroxylase Immunohistochemistry

One series (i.e., every sixth section) was stained with antisera for tyrosine hydroxylase (TH) using the free-floating method. Tissue was blocked in serum and incubated overnight in primary antisera directed against TH (Chemicon MAB318, mouse anti-TH, 1:4000). Cell membranes were permeabilized with the addition of Triton-X (0.3%) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). TH immunoreactive (THir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in Tris buffer. Sections were mounted on subbed slides, dried flat overnight under standard temperature and pressure conditions, dehydrated with ethanol and then xylenes and finally coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Kluver-Barrera Histology

Every sixth section of the subthalamic nucleus (STN) was stained using Kluver-Barrera histochemistry [378] and coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA) to evaluate for appropriate targeting of the electrode to the STN. Only rats with correctly positioned electrodes were included in the data analysis. Electrode location was considered to be appropriate if the tip of the electrode was observed within 250 μm

of the border of the STN within any of the sections based on previous studies in which current spread was determined [135].

α-Synuclein Immunohistochemistry for Vector Transduction Verification

One series (i.e., every sixth section) was stained with antisera for α-synuclein (α-syn) using the free-floating method, as previously described [339]. Tissue was blocked in serum and incubated overnight in primary antisera directed against wild-type human α-syn (mouse monoclonal anti-human α-syn, Invitrogen AHB0261, 1:2000 dilution) in 1.0% normal goat serum (Gibco, Catalog #16210-072). Cell membranes were permeabilized with the addition of Triton-X (0.5%, Sigma X-100) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400 dilution) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). α-Syn immunoreactive (α-syn-ir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in tris-buffered saline (TBS). Sections were mounted on subbed slides, dried flat overnight under standard temperature and pressure conditions, dehydrated with ethanol and then xylenes and finally coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Combined α-Synuclein and Tyrosine Hydroxylase Immunohistochemistry for Near-Infrared Imaging and Optical Density Analysis

One series (i.e., every sixth section) was stained with both antisera for α-syn and antisera for TH using the free-floating method, as previously described [339]. Tissue

was blocked in Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE, 927-40000) with 0.5% Triton-X 100 (Sigma, X-100) at room temperature for one hour followed by overnight incubation in primary antisera directed against TH (rabbit anti-TH antibody, Millipore, Catalog #AB152, 1:1000 dilution) at 4°C. Tissue was then washed for one hour (6 x 10 min) in 0.1M tris-buffered saline (TBS) and incubated in secondary antisera (LiCOR donkey anti-rabbit 800 Catalog #926-32213, 1:500 dilution in blocking buffer) for one hour at room temperature; from this point forward, sections were covered or were kept in a dark room in order to minimize exposure to light. Sections subsequently were rinsed in 0.1 M TBS for forty minutes (4 x 10 min) and blocked again in blocking buffer with 0.5% Triton X-100 (ib.) for one hour. Sections were incubated overnight in primary antisera directed against wild-type human α-syn (mouse anti-human a-syn antibody, Invitrogen Catalog #AHB0261, 1:2000 dilution) at 4°C. Tissue was then washed for one hour (6 x 10 min) in 0.1M TBS and incubated in secondary antisera (LiCOR donkey anti-mouse 680, Catalog #926-68022, 1:500 dilution) for one hour at room temperature. Lastly, sections were rinsed for forty minutes in 0.1M TBS. Sections were mounted on subbed slides, dried flat overnight under standard temperature and pressure conditions, dehydrated with ethanol and then xylenes and finally coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Unbiased Stereology of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to vector injections was quantified using unbiased stereology with the optical fractionator principle. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and

Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X, and THir neurons were counted according to stereological principles at 60X (NA 1.4); estimates of total counts per structure were extrapolated by the software. The Schmitz-Hof Coefficients of Error were less than or equal to 0.10 for all analyses.

Densitometry

A LI-COR Odyssey near-infrared scanner (LI-COR Biosciences) was used to scan every sixth section that was fluorescently labeled for both TH and α-syn using different wavelengths. In order to determine if any unilateral changes in TH or α-syn expression occurred within our groups, the integrated signal intensities were measured for both the intact and lesioned striatal hemispheres on slides normalized to a background measurement taken of the dorsolateral cortex on the intact side. The most rostral and caudal sections used were located AP +2.28 mm and AP -0.24 mm, respectively, as per [415]. For each striatal section analyzed, a dorsolateral, 'pie-shaped' region of interest was defined as previously described [339] due to involvement in forepaw motor function [416-418]; briefly, the striatum was bisected with a vertical line for the medial boundary, and a horizontal line extending from the base of the ipsilateral lateral ventricle for the ventral boundary. The raw integrated intensity values (arbitrary units as defined by the software) from each sampled striatal section were averaged in order to mitigate any differences in the number of sampling sites per animal.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). Statistical significance for all cases was set at $p < 0.05$.

Behavior

A two-way, RM-ANOVA followed by a least significant difference *post hoc* analysis was conducted to assess the presence of functional deficits across the three groups—no electrode implanted ('No Electrode'), DBS lead implanted ('Inactive') and chronic activation of an implanted electrode ('Active')—on spontaneous forelimb use at baseline, pre-lead implantation and at the conclusion of the study at eight weeks without stimulation; a one-way, RM-ANOVA was also used to compare behavioral task iterations within each group. A one-way ANOVA with a least significant difference *post hoc* analysis was conducted to compare forepaw use asymmetry between the Inactive and No Electrode groups both "on" (Day 54, stimulators activated for the Active group on this day, but not for these two groups) and off (Day 55) stimulation; On and Off days were also compared using two-tailed Student's T-Tests within the Inactive and No Electrode groups. A one-way ANOVA with a least significant difference *post hoc* analysis was conducted to compare forepaw use asymmetry both on and off stimulation between the Active group and a combined control group that consisted of both the Inactive and No Electrode groups with cylinder task scores from both on and off stimulation days included.

Histology

Differences in THir neuron survival were determined by a one-way ANOVA comparing the Active, Inactive and No Electrode groups. Differences in striatal α-syn or TH immunoreactivity were determined by one-way ANOVAs comparing the Active, Inactive and No Electrode groups followed by least significant difference *post hoc* comparisons.

Results

Viral Vector Transduction Increases Nigral Human α-Synuclein Expression

The parameters for our rAAV2/5-α-syn model typically result in very robust transduction of SN neurons and production of α-syn protein. Inspection of the SN revealed excellent transduction and adequate visualization of α-syn expression using IHC and brightfield microscopy across all groups. Transduction and α-syn protein expression is illustrated for each group in Figure 9.

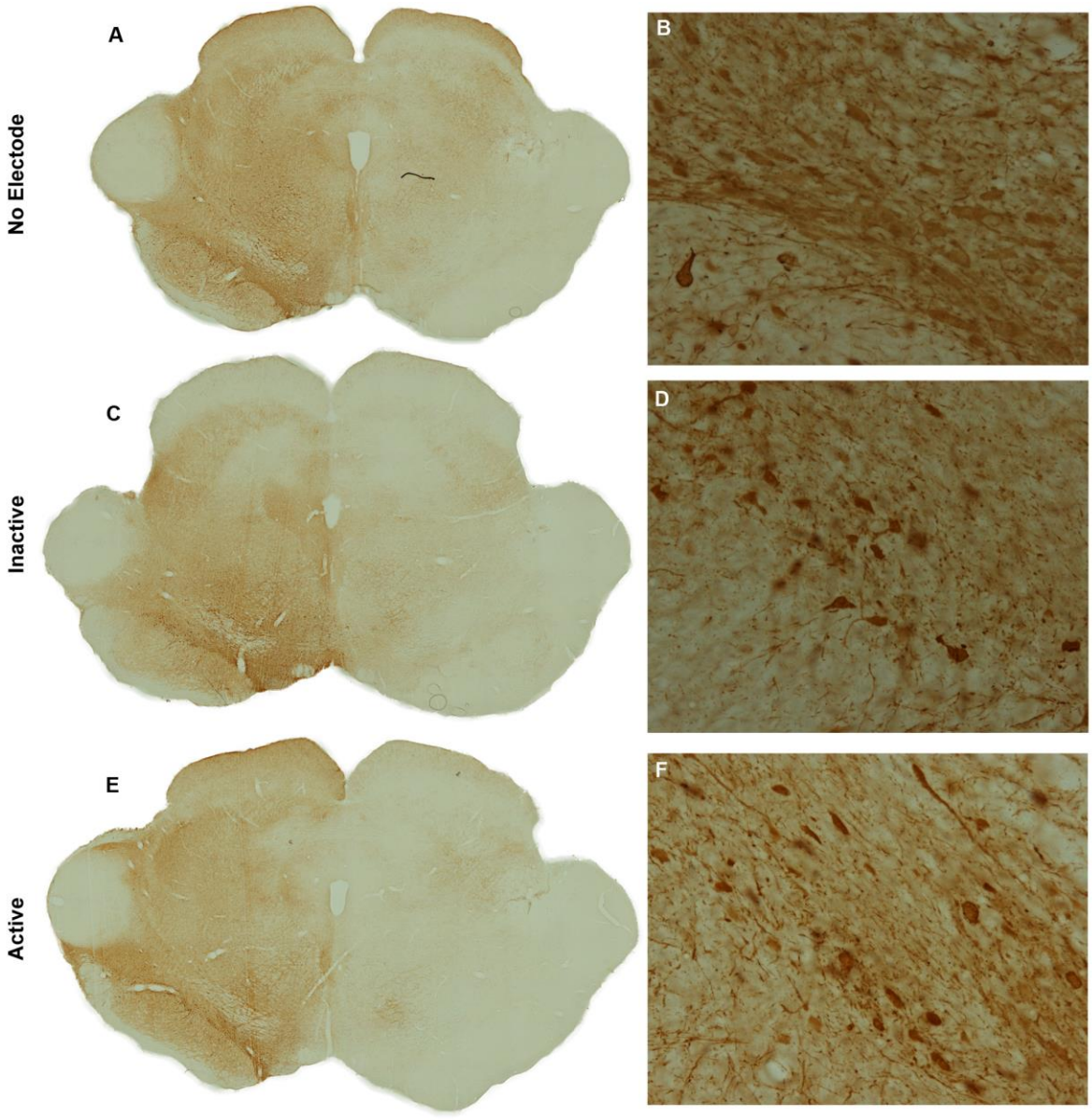

Figure 9. Robust Transduction and α-Synuclein Expression by SNpc Neurons. Immunohistochemistry for α-syn was performed to verify robust transduction and transgene expression by the targeted SNpc neurons, as visualized at low magnification by the transduced, ipsilateral (left) side versus the contralateral, uninjected hemisphere for No Electrode (A), Inactive (C) and Active (E) groups. High magnification images of transduced neurons are shown for No Electrode (B), Inactive (D) and Active (F) groups.

Functional Effects Measured by the Cylinder Task

α-Synuclein Overexpression-Induced Forelimb Asymmetry

Forepaw use asymmetry was measured by the cylinder task across three groups—viz., Active, Inactive and No electrode—at baseline, seventeen days post vector surgery (and before the electrode implantation surgery) and eight weeks (at the end of the study). The group means and standard errors are listed in Table 2. A two-way, RM-ANOVA was conducted to examine if a functional lesion emerged. A test of between-subjects effects showed no significant difference between groups ($F_{(2,17)} = 1.376$, $p > 0.05$). A test of within-subjects effects, however, showed a significant effect of time ($F_{(2,34)} = 14.795$, $p < 0.001$), but no interaction of time and treatment ($F_{(4,34)} = 0.812$, $p > 0.05$). *Post hoc* comparisons showed a significant difference from baseline at seventeen days ($p < 0.05$) and at eight weeks ($p < 0.001$), and the eight-week time also differed from the level of contralateral forelimb impairment at seventeen days ($p < 0.01$).

Table 2: Percent Contralateral Forepaw Use by Treatment.

Group	Percent Contralateral Forepaw Use ± SEM		
	Baseline	**2.5 Weeks**	**8 Weeks**
Active	53.75 ± 3.75	51.67 ± 2.01	40.83 ± 4.41
Inactive	54.56 ± 3.55	53.15 ± 5.39	40.56 ± 2.82
No Electrode	55.50 ± 3.57	41.17 ± 3.75	34.00 ± 2.81
Average	*55.00 ± 2.05*	*49.71 ± 2.80*	*39.00 ± 1.97*

Examination of the group means suggested the effect of time may be driven by one group more than the others. Three separate, one-way, RM-ANOVAs were conducted to examine the effect of time within groups. For the No Electrode group, a test of within-

subjects effects was significant ($F_{(2,8)}$ = 17.389, p < 0.01), and post hoc comparisons showed baseline differed from seventeen days (p < 0.01) and from eight weeks (p < 0.01). For the Inactive group, a test of within-subjects effects was significant ($F_{(2,16)}$ = 5.451, p < 0.05), and *post hoc* comparisons showed baseline differed from eight weeks (p < 0.01) and that seventeen days differed from eight weeks (p < 0.05). For the Active group, a test of within-subjects effects was not significant ($F_{(2,10)}$ = 2.879, p > 0.05). Therefore, results depict that a-syn overexpression resulted in significant deficits in contralateral forelimb use in No Electrode and Inactive rats with Active rats displaying an equivalent, yet non-significant, magnitude of impairment (Figure 10, panel A).

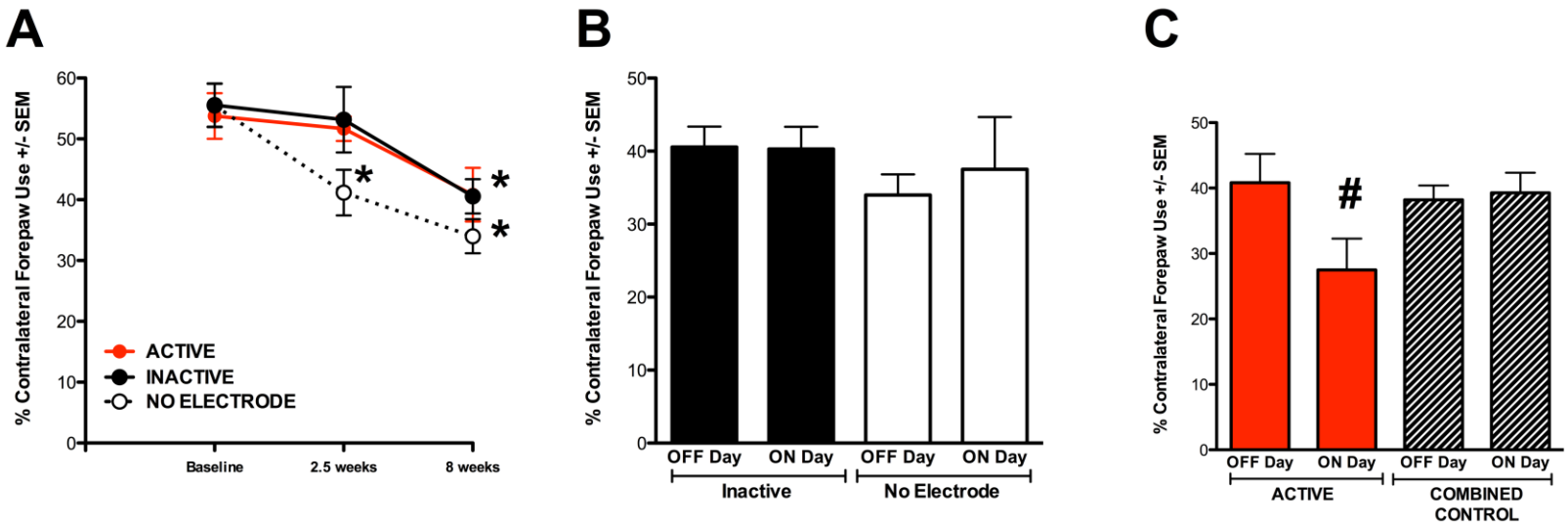

Figure 10. Functional Effects Measured by the Cylinder Task. **A.** Forepaw asymmetry was used as a measure of functional deficits over the course of the study. Under Off stimulation conditions, the No Electrode group was significantly impaired at 2.5 ($p < 0.01$) and 8 weeks ($p < 0.01$) vs. Baseline, and the Inactive group at eight weeks from baseline ($p < 0.01$) and 2.5 weeks ($p < 0.05$). **B.** The ON and OFF stimulation days for the Inactive and No Electrode groups were not significantly different. **C.** Rats receiving stimulation experience a reduction in percentage of affected forepaw use over total forepaw use when compared to the OFF stimulation day or to the ON and OFF stimulation days for the Combined Control group.

The Functional Effect of STN DBS

On the two days preceding sacrifice, the cylinder task was used to assess forelimb asymmetry when rats were on or off stimulation, days 54 and 55, respectively. As rats in the Inactive and No Electrode groups were effectively measured under similar conditions with no stimulation being delivered on either day, data were compared to determine if Inactive and No Electrode forelimb asymmetry scores could be combined. A one-way ANOVA of Inactive and No Electrode groups' data from 'ON Day' (Day 54) and 'Off Day' (Day 55) was conducted and yielded no significant differences between groups ($F_{(3,27)} = 0.565$, $p > 0.05$). Two-tailed Student's T-Tests comparing groups within the same day (e.g., Off Day Inactive vs. Off Day No Electrode) also yielded no significant differences. These results are depicted in Figure 10, panel B. As such, the On Day and Off Day data from each rat in the Inactive and No Electrode groups were combined into a "Combined Control" group for further analysis of behavioral data.

In order to assess the functional effect of stimulation, the forelimb use asymmetry of the Active group was measured by the cylinder task on On Day after which stimulation was ceased for twenty-four hours followed by the Off Day cylinder task. (After the rat was assayed by the Off Day cylinder task, stimulation was started again until the time of sacrifice.) A one-way ANOVA was conducted to compare On Day and Off Day cylinder task measurements from the Active group versus the Combined Control On and Off days: no significant difference was observed ($F_{(3,39)} = 2.234$, $p = 0.101$). However, since there was no expected difference between the Off and On days for the Combined Control, we conducted *post hoc* comparisons in order to examine if there were a

significant effect of stimulation (namely, by the Active group on On day) that was statistically masked by group means that are expected to be equivalent. From this *post hoc* analysis, there was a statistically significant difference between the On Day Active group and the other three groups ($p < 0.05$ in all three cases). These data are depicted in Figure 10, panel C.

Lesion Status of the Substantia Nigra

Unbiased stereological quantification of the ipsilateral and contralateral (to the vector injections) THir SNpc neurons was used to estimate neuronal number for each structure. In this experiment, the Active group had 11324 ± 903 and 10310 ± 570, the Inactive group 12119 ± 552 and 11271 ± 609 and the No Electrode group 12626 ± 411 and 11401 ± 561 remaining THir neurons on the intact and injected sides, respectively. A one-way ANOVA was conducted to assess if the groups significantly differed from one another, and they did not ($F_{(5,37)} = 1.453$, $p > 0.05$). In sum, the Active, Inactive and No Electrode groups all demonstrated the same 5-10% lesion, as depicted in Figure 11.

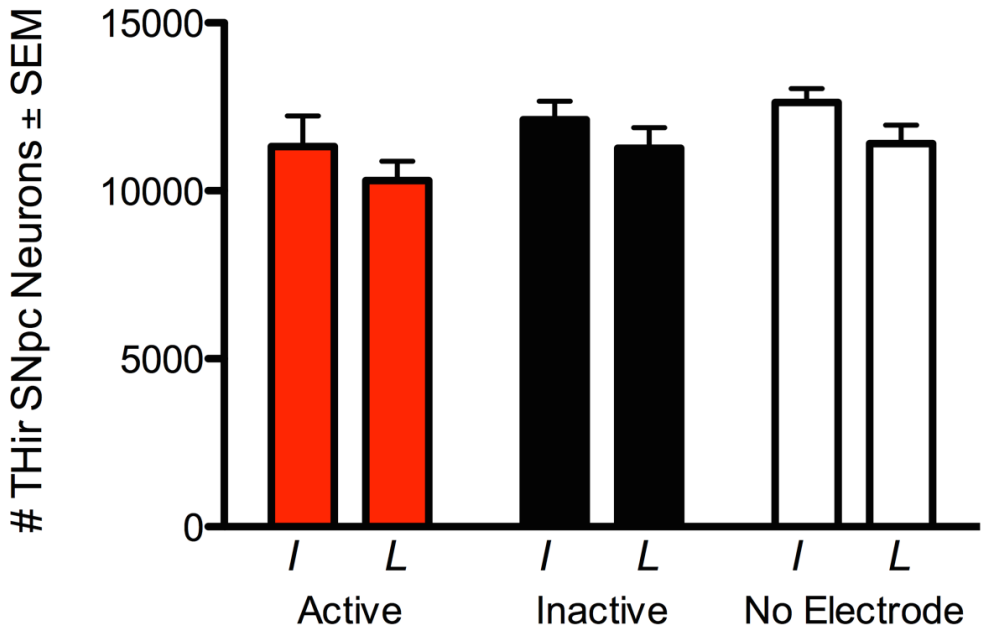

Figure 11. Stereological Counts of SNpc Neurons. Unbiased stereological counts of SNpc neurons on the lesioned side (L) ipsilateral to injections and the intact (I), contralateral side of Active (red), Inactive (black) and No Electrode (white) groups. There was no significant difference between groups or hemispheres ($p > 0.05$).

Viral Vector-Induced Changes in Striatal Proteins

Changes in Striatal α-Synuclein Levels

Densitometric analysis at near infrared wavelengths was used to quantify striatal expression of α-syn and to compare the intact versus the lesioned hemispheres. The raw integrated intensities (arbitrary units) of α-syn and TH expression for the Active, Inactive and No Electrode groups from both hemispheres are listed in Table 3. In order to compare α-syn expression levels between groups and hemispheres, a one-way ANOVA was conducted on the six sets of data (i.e., two hemispheres from each of the three groups), and a significant effect between groups was found ($F_{(5,37)} = 44.021$, $p <$

0.001). *Post hoc* comparisons showed a significant effect of side: each intact hemisphere expressed significantly less α-syn than the lesioned side, regardless of the group assignment ($p < 0.001$ in all cases). Striatal α-syn expression showed robust, equivalent expression across all three groups, as depicted in Figure 12.

Table 3: α-Synuclein or Tyrosine Hydroxylase Striatal Immunoreactivity.

		Integrated Intensity (arbitrary units)			
		A-syn		TH	
Group		**Lesion**	**Intact**	**Lesion**	**Intact**
Active	*Average*	11782370	40368	1957993	2515524
	SEM	1683189	180446	304058	454847
Inactive	*Average*	11080115	319662	2146371	2212872
	SEM	1102163	220626	95383	130899
No Electrode	*Average*	12574044	302475	2411676	3324100
	SEM	1135197	100259	265421	267617

Changes in Striatal Tyrosine Hydroxylase Levels

Densitometric analysis also was used to quantify striatal expression of TH across the intact and lesioned hemispheres. The raw integrated intensities (arbitrary units) of α-TH expression for the Active, Inactive and No Electrode groups from both hemispheres are listed in Table 3. In order to compare TH expression levels between groups and hemispheres, a one-way ANOVA was conducted on the six sets of data (i.e., two hemispheres from each of the three groups), and a significant effect between groups was found ($F_{(5,37)} = 3.034$, $p < 0.05$). *Post hoc* comparisons showed a significant effect between the No Electrode-Intact Hemisphere and all other groups: vs. the lesioned sides of the No Electrode ($p < 0.05$), Inactive ($p < 0.01$) and Active ($p < 0.01$) groups

and vs. the intact sides of the Inactive ($p < 0.01$) and Active ($p < 0.05$) groups. Therefore, a-syn overexpression produced a non-significant reduction in TH immunoreactivity of 2-28% across all treatment groups, as shown in Figure 12.

