

PRECISION MEDICINE AND GRAFT-INDUCED DYSKINESIA (GID):
INVESTIGATING THE CURIOUS SIDE EFFECT OF
DOPAMINE NEURON TRANSPLANTATION IN THE rs6265 BDNF (MET/MET)
PARKINSONIAN BRAIN

By

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ABSTRACT

While dopamine (DA) neuron transplantation is a promising alternative therapy to the current pharmacological agents (e.g., levodopa) prescribed for individuals with Parkinson's disease (PD), significant heterogeneity in clinical outcomes exists. Specifically, the underlying mechanisms responsible for the aberrant side effect, graft-induced dyskinesia (GID), a behavior that develops in a subpopulation of individuals who received primary DA neuron transplants, remains a mystery to be solved. In regard to this heterogeneity in cell therapy, our group previously became interested in the influence of certain genetic risk factors, hypothesizing that the common human single nucleotide polymorphism (SNP), rs6265, which is found in the gene for brain-derived neurotrophic factor (BDNF) and results in decreased BDNF release, is an unrecognized contributor to response variability in cell therapy, specifically the development of GID. Indeed, we previously demonstrated that homozygous rs6265 (Met/Met) parkinsonian rats engrafted with wild-type (WT; Val/Val) DA neurons uniquely exhibited GID compared to their WT counterparts. To further expand these findings, I investigated the impact of rs6265 in both the host and donor on DA neuron transplantation for my thesis research. I additionally studied whether exogenous BDNF treatment would mitigate GID behavior in the Met/Met parkinsonian rats engrafted with WT DA neurons. In both studies, rats were rendered unilaterally parkinsonian using 6-hydroxydopamine (6-OHDA), engrafted with intrastriatal embryonic ventral mesencephalic (eVM) neurons from E14 WT or Met/Met donors, and assessed for amelioration of levodopa-induced dyskinesia (LID) (graft function) and induction of graft-induced dyskinesia (GID) (graft dysfunction). For the second experiment, exogenous BDNF was administered directly above the grafted

DA neurons through a cannula connected to a subcutaneous osmotic minipump for four weeks following engraftment. From these experiments, I first determined that (1) the homozygous rs6265 Met/Met genotype, whether present in the host or donor, elicits superior graft-derived functional benefit compared to WT parkinsonian hosts, and (2) Met/Met parkinsonian rats engrafted with WT DA neurons curiously remain the only host/donor combination to exhibit significant GID behavior. Moreover, I discovered that (3) exogenous BDNF administration is not a feasible treatment for GID as BDNF exacerbated GID in Met/Met parkinsonian rats engrafted with WT DA neurons, and (4) evidence suggests that dysregulated DA/glutamate co-release and/or excess DA release is associated with GID induction, a phenomenon that corresponds with clinical trials where individuals with GID benefited from buspirone (a drug with DA antagonist properties) administration. Because several clinical grafting trials for PD are now planned or ongoing, uncovering the underlying mechanisms responsible for GID will be necessary to optimize cell transplantation as a safe alternative therapeutic in PD. Collectively, the knowledge gained from my research offers guidance moving forward for the development of promising precision-medicine-based therapies that effectively treat the majority, not only a subset, of patients with PD.

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2025

In loving memory of my father, Robert F. Szarowicz

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Dad, I am honored to be your daughter, and I will miss you every day for the rest of my life. But I know I will see you again. *I love you.*

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Thank you, Lord, for these blessings you have brought me.

Colossians 3:17: “And whatever you do, whether in word or deed, do it all in the name of the Lord Jesus, giving thanks to God the Father through him.

PREFACE

Upon completion of this dissertation, manuscripts derived from Chapter 3 and 4 are both finalized and ready for submission. Chapter 3 is intended to be submitted to *Neurobiology of Disease*, and Chapter 4 is intended to be submitted to *Journal for Clinical Investigation*. Additionally, large portions of Chapter 2 were reproduced from my review article that was published in July 2022 in *the International Journal of Molecular Sciences (IJMS)*, PMID: 35887357 (copyright is retained by the authors).

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
3-OMD	3-O-methyldopa
5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
8-OH-DAPT	8-Hydroxy-2-(di-n-propylamino)tetralin
¹⁸ F-DOPA	Fluorodopa
AADC	Aromatic L-amino decarboxylase
AAV	Adeno-associated virus
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AI	Artificial Intelligence
AIMs	Abnormal involuntary movements
ALS	Amyotrophic lateral sclerosis
BBB	Blood-brain-barrier
BDNF	Brain-derived neurotrophic factor
BG	Basal ganglia
Cav1.3	Voltage-dependent, L-type calcium channel, alpha 1D subunit
CMF	Calcium-magnesium free
CNS	Central nervous system
COMT	Catechol-o-methyltransferase
DA	Dopamine

DAB	3,3'-Diaminobenzidine
DAT	Dopamine transporter
DAT1	Dopamine active transporter 1 gene
DaTscan	Dopamine transporter scan
DBS	Deep brain stimulation
DHF	7,8-Dihydroxyflavone
DJ-1	Parkinsonism-associated deglycase or Parkinson disease protein 7
DLB	Dementia with Lewy bodies
dMSNs	Direct pathway Medium Spiny Neuron
DNA	Deoxyribonucleic acid
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
DRD1	Dopamine receptor 1
DRD2	Dopamine receptor 2
DRT	Dopamine replacement therapy
ELLDOPA	Earlier vs. Later Levodopa Therapy in Parkinson's disease
EPA	Environmental Protection Agency
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
eVM	Embryonic ventral mesencephalic
EWAS	Epigenome-wide association study
FBS	Fetal bovine serum
FD	Fluorodopa

FDA	Food and Drug Administration
GABA	Gamma aminobutyric acid
GBA	Glucocerebrosidase A gene
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GID	Graft-induced dyskinesia
GLP-1	Glucagon-like peptide 1
GPe	Globus pallidus externa
GPi	Globus pallidus interna
GSB-106	Bis-(N-monosuccinyl-L-seryl-L-lysine) hexamethylenediamide
GWAS	Genome-wide association study
hAESC	Human amniotic epithelial stem cells
HD	Huntington's disease
hEVMs	Human embryonic ventral mesencephalic cells
hESCs	Human embryonic stem cells
hpNSC	Human parthenogenetic neural stem cells
Hz	Hertz
Iba1	Ionized calcium-binding adaptor molecule 1
IHC	Immunohistochemistry
iMSNs	Indirect pathway medium spiny neuron
iPSCs	Induced pluripotent stem cells
ISH	In situ hybridization
i.p.	Intraperitoneal

JNKs	c-Jun N-terminal kinases
LAT1	L-type amino acid transporter 1
LB	Lewy body
L-DOPA	Levodopa
LN	Lewy neurite
LID	Levodopa-induced dyskinesia
LRRK2	Leucine rich repeat kinase 2
LTD	Long-term depression
LTP	Long-term potentiation
MAO	Monoamine oxidase
MAOBIs	Monoamine oxidase type B inhibitors
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
MDD	Major depressive disorder
MDS	International Parkinson and Movement Disorder Society
MFB	Medial forebrain bundle
MHC-II	Major histocompatibility complex 2
M/M	Homozygous rs6265 Met/Met genotype grafted with Met/Met donor cells
MMP	Matrix metalloproteases
MPTP	1-Methyl,-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSA	Multiple system atrophy

MSCs	Mesenchymal stem cells
MSN	Medium spiny neuron
MTA	Medial terminal nucleus
mTOR	Mechanistic target of rapamycin
M/W	Homozygous rs6265 Met/Met genotype engrafted with WT donor cells
NeuN	Pan neuronal marker
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor
NGS	Normal goat serum
NIH	National Institute of Health
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
OCD	Obsessive compulsive disorder
p75 ^{NTR}	pan 75 neurotrophin receptor
PASCs	Pluripotent stem cells isolated from adipose tissue
PD	Parkinson's disease
PDQ-39	Parkinson's disease questionnaire 39
PET	Positron emission tomography
PI3K	Phosphatidylinositol 3-kinase
PINK1	PTEN-induced putative kinase 1
PLCγ	Phospholipase Cy
PMI	Precision Medicine Initiative

PNS	Peripheral nervous system
PRKN	Parkin gene
PTSD	Post-traumatic stress disorder
PSP	Progressive supranuclear palsy
PWAS	Pesticide-wide association study
QSBB	Queen Square Brain Bank
rAAV	Recombinant adeno-associated virus
REM	Rapid eye movement
RhoA	Ras homolog gene family member A
RNA	Ribonucleic acid
s.c.	Subcutaneous
SERT	Serotonin transporter
shRNA	Short hairpin ribonucleic acid
SN	Substantia nigra
SNCA	Synuclein alpha (α)
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
SNP	Single nucleotide polymorphism
SorCS2	Sortilin-related Vps10p domain containing receptor 2
SPECT	Single photon emission computed tomography
STN	Subthalamic nucleus
STR	Striatum
TBI	Traumatic brain injury

TBS	Tris-buffered saline
TBS-Tx	Tris-buffered saline with Triton-X
TGN	Trans-Golgi network
TH	Tyrosine hydroxylase
Trk	Tropomyosin receptor kinase
TrkA	Tyrosine receptor kinase A
TrkB	Tyrosine receptor kinase B
TrkC	Tyrosine receptor kinase C
UPDRS	Unified Parkinson's Disease Rating Scale
US	United States
UTR	Untranslated region
VACHT	vesicular acetylcholine transporter
VGLUT	Vesicular glutamate transporter
VGLUT2	Vesicular glutamate transporter 2
VGLUT3	Vesicular glutamate transporter 3
VM	Ventral mesencephalon
VMAT2	Vesicular monoamine transporter 2
Vps10	Vacuolar protein sorting 10
VPS35	Vacuolar protein sorting ortholog 35
VTA	Ventral tegmental area
W/M	WT genotype engrafted with Met/Met donor cells
W/W	WT genotype engrafted with WT donor cells

CHAPTER 1: INTRODUCTION TO PARKINSON'S DISEASE (PD)

HISTORY

In 1817, James Parkinson, an English surgeon and apothecary, was the first to describe the disease that came to bear his name, Parkinson's disease (PD). He referred to the disorder as a shaking palsy or paralysis agitans. In his published work entitled, "An Essay on the Shaking Palsy," he made prominent observations of individuals who demonstrated "involuntary tremulous motion, with lessened muscular power...with a propensity to bend the trunk forward, and to pass from a walking to a running pace; the senses and intellect being uninjured" (Parkinson, 2002). Those afflicted with the disease showed slow progression and a profound decrease in quality of life. Along with these symptoms, of which are now considered the classic motor symptoms of PD, Parkinson also remarkably noted the sleep and autonomic (e.g., constipation) components of PD, classified today as common non-motor features (Chaudhuri & Jenner, 2017; Goetz, 2011; Parkinson, 2002).

Fifty years following Dr. Parkinson's observations, a French neurologist, Jean-Martin Charcot, further described the manifestations of PD and distinguished bradykinesia as a primary motor feature. Charcot wisely recognized that not all individuals with PD demonstrated a marked weakness or tremor; therefore, he rejected the title of paralysis agitans or shaking palsy and recommended a name change to "Parkinson's disease" (Charcot, 1892; Goetz, 2011). Many additional valuable observations were made in the years following, one of which was the identification that PD expressed a slight male predominance. This was discovered by William Gowers, a British neurologist, in 1888 (Gowers, 1898).

Although several clinical manifestations were detailed in regard to PD, it was not until the 1920s that significant pathological findings of the disease were determined. For example, Brissaud was the first to propose that damage to the substantia nigra (SN) may be the underlying pathology responsible for PD in 1925 (Edouard Brissaud, 1899). Also in the 1920s, additional pathological studies of the midbrain were separately conducted by Tretiakoff and Foix and Nicolesco (C Trétiakoff, 1921; Foix, 1925). The most comprehensive pathologic analysis which included the demarcation of brain lesions, however, was not performed until 1953 by Greenfield and Bosanquet (see (Greenfield & Bosanquet, 1953) for more details).

Finally, in 1959, Bertler and Rosengren and Sano and colleagues proposed the possibility that dopamine (DA) was involved in the pathogenesis of PD. They demonstrated that the majority of DA in the brain was found in the caudate nucleus and putamen, both in dogs and in humans (Bertler & Rosengren, 1959; Hornykiewicz, 2010; Sano et al., 1959). To confirm, Oleh Hornykiewicz, an Austrian biochemist, analyzed the brains of patients with PD in 1960, discovering that these patients, indeed, had profound loss of DA in the caudate and putamen. Hornykiewicz further observed a loss of DA neurons in the SN, suggesting that this was the cause of the DA terminal loss in the striatum (Birkmayer & Hornykiewicz, 1961; Ehringer & Hornykiewicz, 1960). These findings enabled additional research to be conducted, specifically into the nigrostriatal pathway (Dahlstroem & Fuxe, 1964; Poirier & Sourkes, 1964; Sourkes & Poirier, 1965). The discovery of DA loss in these regions radically changed the field's understanding of PD pathophysiology and remarkably led to the development of successful

pharmacotherapies (e.g., levodopa), some that remain clinically routine to this day (Cotzias et al., 1967).

SYMPTOM PRESENTATION AND CLINICAL DIAGNOSIS

Parkinson's disease is the second most common neurodegenerative disease following Alzheimer's disease (AD), affecting 9.3 million people worldwide (Espay et al., 2017; Maserejian et al., 2020; Schalkamp et al., 2022). If PD maintains its current growth rate, approximately 13 million people are estimated to be diagnosed with PD by the end of 2040 (Dorsey et al., 2018; Straccia et al., 2022). Consequently, PD has placed significant strain on society. Not only does PD cause a poor quality of life, the total economic burden was estimated to be \$51.9 billion in 2017 and projected to surpass \$79 billion by the year 2037 (Yang et al., 2020). In the following paragraphs, the classic motor symptoms, non-motor symptoms, and clinical diagnosis of PD are discussed.

Classic Motor Symptoms

As a movement disorder, PD is known to exhibit several prominent motor features including bradykinesia, resting tremor, rigidity, and postural instability. The most characteristic of these symptoms is bradykinesia, which is defined as slowness of movement. While bradykinesia first manifests as a slowness in daily task performance (J. A. Cooper et al., 1994; Giovannoni et al., 1999), it often progresses to the loss of spontaneous movement, drooling (Bagheri et al., 1999), and a reduction in arm swing whilst walking (Jankovic, 2008). In addition to bradykinesia, another major symptom is resting tremor. These tremors occur at a frequency of 4-6 Hertz (Hz) and tend to affect the distal part of the extremities. While resting tremor does not often impact the neck,

head, or voice, it does involve the chin, jaw, and legs. Interestingly, resting tremor diminishes with action or while an individual is sleeping—a characteristic that helps differentiate PD from other disorders such as essential tremor (Jankovic, 2008).

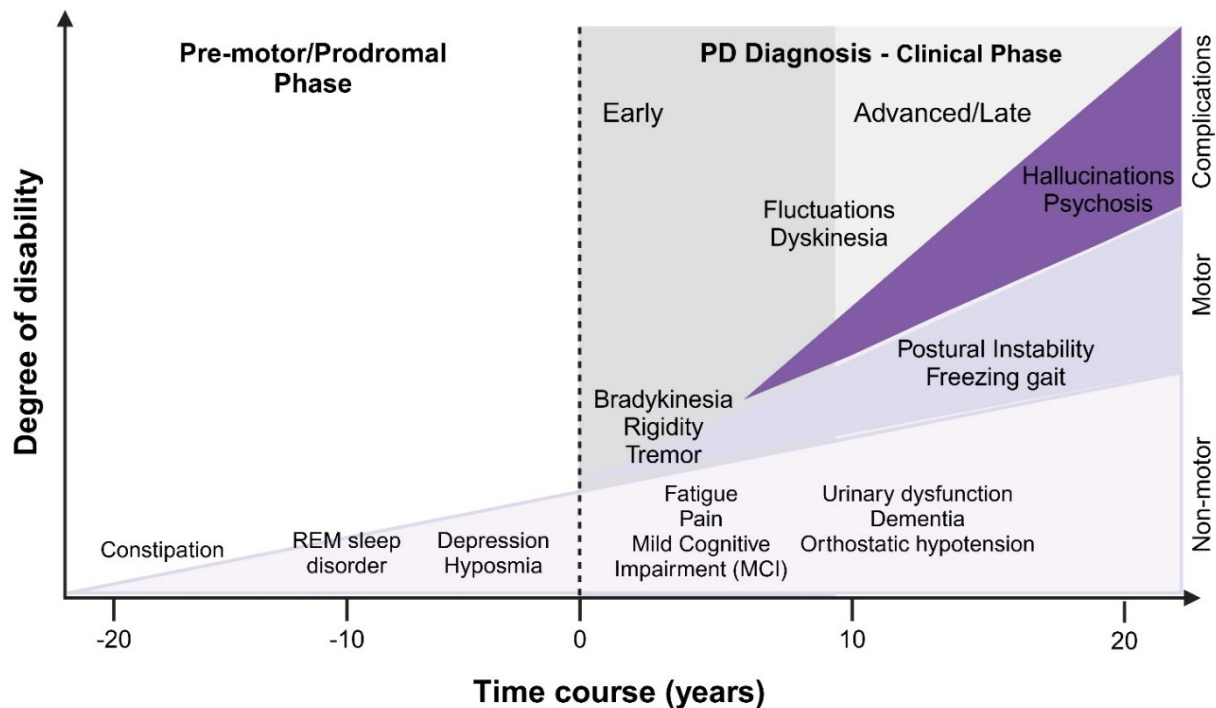


Figure 1.1: Progression time course of PD.

PD is often preceded by premotor symptoms (i.e., prodromal phase) of an estimated 20+ years. This phase is characterized by several non-motor symptoms including constipation, rapid eye movement disorder (REM), and depression. At time of diagnosis, when 50-60% of DA neurons in the substantia nigra (SNpc) have already been lost (Dauer & Przedborski, 2003), motor symptoms including bradykinesia, rigidity, tremor, and/or postural instability are present. Dyskinesias (e.g., LIDs) also develop in approximately 90% of patients by 10 years of treatment with levodopa (Hauser et al., 2017; Huot et al., 2013, 2022). Abbreviations: PD = Parkinson's disease. REM = rapid eye movement. Schematic adapted from (L. V Kalia & Lang, 2015).

Rigidity, another common motor feature, is characterized by stiffness and increased resistance to passive movement. Unfortunately, rigidity can also be accompanied by pain, often getting misdiagnosed as arthritis, bursitis, or a rotator cuff injury (Jankovic, 2008; Riley et al., 1989). The last major motor symptom is postural

instability, which tends to manifest in the later stages of PD. Postural instability involves the loss of postural reflexes, frequently resulting in falls and subsequent hip fractures (Williams, 2006). The later onset of falls, however, can be used to distinguish PD from other parkinsonian disorders including multiple system atrophy (MSA) and progressive supranuclear palsy (PSP). Although the discussed signs are considered to be the “classic hallmarks” of PD, there are additional secondary motor difficulties that several patients with PD can also exhibit. These include, but are not limited to, freezing gait, speech impairment (e.g., microphonia), micrographia (small handwriting), and respiratory disturbances (**Figure 1.1**) (Jankovic, 2008; Jankovic & Tolosa, 2007; Lees et al., 2009; Moustafa et al., 2016).

Non-Motor Symptoms

Despite being primarily considered a movement disorder, PD has long been associated with several non-motor signs and symptoms as well. Indeed, in James Parkinson's report in 1817 (discussed previously), he observed non-motor symptoms alongside the classic motor symptoms in his patients (Parkinson, 2002). To date, numerous non-motor features have been noted in PD including constipation, urinary dysfunction, memory loss, depression, orthostatic hypotension, and sleep disturbances. Markedly, these non-motor symptoms frequently precede motor dysfunction by years or decades (**Figure 1.1**) (G. W. Ross et al., 2012; Tolosa et al., 2021).

One of the most pronounced non-motor symptom individuals with PD exhibit is sleep disturbances, also referred to as rapid eye movement (REM) sleep behavior disorder. Over one-third of individuals will experience this disorder prior to their PD diagnosis, often mentioning an increase in violent dreams (Borek et al., 2007), as well

as sleep talking, kicking, and yelling. Also of note, greater than 50% of patients will have experienced insomnia to some degree (Boeve et al., 2007; Gjerstad et al., 2006). Additional non-motor features that patients with PD can demonstrate include obsessive compulsive disorder (OCD) and impulsive behaviors such as gambling or binge eating. Moreover, sensory abnormalities like olfactory dysfunction, a symptom not often recognized as a parkinsonian feature, recently have been correlated with a 10% increased risk of developing PD (Ponsen et al., 2004). Finally, although cognitive dysfunction is not yet fully understood in PD, a prospective study conducted by Aarsland and colleagues reported that patients with PD are at a sixfold increased risk for developing dementia (Aarsland et al., 2001).

Clinical Diagnosis

Currently, there is no definitive test to diagnose PD. Histopathological postmortem analysis is required to conclusively establish a PD diagnosis by confirming the presence of Lewy bodies (see Neuropathology below) (Jankovic, 2008). Although outside the realm of this thesis discussion, it is important to note that there are exceptions with specific genetic mutations (e.g., G2019S LRRK2) which lack Lewy body pathology at autopsy (see (O'Hara et al., 2020)). Nevertheless, there are clinical diagnostic criteria in place including the Queen Square Brain Bank (QSBB) criteria and the criteria generated by the International Parkinson and Movement Disorder Society (MDS), but these criteria are not without issue. For instance, in clinical practice, error rates for diagnostic misclassification can range from 15-24% (Hughes et al., 1992; Rajput & Rajput, 2014; Schrag, 2002; Tolosa et al., 2021). Furthermore, in over 10% of

cases that are diagnosed by PD neurologists, alternative pathologies were seen to be present upon postmortem autopsy (Tolosa et al., 2021).

The criteria used for clinical PD diagnosis are centered around characterizing motor signs and symptoms. The QSBB criteria, which was initially proposed in the 1980s, has been the most widely used criteria for clinical PD diagnosis up until 2015 when the International Parkinson and MDS added its refinement (Gibb & Lees, 1988; Lees et al., 2009; Marsili et al., 2018; Postuma et al., 2015). Generally, these criteria rely on neurological examination, first identifying bradykinesia as a major motor symptom, in addition to resting tremor and/or rigidity (step one). Step two involves establishing that the patient does not exhibit symptoms or a history that would be indicative of another non-PD disorder (e.g., stepwise decline, repeated head trauma, encephalitis). Step three indicates whether the patient presents supportive criteria including resting tremor, unilateral onset, evidence of progression, a response to levodopa (L-3,4-dihydroxyphenylalanine), development of levodopa-induced dyskinesia (LID), and a long clinical course of 10 years or more (Gibb & Lees, 1988).

In 2015, after scientific advancement, MDS refined the set of diagnostic criteria established by QSBB in order to further improve the diagnostic accuracy of PD. With the new criteria, two new diagnostic categories were created including the “Clinically Established PD” and “Clinically Probable PD” categories. These new categories incorporated what is referred to as “red flags:” factors that would exclude PD. However, when combined with supportive criteria, they do not exclude PD. Two ancillary tests were also added including olfactory dysfunction tests and cardiac imaging (Munhoz et al., 2024). Importantly, the MDS criteria has demonstrated great sensitivity (96%) and

specificity (95%) in a validation study for a clinical diagnosis of “probable PD” (Postuma et al., 2018). For individuals in the earlier stages of the disease (less than 5 years), the specificity of “clinically probable” PD was 87% (Postuma et al., 2018). In order to further enhance accuracy for early (i.e., prodromal) disease stages, additional tests and biomarkers are required.

To reiterate, these criteria are based on motor signs and symptoms of PD. Yet, evidence has demonstrated that pathological and neurochemical markers for PD are established long before the exhibition of these motor symptoms. Because of this, and because non-motor symptoms are difficult to categorize so early-on due to their ambiguous nature, delineating non-motor, prodromal features of the disease (**Figure 1.1**) will be imperative for the development of disease-prevention or disease-reversal therapy for PD. Scientists and clinicians have been making positive strides in this area: ancillary tests are being utilized concomitantly with the clinical diagnostic criteria in order to increase diagnostic accuracy. For example, molecular neuroimaging such as the dopamine transporter scan (DaTscan) can be used to discriminate between PD and essential tremor (Benamer et al., 2000). Unfortunately, however, DaTscan imaging cannot be used to differentiate between PD and MSA or PSP because of their shared degenerative characteristics (Tagare et al., 2017). Genetic testing has also been a useful ancillary test; however, only LRRK2 mutations have been screened successfully (Tolosa et al., 2006) and may be of limited value since over 90% of PD cases are idiopathic. Ultimately, it would be most promising if future research could uncover disease-specific biomarkers that would aid in the delineation between PD and other similar neurodegenerative disorders (Jankovic, 2008).

A correct diagnosis of PD is necessary for proper patient counseling and therapy development. Despite achieving rather high specificity and sensitivity, clinical diagnostic criteria remain fallible and still lead to misdiagnoses. While several ancillary tests have been developed and implemented in order to increase diagnostic accuracy, these tests are costly and not without caveats. Until further discoveries are made (e.g., biomarker development), histopathological confirmation of the presence of Lewy bodies in postmortem tissue remain the criteria for the definitive diagnosis of PD. The underlying neuropathology of PD is further discussed below.

NEUROPATHOLOGY

The Basal Ganglia

The major pathological hallmarks of PD include the loss of DA neurons in the SNpc and the presentation of intracellular inclusion aggregates of the protein α -synuclein, referred to as Lewy bodies (Obeso et al., 2002). Although these two features of PD are widely recognized as the pathological hallmarks of the disease, the underlying pathology is heterogenous and can vary greatly among individuals (Halliday et al., 2008). Despite various other pathologies that may contribute to PD, the present discussion is only focused on these two neuropathological characteristics. The region of the brain that is most affected by these pathologies, subsequently leading to cardinal motor signs and symptoms of PD, is the basal ganglia (BG) (**Figure 1.2a**). Therefore, the structure and function of the BG are reviewed below.

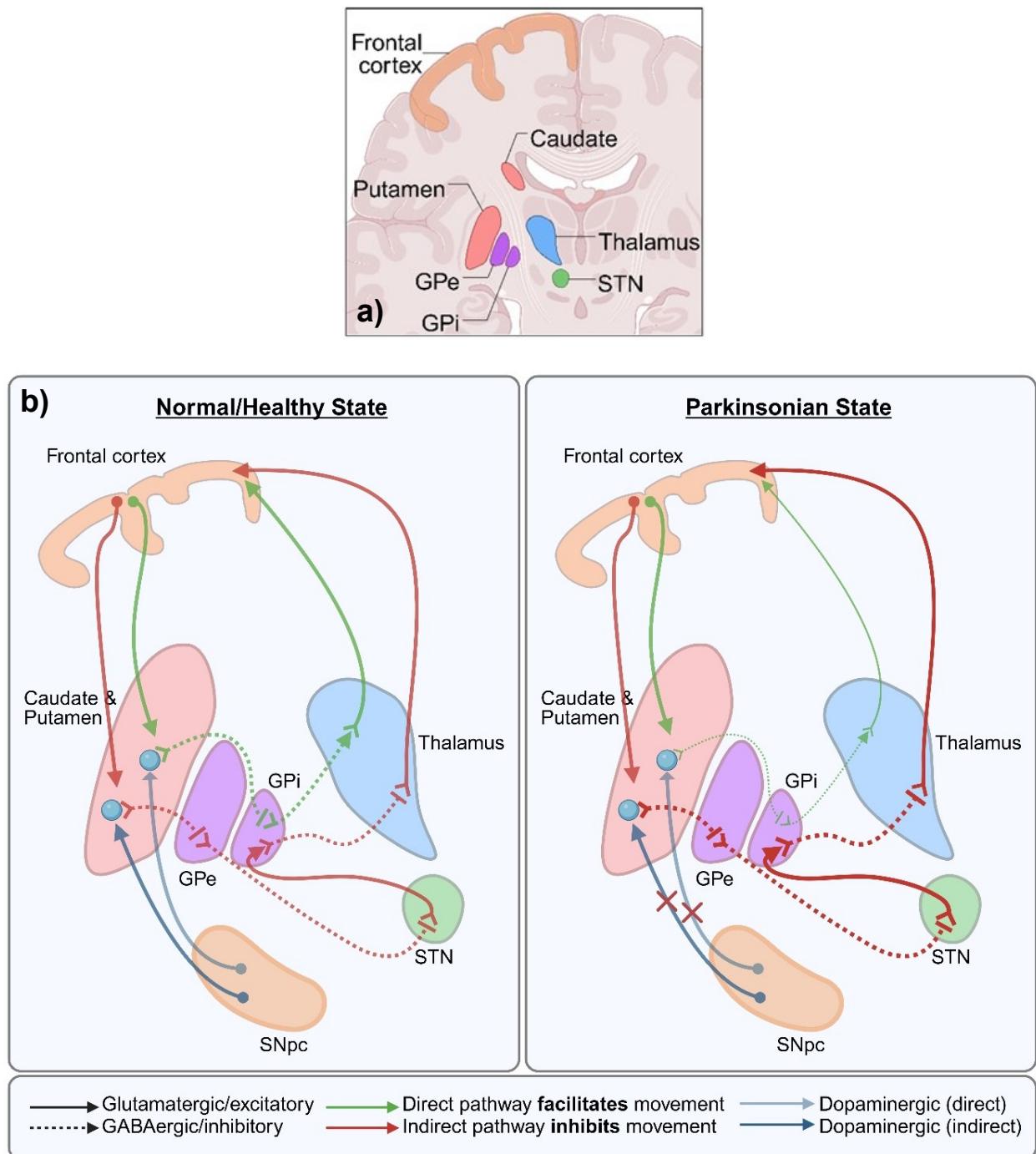


Figure 1.2: Classic Model of Basal Ganglia Circuitry in Normal & Parkinsonian brain.

(a) Coronal diagram of the BG in the human brain, excluding the substantia nigra pars compacta and reticulata, which are combined as SNpc in (b) for simplicity. **(b)** Schematic illustration of the classic BG model, including the direct and indirect pathways in both healthy and parkinsonian brains. The caudate and putamen were condensed for simplicity. This is a limited representation of the mechanisms of the basal ganglia. Black lines indicate glutamatergic (solid) and GABAergic (dashed) neurons. The direct and

Figure 1.2 (cont'd)

indirect pathways are shown in green and red, respectively. Blue arrows demonstrate dopaminergic projections to both the direct (light blue) and indirect (dark blue) pathways. In the parkinsonian state, a red "X" demarcates the degeneration of the DA neurons in the substantia nigra pars compacta. Abbreviations: GPe = globus pallidus externa; GPi = globus pallidus interna; SNpc = substantia nigra pars compacta; STN = subthalamic nucleus.

The BG are a group of seven subcortical nuclei responsible for motor control, reward-based learning, goal-directed behavior, and emotion (Chakravarthy et al., 2010; Lanciego et al., 2012). The seven nuclei include the caudate nucleus, putamen, globus pallidus interna (GPi), globus pallidus externa (GPe), subthalamic nucleus (STN), the substantia nigra pars compacta (SNpc), and the substantia nigra pars reticulata (SNpr) (Chakravarthy et al., 2010). This list can further be categorized into input, output, or intrinsic nuclei (Lanciego et al., 2012). The caudate and putamen make up the input nuclei, and functionally, these nuclei receive information from the cortex, the thalamus, and the SN. The output nuclei, including the GPi and the SNpr, send information to the thalamus. The GPe, the STN, and the SNpc are considered the intrinsic nuclei, and they relay information between the input and output nuclei. In-depth, comprehensive summaries regarding BG anatomy can be found in (Chakravarthy et al., 2010; Gerfen & Wilson, 1996; MINK, 1996; Y. Smith et al., 1998).

Functionally, the BG system requires the release of DA from SNpc neurons to its input nuclei (i.e., caudate and putamen), which are collectively called the striatum (STR) (Lanciego et al., 2012). Approximately 90% of the striatum consists of projection neurons (i.e., medium spiny neurons (MSNs)) and 10% interneurons. Structurally, MSNs are named for their appearance: they are multipolar neurons with medium-sized cell somas (~12-20µm in diameter), and their dendritic processes are covered with dendritic spines (Gerfen & Bolam, 2010). In general, two types of MSNs exist. Some MSNs

express dopaminergic receptor type 1 (DRD1), and some express dopaminergic receptor type 2 (DRD2), which generate two circuits that exert differential effects according to the classical model of the BG system. Both subtypes, however, release the inhibitory neurotransmitter, gamma aminobutyric acid (GABA; GABAergic) upon activation.

The two circuits of the classical BG model include the direct and indirect pathways (**Figure 1.2b**). These circuits are thought to have oppositional effects (Albin et al., 1989; Calabresi et al., 2014; DeLong, 1990; Lanciego et al., 2012) in which the direct pathway proposedly promotes motor movement/selection, whereas activation of the indirect pathway is theorized to inhibit movement/selection. Neurons designated as A9 DA neurons from the SNpc project their axons onto the MSNs in the STR; the DA input on MSNs with DRD1 (dMSNs) exerts a facilitatory effect (direct pathway) and an inhibitory effect on DRD2-expressing MSNs (iMSNs; indirect pathway) (Chakravarthy et al., 2010; D. L. Clark et al., 2010; Lanciego et al., 2012). In this way, activation of the direct pathway will inhibit GPi activity, disinhibiting the thalamus, and promoting neuronal firing. The result is initiation of motor movement. Contrarily, activation of the indirect pathway will inhibit the activation of the GPe, disinhibit the STN, and allow for the GPi neurons to activate, inhibiting the thalamus, and ceasing motor movement. Under normal resting conditions, the indirect pathway is the “active” pathway in which tonically released DA inhibits activation of downstream motor systems (Chakravarthy et al., 2010). Upon phasic DA activation, the increase in striatal DA shifts the balance toward the direct pathway, allowing motor systems to activate (Chevalier et al., 1985; Deniau & Chevalier, 1985).

It is also important to note that glutamatergic excitatory projections from the cortex make synaptic connections generally onto the heads of dendritic spines of MSNs in the STR (Bouyer et al., 1984; Hattori et al., 1979; Z. C. Xu et al., 1989).

Glutamatergic afferents from the thalamus similarly form connections onto MSNs; however, these afferents synapse onto the dendritic shafts of MSNs instead of the head of the spines (Dubé et al., 1988; Lacey et al., 2005; Z. C. Xu et al., 1991). The DA projections that extend from the SNpc make *en passant* synaptic appositions onto the necks of the dendritic spines of the MSNs and then modulate excitatory glutamatergic input coming into the MSNs from the heads of the same dendritic spines (Bamford et al., 2004; Bouyer et al., 1984; T. F. Freund et al., 1984; Gerfen & Surmeier, 2011; W. Shen et al., 2016; Yamamoto & Davy, 1992). In this way, both the glutamatergic input and the dopaminergic modulatory behavior is critical for normal motor function.

In PD, the degeneration of DA neurons in the SNpc results in DA depletion in the STR (**Figure 1.2b**). Consequently, MSN activation in the direct pathway is reduced, resulting in a relative increase in the activity of the indirect circuit. The result is overstimulation of the GPi, ultimately diminishing movement execution, and thus leading to the classic motor features of PD. In the DA neurons that do survive, intracellular α -synuclein inclusions tend to form, representing another pathology of PD. Both DA degeneration and α -synuclein aggregation pathologies are described below.

Nigrostriatal Degeneration and DA Depletion

One of the defining pathological characteristics of PD is the selective degeneration of dopaminergic neurons in the SNpc, specifically in the ventrolateral tier (A9) (Dickson, 2012; Fearnley & Lees, 1991; Kordower et al., 2013; Obeso et al., 2002;

Rudow et al., 2008). Interestingly, the dorsal and medial (i.e., A8 and A10) neurons are less vulnerable to degeneration, which has been demonstrated in both PD patients and animal models (Brooks et al., 1990; Iravani et al., 2005; Kish et al., 1988). From nigrostriatal afferents, DA is released tonically to the STR, with transient bursts of phasic release (A. A. Grace, 1991; A. Grace & Bunney, 1984). Rewarding events will induce brief phasic DA release, while adverse, negative events will decrease DA activity (Redgrave & Gurney, 2006; Schultz, 1998)—a phenomenon central to motor learning. As described above, at resting, tonic release of DA maintains sufficient DA levels in the STR as well as tonic DA receptor stimulation critical for normal BG function (Olanow et al., 2006; Venton et al., 2003). Degeneration of this system thus leads to a decrease in striatal DA, interfering with normal motor movement and action selection, thus resulting in motor symptoms of PD.

The extent of nigrostriatal degeneration in individuals with PD has been studied. For example, Kordower and colleagues demonstrated that, at year one post-diagnosis, a modest loss of dopaminergic terminals in the STR was present visualized by decreased staining of dopaminergic markers (e.g. tyrosine hydroxylase (TH)). At three years post-diagnosis, there was marked loss of DA neuron staining (35-75%), and at four years, there was almost complete loss of DA fibers in the STR. Over the same time period and in the same patients, there was a 50-90% loss of DA neurons in the SNpc (Kordower et al., 2013). Further, other research groups have reported a 44-98% reduction in striatal DA levels in advanced PD (Bernheimer et al., 1973; Ehringer & Hornykiewicz, 1960; Rajput et al., 2008).

The morphological and functional effects of striatal DA depletion has also been studied extensively (see (Villalba & Smith, 2018) for review). More specifically, a significant reduction in spine density of striatal MSNs, both in length and in number (McNeill et al., 1988), has been observed in postmortem tissue from individuals with PD (Stephens et al., 2005; Villalba & Smith, 2018; Zaja-Milatovic et al., 2005), in parkinsonian rodent models (Ingham et al., 1989, 1998; Zhang et al., 2013), and in parkinsonian non-human primates (Villalba 2008). In addition to changes in spine density, glutamatergic reorganization has been observed in the DA-denervated striatum (Arbuthnott et al., 2000; M. Day et al., 2006; Gubellini et al., 2002; Ingham et al., 1998; Liang et al., 2008; Zhang et al., 2013). For instance, parkinsonian rodent models have exhibited a decrease in the quantity of glutamatergic asymmetric synaptic contacts onto MSNs in the striatum (Ingham et al., 1993, 1998). Collectively, this evidence demonstrates that DA plays a critical role in regulating the growth, maintenance, and plasticity of dendritic spines and glutamatergic connections onto MSNs (Arbuthnott et al., 2000; Robinson & Kolb, 1999). It remains to be determined, however, whether dendritic spine loss is an early or late-stage phenomenon in PD. Nevertheless, recent studies that targeted calcium channels in order to block calcium influx have exhibited promising prevention of dendritic spine loss in MSNs (Soderstrom et al., 2010; Steece-Collier et al., 2019); this could, in turn, be used as a therapeutic target to prevent the progression of DA degeneration in PD.