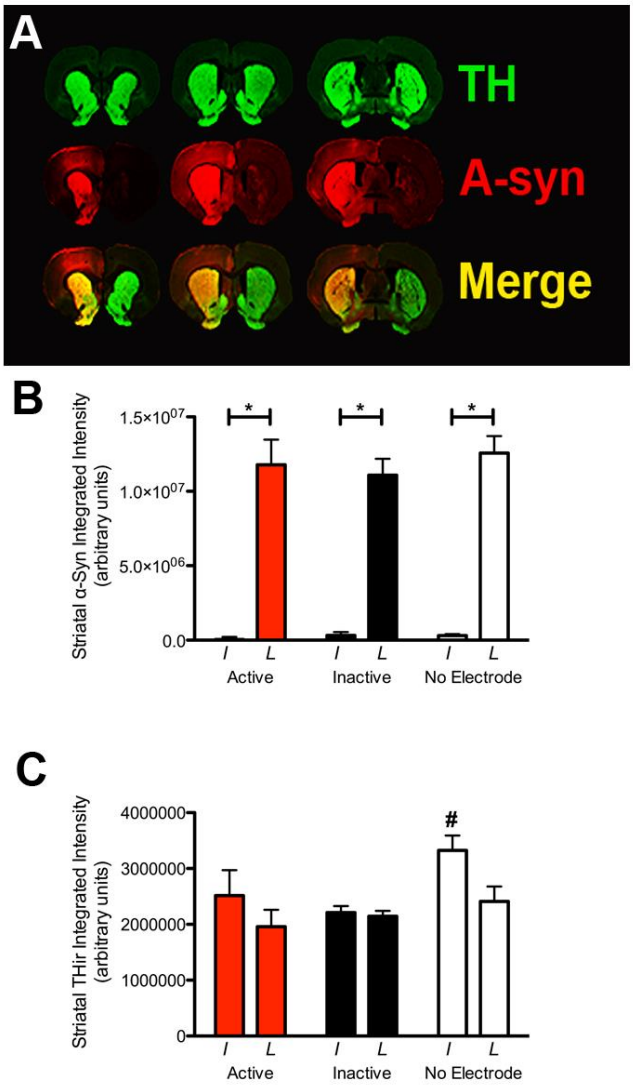

Figure 12. Efficient Striatal Transgene Expression and Altered TH Levels. A. Representative striatal sections expressing TH (green) and α-syn (red) shown individually and as merged images. **B.** Quantification of α-syn transgene expression in the uninjected/intact (I) hemisphere and the injected/lesioned hemisphere (L) of Active, Inactive and No Electrode groups, where there was a significant effect of side in all three groups ($p < 0.001$). **C.** Quantification of striatal TH levels in the intact (I) and lesioned (L) sides across groups, where the uninjected hemisphere of the No Electrode group was significantly different than all other groups ($p \ll 0.05$).

Discussion

We found that human α-syn overexpression targeted to the nigrostriatal system resulted in significant contralateral forelimb deficits and modest, nonsignificant decreases in SNpc THir neurons and TH immunoreactivity in the striatum. Active STN DBS did not provide neuroprotection for any of these metrics. These results suggest that the mechanism(s) whereby STN DBS is neuroprotective against 6-OHDA and MPTP is ineffective in protecting against α-syn-mediated toxicity. As discussed in Chapter 3, STN DBS provided neuroprotection against 6-OHDA via BDNF-trkB signaling. Since STN DBS is neuroprotective against both 6-OHDA and MPTP [134, 242-245] this suggests that enhanced BDNF-trkB signaling is sufficient to attenuate degeneration resulting from these particular oxidative stress insults. As an alternative, while we have verified that STN DBS significantly increases nigral BDNF following 6-OHDA, we have not examined whether nigral or striatal BDNF is similarly increased following STN DBS in the α-syn-overexpressing nigrostriatal system. Of note, viral vector-mediated α-syn overexpression in the nigrstriatal system results in a decreased transcription of a tremendous number of critical genes for DA neuron survival and functioning including Nurr1, Pitx3, TH, vesicular monoamine transporter 2 (VMAT-2), DAT, aromatic-L-amino acid decarboxylase (AADC), GDNF and BDNF [419]; effects on trkB were not reported. This widespread and dramatic effect on gene expression was cited as an indomitable barrier for why GDNF gene therapy failed in this model [335]. Since BDNF is decreased by α-syn overexpression, the mechanism by which STN DBS affords neuroprotection—viz., BDNF-trkB signaling—may not be viable in this model.

In this particular experiment, despite using an identical rAAV2/5 human WT α-syn vector of a titer previously demonstrated to produce a more severe lesion [339], the lesion generated was far less severe than what was expected. A similar titer under an identical surgical protocol in our laboratory has repeatedly resulted in an approximate 60% loss of THir SNpc neurons on the ipsilateral side at two months [339], whereas this experiment showed a far more modest effect of approximately 7-10% loss of nigral neurons. In addition, we had previously observed an approximate 40% loss of THir striatal innervation on the ipsilateral side at two months [339], whereas here we observed half as much loss, around 20% in the No Electrode group. It is unclear why seemingly identical titers and surgical parameters resulted in more modest toxicity in the present case. One possibility is that subtle reductions in titer resulted in differences in vector toxicity between experiments. We have previously observed that rAAV2/5-α-syn-mediated nigrostriatal toxicity is titer dependent [339], and that the threshold at which significant nigral DA neuron toxicity can be observed at two months after injection is between 2.2×10^{12} – 1.0×10^{13} vg/ml. A decrease in vector titer of 10-20% could have resulted in the more modest level of toxicity we observe.

The level of α-syn-mediated nigrostriatal toxicity we observed at eight weeks post vector injections closely resembles the ten day time point described by Decressac et al. [336] using a rAAV2/6 to overexpress human WT α-syn. Specifically, they showed a 10% loss of THir nigral neurons and an approximate 20% loss of striatal THir fibers, a magnitude of loss that was mirrored by loss of striatal DATir fibers and VMAT-2-ir fibers,

as compared to the contralateral side [336]; furthermore, an approximate 10% reduction in contralateral forelimb use was observed at the ten day time point [336]. In addition, TH enzymatic activity and striatal DA turnover was reduced at ten days. In the same laboratory (using the same animals), Lundblad et al. [356] used *in vivo* amperometry to examine if/when changes in DA transmission occur with the rAAV2/6-α-syn model; not too surprisingly, significant reductions in DA neurotransmission were first observed at the ten-day time point. In this experiment, our behavioral deficit is likely a result of decreased DA transmission, as observed by Lundblad et al. [356].

Indeed, this explanation appears supported by the On Day versus Off Day stimulation data. Active rats when receiving stimulation used their contralateral forepaws less than when off stimulation (and less than the Inactive and No Electrode groups), as expressed as a percentage of overall forepaw use. The vector-injected hemisphere experiences deficient expression of DA synthesis enzymes [419] and DA transmission [356], so even though stimulation may improve DA transmission on the affected side, it also does so on the intact hemisphere and disproportionately favors use of the 'unaffected' (ipsilateral to the injected side) forepaw—i.e., overall paw use is increased bilaterally but is increased more on the intact hemisphere, thereby driving the percent of affected forepaw use down. Indeed, a previous study in rats with a partially denervated striatum (by a small dose of intranigral 6-OHDA) using microdialysis measured bilateral increases in release of DA from unilateral STN DBS [420]. In addition, clinical data support the behavioral effect of STN DBS in our model. The rAAV2/5-α-syn model of PD we employed is most analogous to patients with duplicatons or triplications in the

gene that codes for α-syn, *Snca*, and these patients have been treated with STN DBS and enjoyed good outcomes [421, 422]. For our purposes in the laboratory, though, a measure of DA release that can be used to compare one hemisphere to itself under different conditions, like *in vivo* amperometry for striatal DA release, is necessary to test this hypothesis.

Conclusions

Basic and translational research has searched for therapies for PD for decades without any successful disease-modifying therapies to date. The researcher's ability to explore novel therapies is constrained by the methods used to model the human condition. The putative mechanism by which a therapy provides a benefit must answer the chosen model's insult, and the insult must be amenable to therapy in a way that is commensurate with the PD pathophysiology observed in patients. The PD research field has embraced viral vector-mediated α-syn overexpression as a model with good predictive validity because of the central role of α-syn in both the genetic and idiopathic forms of PD. In our hands, STN DBS does not afford neuroprotection against α-syn-mediated toxicity. Whether our findings in this model predict that STN DBS will not be neuroprotective in PD patients can only truly be evaluated in a clinical trial in which STN DBS is started early enough to modify the disease process and maintain striatal DA.

Chapter 6: High-frequency Stimulation of the Rat Entopeduncular Nucleus Does Not Provide Functional or Morphological Neuroprotection from 6-Hydroxydopamine

Abstract

Deep brain stimulation (DBS) is the most common neurosurgical treatment for Parkinson's disease (PD). Whereas the globus pallidus interna (GPi) has been less commonly targeted than the subthalamic nucleus (STN), a recent clinical trial suggests that GPi DBS may provide better outcomes for patients with psychiatric comorbidities. Several laboratories have demonstrated that DBS of the STN provides neuroprotection of substantia nigra pars compacta (SNpc) dopamine neurons in preclinical neurotoxicant models of PD and increases brain-derived neurotrophic factor (BDNF). However, whether DBS of the entopeduncular nucleus (EP), the homologous structure to the GPi in the rat, has similar neuroprotective potential has not been investigated. We investigated the impact of EP DBS on forelimb use asymmetry and SNpc degeneration induced by 6-hydroxydopamine (6-OHDA) and on BDNF levels. EP DBS in male rats received unilateral, intrastriatal 6-OHDA and ACTIVE or INACTIVE stimulation continuously for two weeks. Outcome measures included quantification of contralateral forelimb use, stereological assessment of SNpc neurons and BDNF levels. EP DBS 1) did not ameliorate forelimb impairments induced by 6-OHDA, 2) did not provide neuroprotection for SNpc neurons and 3) did not significantly increase BDNF levels in any of the structures examined. These results are in sharp contrast to the functional improvement, neuroprotection and BDNF-enhancing effects of STN DBS under identical experimental parameters in the rat. The lack of functional response to

EP DBS suggests that stimulation of the rat EP may not represent an accurate model of clinical GPi stimulation.

Introduction

Parkinson's disease (PD) affects nearly one percent of the population over the age of sixty-five [313]. The most common symptoms are bradykinesia, postural instability, rigidity and resting tremor with motor dysfunction being the primary cause for diagnosis, even though a patient may also have depression, cognitive dysfunction, anosmia or other symptoms at clinical presentation [6]. These motor symptoms primarily are a result of degeneration of the dopaminergic cells of the substantia nigra pars compacta (SNpc) and their projections to the striatum. As a result, the current mainstay pharmacotherapy of levodopa (L-DOPA) attempts to bolster nigrostriatal dopaminergic transmission. However, as disease progression continues, dopaminergic pharmacotherapy has decreased symptomatic efficacy and can yield troubling, involuntary movement known as dyskinesia [314], making the identification of neuroprotective therapies critical. Beyond pharmacotherapy, the surgical approach of deep brain stimulation (DBS) of the subthalamic nucleus (STN) is used with increasing frequency as a way to manage many PD motor symptoms with more than 100,000 patients having received DBS [375].

Since the advent of DBS, neurosurgeons have overwhelmingly chosen to target the STN for both surgical and symptomatic goals. STN DBS also results in a reduction of the needed L-DOPA dosage, thereby lessening the severity of drug-induced dyskinesia

[181, 204, 213]. Whereas traditionally the STN has been the preferred implantation site, similar success in treating the motor symptoms of PD with DBS targeting the globus pallidus interna (GPi) has been reported ([181], see also [204-206]). In some instances STN DBS has been associated with depressive symptoms or executive dysfunction post-surgery [423]. The potential for DBS targeted to the STN to exacerbate the existing comorbidities of depression or cognitive dysfunction has led to new consideration of DBS targets based on patient-specific motor and non-motor symptoms [213].

Despite the symptomatic efficacy of DBS, our understanding of its impact on ongoing nigral degeneration remains limited. This is in part due to the practice of using DBS as a last-resort treatment in late-stage PD. Patients who elect surgery receive DBS on average fourteen years after diagnosis [204]. In 2013 the results from a randomized clinical trial in PD patients with mid-stage PD (7.5 years) favored STN DBS over optimized medical therapy [240]. This study will likely shift clinical practice to offer DBS to *mid-stage* PD patients. Yet 50-60% of nigral dopamine (DA) neurons have degenerated seven years post PD diagnosis [46]. Studies in which STN DBS is applied in early-stage PD will be required to investigate its neuroprotective potential. Whether STN DBS is efficacious and safe in early-stage PD is currently the focus of an ongoing clinical trial [409, 411, 412, 424]. The increased focus on early DBS and its possible disease-modifying effects illustrates the significance of preclinical studies aimed at investigating these phenomena. Further, given the difficulties with assessing

neuroprotection in the clinic, preclinical studies can lead the way in the development and assessment of potentially disease-modifying therapies [425, 426].

Previous work in a rat model of long-term STN DBS [135] has yielded three distinct findings. First, STN DBS is associated with significant improvements in contralateral forelimb deficits induced by intrastriatal 6-hydroxydopamine (6-OHDA) injections, an animal model of PD in which DA neurodegeneration is induced by oxidative stress [134]. Second, STN DBS halts ongoing nigral DA neuron degeneration induced by intrastriatal 6-OHDA. Of importance, the degeneration is halted at the stimulation start time (two weeks post lesion) and midway through the course of expected degeneration. Third, STN DBS significantly increased brain-derived neurotrophic factor (BDNF) in the nigrostriatal system, the primary motor cortex (M1) and the entopeduncular nucleus (EP), suggesting its involvement in symptom amelioration and neuroprotection [136]. These preclinical results demonstrate that long-term STN DBS has the potential to impact plasticity within basal ganglia circuitry and may provide neuroprotection against further nigral loss. However, whether DBS targeted to the EP (the homologous structure to the primate GPi in the rat) will similarly provide functional improvements in forelimb use, alter BDNF levels or facilitate nigral DA neuroprotection is unknown. In the present study we examined the impact of long-term EP DBS on the progression of 6-OHDA-induced nigral degeneration and deficits in contralateral forelimb use.

Methods

Animals

A total of thirty-two, male, Sprague-Dawley rats (Harlan, 200-250 g) were used in these studies. Rats were only included in the final analysis if they successfully completed the two-week stimulation interval and exhibited proper electrode placement in the EP. Thirteen rats received intrastriatal 6-hydroxydopamine (6-OHDA) and EP stimulators; nineteen rats were unlesioned with EP stimulators. Animals were allowed food and water *ad libitum* and housed in reverse dark-light cycle conditions in an AAALAC approved facility. This study was specifically approved by the Institutional Animal Care and Use Committee of Michigan State University.

Experimental overview

The following experiments sought to replicate previously published work [134, 136] except that the EP was targeted rather than the STN. Care was taken to conduct the experiments as closely as possible to the original work to allow for comparisons. The overall experimental design is depicted in Figure 13.

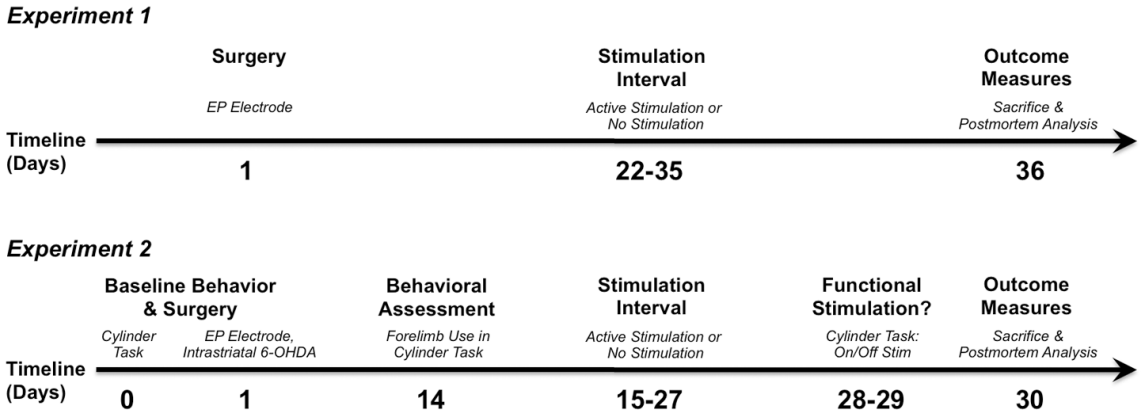

Figure 13. Experimental overview for EP DBS. *Experiment 1.* On Day 1, rats received an electrode implanted in the EP. After three weeks of recovery, rats were

Figure 13 (continued)

randomly assigned to ACTIVE or INACTIVE stimulation for a two-week interval. Rats tolerated stimulation of the EP for two weeks as they otherwise would for STN DBS for the same duration. Rats were sacrificed and perfused on Day 36. *Experiment 2.* On Day 0, rats were assessed for baseline forelimb asymmetry using the cylinder task. On Day 1, rats received unilateral, intrastriatal 6-OHDA and an electrode was implanted during the same surgical session in the EP ipsilateral to the lesion. After two weeks of nigrostriatal degeneration (≈50% loss of SNpc neurons, as determined in [134]), rats were reassessed for forelimb asymmetry, and rats with sufficient deficits in contralateral paw use were randomly assigned to receive ACTIVE or INACTIVE stimulation for a two-week interval. On Day 28, rats were reassessed using the cylinder task ("Stim On" condition), and after a twenty-four-hour washout after the cessation of stimulation, the rats were again assessed using the cylinder task ("Stim Off" condition). Rats were sacrificed and perfused on Day 30.

Experiment 1: stimulation of the EP in unlesioned rats

All rats were implanted unilaterally with electrodes to the EP and allowed a recovery period of three weeks. Rats were then randomly divided into ACTIVE and INACTIVE stimulation groups. Rats assigned to the ACTIVE group were connected to an external stimulation source and received continuous stimulation to the EP for two weeks; the INACTIVE group did not receive stimulation during the same interval. After the two-week period of stimulation, rats were sacrificed within six hours of cessation of stimulation. Brain structures including the M1 cortex (8 ACTIVES vs. 10 INACTIVES),

striatum (8 ACTIVES vs. 9 INACTIVES), hippocampus (3 ACTIVES vs. 7 INACTIVES), thalamus (4 ACTIVES vs. 7 INACTIVES) and substantia nigra (8 ACTIVES vs. 10 INACTIVES) were microdissected and processed for enzyme-linked immunosorbent assay (ELISA) for BDNF.

Experiment 2: stimulation of the EP following intrastriatal 6-OHDA

At least twenty-four hours prior to surgery, forelimb use in the cylinder task was assessed. Rats then received unilateral, intrastriatal injections of 6-OHDA and ipsilateral implantation of an electrode to the EP during the same surgical session. After a two-week recovery period, all rats were reassessed in the cylinder task to determine lesion status, and those that were functionally lesioned (i.e., a minimum 20% reduction in contralateral forelimb use compared to baseline) were randomly divided into ACTIVE and INACTIVE stimulation groups. Rats assigned to the ACTIVE group were connected to an external stimulation source and received continuous stimulation to the EP for two weeks; the INACTIVE group did not receive stimulation during the same interval. At the end of the two-week period of stimulation, rats were assessed using the cylinder task with stimulation and twenty-four hours after cessation of stimulation (3 ACTIVES vs. 7 INACTIVES). All rats were sacrificed within forty-eight hours after cessation of stimulation (4 ACTIVES vs. 7 INACTIVES for morphological analysis).

Intrastriatal 6-OHDA injections

Intrastriatal 6-OHDA injections were conducted as described previously [134]. Specifically, rats were anesthetized prior to surgery with Equithesin (0.3 ml / 100 g body

weight i.p.; chloral hydrate 42.5 mg/ml + sodium pentobarbital 9.72 mg/ml), and then they received two unilateral, intrastriatial injections (AP +1.6 mm, ML +2.4 mm, DV −4.2 mm and AP +0.2 mm, ML +2.6 mm, DV −7.0 mm relative to bregma) of 6-OHDA (MP Biomedicals, Solon, OH; 5.0 µg/µl 6-OHDA in 0.02% ascorbic acid, 0.9% saline solution, injection rate 0.5 µl/minute, 2.0 µl per site). Drill holes were filled with gel foam or bone wax to prevent entry of cement from electrode placement. These 6-OHDA lesion parameters result in ≈50% loss of substantia nigra pars compacta (SNpc) tyrosine hydroxylase immunoreactive (THir) neurons after two weeks that progresses to ≈75% loss after four weeks [134].

Electrode implantation

In 'Experiment 1' naïve rats were implanted with electrodes, whereas in 'Experiment 2' rats were implanted with electrodes immediately following intrastriatal 6-OHDA injections. Specifically, rats were unilaterally implanted (ipsilateral to 6-OHDA injections) with a bipolar, concentric microelectrode (inner electrode projection 1.0 mm, inner insulated electrode diameter 0.15 mm, outer electrode gauge 26, Plastics One, Roanoke, VA) targeted to the dorsal border of the EP (AP −2.4 mm, ML +3.0 mm, relative to bregma and DV −6.6 mm, relative to the dura mater). Burr holes were drilled in the skull, and the electrode was fixed in place using bone screws and dental acrylic. Electrodes were lowered to coordinates corresponding to the dorsal border of the EP in order to minimize damage to the nucleus.

Behavioral testing

Spontaneous forelimb use was assessed using the cylinder task as previously described [99, 136, 405] prior to surgery, two weeks following surgery and four weeks following surgery both on and off stimulation. Other behavioral measures were not employed due to their incompatibility with the external hardware required for continuous, long-term stimulation. Briefly, during the dark cycle rats were videotaped and placed in a clear plexiglass cylinder until twenty, weight-bearing forelimb placements on the side of the cylinder occurred, or until a maximum trial time of five minutes had elapsed. To determine if forelimb preference was present, the number of contralateral, ipsilateral, and simultaneous paw placements was quantified. Data are reported as the percentage of contralateral (to 6-OHDA and electrode) forelimb use: [(contralateral + ½ both)/(ipsilateral + contralateral + both)] x 100%. Rats with a unilateral nigrostriatal lesion will show a bias toward using the ipsilateral limb. Extent of lesion was evaluated two weeks post surgery, and a forelimb deficit was defined as possessing a minimum of a 20% reduction in contralateral forepaw use compared to baseline.

Long-term stimulation

Rats in the ACTIVE group received continuously delivered stimulation in a freely moving setup as previously described [134]. Stimulation was generated by an Accupulser Signal Generator (World Precision Instruments, Sarasota, FL) via a battery-powered Constant Current Bipolar Stimulus Isolator (World Precision Instruments, Sarasota, FL). Stimulation parameters consisted of a frequency of 130 Hz, a pulse width of 60 μs and an intensity of 30-50 μA. At the onset of stimulation, intensity settings were increased until orofacial or contralateral forepaw dyskinesias were observed in order to confirm

stimulation delivery, and immediately following a positive dyskinetic response, the intensity was set below the lower limit of dyskinesias, such that no rat was functionally impaired by stimulation. Rats assigned to INACTIVE stimulation did not receive stimulation at any time but were physically connected within their stimulator bowls to a commutator.

Sacrifice

After the stimulation interval, rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized saline at 37°C followed by ice-cold, 4% paraformaldehyde or ice-cold saline for lesioned and intact brains, respectively. Care was taken to minimize the tissue damage resulting from removing the skull with the electrode still intact. Lesioned brains were post-fixed in 4% paraformaldehyde for 24 hours and transferred to 30% sucrose in 0.1 M phosphate buffer. Unlesioned, saline-perfused brains were immediately flash-frozen in 3-methyl butane and stored at -80°C.

Microdissections

Brains acclimated to -20°C for at least one hour and then sectioned into 1-2 mm slabs using chilled, single-edge razor blades and a chilled, aluminum brain block. The hippocampus, M1 cortex, SN, striatum and the ventrolateral/ventroanterior thalamus were dissected on a cold plate set at −12°C (ThermoElectric Cooling America Corp, Chicago, IL) using a chilled, small tissue punch. Slabs containing the EP were examined for visual verification of electrode placement prior to dissection of this

nucleus. Each structure was placed in a pre-chilled microcentrifuge tube and stored at -80°C.

Protein assay

Total protein levels were quantified by comparison to a bicinchoninic acid (BCA) standard curve. Tissue was first homogenized in T-PER (Pierce, Rockford, IL) using a 300 V/T Ultrasonic Homogenizer (BioLogics, Manassas, VA). From each sample, 20 µl was removed, added to 20 µl of 2% SDS solution and then added to a BD Falcon 96-well Microtest plate (Fisher, Morris Plains, NJ) along with a BSA standard curve (Pierce, Rockford, IL). $CuSO_4$ (4%) was added to each well, and the plate was incubated at 37°C for twenty minutes. Each plate was read at 590 nm on a spectrophotometer (Thermo Scientific).

BDNF ELISA

An ELISA for BDNF was performed in triplicate in Nunc microwell 96-well microplates (Thermo Scientific) as per the manufacturer's instructions (BDNF Emax ImmunoAssay Systems Kit, Promega, Madison, WI). Each plate was read at 450 nm on a spectrophotometer (Thermo Scientific), and unknown values were calculated via interpolation against a BDNF standard curve. Each structure was analyzed individually with ACTIVE and INACTIVE groups present on each plate. Results were calculated as BDNF pg/mg of total protein. The data were normalized relative to the average value of the structure on the intact side (i.e., contralateral to the electrode) of the INACTIVE group.

Tyrosine hydroxylase immunohistochemistry for SNpc neurons

Paraformaldehyde-perfused and postfixed brains were frozen on dry ice and sectioned at 40 μm thickness using a sliding microtome in six series. One series (i.e., every sixth section) was stained with antisera for tyrosine hydroxylase (TH) using the free-floating method. Tissue was blocked in serum and incubated overnight in primary antisera directed against TH (Chemicon MAB318, mouse anti-TH 1:4000). Cell membranes were permeabilized with the addition of Triton-X (0.3%) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). TH immunoreactive (THir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in Tris buffer. Sections were mounted on subbed slides, dehydrated with ethanol and then xylenes and coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Kluver-Barrera histology

Every sixth section of the entopeduncular nucleus (EP) was stained using Kluver-Barrera histochemistry [378] to evaluate for appropriate targeting of the electrode to the EP. Only rats with correctly positioned electrodes were included in the data analysis for Experiment 2. Electrode location was considered to be appropriate if the tip of the electrode was observed within 250 μm of the border of the EP within any of the sections

based on previous studies in which current spread was determined [135]. A representative histological section is shown in Figure 14.

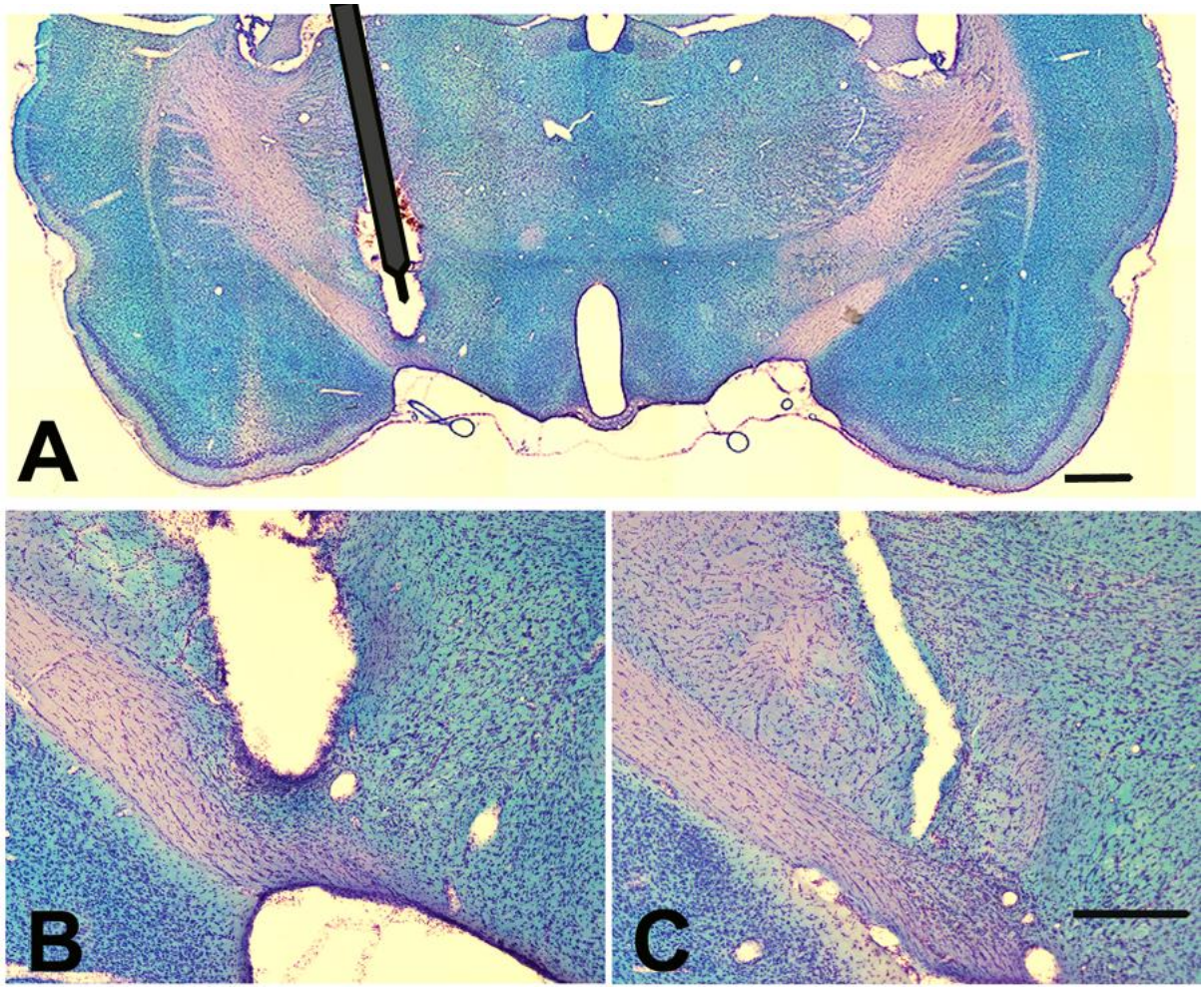

Figure 14. Electrodes implanted in the EP remain in position over the two-week stimulation interval. Representative photomicrographs illustrate unilateral electrode placement in the EP following Kluver-Barrera staining. (**A**) Low magnification image shows the approximate placement of the stimulating electrode prior to its removal post mortem and the tissue damage related to the removal process. The active electrode tip diameter is 150 µm whereas the shaft of the electrode is 400 µm in diameter. (**B**) High magnification of the electrode tip's position in the EP. **(C)** EP neurons are visible in a nearby coronal section (≈160 µm caudal), indicating that a significant portion of the EP

Figure 14 (continued)

remained intact. Rats in which electrodes were found to be positioned more than 250 μm away from the EP were excluded from analysis based on previous estimates of current spread [134]. Scale bar in A = 1000 μm, C = 500 μm.

Unbiased Stereology of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to 6-OHDA injection was quantified using unbiased stereology with the optical fractionator principle. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X, and THir neurons were counted according to stereological principles at 60X (NA 1.4). Percent remaining THir neurons of the ipsilateral, lesioned SNpc relative to the contralateral, intact SNpc were calculated. The Schmitz-Hof Coefficients of Error were less than or equal to 0.15 for all analyses.

Selective Total Enumeration of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to 6-OHDA injection was also quantified using selective total enumeration, a modified stereological method previously established to accurately quantify nigral THir neurons following intrastriatal 6-OHDA injected using identical parameters [379]. SNpc THir neurons from three sections, easily identified by the presence of the medial terminal nucleus of the accessory optic tract (AP -5.04 mm, ML -5.28 mm, DV -5.52 mm relative to bregma

[415]) were quantified. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), selective total enumeration THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X. Virtual markers were then placed on THir neurons at 20X and quantified. Total THir neuron numbers in the intact or lesioned SNpc were summed for the three MTN sections counted. Percent remaining THir neurons of the ipsilateral, lesioned SNpc relative to the contralateral, intact SNpc were calculated.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics (IBM, Armonk, NY). BDNF expression levels in 'Experiment 1' were confirmed by a repeated-measure (RM)-ANOVA. Values presented are normalized to expression levels on the side contralateral to the electrode ± SEM. In 'Experiment 2', a two-way RM-ANOVA followed by a least significant difference *post hoc* analysis was conducted to confirm the presence of functional deficits and the behavioral response to DBS. Differences in THir neuron survival were determined by a two-tailed Student's t-test, either comparing the unlesioned hemisphere to the lesioned hemisphere or comparing lesioned hemispheres between ACTIVE and INACTIVE groups. Statistical significance was set at $p < 0.05$. Statistical analyses are summarized in Table 4.

Table 4: Statistical Table.

	Data Structure	Type of Test	Power
a	Normal Distribution	RM-ANOVA, pairwise comparison	0.68
b	Normal Distribution	RM-ANOVA, pairwise comparison	0.998
c	Normal Distribution	RM-ANOVA, between subjects comparison	0.063
d	Normal Distribution	RM-ANOVA, within subjects comparison	0.574
e	Normal Distribution	RM-ANOVA, pairwise comparison	N/A
f	Normal Distribution	RM-ANOVA, pairwise comparison	N/A
g	Normal Distribution	RM-ANOVA, pairwise comparison	N/A
h	Normal Distribution	RM-ANOVA, pairwise comparison	N/A
i	Normal Distribution	Student's T-Test	1.0
j	Normal Distribution	Student's T-Test	0.57
k	Normal Distribution	Split-Plot ANOVA	0.507
l	Normal Distribution	Split-Plot ANOVA	0.05
m	Normal Distribution	Split-Plot ANOVA	0.095
n	Normal Distribution	Split-Plot ANOVA	0.053
o	Normal Distribution	Split-Plot ANOVA	0.06

Results

Short-Term Behavioral Response to DBS of the EP

Rats receiving ACTIVE stimulation were evaluated for behavioral responses to EP stimulation for a thirty-minute interval upon initiation of stimulation. Stimulation was slowly increased until the onset of dyskinesias or rotations. Contralateral orofacial

dyskinesias often appeared first with stimulation amplitudes between 30-90 μA, though most appeared under 50 μA. By increasing current amplitude, rotations to the ipsilateral side and dyskinesias of the contralateral forepaw were elicited, usually within 20 μA of the amplitude of orofacial dyskinesias, the vast majority observed below 60 μA. These dyskinesias persisted over the initial thirty-minute interval; hence, current intensity was lowered to below the threshold of dyskinesias for long-term stimulation. A similar profiling of behaviors elicited by stimulation has been reported previously [134, 136, 242].

EP stimulation does not improve forelimb asymmetry

Two weeks following intrastriatal 6-OHDA, a significant decrease in contralateral forelimb use was observed in both ACTIVE and INACTIVE groups ($F_{(3,6)} = 5.403$, $p = 0.038$[a] and $F_{(1.718,18)} = 21.137$, $p < 0.001$[b], respectively). Specifically, rats lesioned with 6-OHDA displayed ≈60% reduction in contralateral forelimb use compared to baseline. A two-way RM-ANOVA revealed no significant difference between treatment groups ($F_{(1,8)} = 0.147$, $p = 0.712$[c]) but did reveal a significant main effect within subjects ($F_{(3,24)} = 20.335$, $p < 0.001$[d]); therefore, the ACTIVE and INACTIVE treatment groups were combined for pairwise comparisons within subjects. Intrastriatal 6-OHDA resulted in deficits in contralateral forelimb use compared to baseline ($p < 0.001$[e]) that persisted for the duration of the study ($p = 0.007$[f]). However, contralateral forelimb use was significantly improved (compared to the two-week post lesion time point) at both four-week time points regardless of whether stimulation was 'on' or 'off' ($p = 0.001$[g] and $p = 0.015$[h], respectively). These results demonstrate no functional impact of ACTIVE

stimulation on contralateral forelimb use but a significant improvement over time in both treatment groups. These results are depicted in Figure 15A.

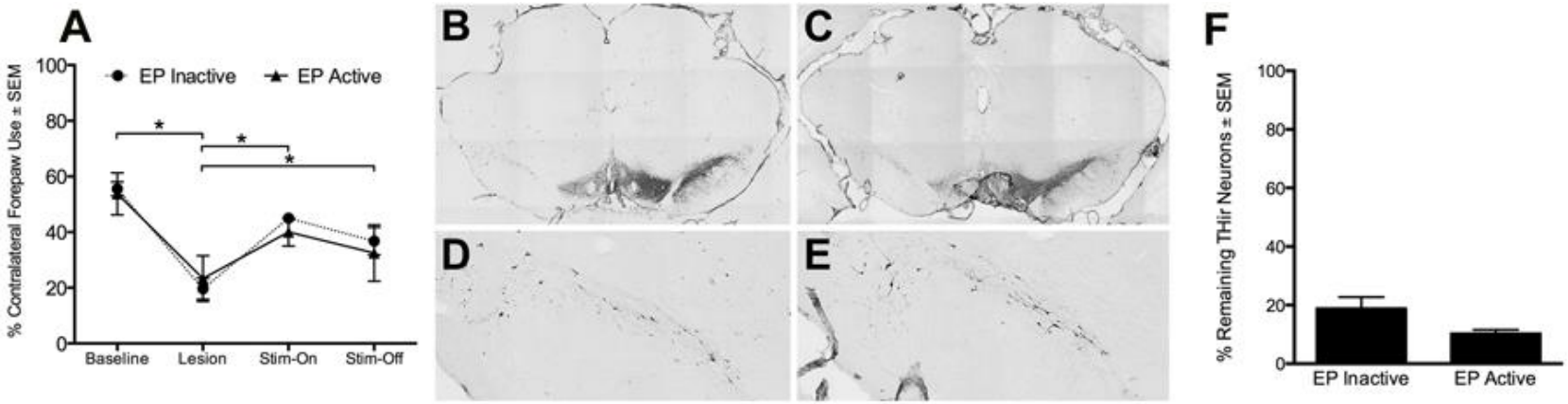

Figure 15. EP DBS does not correct forelimb asymmetry or provide neuroprotection from 6-OHDA. (A) Rats receiving intrastriatal 6-OHDA followed by either ACTIVE or INACTIVE EP DBS were analyzed for forelimb use asymmetry in the cylinder. 6-OHDA led to a significant decrease in contralateral forelimb use. However, ACTIVE EP DBS showed no difference compared to INACTIVE DBS at any time point. Of note, two weeks following electrode implantation in the EP, an improvement in contralateral forepaw use was observed. **(B-E)** EP DBS does not provide neuroprotection from 6-OHDA. Neither ACTIVE nor INACTIVE EP DBS halted ongoing nigral DA neuron loss normally observed between two and four weeks after intrastriatal 6-OHDA. **(B-C)** Representative nigral sections from both INACTIVE **(B)** and ACTIVE **(C)** EP DBS rats labeled with TH antisera reveal significant depletion of nigral DA neurons in the lesioned hemisphere. **(D-E)** At higher magnification, DA neuron loss appears equivalent between the INACTIVE **(D)** and ACTIVE **(E)** treatment groups. **(F)** Stereological assessment of THir neurons revealed no significant difference between ACTIVE and INACTIVE EP DBS groups.

EP Stimulation Does Not Provide Neuroprotection for SNpc Neurons

Rats in the intrastriatal 6-OHDA, INACTIVE treatment group possessed significantly fewer SNpc THir neurons ipsilateral to the injection compared to the contralateral SNpc ($t_{(20)}$ = -12.117, $p < 0.001$[i]). Specifically, the unlesioned SNpc in INACTIVE rats possessed 12255 ± 1099 THir neurons whereas the lesioned SNpc contained 2482 ± 619, or ≈83% fewer THir neurons than the unlesioned SNpc, as expected from this lesion paradigm [134, 379]. Similarly, rats that received two weeks of continuous EP stimulation also displayed a significant depletion of ≈90% THir neurons ipsilateral to 6-OHDA (ACTIVE unlesioned = 13029 ± 897; ACTIVE lesioned = 1379 ± 268). No significant difference was observed in the magnitude of degeneration measured in ACTIVE vs. INACTIVE rats ($t_{(7.193)}$ = 2.136, $p = 0.069$[j]). These results are illustrated in Figure 15, B-F.

Selective total enumeration of THir SNpc neurons [379] was also used to assess lesion status and to compare its utility versus unbiased stereology for our laboratory's future use. As previously reported, direct comparisons of estimates of lesion severity (and SEM) determined using selective total enumeration closely approximated those determined using unbiased stereology: ≈75% (±5.2) and ≈85% (±1.2) fewer THir neurons than the unlesioned SNpc in INACTIVE and ACTIVE rats, respectively.

Impact of EP DBS on BDNF Protein Levels

Five structures—namely, the SN, striatum, M1 cortex, thalamus and hippocampus—were examined bilaterally for levels of BDNF protein expression in unlesioned rats that

received unilateral EP stimulation for two weeks (ACTIVE) or INACTIVE electrode-implanted controls. ACTIVE stimulation of the rat EP was not associated with a significant increase in BDNF protein levels in any of the structures examined (viz., SN, thalamus, hippocampus, striatum and M1; $p > 0.05^{k\text{-}o,\ respectively}$), although BDNF levels in the SN of ACTIVE rats displayed a trend toward increased BDNF ($F_{(1,16)} = 4.426$, $p = 0.052^{k}$). BDNF protein levels were not measured for the EP since they are below the detection threshold for an ELISA [136]. The comprehensive results are summarized in Table 5 and Figure 16.

Table 5: Measured BDNF Levels by Structure.

Structure	BDNF (pg/mg) ± SEM			
	ACTIVE		INACTIVE	
	Left	Right	Left	Right
Substantia Nigra	32.86 ± 5.71	33.53 ± 7.70	18.70 ± 2.05	17.45 ± 2.47
Striatum	7.41 ± 1.50	8.13 ± 2.20	6.67 ± 2.73	5.37 ± 1.79
M1 Cortex	11.49 ± 1.83	12.79 ± 1.66	9.48 ± 1.40	10.04 ± 1.14
Thalamus	5.23 ± 0.65	4.30 ± 1.21	4.12 ± 0.47	5.49 ± 1.16
Hippocampus	24.03 ± 10.38	25.68 ± 9.55	21.09 ± 4.68	17.58 ± 2.86

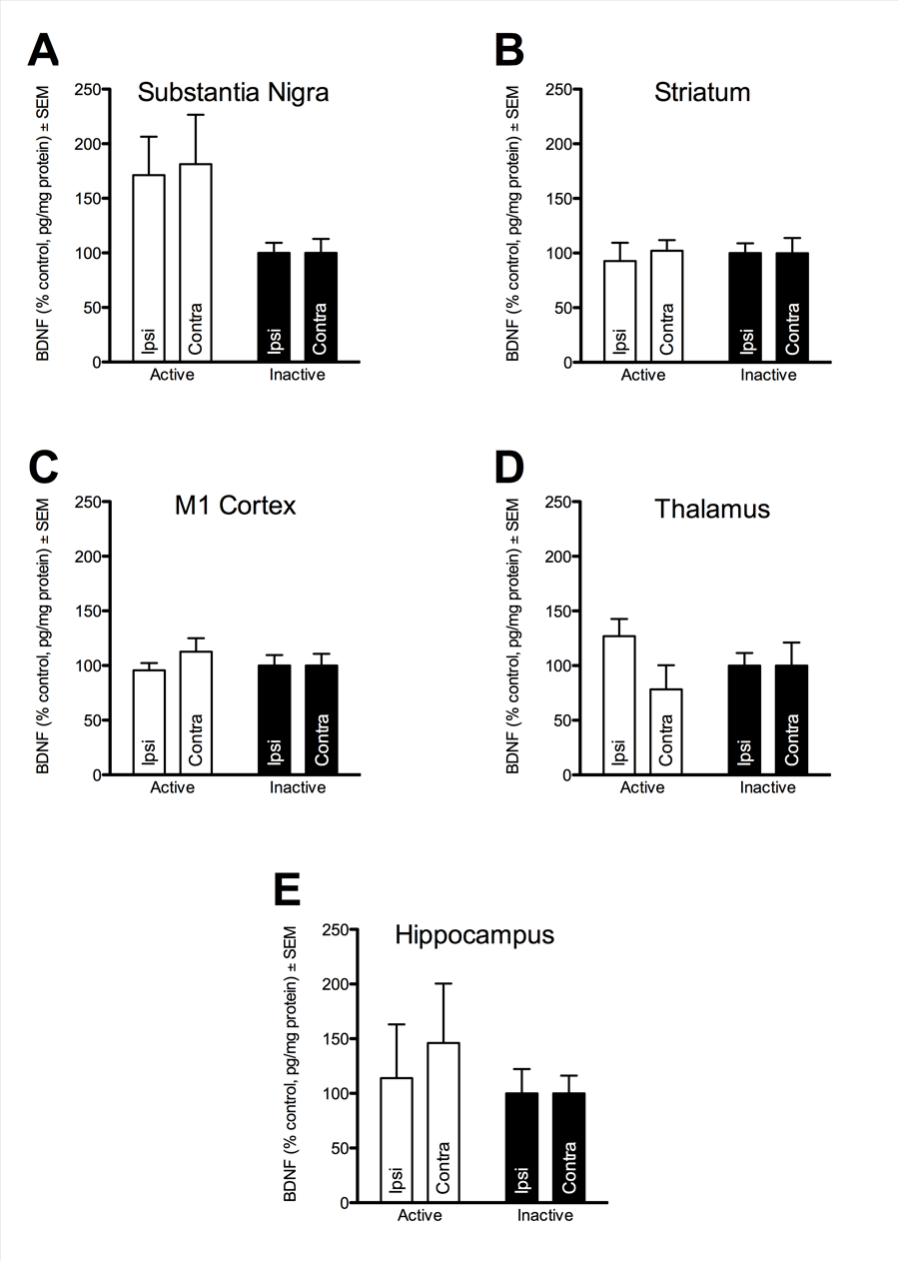

Figure 16. EP DBS does not increase BDNF. BDNF protein levels were normalized to total protein in key basal ganglia structures of intact rats after a two-week stimulation interval. Data from each structure were normalized to the corresponding structure from the INACTIVE right hemisphere to control for the potential effect of dopamine denervation or electrode implantation on BDNF levels. Samples were obtained for the left (L) and right (R) substantia nigra (SN), striatum (STR) and primary motor cortex

Figure 16 (continued)

(M1). No significant difference was observed between ACTIVE and INACTIVE stimulation groups nor within animals between sides, though there was a trend toward significance between the left and right SN.

Discussion

In the present study, we investigated whether long-term EP DBS provides neuroprotection similar to the neuroprotection observed following long-term STN DBS employed in the same lesion paradigm [134]. EP DBS was not associated with stimulation-dependent, functional improvements in contralateral forelimb use. Further, long-term EP DBS during the degenerative process initiated by 6-OHDA did not provide neuroprotection of nigral DA neurons or significant changes in BDNF levels within the brain. These results are in stark contrast with the effects of STN DBS in rodent and non-human primate models. As previously reported by several laboratories [136, 227, 242, 395-403], STN DBS in rats is associated with functional improvement in amphetamine-induced contralateral rotations, treadmill locomotion, walking speed, forelimb akinesia, rearing activity and reaction time following intrastriatal 6-OHDA. In contrast, our present study fails to demonstrate stimulation elicited functional improvement in the cylinder task. The improvement we observed in forelimb use was independent of stimulation status and only observed four weeks post surgery, suggesting that plasticity following the partial lesion of the EP may be responsible for the observed motor improvements, as has been reported previously for lesions of the EP [427] and for microlesions of the thalamus [428, 429]. Of note, under identical 6-

OHDA lesion parameters, functional improvement in contralateral forelimb use does not occur spontaneously or in association with implantation of INACTIVE electrodes in the STN [136, 379]. Therefore, it appears that the functional improvement we observe in the present study is specific to implantation of an electrode in the EP.