Lewy Body Pathology

The other defining pathological characteristic of PD is the accumulation of misfolded α -synuclein protein. α -synuclein is a soluble, heat stable protein

approximately 140 amino acids in length (Jakes et al., 1994; McCann et al., 2014). Its physiological function remains elusive; however, several studies have proposed a role of α -synuclein in the maintenance of synapses, mitochondrial homeostasis, proteasome function, and DA metabolism (McCann et al., 2014; Ramalingam et al., 2023; Uversky, 2003). A-synuclein is known to be highly expressed in neurons of the frontal cortex, hippocampus, and the STR (Iwai et al., 1995; Norris et al., 2004). While under normal conditions, α -synuclein functions properly, in the context of PD, it forms insoluble inclusions within neuronal cell processes or cell bodies, referred to as Lewy neurites (LNs) and Lewy bodies (LBs), respectively.

Frederick Lewy was the first to describe these α -synuclein LN/LB inclusions in 1912. However, it was not until 1997-1998 that significant advancements were made that linked α -synuclein accumulation to LN/LB in PD and other disorders such as MSA or dementia with Lewy bodies (DLB) (McCann et al., 2014; Norris et al., 2004). Lewy bodies are described to contain a dense core of aggregated α -synuclein surrounded by a halo of fibrils that are approximately 10-15 nanometers (nm) in diameter (Arima et al., 1998; Baba et al., 1998; Forno, 1969; Galloway et al., 1992; Spillantini et al., 1998; Tiller-Borich & Forno, 1988). Indeed, cytoplasmic inclusions of α -synuclein are abnormal since α -synuclein is normally localized primarily to presynaptic terminals. Although the mechanistic consequences of Lewy pathology remain to be fully elucidated, it has been postulated that Lewy body inclusions negatively affect protein transport and organelle function (Duffy & Tennyson, 1965; Hill et al., 1991; M. L. Schmidt et al., 1991), often leading to cell death. In confirmation, experiments that have overexpressed α -synuclein in rodent models have demonstrated an inhibition of

neurotransmitter (i.e., DA) release (Gaugler et al., 2012; Nemani et al., 2010), as well as a 60-80% reduction of DA innervation to the STR (Lundblad et al., 2012).

In 2003, Braak and colleagues generated a staging scheme for α -synuclein pathology largely based on the distribution and progression of α -synuclein over time (Braak et al., 2003). In this model, Lewy pathology is described in six stages. Pathology is first proposed to begin in the enteric nervous system and then travel to the dorsal motor nucleus of the vagus nerve in the medulla and to the olfactory nucleus (stage 1 and 2) (Dickson, 2012). Pathology is then proposed to migrate to the locus coeruleus, and then to the DA neurons in the SNpc (stage 3). Later stages (i.e., 4-6) exhibit pathology in the basal forebrain, amygdala, and cortical areas (Dickson, 2012; McCann et al., 2014).

While several clinical studies have reported results in favor of this Braak staging scheme (e.g., (Halliday et al., 2008)), one of which reported a 67% proportion of cases that successfully fit the staging scheme (Dickson et al., 2010), other groups have shown that pathology does not always follow the proposed distribution of α -synuclein. Indeed, in some elderly individuals with PD, Lewy pathology was exclusively found in the olfactory bulb (Beach et al., 2009; Fujishiro et al., 2008) or in the amygdala (Uchikado et al., 2006). Moreover, some neurologically “normal” individuals who were without PD signs or symptoms still exhibited widespread Lewy pathology (Frigerio et al., 2011; Parkkinen et al., 2005). Therefore, Braak staging remains a useful, but tentative, tool for PD pathophysiology.

To date, there is controversy whether α -synuclein accumulation precedes neurodegeneration. While some argue that Lewy pathology is a precursor to neuronal

degeneration (Chu et al., 2024; Gibb & Lees, 1988), others have shown that, even at Braak stage 1 and 2, the quantity of DA neurons is already diminished (Milber et al., 2012). Further challenging this central idea of α -synuclein accumulation toxicity, Lewy pathology is not always detected in PD brains (Buchman et al., 2012, 2019; L. V. Kalia et al., 2015; Milber et al., 2012; Yamashita et al., 2022), therefore suggesting that there could be an earlier, non- α -synuclein-related process involved in the degeneration of the nigrostriatal pathway in PD (Chu et al., 2024). Again, it is clear that PD is a heterogeneous and complex disorder as scientists continue to make strides in this field.

RISK FACTORS AND ETIOLOGY

Parkinson's disease is a complex and multifaceted neurodegenerative disorder of largely unknown etiology. Currently, a combination of genetic and environmental risk factors are thought to contribute to the development of the disorder. Several demographic characteristics have also been associated with PD risk including gender, ethnicity, and advancing age, with age being the greatest risk factor (Collier et al., 2011, 2017; L. V. Kalia et al., 2015; Van Den Eeden, 2003). In addition to age, environmental factors such as toxicant exposure, and genetic susceptibilities, research continues to identify other elements that may influence the likelihood of developing PD. For instance, traumatic brain injury (TBI), lifestyle choices such as diet and exercise, and diabetes have been more recently reported as possible risk factors or comorbidities of PD (Figure 1.3).

Advancing Age

Advancing age is known to be the greatest risk factor for developing PD (Bennett et al., 1996; Collier et al., 2011; J. F. Cooper et al., 2015; Morens et al., 1996; Tanner &

Goldman, 1996; Wyss-Coray, 2016). Yet, advancing age as a risk factor is not specific to PD: it is common in many other neurodegenerative diseases such as AD. Indeed, it is estimated that one in ten individuals over the age of 65 currently has AD, a prevalence that will continue to rise as our aging population increases (Hou et al., 2019). The aging US population (≥ 65 years) is estimated to increase to 88 million in the year 2050 (from 53 million in 2018) (Hou et al., 2019). Therefore, the burden of PD will continue to expand, and identifying ways to halt or slow its progression continues to be a priority in the field of neurodegeneration.

The role of aging in PD pathogenesis remains elusive (Pang et al., 2019). However, the hallmarks of aging, some of which include genome instability, telomere degradation, epigenetic alterations, loss of proteostasis, and mitochondrial dysfunction, share important biological features with PD and have been correlated to an increased PD risk (for review (Hou et al., 2019)). The overlap of the molecular mechanisms of aging and PD continue to allow scientists to make strides in neurodegenerative research. Experimentally, postmortem analyses of neurologically “normal” brains of individuals between the ages of 14 and 92 years old have established a significant decrease of striatal DA with advancing age (Kish et al., 1992). A loss of brain weight and SN volume have also been demonstrated in aging humans and non-human primates (E. Y. Chen et al., 2000; Chu et al., 2002). Aging mechanisms continue to be a target for potential therapies in PD and for other neurodegenerative disorders.

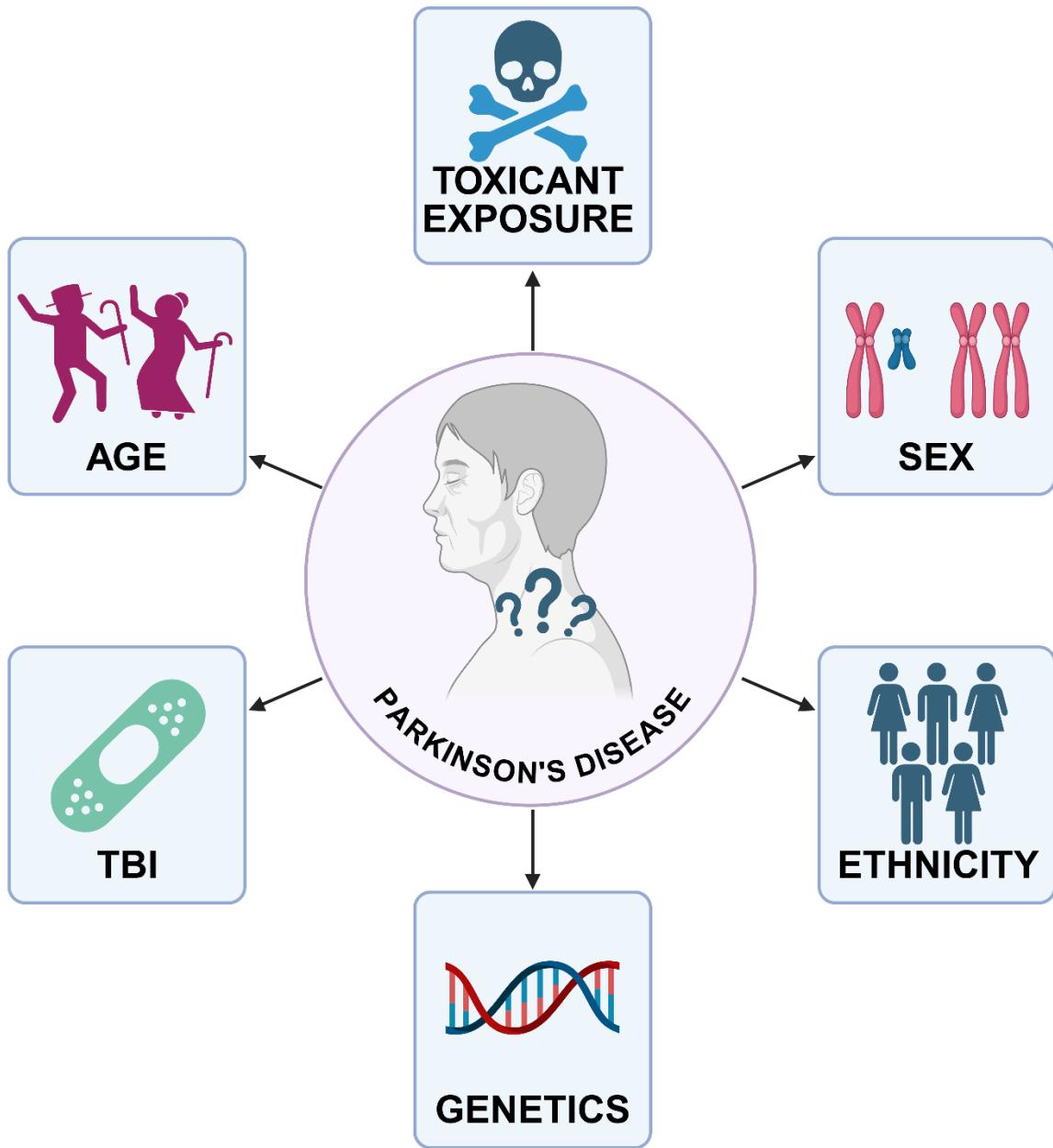


Figure 1.3: Risk Factors for PD.

Schematic representation of risk factors associated with developing PD. While not fully elucidated, a combination and/or interaction of risk factors is thought to contribute to the incidence of PD. The factors presented here are only examples and are not a complete list. Abbreviations: TBI = traumatic brain injury.

Genetic Risk Factors

Approximately 5-10% of patients with PD exhibit a monogenic (i.e., caused by a mutation in a single gene) form of the disorder. As of 2020, over one hundred pathogenic risk loci in PD have been identified using genome-wide association studies (GWAS) (Blauwendraat et al., 2020). Defined below, the major autosomal dominant mutations that have been identified include SNCA, LRRK2, and VPS35, whereas the autosomal recessive mutations are found in PINK1, DJ-1, and Parkin, all of which are known to cause PD with high penetrance. Several other genes with Mendelian inheritance also have been implicated in PD, specifically atypical PD (Lill, 2016; Lunati et al., 2018), but are not as prevalent in the general population. Overall, the vast majority of PD is extraordinarily complex, and it is more likely that PD is caused by a combination of genetic and environmental risk factors.

SNCA

The first autosomal dominant mutation associated with PD was found in the SNCA gene in 1997 (Polymeropoulos et al., 1997). SNCA encodes for α -synuclein, and mutations in SNCA tend to cause abnormal α -synuclein accumulation, leading to LB and LN formation (see Neuropathology section). Moreover, missense mutations or duplications of SNCA produce signs of dementia in patients with PD (Lill, 2016). Interestingly, there is a dosage effect of mutations in this gene. For instance, triplications, compared to duplications, can induce an earlier age of onset of PD of which progresses more rapidly (Lill, 2016; Lunati et al., 2018).

LRRK2

Leucine-rich repeat kinase 2 (LRRK2) is another autosomal dominant mutation that has been implicated in the risk of PD development. To date, nine highly penetrant, pathogenic mutations have been found in LRRK2 (Healy et al., 2008; Paisán-Ruiz et al., 2013; O. A. Ross et al., 2011; Rubio et al., 2012). LRRK2 encodes for a protein called dardarin which is involved in lysosomal and autophagy regulation (Lunati et al., 2018). Consequently, mutations in LRRK2 lead to the hyperactivation of its kinase domain (Alessi & Sammler, 2018); therefore, potential LRRK2 antagonists are currently being studied as a therapeutic for this genetic form of PD. Similar to SNCA mutations, as well as idiopathic PD, individuals with LRRK2 mutations exhibit typical PD symptoms and respond well to levodopa.

VPS35

The third major autosomal dominant mutation associated with PD risk is found in the gene for vacuolar protein sorting 35 (VPS35). This gene encodes for a protein responsible for synaptic endocytosis and retrograde protein transport. In this way, mutations in VPS35 are postulated to disrupt vesicle formation and protein trafficking (Lunati et al., 2018; Trinh & Farrer, 2013). PD patients with VPS35 mutations demonstrate typical PD symptoms and a good response to levodopa, like that of patients with the SNCA and LRRK2 gene mutations.

GBA

The most prominent genetic risk factor for PD is found in the glucocerebrosidase A gene (GBA). GBA encodes for glucocerebrosidase, a lysosomal hydrolase enzyme that catalyzes the breakdown of both glucosylceramide and glucosylsphingosine

(Sidransky & Lopez, 2012; L. Smith & Schapira, 2022). Approximately 5-15% of individuals with PD have GBA mutations, occurring more frequently than any other gene in familial PD (e.g., SNCA, LRRK2) (Sidransky et al., 2009), and well over 300 pathogenic GBA mutations have been identified to date (Beutler et al., 2004; Hruska et al., 2008). Those with GBA mutations have an average age of onset that is estimated to be five years earlier than idiopathic PD (Gan-Or et al., 2008; Malek et al., 2018; Neumann et al., 2009; Sidransky et al., 2009), and their risk of developing cognitive deficits and dementia is also greater (Cilia et al., 2016; Papapetropoulos et al., 2006; Petrucci et al., 2020). Functionally, homozygous GBA mutations have been described as causative factors for Gaucher's disease, which is a lysosomal storage disorder (see **Figure 1.4**).

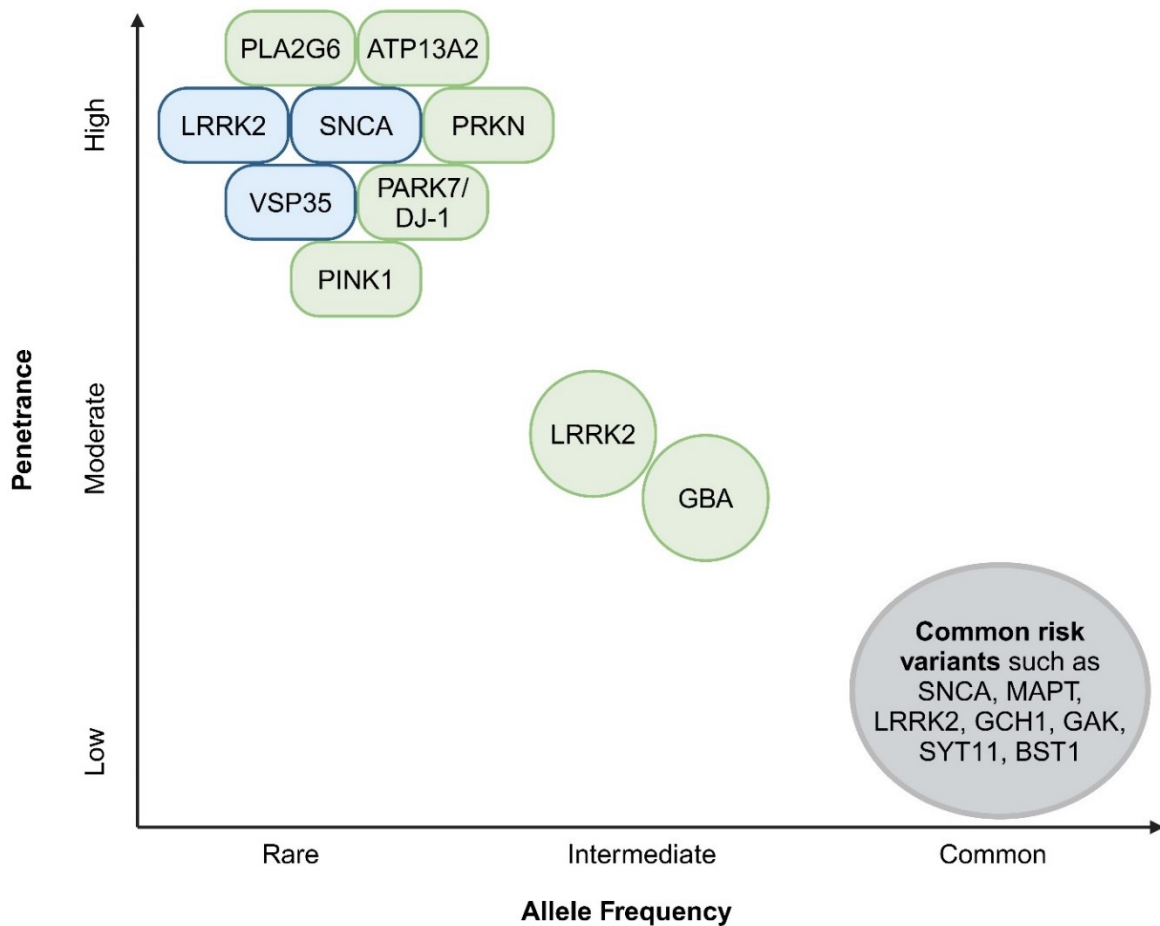


Figure 1.4: Genetic variants in PD.

grouped based on allelic frequency and penetrance. Autosomal dominant genes are labeled blue, and autosomal recessive genes are labeled in green. Risk loci are labeled gray. Adapted from (J. O. Day & Mullin, 2021; Gasser, 2015).

Parkin, PINK1, and PARK7

Autosomal recessive mutations have also been linked to the risk of developing PD. The significant at-risk genes that have currently been mapped include PRKN (Parkin) (Kitada et al., 1998), PINK1 (Valente et al., 2004), and PARK7 (DJ-1) (Bonifati et al., 2003). The most common of these is Parkin, which accounts for 8.6% of early-onset (<50 years) PD; PINK1 accounts for 3.7%, and DJ-1 accounts for 0.4% (Abou-Sleiman et al., 2003; Kilarski et al., 2012). Parkin specifically encodes for E3 ubiquitin ligases, which are enzymes that are responsible for the degradation of damaged

proteins (Shimura et al., 2000; K. Tanaka et al., 2001)). Therefore, mutations in Parkin (and PINK1) are thought to be associated with lysosomal degradation dysfunction (Deliz et al., 2024). In contrast, mutations in DJ-1 cause deficits in protecting neurons from oxidative stress (Kim et al., 2005). Like the major autosomal-dominant mutations, these autosomal recessive mutations (Parkin, PINK1, and DJ-1) all exhibit typical signs and symptoms of PD. However, despite sharing a similar phenotype, those with DJ-1 mutations tend to have more non-motor symptoms including depression, psychosis, and cognitive deficits when compared to those with Parkin and PINK1 mutations (Kasten et al., 2018; Kilarski et al., 2012).

Various other autosomal dominant and recessive mutations implicated in PD risk exist; however, they are outside the scope of this dissertation. Furthermore, it is important to note that the field of epigenetics has been, and continues to be, extensively studied in PD. Epigenetics involves the chemical modification (e.g., methylation) of DNA, resulting in alteration of gene expression. Despite the importance of epigenetics in PD, scientists have yet to conduct an epigenome-wide association study (EWAS) for PD (Lill, 2016).

Environmental Risk Factors

In addition to genetic risk factors of PD, several environmental toxicants have been identified as key risk factors for PD. In 2023, Paul and colleagues conducted a pesticide-wide association study (PWAS). From this study, 39 common pesticides were found to be associated with PD risk, the majority of which are known to induce dopaminergic cell death (Paul et al., 2023). Some of these chemicals/pesticides

associated with increased PD incidence include paraquat, rotenone, cyanide, dieldrin, and manganese (Di Monte et al., 2002; Gorell et al., 1998; Monte, 2003).

MPTP

The idea that contact with various pesticides could increase the risk of PD first came from the observation of 1-methyl,-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exposure. MPTP, which shares structural similarity to the herbicide paraquat (Ball et al., 2019; Kanthasamy et al., 2005; Langston et al., 1983), induced “textbook-like” signs of advanced PD in a small group of drug addicts in 1983 (Ball et al., 2019; Kanthasamy et al., 2005; Langston, 1998; Langston et al., 1983). The “textbook-like” symptoms of PD became understandable upon the discovery that MPTP exposure was determined to induce mitochondrial toxicity in dopaminergic neurons of the SNpc (Chaturvedi & Flint Beal, 2013). This discovery catalyzed additional investigations into other chemicals/pesticides to determine whether their exposures could also be associated with increased incidence of idiopathic PD.

Paraquat

Moving forward from MPTP, scientists began investigating paraquat, again because of its structural similarity to MPTP. Paraquat exposure in individuals has been reported to induce DA neuron cell death, α -synuclein aggregation, and neuroinflammation (Pouchieu et al., 2018; Purisai et al., 2007; Richardson et al., 2007). Likewise, in a rodent model, paraquat administration killed off DA neurons in the SNpc in a dose- and age-dependent manner (McCormack et al., 2002). Consequently, paraquat has been definitively linked to increasing the risk for PD (Kitazawa et al.,

2003). Indeed, in the Agricultural Health study of 110 PD patients, a positive association was discovered between risk of PD and exposure of paraquat (Tanner et al., 2011).

Rotenone

Another at-risk pesticide for PD development is rotenone, a major organophosphate pesticide frequently used in the control of fish populations (Betarbet et al., 2000; Tanner et al., 2011). Mechanistically, rotenone, similar to MPTP, is characterized as a selective inhibitor of the mitochondrial complex I; it is also well-known to promote and accelerate the aggregation of α -synuclein (Silva et al., 2013; Yuan et al., 2015). PD cases have been linked to chronic rotenone exposure in epidemiological studies (Dhillon et al., 2008; Tanner et al., 2011). Particularly, in a French AGRICAN study, an increased risk of PD was reported in farmers who were exposed to rotenone (Pouchieu et al., 2018).

Dieldrin

PD has also been known to be caused by an organochlorine pesticide referred to as dieldrin. In the 1970s, Dieldrin was widely used as an insecticide; however, in 1974, the United States Environmental Protection Agency (US EPA) banned its use due to its propensity for bioaccumulation and its potential carcinogenic effects (Kanthasamy et al., 2005). Several animal models have demonstrated the detrimental effect of dieldrin on the dopaminergic system (Kanthasamy et al., 2005). For example, rodent models have confirmed the targeting of dieldrin to the DA system in a dose-dependent manner (Hatcher et al., 2007; Richardson et al., 2006). Also, in ring doves, significant depletion of DA levels (58.6%) in the brain was reported in response to low-dose dieldrin exposure (Heinz et al., 1980). Most importantly, in postmortem human PD brain tissue,

dieldrin exposure was found to induce cell death in the SNpc (Corrigan et al., 1998; Miller et al., 1999).

Other Risk Factors and Comorbidities

Over the past decades, TBI has emerged as a possible risk factor for developing PD. TBI is known to cause breakdown of the blood-brain-barrier (BBB), as well as chronic inflammation, mitochondrial dysfunction, and α -synuclein accumulation (Marras et al., 2014). This has been confirmed in rodent models of TBI in which the animals developed α -synuclein aggregation and DA cell loss in the SNpc (Acosta et al., 2015). Behaviorally, rodents with TBI exhibited PD-like behavior at six months after injury (Sha et al., 2025). Moreover, individuals with a history of head trauma were at a higher risk of developing PD (Jafari et al., 2013). It must be considered, however, that there is an overall 50% increase in falls/head injuries approximately three months prior to a PD diagnosis; therefore, a correlation/causation of TBI and PD cannot yet be 100% corroborated. Indeed, one study found that there was no association between a TBI experienced 10 or more years prior to a PD diagnosis (Kenborg et al., 2015).

Metabolic syndromes (e.g., diabetes) have recently elicited increased interest as a possible risk factor and/or comorbidity of PD (Chohan et al., 2021; Cullinane et al., 2023; Leibson et al., 2006). It has been postulated that these metabolic syndromes have similar cellular mechanisms, such as mitochondrial dysfunction, to that of PD. A significant increase in the risk of developing PD has been documented in individuals with type 2 diabetes in reports from Finland (Hu et al., 2007), Denmark (Schernhammer et al., 2011), Taiwan (Sun et al., 2012), the Physician health study (Driver et al., 2008), and NIH-AARP (Q. Xu et al., 2011). Correlations between type 2 diabetes and an

increased severity of motor and non-motor symptoms at the time of PD onset has also been noted (Athauda et al., 2022). In contrast, however, this phenomenon was not seen in two large US cohort studies (Palacios et al., 2011; Simon et al., 2007). Despite these findings, pharmaceuticals that treat diabetes have been found to induce neuroprotective effects in PD models (Santiago et al., 2017). Further research in these areas (e.g., TBI, diabetes) is warranted in order to confirm a relationship with PD risk as well as its underlying mechanisms.

In addition to an extensive list of risk factors that increase the incidence of PD, there have been studies that have also revealed possible protective factors that may lower PD risk. Some of these include smoking, caffeine consumption, ibuprofen use, and physical activity (Ascherio & Schwarzschild, 2016; Noyce et al., 2012). While these analyses may seem promising, some of these factors could be contentious (e.g., smoking), and thus require further research to definitively determine whether these habits truly lower the risk of PD. Exercise, as a beneficial example, has been epidemiologically associated with a reduced risk of PD (H. Chen et al., 2005; Thacker et al., 2008), and therefore, may be an up-and-coming, widely-prescribed therapeutic approach to treat PD. Additional therapeutic strategies will be discussed in the next section.

THERAPEUTIC STRATEGIES FOR PD

Pharmacotherapy

The current pharmacological treatments for PD include DA replacement therapies (DRTs) and advanced surgical therapies such as deep brain stimulation (DBS) in the event that DRT becomes difficult to manage due to fluctuating responses. While DRTs are mostly successful in treating the motor symptoms of PD, they unfortunately do little to treat the non-motor symptoms. Moreover, no interventions currently exist that can prevent, delay, or reverse disease progression (i.e., disease-modifying therapies) (Fahn, 2003; Fox et al., 2018; Noyce et al., 2016; Poortvliet et al., 2020). Because gaps in our understanding of the underlying cause of PD remain, it is difficult to create treatments that will modify the pathology of PD (Lang & Espay, 2018). Nevertheless, in addition to optimizing currently available symptomatic treatments, researchers continuously endeavor to discover therapies directed at disease modification; several pathways are being investigated as potential treatment targets. This section will explore the first-line DRTs, advanced therapies, and experimental (potentially disease-modifying) treatments currently available for PD.

Levodopa (L-DOPA) was first isolated in 1910 by Torquato Torquati, but it was not until 1957 that its connection to DA and PD were discovered (A. Carlsson et al., 1957; Hornykiewicz, 2010). In 1957, Arvid Carlsson, a Swedish pharmacologist, remarkably demonstrated that levodopa diminished parkinsonian symptoms in reserpine-treated mice and rabbits. Reserpine, an alkaloid that blocks monoamine transport (A. Carlsson et al., 1957), induces a tranquilizing, parkinsonian-like state, and therefore was a useful model at the time for these studies (A. Carlsson et al., 1957). Within 15-30 minutes of

levodopa administration to the reserpine-treated animals, mice and rabbits returned to almost-normal behavior, ameliorating their parkinsonian state. However, the effect of levodopa only lasted for an hour, and animals returned back to their reserpine-induced parkinsonian-like state (A. Carlsson et al., 1957). Despite dose/timing caveats, these preliminary experiments demonstrated considerable potential for the use of levodopa in the treatment of PD.

A year later, Carlsson's research group determined that DA content in the brain increased upon levodopa administration, initiating their postulations of DA being implicated in motor disorders (e.g., PD). Then, in 1960, Ehringer and Hornykiewicz reported that patients with PD exhibited caudate and putaminal DA depletion (Ehringer & Hornykiewicz, 1960; Fahn, 2008). Following up on this observation, Hornykiewicz and Birkmayer intravenously administered levodopa to patients with PD, notably demonstrating distinct alleviation of their motor symptoms (Birkmayer & Hornykiewicz, 1961; Fahn, 2015). However, levodopa-infused patients developed distressing gastrointestinal-upset in response to the high doses of levodopa. To remedy this problem, Cotzias and colleagues decided to slowly increase the dose overtime, successfully avoiding gastrointestinal side effects (Cotzias et al., 1967).

Today, almost 65 years later, levodopa remains the most effective pharmacological intervention for PD (Cotzias et al., 1967; Poewe et al., 2010; Stoker & Barker, 2020). Compared to other DRTs (discussed below), levodopa demonstrates superior motor improvement when assessed by reductions in the United Parkinson's Disease Rating Scale (UPDRS) scores (Poewe et al., 2010). Importantly, levodopa is also better tolerated than DA agonists, especially in elderly patients (>60 years of age)

(Nutt & Wooten, 2005; Poewe et al., 2010). Mechanistically, in the presence of a peripheral decarboxylase inhibitor, levodopa crosses the BBB and is converted to DA by aromatic L-amino acid decarboxylase (AADC), an enzyme found in catecholaminergic neurons. Following its release into the synapse, DA will then be metabolized by catechol-O-methyltransferase (COMT) or monoamine oxidase (MAO) (**Figure 1.5a**) (National Institute of Diabetes and Digestive and Kidney Diseases, 2012a). Because of its short half-life (≤ 90 minutes) (Poewe et al., 2010) and its mechanism of action, levodopa is given in conjunction with carbidopa (an L-amino acid decarboxylase inhibitor) to prevent its metabolism in the periphery; COMT and MAO inhibitors can also be given alongside levodopa to prolong its half-life in the body (Fahn, 2003).

The success of levodopa can be limited by side effects that can manifest following chronic levodopa administration and continued progression of PD. Like other DRTs, some side effects of levodopa therapy can include nausea, hallucinations, confusion, postural hypotension, constipation, depression, and sleep disturbances (Bastide et al., 2015; National Institute of Diabetes and Digestive and Kidney Diseases, 2012a). The most bothersome and detrimental side effect, however, is the development of levodopa-induced dyskinesia (LID) (Poewe et al., 2010) (see Long-term side effects of Chronic Levodopa Therapy).

Often less tolerated and less effective than levodopa therapy (Stoker & Barker, 2020) are DA agonists, DA metabolism inhibitors (i.e., monoamine oxidase inhibitors; MAOIs), anticholinergics, adenosine antagonists, and β -blockers, all of which are available as additional medications used to treat the motor symptoms of PD (**Figure 1.5b**). Even though levodopa is considered the gold standard, sometimes other DRTs

will be prescribed first, frequently in younger patients (<60 years old), to avoid/delay the side effect of LIDs (Connolly & Lang, 2014). Apomorphine is an example of a DA agonist currently available on the market. Agonists specific to the D2 receptor include ropinirole, pramipexole, and rotigotine (National Institute of Diabetes and Digestive and Kidney Diseases, 2012b). COMT inhibitors have also been developed to block the breakdown of DA by catechol-O-methyltransferase (COMT). COMTs, as well as MAOIs, can be used in conjunction with DA agonists or levodopa to help manage PD symptoms (National Institute of Diabetes and Digestive and Kidney Diseases, 2012b).

Nevertheless, compared to levodopa, DA agonists and MAOIs/COMTs are more likely to induce side effects such as hallucinations, psychosis, compulsive behaviors, sleep disturbances, nausea, and confusion, especially in elderly patients (Armstrong & Okun, 2020; Bloem et al., 2021; Fahn, 2003), and therefore, levodopa is often preferred regardless of the risk of LID development.

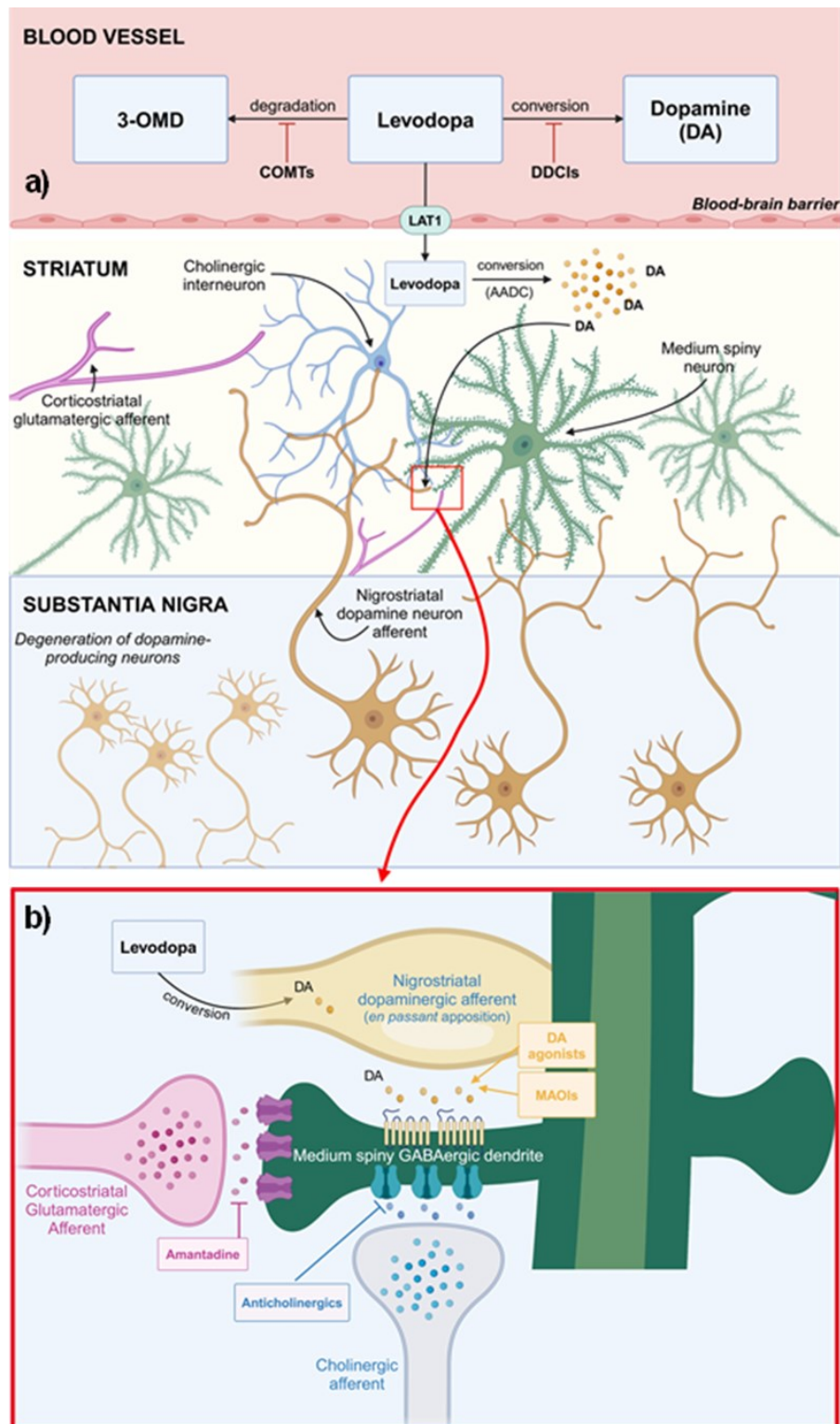


Figure 1.5: Sites of Action for Common Pharmacotherapies to treat PD.

Schematic diagram depicting the sites of action for various PD medications used to treat the motor symptoms of PD. **(a)** Levodopa is converted to dopamine (DA) by aromatic amino acid decarboxylase (AADC) both in the circulatory system and in the brain. Dopa

Figure 1.5 (cont'd)

decarboxylase inhibitors (DDCIs) are used to prevent levodopa's conversion in the periphery, allowing higher concentrations of levodopa across the blood brain barrier (BBB). In the striatum, nigrostriatal dopaminergic afferents, corticostriatal glutamatergic afferents, and cholinergic interneurons converge to regulate the activity of medium spiny GABAergic neurons (MSNs). Once levodopa is converted into DA here in the striatum inside DA terminals, replacing the neurotransmitter deficit in PD, DA will bind and activate DA receptors (D1 and D2) on the resident striatal MSNs, permitting motor movement. **(b)** Likewise, DA agonists and MAOIs also restore motor function as they activate DA receptors and prevent DA degradation, respectively. Other therapeutics such as amantadine (pink) inhibit the activity of N-methyl-D-aspartate (NMDA) receptors to treat dyskinesias. Anticholinergics are another pharmacologic; they are used to treat tremors by blocking nicotinic acetylcholine receptors (blue). Figure has been adapted from (Connolly & Lang, 2014). Abbreviations: 3-OMD = 3-O-methyldopa; DDCIs = dopa decarboxylase inhibitors; AADC = aromatic amino acid decarboxylase; COMTs = catechol-O-methyltransferase inhibitors; DA = dopamine; MAOIs = monoamine oxidase inhibitors; LAT1 = L-type amino acid transporter 1

Long-term Side Effects of Chronic Levodopa Therapy

LIDs are characterized as abnormal involuntary movements in response to chronic levodopa therapy (Poewe et al., 2010). These abnormal movements tend to affect the neck, upper limbs, and torso, triggering chorea, ballism, dystonia, and myoclonus (Kwon et al., 2022; Vijayakumar & Jankovic, 2016), all of which can cause substantial discomfort in individuals with PD (Hung et al., 2010; Khlebtovsky et al., 2012; Prashanth et al., 2011). Estimates of the incidence of LID vary by source but relatively reflect that approximately 50% of patients will develop LID during the first 3-5 years of levodopa treatment (Blanchet et al., 1996; Manson et al., 2012). By 10-15 years of treatment, 50-94% of patients exhibit LID (Ahlskog & Muentner, 2001; Fahn, 2003; Hely et al., 2005; Stoker & Barker, 2020). Moreover, in a large retrospective study referred to as the ELLDOPA study (Earlier vs. Later Levodopa therapy in PD), patients exhibited variable motor improvement ranging from 100% improvement to 242% worsening of symptoms (Hauser et al., 2009).

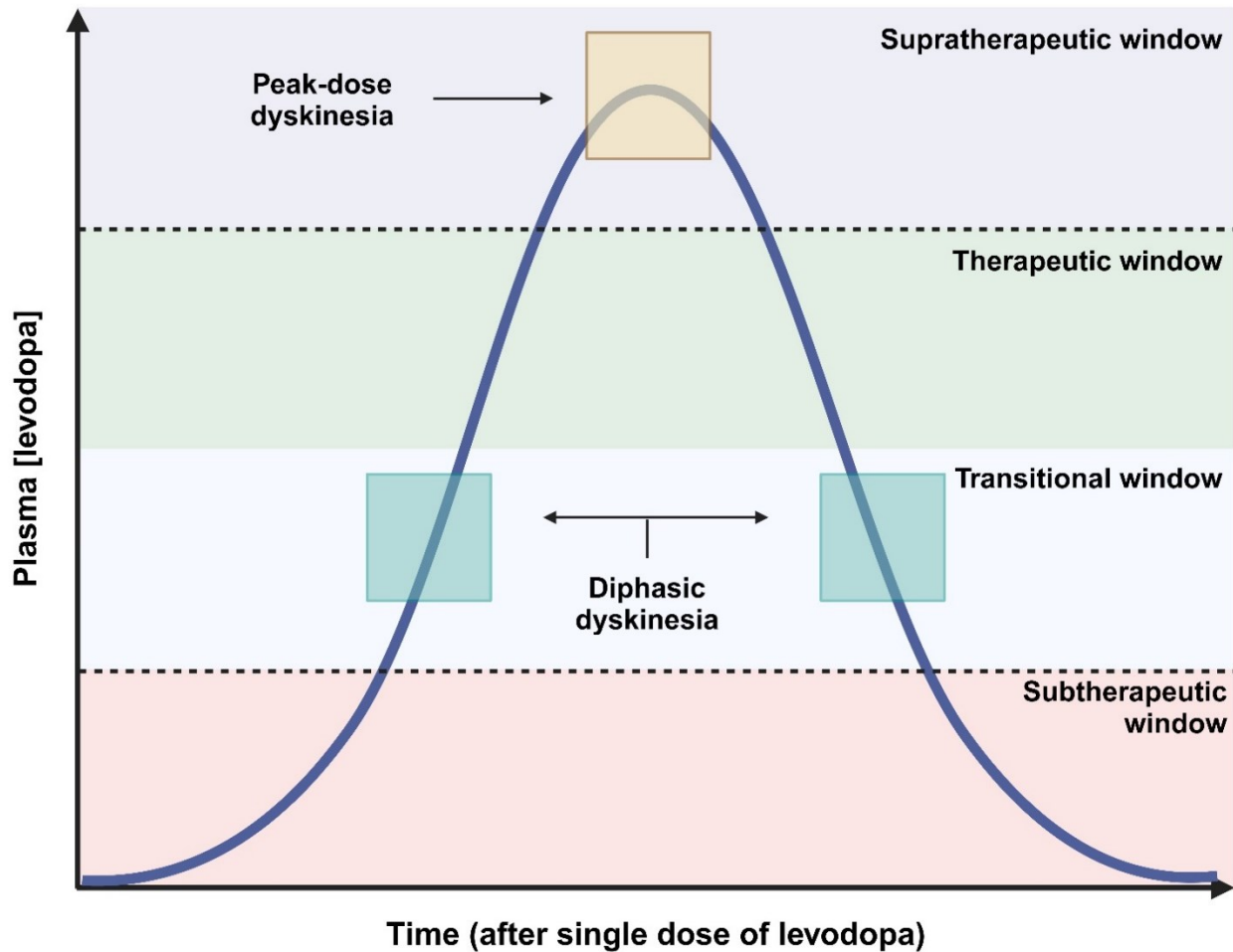


Figure 1.6: Types of Levodopa-induced dyskinesia (LID) and time course.

After a single dose of levodopa, the suprathreshold window is reached and is characterized by peak-dose dyskinesias. Following, the therapeutic window is considered the ON-state when optimal clinical benefit is reached with no dyskinetic behavior. Diphasic (or biphasic) dyskinesias will appear in the transitional window, during the rise and fall of levodopa levels in the plasma. In the OFF-state, subtherapeutic window, parkinsonian symptoms are prevalent again as the effects of levodopa are lost. Adapted from (di Biase et al., 2023). Abbreviations: [levodopa] = plasma concentration of levodopa.

The two most common forms of dyskinesia include peak-dose dyskinesia and diphasic dyskinesia (**Figure 1.6**). Peak-dose dyskinesia is the most prominent with 75-80% of patients experiencing this type of dyskinesia (Zesiewicz et al., 2007). Peak-dose dyskinesia occurs when plasma levels of levodopa are at their highest. Choreiform movements dominate in this form, but other movements including dystonia, myoclonus,

and ballism in the orofacial muscles also occur (Vijayakumar & Jankovic, 2016). In contrast to peak-dose dyskinesia, diphasic dyskinesia appears when plasma concentrations of levodopa are rising and falling (Zesiewicz et al., 2007). Specifically, diphasic dyskinesias manifest when levodopa is first given and when levodopa begins to wear off. Dystonic or ballistic movements most often characterize this form (Rascol et al., 2001).

While the underlying mechanisms of LID are unclear, current research suggests that LIDs manifest due to the non-physiological DA release and activation of striatal DA receptors induced by pharmacological administration of levodopa. To attempt to diminish LID behavior, pharmacologists are investigating various other formulations of levodopa to improve its delivery and its half-life (Poewe et al., 2010). Although LIDs can significantly impact quality of life, it is often said that individuals generally prefer their dyskinetic movements compared to limited movement with PD motor symptoms (Khlebtovsky et al., 2012), however, see (Cenci et al., 2020).

Several underlying mechanisms of LID have been postulated, one of which being the serotonin theory. Briefly, serotonin (5-HT) neurons have the ability to take up and convert exogenous levodopa into DA, but they lack the machinery to reuptake released DA and provide autoreceptor-mediated feedback to the neuron (**Figure 1.7**). Therefore, it is theorized that 5-HT neurons can cause excessive, non-physiological stimulation of the striatal DA receptors, thus resulting in LID (Bezard, 2013; Sellnow et al., 2019). Studies in favor of this theory have utilized 5-HT agonists to reduce LID in animal models (Bezard et al., 2013; Meadows et al., 2018; Stoker & Barker, 2020; Yamada et al., 2007) or genetically expression DAT into 5-HT terminals (Sellnow et al., 2019).

Specifically, eltoprazine, a 5-HT agonist, has been demonstrated to be successful in preventing LID in a parkinsonian rat model and in non-human primates (Fabbrini & Guerra, 2021). These promising results catalyzed a phase I/IIa study in which eltoprazine was administered to patients with PD; eltoprazine was successful in

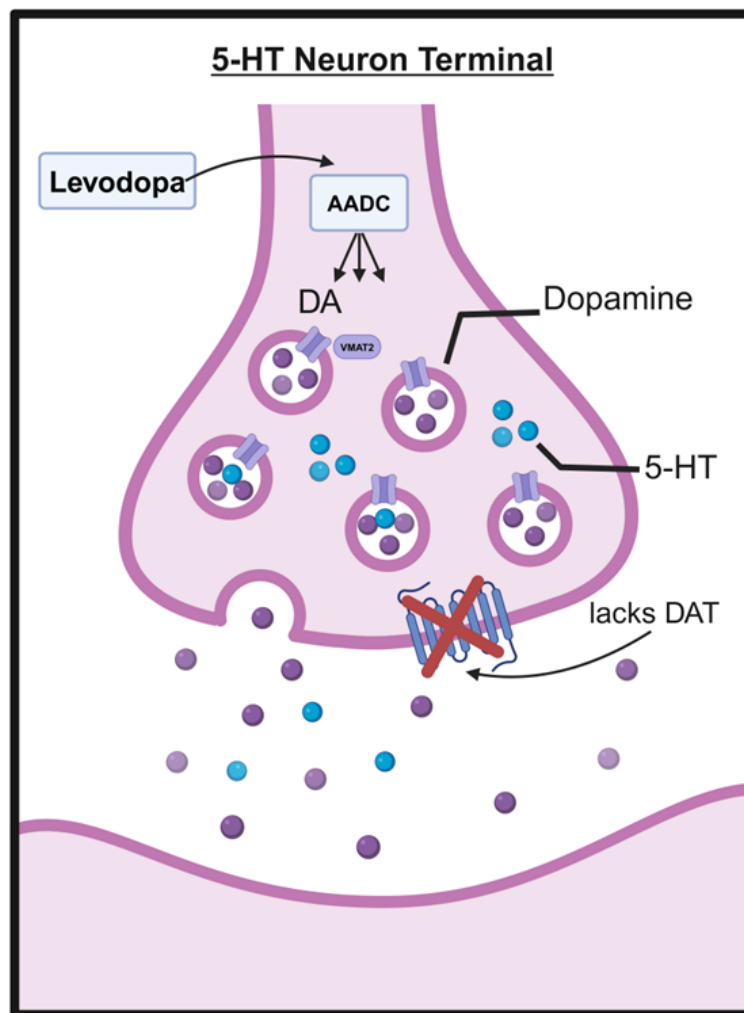


Figure 1.7: Unregulated Release of DA from a 5-HT Terminal.

A simplified schematic illustration of the serotonin theory behind LID behavior. Serotonin neurons contain the same enzymes as DA neurons to convert levodopa to DA. Once converted, the DA displaces serotonin from their storage vesicles, permitting release of DA into the synaptic cleft. However, serotonin neurons do not express DA transporters such as DAT for proper DA reuptake, leading to unregulated DA release and subsequent excessive stimulation of DA receptors. Adapted from (Kwon et al., 2022). Abbreviations: 5-HT = serotonin; AADC = aromatic amino acid decarboxylase; DA = dopamine; DAT = dopamine transporter; VMAT2 = vesicular monoamine transporter 2.

decreasing LID behavior, although a reduction of levodopa efficacy was also reported, contradicting its clinical utility (Bezard, 2013). Ultimately, manipulation of serotonergic neurons will only be used if it becomes more efficacious than other drugs such as amantadine, which, to-date, is considered the best drug to treat LID (Kwon et al., 2022).

Amantadine and clozapine (off-label) are currently the only two pharmaceuticals known to be efficacious in the treatment of LID (Fox et al., 2018), with the extended-release amantadine formulation being the only Food and Drug Administration (FDA)-approved to treat dyskinesias, and marginally most effective, drug (P. Jenner, 2008; Konitsiotis et al., 2000; Vijayakumar & Jankovic, 2016). Amantadine, an N-methyl-D-aspartic acid (NMDA) receptor antagonist, has been shown to stimulate DA release and block DA uptake in addition to blocking NMDA receptors. Its administration in a double-blind, placebo-controlled trial reduced total LID scores by 24% in individuals with low level LID without any change to levodopa efficacy (Snow et al., 2000). Unfortunately, amantadine is also contraindicated in approximately 25% of patients due to significant side effects (see (Hauser et al., 2017)). Because of the mechanism of action of amantadine, glutamatergic overactivity has been hypothesized as a mechanism responsible for dyskinesia development (Kwon et al., 2022). Similar to other pharmaceuticals, amantadine (and clozapine) are not universally effective for all patients (Alvir et al., 1993; Postma & Van Tilburg, 1975) and may result in side effect development such as ankle edema, hallucinations, and confusion in some patients (Fahn, 2003). Fortunately, clinical trials are planned or ongoing to investigate novel drugs and treatments to reduce LID behavior (Huot et al., 2022).

Advanced Therapies

When side effects of pharmacological DRT (e.g., severe LIDs) become unmanageable, or when patients have refractory symptoms like dominant tremor, more advanced options such as deep brain stimulation (DBS) can be considered. It is important to note that DBS, however, is not expected to alleviate levodopa-refractory symptoms (e.g., gait freezing) other than tremor. DBS is an invasive procedure that involves stereotaxic brain surgery to implant electrodes into the STN and GPi (Follett et al., 2010; Grabli et al., 2013; S. K. Kalia et al., 2013; Okun, 2014). The electrodes are then connected to a pulse generator placed in the chest (Espay et al., 2018; Fasano et al., 2012). The STN and GPi have been approved by the FDA as regional targets for DBS, but targeting the GPi has been demonstrated to be superior in reducing LIDs compared to the STN (Mansouri et al., 2018). The thalamus is also a brain region that has been approved, specifically for tremor-dominant symptoms; however, the thalamus is a rarely used DBS target in PD (Bloem 2021). In prospective studies, DBS significantly reduced LID behavior, and decreased the patients' need for medication by 50-60% (Kleiner-Fisman et al., 2004). While the mechanism responsible is not well understood, it is thought that high frequency stimulation of targeted brain regions (i.e., STN and GPi) improve motor function by “normalizing” patterns of neuronal firing (Aum & Tierney, 2018; Lozano et al., 2019; Merola et al., 2015).

To be an eligible candidate to receive DBS, an individual with PD must have a good response to levodopa but exhibit severe LIDs and/or medication-resistant tremor, or have become refractory to DRT (K. A. Smith et al., 2016). Moreover, several risks of DBS exist. Not only are there risks with the surgical procedure itself (e.g., infection,

hemorrhage), or the hardware (e.g., device failure (K. A. Smith et al., 2016; Worth, 2013), side effects such as cognitive dysfunction and adverse speech development can occur (Stoker & Barker, 2020). Also, gait and postural instability symptoms often respond poorly (Grabli et al., 2013). DBS is also extremely costly, and patients require frequent stimulation adjustments following the procedure (Fahn, 2003). Due to the significant side effects and limitations of DBS, clinicians and patients must carefully consider DBS as a therapeutic strategy for the parkinsonian symptoms.

Other advanced treatments are being investigated and implemented as possible therapeutic strategies for PD; however, they will not be discussed in-depth here. An example of one of these treatments includes the infusion of a levodopa-carbidopa intestinal gel which has been FDA approved for almost a decade (Dijk et al., 2020; Olanow et al., 2020; Worth, 2013). The goal of the levodopa-carbidopa gel is to achieve continuous infusion and reliable absorption of levodopa in order to keep levodopa at sufficient levels in the plasma. This procedure, like DBS, is also invasive as it requires the patient to undergo an endoscopy to place a gastrostomy tube in the jejunum of the large intestine (Espay et al., 2018). As with all other treatment, the benefits and drawbacks of advanced therapies must be extensively reviewed by both doctor and patient so that the best approach is chosen (Dijk et al., 2020).

Experimental Disease-Modifying Therapies

The ultimate goal of treatment development for PD is to generate disease-modifying therapies that can prevent, delay, or reverse disease progression (Fahn, 2003; Fox et al., 2018; Noyce et al., 2016; Poortvliet et al., 2020). As was mentioned previously, no interventions of this nature currently exist; however, scientists are

continuing to make progress in this area. For instance, gene therapy is a promising experimental, potentially disease-modifying, therapy that is being investigated.

Gene therapy

Currently, gene therapy has been designed to introduce genes of DA synthesis enzymes so that DA can be replenished in the STR of patients with PD (Schuepbach et al., 2013). Specifically, the research group of Muramatsu and colleagues (Muramatsu et al., 2010) and Christine and colleagues (Christine et al., 2009) utilized gene therapy to introduce an adeno-associated virus (AAV) that expressed aromatic amino decarboxylase (AADC) into the putamen of PD patients, and patients' UPDRS scores were greatly improved (Christine et al., 2009; Muramatsu et al., 2010). Another example is found with lentivirus vector therapy: genes expressing both TH and AADC were administered in an open-label phase I clinical trial to patients with PD (OXB-101, (Palfi et al., 2014; Stoker & Barker, 2020). Twelve months following treatment, patients reported improved UPDRS scores, but not enough to be competitive with other treatments.

A novel gene therapy that has substantial promise to be disease-modifying is presented in the studies conducted by Steece-Collier and colleagues (**Figure 1.8ab**) (Caulfield et al., 2025; Caulfield, Vander Werp, et al., 2023; Steece-Collier et al., 2019). Adeno-associated (AAV)-mediated short-hairpin RNA was administered to parkinsonian rats to silence striatal voltage-gated, L-type Cav1.3 calcium channels. Delivery of Cav1.3 AAV completely prevented LID development, but also strikingly reversed severe LID in parkinsonian rats (**Figure 1.8c**) (Steece-Collier et al., 2019). Because dysregulation of Cav1.3 channels can induce dendritic spine retraction of MSNs (M. Day

et al., 2006; Steece-Collier et al., 2019), it was theorized that blocking these channels could prevent spine retraction, potentially preventing/modifying DA pathophysiology in PD. Although promising, there are limitations to gene therapy. Gene therapy is generally irreversible, and it can also be difficult to determine/regulate the quantity of gene that is delivered (Elkouzi et al., 2019).

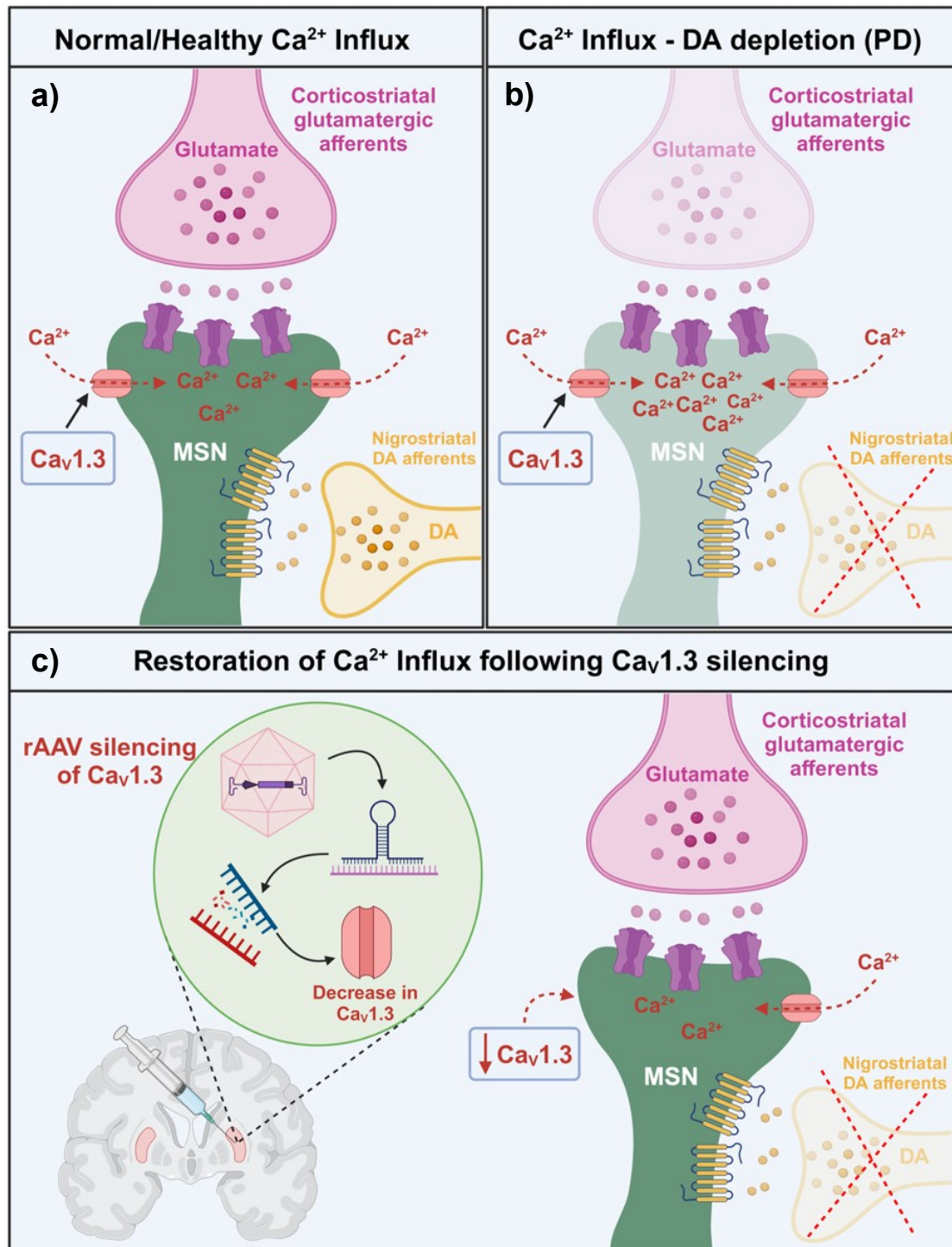


Figure 1.8: Silencing of $\text{Ca}_v1.3$ Channels as a Promising Disease-Modifying Gene Therapy for PD.

Schematic illustration depicting (a) striatal intraspinous $\text{Ca}_v1.3$ channels regulating the influx of Ca^{2+} into the dendritic spines of MSNs. $\text{Ca}_v1.3$ channels are normally regulated by D1 and D2 receptors (shown in yellow) under homeostasis. (b) However, when degeneration of DA neurons from the SNpc occur in PD, the regulation/inhibition of

Figure 1.8 (cont'd)

Cav1.3 channels through DA receptors is diminished, permitting an increased influx of Ca^{2+} . This causes spine retraction and loss of corticostriatal glutamatergic inputs. **(c)** Recent findings have demonstrated that inhibition and/or silencing of Cav1.3 channels using an rAAV- Cav1.3-shRNA allow for the maintenance of normal spine density on MSNs despite severe loss of DAergic neurons, thereby preventing the induction of LID (see Steece-Collier et al., 2019). Figure adapted from (Caulfield, Manfredsson, et al., 2023). Abbreviations: MSN = medium spiny neurons; Ca^{2+} = calcium ions; Cav1.3 = voltage-gated L-type calcium channels; DA = dopamine; AAV = adeno-associated virus; shRNA = short hairpin ribonucleic acid. For more details on these findings, see (Caulfield et al., 2025; Caulfield, Vander Werp, et al., 2023).

Targeting α -synuclein pathology

Along with targeting DA degeneration, other potentially disease-modifying experimental therapies also target α -synuclein pathology. Gene therapy has also been utilized for this: gene-silencing mechanisms that target messenger RNA of α -synuclein has been attempted to reduce the synthesis of α -synuclein (Fields et al., 2019; Savitt & Jankovic, 2019). Immune therapy is also of interest. Specifically, in Phase I clinical trials, a humanized monoclonal antibody that targets aggregated α -synuclein (prasinezumab) resulted in a 97% reduction in free serum α -synuclein (Jankovic et al., 2018; Schenk et al., 2017). Because of the success of the Phase I clinical trial, a Phase II clinical trial is now ongoing (NCT03100149).

Interestingly, several drugs on the market are being repurposed because of their ability to reduce α -synuclein pathology. The glucagon-like peptide 1 (GLP-1) analogue, exenatide, is a fitting example. GLP-1 has historically been used to treat type-2 diabetes; however, individuals with PD have exhibited improved cognitive and motor function following GLP-1 treatment (Stoker & Barker, 2020). Cell and animal models of nigral degeneration have also demonstrated a neuroprotective effect in response to GLP-1 administration (Bertilsson et al., 2008; Harkavyi et al., 2008; Y. Li et al., 2009). Another drug, terazosin, which is an α 1-adrenergic antagonist usually used to treat

benign prostatic hypertrophy, has shown a reduction in α -synuclein in transgenic mice and in neurons from patients with LRRK2 mutations (Cai et al., 2019). Despite the promise of these studies, the entirety of physiological functions of α -synuclein remains to be determined, and thus, it is therefore important to keep in mind that there may be negative consequences of decreasing endogenous α -synuclein too much (Collier et al., 2016; Elkouzi et al., 2019; Gorbatyuk et al., 2010; Stoker & Barker, 2020).

As discussed previously, it is well established that, by the time classic motor symptoms manifest in PD patients, a significant loss of SNpc DA neurons has already occurred (Fearnley & Lees, 1991; Noyce et al., 2016). Therefore, although DRTs (i.e., levodopa) and other therapies are successful at treating motor symptoms, there has yet to be developed a therapy that can prevent or reverse the pathology of PD. Pathways that have been experimentally implicated in PD (e.g., lysosomal and mitochondrial dysfunction, neuroinflammation) are currently being investigated as possible drug targets, with the goal of treating PD prior to motor symptom development (Jankovic, 2008; K. S. P. M. P. Jenner & Olanow, 2007; Pan et al., 2008). A promising experimental therapy aimed at re-establishing the nigrostriatal DA system that remains of worldwide interest is cell transplantation therapy. Understanding factors that impact the benefits and limitations of cell transplantation is the major focus of my dissertation research.

Regenerative Cell Transplantation Therapy

Brief History of Cell Transplantation

The concept of neural transplantation into the adult mammalian brain has been of interest for almost four centuries, but it was not until 1890 that the first experimental attempt at transplantation was successfully conducted. W. Gilman Thompson, an American physician, was the first to attempt transplantation. Briefly, in one of his studies, cortical tissue from the occipital lobe of dogs was excised and subsequently transplanted into the occipital lobe of recipient dogs or cats. Remarkably, when examined histologically after seven weeks, there seemed to be survival of the donor tissue, with a mix of healthy and degenerating cells (Dunnett, 2009; Thompson, 1890a, 1890b). While this seemed to be a promising finding, the methods of the time were limited. Therefore, it was more likely that the transplanted tissue had died and left scar tissue or host-derived immune cells in its place (Bjorklund & Stenevi, 1985; Dunnett, 2009). Despite how profound these findings were at the time, Thompson's studies unfortunately elicited little immediate follow-up experimentation.

Almost twenty years later, in 1907, another attempt was made to demonstrate that grafting into the adult mammalian brain was indeed possible. This was performed by Del Conte who grafted non-neuronal embryonic tissue into the cerebral cortex of adult dogs. Similar to Thompson's work, Del Conte demonstrated partial tissue survival; however, he believed survival to be only temporary (Bjorklund & Stenevi, 1985; Del Conte, 1907). Following these experiments, several grafting studies involving peripheral nerve transplantation or other non-central nervous system (CNS) tissue transplant studies were conducted spanning across the next decade.

Finally, in 1917, Elizabeth Dunn successfully demonstrated that cortical tissue transplanted between neonatal rat pups could survive, albeit with a poor survival rate at less than 10% (Bjorklund & Stenevi, 1985; Dunn, 1917). Despite a minute survival rate, Dunn was credited with the first successful evidence that CNS tissue could survive, at least to some extent, in the brain (Dunnett, 2009). With these findings, Wilfrid Le Gros Clark went on to provide evidence of survival of embryonic cortical tissue into the neonatal brain of immature six-week-old rabbits. He discovered that these cells could not only survive but also differentiate into mature neurons in the host neocortex (W. E. L. G. Clark, 1940; Dunnett, 2009).

Throughout the next few decades, the scientific community still remained skeptical of the ability of transplanted neurons to fully integrate and differentiate into the adult mammalian brain. Then, in the early 70s, Gopal Das and Joseph Altman launched what is now considered the “modern era” of neural transplantation (Bjorklund 1999, Dunnet 2009). In their research, they injected [³H] thymidine into the cerebellar cortex of neonatal rat pups to label still-proliferating cells. Their results demonstrated that the labeled cells successfully survived, migrated, and differentiated into proper neuronal phenotypes when engrafted into the host cerebella (Das & Altman, 1971; Dunnett, 2009). Following Das’ and Altman’s work, additional research teams endeavored to further study the intricacies of cell transplantation. Using new anatomical techniques for the time, groups including Olson and Seiger (Dunnett, 2009; Olson & Seiger, 1972) and a Swedish group at the University of Lund led by Anders Bjorklund (Björklund & Stenevi, 1971; Stenevi et al., 1976) collected evidence that allowed scientists to determine optimal development ages for survival, differentiation, and growth of grafted tissue in the

host brain. A comprehensive exploration of the full history of cell transplantation can be found in (Bjorklund & Stenevi, 1985; Dunnett, 2009).

Preclinical and Early Clinical Trials of Cell Transplantation in PD

In 1979, two independent groups published promising evidence of the functional benefit of SN grafts specifically in a parkinsonian rat model (Bjorklund & Stenevi, 1979; Perlow et al., 1979). Following unilateral lesions of the nigrostriatal DA pathway (6-hydroxydopamine (6-OHDA) injections), Bjorklund and colleagues transplanted embryonic ventral mesencephalic (eVM) tissue containing the developing SN DA neurons into a cerebral cortical cavity overlying the STR (Bjorklund & Stenevi, 1979). Simultaneously, Perlow and colleagues dispersed eVM tissue into the lateral ventricles (Perlow et al., 1979). In both cases, these grafts, which contained the developing DA neurons, reduced rotational asymmetry compared to non-grafted lesioned rats, suggesting a restoration of motor deficit. While nigrostriatal synaptic connectivity was seemingly restored in the study conducted by Bjorklund and colleagues, results from Perlow and colleagues indicated that diffusion of DA from the ventricle was the reason for behavioral improvement, not successful graft-host connectivity (Björklund & Lindvall, 2017b).

Soon after these two experiments, a transplantation technique of stereotaxically inserting eVM neurons directly into the STR (Bjorklund et al., 1980; Björklund et al., 1983; R. H. Schmidt et al., 1981) was developed and proven to achieve widespread reinnervation. Numerous preclinical studies have since been conducted in order to optimize functional outcomes, addressing issues related to experimental protocols of cell transplantation including cell preparation and source, graft delivery method,

immunological responses, and cell storage (Dunnett, 2009; Freeman & Widner, 1998). Although other various cell sources and transplant locations have been studied, the most promising approach to-date has been transplanting eVM DA neurons directly into the STR (Steece-Collier & Collier, 2016). Due to promising evidence of preclinical trials, interest in clinical application of cell transplantation in PD increased rapidly.

The first clinical trials of neural transplantation in individuals with PD occurred in 1982 and 1983, respectively. Two patients received implantations of their own adrenal medulla cells, which secrete catecholamines including DA, into the caudate nucleus. However, only transient improvement of motor function occurred (Backlund et al., 1985; Björklund & Lindvall, 2017a; Lindvall et al., 1987). In another clinical trial conducted by a group in Mexico City, two young patients with PD (35 and 39-years-old) also received adrenal medullary autografts to the caudate nucleus. Results from this study demonstrated a significant reduction in rigidity, tremor, and akinesia in these patients 10 months following transplantation (Madrado et al., 1987). Unfortunately, a larger clinical trial involving 61 patients with PD from the US and Canada who were recipients of adrenal medullary grafts, could not replicate the results from the Mexico City trial: few patients (19%) showed improvement 2 years after surgery, and morbidity/mortality was relatively high in a sizable portion of these patients (Goetz et al., 1991).

Trial ID	Location	Cell Source	Enrollment	Phase	Status
NCT06687837	Boston, MA, USA	Autologous iPSCs	8	Phase I	Recruiting
NCT06482268	La Jolla, CA, USA	Human iPSCs	7	Phase I	Recruiting
NCT06422208	Boston, MA, USA	Autologous iPSC-derived DA neurons	6	Phase I	Enrolling my invitation
NCT06141317	San Jose, Costa Rica	PASCs	40	Phase I/II	Active, not recruiting
NCT05901818	Beijing, China	Autologous induced neural stem cell-derived DA precursor cells	10	Phase I	Recruiting
NCT05699161	Leon, Nicaragua	Adipose-derived stromal vascular fraction cells	10	Phase I/II	Completed
NCT05691114	Shanghai, China	hAESC	18	Phase I	Recruiting
NCT05635409	Lund, Sweden (STEM-PD)	hESCs	8	Phase I	Active, not recruiting
NCT05435755	Shanghai, China	hAESC	12	Early Phase I	Unknown status
NCT05094011	Unknown	Adipose-derived mesenchymal stem cells	9	Phase I	Not yet recruiting
NCT04414813	Shanghai, China	hAESC	3	Early Phase I	Completed
NCT04146519	Minsk, Belarus	Autologous mesenchymal stem cells	50	Phase II/III	Unknown status

Table 1.1: Current Planned or Ongoing Clinical Trials for Cell Transplantation in PD.
Abbreviations: iPSCs = induced pluripotent stem cells; PASCs = pluripotent stem cells

Table 1.1 (cont'd)

isolated from adipose tissue; DA = dopamine; hA ESCs = human amniotic epithelial stem cells; hESC = human embryonic stem cells; MSC = mesenchymal stem cells; ISC-hpNSC = International Stem Cell Corporation human parthenogenetic neural stem cells

Trial ID	Location	Cell Source	Enrollment	Phase	Status
NCT03119636	Zhengzhou, Henan, China	hESCs	50	Phase I/II	Unknown status
NCT02780895	Mexico City, Mexico	hFSCs	8	Phase I	Unknown status
NCT02611167	Houston, TX, USA	Allogenic bone marrow-derived MSCs	20	Phase I	Completed
NCT01860794	Seongnam-si, Gyeonggi-do, Korea	Fetal mesencephalic neuronal precursor cells	15	Phase I/II	Unknown status
NCT01446614	Guangzhou, Guangdong, China	Autologous bone marrow-derived mesenchymal stem cells	20	Phase I/II	Unknown status
NCT00226460	Tampa, FL, USA (Neurocell-PD)	Fetal porcine cells	Unknown	Phase II	Completed
NCT02452723	Melbourne, Victoria, Australia	ISC-hpNSC	12	Phase I	Unknown status
NCT01898390	Unknown (TRANSEURO)	Allografts of fetal ventral mesencephalic tissue	13	N/a, open label	Completed
JMA-IIA00384	Kyoto, Japan	Allogenic human iPSCs	7	Phase I/II	Completed

Following these initial clinical trials, scientists began to shift their focus to utilizing human embryonic neuronal tissue instead of transplanting adrenal medullary cells. In the late 1980s, a group at the University of Lund conducted preclinical transplantation trials of human eVM tissue engrafted into immune-suppressed parkinsonian rats (Björklund & Lindvall, 2017a; Brundin et al., 1986, 1988; Clarke et al., 1988). These DA neurons were shown to successfully survive and reinnervate the STR, providing notable functional benefit to parkinsonian animals. Due to these incredible results in a preclinical rat model, two patients at Lund finally underwent transplantation of eVM neurons into the caudate and putamen in 1989. Remarkably, patients in this open-label trial exhibited successful survival of their grafted neurons, and the grafts rescued both spontaneous and drug-induced DA release (Björklund & Lindvall, 2017b; Lindvall et al., 1990). After a one-year follow-up, patients still exhibited clinical benefit with improved OFF-time motor function (Lindvall et al., 1992). Additional clinical trials confirmed these results, demonstrating that, collectively, individuals with PD who received primary DA neuron transplants can exhibit remarkable functional improvement (Cohen et al., 2003; Freeman et al., 1995; Kordower et al., 1998; Lindvall & Hagell, 2000; Mendez et al., 2002; Peschanski et al., 1994; Piccini et al., 1999).

These notable findings encouraged the National Institute of Health (NIH) to fund two double-blind, placebo-controlled trials of cell transplantation in the mid-1990s in the US (Freed et al., 2001; Olanow et al., 2003). Results from these trials, when categorized by patient age and disease severity, demonstrated that primary DA neuron grafts can survive, function, and restore DA release in the putamen of PD patients (Freed et al., 2001; Olanow et al., 2003). Despite promising results, however, there was

an unfortunate occurrence of novel OFF-medication behaviors known as graft-induced dyskinesia (GID) in 56.5% of the study participants in (Olanow et al., 2003). These GID behaviors developed 6-12 months following the transplantation procedure after cessation of immunosuppression. The manifestation of GID in these and other trials regrettably summoned a worldwide moratorium on all clinical grafting trials for PD (Hagell et al., 2002a). Until the underlying mechanisms, and subsequent prevention, of GID can be elucidated and achieved, grafting cannot be considered a fully optimized option for PD treatment (see **Table 1.1** for currently ongoing/planned clinical trials).

The Unanticipated Side Effect of Cell Transplantation: Graft-Induced Dyskinesia (GID)

Graft-induced dyskinesias (GID) are defined as abnormal involuntary OFF-medication behaviors that develop only in individuals who received primary neural transplants (for review (Maries et al., 2006; Steece-Collier et al., 2012)). These GID profiles develop as the graft matures and as the typical pre-graft LID behaviors disappear (Steece-Collier et al., 2012) both in humans and in animal models (Lane et al., 2006; Maries et al., 2006; Soderstrom et al., 2008). Clinically, GIDs tend to manifest as more focal stereotypic movements in contrast to that of LID (Freed et al., 2001), often localized to either the upper or lower extremities correlating with graft placement (Hagell et al., 2002a; Maries et al., 2006; Olanow et al., 2003). Moreover, GIDs bear resemblance to diphasic drug-induced dyskinesia (Hagell & Cenci, 2005); however, unlike LIDs, GIDs cannot be alleviated by lowering the dose of levodopa. In the clinical trials discussed above, GID severity varied from mild for some patients, to severe, in which some had to undergo STN DBS to reverse the aberrant effects of their grafts (Freed et al., 2001). While several underlying mechanisms of GID have been

postulated, this remains a topic of controversy. These mechanisms include, but are not limited to, pre-graft levodopa history, age of recipient, donor cell source, presence of non-DA neurons (e.g., serotonergic neurons), uneven DA reinnervation/excess DA release, host-immune response, and/or asymmetric synaptic connections between the host and donor (for review (Steece-Collier et al., 2012)). Although not to an exhaustive level, some notable proposed mechanisms of GID are discussed below.

Modeling Graft-Induced Dyskinesia

In preclinical laboratories, rodent models are utilized to experimentally study GID behavior. Commonly, parkinsonism will first be induced in rodent models via unilateral, intranigral 6-OHDA injections in order to lesion the nigrostriatal pathway. Embryonic VM graft tissue will then be transplanted into the parkinsonian striatum. Experimental GID in these animals are then induced with either levodopa or amphetamine administration (**Figure 1.9**).

Following administration of levodopa, grafted rodents will develop focal, stereotypic, and repetitive movements similar to what is seen in human subjects (Hagell & Cenci, 2005; Maries et al., 2006; Soderstrom et al., 2008). Affected bodily regions are also comparable to GID expression, specifically in individuals from the Denver/Columbia clinical trial (Freed et al., 2001). In response to amphetamine administration, GID resemble a more robust, widespread dyskinetic profile similar to LID; however, they are only observed in the presence of a DA graft and as the graft matures. In our laboratory, we have more recently relied on amphetamine administration to induce GID based on the finding that DA-grafted, and not sham-grafted, rats demonstrate robust dyskinetic behavior in response to low-dose amphetamine (Lane, Brundin, et al., 2009b; Lane,

Vercammen, et al., 2009; Shin et al., 2012b; G. A. Smith, Breger, et al., 2012; G. A. Smith, Heuer, et al., 2012).

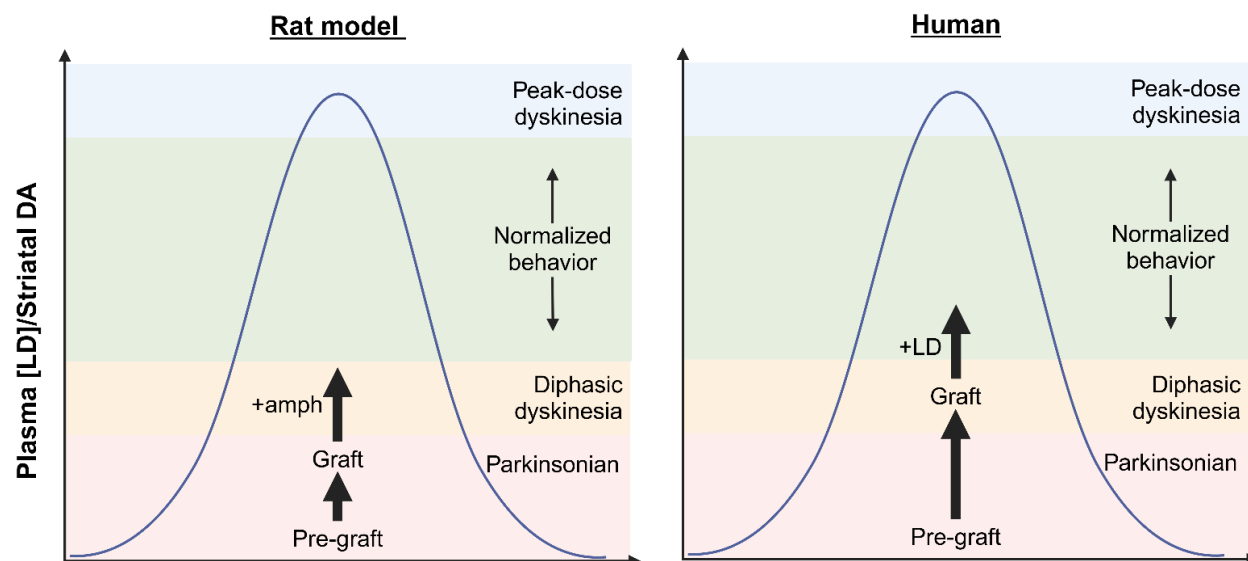


Figure 1.9: Modeling Experimental GID in Rodents.