Whereas there have been numerous reports of STN DBS-mediated functional improvements in rats [136, 227, 242, 395-403], only two reports by Summerson and colleagues demonstrate motor effects following EP DBS [430, 431]. Specifically, amphetamine-induced rotations were acutely attenuated by stimulation amplitudes higher than used in the present study (viz., 65-100 μA [430]). Curiously, although forelimb akinesia was used in the first Summerson report to screen for 6-OHDA-induced dopamine depletion, the cylinder task was not used to assess the functional impact of EP DBS [430]. In their second report, Summerson and colleagues show behavioral improvement in the cylinder task, but again, under conditions of high current amplitudes [431]. In our present study, as reported previously [134, 242], amplitudes above 50 μA elicited contralateral dyskinetic movements, beginning with the orofacial area and forepaw. With even higher stimulation intensity levels, we also observed the tendency to rotate in the contralateral direction. Given that increases in current lead to an expanding volume of tissue activation (termed in [432]), we would expect that at higher amplitudes neurons/circuits outside of the EP would be recruited, as has been previously determined with rat STN stimulation [135]. Modulation of circuitry outside the EP may be responsible for the limited, observed functional effects reported with EP stimulation at higher stimulation amplitudes [404, 430, 431]; indeed, rotational

responses have been associated with direct SN stimulation [433, 434]. Although it is possible that we did not use the precise stimulation intensity required to elicit functional improvements, we can confirm that the current of 65-100 μA reported by Summerson and colleagues would have been incompatible with both long-term stimulation as well as accurate forelimb usage. We speculate that non-specific stimulation outside of the EP may be involved in these previously observed functional effects.

Table 6: Comparison of Primate GPi, Rat EP and Rat SNpr.

Feature	Primate GPi	Rat EP	Rat SNpr	References
GABAergic	Yes	Yes	Yes	[435-437]
Major Basal Ganglia Output	Yes	Yes	Yes	[438, 439]
Afferents from STN, Striatum & GPe	Yes	Yes	Yes	[437, 440-449]
Efferents to Thalamus & PPN	Yes	Yes	Yes	[439, 450-454]
Efferents to Lateral Habenula	Yes	Yes	No	[438, 439, 455, 456]
Fast-Spiking Pacemaker	Yes	No	Yes	[244, 437, 457, 458]

GPi = globus pallidus interna, EP = entopeduncular nucleus, SNpr = substantia nigra pars reticulata, GABA = γ-aminobutyric acid, STN = subthalamic nucleus, GPe = globus pallidus externa, PPN = pedunculopontine tegmental nucleus

In the present study we chose to target the rat EP due to its perceived homology to the primate GPi. Whereas remarkable similarities exist between the rat EP and the primate GPi in both the γ-aminobutyric acid (GABA) phenotype and afferent/efferent connectivity, an important difference in their firing properties remains (Table 6). The primate GPi is composed of fast-spiking, pacemaking neurons, whereas the rat EP is not [458-460]. In this regard the rat SN pars reticulata (SNpr) is more similar to the primate GPi (Table 6); indeed, stimulation of the SNpr in rats has been shown to improvement forelimb akinesia [457]. The disparity in firing properties between the rat EP and primate GPi may underlie the inability of focused EP DBS to ameliorate functional deficits induced by dopamine denervation. Our results suggest that stimulation of the rat EP may not serve as an appropriate model for GPi DBS for PD.

Several laboratories have shown that long-term STN DBS in rats and non-human primates is associated with neuroprotection of nigral DA neurons from 6-OHDA- or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced degeneration, respectively [134, 242-245]. It also has been demonstrated that high-frequency stimulation of the STN provides a significant level of neuroprotection and essentially halts the nigral degeneration that normally manifests during that period [134]. In contrast, continuous EP stimulation over the identical post-6-OHDA interval does not protect SNpc DA neurons from the continuing nigral degeneration induced by 6-OHDA. Both ACTIVE and INACTIVE EP DBS treatment groups displayed equal levels of nigral neuron loss commensurate with the normal lesion magnitude over this time course [134].

The disparity in neuroprotection results between STN and EP DBS may involve BDNF. In unlesioned rats STN DBS is associated with a significant, threefold increase in BDNF in the striatum and a non-significant increase in the SN [136]. Whereas a similar nonsignificant increase in BDNF in the SN is observed with EP DBS, striatal BDNF levels were not affected. We hypothesize that these contrasting results are due to differences in the phenotype and neural circuitry associated with the stimulation sites. The STN sends glutamatergic projections to the SNpc, the SNpr and the striatum [440, 441, 461, 462]. Stimulation of glutamatergic, hippocampal neurons *in vitro* results in activity-dependent BDNF release [285, 286]. Ergo, high-frequency stimulation of the glutamatergic neurons of the STN, known to contain BDNF [463, 464], may similarly mediate an increase in BDNF in STN target sites and provide trophic support for nigral

neurons [465]. The EP sends GABAergic projections to the pedunculopontine nucleus (PPN), the lateral habenula, the centromedian nucleus and the ventral anterior and the anterior part of the ventral lateral thalamic nuclei [438, 439, 452] (Table 6). Although we did not measure BDNF levels in the PPN in the present study, we show that EP DBS does not significantly increase BDNF in either the striatum or the SN. Further, while it is well established that glutamatergic neurons secrete BDNF [288], it is unclear whether GABAergic EP neurons possess or release BDNF. Analysis of BDNF expression levels in an online atlas [464] indicates expression of BDNF in the STN but levels indistinguishable from background in the EP. Other studies have demonstrated a differential effect on BDNF signaling within the hippocampus following STN or EP stimulation [136, 404]. Further studies are required to elucidate the source of elevated nigrostriatal BDNF following STN DBS and whether this phenomenon contributes to the neuroprotection observed.

Conclusions

In summary, EP DBS in our rat model of PD does not result in functional improvements nor morphological neuroprotection. Further, EP DBS in the rodent does not result in significant increases in BDNF protein levels in the nigrostriatal system or M1 cortex. Studies directly comparing STN vs. GPi stimulation in the non-human primate are warranted to ascertain whether our rat findings are due to the neuroanatomical differences between the rodent EP and the primate GPi. STN DBS has been shown to be neuroprotective in the non-human primate [245]. If GPi stimulation fails to provide

neuroprotection in the non-human primate, this would suggest that stimulation of the STN but not the GPi may offer a disease-modifying effect in PD patients.

Chapter 7: Conclusions and Future Directions

Conclusions, In Brief

The present work has accomplished several tasks. First, an overview of the literature on PD, DBS and BDNF was presented. Second, the use of gene therapy methods for modeling PD was critically evaluated. Lastly, this work has shed light on several aspects of DBS for PD. In this last regard, we have shown using the 6-OHDA rat model of PD that BDNF is increased and that STN DBS-mediated neuroprotection requires signaling through trkB, likely through canonical pathways, and trkB signaling mediates a component of the therapeutic effects from stimulation, likely through a non-canonical signaling pathway. We have also demonstrated that STN DBS is not neuroprotective in a viral vector-mediated nigrostriatal α-syn overexpression model, albeit effects on BDNF in this model remain unclear. Finally, we have demonstrated that while EP DBS does not provide a functional benefit, neuroprotection or an increase in BDNF, the paradigm is not analogous to the clinical scenario and thus deserves repeating in a non-human primate model. These studies have advanced our prior work and will inform future work in our laboratory as well as have implications for clinical practice.

Implications for Future Research and Clinical Practice

The Roles of BDNF in STN DBS – But Where?

As discussed in earlier chapters, the use of ANA-12 was a double-edged sword. Systemic administration of ANA-12 targets all trkB receptors, thereby implicating most neurons in the brain. For the results found using ANA-12—viz., abolishment of neuroprotection and attenuation of functional recovery—two main questions remain for

both cases: (1) where is the source of BDNF and (2) where are the trkB receptors that matter?

Testing Where BDNF-trkB Signaling is Needed for Neuroprotection

Since a near complete loss of THir terminals in the striatum is observed within twenty-four hours of 6-OHDA administration [134], it is likely that the neuroprotective trkB signaling occurs at the soma of SNpc neurons. As for the source of the BDNF, its transcript was increased in the SN regardless of 6-OHDA lesion status in our previous work [136]. STN DBS likely increases glutamate release at the SNpc, resulting in BDNF production and release to act in an autocrine/paracrine manner. However, it may be that the putative BDNF is released from subthalamic projections to the SNpc.

One of Nature's Tools: BDNF rs6265 Single Nucleotide Polymorphism (SNP)

These hypotheses could be tested using a gene therapy approach. A relatively common SNP in the BDNF gene is present in the human population (rs6265, 5' proregion at nucleotide 196, hereafter referred to as "BDNF SNP") [466, 467]. The BDNF SNP has a prevalence of 40.6% in the human population (codon 66: val66met 35.4%, met66met 5.2%, allelic frequency assuming Hardy-Weinberg) [468]. Both the heterozygous major allele and homozygous minor allele of the BDNF SNP results in a disruption of packaging and release of activity-dependent BDNF, whereas constitutive release of BDNF is unaffected [469]. The majority of BDNF in the brain is released from neurons via this regulated secretory pathway; hence, the BDNF SNP leads to a significant decrease in available BDNF [469]. BDNF is a critical modulator of gamma

aminobutyric acid (GABA)-ergic and glutamatergic synapses. BDNF facilitates long-term potentiation (LTP) and mediates use-dependent plasticity [288, 470, 471]. Within the nigrostriatal system, BDNF has the potential to exert a multitude of effects. BDNF plays an essential role in the maintenance of postsynaptic spine density of the striatal medium spiny neurons (MSNs) that are the targets of dopaminergic innervation [472]. Loss of MSN spines has been demonstrated in both preclinical models of DA depletion and postmortem PD patients [473, 474]. Striatal MSN spines are the site of interaction for nigral DA and glutamatergic cortical and thalamic neurons, and this interaction is necessary for normal basal ganglia functioning. BDNF is known to increase DA turnover and/or release in the nigrostriatal system [475]. Therefore, a decrease in activity-dependent release of BDNF as a result of the BDNF SNP has significant potential to affect basal ganglia functioning.

In our STN DBS platform and 6-OHDA model, pre-treatment with a viral vector expressing the BDNF variant could be used in lieu of ANA-12. This approach would be advantageous in that through stereotactic injection and viral vector construction, a specific cell population can be targeted. Injection into the SNpc or STN would test either one is the source of BDNF; if both structures are sources, then targeting both sites would test that possibility.

Testing Where BDNF-trkB Signaling is Needed for Behavioral Effects

Our laboratory previously showed that STN DBS increases BDNF in the primary motor (M1) cortex. Optogenetic approaches have demonstrated that the functional efficacy of

STN DBS can be explained through activation of the hyperdirect pathway that extends from the M1 cortex to the STN [228]. These M1 fibers are glutamatergic and may release BDNF to act on trkB receptors on STN neurons. However, glutamatergic STN projections to the M1 cortex unique to the rat ([407], cf. [408]) may release BDNF to act on M1 cortex, trkB-expressing neurons.

Discovering the source of BDNF mediating this effect could be done through the same overall approach discussed above. A BDNF SNP expressing viral vector could be injected into the STN or the layer V pyramidal neurons of the M1 cortex or both in order to examine if decreased activity-dependent release in any of these cases significantly attenuates the functional efficacy of STN DBS. Given that these effects are measured acutely by the cylinder task, an additional feature to the viral vector would make for a more elegant experiment: constructing the vector to conditionally express the variant gene with the addition of tetracycline would allow for a similar experimental paradigm as used in the present work with ANA-12 (i.e., tetracycline administration would parallel ANA-12 administration and an injected vehicle would result in no attenuation of the behavioral response to stimulation).

Is DBS Disease-Modifying?

The present work sought to evaluate the neuroprotective potential of DBS of the STN and the GPi using rat models of PD. Whether STN DBS is neuroprotective has been examined before in animal models [134, 242-245], but it has not been vetted in the rAAV2/5-α-syn rat model of PD. This model has greater construct validity and therefore

is considered to have greater predictive validity as well, although the complete absence of neuroprotective therepies in the PD patient population ultimately limits this contention. On one hand, we have observed that in one model (viz., intrastriatal 6-OHDA) STN DBS is neuroprotective in association with increased nigrostriatal BDNF, and this neuroprotection is abrogated by blockade of trkB. In another model (viz., nigrostriatal α-syn overexpression) STN DBS is not neuroprotective, and the effects of stimulation on BDNF are unknown. Conclusions drawn regarding neuroprotective strategies in preclinical models are only as generalizable to the patient population as the model is predictive, and it remains unclear which of these two models more accurately represents the disease-modifying potential of STN DBS in the clinic.

In examining if GPi DBS results in similar effects as STN DBS, we investigated the potential for the rodent EP to serve as a homologous structure to the primate GPi. EP DBS in our 6-OHDA model of PD did not afford any neuroprotection nor did it increase BDNF levels in any of the basal ganglia structures examined; however, as we were unable to replicate the clinical scenario of a symptomatic benefit of GPi DBS in our rodent DBS platform, we are unable to inform the PD field as to whether there is preclinical support for a disease-modifying effect of GPi DBS. In order to answer this question and other questions regarding GPi DBS for PD, researchers should abandon their work in rodents in favor of non-human primate models or conduct clinical research if warranted.

Impact of BDNF rs6265 SNP on Patient Response to Dopaminergic Pharmacotherapy

Heterogeneity in the Clinical Response to Antiparkinsonian Therapies

Levodopa (L-DOPA) and STN DBS have become the mainstay pharmacological and surgical therapies for PD, respectively. Although generally effective in treating the motor symptoms of PD, the clinical response range to both of these therapies is exceedingly wide. For example, early-stage PD subjects receiving equivalent L-DOPA dosages experienced a magnitude of response ranging from a 100% improvement to a 242% worsening as assessed using the United Parkinson's Disease Rating Scale part III (UPDRS motor, [476]). In one of the largest clinical trials of bilateral STN DBS, late-stage PD subjects experienced improvements in UPDRS part III that ranged from 3% to 63% improvement [477]. In a recent clinical trial in mid-stage PD subjects comparing STN DBS to medical therapy, UPDRS-III scores in the STN DBS treatment group ranged from a worsening of 7% to an improvement of 83%, whereas in the medication arm, UPDRS-III scores ranged from a worsening of 42% to an improvement of 50% [240]. These examples underscore the incredible heterogeneity in the clinical response to standard of care antiparkinsonian therapies even when disease severity is taken into account. Indeed, as pointed out in the Parkinson's Disease 2014: Conference and Recommendations Report to the National Advisory Neurological Disorders and Stroke Council, "Recent research has begun to reveal the molecular and clinical heterogeneity of PD, a complex neurodegenerative process that likely derives from multiple molecular drivers that vary among individuals, act over years prior to clinical expression, underlie an individual's ensemble of motor and non-motor symptoms, and likely dictate response to treatment and its complications" [394]. From this fact, an overall goal of the PD2014 report is to "deliver the right treatment to the right person at the right time[, and] to

implement safe, effective, and precise interventions with minimal complications" [394]. Given the data in the present work on the importance of BDNF-trkB signaling in the therapeutic response to STN DBS, it is plausible that gauging BDNF-trkB signaling within a specific patient could predict his or her clinical outcome; a specific genetic variation in BDNF may be one way to accomplish this.

BDNF SNP: Brain Structure, Function and PD

To investigate whether BDNF plays a role in the therapeutic efficacy of STN DBS in PD subjects, our laboratory is exploring the BDNF SNP. Individuals with the BDNF SNP have significantly reduced grey matter volumes in a number of structures [478] and decreased performance and hippocampal engagement during memory tasks [467, 479]. A similar BDNF SNP-associated difference is observed in age-related hippocampal and whole brain atrophy, although the BDNF SNP is not associated with a diagnosis of AD [480, 481]. BDNF SNP mice recapitulate this phenotype in that they exhibit decreased hippocampal dendritic complexity, decreased hippocampal volume and deficits in hippocampal-dependent learning [469]. A meta-analysis of several case-control studies concluded that no association exists between the BDNF SNP and the risk of developing PD [482]. However, in PD subjects treated with L-DOPA, the BDNF SNP has been associated with earlier development of L-DOPA-induced dyskinesias (LIDs) [483]; although, this does not hold true in all patient populations [484]. Experience-dependent plasticity in the motor cortex as well as volume of the caudate nucleus and motor cortex is reduced in subjects with the BDNF SNP [478, 485, 486]. The motor cortex processes information for planning and controlling voluntary movement. Reductions in motor

cortex excitability and output have been observed in PD and are thought to underlie bradykinesia [17, 487].

BDNF SNP Brain Environment: Compensatory Changes In Response to Less BDNF

The decrease in activity-dependent BDNF release in BDNF SNP mice results in a multitude of downstream, perhaps compensatory, alterations in the brain environment. These alterations include changes in the expression of numerous genes involved in glutamate signaling, ubiquitin-mediated proteolysis, SNARE interactions in vesicular transport, p53 and ribosomal signaling pathways and cell adhesion [488, 489]. Of particular interest, mice with the BDNF SNP have *increased* BDNF and trkB mRNA expression [490]. In this regard, the BDNF SNP brain appears to compensate for decreased BDNF release by becoming "hyperresponsive" to BDNF in an attempt to normalize BDNF signaling. This compensatory response falls short, however, as impaired synaptic plasticity is still evident under baseline conditions in the BDNF SNP cortex and hippocampus [491, 492]. Under pathological conditions a different picture has emerged. Evidence supports the concept that the BDNF SNP, perhaps due to a BDNF hyper-responsive environment, may confer an advantage to brain repair and remodeling. Superior recovery from traumatic brain injury has been reported in BDNF SNP subjects [493], and enhanced plasticity and recovery of function has been observed in BDNF SNP mice following middle cerebral artery occlusion [488].

Rationale for Investigating the Role of the BDNF SNP in Response to Anti-parkinsonian Therapies

Previous findings in the literature collectively demonstrate that the BDNF SNP brain environment is markedly different from the more frequent (val66val) BDNF genotype. Under conditions in which BDNF participates, as we have observed in our STN DBS rat model, the response of the BDNF SNP brain may deviate significantly. Examples of this include that BDNF SNP individuals are more likely to be nonresponsive to antidepressant or antipsychotic treatments [494, 495], pharmacotherapies that can be influenced by BDNF signaling. In addition, the findings that the BDNF SNP: a) may predispose for developing LIDs, b) drives PD-relevant striatal and motor cortex plasticity and c) negatively interacts with the aged brain environment, are relevant. Taken together, it is plausible that the BDNF SNP confers a differential treatment response to pharmacotherapy in PD subjects and represents an importance future direction for this line of research, as it could inform optimal patient care for the one in three PD patients possessing the BDNF SNP. Our laboratory has begun to explore whether this is true through a collaborative clinical project.

Impact of the BDNF SNP on Response to Optimized Drug Therapy (ODT)

To examine whether the BDNF SNP confers a differential treatment response to dopaminergic pharmacotherapy, our laboratory collaborated with Dr. P. David Charles to genotype early-stage PD subjects enrolled in the Vanderbilt DBS for Early Stage PD clinical trial (Clinical Trials.gov identifier NCT00282152) [410, 411, 496]. Our collaboration has been presented as a meeting abstract [497], but as it is not yet published, the methods and results will be described herein.

The study population consisted of thirty, early-stage PD subjects treated for two years with bilateral STN DBS plus medication (DBS+ODT) or optimized drug therapy (ODT). Subjects were age 50-75 years with idiopathic PD, Hoehn and Yahr Stage II when OFF medication, treated with antiparkinsonian medications for greater than six months but less than or equal to four years and with no current or prior history of motor fluctuations. Additional subject characteristic details are currently published [496].

Subjects from both the STN DBS and ODT treatment arms of the Vanderbilt DBS for Early Stage PD clinical trial were genotyped. BDNF SNP genotyping was performed using the 59 exonuclease allelic discrimination Taqman assay. Comparisons were made between ON baseline and ON two-year scores in total UPDRS, individual UPDRS subscores and PDQ-39. Five of fifteen subjects (33%) in the DBS treatment arm and six of thirteen subjects (46%) in the ODT treatment arm possessed the BDNF SNP. At baseline no significant differences were observed in any metric. However, at two years after the initiation of either treatment, PD subjects with the BDNF SNP in the ODT treatment arm exhibited significantly worse scores (twenty points higher on Total UPDRS) compared to BDNF Val/Val ODT patients ($p < 0.05$). In contrast, DBS BDNF SNP subjects exhibited lower total UPDRS scores compared to ODT BDNF SNP subjects, though this effect did not achieve significance ($p = 0.06$). No significant differences were observed between BDNF genotypes in the DBS treatment arm and the ODT Val/Val subjects. Our results suggest that PD patients possessing the BDNF SNP (i.e., one in three PD patients) will experience superior therapeutic benefit from STN DBS than from ODT. If findings from our small, early-stage PD cohort hold true in larger

and more diverse patient populations, this would provide compelling support for using the BDNF rs6265 SNP as a screen to inform optimal personalized patient care for the medical vs. surgical management of PD. (This paragraph was submitted by the author as part of an abstract for the NINDS Udall Centers Directors' Meeting in 2014.)

Final Remarks

PD is a relentlessly progressive disease for which there are no known disease-modifying therapies. The work described herein has sought to characterize the effects of STN DBS, a therapy currently used with much acclaim. The dramatic results from DBS that are so often observed have captivated the PD patient community, PD researchers and the general public. Exploring the mechanism(s) of DBS is of critical importance if they are to be harnessed through development of less invasive therapies (e.g., trkB agonists) or optimized through refined criteria for patient selection, like genetic testing for BDNF variants. Beyond its use for symptom alleviation, STN DBS may offer a disease-modifying effect, but inappropriately designed clinical trials have been unable to offer a definitive conclusion. The data presented in this work show that the neuroprotective potential of STN DBS is specific to the model. To put an end to this debate, the time for a well-designed clinical trial could not come sooner. The PD research field owes such an effort to the many patients and their families who still fight for their personal victories with each passing day.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Parkinson, J., *An Essay On The Shaking Palsy*. 1817, London: Sherwood, Neely and Jones. 66.

2. Parent, M. and A. Parent, *Substantia nigra and Parkinson's disease: a brief history of their long and intimate relationship.* Can J Neurol Sci, 2010. **37**(3): p. 313-9.

3. Goetz, C.G., *Jean Martin Charcot and Movement Disorders: Historical Legacies to the 21st Century*, in *Stanley Fahn Lecture*. 2009, International Parkinson and Movement Disorder Society.

4. Carlsson, A., *Treatment of Parkinson's with L-DOPA. The early discovery phase, and a comment on current problems.* J Neural Transm, 2002. **109**(5-6): p. 777-87.

5. Cotzias, G.C., M.H. Van Woert, and L.M. Schiffer, *Aromatic amino acids and modification of parkinsonism.* N Engl J Med, 1967. **276**(7): p. 374-9.

6. Jankovic, J., *Parkinson's disease: clinical features and diagnosis.* J Neurol Neurosurg Psychiatry, 2008. **79**(4): p. 368-76.

7. Marinus, J. and J.J. van Hilten, *The significance of motor (A)symmetry in Parkinson's disease.* Mov Disord, 2015. **30**(3): p. 379-85.

8. Nandhagopal, R., et al., *Longitudinal progression of sporadic Parkinson's disease: a multi-tracer positron emission tomography study.* Brain, 2009. **132**(Pt 11): p. 2970-9.

9. Gelb, D.J., *Introduction to Clinical Neurology*. 4th ed. 2011, New York: Oxford University Press. 504.

10. Gelb, D.J., E. Oliver, and S. Gilman, *Diagnostic criteria for Parkinson disease.* Arch Neurol, 1999. **56**(1): p. 33-9.

11. Dalvi, A., *Premotor Symptoms*, in *Handbook of Parkinson's Disease*, R. Pahwa and K.E. Lyons, Editors. 2013, CRC Press: New York. p. 93-102.

12. Bolam, J.P., et al., *Synaptic organisation of the basal ganglia.* Journal of Anatomy, 2000. **196**: p. 527-542.

13. Obeso, J.A., et al., *Functional organization of the basal ganglia: Therapeutic implications for Parkinson's disease.* Movement Disorders, 2008. **23**: p. S548-S559.

14. Albin, R.L., A.B. Young, and J.B. Penney, *The functional anatomy of basal ganglia disorders.* Trends Neurosci, 1989. **12**(10): p. 366-75.

15. Okun, M.S., *Deep-brain stimulation--entering the era of human neural-network modulation.* N Engl J Med, 2014. **371**(15): p. 1369-73.

16. Chevalier, G., et al., *DISINHIBITION AS A BASIC PROCESS IN THE EXPRESSION OF STRIATAL FUNCTIONS.* Trends in Neurosciences, 1990. **13**(7): p. 277-280.

17. DeLong, M.R., *Primate models of movement disorders of basal ganglia origin.* Trends Neurosci, 1990. **13**(7): p. 281-5.

18. Lewis, S.J., M.A. Caldwell, and R.A. Barker, *Modern therapeutic approaches in Parkinson's disease.* Expert Rev Mol Med, 2003. **5**(10): p. 1-20.

19. Shink, E., et al., *The subthalamic nucleus and the external pallidum: Two tightly interconnected structures that control the output of the basal ganglia in the monkey.* Neuroscience, 1996. **73**(2): p. 335-357.

20. Bernheimer, H., et al., *Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations.* J Neurol Sci, 1973. **20**(4): p. 415-55.

21. Agid, Y., *Parkinson's disease: pathophysiology.* Lancet, 1991. **337**(8753): p. 1321-4.

22. Hornykiewicz, O., *Chemical neuroanatomy of the basal ganglia--normal and in Parkinson's disease.* J Chem Neuroanat, 2001. **22**(1-2): p. 3-12.

23. Gibb, W.R. and A.J. Lees, *Anatomy, pigmentation, ventral and dorsal subpopulations of the substantia nigra, and differential cell death in Parkinson's disease.* J Neurol Neurosurg Psychiatry, 1991. **54**(5): p. 388-96.

24. Nyberg, P., et al., *Dopaminergic deficiency is more pronounced in putamen than in nucleus caudatus in Parkinson's disease.* Neurochemical Pathology, 1983. **1**(3): p. 193-202.

25. Kish, S.J., K. Shannak, and O. Hornykiewicz, *Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications.* N Engl J Med, 1988. **318**(14): p. 876-80.

26. Bernheimer, H. and O. Hornykiewicz, *[Decreased homovanillic acid concentration in the brain in parkinsonian subjects as an expression of a disorder of central dopamine metabolism].* Klin Wochenschr, 1965. **43**(13): p. 711-5.

27. Hornykiewicz, O. and O. Hornykiewicz, *Biochemical aspects of Parkinson's disease.* Neurology, 1998. **51**(2): p. S2-S9.

28. Piggott, M.A., et al., *Striatal dopaminergic markers in dementia with Lewy bodies, Alzheimer's and Parkinson's diseases: rostrocaudal distribution.* Brain, 1999. **122**: p. 1449-1468.

29. Buonamici, M., et al., *D-1 RECEPTOR SUPERSENSITIVITY IN THE RAT STRIATUM AFTER UNILATERAL 6-HYDROXYDOPAMINE LESIONS.* European Journal of Pharmacology, 1986. **126**(3): p. 347-348.

30. Rinne, U.K., et al., *POSITRON EMISSION TOMOGRAPHY DEMONSTRATES DOPAMINE-D2 RECEPTOR SUPERSENSITIVITY IN THE STRIATUM OF PATIENTS WITH EARLY PARKINSONS-DISEASE.* Movement Disorders, 1990. **5**(1): p. 55-59.

31. Gerfen, C.R., et al., *D1 dopamine receptor supersensitivity in the dopamine-depleted striatum results from a switch in the regulation of ERK1/2/MAP kinase.* J Neurosci, 2002. **22**(12): p. 5042-54.

32. Mizuno, Y., *Pathophysiology of Parkinson's Disease, Neurochemical Pathology: Other Neurotransmitters*, in *Parkinson's Disease*, R.F. Pfeiffer, Z.K. Wszolek, and M. Ebadi, Editors. 2013, CRC Press: Boca Raton. p. 529-555.

33. Shults, C.W., *Lewy bodies.* Proc Natl Acad Sci U S A, 2006. **103**(6): p. 1661-8.

34. Wakabayashi, K., et al., *The Lewy body in Parkinson's disease and related neurodegenerative disorders.* Mol Neurobiol, 2013. **47**(2): p. 495-508.

35. Gibb, W. and A. Lees, *Pathological Clues to the cause of Parkinson's disease*, in *Movement Disorders 3*, C. Marsden and S. Fahn, Editors. 1994, Butterworth-Heinemann: Oxford. p. 147-166.

36. Holdorff, B., *Friedrich Heinrich Lewy (1885-1950) and his work.* J Hist Neurosci, 2002. **11**(1): p. 19-28.

37. Leverenz, J.B., et al., *Proteomic identification of novel proteins in cortical lewy bodies.* Brain Pathol, 2007. **17**(2): p. 139-45.

38. Roy, S. and L. Wolman, *Ultrastructural observations in Parkinsonism.* J Pathol, 1969. **99**(1): p. 39-44.

39. Dickson, D.W., *Neuropathology of Parkinsonism*, in *Handbook of Parkinson's Disease*, R. Pahwa and K.E. Lyons, Editors. 2013, CRC Press: New York. p. 239-257.

40. Braak, H. and K. Del Tredici, *Neuroanatomy and pathology of sporadic Parkinson's disease*, in *Advances in anatomy, embryology, and cell biology,*. 2009, Springer Verlag,: Berlin. p. vi, 119 p.

41. Steiner, J.A., E. Angot, and P. Brundin, *A deadly spread: cellular mechanisms of alpha-synuclein transfer.* Cell Death Differ, 2011. **18**(9): p. 1425-33.

42. Parashos, S.A., et al., *Measuring disease progression in early Parkinson disease: the National Institutes of Health Exploratory Trials in Parkinson Disease (NET-PD) experience.* JAMA Neurol, 2014. **71**(6): p. 710-6.

43. Writing Group for the, N.E.T.i.P.D.I., et al., *Effect of creatine monohydrate on clinical progression in patients with Parkinson disease: a randomized clinical trial.* JAMA, 2015. **313**(6): p. 584-93.

44. Kingsbury, A.E., et al., *Brain stem pathology in Parkinson's disease: an evaluation of the Braak staging model.* Mov Disord, 2010. **25**(15): p. 2508-15.

45. Cheng, H.C., C.M. Ulane, and R.E. Burke, *Clinical progression in Parkinson disease and the neurobiology of axons.* Ann Neurol, 2010. **67**(6): p. 715-25.

46. Kordower, J.H., et al., *Disease duration and the integrity of the nigrostriatal system in Parkinson's disease.* Brain, 2013. **136**(Pt 8): p. 2419-31.

47. Fujioka, S., et al., *Genetics*, in *Handbook of Parkinson's Disease*, R. Pahwa and K.E. Lyons, Editors. 2013, CRC Press: New York. p. 317-340.

48. Wirdefeldt, K., et al., *Epidemiology and etiology of Parkinson's disease: a review of the evidence.* Eur J Epidemiol, 2011. **26 Suppl 1**: p. S1-58.

49. Polymeropoulos, M.H., et al., *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease.* Science, 1997. **276**(5321): p. 2045-7.

50. Ibáñez, P., et al., *Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease.* Lancet, 2004. **364**(9440): p. 1169-71.

51. Kruger, R., et al., *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease.* Nat Genet, 1998. **18**(2): p. 106-8.

52. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease.* Science, 2003. **302**(5646): p. 841.

53. Gasser, T., J. Hardy, and Y. Mizuno, *Milestones in PD genetics.* Mov Disord, 2011. **26**(6): p. 1042-8.

54. Lees, A.J., J. Hardy, and T. Revesz, *Parkinson's disease.* Lancet, 2009. **373**(9680): p. 2055-2066.

55. Giasson, B.I., et al., *Biochemical and pathological characterization of Lrrk2.* Ann Neurol, 2006. **59**(2): p. 315-22.

56. Paisan-Ruiz, C., et al., *Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease.* Neuron, 2004. **44**(4): p. 595-600.

57. Ross, O.A., et al., *Lrrk2 and Lewy body disease.* Ann Neurol, 2006. **59**(2): p. 388-93.

58. Zimprich, A., et al., *The PARK8 locus in autosomal dominant parkinsonism: confirmation of linkage and further delineation of the disease-containing interval.* Am J Hum Genet, 2004. **74**(1): p. 11-9.

59. Kitada, T., et al., *Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism.* Nature, 1998. **392**(6676): p. 605-8.

60. Lucking, C.B., et al., *Association between early-onset Parkinson's disease and mutations in the parkin gene.* N Engl J Med, 2000. **342**(21): p. 1560-7.

61. Houlden, H. and A.B. Singleton, *The genetics and neuropathology of Parkinson's disease.* Acta Neuropathol, 2012. **124**(3): p. 325-38.

62. Trempe, J.F. and E.A. Fon, *Structure and Function of Parkin, PINK1, and DJ-1, the Three Musketeers of Neuroprotection.* Front Neurol, 2013. **4**: p. 38.

63. *Genetics and Parkinson's Disease*. [cited 2015 April 4]; Available from: https://www.michaeljfox.org/understanding-parkinsons/living-with-pd/topic.php?genetics.

64. Nelson, G. and B.A. Racette, *Environmental Risk Factors*, in *Handbook of Parkinson's Disease*, R. Pahwa and K.E. Lyons, Editors. 2013, CRC Press: New York. p. 341-357.

65. Langston, J.W., et al., *CHRONIC PARKINSONISM IN HUMANS DUE TO A PRODUCT OF MEPERIDINE-ANALOG SYNTHESIS.* Science, 1983. **219**(4587): p. 979-980.

66. Haubrich, W.S., *Medical meanings : a glossary of word origins*. 2nd ed. 2003, Philadelphia: American College of Physicians. xii, 267 p.

67. Bennett, D.A., et al., *Prevalence of parkinsonian signs and associated mortality in a community population of older people.* N Engl J Med, 1996. **334**(2): p. 71-6.

68. Morens, D.M., et al., *Epidemiologic observations on Parkinson's disease: incidence and mortality in a prospective study of middle-aged men.* Neurology, 1996. **46**(4): p. 1044-50.

69. Tanner, C.M. and S.M. Goldman, *Epidemiology of Parkinson's disease.* Neurol Clin, 1996. **14**(2): p. 317-35.

70. Collier, T.J., N.M. Kanaan, and J.H. Kordower, *Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates.* Nat Rev Neurosci, 2011. **12**(6): p. 359-66.

71. Feany, M.B. and W.W. Bender, *A Drosophila model of Parkinson's disease.* Nature, 2000. **404**(6776): p. 394-398.

72. Lakso, M., et al., *Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human alpha-synuclein.* Journal of Neurochemistry, 2003. **86**(1): p. 165-172.

73. Masliah, E., et al., *Dopaminergic loss and inclusion body formation in alpha-synuclein mice: Implications for neurodegenerative disorders.* Science, 2000. **287**(5456): p. 1265-1269.

74. Lo Bianco, C., et al., *alpha -Synucleinopathy and selective dopaminergic neuron loss in a rat lentiviral-based model of Parkinson's disease.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10813-8.

75. Dawson, T.M., H.S. Ko, and V.L. Dawson, *Genetic animal models of Parkinson's disease.* Neuron, 2010. **66**(5): p. 646-61.

76. Nuber, S., et al., *A progressive dopaminergic phenotype associated with neurotoxic conversion of alpha-synuclein in BAC-transgenic rats.* Brain, 2013. **136**(Pt 2): p. 412-32.

77. Lee, K.H., et al., *High-frequency stimulation of the subthalamic nucleus increases glutamate in the subthalamic nucleus of rats as demonstrated by in vivo enzyme-linked glutamate sensor.* Brain Research, 2007. **1162**: p. 121-129.

78. Saha, R.N., et al., *HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis.* Cell Death and Differentiation, 2006. **13**(4): p. 539-550.

79. Li, Y.P., et al., *Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease.* Nature Neuroscience, 2009. **12**(7): p. 826-828.

80. Dave, K.D., et al., *Phenotypic characterization of recessive gene knockout rat models of Parkinson's disease.* Neurobiol Dis, 2014. **70**: p. 190-203.

81. Bergman, H., et al., *THE PRIMATE SUBTHALAMIC NUCLEUS .2. NEURONAL-ACTIVITY IN THE MPTP MODEL OF PARKINSONISM.* Journal of Neurophysiology, 1994. **72**(2): p. 507-520.

82. Hassani, O.K., et al., *Increased subthalamic neuronal activity after nigral dopaminergic lesion independent of disinhibition via the globus pallidus.* Neuroscience, 1996. **72**(1): p. 105-115.

83. Hutchison, W.D., et al., *Neurophysiological identification of the subthalamic nucleus in surgery for Parkinson's disease.* Annals of Neurology, 1998. **44**(4): p. 622-628.

84. Wichmann, T., et al., *Comparison of MPTP-induced changes in spontaneous neuronal discharge in the internal pallidal segment and in the substantia nigra pars reticulata in primates.* Experimental Brain Research, 1999. **125**(4): p. 397-409.

85. Magnin, M., et al., *Single-unit analysis of the pallidum, thalamus and subthalamic nucleus in parkinsonian patients.* Neuroscience, 2000. **96**(3): p. 549-564.

86. Difazio, M.C., et al., *GLUTAMATE RECEPTORS IN THE SUBSTANTIA-NIGRA OF PARKINSONS-DISEASE BRAINS.* Neurology, 1992. **42**(2): p. 402-406.

87. Vila, M., et al., *Consequences of nigrostriatal denervation on the functioning of the basal ganglia in human and nonhuman primates: An in situ hybridization study of cytochrome oxidase subunit I mRNA.* Journal of Neuroscience, 1997. **17**(2): p. 765-773.

88. Rodriguez, M.C., et al., *Subthalamic nucleus-mediated excitotoxicity in Parkinson's disease: A target for neuroprotection.* Annals of Neurology, 1998. **44**(3): p. S175-S188.

89. Ni, Z.G., et al., *Time-course of changes in firing rates and firing patterns of subthalamic nucleus neuronal activity after 6-OHDA-induced dopamine depletion in rats.* Brain Research, 2001. **899**(1-2): p. 142-147.

90. Breit, S., et al., *Unilateral lesion of the pedunculopontine nucleus induces hyperactivity in the subthalamic nucleus and substantia nigra in the rat.* European Journal of Neuroscience, 2005. **22**(9): p. 2283-2294.

91. Gajendiran, M., et al., *Electrophysiological alterations in subthalamic neurons after unilateral dopamine depletion in the rat.* Journal of Neuroscience Research, 2005. **80**(2): p. 203-210.

92. Crossman, A.R., et al., *REGIONAL BRAIN UPTAKE OF 2-DEOXYGLUCOSE IN N-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP)-INDUCED PARKINSONISM IN THE MACAQUE MONKEY.* Neuropharmacology, 1985. **24**(6): p. 587-&.

93. Schwartzman, R.J., et al., *CHANGES IN THE LOCAL CEREBRAL METABOLIC-RATE FOR GLUCOSE IN THE 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE (MPTP) PRIMATE MODEL OF PARKINSONS-DISEASE.* Brain Research, 1985. **358**(1-2): p. 137-143.

94. Porter, R.H.P., et al., *POLYSYNAPTIC REGULATION OF GLUTAMATE RECEPTORS AND MITOCHONDRIAL ENZYME-ACTIVITIES IN THE BASAL*

GANGLIA OF RATS WITH UNILATERAL DOPAMINE DEPLETION. Journal of Neuroscience, 1994. **14**(11): p. 7192-7199.

95. Wullner, U., et al., *GLUTAMATE RECEPTORS IN STRIATUM AND SUBSTANTIA-NIGRA - EFFECTS OF MEDIAL FOREBRAIN-BUNDLE LESIONS.* Brain Research, 1994. **645**(1-2): p. 98-102.

96. Kaneda, K., et al., *Down-regulation of metabotropic glutamate receptor 1 alpha in globus pallidus and substantia nigra of parkinsonian monkeys.* European Journal of Neuroscience, 2005. **22**(12): p. 3241-3254.

97. Blandini, F., et al., *Time-course of nigrostriatal damage, basal ganglia metabolic changes and behavioural alterations following intrastriatal injection of 6-hydroxydopamine in the rat: new clues from an old model.* Eur J Neurosci, 2007. **25**(2): p. 397-405.

98. Schallert, T., et al., *CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury.* Neuropharmacology, 2000. **39**(5): p. 777-87.

99. Schallert, T. and J.L. Tillerson, *Intervention Strategies for Degeneration of Dopamine Neurons in Parkinsonism: Optimizing Behavioral Assessment of Outcome*, in *Central Nervous System Diseases: Innovative Models of CNS Diseases from Molecule to Therapy*, D.F. Emerich, R.L.D. III, and P.R. Sanberg, Editors. 2000, Humana Press: Totowa, NJ. p. 131-151.

100. Meredith, G.E., et al., *Behavioral models of Parkinson's disease in rodents: A new look at an old problem.* Movement Disorders, 2006. **21**(10): p. 1595-1606.

101. Fleming, S.M., *Behavioral outcome measures for the assessment of sensorimotor function in animal models of movement disorders.* Int Rev Neurobiol, 2009. **89**: p. 57-65.

102. Lundblad, M., et al., *Pharmacological validation of behavioural measures of akinesia and dyskinesia in a rat model of Parkinson's disease.* Eur J Neurosci, 2002. **15**(1): p. 120-32.

103. Winkler, C., et al., *L-DOPA-induced dyskinesia in the intrastriatal 6-hydroxydopamine model of Parkinson's disease: Relation to motor and cellular parameters of nigrostriatal function.* Neurobiology of Disease, 2002. **10**(2): p. 165-186.

104. Petzinger, G.M. and M.W. Jakowec, *Animal Models of Parkinson's Disease and Related Disorders*, in *Handbook of Parkinson's Disease*, R. Pahwa and K.E. Lyons, Editors. 2013, CRC Press: Boca Raton, FL. p. 281-316.

105. Sanders, L.H. and J.T. Greenamyre, *Oxidative damage to macromolecules in human Parkinson disease and the rotenone model.* Free Radic Biol Med, 2013. **62**: p. 111-20.

106. Johnson, M.E. and L. Bobrovskaya, *An update on the rotenone models of Parkinson's disease: Their ability to reproduce the features of clinical disease and model gene-environment interactions.* Neurotoxicology, 2015. **46C**: p. 101-116.

107. Porter, C.C., et al., *EFFECT OF 6-HYDROXYDOPAMINE AND SOME OTHER COMPOUNDS ON CONCENTRATION OF NOREPINEPHRINE IN HEARTS OF MICE.* Journal of Pharmacology and Experimental Therapeutics, 1963. **140**(3): p. 308-&.

108. Ungerste.U and Ungerste.U, *6-HYDROXY-DOPAMINE INDUCED DEGENERATION OF CENTRAL MONOAMINE NEURONS.* European Journal of Pharmacology, 1968. **5**(1): p. 107-&.

109. Ungerstedt, U. and G.W. Arbuthnott, *QUANTITATIVE RECORDING OF ROTATIONAL BEHAVIOR IN RATS AFTER 6 HYDROXY DOPAMINE LESIONS OF THE NIGROSTRIATAL DOPAMINE SYSTEM.* Brain Research, 1970. **24**(3): p. 485-493.

110. Ungerstedt, U., *Adipsia and aphagia after 6-hydroxydopamine induced degeneration of the nigro-striatal dopamine system.* Acta physiologica Scandinavica. Supplementum, 1971. **367**: p. 95-122.

111. Blandini, F., M.-T. Armentero, and E. Martignoni, *The 6-hydroxydopamine model: news from the past.* Parkinsonism & related disorders, 2008. **14 Suppl 2**: p. S124-9.

112. Simola, N., et al., *The 6-hydroxydopamine model of Parkinson's disease.* Neurotoxicity Research, 2007. **11**(3-4): p. 151-167.

113. Glinka, Y.Y., et al., *INHIBITION OF MITOCHONDRIAL COMPLEX-I AND COMPLEX-IV BY 6-HYDROXYDOPAMINE.* European Journal of Pharmacology-Environmental Toxicology and Pharmacology Section, 1995. **292**(3-4): p. 329-332.

114. Rodrigues, R.W.P., et al., *Astroglial and microglial reaction after a partial nigrostriatal degeneration induced by the striatal injection of different doses of 6-hydroxydopamine.* International Journal of Neuroscience, 2001. **109**(1-2): p. 91-126.

115. Cicchetti, F., et al., *Neuroinflammation of the nigrostriatal pathway during progressive 6-OHDA dopamine degeneration in rats monitored by immunohistochemistry and PET imaging.* European Journal of Neuroscience, 2002. **15**(6): p. 991-998.

116. Mogi, M. and T. Nagatsu, *Neurotrophins and cytokines in Parkinson's disease.* Advances in neurology, 1999. **80**: p. 135-9.

117. Wilms, H., et al., *Involvement of benzodiazepine receptors in neuroinflammatory and neurodegenerative diseases: evidence from activated microglial cells in vitro.* Neurobiology of Disease, 2003. **14**(3): p. 417-424.

118. Wilms, H., et al., *Activation of microglia by human neuromelanin is NF-kappa B-dependent and involves p38 mitogen-activated protein kinase: implications for Parkinson's disease.* Faseb Journal, 2003. **17**(1): p. 500-+.

119. Nagatsu, T., et al., *Inflammatory process in Parkinson's disease: Role for cytokines.* Current Pharmaceutical Design, 2005. **11**(8): p. 999-1016.

120. McCoy, M.K., et al., *Blocking soluble tumor necrosis factor signaling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease.* Journal of Neuroscience, 2006. **26**(37): p. 9365-9375.

121. Armentero, M.T., et al., *Peripheral inflammation and neuroprotection: Systemic pretreatment with complete Freund's adjuvant reduces 6-hydroxydopamine toxicity in a rodent model of Parkinson's disease.* Neurobiology of Disease, 2006. **24**(3): p. 492-505.

122. Na, S.J., et al., *Molecular Profiling of a 6-Hydroxydopamine Model of Parkinson's Disease.* Neurochemical Research, 2010. **35**(5): p. 761-772.

123. Sanchez-Guajardo, V., et al., *Neuroimmunological processes in Parkinson's disease and their relation to alpha-synuclein: microglia as the referee between neuronal processes and peripheral immunity.* ASN Neuro, 2013. **5**(2): p. 113-39.

124. Mogi, M., et al., *Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients.* Neurosci Lett, 1994. **180**(2): p. 147-50.

125. Mogi, M., et al., *Tumor necrosis factor-alpha (TNF-alpha) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients.* Neurosci Lett, 1994. **165**(1-2): p. 208-10.

126. McGeer, P.L., et al., *Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains.* Neurology, 1988. **38**(8): p. 1285-91.

127. McGeer, P.L., S. Itagaki, and E.G. McGeer, *Expression of the histocompatibility glycoprotein HLA-DR in neurological disease.* Acta Neuropathol, 1988. **76**(6): p. 550-7.

128. Imamura, K., et al., *Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains.* Acta Neuropathol, 2003. **106**(6): p. 518-26.

129. Croisier, E., et al., *Microglial inflammation in the parkinsonian substantia nigra: relationship to alpha-synuclein deposition.* J Neuroinflammation, 2005. **2**: p. 14.

130. Gerhard, A., et al., *In vivo imaging of microglial activation with [11C](R)-PK11195 PET in idiopathic Parkinson's disease.* Neurobiol Dis, 2006. **21**(2): p. 404-12.

131. Doorn, K.J., et al., *Microglial phenotypes and toll-like receptor 2 in the substantia nigra and hippocampus of incidental Lewy body disease cases and Parkinson inverted question marks disease patients.* Acta Neuropathol Commun, 2014. **2**(1): p. 90.

132. Deumens, R., et al., *Modeling Parkinsn's disease in rats: An evaluation of 6-OHDA lesions of the nigrostriatal pathway.* Experimental Neurology, 2002. **175**(2): p. 303-317.

133. Berger, K., et al., *RETROGRADE DEGENERATION OF NIGROSTRIATAL NEURONS INDUCED BY INTRASTRIATAL 6-HYDROXYDOPAMINE INJECTION IN RATS.* Brain Research Bulletin, 1991. **26**(2): p. 301-307.

134. Spieles-Engemann, A.L., et al., *Stimulation of the rat subthalamic nucleus is neuroprotective following significant nigral dopamine neuron loss.* Neurobiol Dis, 2010. **39**(1): p. 105-15.

135. Spieles-Engemann, A.L., T.J. Collier, and C.E. Sortwell, *A functionally relevant and long-term model of deep brain stimulation of the rat subthalamic nucleus: advantages and considerations.* Eur J Neurosci, 2010. **32**(7): p. 1092-9.