Schematic diagram illustrating that plasma DA levels must be elevated with amphetamine (or levodopa) administration in rats to “push” the animal into a diphasic-like dyskinesia range, phenotypically like the GID behavior seen in engrafted patients with PD. Upon low-dose amphetamine administration, grafted parkinsonian rats will develop focal, stereotypic movements that characterize GID comparable to grafted human patients. Adapted from (Steece-Collier et al., 2012). Abbreviations: LD = levodopa; amph = amphetamine.

While spontaneous, non-medicated GIDs can occur in rodent models, they occur sporadically, and in their active phase (i.e., dark), making behavioral evaluation almost impossible. The phenomenon of requiring pharmacological agents to raise plasma DA levels in animal models arguably remains the only major discrepancy between experimental preclinical studies and clinical human trials of GID. Regardless, the appearance of GID, both in humans and in rodents, only manifests after grafting as the cells mature and is not seen preoperatively (T. Carlsson et al., 2006; Lane et al., 2006;

Lane, Vercammen, et al., 2009; Maries et al., 2006; Soderstrom et al., 2008). Further limitations of neural grafting will be discussed later on.

Postulated Mechanisms Underlying GID Behavior

There remains contention in the field of neural transplantation as the underlying mechanisms responsible for GID behavior have not yet been elucidated. While some are confident that the presence of non-dopaminergic neurons in the grafts are the culprit (see below), preclinical and clinical evidence suggests against this notion. Some have shown that the size of the graft itself impacts GID: grafted parkinsonian rats with large grafts demonstrated increased GID severity compared to smaller grafts (Lane et al., 2006). In contrast, another study demonstrated that focal, not widespread, grafts induce GID behavior in parkinsonian rats (Maries et al., 2006). Other groups have also revealed that the degree of disease severity or the severity of preoperative LID behavior correlates the development of GID (García et al., 2011; Lane, Brundin, et al., 2009b; Rylander Ottosson & Lane, 2016; Tronci et al., 2015). Additional components that have been considered include immune response (Soderstrom et al., 2008), age of the graft recipient, and preoperative cell storage. Consequently, clinical researchers have endeavored to modify and optimize factors such as patient selection and cell composition prior to transplantation; however, the mystery of GID remains. In the following section, five prominent postulated GID mechanisms relating to my studies are discussed: the presence of non-DA neurons, the immune response, abnormal graft-host synaptic circuitry, uneven dopamine innervation/excessive DA release, and DA/glutamate co-transmission.

Presence of Non-DA Neurons/Cellular Components

The cellular composition of eVM grafts, specifically the presence of 5-HT neurons, has been suggested as a possible underlying factor responsible for GID behavior. While the 5-HT system in the DA-denervated brain (i.e., PD) has been linked to LID following administration of levodopa (Carta et al., 2007; Lindgren et al., 2010; Rylander et al., 2010; Sellnow et al., 2019; H. Tanaka et al., 1999), there remains a lack of consensus on the role of this system in the development of GID. The hypothesis of 5-HT and GID is largely based on the biological ability of 5-HT neurons to convert, store, and release DA due to having similar cellular machinery. For instance, it is well-known that 5-HT neurons can take up exogenous levodopa, convert it into DA, and store DA in its vesicles via the vesicular monoamine transporter 2 (VMAT2) found in both DA and 5-HT neurons (Tronci et al., 2015). However, because 5-HT neurons do not possess dopamine transporters (DAT) for DA reuptake, and do not have regulatory DA autoreceptors on their terminals, DA continues to be released and left in the synaptic cleft, theoretically leading to GID behavior (Politis, 2010).

Many preclinical rodent model studies have collected evidence in favor of this hypothesis, demonstrating the presence of 5-HT+ neurons within intrastriatal eVM transplants (Winkler et al., 2005). Using bimodal chemogenetic (DREADD) activation of 5-HT receptors, Aldrin-Kirk and colleagues observed substantial GID induction in 6-OHDA-lesioned rats (Aldrin-Kirk et al., 2016). Likewise, in grafted patients with PD, two individuals who exhibited significant GID behavior developed excessive 5-HT innervation from their grafts. Following administration of buspirone, which is a partial 5-HT_{1a} agonist, GID behavior in these patients was attenuated (Politis et al., 2010; Shin

et al., 2012a). In another study also conducted by Politis, positron emission topography (PET) and single photon emission computed tomography (SPECT) imaging revealed an elevated 5-HT/DA ratio within eVM neurons that were transplanted into an individual with PD (Politis et al., 2011). It is important to note that, in another study where grafted patients had abundant 5-HT neurons in their grafts, they did not develop GID (Mendez et al., 2008).

To contrast the evidence in favor of the role of the 5-HT system in GID, other research has shown that GID can develop in the absence of 5-HT neurons. For instance, histological results collected by Lane and colleagues of 6-OHDA-lesioned rats following intrastriatal VM transplantation revealed very low numbers of 5-HT+ neurons despite GID expression (Lane, Brundin, et al., 2009a). Further, the 5-HT/DA cell ratio within grafted VM grafts was not shown to be significantly correlated with GID behavior in parkinsonian rat models (García et al., 2012; Mercado et al., 2021). Lastly, an experiment that transplanted DA-only, DA + 5-HT, or 5-HT-only grafted neurons into 6-OHDA-lesioned rats demonstrated that only the recipients of either DA-only or DA + 5-HT neurons exhibited amphetamine-mediated GIDs. 5-HT alone did not induce aberrant behavior (Shin et al., 2012b), suggesting that the DA system within grafted neurons may provide more of a contribution to GIDs (Aldrin-Kirk et al., 2016; García et al., 2012; Lane, Brundin, et al., 2009b; Rylander Ottosson & Lane, 2016).

A reasonable, biological explanation for the divergency of the above studies is that 5-HT neurons may, instead, play more of a modulatory, instead of a direct, role in GID development. When given concomitantly with a DAT blocker, fluvoxamine (i.e., serotonin transporter (SERT) blocker) administration significantly increased GID

expression in 6-OHDA-lesioned parkinsonian rats (Lane et al., 2006). Another experiment similarly co-administered 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; a 5-HT agonist) with raclopride (D2 receptor antagonist) to parkinsonian rodents, and this co-administration suppressed amphetamine-mediated axial and limb GIDs (Lane, Brundin, et al., 2009b). Moreover, eticlopride administration alone, also a D2 antagonist, suppressed GIDs in parkinsonian rats (Shin et al., 2012a). Lastly, while buspirone is a 5-HT receptor partial agonist, it also displays DA D2 antagonism (for review (Steece-Collier et al., 2012)). Collectively, the evidence seemingly points to more of a key role of the dopaminergic system in GID behavior (discussed further in the “Uneven DA reinnervation/DA release” section). While the 5-HT-GID hypothesis remains controversial as patients still express GID behavior with low 5-HT expression, clinical trials have since attempted to minimize the inclusion of 5-HT neurons prior to transplantation to lower the potential risk of GID exhibition in patients with PD (Lane & Lelos, 2022).

Immune Response

The host immune response has been a major area of debate in the field of neural transplantation (Tronci et al., 2015). Historically, the CNS was considered to be an immuno-privileged site; however, moderate immune activation does occur in the brain following ectopic cell engraftment, mostly due to the necessity of having to use non-genetically identical allografts in human subjects (for review (Steece-Collier et al., 2012)). Several clinical trials have reported the presence of immune markers such as activated microglia surrounding the grafted cells in immunohistochemical postmortem analyses of grafted patients with PD (Freed et al., 2001; Kordower et al., 1997; Olanow

et al., 2003; Winkler et al., 2005). Significantly elevated levels of activated microglia and astrocytes surrounding DA grafts transplanted into parkinsonian rat models has also been reported, sharply contrasting a lack of microglia and astrocytes in non-DA control grafts (Lane & Lelos, 2022; Soderstrom et al., 2008).

Not only have these studies marked the presence of microglia and astrocytes surrounding grafted DA neurons, research has also pointed to a probable role of the immune response in the induction of GID behavior clinically. For instance, in the Tampa-Mount Sinai trial in which low-dose immunosuppressive medication was given for six months following grafting surgery, patients developed GID behavior only after immunosuppression was ceased (Olanow et al., 2003). The Denver/Columbia trial, which did not offer any immunosuppression, similarly reported GID behavior in grafted patients with PD (Freed et al., 2001). Likewise, in our primary DA-grafted parkinsonian rat model, GID behavior emerged, and increased, following exposure to immune activation via injections of peripheral spleen cells (Soderstrom et al., 2008). Curiously, GID did not manifest in grafted parkinsonian rats who received tissue from the same inbred strain (i.e., syngeneic grafts), further confirming a role of the host-immune response in GID development (Soderstrom et al., 2008; Steece-Collier et al., 2012).

Mechanistically, immune activation has been proposed to cause GID by a few mechanisms. First, immune activation has potential to cause diminished graft cell survival, ultimately affecting the ability of the graft to effectively integrate into the host and successfully restore DA levels in the STR (Hagell & Cenci, 2005; Hudson et al., 1994; Tronci et al., 2015). Another possibility is the release of pro-inflammatory cytokines that could activate specific signaling pathways or remodel synaptic

connections, both of which could lead to the development of dyskinetic behavior (for review (Hagell & Cenci, 2005)). Indeed, cytokines released from inflammatory immune cells activated nuclear signaling pathways that increased Fos protein expression in striatal neurons, which was correlated with the development of LID in animal models (Andersson et al., 1999; Hagell et al., 2002a; Winkler et al., 2002). Moreover, DA is known to have a modulatory effect on astrocytes and microglia, both of which express D1- and D2-like receptors (Boyson et al., 1986; Färber et al., 2005; Miyazaki et al., 2004). In this way, transplanting exogenous DA-producing cells would be expected to induce immune cell infiltration, activation, and cytokine release from astrocytes and microglia in the host (Lane & Lelos, 2022).

As more research is conducted on the connection between the host-immune response and GID induction, understanding whether immunosuppressive therapies in preclinical animal models eliminates GID may be an important next-step in elucidating its underlying mechanisms. It would also be important to reveal which specific immune components (e.g., microglia, complement factors) are seemingly permissive to these aberrant GID behaviors. More specifically, some immune factors could affect synapse formation between the grafted DA neurons and the host MSNs, potentially leading to GID (for review (Steece-Collier et al., 2012)). This could offer an explanation as to why there is an increase in the percentage of atypical, asymmetric synapses formed by engrafted DA neurons in GID+ patients with PD and grafted parkinsonian rats (see the “Abnormal Graft-Host Synaptic Circuitry” section). In Chapter 3 and 4, correlations between well-known immune markers and GID expression is experimentally investigated in our DA-grafted parkinsonian rat model.

Abnormal Graft-Host Synaptic Circuitry

Another hypothesis theorized to be responsible for GID behavior includes abnormal graft-host synaptic circuitry. Grafted eVM neurons establish synaptic connections with host striatal MSNs (Bolam et al., 1987; Kordower et al., 1996); however, it is more than possible that the grafted neurons fail to restore proper synaptic circuitry onto host MSNs, leading to signaling abnormalities that could potentially underlie GID (Hagell & Cenci, 2005). While DA neurons normally form *en passant* (i.e., in passing), symmetrical appositions, largely devoid of defined synaptic characteristics, onto the dendritic spines of MSNs (Gerfen & Surmeier, 2011; W. Shen et al., 2016), increasing evidence indicates that DA neurons in grafted subjects exhibit abnormal, atypical asymmetric synaptic connections (i.e., axodendritic or axosomatic) onto host neurons. The functional asymmetric synaptic connections are characteristic of excitatory neurotransmission (e.g., glutamatergic transmission) (Peters & Palay, 1996). In this way, it is reasonable to suggest that an increase in the formation of asymmetric synapses between grafted DA and host neurons could lead to the development of dyskinesia (i.e., GID) (Morgante et al., 2006; Picconi et al., 2003).

Various research groups have collected evidence in favor of the abnormal synaptic circuitry hypothesis. For instance, using ultrastructural and immunohistochemical analysis, Freund and colleagues and Mahalik and colleagues demonstrated that striatal DA grafts formed aberrant connections with host MSNs in a 6-OHDA parkinsonian rat model, specifically onto host cell bodies (T. Freund et al., 1985; Mahalik et al., 1985). Arguably the most promising findings were demonstrated by the Steece-Collier group in 2008. In a DA-grafted parkinsonian rat model, our group

demonstrated, ultrastructurally, that the grafted DA neurons made asymmetric synapses directly onto the host dendrites or the cell somas, and this was strongly correlated with the exhibition of GID behavior in these animals (Soderstrom et al., 2008). Interestingly, this phenomenon has also been importantly noted in human postmortem tissue from grafted patients with PD; asymmetric connections made by grafted DA neurons were also observed ultrastructurally (Kordower et al., 1997).

Not only have asymmetric synapses been detected in preclinical rodent models and in patients with PD, atypical synapses have been observed in non-human primate parkinsonian models as well. In MPTP-lesioned primates, 67% of transplanted DA neurons exhibited axodendritic connections, 32% axosomatic connections, and only 1.33% onto dendritic spines (Leranth et al., 1998). This is in comparison to the control primates that had 97% of DA terminals that terminated onto the host MSN spines (i.e., normal symmetric associations). Despite the evidence of abnormal graft-host circuitry in this non-human primate model of neural grafting, it is important to mention that GIDs have never been observed in primates, even after levodopa or amphetamine treatment, so the phenomenon in this model cannot be correlated, yet, to GID behavior (Kordower, Vinuela, et al., 2017). Nevertheless, the abundance of evidence collected thus far deems in favor of the abnormal graft-host synaptic connectivity hypothesis underlying GID, and if correct, enhancement of physiological synapse formation between grafted DA and host neurons could effectively ameliorate GID in both patients and animal models of PD.

Uneven DA reinnervation/DA release

Preclinical animal models and clinical grafting trials have pointed to the possibility of uneven DA reinnervation and/or excess DA release in association with GID development following engraftment of eVM DA neurons. However, study results remain indefinite or contradictory. In grafted individuals with PD, clinicians have performed ^{18}F -DOPA (fluorodopa; FD) PET scans in order to directly measure DA storage capacity and indirectly assess DA innervation (Hagell & Cenci, 2005). With these scans, researchers have demonstrated that VM grafts can normalize FD uptake in the grafted striatum (Piccini et al., 1999). Further, FD values of grafted patients were found to be significantly increased in patients who also developed GID compared to those who did not (Ma et al., 2002). Remarkably, in the Denver/Columbia clinical grafting trial, patients who expressed GID behavior had twice the amount of FD PET signals compared to patients who did not develop GID at 12 months following transplantation; at 24 month post-transplantation, levels were almost three times larger (Ma et al., 2002).

Despite demonstrable promise for the role of excess DA release, other clinical trials have failed to provide comparable results. For instance, in a retrospective analysis conducted by Hagell and colleagues, GID scores were not found to be correlated with postoperative FD uptake (Hagell et al., 2002b). Similarly, Olanow and colleagues reported a lack of correlation between GID and FD uptake in the putamen (Olanow et al., 2003). Not only were they not able to find a correlation in patients, preclinical animal studies have likewise demonstrated a lack of association between GID and FD (Cragg et al., 2000; Doucet et al., 1990; Kirik et al., 2001). A key issue of these collective clinical trials and preclinical animal studies is, even if an increase in FD uptake was

demonstrated (Ma et al., 2002), DA uptake failed to exceed supranormal DA levels or innervation of the intact STR (Hagell et al., 2002a; Ma et al., 2002; Olanow et al., 2003), arguing against the theory of excess DA release, or at least widespread excess release. Because of this, Hagell et al., 2002 has posited that OFF-medication dyskinesia (i.e., GID) do not result from excessive innervation of grafted DA neurons (Hagell et al., 2002b).

Although the postulation from Hagell and colleagues is not in favor of excessive DA release from the grafts, the possibility of this phenomenon underlying GID behavior should not be completely denied. An alternative explanation that has been offered is that GIDs result from of so-called “hotspots” of DA activity due to uneven patterns of DA release and/or reinnervation. Indeed, evidence from Ma Y and colleagues showed that FD uptake was increased in GID+ patients but signals were localized to only two zones within the left putamen (Ma et al., 2002). Additional evidence has established that more focal VM grafts, either transplanted at two separate striatal sites (Lane et al., 2006) or at a single “hotspot” site (Maries et al., 2006), induced GID behavior in parkinsonian rats. In contrast, VM tissue transplanted and distributed at six sites, which provided more widespread graft-derived reinnervation, significantly reduced GID induction (Maries et al., 2006). In this way, imbalanced DA reinnervation may be a more appropriate pathogenic theory potentially underlying GIDs.

While current clinical trials and preclinical animal studies exhibit contrarian evidence, it is imperative to note the limitations of these studies. Most importantly, FD PET uptake widely used in the above studies does not directly show DA release. It only measures the capacity of the grafted neurons to uptake and synthesize DA.

Undoubtedly, this technique is a valuable tool; however, it does not illustrate the intricacies of DA signaling/release occurring in grafted VM neurons. Certainly, in spite of the lack of evidence for VM grafts releasing excess DA, other clinical trials have revealed that buspirone administration (a DA D2 receptor antagonist) successfully reduced GID severity in grafted patients (Politis et al., 2010, 2011; Steece-Collier et al., 2012), yet another piece of evidence in favor of a role for DA release. Therefore, the connection between DA release and/or uneven DA innervation and GID behavior should continue to be investigated in clinical research until fully elucidated.

DA/glutamate co-transmission

Due to the complex nature of GID behavior, it is more than likely that one mechanism alone does not solely cause GID. For example, excessive DA release/DA reinnervation alone may not cause GID, but in combination with abnormal synaptic circuitry, it could underlie GID. Of relevance, DA neurons have been shown to have the potential to co-release DA and other neurotransmitters, including glutamate. While this has been known for some time, the functional significance of dual neurotransmission remains unclear. Many research groups have reported that a subpopulation of DA neurons can co-express both DA and glutamate, evidenced by co-expression of vesicular glutamate transporter 2 (VGLUT2) in tyrosine hydroxylase-positive (TH+) neurons (Bérubé-Carrière et al., 2009; Buck et al., 2022; Dal Bo et al., 2004; Fortin et al., 2019; Mercado et al., 2021, 2024; Mingote et al., 2019; Root et al., 2016; H. Shen et al., 2018; Sulzer et al., 1998; Trudeau et al., 2014). Confirmed behaviorally, reduced locomotion in mice following cocaine administration (Hnasko et al., 2010) and methamphetamine (H. Shen et al., 2021) was reported in knock-out mice of VGLUT2

expression in DA neurons. Hnasko and colleagues also demonstrated, in slice culture of VGLUT2 knock-out DA neurons, that glutamate and DA release was significantly decreased, further supporting an important function of DA/glutamate co-transmission (Hnasko et al., 2010). Despite evidence of co-neurotransmitter release, it is uncertain how DA/glutamate co-release may contribute to GID behavior.

A promising phenomenon that could provide a logical functional explanation for the role of DA/glutamate release in GID is known as vesicular synergy. The general idea behind vesicular synergy involves a loading enhancement of non-glutamate neurotransmitters into secretory vesicles via the co-localization of a vesicular glutamate (VGLUT) protein (El Mestikawy et al., 2011). This phenomenon has been well documented in cholinergic neurons: the presence of vesicular glutamate transporter 3 (VGLUT3) and vesicular acetylcholine transporter (VACHT) on the same synaptic vesicle enhances packaging and release of acetylcholine (Gras et al., 2008). Vesicular synergy in other systems, however, such as DA and GABAergic neurons, has only recently begun to be explored (see (Prévost et al., 2024, 2025)). In dopaminergic systems, it is hypothesized that, if VGLUT2 protein and VMAT2 are present on the same synaptic vesicle, enhanced packaging of DA will occur and lead to increased DA release (Aguilar et al., 2017; Hnasko et al., 2010; H. Shen et al., 2018). The data described above in VGLUT2 knock-out mice supports the occurrence of this phenomenon in DA neurons (Hnasko et al., 2010; H. Shen et al., 2021).

If VMAT2/VGLUT2 are co-localized on the same vesicle in grafted DA neurons, increased DA release could potentially lead to the development of GID. While promising, current clinical trials have exhibited varying support behind increased DA

release and GID behavior (see above); however, researchers are limited by the proper tools for analysis. Although postmortem evidence of asymmetric synapses formed by grafted DA neurons in PD subjects (T. Freund et al., 1985; Kordower et al., 1997; Mahalik et al., 1985; Mercado et al., 2021; Soderstrom et al., 2008) support this idea, future experiments are still needed to definitively determine whether VMAT2 and VGLUT2 are found on the same vesicle in the grafted striatal environment. As will be discussed in later portions of my thesis, my preclinical evidence suggests that the theory of vesicular synergy provides a compelling mechanism of how DA/glutamate co-transmission (and excessive DA release) could underlie GID behavior. Furthermore, as mentioned above, GIDs are a complex behavioral malady in which not only one mechanism is likely responsible. The theory of vesicular synergy provides one parsimonious explanation for both the phenomenon of excess DA and the phenomenon of abnormal graft-host synaptic circuitry.

Overall, the manifestation of aberrant GID behavior in a subpopulation of PD patients who received VM transplants has limited neural grafting as an effective therapeutic approach for PD. While promising preclinical and clinical studies have investigated the possible underlying mechanisms of GID behavior, its true pathogenesis remains elusive, as does the solution to its successful amelioration. Most recently, and for many reasons, clinical grafting trials have begun utilizing different cell sources including induced pluripotent stem cells (iPSCs). Because VM transplants are the only cell source to demonstrate GID induction thus far, clinical outcomes of iPSC grafting trials remain a gap in our knowledge, adding another obstacle toward optimization of cell transplantation therapy for patients with PD.

Alternative Cell Sources

To date, the most successful cell source in neural transplantation is eVM tissue that contains developing SN DA neurons. Collectively, the studies addressed above have provided sufficient evidence that transplanted eVM tissue can survive long term, successfully produce DA, and induce behavioral improvement in individuals with PD. However, using this cell source is not without caveat: utilizing eVM tissue is encumbered with several practical and ethical concerns. Ethically, the use of eVM neurons from aborted tissue is highly controversial and not accepted in many countries (Brundin et al., 2010). As a practical concern, trying to procure sufficient amounts of tissue (approximately 4-10 embryos per patient) on a nation-wide scale is nearly impossible (Barker et al., 2017; Stoddard-Bennett & Reijo Pera, 2019) and contributes further to the immunological concerns of multiple allograft donors. Indeed, in the TRANSEURO clinical grafting trial, only 20 of the planned 90 surgeries were conducted due to low tissue supply (human embryonic ventral mesencephalic, hEVMs) (Barker et al., 2017).

To combat these issues, a number of different cell sources are being investigated as alternatives to eVM tissue. Some of these alternative sources include neural stem cells and bone marrow mesenchymal cells. Most recently, the field has shifted its attention to the use of human pluripotent stems cells (hPSCs), which include human embryonic stem cells (hESCs) and iPSCs, as promising cell sources in clinical grafting trials. Therefore, a brief discussion on each source can be found below.

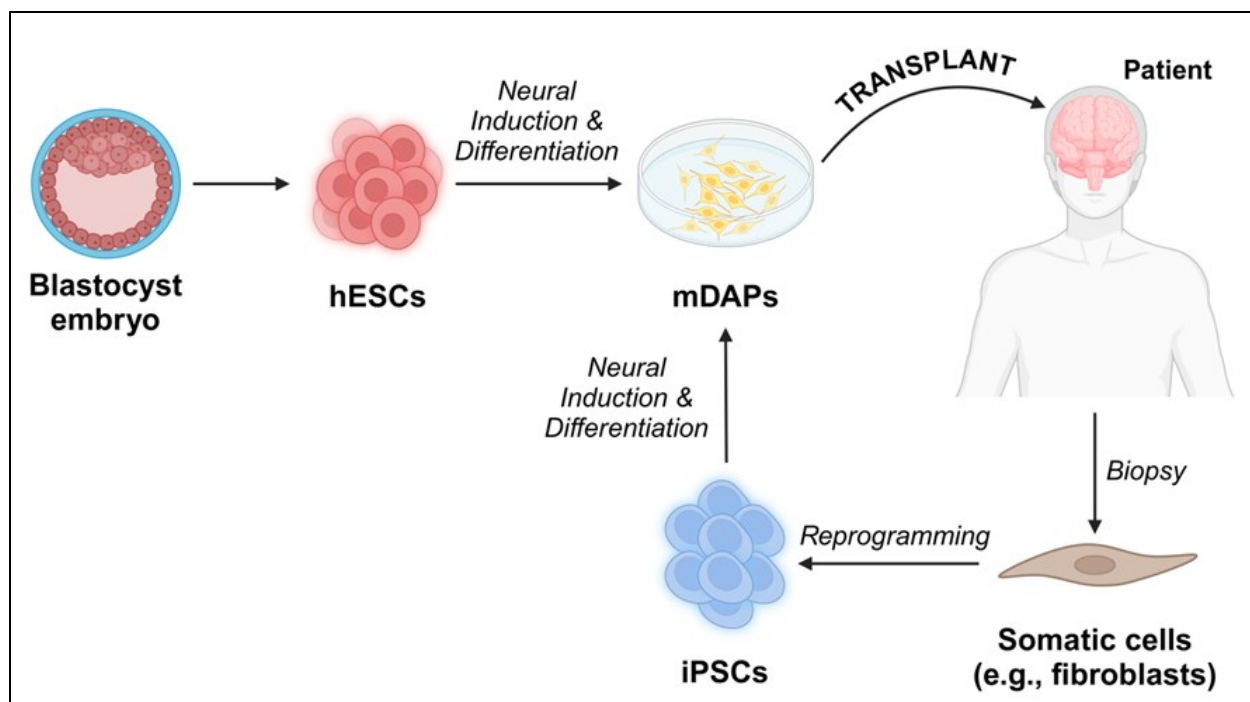


Figure 1.10: hESCs vs. iPSCs as cell transplantation sources for PD.

Two sources of dopaminergic progenitors currently being utilized as cell sources for transplantation in PD include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (iPSCs). hESCs are harvested from human blastocysts and differentiated into midbrain dopaminergic progenitor cells (mDAPs) for transplantation. iPSCs are reprogrammed from somatic cells (e.g., fibroblasts) from adult donors, differentiated into mDAPs, and then transplanted into the patient's brain. Adapted from (Parmar et al., 2020). Abbreviations: hESCs = human embryonic stem cells; mDAPs: midbrain dopaminergic progenitor cells (mDAPs); iPSCs = induced pluripotent stem cells.

Human Embryonic Stem Cells (hESCs)

Human embryonic stem cells (hESCs) are derived from pre-implantation embryos and can be successfully differentiated into authentic midbrain dopaminergic neurons (**Figure 1.10**) (Barker et al., 2017). In 1998, Thompson and colleagues reported the first successful hESC derivation, stimulating interest in the use of hESCs due to its unlimited capacity for self-renewal and pluripotent differentiation (Brundin et al., 2010; Thomson et al., 1998). Later on, hESCs were shown to successfully survive and provide functional benefit following engraftment into mouse, rat, and non-human

primate models of PD (Kirkeby et al., 2012; Kriks et al., 2011; Roy et al., 2006). Most importantly, hESCs were found to be molecularly and functionally identical to human eVM DA neurons (Grealish et al., 2014; Parmar et al., 2020). In a 2017 trial of parkinsonian non-human primates that received intrastriatal hESC-transplants, behavioral improvement was demonstrated for at least 24 months following engraftment, and there were slight increases in DA which correlated with behavioral improvement (Cyranoski, 2017). These findings provided preclinical data for a phase I/IIa ESC-based clinical transplantation study in China, although results from the trial are not yet available (Wang et al., 2018).

The current limitations of utilizing hESCs as a cell source for transplantations in PD include the necessity for immunosuppression and the possibility for tumorigenesis (Stoddard-Bennett & Reijo Pera, 2019). Because of the unlimited capacity for self-renewal, hESCs have the ability to differentiate into various somatic cell types. In this way, it is possible for hESCs to form teratomas in the host brain; thus, particular care must be taken in order to avoid differentiation into non-neuronal cells (Brundin et al., 2010). Also with hESCs, strong immunosuppressants must be administered prior to transplant surgery to avoid graft rejection and human leukocyte antigen (HLA) matches are required.

Human Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are another source being investigated as an alternative to eVM neuron transplants. iPSCs can be generated by reprogramming differentiated cells taken from the patient (e.g., fibroblasts) into an embryonic state (**Figure 1.10**) (J. Takahashi, 2018; K. Takahashi et al., 2007; K. Takahashi & Yamanaka,

2006) and then are pushed directly into DA neurons (Doi et al., 2014; Hargus et al., 2010; Rhee et al., 2011; Swistowski et al., 2010; Theka et al., 2013). Using iPSCs, which come from non-embryonic tissue, removes the ethical obstacles that are present with eVM neurons. Additionally, the need for postoperative immunosuppressants is greatly reduced because iPSCs permit HLA matches (i.e., autologous transplantation).

Several preclinical animal studies of PD have demonstrated that transplanted human iPSC-derived DA neurons can survive long-term and enable functional motor benefit (Doi et al., 2014; Hargus et al., 2010; Kikuchi et al., 2011; Rhee et al., 2011; Swistowski et al., 2010). Human iPSCs were also successfully transplanted into parkinsonian non-human primates, revealing robust growth, proliferation, and integration two years following surgery (Kikuchi et al., 2017). These studies led to the first clinical trial of iPSC-derived DA neurons grafted into patients with PD held in Kyoto, Japan in 2018. Since this trial, only one additional human case-study has been conducted. Autologous iPSC-derived DA neuron transplants were engrafted into a single individual with PD; the cells survived two years, but there were no significant changes in the patient's MDS-UPDRS Part III scores. However, the patient did show an improvement in the Parkinson's Disease questionnaire 39 (PDQ-39) (Schweitzer et al., 2020).

Unfortunately, one of the greatest limitations of using iPSCs for neural transplantation is the cost: reprogramming a patient's cells may be not only a lengthy process but also expensive for the patient (Stoddard-Bennett & Reijo Pera, 2019). Moreover, like hESCs, iPSCs also possess a substantial proliferative capacity. Therefore, incomplete, or uncontrolled differentiation is possible, increasing the potential

risk for tumor formation in the grafted neurons (Brundin et al., 2010; J.-Y. Li, Christophersen, et al., 2008). To date, neither in the non-human primate study nor the Kyoto clinical trial have iPSC transplants resulted in tumor formation; however, the use of iPSCs is only in its infancy, so further research is warranted. Please see **Table 1.1** for a list of the current planned or ongoing clinical trials that are utilizing hESCs or human iPSCs for transplantation in patients with PD.

Additional Limitations of Cell Transplantation Therapy

Although still a promising alternative therapeutic for PD, like most therapies, cell transplantation is not without limitation. While the overall goal of neural transplantation in PD is to repair the loss of dopaminergic neurons by engrafting new ones, this will not “cure” PD. In many cases, drug treatment and rehabilitation will still be required following transplantation surgery (Mishima et al., 2021). Additionally, this method cannot holistically treat all signs and symptoms of PD: non-motor dysfunction remains following transplantation as these symptoms stem from various other pathways in the brain (Barker et al., 2024).

Not only are non-motor symptoms not targeted with neural transplantation, motor recovery in response to DA grafts can also be variable. Variability has been demonstrated both between different clinical trials and among individuals within the same trial (Barker et al., 2013; Winkler et al., 2005). Following engraftment, some patients have demonstrated great graft-derived motor benefit, while others have exhibited limited to no benefit (e.g., (Freed et al., 2001)). For example, one individual with PD who received an embryonic VM DA neuron transplant demonstrated dramatic recovery for 12 years after engraftment. However, by year 18, graft-derived motor

benefit was almost non-existent for this patient, despite robust graft survival and extensive innervation (W. Li et al., 2016). Postmortem analysis of another patient who received an embryonic VM DA graft revealed a significantly dense and widespread graft; however, the patient never experienced motor benefit and had to receive DBS for GID (Kordower, Goetz, et al., 2017).

Another shortcoming of this experimental therapy concerns the development of α -synuclein pathology within the grafted cells. α -synuclein-positive protein inclusions were found to develop in human embryonic VM midbrain tissue engrafted into patients with PD 10+ years post-transplantation (Barker et al., 2024; Kordower et al., 2008; J.-Y. Li, Englund, et al., 2008). It was reported that α -synuclein in these grafts was phosphorylated at Serine residue 129, indicative of disease-related, aggregated α -synuclein (Anderson et al., 2006; J.-Y. Li, Englund, et al., 2008). Statistically, in the Li et al. report, only 1.9% of a patient's 12-year-old graft contained Lewy bodies—a number that increased to approximately 5% in another patient with a 16-year-old graft (J. Li et al., 2010). Some studies have found no Lewy pathology in long-term grafts up to 14 years old (Hallett et al., 2014; Mendez et al., 2008). While potentially problematic, these data argue that only a small portion of the transplanted dopaminergic neurons will develop PD pathology, and despite the presence of pathological α -synuclein inclusions, some patients have still demonstrated motor benefit. Therefore, researchers believe that the presence of this pathology should not invalidate cell transplantation as a therapy for PD.

Scientists remain uncertain why and how Lewy pathology occurs in grafted neurons, theorizing that pathology spreads to the transplanted neurons via a prion-like

mechanism (Brundin et al., 2010; Brundin & Kordower, 2012; Brundin & Melki, 2017; Kordower & Brundin, 2009; J.-Y. Li, Englund, et al., 2008; Olanow & Brundin, 2013; Olanow & Prusiner, 2009; Surmeier et al., 2017) or that α -synuclein is upregulated and aggregated in response to inflammation (Brundin et al., 2010). Indeed, α -synuclein pathology has been known to transfer from host to graft in parkinsonian mouse and rat models (Hansen et al., 2011; Kordower et al., 2011). Although the significance of α -synuclein pathology in neural transplantation has yet to be determined, it will be critical to consider with the future use of autologous stem cell transplants as there may be a possibility of pathology spread from the host to donor cells (Parmar et al., 2020).

Lastly, a limitation that has emerged more recently is the lack of a standard surgical device used to stereotactically deliver DA cells to the brain. For instance, it is thought that the variety of different devices used in the original human embryonic VM transplant trials could have exacerbated the negative outcomes or heterogeneity in clinical responsiveness. Without a regulated global standard surgical device, developing a consistent, effective transplantation protocol will be challenging. Therefore, deciding on a device for cell implantation will have to be carefully considered moving forward, especially with stem cell trials commencing, in order to greatly reduce heterogeneity in clinical outcomes (Barker et al., 2024).

As the field of regenerative medicine continues to evolve, especially with the increasing use of stem cells in ongoing clinical trials, it will be imperative to continue to carefully consider the limitations and concerns surrounding cell therapy. As addressed above, the aberrant side effect of GID remains a significant obstacle, and understanding its underlying pathology will be necessary for this field to continue to advance. While it is

true that a large number of factors have been addressed in prior clinical grafting trials (e.g., disease severity, patient age, and removal of 5-HT neurons), other factors that could affect patient outcomes in response to transplantation remain relatively unexplored (e.g., genetic risk factors). Ultimately, PD is a disease of complex heterogeneity. Therefore, moving toward a precision-medicine-based approach could be crucial in effectively developing and optimizing therapies that will provide maximum benefit for each and every patient, particularly in the context of neural transplantation.

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CHAPTER 2: ADVANCING CELL-BASED THERAPY FOR PARKINSON'S DISEASE THROUGH THE SCOPE OF PRECISION MEDICINE

UNDERSTANDING THE COMPLEXITY OF PATIENT RESPONSE TO PD THERAPY

Introduction to Precision Medicine

Precision medicine, also referred to as personalized medicine, is a conceptual framework that aims to tailor treatment for an individual based on his or her characteristics (Collins & Varmus, 2015; Schneider & Alcalay, 2020). While the traditional approach is to prescribe one established treatment for all patients (**Figure 2.1a**), using a precision medicine approach considers an individual's biology, environment, and lifestyle when developing or prescribing treatment (**Figure 2.1b**). Precision medicine may additionally focus more specifically on genetic profiles, cell types, biomarkers, and molecular pathways in order to achieve the most effective therapeutic intervention for the patient (Collins & Varmus, 2015; Payami, 2017). One long-term goal of precision medicine, especially for neurodegenerative disease, is to diagnose a patient at the earliest stages of the disease so that the proper, most effective treatment can be initiated as soon as possible. As clinical medicine continues to advance, using a precision medicine approach will likely evolve from a diagnosis/treatment focus to more of an emphasis on prevention of disease.

While precision medicine is not a new concept, it has recently begun to be put into practice more regularly in healthcare. Just a decade ago, in 2015, the Precision Medicine Initiative (PMI) was launched in the United States by former President Barack Obama. The NIH awarded this initiative approximately \$55 million in order to build its infrastructure so that advances could be made toward a new era of precision medicine (Payami, 2017). The advancements we have made since then with technologies such as genome sequencing, pharmacogenetics, Big Data, and artificial intelligence (AI) have

drastically accelerated our progress of implementing a precision-medicine-based approach to clinical care and preclinical research. A powerful example of the impact that precision medicine has had thus far is demonstrated in the field of oncology: oncologists work to identify anatomical spread, biology, and possible genetic changes that could have triggered the growth of cancer cells in a specific patient (Espay et al., 2017; Sherer et al., 2016). In this way, scientists and doctors have been able to develop precise, successful treatments based on certain characteristics of a patient's cancer.

Precision Medicine in Parkinson's Disease

Although precision medicine in oncology has had substantial success, precision medicine approaches for other diseases and disorders such as PD require more attention. Unlike oncology, one of the challenges in neurology is the limited availability of tissue biopsies for histological and biochemical analyses of individual patients. This, unfortunately, makes it difficult to identify biomarkers for neurological and neurodegenerative diseases (Keller et al., 2012; Schalkamp et al., 2022). Specifically, in PD, personalized medicine has not yet been fully realized largely due to the immense heterogeneity in the clinical manifestation of the disorder (Mishima et al., 2021). Among the 9.3 million people who live with PD worldwide, age of onset, rate of progression, and severity of symptoms vary dramatically, even in individuals who have the same mutations in at-risk genes (e.g., LRRK2) (Espay et al., 2017; Maserejian et al., 2020; Schalkamp et al., 2022). The ultimate problem for PD, then, is trying to get one treatment to work for all patients (Payami, 2017).