136. Spieles-Engemann, A.L., et al., *Subthalamic Nucleus Stimulation Increases Brain Derived Neurotrophic Factor in the Nigrostriatal System and Primary Motor Cortex.* J Parkinsons Dis, 2011. **1**(1): p. 123-136.

137. Betarbet, R., et al., *Animal models of Parkinson's disease.* Bioessays, 2002. **24**(4): p. 308-318.

138. Del Tredici, K., et al., *Where does Parkinson disease pathology begin in the brain?* Journal of Neuropathology and Experimental Neurology, 2002. **61**(5): p. 413-426.

139. Schober, A. and A. Schober, *Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP.* Cell and Tissue Research, 2004. **318**(1): p. 215-224.

140. Espay, A.J., *Seminar: Clinical Research in Parkinson's Disease and Other Movement Disorders*. 2011, Michigan State University: Secchia Center, Grand Rapids, MI, USA.

141. Goodman, L.S., et al., *Goodman & Gilman's pharmacological basis of therapeutics*. 2011, McGraw-Hill,: New York.

142. Agid, Y., et al., *Adverse reactions to levodopa: drug toxicity or progression of disease?* Lancet, 1998. **351**(9106): p. 851-852.

143. Miyasaki, J.M., et al., *Practice parameter: Initiation of treatment for Parkinson's disease: An evidence-based review - Report of the quality standards subcommittee of the American Academy of Neurology.* Neurology, 2002. **58**(1): p. 11-17.

144. Goudreau, J.L. and J.E. Ahlskog, *Symptomatic Treatment of Parkinson's Disease: Levodopa*, in *Parkinson's disease*, R. Pfeiffer, Z.K. Wszolek, and M.S. Ebadi, Editors. 2013, CRC Press/Taylor & Francis Group: Boca Raton, FL. p. 847-863.

145. Schrag, A., et al., *Dyskinesias and motor fluctuations in Parkinson's disease - A community-based study.* Brain, 2000. **123**: p. 2297-2305.

146. Hely, M.A., et al., *The Sydney multicentre study of Parkinson's disease: progression and mortality at 10 years.* Journal of Neurology Neurosurgery and Psychiatry, 1999. **67**(3): p. 300-307.

147. Pahwa, R., et al., *Practice Parameter: treatment of Parkinson disease with motor fluctuations and dyskinesia (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology.* Neurology, 2006. **66**(7): p. 983-95.

148. Zesiewicz, T.A., et al., *Neurosurgery for Parkinson's disease.* Seminars in Neurology, 2001. **21**(1): p. 91-101.

149. Tasker, R.R., *Thalamotomy.* Neurosurg Clin N Am, 1990. **1**(4): p. 841-64.

150. Lozano, A.M., *Pallidotomy: Where are we?* Parkinsonism & related disorders, 1996. **2**(2): p. 53-4.

151. Sassi, M. and M.S. Okun, *Stereotactic Ablative Procedures for Intractable Parkinson's Disease*, in *Parkinson's Disease*, R.F. Pfeiffer, Z.K. Wszolek, and M. Ebadi, Editors. 2013, CRC Press: Boca Raton, FL. p. 893-902.

152. Collins, K.L., et al., *Deep brain stimulation for movement disorders.* Neurobiology of Disease, 2010. **38**(3): p. 338-345.

153. Horsley, V., et al., *The structure and functions of the cerebellum examined by a new method.* Brain, 1908. **31**: p. 45-124.

154. Horsley, V., *The Linacre Lecture ON THE FUNCTION OF THE SO-CALLED MOTOR AREA OF THE BRAIN: Delivered to the Master and Fellows of St. John's College, Cambridge, May 6th, 1909.* Br Med J, 1909. **2**(2533): p. 121-32.

155. Bucy, P.C., et al., *Athetosis.* Brain, 1932. **55**: p. 479-492.

156. Bucy, P.C., et al., *Tremor - Physiologic mechanism and abolition by surgical means.* Archives of Neurology and Psychiatry, 1939. **41**(4): p. 721-746.

157. Spiegel, E.A., et al., *Stereotaxic Apparatus for Operations on the Human Brain.* Science, 1947. **106**(2754): p. 349-50.

158. Cooper, I.S. and G. Bravo, *Chemopallidectomy and chemothalamectomy.* J Neurosurg, 1958. **15**(3): p. 244-50.

159. Hassler, R. and T. Riechert, *[Indications and localization of stereotactic brain operations].* Nervenarzt, 1954. **25**(11): p. 441-7.

160. Hassler, R., *Extrapyramidal cortical systems and central regulation of motor function.* Deutsche Zeitschrift fur Nervenheilkunde, 1956. **175**(3): p. 233-58.

161. Narabayashi, H., T. Okuma, and S. Shikiba, *Procaine oil blocking of the globus pallidus.* AMA Arch Neurol Psychiatry, 1956. **75**(1): p. 36-48.

162. Svennilson, E., et al., *Treatment of parkinsonism by stereotatic thermolesions in the pallidal region. A clinical evaluation of 81 cases.* Acta Psychiatr Scand, 1960. **35**: p. 358-77.

163. Whitty, C.W., et al., *Anterior cingulectomy in the treatment of mental disease.* Lancet, 1952. **1**(6706): p. 475-81.

164. Ballantine, H.T., Jr., et al., *Stereotaxic anterior cingulotomy for neuropsychiatric illness and intractable pain.* J Neurosurg, 1967. **26**(5): p. 488-95.

165. Knight, G., *Stereotactic Tractotomy in the Surgical Treatment of Mental Illness.* J Neurol Neurosurg Psychiatry, 1965. **28**: p. 304-10.

166. Kelly, D., A. Richardson, and N. Mitchell-Heggs, *Stereotactic limbic leucotomy: neurophysiological aspects and operative technique.* Br J Psychiatry, 1973. **123**(573): p. 133-40.

167. Kopell, B.H., A.G. Machado, and A.R. Rezai, *Not your father's lobotomy: psychiatric surgery revisited.* Clin Neurosurg, 2005. **52**: p. 315-30.

168. Heller, A.C., et al., *Surgery of the mind and mood: a mosaic of issues in time and evolution.* Neurosurgery, 2006. **59**(4): p. 720-33; discussion 733-9.

169. Hassler, R., et al., *Physiological observations in stereotaxic operations in extrapyramidal motor disturbances.* Brain, 1960. **83**: p. 337-50.

170. Rezai, A.R., et al., *Surgery for movement disorders.* Neurosurgery, 2008. **62 Suppl 2**: p. 809-38; discussion 838-9.

171. Bechtereva, N.P., et al., *Method of electrostimulation of the deep brain structures in treatment of some chronic diseases.* Confin Neurol, 1975. **37**(1-3): p. 136-40.

172. Schwalb, J.M., et al., *The history and future of deep brain stimulation.* Neurotherapeutics, 2008. **5**(1): p. 3-13.

173. Benabid, A.L., et al., *Combined (thalamotomy and stimulation) stereotactic surgery of the VIM thalamic nucleus for bilateral Parkinson disease.* Appl Neurophysiol, 1987. **50**(1-6): p. 344-6.

174. Benabid, A.L., et al., *LONG-TERM SUPPRESSION OF TREMOR BY CHRONIC STIMULATION OF THE VENTRAL INTERMEDIATE THALAMIC NUCLEUS.* Lancet, 1991. **337**(8738): p. 403-406.

175. Laitinen, L.V., et al., *VENTROPOSTEROLATERAL PALLIDOTOMY CAN ABOLISH ALL PARKINSONIAN SYMPTOMS.* Stereotactic and Functional Neurosurgery, 1992. **58**(1-4): p. 14-21.

176. Siegfried, J., et al., *BILATERAL CHRONIC ELECTROSTIMULATION OF VENTROPOSTEROLATERAL PALLIDUM - A NEW THERAPEUTIC APPROACH FOR ALLEVIATING ALL PARKINSONIAN SYMPTOMS.* Neurosurgery, 1994. **35**(6): p. 1126-1129.

177. Delong, M.R., et al., *FUNCTIONAL-ORGANIZATION OF THE BASAL GANGLIA - CONTRIBUTIONS OF SINGLE-CELL RECORDING STUDIES.* Ciba Foundation Symposia, 1984. **107**: p. 64-82.

178. Benabid, A.L., et al., *Acute and long-term effects of subthalamic nucleus stimulation in Parkinson's disease.* Stereotactic and Functional Neurosurgery, 1994. **62**(1-4): p. 76-84.

179. Krack, P., et al., *Subthalamic nucleus or internal pallidal stimulation in young onset Parkinson's disease.* Brain, 1998. **121 (Pt 3)**: p. 451-7.

180. Kumar, R., et al., *Pallidotomy and deep brain stimulation of the pallidum and subthalamic nucleus in advanced Parkinson's disease.* Movement Disorders, 1998. **13**: p. 73-82.

181. Follett, K.A., et al., *Pallidal versus subthalamic deep-brain stimulation for Parkinson's disease.* N Engl J Med, 2010. **362**(22): p. 2077-91.

182. Ellenbogen, R.G., S.I. Abdulrauf, and L.N. Sekhar, *Principles of neurological surgery*. 2012, Saunders/Elsevier,: Philadelphia, PA. p. p.

183. Benabid, A.L., et al., *Deep brain stimulation of the subthalamic nucleus for the treatment of Parkinson's disease.* Lancet Neurol, 2009. **8**(1): p. 67-81.

184. Binder, D.K., G.M. Rau, and P.A. Starr, *Risk factors for hemorrhage during microelectrode-guided deep brain stimulator implantation for movement disorders.* Neurosurgery, 2005. **56**(4): p. 722-32; discussion 722-32.

185. Deep-Brain Stimulation for Parkinson's Disease Study, G., *Deep-brain stimulation of the subthalamic nucleus or the pars interna of the globus pallidus in Parkinson's disease.* N Engl J Med, 2001. **345**(13): p. 956-63.

186. Kleiner-Fisman, G., et al., *Subthalamic nucleus deep brain stimulation: Summary and meta-analysis of outcomes.* Movement Disorders, 2006. **21**: p. S290-S304.

187. Parsons, T.D., et al., *Cognitive sequelae of subthalamic nucleus deep brain stimulation in Parkinson's disease: a meta-analysis.* Lancet Neurology, 2006. **5**(7): p. 578-588.

188. Smeding, H.M.M., et al., *Neuropsychological effects of bilateral STN stimulation in Parkinson disease - A controlled study.* Neurology, 2006. **66**(12): p. 1830-1836.

189. Castelli, L., et al., *Neuropsychological changes 1-year after subthalamic DBS in PD patients: A prospective controlled study.* Parkinsonism & Related Disorders, 2010. **16**(2): p. 115-118.

190. Fasano, A., et al., *Motor and cognitive outcome in patients with Parkinson's disease 8 years after subthalamic implants.* Brain, 2010. **133**: p. 2664-2676.

191. Mikos, A., et al., *Patient-specific analysis of the relationship between the volume of tissue activated during DBS and verbal fluency.* Neuroimage, 2011. **54**: p. S238-S246.

192. Saint-Cyr, J.A., et al., *Neuropsychological consequences of chronic bilateral stimulation of the subthalamic nucleus in Parkinson's disease.* Brain, 2000. **123**: p. 2091-2108.

193. Krack, P., et al., *Five-year follow-up of bilateral stimulation of the subthalamic nucleus in advanced Parkinson's disease.* New England Journal of Medicine, 2003. **349**(20): p. 1925-1934.

194. Herzog, E.D. and R.M. Huckfeldt, *Circadian entrainment to temperature, but not light, in the isolated suprachiasmatic nucleus.* J Neurophysiol, 2003. **90**(2): p. 763-70.

195. Rodriguez-Oroz, M.C., et al., *Efficacy of deep brain stimulation of the subthalamic nucleus in Parkinson's disease 4 years after surgery: double blind and open label evaluation.* Journal of Neurology Neurosurgery and Psychiatry, 2004. **75**(10): p. 1382-1385.

196. Schupbach, W.M.M., et al., *Stimulation of the subthalamic nucleus in Parkinson's disease: a 5 year follow up.* Journal of Neurology Neurosurgery and Psychiatry, 2005. **76**(12): p. 1640-1644.

197. Ostergaard, K., et al., *Evolution of Parkinson's disease during 4 years of bilateral deep brain stimulation of the subthalamic nucleus.* Movement Disorders, 2006. **21**(5): p. 624-631.

198. Limousin, P., et al., *Deep brain stimulation for Parkinson's disease.* Neurotherapeutics, 2008. **5**(2): p. 309-319.

199. Martinez-Martin, P., et al., *Effect of medical and surgical interventions on health-related quality of life in Parkinson's disease.* Movement Disorders, 2007. **22**(6): p. 757-765.

200. Deuschl, G., et al., *A randomized trial of deep-brain stimulation for Parkinson's disease.* New England Journal of Medicine, 2006. **355**(9): p. 896-908.

201. Charles, P.D., et al., *Deep brain stimulation of the subthalamic nucleus reduces antiparkinsonian medication costs.* Parkinsonism & Related Disorders, 2004. **10**(8): p. 475-479.

202. Meissner, W., et al., *Deep brain stimulation in late stage Parkinson's disease: a retrospective cost analysis in Germany.* J Neurol, 2005. **252**(2): p. 218-23.

203. Moum, S.J., et al., *Effects of STN and GPi Deep Brain Stimulation on Impulse Control Disorders and Dopamine Dysregulation Syndrome.* Plos One, 2012. **7**(1): p. 8.

204. Bronstein, J.M., et al., *Deep brain stimulation for Parkinson disease: an expert consensus and review of key issues.* Arch Neurol, 2011. **68**(2): p. 165.

205. Moro, E., et al., *Long-term results of a multicenter study on subthalamic and pallidal stimulation in Parkinson's disease.* Mov Disord, 2010. **25**(5): p. 578-86.

206. Andrade, P., J.D. Carrillo-Ruiz, and F. Jimenez, *A systematic review of the efficacy of globus pallidus stimulation in the treatment of Parkinson's disease.* J Clin Neurosci, 2009. **16**(7): p. 877-81.

207. Adler, C.H., et al., *Low clinical diagnostic accuracy of early vs advanced Parkinson disease: clinicopathologic study.* Neurology, 2014. **83**(5): p. 406-12.

208. Chang, V.C. and K.L. Chou, *Deep brain stimulation for Parkinson's disease: patient selection and motor outcomes.* Med Health R I, 2006. **89**(4): p. 142-4.

209. Espay, A.J., et al., *Surgical treatment of movement disorders.* Clinics in Geriatric Medicine, 2006. **22**(4): p. 813-+.

210. Neimat, J.S., C. Hamani, and A.M. Lozano, *Neural stimulation for Parkinson's disease: current therapies and future directions.* Expert review of neurotherapeutics, 2006. **6**(1): p. 101-9.

211. Shih, L.C. and D. Tarsy, *Deep brain stimulation for the treatment of atypical parkinsonism.* Mov Disord, 2007. **22**(15): p. 2149-55.

212. Okun, M.S., *Deep-brain stimulation for Parkinson's disease.* N Engl J Med, 2012. **367**(16): p. 1529-38.

213. Okun, M.S. and K.D. Foote, *Parkinson's disease DBS: what, when, who and why? The time has come to tailor DBS targets.* Expert Rev Neurother, 2010. **10**(12): p. 1847-57.

214. Williams, N.R., K.D. Foote, and M.S. Okun, *STN vs. GPi Deep Brain Stimulation: Translating the Rematch into Clinical Practice.* Mov Disord Clin Pract (Hoboken), 2014. **1**(1): p. 24-35.

215. Rodriguez-Oroz, M.C., et al., *Bilateral deep brain stimulation in Parkinson's disease: a multicentre study with 4 years follow-up.* Brain, 2005. **128**(Pt 10): p. 2240-9.

216. Krause, M., et al., *Deep brain stimulation for the treatment of Parkinson's disease: subthalamic nucleus versus globus pallidus internus.* J Neurol Neurosurg Psychiatry, 2001. **70**(4): p. 464-70.

217. Deuschl, G., et al., *Deep-brain stimulation for Parkinson's disease.* Journal of Neurology, 2002. **249**(Suppl. 3): p. III.36-III.39.

218. Benazzouz, A., et al., *RESPONSES OF SUBSTANTIA-NIGRA PARS RETICULATA AND GLOBUS-PALLIDUS COMPLEX TO HIGH-FREQUENCY STIMULATION OF THE SUBTHALAMIC NUCLEUS IN RATS - ELECTROPHYSIOLOGICAL DATA.* Neuroscience Letters, 1995. **189**(2): p. 77-80.

219. Benazzouz, A., et al., *Effect of high-frequency stimulation of the subthalamic nucleus on the neuronal activities of the substantia nigra pars reticulata and ventrolateral nucleus of the thalamus in the rat.* Neuroscience, 2000. **99**(2): p. 289-295.

220. Dostrovsky, J.O., et al., *Mechanisms of deep brain stimulation.* Movement Disorders, 2002. **17**: p. S63-S68.

221. McIntyre, C.C., et al., *Uncovering the mechanism(s) of action of deep brain stimulation: activation, inhibition, or both.* Clinical Neurophysiology, 2004. **115**(6): p. 1239-1248.

222. Ceballos-Baumann, A.O., et al., *A positron emission tomographic study of subthalamic nucleus stimulation in Parkinson disease: enhanced movement-related activity of motor-association cortex and decreased motor cortex resting activity.* Arch Neurol, 1999. **56**(8): p. 997-1003.

223. Hershey, T., et al., *Cortical and subcortical blood flow effects of subthalamic nucleus stimulation in PD.* Neurology, 2003. **61**(6): p. 816-21.

224. Windels, F., et al., *Effects of high frequency stimulation of subthalamic nucleus on extracellular glutamate and GABA in substantia nigra and globus pallidus in the normal rat.* Eur J Neurosci, 2000. **12**(11): p. 4141-6.

225. Boulet, S., et al., *Subthalamic stimulation-induced forelimb dyskinesias are linked to an increase in glutamate levels in the substantia nigra pars reticulata.* J Neurosci, 2006. **26**(42): p. 10768-76.

226. Garcia, L., et al., *Dual effect of high-frequency stimulation on subthalamic neuron activity.* J Neurosci, 2003. **23**(25): p. 8743-51.

227. Shi, L.H., et al., *Basal ganglia neural responses during behaviorally effective deep brain stimulation of the subthalamic nucleus in rats performing a treadmill locomotion test.* Synapse, 2006. **59**(7): p. 445-57.

228. Gradinaru, V., et al., *Optical deconstruction of parkinsonian neural circuitry.* Science, 2009. **324**(5925): p. 354-9.

229. Johnson, L.A., et al., *Modulation of motor cortex neuronal activity and motor behavior during subthalamic nucleus stimulation in the normal primate.* J Neurophysiol, 2015: p. jn 00997 2014.

230. Zheng, F., et al., *Axonal failure during high frequency stimulation of rat subthalamic nucleus.* J Physiol, 2011.

231. Brown, P., et al., *Effects of stimulation of the subthalamic area on oscillatory pallidal activity in Parkinson's disease.* Experimental Neurology, 2004. **188**(2): p. 480-490.

232. Meissner, W., et al., *Subthalamic high frequency stimulation resets subthalamic firing and reduces abnormal oscillations.* Brain, 2005. **128**: p. 2372-2382.

233. Silberstein, P., et al., *Cortico-cortical coupling in Parkinson's disease and its modulation by therapy.* Brain, 2005. **128**: p. 1277-1291.

234. Wingeier, B., et al., *Intra-operative STN DBS attenuates the prominent beta rhythm in the STN in Parkinson's disease.* Experimental Neurology, 2006. **197**(1): p. 244-251.

235. Bronte-Stewart, H., et al., *The STN beta-band profile in Parkinson's disease is stationary and shows prolonged attenuation after deep brain stimulation.* Experimental Neurology, 2009. **215**(1): p. 20-28.

236. Blumenfeld, Z., et al., *Sixty hertz neurostimulation amplifies subthalamic neural synchrony in Parkinson's disease.* PLoS One, 2015. **10**(3): p. e0121067.

237. Tagliati, M., C. Martin, and R. Alterman, *Lack of motor symptoms progression in Parkinson's disease patients with long-term bilateral subthalamic deep brain stimulation.* Int J Neurosci, 2010. **120**(11): p. 717-23.

238. Hilker, R., et al., *Deep brain stimulation of the subthalamic nucleus does not increase the striatal dopamine concentration in parkinsonian humans.* Mov Disord, 2003. **18**(1): p. 41-8.

239. Volkmann, J. and J. Volkmann, *Deep brain stimulation for the treatment of Parkinson's disease.* Journal of Clinical Neurophysiology, 2004. **21**(1): p. 6-17.

240. Schuepbach, W.M., et al., *Neurostimulation for Parkinson's disease with early motor complications.* N Engl J Med, 2013. **368**(7): p. 610-22.

241. Charles, D., et al., *Subthalamic nucleus deep brain stimulation in early stage Parkinson's disease.* Parkinsonism Relat Disord, 2014. **20**(7): p. 731-7.

242. Maesawa, S., et al., *Long-term stimulation of the subthalamic nucleus in hemiparkinsonian rats: neuroprotection of dopaminergic neurons.* J Neurosurg, 2004. **100**(4): p. 679-87.

243. Temel, Y., et al., *Protection of nigral cell death by bilateral subthalamic nucleus stimulation.* Brain Res, 2006. **1120**(1): p. 100-5.

244. Harnack, D., et al., *Placebo-controlled chronic high-frequency stimulation of the subthalamic nucleus preserves dopaminergic nigral neurons in a rat model of progressive Parkinsonism.* Exp Neurol, 2008. **210**(1): p. 257-60.

245. Wallace, B.A., et al., *Survival of midbrain dopaminergic cells after lesion or deep brain stimulation of the subthalamic nucleus in MPTP-treated monkeys.* Brain, 2007. **130**(Pt 8): p. 2129-45.

246. Friedman, A., et al., *Programmed acute electrical stimulation of ventral tegmental area alleviates depressive-like behavior.* Neuropsychopharmacology, 2009. **34**(4): p. 1057-66.

247. Hamani, C., et al., *Deep brain stimulation reverses anhedonic-like behavior in a chronic model of depression: role of serotonin and brain derived neurotrophic factor.* Biol Psychiatry, 2012. **71**(1): p. 30-5.

248. Gersner, R., et al., *Site-specific antidepressant effects of repeated subconvulsive electrical stimulation: potential role of brain-derived neurotrophic factor.* Biol Psychiatry, 2010. **67**(2): p. 125-32.

249. Hamburger, V., *The history of the discovery of the nerve growth factor.* J Neurobiol, 1993. **24**(7): p. 893-7.

250. Levi-Montalcini, R., *Developmental neurobiology and the natural history of nerve growth factor.* Annu Rev Neurosci, 1982. **5**: p. 341-62.

251. Levi-Montalcini, R. and V. Hamburger, *Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo.* J Exp Zool, 1951. **116**(2): p. 321-61.

252. Levi-Montalcini, R., *Effects of mouse tumor transplantation on the nervous system.* Ann N Y Acad Sci, 1952. **55**(2): p. 330-44.

253. Levi-Montalcini, R., *The nerve growth factor: thirty-five years later.* Biosci Rep, 1987. **7**(9): p. 681-99.

254. Levi-Montalcini, R. and S. Cohen, *Effects of the extract of the mouse submaxillary salivary glands on the sympathetic system of mammals.* Ann N Y Acad Sci, 1960. **85**: p. 324-41.

255. McDonald, N.Q. and W.A. Hendrickson, *A structural superfamily of growth factors containing a cystine knot motif.* Cell, 1993. **73**(3): p. 421-4.

256. Fandl, J.P., et al., *Characterization and crystallization of recombinant human neurotrophin-4.* J Biol Chem, 1994. **269**(1): p. 755-9.

257. Robinson, R.C., et al., *Structure of the brain-derived neurotrophic factor/neurotrophin 3 heterodimer.* Biochemistry, 1995. **34**(13): p. 4139-46.

258. Butte, M.J., et al., *Crystal structure of neurotrophin-3 homodimer shows distinct regions are used to bind its receptors.* Biochemistry, 1998. **37**(48): p. 16846-52.

259. Bothwell, M., *Functional interactions of neurotrophins and neurotrophin receptors.* Annu Rev Neurosci, 1995. **18**: p. 223-53.

260. Huang, E.J. and L.F. Reichardt, *Neurotrophins: roles in neuronal development and function.* Annu Rev Neurosci, 2001. **24**: p. 677-736.

261. Barde, Y.A., et al., *New factor released by cultured glioma cells supporting survival and growth of sensory neurones.* Nature, 1978. **274**(5673): p. 818.

262. Barde, Y.A., D. Edgar, and H. Thoenen, *Sensory neurons in culture: changing requirements for survival factors during embryonic development.* Proc Natl Acad Sci U S A, 1980. **77**(2): p. 1199-203.

263. Barde, Y.A., D. Edgar, and H. Thoenen, *Purification of a new neurotrophic factor from mammalian brain.* EMBO J, 1982. **1**(5): p. 549-53.

264. Leibrock, J., et al., *MOLECULAR-CLONING AND EXPRESSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR.* Nature, 1989. **341**(6238): p. 149-152.

265. Johnson, J.E., et al., *BRAIN-DERIVED NEUROTROPHIC FACTOR SUPPORTS THE SURVIVAL OF CULTURED RAT RETINAL GANGLION-CELLS.* Journal of Neuroscience, 1986. **6**(10): p. 3031-3038.

266. Levi-Montalcini, R. and B. Booker, *Destruction of the Sympathetic Ganglia in Mammals by an Antiserum to a Nerve-Growth Protein.* Proc Natl Acad Sci U S A, 1960. **46**(3): p. 384-91.

267. Crowley, C., et al., *Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons.* Cell, 1994. **76**(6): p. 1001-11.

268. Oppenheim, R.W., *Pathways in the emergence of developmental neuroethology: antecedents to current views of neurobehavioral ontogeny.* J Neurobiol, 1992. **23**(10): p. 1370-403.

269. Ernfors, P., K.F. Lee, and R. Jaenisch, *Mice lacking brain-derived neurotrophic factor develop with sensory deficits.* Nature, 1994. **368**(6467): p. 147-50.

270. Ernfors, P., et al., *Complementary roles of BDNF and NT-3 in vestibular and auditory development.* Neuron, 1995. **14**(6): p. 1153-64.

271. Wetmore, C., et al., *LOCALIZATION OF BRAIN-DERIVED NEUROTROPHIC FACTOR MESSENGER-RNA TO NEURONS IN THE BRAIN BY INSITU HYBRIDIZATION.* Experimental Neurology, 1990. **109**(2): p. 141-152.

272. Hofer, M., et al., *REGIONAL DISTRIBUTION OF BRAIN-DERIVED NEUROTROPHIC FACTOR MESSENGER-RNA IN THE ADULT-MOUSE BRAIN.* Embo Journal, 1990. **9**(8): p. 2459-2464.

273. Yamamoto, H., et al., *HUMAN PLATELETS CONTAIN BRAIN-DERIVED NEUROTROPHIC FACTOR.* Journal of Neuroscience, 1990. **10**(11): p. 3469-3478.

274. Acheson, A., et al., *DETECTION OF BRAIN-DERIVED NEUROTROPHIC FACTOR-LIKE ACTIVITY IN FIBROBLASTS AND SCHWANN-CELLS - INHIBITION BY ANTIBODIES TO NGF.* Neuron, 1991. **7**(2): p. 265-275.

275. Seroogy, K.B. and C.M. Gall, *Expression of neurotrophins by midbrain dopaminergic neurons.* Exp Neurol, 1993. **124**(1): p. 119-28.

276. Hyman, C., et al., *BDNF IS A NEUROTROPHIC FACTOR FOR DOPAMINERGIC-NEURONS OF THE SUBSTANTIA-NIGRA.* Nature, 1991. **350**(6315): p. 230-232.

277. Evans, S.F., et al., *Neuronal brain-derived neurotrophic factor is synthesized in excess, with levels regulated by sortilin-mediated trafficking and lysosomal degradation.* J Biol Chem, 2011. **286**(34): p. 29556-67.

278. Lindholm, D., et al., *ACTIVITY-DEPENDENT AND HORMONAL-REGULATION OF NEUROTROPHIN MESSENGER-RNA LEVELS IN THE BRAIN - IMPLICATIONS FOR NEURONAL PLASTICITY.* Journal of Neurobiology, 1994. **25**(11): p. 1362-1372.

279. Bustos, G., et al., *Functional interactions between somatodendritic dopamine release, glutamate receptors and brain-derived neurotrophic factor expression in mesencephalic structures of the brain.* Brain Res Brain Res Rev, 2004. **47**(1-3): p. 126-44.

280. Zafra, F., et al., *ACTIVITY DEPENDENT REGULATION OF BDNF AND NGF MESSENGER-RNAS IN THE RAT HIPPOCAMPUS IS MEDIATED BY NON-NMDA GLUTAMATE RECEPTORS.* Embo Journal, 1990. **9**(11): p. 3545-3550.

281. Lindefors, N., et al., *STIMULATION OF GLUTAMATE RECEPTORS INCREASES EXPRESSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR MESSENGER-RNA IN RAT HIPPOCAMPUS*. Neurotoxins and Neurodegenerative Disease, ed. J.W. Langston and A. Young. Vol. 648. 1992, New York: New York Acad Sciences. 296-299.

282. Favaron, M., et al., *NMDA-STIMULATED EXPRESSION OF BDNF MESSENGER-RNA IN CULTURED CEREBELLAR GRANULE NEURONS.* Neuroreport, 1993. **4**(10): p. 1171-1174.

283. Gwag, B.J., et al., *ACTIVATION OF NMDA RECEPTORS INCREASES BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) MESSENGER-RNA EXPRESSION IN THE HIPPOCAMPAL-FORMATION.* Neuroreport, 1993. **5**(2): p. 125-128.

284. Lapchak, P.A., et al., *CHOLINERGIC REGULATION OF HIPPOCAMPAL BRAIN-DERIVED NEUROTROPHIC FACTOR MESSENGER-RNA EXPRESSION - EVIDENCE FROM LESION AND CHRONIC CHOLINERGIC DRUG-TREATMENT STUDIES.* Neuroscience, 1993. **52**(3): p. 575-585.

285. Hartmann, M., R. Heumann, and V. Lessmann, *Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses.* EMBO J, 2001. **20**(21): p. 5887-97.

286. Matsuda, N., et al., *Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite.* J Neurosci, 2009. **29**(45): p. 14185-98.

287. Lewin, G.R., B.D. Carter, and SpringerLink (Online service), *Neurotrophic Factors*, in *Handbook of Experimental Pharmacology,*. 2014, Springer Berlin Heidelberg : Imprint: Springer,: Berlin, Heidelberg. p. VIII, 520 p. 37 illus., 27 illus. in color.

288. Gottmann, K., T. Mittmann, and V. Lessmann, *BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses.* Exp Brain Res, 2009. **199**(3-4): p. 203-34.

289. Mowla, S.J., et al., *Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons.* Journal of Neuroscience, 1999. **19**(6): p. 2069-2080.

290. Nagappan, G., et al., *Control of extracellular cleavage of ProBDNF by high frequency neuronal activity.* Proc Natl Acad Sci U S A, 2009. **106**(4): p. 1267-72.

291. Cobb, M.H. and M.H. Cobb, *MAP kinase pathways.* Progress in Biophysics & Molecular Biology, 1999. **71**(3-4): p. 479-500.

292. Airaksinen, M.S., et al., *The GDNF family: Signalling, biological functions and therapeutic value.* Nature Reviews Neuroscience, 2002. **3**(5): p. 383-394.

293. Chao, M.V. and M.V. Chao, *Neurotrophins and their receptors: A convergence point for many signalling pathways.* Nature Reviews Neuroscience, 2003. **4**(4): p. 299-309.

294. Bibel, M., et al., *Biochemical and functional interactions between the neurotrophin receptors trk and p75(NTR).* Embo Journal, 1999. **18**(3): p. 616-622.

295. Chen, W., et al., *BDNF released during neuropathic pain potentiates NMDA receptors in primary afferent terminals.* Eur J Neurosci, 2014. **39**(9): p. 1439-54.

296. Nakai, T., et al., *Girdin phosphorylation is crucial for synaptic plasticity and memory: a potential role in the interaction of BDNF/TrkB/Akt signaling with NMDA receptor.* J Neurosci, 2014. **34**(45): p. 14995-5008.

297. Li, J., et al., *Experimental colitis modulates the functional properties of NMDA receptors in dorsal root ganglia neurons.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(2): p. G219-28.

298. Fenner, M.E., C.L. Achim, and B.M. Fenner, *Expression of full-length and truncated trkB in human striatum and substantia nigra neurons: implications for Parkinson's disease.* J Mol Histol, 2014. **45**(3): p. 349-61.

299. Bosse, K.E., et al., *Aberrant striatal dopamine transmitter dynamics in brain-derived neurotrophic factor-deficient mice.* J Neurochem, 2012. **120**(3): p. 385-95.

300. Besusso, D., et al., *BDNF-TrkB signaling in striatopallidal neurons controls inhibition of locomotor behavior.* Nat Commun, 2013. **4**: p. 2031.

301. Rizzone, M., et al., *Deep brain stimulation of the subthalamic nucleus in Parkinson's disease: effects of variation in stimulation parameters.* Journal of Neurology Neurosurgery and Psychiatry, 2001. **71**(2): p. 215-219.

302. Moro, E., et al., *The impact on Parkinson's disease of electrical parameter settings in STN stimulation.* Neurology, 2002. **59**(5): p. 706-713.

303. Garcia, L., et al., *High-frequency stimulation in Parkinson's disease: more or less?* Trends in Neurosciences, 2005. **28**(4): p. 209-216.

304. Parain, K., et al., *Reduced expression of brain-derived neurotrophic factor protein in Parkinson's disease substantia nigra.* Neuroreport, 1999. **10**(3): p. 557-61.

305. Howells, D.W., et al., *Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra.* Exp Neurol, 2000. **166**(1): p. 127-35.

306. Spina, M.B., et al., *Brain-derived neurotrophic factor protects dopaminergic cells from 6-hydroxydopamine toxicity.* Ann N Y Acad Sci, 1992. **648**: p. 348-50.

307. Altar, C.A., et al., *BRAIN-DERIVED NEUROTROPHIC FACTOR AUGMENTS ROTATIONAL BEHAVIOR AND NIGROSTRIATAL DOPAMINE TURNOVER INVIVO.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(23): p. 11347-11351.

308. Frim, D.M., et al., *IMPLANTED FIBROBLASTS GENETICALLY-ENGINEERED TO PRODUCE BRAIN-DERIVED NEUROTROPHIC FACTOR PREVENT 1-METHYL-4-PHENYLPYRIDINIUM TOXICITY TO DOPAMINERGIC-NEURONS IN THE RAT.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(11): p. 5104-5108.

309. Yoshimoto, Y., et al., *ASTROCYTES RETROVIRALLY TRANSDUCED WITH BDNF ELICIT BEHAVIORAL IMPROVEMENT IN A RAT MODEL OF PARKINSONS-DISEASE.* Brain Research, 1995. **691**(1-2): p. 25-36.

310. Tsukahara, T., et al., *EFFECTS OF BRAIN-DERIVED NEUROTROPHIC FACTOR ON 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE-INDUCED PARKINSONISM IN MONKEYS.* Neurosurgery, 1995. **37**(4): p. 733-739.

311. Yurek, D.M., et al., *BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons.* Experimental Neurology, 1996. **137**(1): p. 105-118.

312. Pahwa, R. and K.E. Lyons, *Handbook of Parkinson's disease*. 2013, CRC Press, Taylor & Francis Group,: Boca Raton, FL. p. xiv, 605 pages.

313. Hirtz, D., et al., *How common are the "common" neurologic disorders?* Neurology, 2007. **68**(5): p. 326-37.

314. Aminoff, M.J., *Parkinson's disease.* Neurol Clin, 2001. **19**(1): p. 119-28, vi.

315. Pollanen, M.S., D.W. Dickson, and C. Bergeron, *Pathology and biology of the Lewy body.* J Neuropathol Exp Neurol, 1993. **52**(3): p. 183-91.

316. Bendor, J.T., T.P. Logan, and R.H. Edwards, *The function of alpha-synuclein.* Neuron, 2013. **79**(6): p. 1044-66.

317. Kirik, D. and A. Bjorklund, *Modeling CNS neurodegeneration by overexpression of disease-causing proteins using viral vectors.* Trends Neurosci, 2003. **26**(7): p. 386-92.

318. Chesselet, M.F., *In vivo alpha-synuclein overexpression in rodents: a useful model of Parkinson's disease?* Exp Neurol, 2008. **209**(1): p. 22-7.

319. Schneider, B., R. Zufferey, and P. Aebischer, *Viral vectors, animal models and new therapies for Parkinson's disease.* Parkinsonism Relat Disord, 2008. **14 Suppl 2**: p. S169-71.

320. Ulusoy, A., et al., *In vivo gene delivery for development of mammalian models for Parkinson's disease.* Exp Neurol, 2008. **209**(1): p. 89-100.

321. Lindgren, H.S., M.J. Lelos, and S.B. Dunnett, *Do alpha-synuclein vector injections provide a better model of Parkinson's disease than the classic 6-hydroxydopamine model?* Exp Neurol, 2012. **237**(1): p. 36-42.

322. Low, K. and P. Aebischer, *Use of viral vectors to create animal models for Parkinson's disease.* Neurobiol Dis, 2012. **48**(2): p. 189-201.

323. Van der Perren, A., C. Van den Haute, and V. Baekelandt, *Viral Vector-Based Models of Parkinson's Disease.* Curr Top Behav Neurosci, 2014.

324. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.

325. Kirik, D., et al., *Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system.* J Neurosci, 2002. **22**(7): p. 2780-91.

326. Klein, R.L., et al., *Dopaminergic cell loss induced by human A30P alpha-synuclein gene transfer to the rat substantia nigra.* Hum Gene Ther, 2002. **13**(5): p. 605-12.

327. Gorbatyuk, O.S., et al., *The phosphorylation state of Ser-129 in human alpha-synuclein determines neurodegeneration in a rat model of Parkinson disease.* Proc Natl Acad Sci U S A, 2008. **105**(2): p. 763-8.

328. Azeredo da Silveira, S., et al., *Phosphorylation does not prompt, nor prevent, the formation of alpha-synuclein toxic species in a rat model of Parkinson's disease.* Hum Mol Genet, 2009. **18**(5): p. 872-87.

329. McFarland, N.R., et al., *Alpha-synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease.* J Neuropathol Exp Neurol, 2009. **68**(5): p. 515-24.

330. Winner, B., et al., *In vivo demonstration that alpha-synuclein oligomers are toxic.* Proc Natl Acad Sci U S A, 2011. **108**(10): p. 4194-9.

331. Yamada, M., et al., *Overexpression of alpha-synuclein in rat substantia nigra results in loss of dopaminergic neurons, phosphorylation of alpha-synuclein and activation of caspase-9: resemblance to pathogenetic changes in Parkinson's disease.* J Neurochem, 2004. **91**(2): p. 451-61.

332. Yamada, M., Y. Mizuno, and H. Mochizuki, *Parkin gene therapy for alpha-synucleinopathy: a rat model of Parkinson's disease.* Hum Gene Ther, 2005. **16**(2): p. 262-70.

333. Ulusoy, A., et al., *Co-expression of C-terminal truncated alpha-synuclein enhances full-length alpha-synuclein-induced pathology.* Eur J Neurosci, 2010. **32**(3): p. 409-22.

334. Sanchez-Guajardo, V., et al., *Microglia acquire distinct activation profiles depending on the degree of alpha-synuclein neuropathology in a rAAV based model of Parkinson's disease.* PLoS One, 2010. **5**(1): p. e8784.

335. Decressac, M., et al., *GDNF fails to exert neuroprotection in a rat alpha-synuclein model of Parkinson's disease.* Brain, 2011. **134**(Pt 8): p. 2302-11.

336. Decressac, M., et al., *Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of alpha-synuclein in midbrain dopamine neurons.* Neurobiol Dis, 2012. **45**(3): p. 939-53.

337. Decressac, M., B. Mattsson, and A. Bjorklund, *Comparison of the behavioural and histological characteristics of the 6-OHDA and alpha-synuclein rat models of Parkinson's disease.* Exp Neurol, 2012. **235**(1): p. 306-15.

338. Mulcahy, P., et al., *Development and characterisation of a novel rat model of Parkinson's disease induced by sequential intranigral administration of AAV-alpha-synuclein and the pesticide, rotenone.* Neuroscience, 2012. **203**: p. 170-9.

339. Gombash, S.E., et al., *Morphological and behavioral impact of AAV2/5-mediated overexpression of human wildtype alpha-synuclein in the rat nigrostriatal system.* PLoS One, 2013. **8**(11): p. e81426.

340. St Martin, J.L., et al., *Dopaminergic neuron loss and up-regulation of chaperone protein mRNA induced by targeted over-expression of alpha-synuclein in mouse substantia nigra.* J Neurochem, 2007. **100**(6): p. 1449-57.

341. Theodore, S., et al., *Targeted overexpression of human alpha-synuclein triggers microglial activation and an adaptive immune response in a mouse model of Parkinson disease.* J Neuropathol Exp Neurol, 2008. **67**(12): p. 1149-58.

342. Oliveras-Salva, M., et al., *rAAV2/7 vector-mediated overexpression of alpha-synuclein in mouse substantia nigra induces protein aggregation and progressive dose-dependent neurodegeneration.* Mol Neurodegener, 2013. **8**: p. 44.

343. Oliveras-Salva, M., et al., *Alpha-synuclein-induced neurodegeneration is exacerbated in PINK1 knockout mice.* Neurobiol Aging, 2014.

344. Kirik, D., et al., *Nigrostriatal alpha-synucleinopathy induced by viral vector-mediated overexpression of human alpha-synuclein: a new primate model of Parkinson's disease.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2884-9.

345. Eslamboli, A., et al., *Long-term consequences of human alpha-synuclein overexpression in the primate ventral midbrain.* Brain, 2007. **130**(Pt 3): p. 799-815.

346. Yasuda, T., et al., *Neuronal specificity of alpha-synuclein toxicity and effect of Parkin co-expression in primates.* Neuroscience, 2007. **144**(2): p. 743-53.

347. Lo Bianco, C., et al., *Lentiviral nigral delivery of GDNF does not prevent neurodegeneration in a genetic rat model of Parkinson's disease.* Neurobiol Dis, 2004. **17**(2): p. 283-9.

348. Lo Bianco, C., et al., *Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease.* Proc Natl Acad Sci U S A, 2004. **101**(50): p. 17510-5.

349. Lauwers, E., et al., *Non-invasive imaging of neuropathology in a rat model of alpha-synuclein overexpression.* Neurobiol Aging, 2007. **28**(2): p. 248-57.

350. Lauwers, E., et al., *Neuropathology and neurodegeneration in rodent brain induced by lentiviral vector-mediated overexpression of alpha-synuclein.* Brain Pathol, 2003. **13**(3): p. 364-72.

351. Maingay, M., et al., *Ventral tegmental area dopamine neurons are resistant to human mutant alpha-synuclein overexpression.* Neurobiol Dis, 2006. **23**(3): p. 522-32.

352. Chung, C.Y., et al., *Dynamic changes in presynaptic and axonal transport proteins combined with striatal neuroinflammation precede dopaminergic neuronal loss in a rat model of AAV alpha-synucleinopathy.* J Neurosci, 2009. **29**(11): p. 3365-73.

353. Koprich, J.B., et al., *Progressive neurodegeneration or endogenous compensation in an animal model of Parkinson's disease produced by decreasing doses of alpha-synuclein.* PLoS One, 2011. **6**(3): p. e17698.

354. Barkholt, P., et al., *Long-term polarization of microglia upon alpha-synuclein overexpression in nonhuman primates.* Neuroscience, 2012. **208**: p. 85-96.

355. Febbraro, F., et al., *Ser129D mutant alpha-synuclein induces earlier motor dysfunction while S129A results in distinctive pathology in a rat model of Parkinson's disease.* Neurobiol Dis, 2013. **56**: p. 47-58.

356. Lundblad, M., et al., *Impaired neurotransmission caused by overexpression of alpha-synuclein in nigral dopamine neurons.* Proc Natl Acad Sci U S A, 2012.

357. Ulusoy, A., et al., *Viral vector-mediated overexpression of alpha-synuclein as a progressive model of Parkinson's disease.* Prog Brain Res, 2010. **184**: p. 89-111.

358. Mata, I.F., et al., *LRRK2 in Parkinson's disease: protein domains and functional insights.* Trends Neurosci, 2006. **29**(5): p. 286-93.

359. Cookson, M.R., J. Hardy, and P.A. Lewis, *Genetic neuropathology of Parkinson's disease.* Int J Clin Exp Pathol, 2008. **1**(3): p. 217-31.

360. Dusonchet, J., et al., *A rat model of progressive nigral neurodegeneration induced by the Parkinson's disease-associated G2019S mutation in LRRK2.* J Neurosci, 2011. **31**(3): p. 907-12.

361. Lee, B.D., et al., *Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease.* Nat Med, 2010. **16**(9): p. 998-1000.

362. Shimura, H., et al., *Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase.* Nat Genet, 2000. **25**(3): p. 302-5.

363. Takahashi, H., et al., *Familial juvenile parkinsonism: clinical and pathologic study in a family.* Neurology, 1994. **44**(3 Pt 1): p. 437-41.

364. Dong, Z., et al., *Dopamine-dependent neurodegeneration in rats induced by viral vector-mediated overexpression of the parkin target protein, CDCrel-1.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12438-43.

365. Kitao, Y., et al., *Pael receptor induces death of dopaminergic neurons in the substantia nigra via endoplasmic reticulum stress and dopamine toxicity, which is enhanced under condition of parkin inactivation.* Hum Mol Genet, 2007. **16**(1): p. 50-60.

366. Dusonchet, J., et al., *Targeted overexpression of the parkin substrate Pael-R in the nigrostriatal system of adult rats to model Parkinson's disease.* Neurobiol Dis, 2009. **35**(1): p. 32-41.

367. Ko, H.S., et al., *Accumulation of the authentic parkin substrate aminoacyl-tRNA synthetase cofactor, p38/JTV-1, leads to catecholaminergic cell death.* J Neurosci, 2005. **25**(35): p. 7968-78.

368. Krenz, A., et al., *Aggregate formation and toxicity by wild-type and R621C synphilin-1 in the nigrostriatal system of mice using adenoviral vectors.* J Neurochem, 2009. **108**(1): p. 139-46.

369. Hommel, J.D., et al., *Local gene knockdown in the brain using viral-mediated RNA interference.* Nat Med, 2003. **9**(12): p. 1539-44.

370. Ulusoy, A., et al., *Dose optimization for long-term rAAV-mediated RNA interference in the nigrostriatal projection neurons.* Mol Ther, 2009. **17**(9): p. 1574-84.

371. Haque, M.E., et al., *Inactivation of Pink1 gene in vivo sensitizes dopamine-producing neurons to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and can be rescued by autosomal recessive Parkinson disease genes, Parkin or DJ-1.* J Biol Chem, 2012. **287**(27): p. 23162-70.

372. Polinski, N.K., et al., *Recombinant adenoassociated virus 2/5-mediated gene transfer is reduced in the aged rat midbrain.* Neurobiology of Aging.

373. Wu, K., et al., *The effects of rAAV2-mediated NGF gene delivery in adult and aged rats.* Mol Ther, 2004. **9**(2): p. 262-9.

374. Klein, R.L., et al., *Pronounced microgliosis and neurodegeneration in aged rats after tau gene transfer.* Neurobiol Aging, 2010. **31**(12): p. 2091-102.

375. Medtronic. *Deep Brain Stimulation for Movement Disorders*. [cited 2013 April 24]; Available from: http://professional.medtronic.com/pt/neuro/dbs-md/prod/ -.UXiVipXHPXQ.

376. Hilker, R., et al., *Disease progression continues in patients with advanced Parkinson's disease and effective subthalamic nucleus stimulation.* J Neurol Neurosurg Psychiatry, 2005. **76**(9): p. 1217-21.