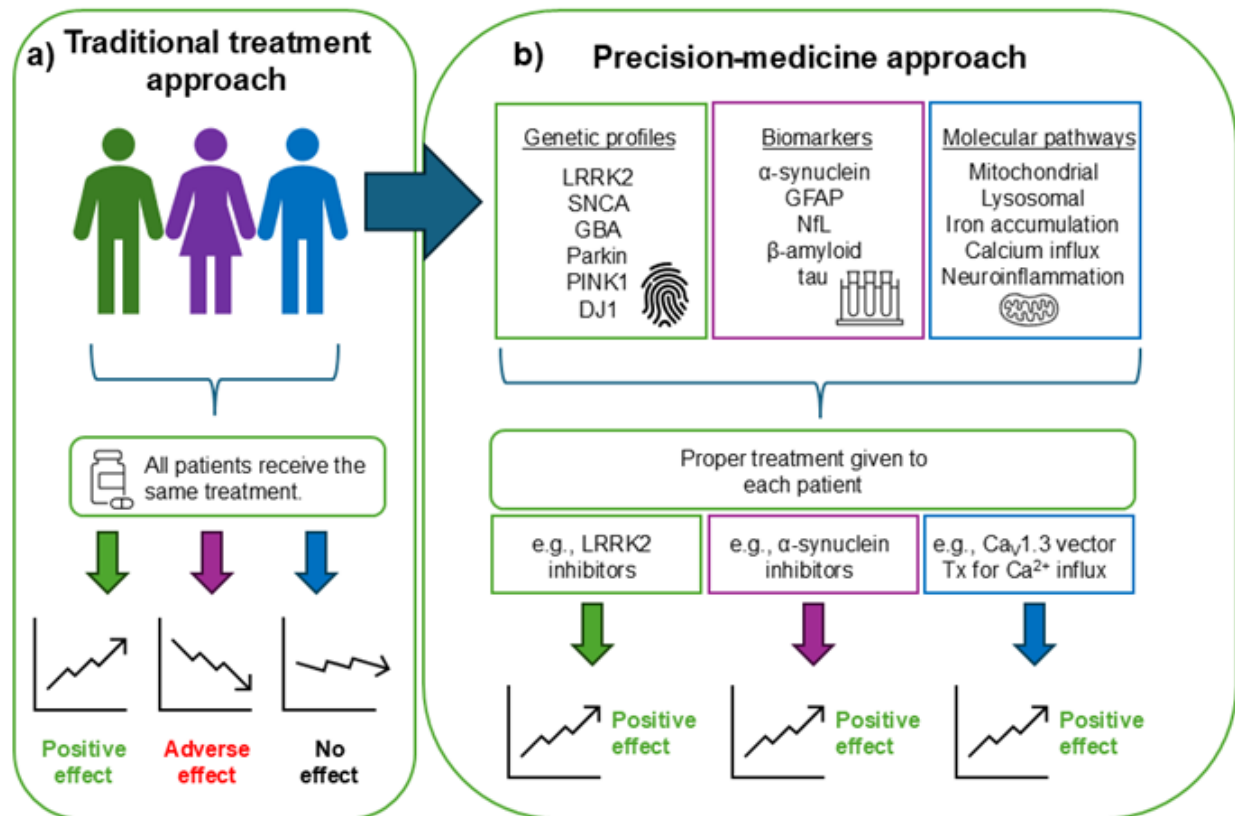


Figure 2.1: Precision medicine in Parkinson’s disease (PD).

(a) The traditional approach to treating all patients with PD. This is considered a “one-size-fits-all” approach in which, despite differences in age of onset, disease severity, sex, patients receive similar pharmacological interventions (e.g., levodopa or dopamine agonists). With this approach, only a small population of patients will demonstrate significant efficacy of the prescribed therapeutic. Others may develop adverse reactions, and another subpopulation of patients may experience no benefit or detriment at all. **(b)** Examples of a precision-medicine-based approach for patients with PD. Each individual patient may exhibit differences in genetic profiles, biomarkers, and/or molecular pathways and should be treated accordingly. As the scientific community continues to investigate the intricacies of PD, more precise treatments are being developed which will provide safe and effective treatments for all patients, not just a small population.

Heterogeneity in Clinical Response to PD-related Therapy

A prominent example of the heterogeneous nature of PD is an individual's differential response to levodopa, the mainstay pharmacological therapeutic for PD. Levodopa is generally effective in treating motor symptoms of PD; however, the clinical response for each patient remains highly variable. As mentioned previously in Chapter 1, in early-stage PD patients who received the same dose of levodopa, responses ranged from a 100% improvement to a 242% worsening of UPDRS Part III scores (Hauser et al., 2009). This variability suggests that various biochemical mechanisms are involved, requiring differential treatment approaches (i.e., precision medicine) between patients (Stoddard-Bennett & Pera, 2019).

Currently, the heterogeneity in PD is being extensively studied. Yet, underlying characteristics and mechanisms remain unclear. In line with differential responses to levodopa, some studies have pointed to certain mutations and/or single nucleotide polymorphisms (SNPs) that have been associated with side effect development from chronic levodopa use including LID. Specifically, carriers of a polymorphism in the DA active transporter 1 gene (DAT1), a gene involved in DA reuptake, are 2.5 times more likely to develop LID (Cacabelos, 2017; Moreau et al., 2015; Stoddard-Bennett & Pera, 2019). Another study recounted that there was a dose-dependent association between a variant in the GRIN2A gene (which encodes for the NR2A subunit of the N-methyl-D-aspartate (NMDA) glutamatergic receptor) and susceptibility to LID behavior (Ivanova et al., 2012).

One of the most prominent SNPs that has been linked to differential patient responses to levodopa (e.g., LID) is a common SNP known as rs6265 found within the

gene for brain-derived neurotrophic factor (BDNF) (see (Martinez-Carrasco et al., 2023) for other SNPs). In a retrospective analysis conducted by Fischer and colleagues, rs6265-carriers (aka heterozygous Val/Met or homozygous Met/Met) who received levodopa monotherapy reported worse UPDRS scores compared to wild-type (Val/Val; WT) subjects (~6 points worse) (Fischer et al., 2020). Met-allele carriers also exhibited a higher risk of developing LID earlier in treatment in contrast to their WT counterparts (Fischer et al., 2020; Foltynie et al., 2009). Alternatively to levodopa monotherapy, however, unmedicated Met-allele carriers presented a lower severity of motor symptoms compared to WT patients. Disease progression was slower for unmedicated Met-allele carriers, confirmed by a delayed need for levodopa (Fischer et al., 2018). Met-allele carriers also had a 5.3-year later age of onset of PD (Bialecka et al., 2014; Karamohamed et al., 2005a).

ROLE OF BDNF IN HETEROGENETIY OF CLINICAL RESPONSE TO PD THERAPY

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Current research has added to our understanding of the global risk factors (e.g., age, disease severity) of cell transplantation (see Chapter 1). However, the role of specific genetic variations remained entirely unexplored until recent studies conducted by our group which focused on the rs6265 SNP in the BDNF gene (see (Mercado et al., 2021, 2024). Because of promising evidence of the role of rs6265 in heterogenetic responses to levodopa therapy, and the biological relevance of BDNF (detailed below), our laboratory utilized a precision-medicine-based approach to investigate whether rs6265 was a risk factor that impacts therapeutic efficacy of DA neuron transplantation

therapy for PD. Structure, function, and significance of BDNF as a critical neurotrophic factor is first discussed in-depth below.

Introduction to BDNF

BDNF is a neurotrophin that functions to regulate and promote neuronal survival, differentiation, and outgrowth of central and peripheral neurons (Gonzalez et al., 2016; Kowiański et al., 2018; Liu et al., 2018; Park & Poo, 2013; Sasi et al., 2017; Urbina-Varela et al., 2020; Zagrebelsky et al., 2020). Other members of the mammalian neurotrophin family include nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5), and they share more than a 50% sequence homology in their primary structure with BDNF (Al-Qudah & Al-Dwairi, 2016). NGF was the first neurotrophin to be discovered by Rita Levi-Montalcini and Viktor Hamburger in the 1950s (Levi-Montalcini & Hamburger, 1951, 1953). Using chick embryos, their work described the observation that neurons die when they lack contact with their targets; research which led to their later revelation that the target was a critical source of a diffusible growth factor eventually identified as NGF (Levi-Montalcini & Hamburger, 1951, 1953). In 1982, a few decades following this discovery, BDNF was isolated by Yves-Alain Barde and Hans Thoenen from pig brain (Barde et al., 1982). Their research demonstrated that this novel growth factor could induce neuronal outgrowth and survival of cultured embryonic chick sensory neurons (Barde et al., 1982), supporting the “neurotrophic hypothesis” developed by Levi-Montalcini and Hamburger (Levi-Montalcini & Hamburger, 1951). Although BDNF had a similar molecular weight to NGF, its functional capacities were distinct, and NGF neutralizing antibodies were not able to block its survival-promoting activity (Levi-Montalcini & Hamburger, 1953). Follow-up

cloning experiments established the identity of BDNF with a unique sequence and structure (Leibrock et al., 1989).

Nearly all brain regions have been reported to contain BDNF at varying concentrations, but its specific function depends on stage of development as well as the composition of neuronal, glial, and vascular constituents present in the anatomical region (Kowiański et al., 2018). BDNF is abundant in the cortex, hippocampus, and visual cortex. It is also found in the STR, the SN, and ventral tegmental areas (VTA), though BDNF found in the STR is supplied by cortical and nigral DA neuron afferent projections and not the local neurons themselves (Baydyuk & Xu, 2014). This trophic factor is not solely abundant in the central nervous system (CNS) but is also released in appreciable amounts in the peripheral nervous system (PNS) and by other non-neuronal cells including lymphocytes, microglia, megakaryocytes, endothelial cells, and smooth muscle cells (Brigadski & Lessmann, 2020). BDNF production and signaling is critical for a vast array of neurophysiological processes including, but not limited to, neuronal survival, dendritic spine development, synaptogenesis, neurite outgrowth, neuroprotection, long-term potentiation (LTP), and long-term depression (LTD) (for review (Gonzalez et al., 2016; Kowiański et al., 2018; Park & Poo, 2013; Sasi et al., 2017; Zagrebelsky et al., 2020)). BDNF has also been found to be a necessary factor in neurogenesis and osteogenesis in human bone both in vitro and in vivo (Liu et al., 2018; Urbina-Varela et al., 2020).

BDNF Gene Structure and Isoform Processing

The human BDNF gene is located on chromosome 11p13-14 and is composed of multiple noncoding exons and one coding exon. There are 11 exons that can be

alternatively spliced to produce a minimum of 17 transcripts, but each transcript generates the same final protein product (Aid et al., 2007; Cattaneo et al., 2016; Vaghi et al., 2014). Of the 11 exons, 9 fall within the 5' region (Notaras & van den Buuse, 2019). The BDNF messenger ribonucleic acid (mRNA) transcripts that contain exons II and VII are exclusively expressed in the brain, whereas the transcripts containing exons I, IV, and V are expressed in peripheral tissue; exons VI and IX are broadly expressed (Urbina-Varela et al., 2020). BDNF transcription terminates at two polyadenylation sites within exon IX, thus giving rise to two distinct mRNA populations including short (0.35 kb) or long (2.85 kb) 3' untranslated regions (UTR) (Cohen-Cory et al., 2010; Notaras & van den Buuse, 2019; Urbina-Varela et al., 2020). These two distinct populations have differing localizations: short UTR BDNF (exon I and IV) transcripts are found in the cell soma, whereas long UTR BDNF transcripts (exon II and IV) are trafficked to dendrites to regulate dendritic morphology and affect LTP (Chiaruttini et al., 2009; Notaras & van den Buuse, 2019).

The major coding sequence of BDNF is present in exon IX at the 3' end and is translated into an inactive precursor polypeptide (i.e., preproBDNF) in the rough endoplasmic reticulum (ER) (Brigadski & Lessmann, 2020; Cattaneo et al., 2016; Pruunsild et al., 2007). Within the rough ER, the signal sequence is immediately cleaved to yield the 28- to 32-kDa isoform proBDNF (Brigadski & Lessmann, 2020; Notaras & van den Buuse, 2019) which is comprised of an N-terminal prodomain and C-terminal mature domain (**Figure 2.2a**). Post-translational modifications including N-linked glycosylation of the prodomain, as well as sulfation of the N-linked oligosaccharides, can take place as the proBDNF neurotrophins migrate from the Golgi

apparatus to the trans-Golgi network (TGN). The processing of proBDNF continues via cleavage by intracellular proteolytic enzymes in the TGN (i.e., furin) or by convertases present in intracellular secretory vesicles for extracellular export (Pang et al., 2016). A portion of full-length proBDNF proteins is also released and can subsequently bind the high affinity receptor, p75^{NTR} (R. Lee et al., 2001). After release from the cell, extracellular processing of proBDNF by plasmin or matrix metalloproteases (e.g., MMP-2, MMP-9) can also occur (**Figure 2.2b**) (Brigadski & Lessmann, 2020; R. Lee et al., 2001; Mizoguchi et al., 2011; Pang et al., 2016). Processing of the preproBDNF yields three distinct active isoforms: the ~30kDa proBDNF, the ~13kDa mature BDNF (mBDNF), and the ~17kDa BDNF pro-peptide (McGregor & English, 2019) (**Figure 2.2**).

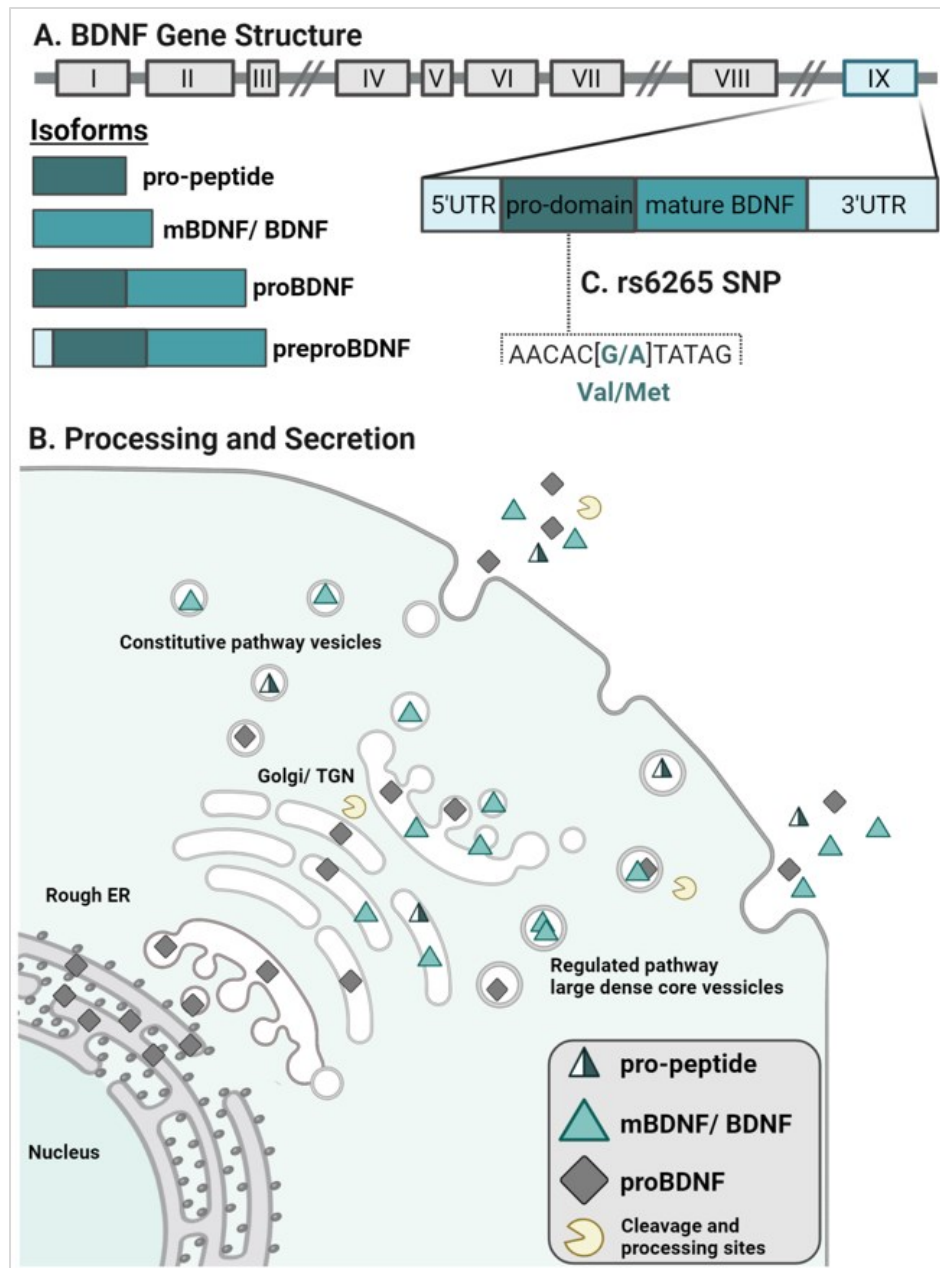


Figure 2.2: BDNF Gene Structure, Processing, and Secretion.

a) Schematic representation of human BDNF gene structure and isoforms. Grey boxes represent exons; exon IX (blue) contains the major coding sequence of BDNF (Brigadski & Lessmann, 2020; Cattaneo et al., 2016; Pruunsild et al., 2007). **b)** Following translation into preproBDNF in the ER, the signaling sequence is cleaved, and proBDNF is transported through the Golgi apparatus to the trans-Golgi network. Here, proBDNF can be cleaved by intracellular proteolytic enzymes sorting into the constitutive or regulated pathways (Brigadski & Lessmann, 2020; Pang et al., 2016). ProBDNF can also be cleaved within the vesicles or extracellularly, generating mBDNF and the BDNF pro-peptide (McGregor & English, 2019). **c)** The common SNP rs6265 (aka Val66Met) is located within the prodomain region of the BDNF gene and results a substitution of valine

Figure 2.2 (cont'd)

(Val) for methionine (Met) at codon (G/A) 66. (Baj & Tongiorgi, 2009; Colucci-D'amato et al., 2020). Abbreviations: pro-peptide = cleaved BDNF pro-peptide; mBDNF/BDNF = mature BDNF; proBDNF = BDNF isoform with pro-domain and mature domain.

BDNF Sorting and Release

Two distinct pathways of secretion exist for proBDNF and mBDNF: the constitutive and the regulated pathways. The constitutive pathway involves packaging BDNF into small-diameter granules that release BDNF independently of calcium fluctuation (Al-Qudah & Al-Dwairi, 2016). The majority of BDNF is packaged for release via the regulated pathway into larger granules that fuse to the plasma membrane in response to a calcium-dependent trigger (**Figure 2.2b**). Thus, the regulated release of BDNF occurs during activity-dependent depolarization (Al-Qudah & Al-Dwairi, 2016; Brigadski & Lessmann, 2020; Lessmann & Brigadski, 2009; Wong et al., 2015) (**Figure 2.2b**). Proper sorting and secretion of BDNF is critical for the maintenance of synaptic plasticity, neuronal survival, and CNS homeostasis (Al-Qudah & Al-Dwairi, 2016; Brigadski & Lessmann, 2020; Cunha et al., 2010; Mizui et al., 2015). As such, disruption of BDNF sorting and/or secretion has been implicated in various neurodegenerative and psychiatric diseases. While the specific molecular mechanisms associated with improper BDNF secretion remain largely uncertain, current evidence correlates reductions of hippocampal and cortical volumes (Frodl et al., 2006), formation of abnormal synapses (Mercado et al., 2021), and decreases in dendritic complexity (Z. Y. Chen et al., 2006; Egan et al., 2003) as consequences of dysfunctional BDNF sorting and reduced secretion.

For the regulated pathway, two binding interactions drive sorting of BDNF into vesicles. The BDNF prodomain/pro-peptide region binds directly to either sortilin, a

vacuolar protein sorting 10 (Vps10) domain-containing molecule, or carboxypeptidase E (Brigadski & Lessmann, 2020; Notaras & van den Buuse, 2019). Sortilin contains a transmembrane region and a cytoplasmic tail responsible for signaling endosome sorting in the Golgi apparatus (Notaras & van den Buuse, 2019). Sortilin and BDNF have been observed to colocalize within large dense-core vesicles, and sortilin truncation mutations result in impaired sorting of BDNF to the regulated pathway, subsequently decreasing activity-dependent release (Z. Y. Chen et al., 2005). Similarly, membrane-bound carboxypeptidase E is a glycoprotein that binds BDNF, and knockdown of carboxypeptidase E in mice has also demonstrated a reduction of downstream activity-dependent BDNF release (Lou et al., 2005; Notaras & van den Buuse, 2019). After being sorted into large dense-core vesicles of the regulated pathway, BDNF is generally trafficked to the axon where it can be degraded by the lysosome (Evans et al., 2011) or secreted into the synaptic cleft in response to neuronal activation where it can activate two classes of receptors, TrkB and p75^{NTR} (defined below) (Carvalho et al., 2008; Lu et al., 2014; Skaper, 2018). While the majority of BDNF is transported anterogradely, approximately 23% of BDNF is retrogradely transported to dendrites, although the biological significance of its retrograde trafficking has yet to be elucidated (Adachi et al., 2005; Dieni et al., 2012; Notaras & van den Buuse, 2019).

BDNF Signaling

Neurotrophins are known to bind to two classes of receptors: a tropomyosin receptor kinase (Trk) and a pan neurotrophin receptor (p75^{NTR}) which is a member of the tumor necrosis factor super family (Reichardt, 2006) (**Figure 2.3**). More specifically,

proBDNF binds with high affinity to p75^{NTR} (R. B. Meeker & Williams, 2015; Reichardt, 2006) (**Figure 2.3a**). In contrast, mBDNF preferentially binds to its high affinity receptor, TrkB, following its release into the synapse (Carvalho et al., 2008; Skaper, 2018) (**Figure 2.3b**). While mBDNF can also bind p75^{NTR}, it does so with low affinity (Binder & Scharfman, 2004). Additionally, the BDNF prodomain/pro-peptide region binds directly to sortilin, thereby participating in proper sorting of this molecule to its regulated pathway (Z. Y. Chen et al., 2005).

proBDNF and p75^{NTR}

ProBDNF binds to p75^{NTR} upon release, stimulating nuclear factor kappa B (NF- κ B), c-Jun N-terminal Kinases (JNKs), and Ras homolog family member A (RhoA) signaling that modulate survival, apoptosis, and growth cone motility, respectively (M. V. Chao, 2003; Kowiański et al., 2018; Reichardt, 2006; Teng et al., 2005) (**Figure 2.3a**). The specific cascade that is activated is dependent on which receptors are complexed with p75^{NTR}. For instance, when complexed with sortilin, pro-apoptotic pathways are activated (Friedman, 2000; R. B. Meeker & Williams, 2015). Recent evidence indicates that signaling through p75^{NTR} can also synergistically aid in TrkB activation (Hempstead, 2006; R. Meeker & Williams, 2014; Zanin et al., 2019). Specifically, p75^{NTR} can heterodimerize with TrkB, increasing TrkB binding affinity for mBDNF, thus promoting neuronal growth and survival (R. B. Meeker & Williams, 2015; R. Meeker & Williams, 2014; Zanin et al., 2019).

mBDNF and TrkB

Upon mBDNF binding to full-length TrkB, TrkB dimerizes and autophosphorylates several of its tyrosine kinase residues including Y705 and Y706 in the cytoplasmic loop

of the kinase domain, as well as Y515 and Y816 (Diniz et al., 2018; Notaras & van den Buuse, 2019). Multiple signaling pathways can be triggered once TrkB is activated including the phosphatidylinositol 3-kinase (PI3K), the phospholipase-C- γ 1 (PLC- γ 1), the guanosine triphosphate hydrolases of RhoA, and the mitogen-activated protein kinase (MAPK)/Ras cascades (reviewed in (M. V. Chao, 2003; Reichardt, 2006; Segal, 2003)). The PI3K pathway engages in pro-survival activity and enhances dendritic growth and branching (Jaworski et al., 2005; Kumar et al., 2005). The MAPK/Ras signaling cascade controls protein synthesis during neuronal differentiation (Molina & Adjei, 2006). Lastly, growth of neuronal fibers is activated via activation of RhoA (**Figure 2.3b**) (Kowiański et al., 2018; Reichardt, 2006).

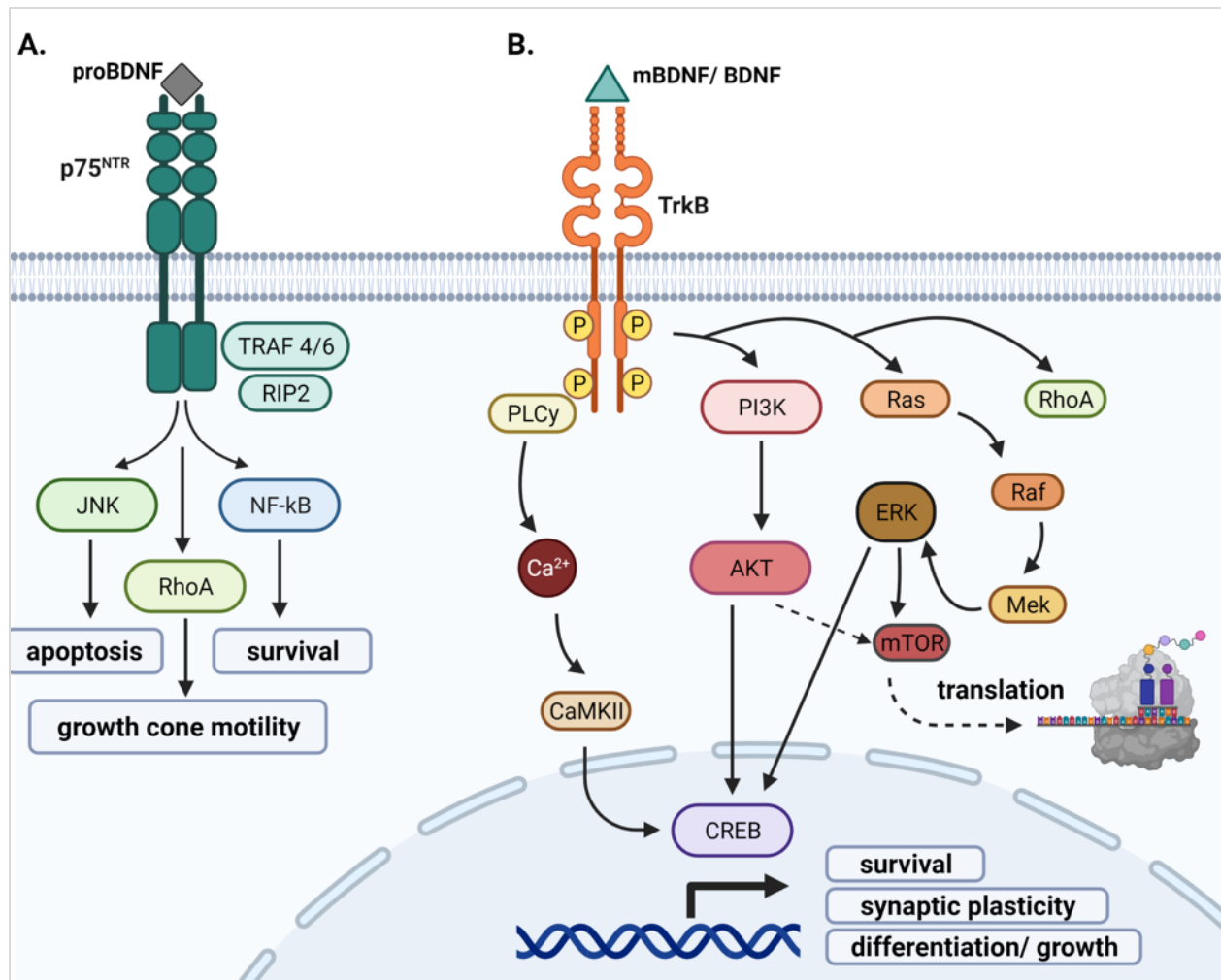


Figure 2.3: Schematic representations of conventional proBDNF and mBDNF signaling cascades.

a) ProBDNF binds with high affinity to p75^{NTR}, initiating downstream JNK, RhoA, and NF-κB signaling (M. V. Chao, 2003; Kowiański et al., 2018; Reichardt, 2006; Teng et al., 2005). **b)** mBDNF binds with high affinity to TrkB, inducing its dimerization and autophosphorylation and activating three main signaling pathways, PI3K, PLCγ, and Ras/MAPK, all of which lead to activation of the transcription factor CREB, driving transcription of genes crucial for neuronal growth and survival (Mitre et al., 2017; Reichardt, 2006; Segal, 2003). RhoA signaling and mTOR pathways can also be activated leading to growth cone modulation and translation of proteins involved in the regulation of cellular proliferation (Diniz et al., 2018; Kumar et al., 2005; R. B. Meeker & Williams, 2015; Notaras & van den Buuse, 2019).

It is widely accepted that the proBDNF and mBDNF ligands induce opposing outcomes through their preferential binding to different receptors in order to promote neurological homeostasis (Kowiański et al., 2018). Specifically, mBDNF-TrkB signaling stimulates neuronal growth and synaptic plasticity, whereas signaling through p75^{NTR} tends to initiate apoptosis thought to be important in development for eliminating inessential neurons (Friedman, 2000; Teng et al., 2005). Moreover, while mBDNF signaling is instrumental in driving hippocampal LTP, proBDNF promotes LTD (Deinhardt et al., 2011; Sakuragi et al., 2013; Woo et al., 2005; Yang et al., 2014). Because of homeostatic regulation, the expression of p75^{NTR} and TrkB are known to be tightly linked where they are co-expressed on the surface of the cell to establish signaling between cell survival and cell death (Notaras & van den Buuse, 2019). Homeostasis can therefore be disrupted when there is an imbalance in the expression of these receptors or an imbalance in the levels of proBDNF and mBDNF isoforms. For example, research conducted by Suelves and colleagues (Suelves et al., 2019) examined the consequences of BDNF/TrkB/p75^{NTR} imbalance in a Huntington's disease (HD) mouse model, showing that the reduction of BDNF and TrkB levels, along with an increase in p75^{NTR} expression, correlated with striatal neuropathology and motor dysfunction. Pharmacological normalization of p75^{NTR} levels rescued neuropathology (e.g., dendritic spine density) and motor deficits (Brito et al., 2013; Suelves et al., 2019).

In addition to changes in receptor levels/balance, increased proBDNF levels have been correlated with adverse outcomes in neurodegenerative disorders. Specifically, in mice expressing one BDNF allele with a mutated cleavage site, hippocampal proBDNF levels rose and promoted a decrease in dendritic arborization as

well as hippocampal volume (Diniz et al., 2018; Yang et al., 2014). Further reinforcing the importance of homeostatic balance in brain health, in PD, serum levels of proBDNF have been reported to be significantly higher in individuals with early PD as compared to healthy controls, whereas mBDNF levels were significantly lower (X. Yi et al., 2021). Collectively, an abundance of data indicate that tight control of both BDNF ligands and their receptors is critical for proper neuronal function and/or survival.

BDNF Pro-Peptide and Sortilin

It has been demonstrated that BDNF pro-peptide binding to sortilin drives proper sorting of BDNF into vesicles of the regulated secretory pathway (Z. Y. Chen et al., 2005). In addition, the BDNF prodomain (pro-peptide), once cleaved from proBDNF, appears to function as an independent ligand similar to proBDNF and mBDNF isoforms (Anastasia et al., 2013; Mizui et al., 2016, 2017). Upon cleavage from proBDNF and its subsequent release, the BDNF pro-peptide binds to sortilin and complexes with p75^{NTR}, resulting in various effects on the BDNF signaling cascade, neuronal survival, and synaptic plasticity (Anastasia et al., 2013; Z. Y. Chen et al., 2005; Giza et al., 2018; Mizui et al., 2016, 2017), although specific mechanisms and downstream pathways remain to be elucidated.

PD and BDNF

While dysfunction in BDNF signaling is not considered a primary cause of PD, it has long been known to be important for survival and development of SNpc DA neurons (Hyman et al., 1991; Yurek & Fletcher-Turner, 2001). In addition, there is abundant literature demonstrating that, in the aged brain, there is diminished BDNF, diminished upregulation in response to stress, reduced expression of several BDNF transcription

factors, and decreased expression of its TrkB receptor (for review (Mercado et al., 2017)). Given that the primary risk factor for PD is aging, and given the critical role of BDNF in the well-being of SNpc DA neurons, BDNF dysfunction has been abundantly explored in PD.

Current evidence has demonstrated reduced expression of BDNF mRNA transcripts in the SNpc in PD (Howells et al., 2000; Murer et al., 2001) as well as lower levels of BDNF protein specifically in the SN of individuals with PD compared to other brain regions, and significantly reduced serum BDNF (Scalzo et al., 2010). In addition to decreases in BDNF transcript levels, Scalzo and colleagues (Scalzo et al., 2010) have demonstrated that decreased BDNF levels are also detectable in serum of individuals with PD compared to healthy individuals and that concentrations were correlated with PD symptom severity (Scalzo et al., 2010) (**Figure 2.4a**). However, as the disease progresses, BDNF levels have been shown to increase (Knott et al., 2002; Scalzo et al., 2010; Ventriglia et al., 2013), thought to be a compensatory mechanism in later disease states.

In addition to changes in BDNF in PD, expression of TrkB receptors, which have high expression in SNpc neurons (Jin, 2020), has been shown to be altered in individuals with PD with evidence of isoform-specific alterations. For instance, levels of truncated TrkB have been reported to decrease in axons of the striatum, whereas levels were reported to increase in the striatal soma and distal dendrites of the SN in individuals with PD (Fenner et al., 2014). Full-length TrkB levels, in contrast, were found to be decreased in striatal neurites and in the cell soma of dendrites, but levels were

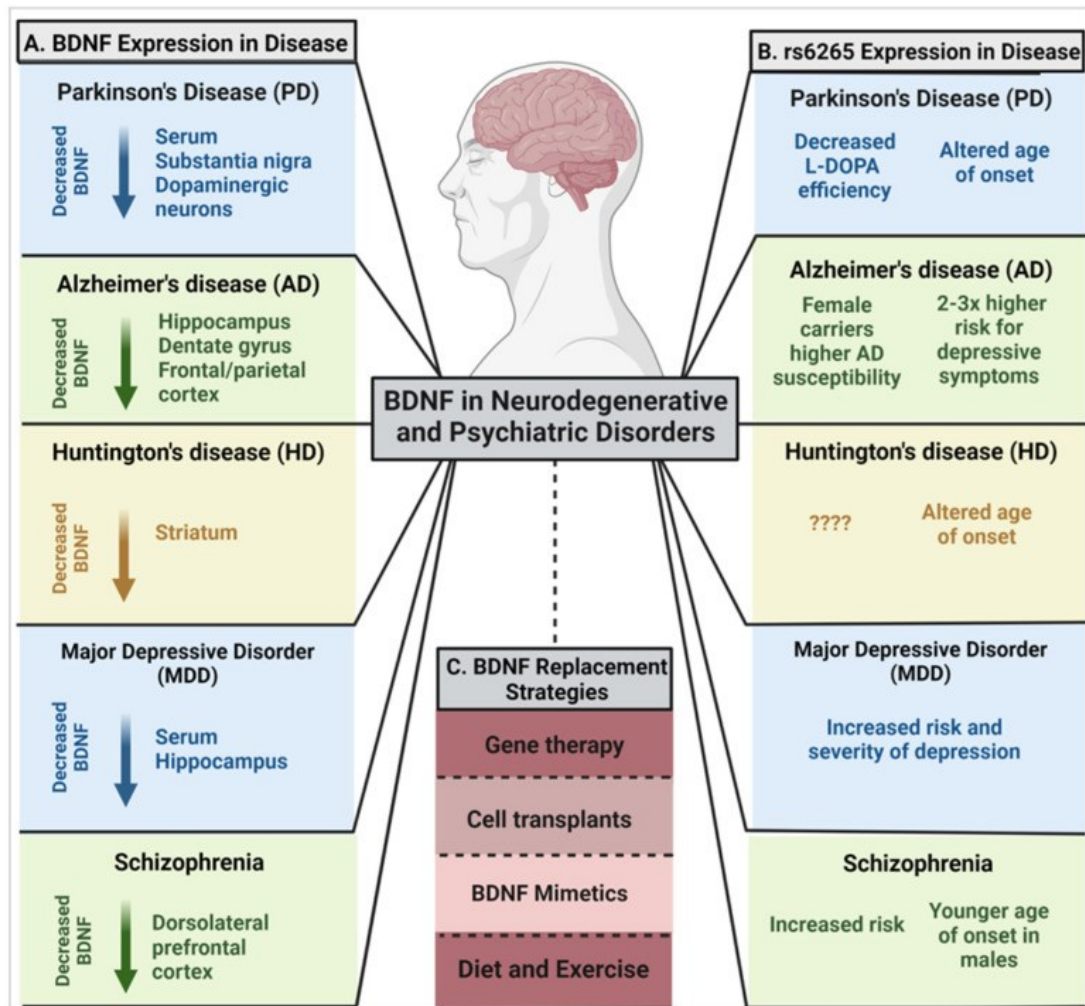


Figure 2.4: Summary of altered BDNF expression levels and consequences of the rs6265 SNP in neurodegenerative and psychiatric disorders.

(a) Decreased BDNF mRNA and protein expression in various regions of the brain in PD (Baquet et al., 2004; Howells et al., 2000; Y. Huang et al., 2018; Razgado-Hernandez et al., 2015; Scalzo et al., 2010), AD (Hock et al., 2000; Narisawa-Saito et al., 1996; Peng et al., 2005; Phillips et al., 1991), HD (Ferrer et al., 2000; Knott et al., 2002), MDD (Dwivedi et al., 2003; Januar et al., 2015; Lima Giacobbo et al., 2019; Molendijk et al., 2014; Pandey et al., 2008; Shimizu et al., 2003), and schizophrenia (Hashimoto et al., 2005; Reinhart et al., 2015; Weickert et al., 2003, 2005; Xiu et al., 2009). **(b)** Associations of rs6265 SNP expression and disease state including therapeutic efficacy, age of onset, and susceptibility to the disease: PD (Drozdzik et al., 2014; Fischer et al., 2018; Karamohamed et al., 2005b; Sortwell et al., 2021), AD (Borroni et al., 2009; Fukumoto et al., 2010; Laing et al., 2012), HD (Alberch et al., 2005), MDD (Hosang et al., 2014; Losenkov et al., 2020; Pei et al., 2012), Schizophrenia (H. M. Chao et al., 2008; Gratacòs et al., 2007; Kheirollahi et al., 2016; Suchanek et al., 2013; Z. Yi et al., 2011). **(c)** BDNF replacement strategies currently being implemented preclinically and clinically (reviewed in (Zuccato & Cattaneo, 2009)).

higher in cell somas and axons of the striatum and SNpc, respectively (Fenner et al., 2014; Mitre et al., 2017). These findings are corroborated in mouse models of PD where reduced levels of BDNF protein in the SNpc results in a reduction in DA neurons as well as a subsequent decrease in striatal DA (Baquet et al., 2004; Porritt et al., 2005). Further, haplo-insufficiency of the BDNF receptor, TrkB, in transgenic mice has been associated with degeneration of SNpc DA neurons over time and in association with aging (for review (Mercado et al., 2017)).