377. Spieles-Engemann, A.L., et al., *Deep Brain Stimulation in a Rodent Model of Parkinson's Disease Upregulates BDNF in Subthalamic Nucleus Target Structures*, in *Society for Neuroscience Abstracts 46.1*. 2009: Chicago, IL.

378. Kluver, H. and E. Barrera, *A method for the combined staining of cells and fibers in the nervous system.* J Neuropathol Exp Neurol, 1953. **12**(4): p. 400-3.

379. Gombash, S.E., et al., *Striatonigral pleiotrophin overexpression provides superior protection compared to overexpression in both striatonigral and nigrostriatal pathways.* Gene Therapy, 2014.

380. Cazorla, M., et al., *Identification of a low-molecular weight TrkB antagonist with anxiolytic and antidepressant activity in mice.* J Clin Invest, 2011. **121**(5): p. 1846-57.

381. Merlio, J.P., et al., *Increased production of the TrkB protein tyrosine kinase receptor after brain insults.* Neuron, 1993. **10**(2): p. 151-64.

382. Conner, J.M., et al., *Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport.* J Neurosci, 1997. **17**(7): p. 2295-313.

383. Yan, Q., et al., *Immunocytochemical localization of TrkB in the central nervous system of the adult rat.* J Comp Neurol, 1997. **378**(1): p. 135-57.

384. Yan, Q., et al., *Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system.* Neuroscience, 1997. **78**(2): p. 431-48.

385. Drake, C.T., T.A. Milner, and S.L. Patterson, *Ultrastructural localization of full-length trkB immunoreactivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity.* J Neurosci, 1999. **19**(18): p. 8009-26.

386. Binder, D.K. and H.E. Scharfman, *Brain-derived neurotrophic factor.* Growth Factors, 2004. **22**(3): p. 123-31.

387. Kryl, D., et al., *Subcellular localization of full-length and truncated Trk receptor isoforms in polarized neurons and epithelial cells.* J Neurosci, 1999. **19**(14): p. 5823-33.

388. Jang, S.W., et al., *A selective TrkB agonist with potent neurotrophic activities by 7,8-dihydroxyflavone.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(6): p. 2687-2692.

389. Lu, B., et al., *BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases.* Nature Reviews Neuroscience, 2013. **14**(6): p. 401-416.

390. di Nuzzo, L., et al., *Molecular pharmacodynamics of new oral drugs used in the treatment of multiple sclerosis.* Drug Design Development and Therapy, 2014. **8**: p. 555-567.

391. Deogracias, R., et al., *Fingolimod, a sphingosine-1 phosphate receptor modulator, increases BDNF levels and improves symptoms of a mouse model of Rett syndrome.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(35): p. 14230-14235.

392. Doi, Y., et al., *Fingolimod Phosphate Attenuates Oligomeric Amyloid beta-Induced Neurotoxicity via Increased Brain-Derived Neurotrophic Factor Expression in Neurons.* Plos One, 2013. **8**(4): p. 10.

393. Fukumoto, K., et al., *Fingolimod increases brain-derived neurotrophic factor levels and ameliorates amyloid beta-induced memory impairment.* Behavioural Brain Research, 2014. **268**: p. 88-93.

394. *Parkinson's Disease 2014: Advancing Research, Improving Lives*. 2014; Available from: http://www.ninds.nih.gov/research/parkinsonsweb/PD2014/.

395. Meissner, W., et al., *Deep brain stimulation of subthalamic neurons increases striatal dopamine metabolism and induces contralateral circling in freely moving 6-hydroxydopamine-lesioned rats.* Neurosci Lett, 2002. **328**(2): p. 105-8.

396. Chang, J.Y., et al., *High frequency stimulation of the subthalamic nucleus improves treadmill locomotion in unilateral 6-hydroxydopamine lesioned rats.* Brain Res, 2003. **983**(1-2): p. 174-84.

397. Darbaky, Y., et al., *High frequency stimulation of the subthalamic nucleus has beneficial antiparkinsonian effects on motor functions in rats, but less efficiency in a choice reaction time task.* Eur J Neurosci, 2003. **18**(4): p. 951-6.

398. Shi, L.H., et al., *High-frequency stimulation of the subthalamic nucleus reverses limb-use asymmetry in rats with unilateral 6-hydroxydopamine lesions.* Brain Res, 2004. **1013**(1): p. 98-106.

399. Fang, X., et al., *Improvements in motor behavioral tests during deep brain stimulation of the subthalamic nucleus in rats with different degrees of unilateral parkinsonism.* Brain Res, 2006. **1120**(1): p. 202-10.

400. Vlamings, R., et al., *High frequency stimulation of the subthalamic nucleus improves speed of locomotion but impairs forelimb movement in Parkinsonian rats.* Neuroscience, 2007. **148**(3): p. 815-23.

401. Temel, Y., et al., *Acute and separate modulation of motor and cognitive performance in parkinsonian rats by bilateral stimulation of the subthalamic nucleus.* Exp Neurol, 2005. **193**(1): p. 43-52.

402. Akita, H., et al., *Activation of the NMDA receptor involved in the alleviating after-effect of repeated stimulation of the subthalamic nucleus on motor deficits in hemiparkinsonian rats.* Brain Res, 2010. **1306**: p. 159-67.

403. Li, X.H., et al., *High-frequency stimulation of the subthalamic nucleus restores neural and behavioral functions during reaction time task in a rat model of Parkinson's disease.* J Neurosci Res, 2010. **88**(7): p. 1510-21.

404. Creed, M.C., C. Hamani, and J.N. Nobrega, *Effects of repeated deep brain stimulation on depressive- and anxiety-like behavior in rats: comparing entopeduncular and subthalamic nuclei.* Brain Stimul, 2013. **6**(4): p. 506-14.

405. Schallert, T., *Behavioral tests for preclinical intervention assessment.* NeuroRx, 2006. **3**(4): p. 497-504.

406. Quintana, A., V. Sgambato-Faure, and M. Savasta, *Effects of L-DOPA and STN-HFS dyskinesiogenic treatments on NR2B regulation in basal ganglia in the rat model of Parkinson's disease.* Neurobiol Dis, 2012. **48**(3): p. 379-90.

407. Degos, B., et al., *Evidence for a direct subthalamo-cortical loop circuit in the rat.* Eur J Neurosci, 2008. **27**(10): p. 2599-610.

408. Parent, A. and Y. Smith, *Organization of efferent projections of the subthalamic nucleus in the squirrel monkey as revealed by retrograde labeling methods.* Brain Res, 1987. **436**(2): p. 296-310.

409. Gill, C.E., et al., *Deep brain stimulation for early-stage Parkinson's disease: an illustrative case.* Neuromodulation, 2011. **14**(6): p. 515-21; discussion 521-2.

410. Charles, D., et al., *Pilot study assessing the feasibility of applying bilateral subthalamic nucleus deep brain stimulation in very early stage Parkinson's disease: study design and rationale.* J Parkinsons Dis, 2012. **2**(3): p. 215-23.

411. Charles, P.D., et al., *Deep brain stimulation in early Parkinson's disease: enrollment experience from a pilot trial.* Parkinsonism Relat Disord, 2012. **18**(3): p. 268-73.

412. Charles, P.D.e.a., *Safety and Tolerability of Neurostimulation in Early Stage Parkinson's Disease*. ClinicalTrials.gov [Internet].

413. Gombash, S.E., et al., *Striatal Pleiotrophin Overexpression Provides Functional and Morphological Neuroprotection in the 6-Hydroxydopamine Model.* Mol Ther, 2012. **20**(3): p. 544-54.

414. Zolotukhin, S., et al., *Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield.* Gene Ther, 1999. **6**(6): p. 973-85.

415. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates*. 6th ed. 2007, Amsterdam ; Boston: Elsevier.

416. Brown, L.L., et al., *Differential metabolic activity in the striosome and matrix compartments of the rat striatum during natural behaviors.* J Neurosci, 2002. **22**(1): p. 305-14.

417. Kelley, A.E., C.G. Lang, and A.M. Gauthier, *Induction of oral stereotypy following amphetamine microinjection into a discrete subregion of the striatum.* Psychopharmacology (Berl), 1988. **95**(4): p. 556-9.

418. Pisa, M., *Motor somatotopy in the striatum of rat: manipulation, biting and gait.* Behav Brain Res, 1988. **27**(1): p. 21-35.

419. Decressac, M., et al., *alpha-Synuclein-induced down-regulation of Nurr1 disrupts GDNF signaling in nigral dopamine neurons.* Sci Transl Med, 2012. **4**(163): p. 163ra156.

420. Bruet, N., et al., *High frequency stimulation of the subthalamic nucleus increases the extracellular contents of striatal dopamine in normal and partially dopaminergic denervated rats.* J Neuropathol Exp Neurol, 2001. **60**(1): p. 15-24.

421. Shimo, Y., et al., *Subthalamic Deep Brain Stimulation for a Parkinson's Disease Patient With Duplication of SNCA.* Neuromodulation, 2013.

422. Antonini, A., et al., *Successful subthalamic stimulation in genetic Parkinson's disease caused by duplication of the alpha-synuclein gene.* J Neurol, 2012. **259**(1): p. 165-7.

423. Daniels, C., et al., *Risk factors for executive dysfunction after subthalamic nucleus stimulation in Parkinson's disease.* Mov Disord, 2010. **25**(11): p. 1583-9.

424. Kahn, E., et al., *Deep brain stimulation in early stage Parkinson's disease: operative experience from a prospective randomised clinical trial.* J Neurol Neurosurg Psychiatry, 2012. **83**(2): p. 164-70.

425. Charles, P.D., et al., *Is deep brain stimulation neuroprotective if applied early in the course of PD?* Nat Clin Pract Neurol, 2008. **4**(8): p. 424-6.

426. Harnack, D. and A. Kupsch, *The impact of subthalamic deep brain stimulation on nigral neuroprotection-myth or reality?* Neuromodulation, 2010. **13**(3): p. 160-7.

427. Olds, M.E., D.B. Jacques, and O. Kopyov, *Entopeduncular lesions facilitate and thalamic lesions depress spontaneous and drug-evoked motor behavior in the hemiparkinsonian rat.* Synapse, 2001. **40**(3): p. 215-24.

428. Tasker, R.R., *Deep brain stimulation is preferable to thalamotomy for tremor suppression.* Surg Neurol, 1998. **49**(2): p. 145-53; discussion 153-4.

429. Kondziolka, D. and J.Y. Lee, *Long-lasting microthalamotomy effect after temporary placement of a thalamic stimulating electrode.* Stereotact Funct Neurosurg, 2004. **82**(2-3): p. 127-30.

430. Summerson, S.R., C.T. Kemere, and B. Aazhang, *Current amplitude-dependent modulation of rotational behavior with GPi stimulation in the rodent model of Parkinson's Disease.* Conf Proc IEEE Eng Med Biol Soc, 2013. **2013**: p. 783-6.

431. Summerson, S., B. Aazhang, and C. Kemere, *Characterizing Motor and Cognitive Effects Associated with Deep Brain Stimulation in the GPi of Hemi-Parkinsonian Rats.* IEEE Trans Neural Syst Rehabil Eng, 2014.

432. Butson, C.R. and C.C. McIntyre, *Tissue and electrode capacitance reduce neural activation volumes during deep brain stimulation.* Clin Neurophysiol, 2005. **116**(10): p. 2490-500.

433. Arbuthnott, G.W. and U. Ungerstedt, *Turning behavior induced by electrical stimulation of the nigro-neostriatal system of the rat.* Exp Neurol, 1975. **47**(1): p. 162-72.

434. Watanabe, H., S. Hata, and K. Watanabe, *[Pharmacological investigation of rotational behaviors produced by unilateral electrical stimulation of the substantia nigra in the rat].* Nihon Yakurigaku Zasshi, 1983. **82**(4): p. 247-57.

435. Oertel, W.H., C. Nitsch, and E. Mugnaini, *Immunocytochemical demonstration of the GABA-ergic neurons in rat globus pallidus and nucleus entopeduncularis and their GABA-ergic innervation.* Adv Neurol, 1984. **40**: p. 91-8.

436. Rajakumar, N., K. Elisevich, and B.A. Flumerfelt, *Parvalbumin-containing GABAergic neurons in the basal ganglia output system of the rat.* J Comp Neurol, 1994. **350**(2): p. 324-36.

437. Zhou, F.M. and C.R. Lee, *Intrinsic and integrative properties of substantia nigra pars reticulata neurons.* Neuroscience, 2011. **198**: p. 69-94.

438. van der Kooy, D. and D.A. Carter, *The organization of the efferent projections and striatal afferents of the entopeduncular nucleus and adjacent areas in the rat.* Brain Res, 1981. **211**(1): p. 15-36.

439. Parent, A. and L.N. Hazrati, *Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop.* Brain Res Brain Res Rev, 1995. **20**(1): p. 91-127.

440. Kita, H., H.T. Chang, and S.T. Kitai, *The morphology of intracellularly labeled rat subthalamic neurons: a light microscopic analysis.* J Comp Neurol, 1983. **215**(3): p. 245-57.

441. Kita, H. and S.T. Kitai, *Efferent projections of the subthalamic nucleus in the rat: light and electron microscopic analysis with the PHA-L method.* J Comp Neurol, 1987. **260**(3): p. 435-52.

442. Smith, Y. and A. Parent, *Neurons of the subthalamic nucleus in primates display glutamate but not GABA immunoreactivity.* Brain Res, 1988. **453**(1-2): p. 353-6.

443. Kincaid, A.E., et al., *Evidence for a projection from the globus pallidus to the entopeduncular nucleus in the rat.* Neurosci Lett, 1991. **128**(1): p. 121-5.

444. Kincaid, A.E., et al., *6-Hydroxydopamine lesions of the nigrostriatal pathway alter the expression of glutamate decarboxylase messenger RNA in rat globus pallidus projection neurons.* Neuroscience, 1992. **51**(3): p. 705-18.

445. Smith, Y., T. Wichmann, and M.R. DeLong, *Synaptic innervation of neurones in the internal pallidal segment by the subthalamic nucleus and the external pallidum in monkeys.* J Comp Neurol, 1994. **343**(2): p. 297-318.

446. Shink, E. and Y. Smith, *Differential synaptic innervation of neurons in the internal and external segments of the globus pallidus by the GABA- and glutamate-containing terminals in the squirrel monkey.* J Comp Neurol, 1995. **358**(1): p. 119-41.

447. Deniau, J.M., A. Menetrey, and S. Charpier, *The lamellar organization of the rat substantia nigra pars reticulata: segregated patterns of striatal afferents and relationship to the topography of corticostriatal projections.* Neuroscience, 1996. **73**(3): p. 761-81.

448. Sato, F., et al., *Axonal branching pattern of neurons of the subthalamic nucleus in primates.* J Comp Neurol, 2000. **424**(1): p. 142-52.

449. Tachibana, Y., et al., *Motor cortical control of internal pallidal activity through glutamatergic and GABAergic inputs in awake monkeys.* Eur J Neurosci, 2008. **27**(1): p. 238-53.

450. Spann, B.M. and I. Grofova, *Nigropedunculopontine projection in the rat: an anterograde tracing study with phaseolus vulgaris-leucoagglutinin (PHA-L).* J Comp Neurol, 1991. **311**(3): p. 375-88.

451. Steininger, T.L., D.B. Rye, and B.H. Wainer, *Afferent projections to the cholinergic pedunculopontine tegmental nucleus and adjacent midbrain extrapyramidal area in the albino rat. I. Retrograde tracing studies.* J Comp Neurol, 1992. **321**(4): p. 515-43.

452. Kha, H.T., et al., *Study of projections from the entopeduncular nucleus to the thalamus of the rat.* J Comp Neurol, 2000. **426**(3): p. 366-77.

453. Parent, M., M. Levesque, and A. Parent, *Two types of projection neurons in the internal pallidum of primates: single-axon tracing and three-dimensional reconstruction.* J Comp Neurol, 2001. **439**(2): p. 162-75.

454. Gulcebi, M.I., et al., *Topographical connections of the substantia nigra pars reticulata to higher-order thalamic nuclei in the rat.* Brain Res Bull, 2012. **87**(2-3): p. 312-8.

455. Rajakumar, N., et al., *Neurochemical compartmentalization of the globus pallidus in the rat: an immunocytochemical study of calcium-binding proteins.* J Comp Neurol, 1994. **346**(3): p. 337-48.

456. Bianco, I.H. and S.W. Wilson, *The habenular nuclei: a conserved asymmetric relay station in the vertebrate brain.* Philos Trans R Soc Lond B Biol Sci, 2009. **364**(1519): p. 1005-20.

457. Sutton, A.C., et al., *Deep brain stimulation of the substantia nigra pars reticulata improves forelimb akinesia in the hemiparkinsonian rat.* J Neurophysiol, 2013. **109**(2): p. 363-74.

458. Benhamou, L. and D. Cohen, *Electrophysiological characterization of entopeduncular nucleus neurons in anesthetized and freely moving rats.* Front Syst Neurosci, 2014. **8**: p. 7.

459. DeLong, M.R., *Activity of pallidal neurons during movement.* J Neurophysiol, 1971. **34**(3): p. 414-27.

460. Mink, J.W. and W.T. Thach, *Basal ganglia motor control. I. Nonexclusive relation of pallidal discharge to five movement modes.* J Neurophysiol, 1991. **65**(2): p. 273-300.

461. Parent, A. and L.N. Hazrati, *Functional anatomy of the basal ganglia. II. The place of subthalamic nucleus and external pallidum in basal ganglia circuitry.* Brain Res Brain Res Rev, 1995. **20**(1): p. 128-54.

462. Kitai, S. and H. Kita, *Anatomy and physiology of the subthalamic nucleus: a driving force of the basal ganglia*, in *The Basal Ganglia II - Structure and Function: Current Concepts*, C. M and J. A, Editors. 2006, Plenum Press: New York. p. 357-373.

463. Zhang, X., P.E. Andren, and P. Svenningsson, *Repeated l-DOPA treatment increases c-fos and BDNF mRNAs in the subthalamic nucleus in the 6-OHDA rat model of Parkinson's disease.* Brain Res, 2006. **1095**(1): p. 207-10.

464. *Allen Mouse Brain Atlas*. 2012; Available from: http://mouse.brain-map.org/.

465. Collier, T.J. and C.E. Sortwell, *Therapeutic potential of nerve growth factors in Parkinson's disease.* Drugs Aging, 1999. **14**(4): p. 261-87.

466. Bath, K.G. and F.S. Lee, *Variant BDNF (Val66Met) impact on brain structure and function.* Cogn Affect Behav Neurosci, 2006. **6**(1): p. 79-85.

467. Egan, M.F., et al., *The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function.* Cell, 2003. **112**(2): p. 257-69.

468. *dbSNP Build ID: rs6265*. Available from: http://www.ncbi.nlm.nih.gov/SNP/.

469. Chen, Z.Y., et al., *Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior.* Science, 2006. **314**(5796): p. 140-3.

470. Cirillo, J., et al., *Differential modulation of motor cortex excitability in BDNF Met allele carriers following experimentally induced and use-dependent plasticity.* Eur J Neurosci, 2012. **36**(5): p. 2640-9.

471. Schinder, A.F. and M. Poo, *The neurotrophin hypothesis for synaptic plasticity.* Trends Neurosci, 2000. **23**(12): p. 639-45.

472. Rauskolb, S., et al., *Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth.* J Neurosci, 2010. **30**(5): p. 1739-49.

473. Ingham, C.A., et al., *Morphological changes in the rat neostriatum after unilateral 6-hydroxydopamine injections into the nigrostriatal pathway.* Exp Brain Res, 1993. **93**(1): p. 17-27.

474. McNeill, T.H., et al., *Atrophy of medium spiny I striatal dendrites in advanced Parkinson's disease.* Brain Res, 1988. **455**(1): p. 148-52.

475. Altar, C.A., et al., *Efficacy of brain-derived neurotrophic factor and neurotrophin-3 on neurochemical and behavioral deficits associated with partial nigrostriatal dopamine lesions.* J Neurochem, 1994. **63**(3): p. 1021-32.

476. Hauser, R.A., P. Auinger, and D. Oakes, *Levodopa response in early Parkinson's disease.* Mov Disord, 2009. **24**(16): p. 2328-36.

477. Weaver, F.M., et al., *Randomized trial of deep brain stimulation for Parkinson disease: thirty-six-month outcomes.* Neurology, 2012. **79**(1): p. 55-65.

478. Pezawas, L., et al., *The brain-derived neurotrophic factor val66met polymorphism and variation in human cortical morphology.* J Neurosci, 2004. **24**(45): p. 10099-102.

479. Hariri, A.R., et al., *Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance.* J Neurosci, 2003. **23**(17): p. 6690-4.

480. Honea, R.A., et al., *Characterizing the role of brain derived neurotrophic factor genetic variation in Alzheimer's disease neurodegeneration.* PLoS One, 2013. **8**(9): p. e76001.

481. Voineskos, A.N., et al., *The brain-derived neurotrophic factor Val66Met polymorphism and prediction of neural risk for Alzheimer disease.* Arch Gen Psychiatry, 2011. **68**(2): p. 198-206.

482. Zintzaras, E. and G.M. Hadjigeorgiou, *The role of G196A polymorphism in the brain-derived neurotrophic factor gene in the cause of Parkinson's disease: a meta-analysis.* J Hum Genet, 2005. **50**(11): p. 560-6.

483. Foltynie, T., et al., *BDNF val66met influences time to onset of levodopa induced dyskinesia in Parkinson's disease.* J Neurol Neurosurg Psychiatry, 2009. **80**(2): p. 141-4.

484. Kaplan, N., et al., *Sequence Variants in SLC6A3, DRD2, and BDNF Genes and Time to Levodopa-Induced Dyskinesias in Parkinson's Disease.* J Mol Neurosci, 2014. **53**(2): p. 183-8.

485. Kleim, J.A., et al., *BDNF val66met polymorphism is associated with modified experience-dependent plasticity in human motor cortex.* Nat Neurosci, 2006. **9**(6): p. 735-7.

486. McHughen, S.A., et al., *BDNF val66met polymorphism influences motor system function in the human brain.* Cereb Cortex, 2010. **20**(5): p. 1254-62.

487. Ni, Z., et al., *Increased motor cortical facilitation and decreased inhibition in Parkinson disease.* Neurology, 2013. **80**(19): p. 1746-53.

488. Qin, L., et al., *An adaptive role for BDNF Val66Met polymorphism in motor recovery in chronic stroke.* J Neurosci, 2014. **34**(7): p. 2493-502.

489. Wang, D.D., et al., *Transcriptome profiling analysis of the mechanisms underlying the BDNF Val66Met polymorphism induced dysfunctions of the central nervous system.* Hippocampus, 2014. **24**(1): p. 65-78.

490. Spencer, J.L., et al., *BDNF variant Val66Met interacts with estrous cycle in the control of hippocampal function.* Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4395-400.

491. Pattwell, S.S., et al., *The BDNF Val66Met polymorphism impairs synaptic transmission and plasticity in the infralimbic medial prefrontal cortex.* J Neurosci, 2012. **32**(7): p. 2410-21.

492. Ninan, I., et al., *The BDNF Val66Met polymorphism impairs NMDA receptor-dependent synaptic plasticity in the hippocampus.* J Neurosci, 2010. **30**(26): p. 8866-70.

493. Krueger, F., et al., *The role of the Met66 brain-derived neurotrophic factor allele in the recovery of executive functioning after combat-related traumatic brain injury.* J Neurosci, 2011. **31**(2): p. 598-606.

494. Kocabas, N.A., et al., *Brain-derived neurotrophic factor gene polymorphisms: influence on treatment response phenotypes of major depressive disorder.* Int Clin Psychopharmacol, 2011. **26**(1): p. 1-10.

495. Perkovic, M.N., et al., *Association between the brain-derived neurotrophic factor Val66Met polymorphism and therapeutic response to olanzapine in schizophrenia patients.* Psychopharmacology, 2014. **Mar 5. [Epub ahead of print]**.

496. Charles, D., et al., *Subthalamic nucleus deep brain stimulation in early stage Parkinson's disease.* Parkinsonism Relat Disord, 2014.

497. Hacker, M.L., et al., *Outcomes in Early Stage Parkinson's Disease Patients May Be Influenced by the BDNF Val66Met Polymorphism.* Annals of Neurology, 2014. **76**: p. S115-S115.