Utilizing BDNF as a Potential Therapeutic

Overall, BDNF levels are negatively correlated in neurodegenerative and psychiatric disorders (**Figure 2.4a**). Therefore, many BDNF-targeted therapies aim to raise the levels of BDNF either exogenously or endogenously. Exogenous application of BDNF through direct infusion has been demonstrated to be beneficial to varying degrees in numerous animal studies (Altar et al., 1994; Arancibia et al., 2008; Deng et al., 2016; Hung & Lee, 1996). As a neuroprotective agent in PD models against DA neuron toxins such as 6-OHDA or MPTP, BDNF is effective at protecting SH-SY5Y neuroblastoma neurons in vitro and can modestly protect against 6-OHDA in vivo (Altar et al., 1994). Despite promising outcomes from select research conducted in preclinical animal models, a large-scale clinical trial involving oral BDNF supplementation at dosages of 50–100 mg/day in patients with amyotrophic lateral sclerosis (ALS) did not significantly increase patient survival (Bradley, 1999). In a clinical trial involving intrathecal delivery of BDNF to ALS patients, doses of 150 mg/day were well tolerated; however, conclusions about treatment efficacy were unable to be drawn due to small sample sizes (Ochs et al., 2000). However, a later trial also using intrathecal BDNF for

ALS found a lack of clinical efficacy (Kalra et al., 2003). These disappointing clinical trial results could, in part, be due to the poor pharmacokinetics of BDNF.

The pharmacokinetics of neurotrophins are complex, making BDNF administration for brain therapeutics especially difficult. Neurotrophins are large, sticky molecules that cannot readily cross the blood-brain-barrier, have short half-lives reported to be 30 min or less (Habtemariam, 2018), inefficiently diffuse into tissues (Zuccato & Cattaneo, 2009), and approaches like intrathecal delivery result in broad exposure to nontargeted structures, thus limiting their scope of effectiveness (Zuccato & Cattaneo, 2009). If pharmacokinetic barriers could be overcome, consideration needs to be given to therapeutic concentrations of BDNF intended for delivery as well as the availability and status of TrkB receptors. Specifically, exogenous administration of BDNF in regions with significant reductions in TrkB expression, which is known to occur in PD and AD, could severely limit therapeutic benefit. In addition, excessive levels of BDNF could also have a negative impact. Not only can higher concentrations of BDNF downregulate TrkB expression, but excessive amounts of BDNF can lead to unwanted side effects such as seizures, fever, weight loss, fatigue, and diarrhea (Mitre et al., 2017). Molecularly, excess BDNF can likewise have a negative effect on synaptic circuitry, learning, and memory by inducing hyper-excitation in regions such as the hippocampus (Yeom et al., 2016). Keeping the above challenges in mind, non-pharmacological methods of BDNF delivery bear potential.

BDNF Gene- and Cell-Based Therapy

A promising non-pharmacological therapeutic technique is in vivo BDNF gene delivery. This technique involves utilizing viral vectors to transduce host cells with the

BDNF gene for downstream endogenous in situ mRNA and protein production. In this way, the high concentrations of local BDNF production in specific regions will ideally protect degenerating neurons in diseases such as PD, HD, and AD (Nagahara & Tuszynski, 2011). Preclinically, in a post-stroke depression rat model, intranasal delivery of a BDNF-encoding adeno-associated viral vector (AAV-BDNF) increased BDNF mRNA and protein in the prefrontal cortex, alleviating depressive-like symptoms (C. Chen et al., 2020). Additionally, preventative intrastriatal injections of AAV-BDNF reduced the loss of NeuN, a pan neuronal marker, in a lesioned rat model of HD, therefore providing neural protection (Kells et al., 2004). Although clinical trials of gene therapy that intended to supplement another neurotrophic factor (i.e., GDNF or neurturin) for neuroprotection against PD have been conducted, results are not yet promising (Manfredsson et al., 2020; Marks et al., 2010; Merola et al., 2020). Moreover, it remains unknown if it is clinically viable to target low BDNF levels in neurodegenerative or psychiatric disorders via gene therapy.

Another available BDNF-targeting gene therapy involves an ex vivo autologous approach for neuroregeneration. Briefly, cells such as fibroblasts are taken from the subject, genetically modified to produce BDNF, and then transplanted back into the cell donor's brain. Like in vivo methods, this strategy could allow for the sustained release of BDNF locally in specific brain regions but advantageously would be poised to avoid immune rejection. Levivier and colleagues showed that genetically modified fibroblasts were able to prevent degeneration induced by 6-OHDA in a rat model of PD (Levivier et al., 1995). Likewise, in a quinolinic acid toxin model of HD, rat fibroblasts were genetically engineered to produce BDNF and transplanted back into the rat brain,

resulting in the protection of striatal neurons as compared to control animals (Kells et al., 2004). Similarly, mesenchymal stem cells (MSCs) genetically altered to overexpress BDNF have been shown to reduce neuropathological and behavioral deficits in rodent models of HD, suggesting that these approaches have considerable potential for clinical use (for review (Crane et al., 2014)).

Gene therapy, whether viral vector-mediated or autologous transplantation of genetically modified cells, holds strong promise but is not without caveats (Baum et al., 2003, 2004). In general, local release of BDNF is difficult to tightly regulate genetically, and as introduced above, overproduction of BDNF can be detrimental to the circuitry of the brain (Yeom et al., 2016; Zuccato & Cattaneo, 2009). In addition, both approaches involve invasive surgical protocols; however, in the scope of neurosurgery methods that are much more aggressive (e.g., tumor resection), the approach for vector or cell graft delivery is minimally invasive and straightforward. Of additional concern is immune response to viral vectors and the associated products of foreign transgenes (Bulaklak & Gersbach, 2020). However, as recently reviewed, current efforts and advances in clinical trials have led to advances to circumvent immune obstacles including modifying AAV capsids to evade pre-existing neutralizing antibodies and development of new methods for clearing of antibodies from circulation (for review (Bulaklak & Gersbach, 2020)). With the advent of new DNA modification techniques, it is not beyond the realm of possibilities that novel gene therapy approaches could be applied in the future. In addition, given that ex vivo autologous treatment was well tolerated, and symptom improvement was demonstrated in AD (Nagahara & Tuszynski, 2011; Tuszynski et al.,

2005), this approach remains hopeful to those suffering from neurodegenerative or neurological disorders.

BDNF Mimetics

One of the most promising BDNF-related administration strategies involves the use of BDNF mimetics. These are small molecules designed to mimic the binding loops of BDNF, resulting in the phosphorylation and activation of TrkB and its downstream effectors, AKT and ERK (Du & Hill, 2015; Kazim & Iqbal, 2016; Zuccato & Cattaneo, 2009). The use of small molecules allows for the delivery of controlled dosages with improved pharmacokinetics compared to full-length BDNF. Mimetics have shown improved diffusivity, blood-brain-barrier permeability, and augmented receptor specificity with less promiscuity (Cardenas-Aguayo et al., 2013; Du & Hill, 2015; Kazim & Iqbal, 2016; Zainullina et al., 2021). These compounds, however, would require repeat dosing and would not be brain region-specific in targeting, potentially trafficking to areas where their engagement is not advantageous (Kazim & Iqbal, 2016; Longo & Massa, 2013).

Two common BDNF mimetics are 7,8-dihydroxyflavone (DHF) and GSB-106. 7,8-DHF is a naturally occurring flavonoid responsible for binding and initiating TrkB signaling pathways. 7,8-DHF application has been investigated in many neurodegenerative and neurological disorders including PD and AD (Bollen et al., 2013; Devi & Ohno, 2012; Jang et al., 2010). For example, in a comprehensive report by Jang and colleagues (Jang et al., 2010), 7,8-DHF was documented in mice to specifically activate TrkB in the brain, to diminish kainic acid-induced toxicity in the hippocampus, to decrease infarct volumes in a middle cerebral artery occlusion model of stroke, and it was neuroprotective in a MPTP model of Parkinson's disease (Jang et al., 2010).

Additionally, in a mouse model of AD, cognitive deficits were restored after 7,8-DHF administration (Bollen et al., 2013; Devi & Ohno, 2012). Collectively, these studies support the idea that 7,8-DHF may be a therapeutic mimetic worth implementing in a wide range of disorders.

Another common mimetic is bis-(N-monosuccinyl-L-seryl-L-lysine) hexamethylenediamide, also referred to as GSB-106, and it mimics the interaction between the TrkB receptor and BDNF via loop 4 of BDNF. Like 7,8-DHF, GSB-106 administration elicits neuroprotective properties by preventing apoptosis in SH-SY5Y cells through the suppression of caspase-3 activity (Zainullina et al., 2021). As reviewed in (Gudasheva, Povarnina, et al., 2021), GSB-106 has also been shown to have a variety of TrkB-mediated neuroprotective effects as well as reduce depressive-like symptoms in a mouse model of depression where administration increased locomotor activity and reduced signs of anhedonia (Gudasheva, Povarnina, et al., 2021; Gudasheva, Tallerova, et al., 2021). Studies focused on these two BDNF mimetics demonstrate that these small molecules represent potentially useful treatment approaches for those with neurodegenerative diseases such as PD. Continued preclinical and clinical development are needed so that their therapeutic effects can be optimized to the greatest extent.

Diet and Exercise

Diet and exercise are widely accessible, non-invasive, low-cost treatments that are of interest for neurodegenerative and neurological conditions. Preclinical studies in various animal models confirm that dietary and exercise regimens increase BDNF levels in the brain and improve cognitive and behavioral functions (Duan et al., 2001;

Fahnestock et al., 2012; Maswood et al., 2004; Mattson et al., 2002; Zuccato & Cattaneo, 2007, 2009). For example, Fahnestock and colleagues (Fahnestock et al., 2012) demonstrated that implementing a diet high in antioxidants in aged dogs increased BDNF (Fisher et al., 2008; Herman et al., 2007; Stuckenschneider et al., 2016) transcripts to levels which were comparative to the young dog cohort (Fahnestock et al., 2012). Additionally, restricting the diet of 3-month-old male Sprague Dawley rats to an alternate day feeding regimen compared to ad libitum increased BDNF levels in multiple brain regions including the cortex, striatum, and hippocampus (Duan et al., 2001). There also is a wealth of data suggesting that exercise provides neuroprotection in multiple animal models of PD (Fredriksson et al., 2011; Lau et al., 2011; Petzinger et al., 2007; Tajiri et al., 2010; Toy et al., 2014; Tuon et al., 2012; Wu et al., 2011) with additional indications that it improves motor symptoms and quality of life in individuals with PD. Studies using heterozygous deletion of BDNF (Gerecke et al., 2012) or inhibition of BDNF TrkB receptors (Real et al., 2013) demonstrate that BDNF is essential for the beneficial effects of exercise on the neuroprotection of the nigrostriatal DA system in PD rodent models.

In patients with depression, exercise was found to induce significant increases in serum levels of BDNF levels in all assessed participants (Szuhany et al., 2015). After sprint interval training, BDNF levels were increased directly afterward, then returned to baseline within 90 minutes in eight male subjects (Reycraft et al., 2020). A number of genes, including BDNF, are associated with risk for post-traumatic stress disorder (PTSD) (Voisey et al., 2019). Intriguingly, in combat veterans with PTSD, active exercise reduced methylation of the BDNF gene at specific CpG sites, resulting in normalized

gene expression of BDNF as compared to those without active exercise (Voisey et al., 2019). Although there are many studies reporting that diet and exercise lead to increased BDNF levels (Duan et al., 2001; Fahnestock et al., 2012; Reycraft et al., 2020), the specific mechanisms responsible have yet to be elucidated. In the context of BDNF as a therapeutic target, understanding and harnessing the benefits of diet and exercise on BDNF function could lead to vital non-invasive treatments geared toward improving not only neurodegenerative or psychiatric conditions but general patient quality of life.

Genetic Polymorphisms of BDNF

Remarkably, more than one hundred polymorphisms have been described in the BDNF gene (Tudor et al., 2018; Urbina-Varela et al., 2020). While many known variants exist within non-coding regions, understanding of their functional consequences remains limited. However, the most extensively studied SNP is the Val66Met (G196A, rs6265) polymorphism within the prodomain region of the BDNF gene. Other less well-studied variants exist within this region including Thr211e (rs8192466), Gln75His (rs1048221), Arg125Met (rs1048220), and Arg127Leu (rs1048221) and are reviewed elsewhere (R. Huang et al., 2007; Notaras & van den Buuse, 2019; Shen et al., 2018; Urbina-Varela et al., 2020).

rs6265 (Val66Met)

The rs6265 BDNF SNP, or Val66Met, results from a nucleotide exchange from guanine to adenine at position 196 (G196A). This change results in a substitution of valine to methionine at codon 66, thus referred to as Val66Met (Anastasia et al., 2013) (see **Figure 2.2c**). An individual can be heterozygous (Val66Met) or homozygous

(Met66Met) for this SNP. The prevalence of this SNP worldwide is approximately 20%, with certain populations in East Asia reporting an incidence up to 72% (Mercado et al., 2021; Petryshen et al., 2010; Tsai, 2018). Found in the prodomain region of the BDNF gene, this substitution creates binding interference between the BDNF prodomain/pro-peptide of proBDNF to sortilin. The consequential result, and subsequent hallmark of this polymorphism, is a decrease in activity-dependent release of BDNF, with no reported alterations in constitutive release (Egan et al., 2003; Urbina-Varela et al., 2020). The reduction in BDNF release is dose-dependent with homozygous subjects showing significantly less release compared to heterozygous subjects (Met/Met > Val/Met > Val/Val) (Mercado et al., 2021).

Several studies have documented a variety of neuropathologies associated with the decrease in secreted mBDNF linked to rs6265 including reduction of hippocampal and cortical volume, abnormal synaptic connections, and decreased dendritic complexity and arborization (Z. Y. Chen et al., 2006; Chiaruttini et al., 2009; Egan et al., 2003; Frodl et al., 2006; Y. Lee et al., 2013; Mercado et al., 2021). The functional consequences of this common genetic variant are wide-reaching and have been documented to impact memory and cognition, anxiety, and depression, and have been associated with obsessive compulsive disorder (OCD), attention deficit hyperactivity disorder (ADHD), schizophrenia, multiple sclerosis (MS), blepharospasm, and migraines (Cai et al., 2017; Z. Y. Chen et al., 2006; Di Carlo et al., 2019; Egan et al., 2003; Frodl et al., 2006; Y. Lee et al., 2013; Mei et al., 2022; Shang et al., 2022; Siokas et al., 2019). Such pathology may be linked to evidence demonstrating that the BDNF Val66Met substitution can result in binding disruption of the translin/trax complex to BDNF mRNA

transcripts, subsequently compromising transport of transcripts to dendrites which is critical for synaptic plasticity and dendritic complexity (Chiaruttini et al., 2009; Cohen-Cory et al., 2010; Notaras & van den Buuse, 2019). As a consequence, decreased BDNF trafficking to dendrites may have negative implications in multiple neurodevelopmental and neurological disorders (Chiaruttini et al., 2009; Di Carlo et al., 2019).

In addition, decreased BDNF levels/signaling (i.e., rs6265) have been implicated in several neurodegenerative disorders including AD, PD, and HD (**Figure 2.4b**). How the expression of this common human genetic variant impacts PD is highlighted below as the aforementioned brain maladies (e.g., AD, HD) are beyond the scope of this thesis research. Please see **Figure 2.4** for more details regarding BDNF in these other neurodegenerative and neurological disorders.

rs6265 and PD

Although expression of the BDNF rs6265 Met allele is not correlated with an increased incidence of PD, it may contribute to worsening non-motor symptomology (Fedosova et al., 2021; Gorzkowska et al., 2021; Shen et al., 2018). For example, apathy is one of the most common non-motor neuropsychiatric symptoms of PD (Gorzkowska et al., 2021), and although not statistically significant, PD individuals who were homozygous for the Met allele (i.e., Met/Met) were reportedly more likely to display apathetic emotions compared to those without the Met/Met genotype. Moreover, the risks of impulsive-compulsive and related behavioral disorders are also statistically correlated in individuals with PD when expressing the rs6265 SNP (Fedosova et al., 2021).

An important distinction of Met allele carriers with PD has been in their response to certain pharmacotherapies including levodopa treatment (see full discussion above in the “Heterogeneity” section). Specifically, it has recently been reported that Met allele carriers, homozygous or heterozygous, reported worse UPDRS scores when administered levodopa monotherapy compared to their homozygous Val allele carrier counterparts (Fischer et al., 2020; Sortwell et al., 2021). Individuals expressing the Met allele were also found to have a higher risk of developing the often debilitating side effect known as LID earlier in their treatment compared to homozygous Val allele carriers (Drozdziak et al., 2014; Foltynie et al., 2009).

To contrast these negative correlations of the Met allele, in unmedicated PD patients, a lower severity of motor symptoms has been observed in the initial stages of the disease in BDNF variant individuals (Fischer et al., 2018). Although homozygous Met-allele carriers tended to have more tremor-like symptoms, the progression of the disease was slower, with delayed need for levodopa administration compared to Val allele carriers (Fischer et al., 2018). Along with this notable decrease in severity of motor symptoms, a later age of onset of PD was reported in homozygous Met allele individuals compared to their Val/Val and Val/Met counterparts with one cohort reporting a 5.3-year later age of onset (Białecka et al., 2014; Karamohamed et al., 2005a) (**Figure 2.4b**). In contrast, Svetel et al., 2013 reported that the presence of the Met allele was not associated with clinical characteristics of PD including age of onset and disease severity (Svetel et al., 2013).

HETEROGENEITY IN SIDE EFFECT LIABILITY OF CELL TRANSPLANTATION

GID and the rs6265 BDNF SNP

As discussed previously in Chapter 1, a subpopulation of patients developed graft-induced dyskinesia (GID) as a side effect following primary DA neuron transplantation in clinical trials for PD (Freed et al., 2001; Olanow et al., 2003). Now, after decades of rigorous preclinical research following the enacted moratorium in the early 2000s (Hagell & Cenci, 2005), several clinical grafting trials for PD are now planned or ongoing (Barker et al., 2019); example clinical trial identifiers NCT04802733, NCT01898390, NCT03309514, NCT03119636, NCT04146519). A comprehensive list of the current planned/ongoing clinical cell transplantation trials are listed in **Table 1.1** in Chapter 1. While these experiments have strived to optimize patient selection (i.e., age, disease severity, cell preparation) prior to transplantation (Barker et al., 2024), the underlying mechanisms of aberrant GID behavior remain, to this day, unknown. Until GIDs are addressed, neural grafting for PD will not be considered a safe or optimized therapeutic option for PD patients. For a comprehensive discussion of the postulated mechanisms underlying GID behavior, please see Chapter 1.

Goals of Current Research

Because the underlying mechanisms of GID remain a gap in our knowledge, taking the necessary actions to fully understand its underlying pathology is the first step in developing a precision-medicine approach for neural therapy. The rs6265 SNP, which has been implicated in clinical outcomes for levodopa treatment (Fischer et al., 2020), and now cell-based therapy (e.g., (Mercado et al., 2021, 2024), points to the rationale for continuing research in this area. Therefore, the overarching hypothesis for my

dissertation research centers around a probable role for the rs6265 SNP in the underlying mechanisms responsible for the substantial heterogeneity demonstrated in grafted patients with PD (i.e., GID development). Indeed, while my predecessor, Dr. Natosha Mercado, successfully demonstrated that DA-grafted homozygous rs6265 (Met/Met) parkinsonian rats exhibit enhanced functional recovery following engraftment of WT DA neurons (i.e., earlier and more robust amelioration of LID), she conversely demonstrated that DA-grafted Met/Met parkinsonian rats uniquely develop aberrant GID compared to WT subjects (Mercado et al., 2021). In order to further her investigations into the benefit and detriment of the rs6265 SNP, I endeavored to:

- (1) examine additional host/donor genotype combinations and their impact on graft-derived efficacy and side effect liability (i.e., GID) (Chapter 3) and
- (2) investigate whether exogenous intrastriatal administration of BDNF would replenish the decreased BDNF release in rs6265 Met/Met carriers, induce maturation/integration of grafted DA neurons, and ameliorate GID (Chapter 4).

Considering that BDNF plays a crucial role in proper synapse formation and maturation of DA neurons (Gonzalez et al., 2016; Kowiański et al., 2018; Liu et al., 2018; Park & Poo, 2013; Sasi et al., 2017; Urbina-Varela et al., 2020; Zagrebelsky et al., 2020), it is biologically reasonable to hypothesize that aberrant and/or immature synaptic connectivity between host and donor, permitted by a decrease in activity-dependent BDNF release (i.e., rs6265), underlies GID behavior. Specifically, as introduced in Chapter 1, Soderstrom and colleagues previously demonstrated that GID development in DA-grafted parkinsonian rats was associated with atypical, asymmetric (presumed glutamatergic) synaptic connections made by the grafted DA neurons

(Soderstrom et al., 2008). Moreover, Dr. Mercado further showed that the DA-grafted Met/Met parkinsonian rats that developed GID behavior demonstrated expression of vesicular glutamate transporter 2 (VGLUT2) in grafted DA neurons, indicative of an immature graft phenotype, and showed immunohistochemical evidence of atypical glutamatergic synapse formation.

Using these findings as a basis for my thesis research, I will provide evidence in the next two chapters demonstrating that the homozygous rs6265 (Met/Met) genotype, whether found in the host or donor, confers a degree of graft-derived benefit; however, the Met/Met parkinsonian hosts engrafted with WT DA neurons remain the only host/donor combination to exhibit significant GID behavior (Chapter 3). Additionally, I will also demonstrate that, contrary to my hypothesis that BDNF supplementation would promote graft maturation and reduction of GID, BDNF supplementation instead exacerbated GID behavior in the Met/Met hosts engrafted with WT DA neurons (Chapter 4). Finally, my research provides evidence in support of the contention that dysregulated DA/glutamate co-transmission and/or excess DA release appear to contribute to GID induction.

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**CHAPTER 3: PRECISION MEDICINE IN PARKINSON'S DISEASE: HOST/DONOR
INTERACTIONS AND GRAFT-INDUCED DYSKINESIA LIABILITY IN HOMOZYGOUS
rs6265 (MET/MET) BDNF PARKINSONIAN RATS**

ABSTRACT

Transplanting replacement dopamine (DA) neurons remains of worldwide interest as an experimental treatment for Parkinson's disease (PD). However, like other PD therapies, heterogeneity in clinical responsiveness exists. To deconstruct this variability, our laboratory focuses on the common single nucleotide polymorphism (SNP), rs6265, present in the brain-derived neurotrophic factor (BDNF) gene. Our group previously reported that homozygous rs6265 (Met/Met) knock-in parkinsonian rats engrafted with embryonic wild-type (WT) DA neurons demonstrate paradoxical enhancement of graft function compared to their WT counterparts but uniquely develop the side effect known as graft-induced dyskinesia (GID). To expand our understanding of the impact of rs6265 in DA neuron transplantation, I have examined the effect of rs6265 in both host and donor as part of my thesis research. Results indicate that functional benefit continues to occur more rapidly in the presence of the Met allele regardless of whether found in the host or donor. Curiously, Met/Met hosts engrafted with WT DA neurons remain the only group to exhibit significant GID behavior.

INTRODUCTION

Parkinson's disease is a relentlessly progressive neurodegenerative disorder that continues to place an immense burden on society (Dorsey et al., 2018; Straccia et al., 2022; Yang et al., 2020). At its current growth rate, it is estimated that approximately 13 million people will be diagnosed with PD by the year 2040 (Dorsey et al., 2018; Straccia et al., 2022). To treat the symptoms of PD, several pharmacological options are available including anticholinergic agents, DA agonists such as levodopa, monoamine oxidase inhibitors (MAOIs), and catechol-O-methyltransferase (COMT) inhibitors (Stoker & Barker, 2020). Despite the extensive competition, levodopa remains the most tolerated and effective pharmaceutical intervention for motor symptoms of PD, even after over six decades (Cotzias et al., 1967; Nutt & Wooten, 2005; Poewe et al., 2010; Stoker & Barker, 2020).

Levodopa therapy, however, is not without limitations. While levodopa works well for PD patients for some time, individuals eventually are plagued with significant side effects (i.e., levodopa-induced dyskinesia (LID)) and waning efficacy as their disease progresses. Indeed, based on the results from a large retrospective analysis (Earlier versus Later Levodopa therapy in PD study; ELLDOPA), patients reported a range of responses to levodopa administration, from 100% improvement to a 242% worsening of symptoms assessed by the United Parkinson's Disease Rating Scale (UPDRS) (Hauser et al., 2009). Collectively, the scientific community has recognized that PD is a complicated and heterogeneous disease with substantial variability in clinical responsiveness to existing therapeutic interventions.

An alternative approach aimed at mitigating the heterogeneous nature of several PD therapies is the regenerative approach of DA neuron transplantation. Currently, the method that has had the most success clinically is primary embryonic ventral mesencephalic (eVM) DA neuron transplantation into the caudate/putamen (Olanow et al., 2009; Steece-Collier & Collier, 2016; Stoker et al., 2017). Unfortunately, similar to the heterogeneity demonstrated with levodopa administration, variability in clinical responsiveness also exists following cell transplantation. Further, a subpopulation of patients who received eVM transplants developed a novel dyskinetic side effect known as GID (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). The mechanisms underlying GID behavior remain unknown and controversial.

While the field has historically gained understanding of the role of global risk factors (e.g., age, disease severity) in response to cellular transplantation, the role of genetic risk factors has been relatively unexplored until two recent studies conducted by our group (Mercado et al., 2021, 2024). We explored the common SNP, rs6265, found in the BDNF gene, which results in the decrease of activity-dependent release of BDNF (Chen et al., 2005; Egan et al., 2003; Urbina-Varela et al., 2020). Also referred to as Val66Met, the rs6265 SNP involves a valine to methionine substitution at codon 66 (Anastasia et al., 2013). Although not correlated with an increased incidence of PD (Fedosova et al., 2021; Gorzkowska et al., 2021; Mariani et al., 2015; Shen et al., 2018), rs6265 has been shown to reduce the therapeutic efficacy of levodopa in PD patients (Drozdziak et al., 2014; Fischer et al., 2020; Foltynie et al., 2009; Sortwell et al., 2021). In the general worldwide population, the prevalence of rs6265 is approximately 20%; however, in certain East Asian populations, prevalence can reach 72% (Petryshen

et al., 2010; Tsai, 2018). The impact of rs6265 leads to a substantial decrease in BDNF release (Egan et al., 2003; Urbina-Varela et al., 2020) in roughly 20% of the general population.

Due to the critical role BDNF plays in promoting dendritic spine growth, synapse formation, and maturation of DA neurons (Gonzalez et al., 2016; Kowiański et al., 2018; Liu et al., 2018; Park & Poo, 2013; Sasi et al., 2017; Urbina-Varela et al., 2020; Zagrebelsky et al., 2020), our group previously hypothesized that the rs6265 SNP underlies the variability (e.g., GID) in clinical response to DA neuron transplantation in PD patients. Using a CRISPR rs6265 knock-in parkinsonian rat model, we demonstrated that homozygous rs6265 (aka Met/Met) parkinsonian rats engrafted with WT (Val/Val) DA neurons paradoxically exhibited enhanced neurite outgrowth and functional recovery compared to WT subjects. However, WT-grafted Met/Met rats uniquely demonstrated significant GID induction compared to WT hosts engrafted with cells from the same source (i.e., WT DA neurons). Interestingly, GID behavior was strongly correlated to expression of vesicular glutamate transporter 2 (VGLUT2), a marker of immature DA neurons, in Met/Met host parkinsonian rats (see (Mercado et al., 2021)).

Because of the relatively high prevalence of rs6265 in the general population, and because only WT DA neuron grafts have been studied, I endeavored to investigate functional outcomes of DA transplantation in both the host and donor carrying the rs6265 allele. Accordingly, both WT and Met/Met hosts engrafted with either WT or Met/Met donor neurons were studied to uncover a potentially optimal host/donor combination that would exhibit superior functional benefit with limited side effect liability.

Since enhanced functional benefit of the Met allele was reported previously (Barbey et al., 2014; Finan et al., 2018; Kailainathan et al., 2016; Krueger et al., 2011; McGregor et al., 2019; McGregor & English, 2019; Mercado et al., 2021; Qin et al., 2014; Voineskos et al., 2011; Zivadinov et al., 2007), we hypothesized that the Met/Met hosts and/or donor neurons would induce significant graft efficacy but also develop the highest GID severity. In this study, we report that the homozygous Met/Met genotype, whether found in the host or donor, produces a modest, but significant, enhanced behavioral benefit (i.e., earlier amelioration of LID) compared to WT hosts engrafted with WT donor neurons, indicating that the Met allele does indeed retain a mechanistic benefit in a DA-grafted parkinsonian rat model similar to our first report (Mercado et al., 2021). Unexpectedly, the Met/Met parkinsonian recipients of WT DA grafts remained the only host/donor combination to develop significant GID compared to all other grafted host/donor groups. While a correlation between VGLUT2 expression and GID severity was no longer apparent as in our previous experiment (Mercado et al., 2021), evidence collected from this study suggests that there is a possible complex association between DA release and GID behavior, warranting further investigation into this phenomenon as a promising underlying mechanism of GID.

METHODS

Animals

Sprague-Dawley homozygous rs6265 (i.e., Met/Met) male rats (8-9 months at lesioning, 13-14 months at sacrifice) were utilized from our in-house colony derived from CRISPR knock-in rats generated by Cyagen Biosciences (Santa Clara, CA). These knock-in rats carry the valine to methionine substitution in the rat BDNF gene (Val68Met). Equivalent to the human Val66Met SNP, the rat rs6265 SNP is located at codon 68 (Val68Met) because rats have two additional threonine amino acid residues. Moreover, the rat BDNF gene has a 96.8% sequence homology with the human BDNF gene (BLAST queries: P23560 and P23363). For this study, both WT and homozygous rs6265 Met/Met rat hosts were employed. The Michigan State University Institutional Animal Care and Use Committee approved all animal experimental procedures.

Eight animals were excluded due to failed lesion surgeries. Additional animals (N=5) were excluded a priori (i.e., prior to grafting) due to failure to develop effective LID prior to cell transplantation. Following postmortem analysis of the transplanted grafts, a small number (N=4) of grafted rats were excluded for having few surviving grafted cells (<100) or misplaced/cortically-placed grafts. One animal was excluded from analysis as a biological outlier (i.e., having a “hotspot” graft) (Maries et al., 2006). Final experimental cohorts included N=7 (WT host/sham-graft), N=8 (Met/Met host, sham-graft), N=7 (Met/Met host, WT graft), N=9 (Met/Met host, Met/Met graft), N=7 (WT host, Met/Met graft), and N=6 (WT host, WT graft) (see **Figure 3.1**).

Experimental Design and Timeline

As shown in the experimental timeline schematic (**Figure 3.1**), rats were rendered unilaterally parkinsonian by an injection of the DA neurotoxin, 6-hydroxydopamine (6-OHDA), delivered stereotactically to the substantia nigra pars compact (SNpc) and medial forebrain bundle (MFB). Two weeks following stereotaxic lesioning surgeries, amphetamine-mediated rotational behavior was analyzed to confirm the lesion status of each animal subject. Following lesion confirmation, successfully lesioned rats were primed with daily (M-F) levodopa two weeks later to generate significant and stable LID, our primary behavioral measure of graft function (i.e., amelioration of LID). Levodopa priming lasted for a total of four weeks, after which rats underwent neural transplantation surgery. Rats received intrastrially placed embryonic VM DA neurons from WT (Val/Val), rs6265 (Met/Met), or sham-grafted (cell-free) donors. Immediately following transplantation surgeries, levodopa was withdrawn for one week but then reinitiated for the remainder of the experiment. For a total of 10 weeks following engraftment, parkinsonian rats were evaluated for amelioration of LID behavior, rated every two weeks. At 10 weeks post-engraftment, amphetamine-mediated rotational behavior was measured as a secondary assessment of graft function. As an indication of graft dysfunction, total and peak (70 minutes) amphetamine-mediated GID behavior was evaluated at 10 weeks 24 hours after final LID assessment.

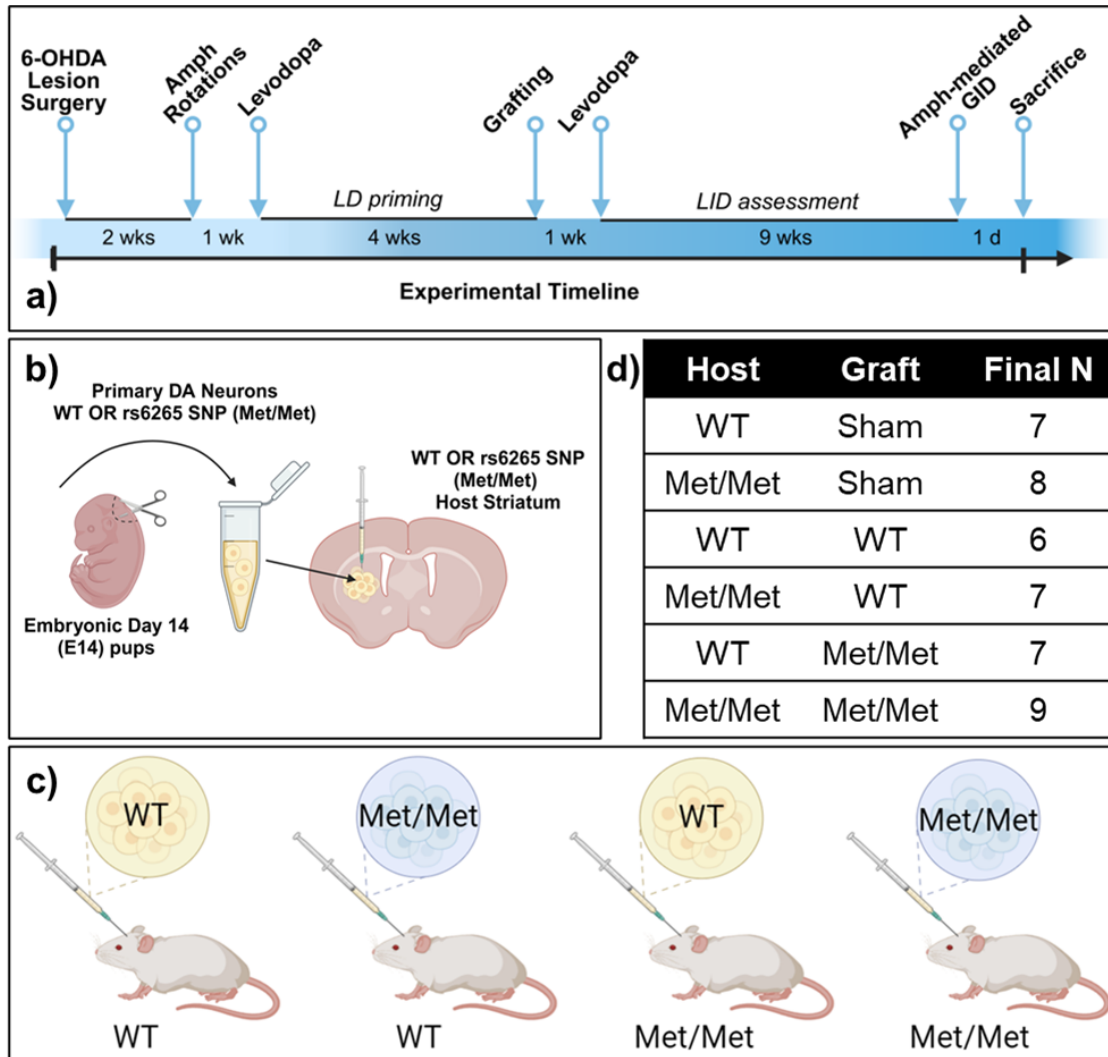


Figure 3.1: Experimental timeline and design.

(a) Timeline of lesion and grafting surgeries, behavioral assessment, and drug administration. **(b)** Schematic diagram demonstrating cell transplantation of embryonic day 14 (E14) ventral mesencephalic (eVM) tissue from either WT (Val/Val) Sprague-Dawley male rats. eVM tissue was dissected and transplanted into either WT or homozygous rs6265 (Met/Met) host parkinsonian rats. **(c)** Experimental schematic depicting the various host/donor combination groups following cell transplantation. **(d)** Table including the genotype of the graft, donor, and final group sizes. Abbreviations: 6-OHDA = 6-hydroxydopamine, amph = amphetamine, LD = levodopa, LID = levodopa-induced dyskinesia, GID = graft-induced dyskinesia, WT = wild-type, wk = week.

Nigrostriatal 6-OHDA Stereotaxic Surgery

Rats were anesthetized with 2% isoflurane (Sigma St. Louis, MO, USA) and positioned in a stereotaxic frame. A total of 2 μ L of 6-OHDA was administered at a flow rate of 0.5 μ L/min to the SNpc (coordinates of 4.8 mm posterior, 2.0 mm lateral, and 8.0 mm ventral relative to bregma) and the MFB (coordinates of 4.3 mm posterior, 1.6 mm lateral, and 8.4 mm ventral relative to bregma). Immediately following lesion surgery, rats were given an intraperitoneal (i.p.) injection of 5 mg/kg carprofen (Rimadyl) for pain relief. Histological confirmation of successful nigral lesions was performed postmortem using the stereological medial terminal nucleus (MTN) DA cell enumeration method (Gombash et al., 2014).

Amphetamine-mediated Rotational Behavior

As a method to assess lesion status after stereotaxic 6-OHDA lesion surgeries, as well as graft function (LID) and dysfunction (GID; see below) following grafting surgeries, amphetamine-induced rotational behavior was employed since it is a reliable measure of both nigrostriatal DA depletion and function of the graft (e.g., (Collier et al., 1999, 2015; Dunnett & Torres, 2011; Soderstrom et al., 2008)). Two weeks following stereotaxic lesion surgeries, amphetamine rotations were assessed to confirm lesion status in each rat subject. Amphetamine sulfate (2.5 mg/kg) was administered i.p. into each rat, and rotational behavior was subsequently recorded for 90 minutes with the automated Rotameter System (TSE-Systems, Chesterfield, MO, USA). In order to be included for the continuation of the experiment, rats were required to rotate ≥ 5 ipsilateral rotations per minute. Additionally, at 10 weeks post-engraftment, amphetamine rotations were manually quantified at one-minute time intervals in the rat's home cage at 70

minutes post-amphetamine injection during the assessment of GID behavior as a secondary readout of graft function.

Levodopa Administration and LID Ratings

For a total of four weeks following 6-OHDA lesion surgeries, rats were primed with daily (M-F) levodopa (12 mg/kg levodopa/benserazide (1:1); subcutaneous) administration. One week after neural transplantation surgeries, levodopa was withdrawn from rat subjects in order to prevent any possible toxic interactions between levodopa and the grafted DA neurons (Steece-Collier et al., 1990). After the one-week interval of no levodopa administration, levodopa was introduced again daily (M-F) throughout the remainder of the study.

We employed a well-established rat LID model as a measure of graft function as this behavioral side effect can be improved by dopaminergic neuron grafts in both parkinsonian rats (Lane et al., 2006; Lee et al., 2000; Maries et al., 2006; Mercado et al., 2021; Soderstrom et al., 2008, 2010) and individuals with PD (Hagell & Cenci, 2005). LIDs were assessed on days 1, 7, 14, and 21 prior to grafting, and at five post-graft intervals including weeks 2, 4, 6, 8, and 10. The LID rating scale utilized was developed in our lab based on specific criteria aligned with attributes of dyskinesia (refer to (Caulfield et al., 2021; Maries et al., 2006)). A blinded investigator assessed LID behavior at one-minute intervals at 20, 70, 120, 170, and 220 minutes after levodopa administration, following the method previously detailed in (Mercado et al., 2021, 2024). A total LID severity score, determined as the sum of the severity and frequency of each assessed behavior, was calculated for each animal at each timepoint (Mercado et al., 2021, 2024).

Donor Tissue Preparation and Neural Cell Transplantation

After the completion of levodopa priming, rats were assigned to one of six groups based on pre-grafted LID severity scores. Rats were blindly and randomly assigned to a group in order to ensure that the average LID severity score was statistically similar between host/donor groups. The six host/donor combinations groups included two sham-grafted groups (WT or Met/Met hosts), Met/Met hosts engrafted with WT DA neurons (M/W), Met/Met hosts engrafted with Met/Met DA neurons (M/M), WT hosts engrafted with WT DA neurons (W/W) and WT hosts engrafted with Met/Met DA neurons (W/M). Rat hosts in each group received intrastriatal transplantations of 200,000 VM neurons from embryonic day 14 (E14) timed-pregnant donors corresponding to the assigned genotype. First, the VM was harvested in cold calcium-magnesium free (CMF) buffer, and the cells were dissociated according to our standard, previously reported protocol (Collier et al., 2015, and Mercado et al., 2021). Briefly, the tissue was incubated for 10 minutes at 37°C in CMF buffer containing 0.125% trypsin. Cells were then triturated with 0.005% DNase using a 2.0 mm tip Pasteur pipette, followed by further trituration with a sterile 3cc, 22-gauge syringe. The resulting cell suspension was layered onto sterile fetal bovine serum (FBS) and centrifuged at 1,000 rpm for 10 minutes at 4°C, then resuspended in 1.0 mL of Neurobasal medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cell number and viability were assessed using the trypan blue exclusion method, and the final concentration was adjusted to 33,333 cells/ μ L. Cells were kept on ice throughout the surgery and transplanted within five hours of preparation. The cells were injected into the striatum at a single rostral-caudal site (0.2 mm anterior, 3.0 mm lateral to bregma) and distributed at three dorsal-

ventral (DV) locations of 5.7, 5.0, and 4.3 mm ventral to the skull base (Collier et al., 2015; Mercado et al., 2021, 2024). A total of 2 μ L of the VM cell suspension was injected at each DV site (injected at 0.5 μ L/min), for a total of 6 μ L per rat. WT and Met/Met sham-grafted rats received cell-free Neurobasal™ medium using the same injection paradigm.

Graft-induced Dyskinesia (GID)

Low-dose amphetamine was implemented to assess GID, with rats receiving a single 2mg/kg dose of amphetamine sulfate (i.p.). The amphetamine-mediated GID behavioral method was utilized based on evidence that DA-grafted, but not sham-grafted, animals exhibit dyskinetic behaviors in response to low-dose amphetamine (Lane et al., 2009; Shin et al., 2012; Smith et al., 2012). A blinded investigator rated GID behavior in the same manner and using the same rating scale as described for LID since GID appears phenotypically similar to LID in DA-grafted rats. Amphetamine-induced GIDs were evaluated at 10 weeks post-engraftment since GID are only notable upon graft maturation. GID were examined following the final LID assessment.

The incidence of “total” GID severity and “peak” GID severity, which are both illustrated in **Figure 3.4**, are defined as the number of animals which exhibited a total GID severity score of 4 or higher (total) or a peak GID score of 2 or higher (peak). Peak GID incidence was observed at 70 minutes post-amphetamine administration. Total and peak GID incidence scores were determined in this way based on the fact that a score less than four (total) or two (peak) reflects stereotypic behavioral profiles that can occur in non-grafted/non-lesioned rats such as typical intermittent licking and chewing behavior.

Necropsy

Euthanasia of the rats was achieved as detailed previously (Mercado et al., 2021 and 2024). Briefly, rats were deeply anesthetized with phenytoin/pentobarbital (250 mg/kg; i.p., VetOne, Boise, ID, USA) followed by intracardiac perfusions with room temperature 0.9% saline (heparinized) and cold 4% paraformaldehyde. Following intracardiac perfusion, each brain was carefully removed and placed into a 4% paraformaldehyde solution. The brains remained in this solution for 24 hours at 4°C. Next, brains were then submerged into 30% sucrose (4°C); the brains remained in sucrose until sectioning. When sectioned, coronal cuts of the brains were made at a thickness of 40 µm using a sliding microtome. Cut tissue sections were stored at -20°C in cryoprotectant.

Histology

Tyrosine hydroxylase (TH) Immunohistochemistry for Stereological Quantification of Graft Cell Number and Volume

Tissue sections were rinsed in Tris-buffered saline containing 0.3% Triton-X (TBS-Tx), and then incubated in 0.3% hydrogen peroxide. Afterward, they were blocked with 10% normal goat serum (NGS) for 90 minutes. For primary antibody incubation, the sections were exposed overnight at room temperature to rabbit anti-TH (see **Table 3.1**). Following primary antibody incubation, the sections were rinsed, incubated with biotinylated goat anti-rabbit secondary antibody (**Table 3.1**), and then developed using the avidin/biotin enzyme complex and 3,3'-diaminobenzidine (DAB; 0.5 mg/mL).

A blinded investigator employed the Stereo Investigator® Optical Fractionator method (MBF Bioscience, Williston, VT, USA) to quantify the number of TH-positive

(TH+) cells in the grafted striatum. The 20x objective (numerical aperture 0.75) was utilized to count cells on a Nikon Eclipse 80i microscope with a 200 μm x 200 μm counting frame. The optical dissector height was set to 20 μm , with a 2.0 μm guard zone. This process was performed on 4-12 serial (1:6) TH+ sections, with the number of sections varying based on the rostral-caudal spread of the graft.

To estimate the total graft volume, a blinded investigator used the Stereo Investigator® Cavalieri Estimator on the same tissue sections described above. The central region of the graft was outlined, and a grid with random sampling points (50 μm spacing) was overlaid on the contours. The calculated total estimated graft volume is reported in mm^3 .

Stereological Quantification of Neurite Outgrowth

The Stereo Investigator® Spaceballs workflow was employed to stereologically determine the extent of graft-derived innervation in the host striatum. The TH+ immunolabeled tissue section that contained the largest portion of the graft was selected for analysis. Contours (345 μm x 265 μm) were manually drawn proximal and distal to the graft in four directions including medial, dorsal, lateral, and ventral. We defined the proximal region as 100 μm from the graft and the distal region measuring 700 μm from the edge of the graft (per (Mercado et al., 2021, 2024)); this generated a total of eight contoured measurement sites. Spaceballs was applied to each of the eight contours which generated random sampling sites throughout the contour. The spherical probe that the Spaceballs workflow employs had a radius measuring 5.0 μm with guard zones of 1.0 μm . A blinded investigator collected all neurite density measurements using the 60x oil immersion objective on the Nikon Eclipse 80i stereotaxic microscope. The

numerical aperture was 1.40. Data are reported as the estimated average neurite length per the volume of the probe ($\mu\text{m}/\text{mm}^3$) per grafted TH+ neuron (i.e., neurite density per grafted cell).

Immunofluorescence (IF)

Full series, DAB-developed TH-labeled sections as described above were used as a guide when choosing one representative grafted striatal section for each immunofluorescent and in situ hybridization assay. For all protein staining procedures, tissue sections were rinsed in TBS-Tx, blocked in 10% NGS/0.3% TBS-Tx, and then incubated overnight at 4°C. Tissue sections were then labeled with their respective Alexa Fluor™ secondary antibodies (1:500 dilution; see Table 3.1) for 90 minutes at room temperature, protected from light exposure. Sections were mounted and coverslipped with Vectashield® anti-fade mounting medium with DAPI (H-1500; Vector Laboratories, Inc. Burlingame, CA, USA).

Antibody	Assay	Primary ab	Vendor	Catalog #	Dilution	2° Ab
Tyrosine hydroxylase	TH-VGLUT2	Rabbit anti-TH	Millipore	AB152	1:4000	A-11008
Tyrosine hydroxylase	TH-DAT	Mouse anti-TH	Millipore	MAB318	1:1000	A-11032
Tyrosine hydroxylase	TH-Iba1-GFAP	Sheep anti-TH	Novus Biologicals	NB300-110	1:1000	A-11015
VGLUT2	TH-VGLUT2	Mouse anti-VGLUT2	Abcam	Ab79157	1:1000	A-21124
DAT	TH-DAT	Rabbit anti-DAT	Millipore	D6944	1:1000	A-32733
Iba1	TH-Iba1-GFAP	Rabbit anti-Iba1	FUJIFILM Wako Chemicals	019-19741	1:1000	A-32740
GFAP	TH-Iba1-GFAP	Mouse anti-GFAP	Millipore	MAB360	1:1000	A-21240

Table 3.1: Targeted Antigens with corresponding antibodies.

Secondary antibody catalog numbers are Alexa Fluor®-conjugated and purchased from Invitrogen®.

Fluorescent In Situ Hybridization (FISH) using RNAscope™

In order to examine the impact of the various host/donor genotypes on mRNA expression of the two common BDNF receptors, TrkB and p75^{NTR} (**Table 3.2**) within the grafted DA neurons and host striatum, RNAscope™ in situ hybridization was performed according to the manufacturer's instructions for the RNAscope™ Multiplex Fluorescent V2 Assay kit (Advanced Cell Diagnostics Inc., Hayward, CA, USA). Immunofluorescent staining for TH was followed by the completion of RNAscope™ to stain for grafted DA

neurons. Similarly, RNAscopeTM/TH-treated tissue sections were mounted and coverslipped using Vectashield® anti-fade mounting medium with DAPI (H-1500; Vector Laboratories, Inc. Burlingame, CA, USA).

RNA Target	Probe	Accession Number	Catalog Number
TrkB	Rn-Ntrk2-ver2	NM_012731.2	406381
p75 ^{NTR}	Rn-Ngfr	NM_012610.2	406351-C2

Table 3.2: RNA Targets and RNAscopeTM probes.

Fluorescent Image Acquisition

All fluorescent images (1024 x 1024) of immunofluorescent and in situ hybridization-treated tissue sections were acquired using a Nikon A1 laser scanning confocal microscope system that was equipped with a Nikon Eclipse Ti microscope and Nikon NIS-Elements AR software. For the TrkB/p75^{NTR}/TH protocol, the 20x magnification objective was employed to collect images of inside the graft, outside the graft, and the intact striatum of all animals. The 4x objective was used for dopamine transporter (DAT)/TH immunohistochemistry staining to allow collection of a full image of the entire graft in each striatal section. One image of the intact striatum was used for comparison. For both DAT/TH and TrkB/p75^{NTR} mRNA, 2 µm z-stacks with a scan speed of 1/8 frame/second were taken. Likewise, 2 µm z-stacks with a scan speed of 1/8 frame/second were taken of the tissue sections stained for Iba1/GFAP/TH. Z-stacks for Iba1/GFAP/TH were acquired using the 10x objective, and two images were taken to capture the entirety of the graft. Additional images of the intact striatum were taken for comparison. For the VGLUT2 colocalization inside TH+ neurons, the 60x oil-immersion objective (numerical aperture of 1.40) was used, and 1.5 µm-thick z-stacks were

acquired. The scan speed was 1/8 frame/second. Two representative images for this experiment were taken within the dorsolateral area of the DA graft, and one image was acquired of the intact striatum for comparison.

Imaris® Fluorescent Image Quantification

Triple-label protein mRNA analysis: TH protein and TrkB, p75^{NTR} mRNA

Three-dimensional (3D) images of the grafted tissue sections labeled for TrkB mRNA, p75^{NTR} mRNA, and TH protein were imported into Imaris® and converted to the native Imaris® file format. In order to minimize any background or off-target fluorescence, background subtraction was employed. A 3D surface object for the TH+ graft was generated using the surface function Imaris® plugin. The spots function was then used to select all mRNA puncta for TrkB and p75^{NTR} both inside and outside of the grafted neurons. Once created, the same exact parameters were used across all images. The “Find Spots Close to surface” MATLAB plugin was utilized to quantify TrkB and p75^{NTR} mRNA puncta within the DA graft. Data are represented as the number of TrkB and p75^{NTR} mRNA puncta inside TH+ neurons (μm^3), as well as total number of TrkB and p75^{NTR} mRNA puncta per cell (TH+ and TH- cells) in the striatum. The ratio of the quantity of TrkB mRNA transcripts to the quantity of p75^{NTR} mRNA transcripts within the TH+ neurons is also reported.

Dual-label protein analysis: VGLUT2/TH

Z-stacks of grafted tissue dual-immunolabeled for VGLUT2 and TH proteins were imported into Imaris® and converted into its native file format. Background subtraction was applied to each image to reduce background fluorescence. The surface function was used to generate a precise 3D reconstruction of TH+ neuron fibers within the graft

(μm^3). The spots function was employed to detect VGLUT2 protein puncta, with consistent parameters applied across all images. Colocalized VGLUT2 puncta were then filtered through the Object-Object statistics “Shortest Distance to Surface” function to retain only those located within the TH surface. Data are presented as the number of VGLUT2 protein puncta within the grafted TH surface (μm^3).

Dual-label protein analysis: Dopamine transporter (DAT) and TH

Confocal 2D images of dual-immunolabeled tissue for TH and DAT proteins were imported into Imaris® and converted into its native file format. To reduce background fluorescence in each channel, background subtraction was applied to all images. The surface function was then utilized to generate an accurate reconstruction of TH and DAT fibers within the graft. Data are expressed as the ratio of the sum of DAT fluorescence intensity to DAT surface area (μm^2) relative to the sum of TH fluorescence intensity to TH surface area (μm^2).

Triple-label protein analysis: Iba1/GFAP/TH

3D z-stacks of triple-immunolabeled grafted tissue sections for TH, Iba1, and GFAP proteins were imported into Imaris® and converted into its native file format. Background subtraction was applied to each image to reduce background fluorescence. Using semi-automatic thresholding and the surface function plugin, 3D surface objects were generated for TH, Iba1, and GFAP. Data are expressed as the Iba1 surface volume (μm^3) normalized to the graft surface volume (TH; μm^3), with GFAP reported using the same approach.

Statistical Analysis

LID and GID data are created using ordinal rating scales and were statistically analyzed using non-parametric tests including the Kruskal-Wallis test followed by Dunn's multiple comparisons, or the Mann-Whitney U tests with Dunn's multiple comparisons (between subjects). Pre-graft amphetamine-mediated rotational behavior was analyzed using a Mann-Whitney test, and post-graft amphetamine-mediated rotational behavior was analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. The amphetamine-mediated time course analysis at each time point (i.e., 20, 70, 120, 170, and 220 minutes post-administration) was also analyzed using non-parametric Kruskal-Wallis with Dunn's multiple comparisons.

An ordinary One-way ANOVA test with Tukey's multiple comparisons was performed to analyze total enumeration and volume (μm^3) of the graft. Results that were also analyzed using this test included quantity of VGLUT2 protein/ μm^3 TH, TrkB:p75^{NTR} per TH neuron, DAT sum intensity/ μm^3 , and Iba1 and GFAP volume (μm^3) per TH neuron. An ordinary One-way ANOVA with Šidák's post-hoc comparisons was conducted for average neurite density both proximal and distal to the graft.

Mann-Whitney two-tailed tests were conducted for the total of p75^{NTR} mRNA puncta inside the TH+ graft comparing the GID+ M/W host/donor group and the three other GID- host/donor combinations (i.e., M/M, W/W, W/M; combined based on no statistical differences between these GID- groups). In addition, a two-way ANOVA test with Tukey's multiple comparisons was implemented to analyze the quantity of TrkB and the quantity of p75^{NTR} mRNA transcripts per cell in the striatum (TH+ and TH- cells).

The statistical test used to compare the quantity of VGLUT2 protein per TH volume (μm^3) between the M/W host/donor group and the combined host/donor group of M/M, W/W, W/M was an unpaired, two-tailed t-test to determine specific GID+ vs. GID- group comparisons. Also between these groups, this same statistical test was used to analyze the ratio of TrkB:p75^{NTR} mRNA transcripts inside TH+ DA neurons. Unpaired, two-tailed t-tests were also employed for the total of TrkB mRNA puncta inside the TH+ graft between the GID+ M/W host/donor group and the GID- M/M, W/W, W/M combined groups. The DAT sum intensity/ μm^3 was similarly analyzed with this statistical test.

For all correlations between protein expressions and GID behavior, a non-parametric Spearman correlation was applied. Statistical outliers, although rare, were identified and removed using both the ROUT and Grubb's outlier tests. If data met assumptions for normality and homogeneity of variances, parametric statistical tests were employed. All statistical analyses for this study were successfully completed using the GraphPad Prism software created for Windows (v.10.4.1).

RESULTS

The homozygous rs6265 (Met/Met) genotype, in either host or donor, demonstrates superior graft efficacy and earlier amelioration of LID behavior

Based on results from previous studies that revealed behavioral benefit of the homozygous rs6265 (Met/Met) genotype in a parkinsonian rat model (see (Mercado et al., 2021)), traumatic brain injury (TBI) (Barbey et al., 2014; Finan et al., 2018; Krueger et al., 2011), and multiple sclerosis (MS) (Zivadinov et al., 2007), I continued to hypothesize that the Met allele, whether found in the host or donor, would confer a greater degree of graft-derived benefit compared to the WT genotype in response to neural transplantation. I theorized that the Met/Met hosts engrafted with Met/Met DA neurons would exhibit the greatest degree of benefit with the earliest amelioration of LID behavior.

In keeping with this hypothesis, Met/Met allele carriers, either in the host or in the donor neurons, exhibited enhanced behavioral recovery demonstrated by a four-week-earlier amelioration of LID behavior. Compared to sham-grafted parkinsonian subjects, Met/Met hosts engrafted with WT DA neurons (M/W), Met/Met hosts engrafted with Met/Met DA neurons (M/M), and WT hosts engrafted with Met/Met DA neurons (W/M) showed significant reductions in LID behavior at week 4 post-engraftment (**Figure 3.2a**; Week 4: $p = 0.0033$ M/W vs. sham, $p = 0.0044$ M/M vs. sham, $p = 0.0301$ W/M vs. sham). In contrast, it took an additional four weeks for WT hosts engrafted with WT DA donor neurons (W/W) to exhibit significant amelioration of LID compared to sham-grafted animals (**Figure 3.2a**; Week 8: $p = 0.0266$ W/W vs. sham). This significant difference in WT hosts engrafted with WT donor neurons, however, was lost at the

conclusion of week 10 for this host/donor combination (**Figure 3.2a**; Week 10: $p = 0.0940$), while other Met-allele host/donor combinations exhibited significantly lower LID compared to sham-grafted animals for the duration of the study (**Figure 3.2a**; Week 10: $p = 0.0003$ M/W; $p = 0.0202$ M/M; $p = 0.0217$ W/M). Met-allele-carriers (host or donor) notably had higher percentages of improvement from pre-graft LID behavior, with the Met/Met hosts engrafted with WT DA neurons generating the highest percentage of improvement (**Figure 3.2b**; M/W $75.26 \pm 5.67\%$, M/M $60.00 \pm 11.37\%$, W/M $50.67 \pm 13.74\%$ vs. W/W $42.60 \pm 14.34\%$, Mean $\pm$ SEM).

Our secondary assessment of DA neuron graft function, amphetamine-induced rotational behavior, demonstrated that pre-graft amphetamine-mediated ipsilateral rotations were not statistically different between WT or Met/Met host rats (**Figure 3.2ei**; $p = 0.1465$). At 10 weeks post-engraftment, similar to total LID scores, amphetamine rotational behavior was significantly reduced in only Met-allele carriers when compared to sham-grafted animals (**Figure 3.2eiii**; $p = 0.0013$ M/W; $p = 0.0010$ M/M; $p = 0.0088$ W/M). Conversely, the number of amphetamine-mediated ipsilateral rotations in the WT hosts engrafted with WT donor neurons was not significantly different than sham-grafted subjects (**Figure 3.2eiii**; $p = 0.0539$).

Despite enhanced recovery seen in Met-allele carriers compared to sham-grafted subjects, total LID scores and number of amphetamine rotations of the four host/donor combinations were not significantly different from each other (**Figure 3.2e**; $p > 0.9999$ between grafted animals from Weeks 4-10 post-engraftment (LID) and $p > 0.9999$ at Week 10 (amphetamine rotations). Nevertheless, these results, along with an earlier reduction in LIDs (i.e., Week 4 vs. Week 8) and fewer rotations per minute at 10 weeks

in Met-allele carriers, support that the Met allele remains to confer a degree of benefit compared to the WT allele, at least in the context of neural transplantation.

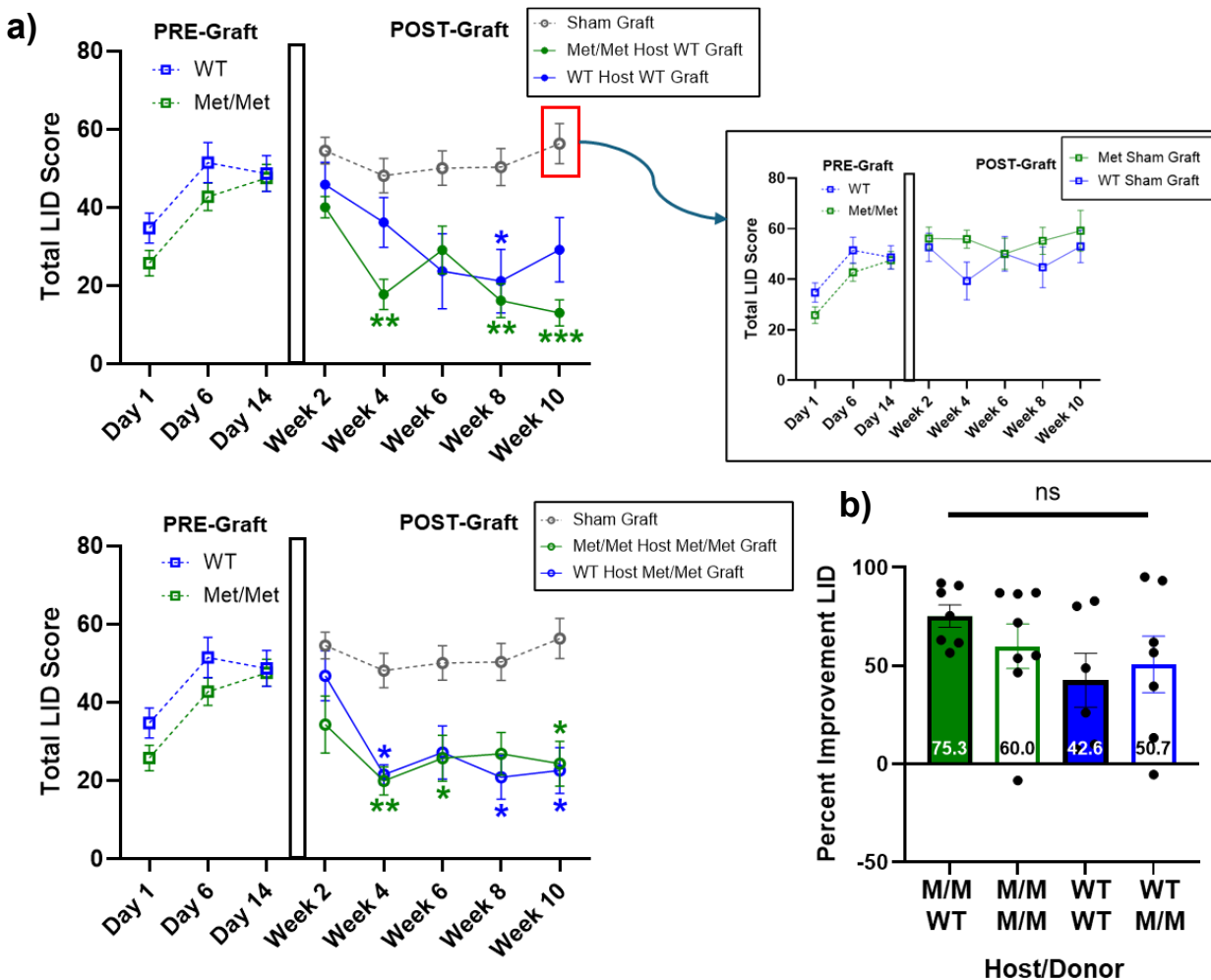


Figure 3.2: Impact of host/donor genotype on LID behavior and amphetamine-rotational asymmetry in DA-grafted parkinsonian rats.

(a) Total LID severity scores for each host/donor combination throughout the duration of the experiment, including pre- and post-engraftment. LID severity scores were not significantly different between sham-grafted groups; therefore, sham-grafted groups were combined post-engraftment (see inset graph separated by genotype) ($p \geq 0.0999$ for all time points; Mann-Whitney unpaired two-tailed t-test). Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons at each timepoint. **Week 4:** **p = 0.0033 M/W host/donor vs. sham-graft, **p = 0.0044 M/M vs. sham-graft, *p = 0.0301 W/M vs. sham-graft. **Week 6:** *p = 0.0418 M/M vs. sham-graft; **Week 8:** **p = 0.0028 M/W vs. sham-graft, *p = 0.0266 W/W vs. sham-graft, *p = 0.0322 W/M vs. sham-graft; **Week 10:**

Figure 3.2 (cont'd)

***p = 0.0003 M/W vs. sham-graft, *p = 0.0202 M/M vs. sham-graft, *p = 0.0217 W/M vs. sham-graft. At no time point were the grafted groups significantly different from each other ($p \geq 0.3644$ for all time points). **(b)** Percent improvement in LID behavior for each host/donor group, from pre-graft LID scores to LID scores at 10 weeks post-engraftment. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons, $p \geq 0.2778$ for all groups. **(c)** Time course of LID severity scores for individual animal responses at Week 4-10 following levodopa administration. Rats were rated at 20, 70-, 120-, 170-, and 220-minutes post-injection. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons at each time point post-levodopa injection. **Week 4** (20 minutes): *p = 0.0243 M/W vs. sham, **p = 0.0097 W/M vs. sham; (70 minutes) **p = 0.0057 M/M vs. sham; (120 minutes) ***p = 0.0006 M/W vs. sham, *p = 0.0260 W/M vs. sham; **Week 6** (20 minutes) ***p = 0.0041 M/W vs. sham, **p = 0.0066 W/W vs. sham, **p = 0.0028 M/M vs. sham; (70 minutes) *p = 0.0196 W/W vs. sham; **Week 8** (20 minutes) *p = 0.0107 M/W vs. sham, **p = 0.0099 W/M vs. sham; (70 minutes) *p = 0.0195 W/W vs. sham; (120 minutes) **p = 0.0028 M/W vs. sham; (170 minutes) *p = 0.0235 M/W vs. sham, *p = 0.0132 M/M vs. sham, *p = 0.0295 W/M vs. sham; **Week 10** (20 minutes) *p = 0.0245 M/W vs. sham, **p = 0.0091 W/M vs. sham; (70 minutes) **p = 0.0012 M/W vs. sham, *p = 0.0135 M/M vs. sham, **p = 0.0093 W/M vs. sham; (120 minutes) ***p = 0.0006 M/W vs. sham, *p = 0.0217 M/M vs. sham; (170 minutes) *p = 0.0228 M/W vs. sham.

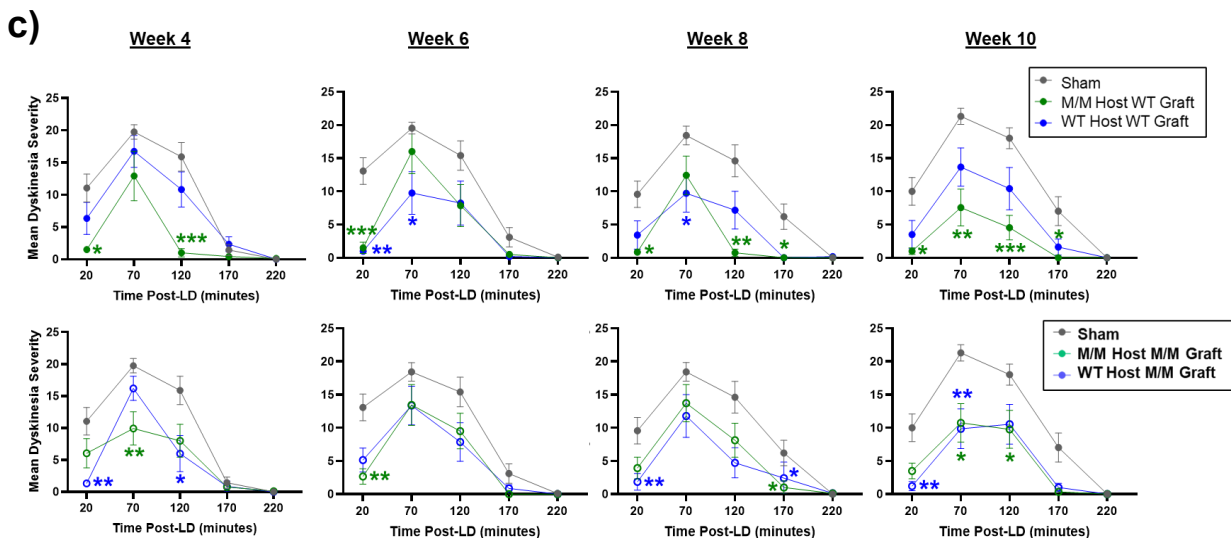
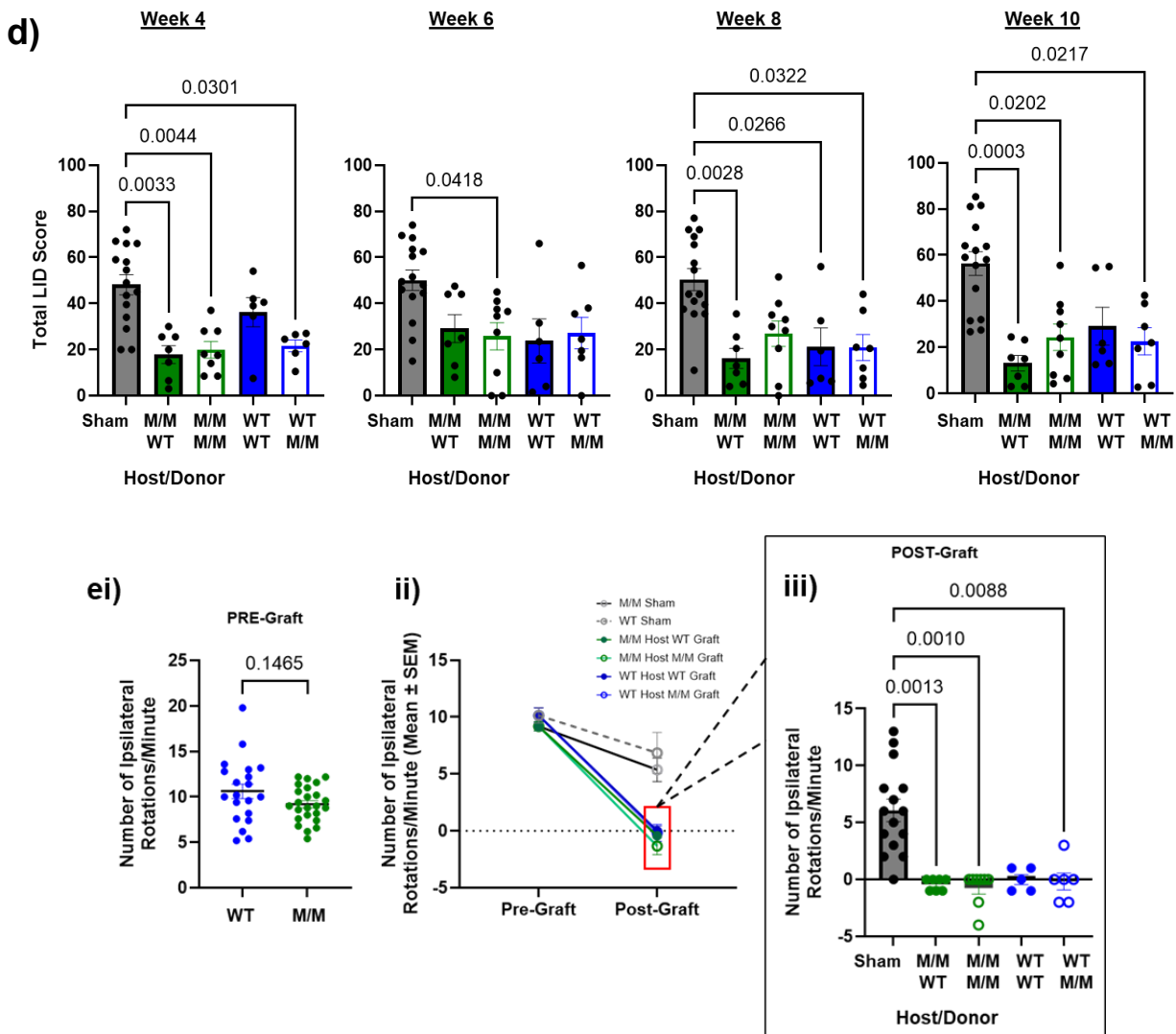


Figure 3.2 (cont'd)

(d) Total LID score for each individual animal at Week 4, 6, 8, and 10 post-engraftment. Statistics (listed within graph): Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons at each time point. **(e)** Amphetamine rotational asymmetry at pre-graft and 10 weeks post-engraftment. Data are expressed as (i) number of ipsilateral rotations per minute (at the 70-minute time point), and (ii, iii) average number of ipsilateral rotations per minute at 70 minutes (Mean $\pm$ SEM). Statistics: (i) Mann-Whitney U unpaired two-tailed t-test, (ii) Two-way ANOVA with Tukey's multiple comparisons, **** $p < 0.0001$ W/W vs. WT sham and W/M vs. WT sham, *** $p = 0.0002$ M/W vs. Met sham, **** $p < 0.0001$ M/M vs. Met Sham. Pre-graft vs. post-graft for all groups $p \geq 0.0034$. No significant differences in rotations were found between grafted groups, $p \geq 0.9314$. (iii) Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons. Abbreviations: LID = levodopa-induced dyskinesia, M/M = Met/Met, LD = levodopa, ns = not significant.



Cell survival, graft volume, and neurite outgrowth are not significantly affected by the WT and/or homozygous rs6265 (Met/Met) genotype in host or donor

Stereological quantification of TH immunoreactivity indicated that the estimated number of surviving transplanted DA neurons was not different between WT and homozygous rs6265 (Met/Met) host/donor combinations (**Figure 3.3b**; Mean $\pm$ SEM, M/W 2492 ± 508.9 ; M/M 2284 ± 294.9 ; W/W 2115 ± 406.7 ; W/M 1915 ± 219.5 , $p \geq 0.7060$ for all comparisons). Likewise, graft volume (mm^3) of the DA grafts was not statistically significant between genotypic host/donor combinations either (**Figure 3.3c**; Mean $\pm$ SEM, M/W $0.3352 \pm 0.0661 \text{ mm}^3$, M/M $0.2728 \pm 0.0257 \text{ mm}^3$, W/W $0.2231 \pm 0.0252 \text{ mm}^3$, W/M $0.3257 \pm 0.0378 \text{ mm}^3$; $p \geq 0.2925$ for all combinations).

Previously, Met/Met parkinsonian rats paradoxically demonstrated more extensive graft-derived neurite outgrowth in the distal regions of the graft in contrast to WT host rats (Mercado et al., 2021). In a Met/Met environment, there is reduced activity-dependent BDNF release (Egan et al., 2003), and therefore, this finding is unexpected. Nonetheless, based on this previous finding, I hypothesized that neurite outgrowth would be most extensive in the Met-allele host/donor combinations. However, all host/donor combinations, both proximally and distally to the graft, stereologically exhibited no differences in neurite outgrowth, reported as the average neurite density ($\mu\text{m}/\text{mm}^3$) (**Figure 3.3e**; Proximal average: M/W vs. M/M $p = 0.9667$; M/W vs. W/W $p > 0.9999$; W/W vs. W/M $p = 0.9043$; M/M vs. W/M $p = 0.9986$). Data also reveal no differences in neurite outgrowth located distal to the graft at any of the regions surrounding the graft (i.e., dorsal, ventral, lateral, medial) (**Figure 3.3f**; Distal average:

M/W vs. M/M $p > 0.9999$; M/W vs. W/W $p > 0.9999$; W/W vs. W/M $p = 0.9967$; M/M vs. W/M $p = 0.9987$).

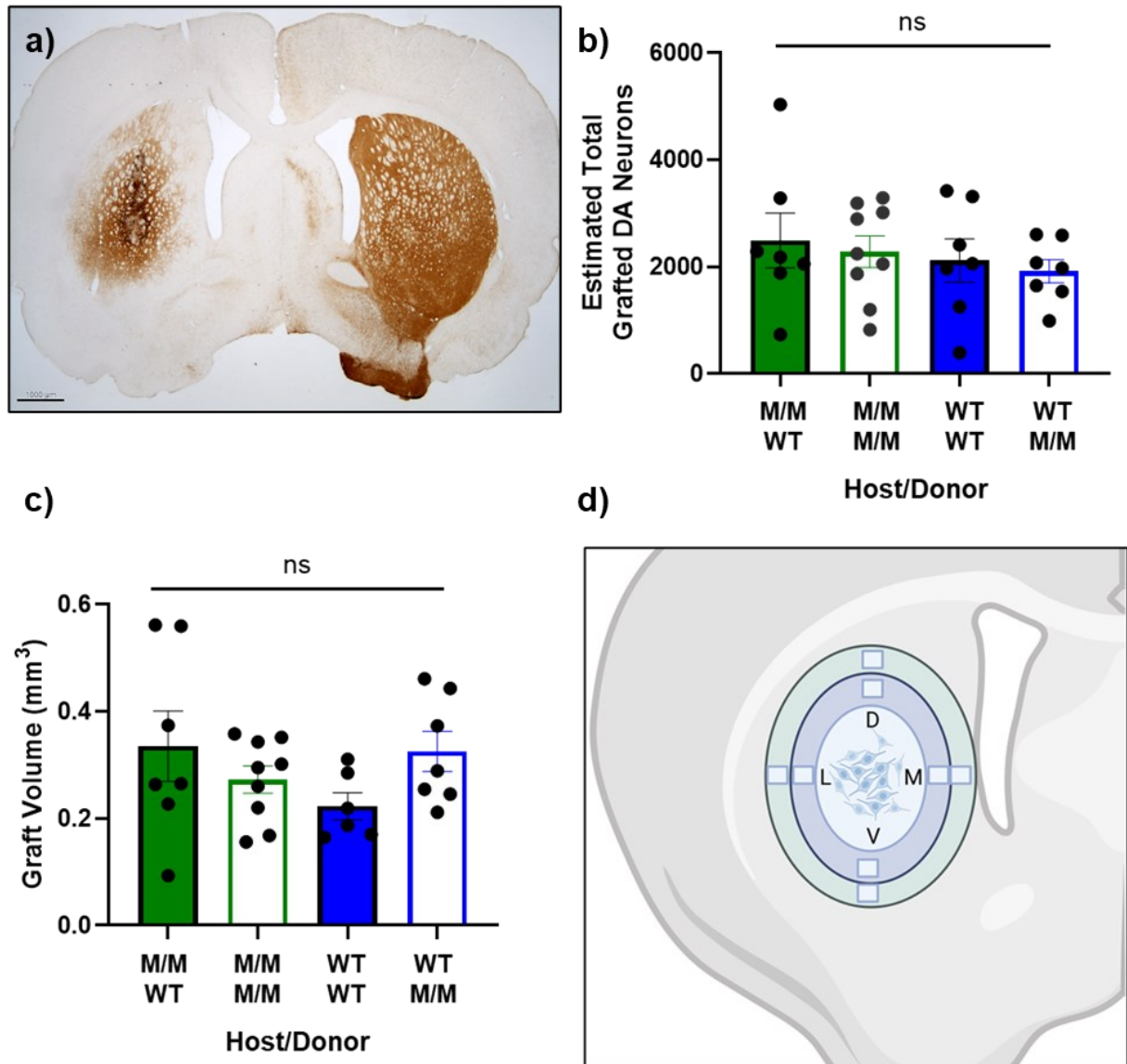
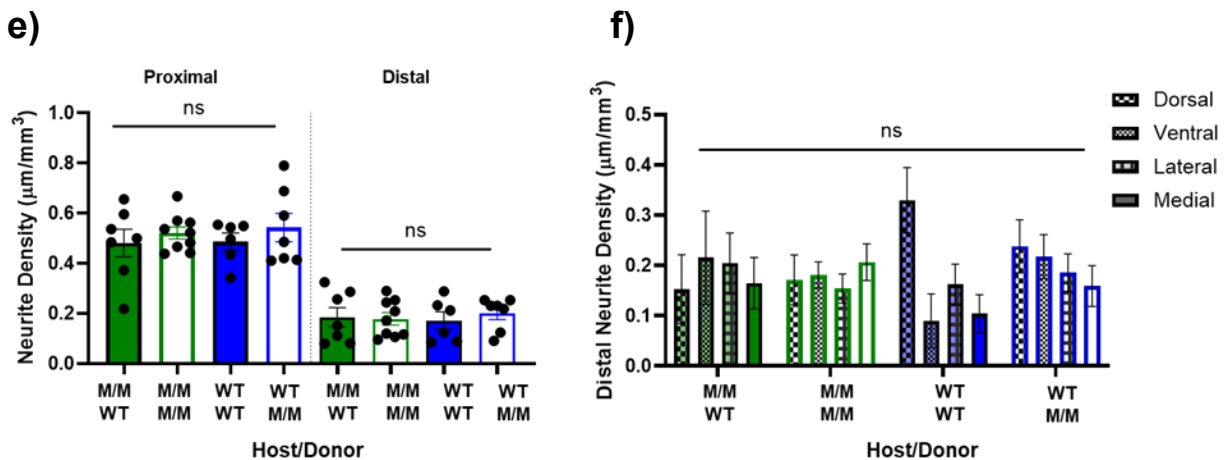


Figure 3.3: Impact of host/donor genotype on graft survival and neurite outgrowth in DA-grafted parkinsonian rats.

(a) Histological representation of the DA-grafted parkinsonian striatum (4x) micrograph (Scale bar = 1000 μ m). **(b)** Stereologically estimated number of surviving grafted DA neurons. Statistics: Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons **(c)** Stereologically estimated graft volume (μ m³). Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons. **(d)** Schematic depiction of grafted DA neurite outgrowth analysis. Proximal regions are depicted in blue, and distal regions are depicted in green. **(e)** Average neurite density of grafted DA neurons both proximal and distal to the border of the graft. Statistics: Mean $\pm$ SEM. Two-way repeated measures ANOVA with Šidák's

Figure 3.3 (cont'd)

post-hoc test; proximal ($p \geq 0.8276$) and distal ($p \geq 0.9967$). **(f)** Distal neurite density comparison between DA-grafted groups separated into each region surrounding the graft. Statistics: Mean $\pm$ SEM. Two-way ANOVA with Tukey's multiple comparisons, $p \geq 0.0915$ for all host/donor grafted groups. Abbreviations: Ctx = cortex, Str = striatum, D = dorsal, L = lateral, M = medial, V = ventral, ns = not significant.



Homozygous rs6265 (Met/Met) parkinsonian rats engrafted with WT DA neurons remain the only host/donor combination to develop aberrant GID behavior

We previously demonstrated that homozygous rs6265 (Met/Met) parkinsonian rats engrafted with WT DA neurons uniquely developed aberrant GID behavior compared to WT rats engrafted with WT DA neurons (Mercado et al., 2021). Since the Met/Met genotype has a reduction in activity-dependent release of BDNF (Egan et al., 2003), I postulated that the Met/Met parkinsonian host rats engrafted with Met/Met donor DA neurons (M/M) would be the host/donor combination that develops the most severe GID behavior compared to other host/donor combinations. In contrast, the parkinsonian Met/Met hosts engrafted with WT DA neurons (M/W) strikingly remain the only host/donor combination to develop significant GIDs (**Figure 3.4**). When analyzed against sham-grafted parkinsonian subjects, the M/W host/donor group exhibited

approximately an 8-fold increase in total GID severity (**Figure 3.4a**; M/W 18.21 ± 6.59 vs. sham 2.16 ± 0.71 $p = 0.0406$, Mean $\pm$ SEM) and peak GID severity (**Figure 3.4b**; M/W 6.79 ± 1.61 vs. sham 0.78 ± 0.35 $p = 0.0071$, Mean $\pm$ SEM). Total GID severity and peak GID behavior was not statistically different between other DA-grafted host/donor combinations (**Figure 3.4a,b**, $p \geq 0.6774$ (total); $p \geq 0.6318$).

In complement to GID severity, the incidence of GID behavior was also reported. Confirming the results of total and peak GID severity, percent GID incidence for total and peak GID was the highest in Met/Met parkinsonian rats engrafted with WT DA neurons (M/W) (**Figure 3.4c**, total GID: Mean $\pm$ SEM; sham 20.0%, M/W 71.4%, M/M 66.7%, W/W 33.3%, W/M 42.9%; peak GID: sham 20%, M/W 85.7%, M/M 44.4%, W/W 33.3%, W/M 28.6%). When amphetamine-mediated GID behavior at 10 weeks post-engraftment was reported at the rating time points of 20, 70, 120, 170, and 220 minutes following amphetamine administration (**Figure 3.4d**), a statistical difference was prevalent at 70-minutes post-injection between the Met/Met hosts engrafted with WT DA grafts (M/W) and sham-grafted subjects (**Figure 3.4d**; M/W vs. sham, $p = 0.0071$, 70-minutes). Despite no significant differences exhibited between the GID+ M/W host/donor combination and the other GID- DA-grafted host/donor combinations, these data further corroborate the findings demonstrated in total and peak GID severity between M/W host/donors and sham-grafted animals, ultimately signifying that only Met/Met parkinsonian recipients of WT donor neurons display significant aberrant GID.

While the underlying mechanism of GID behavior remains elusive, our group previously demonstrated that grafted DA neurons transplanted into parkinsonian rats expressed morphological evidence of atypical, excitatory synapses observed with

positive immunoreactivity to VGLUT2, a marker of glutamatergic neurons (Mercado et al., 2021; Soderstrom et al., 2008). Normally, VGLUT2 is expressed in immature embryonic DA neurons; however, as the neurons mature, the VGLUT2 phenotype disappears (El Mestikawy et al., 2011). In Mercado et al., 2021, not only did the transplanted DA neurons retain an immature phenotype, a statistically positive correlation between GID severity and VGLUT2 expression was previously reported in the Met/Met hosts engrafted with WT DA neurons (Mercado et al., 2021). Based on this evidence, I endeavored to investigate, in this current study, whether all host/donor grafts retained an immature phenotype (i.e., VGLUT2 expression) and if VGLUT2 expression in the Met/Met-WT host/donor parkinsonian rats remained strongly correlated to GID behavior.

Unsurprisingly, as was apparent in (Mercado et al., 2021), each host/donor combination (M/W, M/M, W/W, W/M) did not express statistically different quantities of VGLUT2 protein within the transplanted DA graft (**Figure 3.4f**; M/W vs. M/M $p = 0.7323$; M/W vs. W/W $p = 0.9978$; M/M vs. W/M $p = 0.9928$; W/W vs. W/M $p = 0.8905$). Because the M/W host/donor group uniquely exhibited GID behavior, the additional groups (i.e., M/M, W/W, W/M) were consolidated to compare to the M/W group (**Figure 3.4g**; GID+ group vs. GID- group). No significant differences were observed when reported in this manner (GID+ M/W vs. 3 other GID- host/donor groups $p = 0.4587$). Further, to determine whether a correlation still exists between VGLUT2 expression in Met/Met hosts with WT grafts and GID behavior, a Spearman correlation was performed (**Figure 3.4h**). In this study, the number of VGLUT2 protein expressed within the grafted DA neurons was not significantly correlated with total GID severity at 10 weeks post-

engraftment (**Figure 3.4h**; $r = 0.3571$, $p = 0.4444$). Despite no longer being statistically correlated, a positive trend remains. As the number of VGLUT2 protein expression increased, the total GID severity score also increased. As presented below, additional evidence suggests that VGLUT2 expression in grafted DA neurons has a complex relationship to GID.

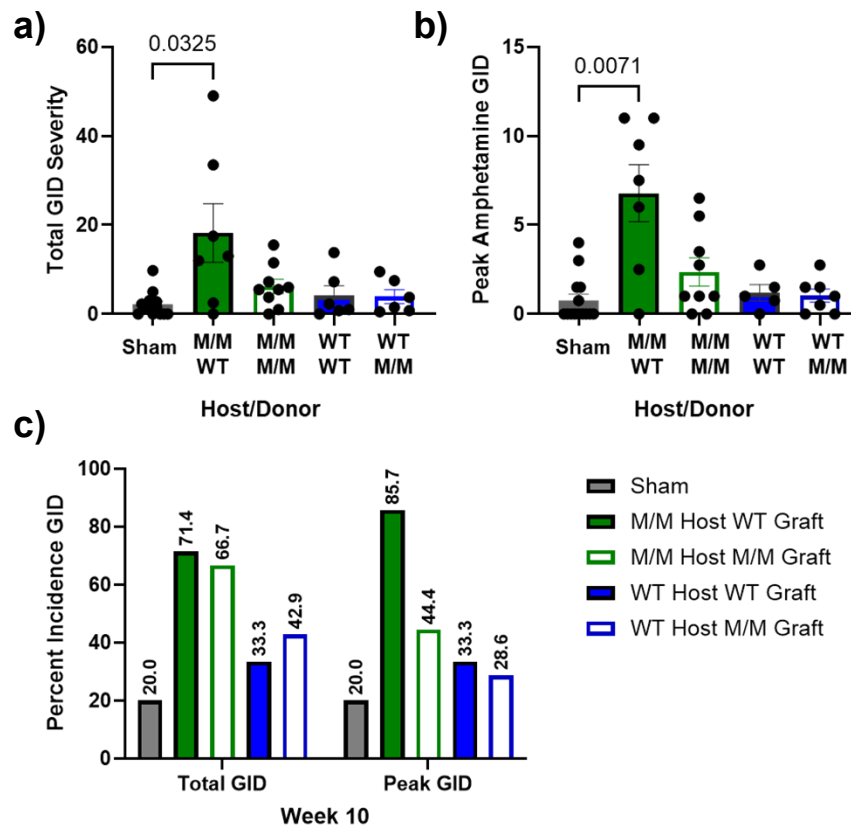


Figure 3.4: Impact of host/donor genotype on development of GID behavior and association with VGLUT2 expression.

(a) Total and **(b)** peak amphetamine-induced GID severity scores for all host/donor groups at week 10 post-engraftment. Statistics: Mean $\pm$ SEM. Non-parametric Kruskal-Wallis with Dunn's multiple comparisons, $p = 0.0325$ M/W vs. sham-graft (total) and $p = 0.0071$ M/W vs. sham-graft (peak). **(c)** Percent incidence of total (≥ 4) and peak (≥ 2) GID severity score in all host/donor groups at 10 weeks post-engraftment. Percentages are listed above each bar. **(d)** Time course of amphetamine-mediated GID behavior in each host/donor at week 10 post-engraftment at 20, 70-, 120-, 170-, and 220-minutes post-amphetamine administration. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons at each time point post-amphetamine injection. 70 minutes: $p = 0.0071$ M/W vs. sham-graft. **(e)** Fluorescent image and Imaris 3D reconstruction of DA

Figure 3.4 (cont'd)

(TH+) neurons positive for VGLUT2 protein co-expression. Scale bar = 5 μm . **(f)** Total quantification of the number of VGLUT2 protein co-localized in TH+ grafted DA neurons normalized to the surface volume (μm^3) of the graft. Statistics: Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons, $p \geq 0.6513$ for all host/donor groups. **(g)** Total quantification of VGLUT2 co-localization inside TH+ grafted DA neurons with the 3 GID-host/donor groups combined (i.e., M/M, W/W, W/M host/donor). Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, not significant.

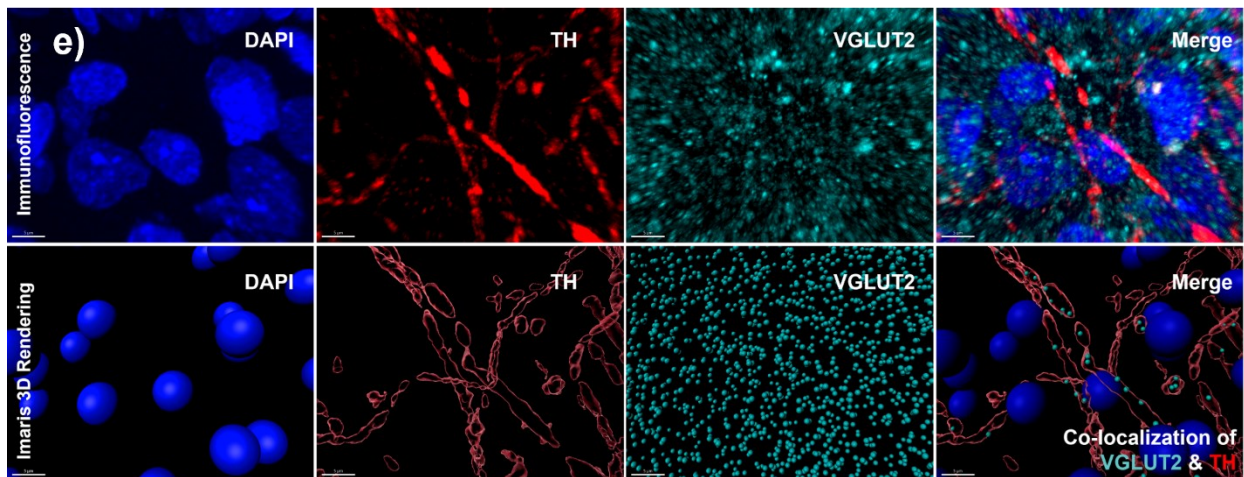
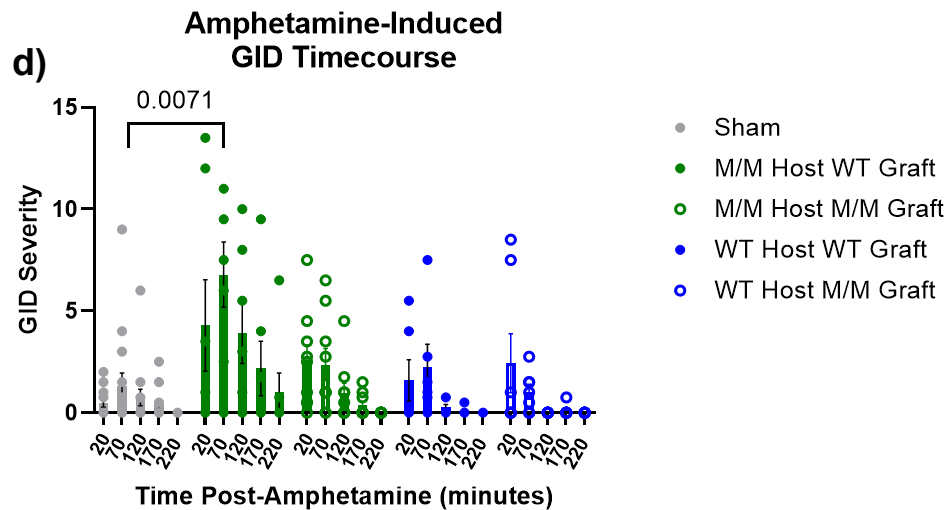
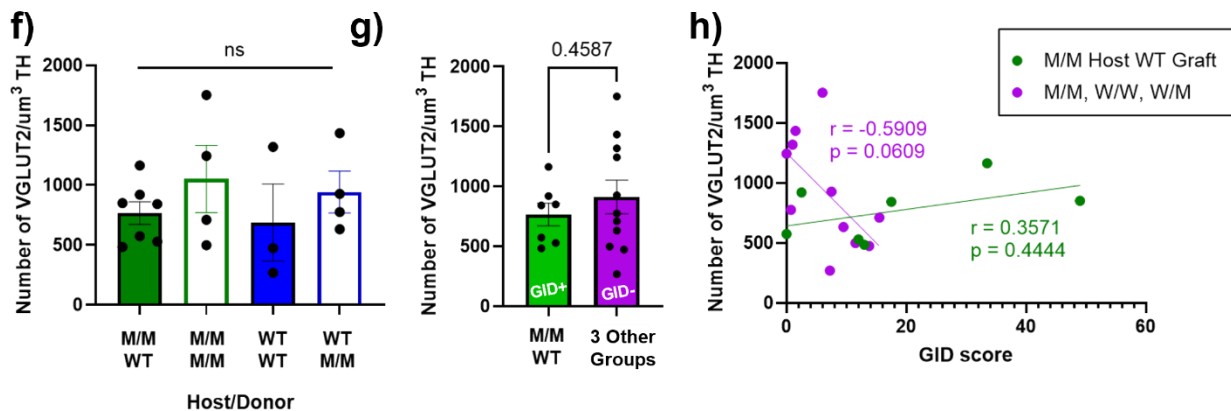


Figure 3.4 (cont'd)

(h) Spearman correlation comparing the total quantity of VGLUT2 co-localized inside TH+ neurons and total amphetamine-mediated GID severity scores at 10 weeks post-engraftment. No significance. Abbreviations: GID = graft-induced dyskinesia, VGLUT2 = vesicular glutamate transporter 2, TH = tyrosine hydroxylase, ns = not significant, 3OR = 3 other groups combined (i.e., M/M, W/W, W/M host/donor).



Homozygous rs6265 (Met/Met) parkinsonian rats engrafted with WT DA neurons express lower BDNF receptor transcript ratios (TrkB to p75^{NTR})

The two predominant receptors that BDNF binds to include tropomyosin receptor kinase B (TrkB) and the pan neurotrophin receptor (p75^{NTR}) (Reichardt, 2006). Upon activation of the TrkB receptor, multiple signaling pathways involved in pro-survival and dendritic growth/branching are activated (Jaworski et al., 2005; Kumar et al., 2005). In contrast, when BDNF is bound to p75^{NTR}, it is generally accepted that pro-apoptotic pathways are activated (Friedman, 2000; Meeker & Williams, 2015). In the literature, an imbalance between TrkB/ p75^{NTR} proteins has been implicated in neurodegenerative rodent models of Huntington's disease (HD) spine density (Brito et al., 2013; Suelves et al., 2019). Therefore, I hypothesized that an imbalance between TrkB and p75^{NTR} transcript expression exists, with a prominent upregulation in p75^{NTR} mRNA, and that

this imbalance is correlated with GID in Met/Met parkinsonian rats engrafted with WT DA neurons.

The average quantity of the TrkB mRNA transcripts per cell in the striatum (TH+ and non-TH+ cells) was significantly increased in the Met/Met hosts engrafted with Met/Met DA neurons, however, only on the intact side (**Figure 3.5b**; M/W vs. M/M $p = 0.0481$; M/M vs. W/M $p = 0.0424$; M/M vs. W/W $p = 0.0383$). On the grafted side, TrkB transcript expression was normalized in the presence of all DA neuron grafts (i.e., not significantly different between DA-grafted groups). Interestingly, p75^{NTR} transcript expression was only found to be significantly upregulated between the M/M and W/W host/donor groups within the grafted DA neurons (**Figure 3.5c**; M/M vs. W/W graft $p = 0.0399$). Further, slightly increased expression of p75^{NTR} transcripts was present outside the DA graft (i.e., in TH- cells located dorsolateral from the graft), albeit this was not found to be statistically significant (data not shown).

Since the imbalance of p75^{NTR}/TrkB receptor expression has specifically been implicated in neurodegenerative diseases, we also reported the ratio of TrkB to p75^{NTR} mRNA within the grafted TH+ neurons of each host/donor combination. When analyzed separately, no significant differences exist between groups (**Figure 3.5d**). However, when the three GID- groups are combined and compared to the GID+ M/W host/donor group, there is a notable decrease in the TrkB:p75^{NTR} mRNA ratio in the grafted TH+ neurons (**Figure 3.5f**; M/W vs. M/M, W/M, W/W $p = 0.0472$), indicative of a relative increase in p75^{NTR} receptors. While this was not significantly correlated with GID behavior at week 10 post-engraftment (**Figure 3.5e**; M/W $r = -0.4058$, $p = 0.4333$), a negative trend is apparent between the TrkB:p75^{NTR} mRNA ratio and GID behavior

where a lower ratio was associated with a higher GID score. Moreover, total p75^{NTR} mRNA expression inside grafted TH+ neurons was higher in the GID+ M/W host/donor group compared to the three other GID- host/donor combinations, albeit not statistically significant (**Figure 3.5h**; M/W vs. M/M, W/M, W/W $p = 0.2824$). Total TrkB mRNA expression inside grafted TH+ neurons was not different between groups (**Figure 3.5g**). These results suggest there is a trend toward upregulation of p75^{NTR} mRNA expression in the M/W host/donor group which could potentially be associated with GID behavior; however, additional analyses such as protein expression are warranted.

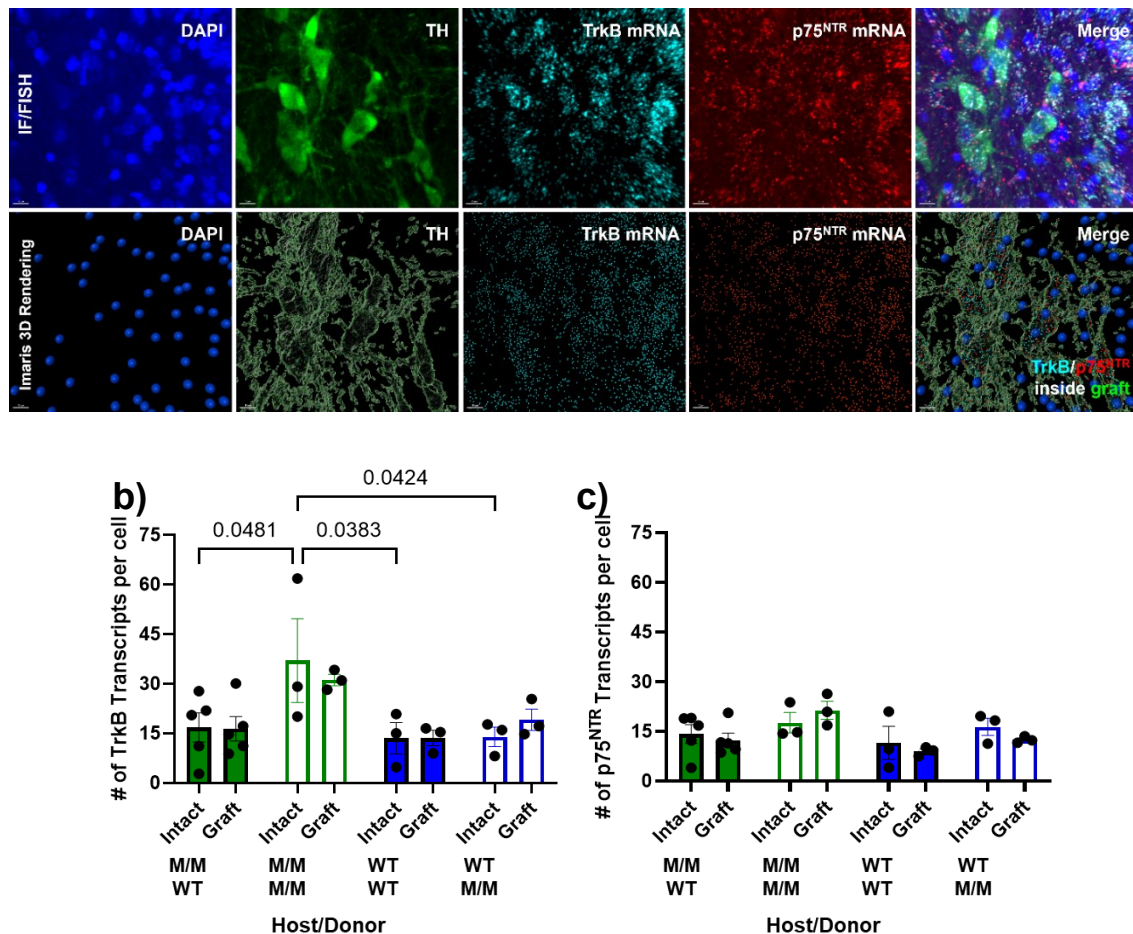


Figure 3.5: Impact of host/donor genotype on TrkB and p75^{NTR} BDNF receptor transcript expression in DA-grafted parkinsonian rats.

(a) Confocal fluorescent image and Imaris 3D reconstruction of TrkB and p75^{NTR} mRNA puncta inside DA (TH+) neurons. Scale bar = 10 μ m. (b) Total quantity of TrkB mRNA

Figure 3.5 (cont'd)

transcripts per cell (TH+ and TH-) in the intact and grafted striatum of each host/donor combination. Statistics: Mean $\pm$ SEM. Two-way ANOVA with Tukey's' multiple comparisons, $p = 0.0481$ M/W vs. M/M, $p = 0.0383$ M/M vs. W/W, $p = 0.0424$ M/M vs. W/M in the intact striatum. No significance was found in the grafted striatum between host/donor groups, $p \geq 0.1612$ for all groups. **(c)** Total quantity of $p75^{NTR}$ mRNA transcripts per cell (TH+ and TH-) in the intact and grafted striatum of each host/donor combination. Statistics: Mean $\pm$ SEM. Two-way ANOVA with Tukey's' multiple comparisons, $p = 0.0399$ M/M vs. W/W in the grafted striatum, $p \geq 0.4991$ in the intact striatum for all host/donor combinations. **(d)** Ratio of TrkB:p75^{NTR} mRNA per TH+ grafted DA neuron. Statistics: Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons, $p \geq 0.4189$ for all host/donor groups. **(e)** Ratio of TrkB:p75^{NTR} mRNA per TH+ grafted DA neuron with the 3 GID- host/donor groups combined (i.e., M/M, W/W, W/M host/donor). Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, $p = 0.0472$ M/W vs. 3OR. **(f)** Correlation of the ratio of TrkB:p75^{NTR} mRNA per TH+ grafted DA neuron and GID score at 10 weeks post-engraftment in M/W host/donors and 3OR combined. Statistics: Spearman correlation, not significant.

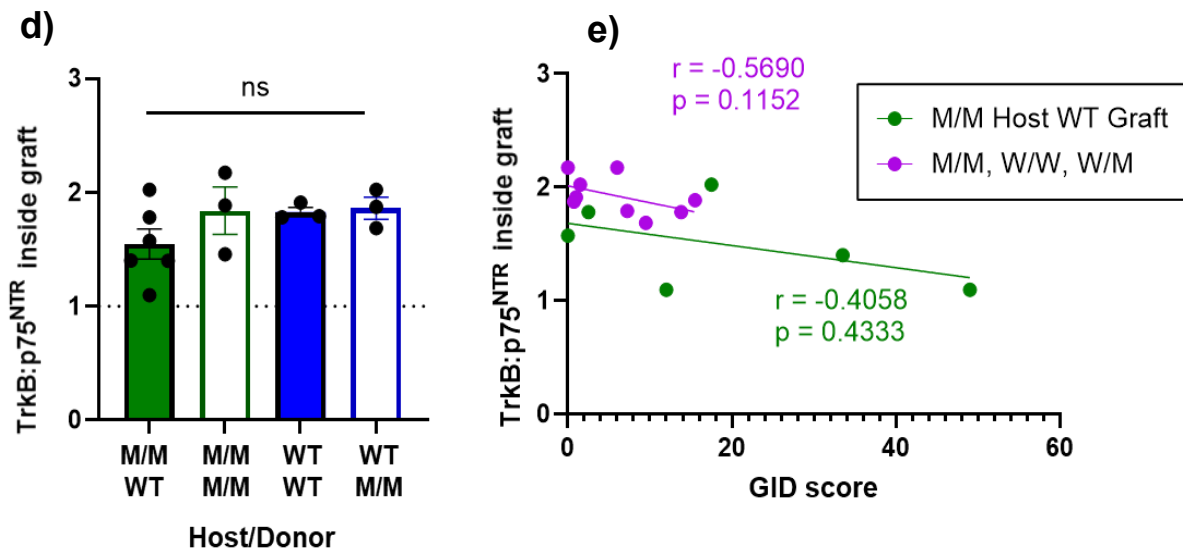
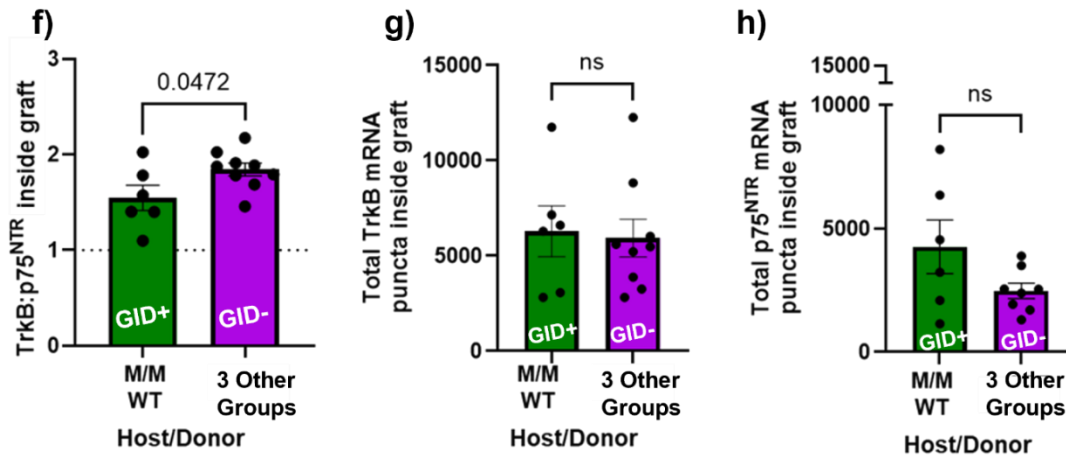


Figure 3.5 (cont'd)

(g) Total TrkB mRNA transcripts and (h) p75^{NTR} alone inside TH+ grafted DA neurons between M/W host/donor and 3 other host/donor groups. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-tests, no significance. Abbreviations: TrkB = tropomyosin receptor kinase B, p75^{NTR} = pan neurotrophin receptor, TH = tyrosine hydroxylase, mRNA = messenger ribonucleic acid, 3OR = 3 other host/donor groups combined.



Aberrant GID behavior in homozygous rs6265 (Met/Met) parkinsonian recipients of WT DA grafts is associated with excess DA release

Excess DA release is one of the mechanisms that has been postulated as an underlying cause of GID behavior (Politis, 2010b; Politis et al., 2011; Steece-Collier et al., 2012). To gain initial insight into this possible mechanism, I indirectly examined DA release in our host/donor combinations using immunohistochemical postmortem expression of the DAT protein. DAT is a transmembrane receptor that clears DA from the extracellular space following its release into the synapse. In order to clear increased concentrations of DA from the synapse, a compensatory upregulation of DAT is required (Lohr et al., 2017; Zhu & Reith, 2008). Thus, an increase in DAT expression is a surrogate marker indicative of an increase in DA release.

Although not statistically significant when examined as separate host/donor combination groups (**Figure 3.6b**), when combined by GID status, Met/Met hosts engrafted with WT DA neurons demonstrated a significant increase in DAT expression (i.e., DAT sum intensity/ μm^2) in comparison to the GID- M/M, W/M, and W/W host/donor groups (**Figure 3.6c**; M/W vs. M/M, W/M, W/W $p = 0.0085$), suggestive of an increase in DA release in the GID+ group. Fluorescent intensity in these postmortem analyses is equivalent to DAT protein expression since the fluorescent staining pattern of DAT is ubiquitous and fills the entire neuron. Although exhibiting increased expression, DAT intensity in the M/W host/donor animals was not statistically correlated with total GID severity at the conclusion of the study (i.e., 10 weeks post-engraftment, **Figure 3.6d**), suggesting that, in these animals, while enhanced DA release may exist, it alone may not be sufficient for GID induction.

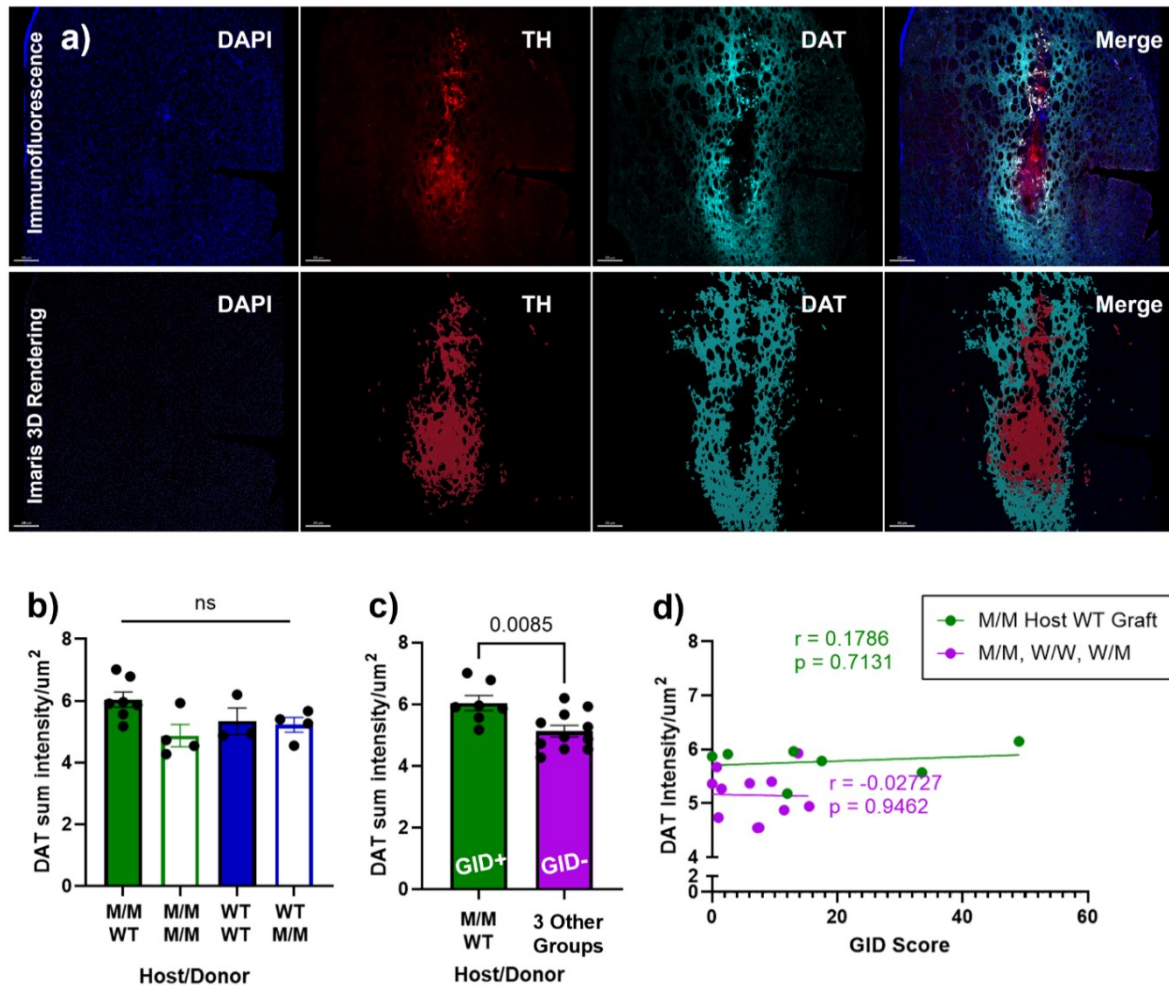


Figure 3.6: Impact of host/donor genotype on DAT expression in DA-grated parkinsonian rats.

(a) Representative confocal fluorescent micrograph of depicting staining patterns of the dopamine transporter (DAT) and TH in the grafted parkinsonian striatum (cyan = DAT, red = TH). Scale bar = 300 μm; 50 μm for the inset image. **(b)** DAT expression/fluorescent intensity quantification in grafted DA neurons. Data are expressed as the sum DAT intensity per DAT surface area (μm²). Statistics: Mean ± SEM. One-way ANOVA with Tukey's multiple comparisons, $p \geq 0.2313$ in all host/donor groups. **(c)** DAT expression/fluorescent intensity quantification in grafted DA neurons, demonstrated between the M/W host/donor group and the other host/donor combinations combined. Statistics: Mean ± SEM. Unpaired two-tailed t-test, $p = 0.0085$. **(d)** Non-parametric Spearman correlation between DAT sum intensity/DAT surface area (μm²) and GID score at 10 weeks post-engraftment. Statistics: Spearman correlation, no significance. Abbreviations: DAT = dopamine transporter, TH = tyrosine hydroxylase, GID = graft-induced dyskinesia.

GID behavior in homozygous rs6265 (Met/Met) parkinsonian rats engrafted with WT DA neurons is not correlated to immune marker expression in the parkinsonian striatum

Another mechanism that has been speculated to underlie GID induction is increased activation of the immune system as detailed in Chapter 1 (Freed et al., 2001; Olanow et al., 2003; Soderstrom et al., 2008; Steece-Collier et al., 2012). Since existing evidence points to a promising influential role of the immune system in GID induction (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003; Soderstrom et al., 2008), I investigated the expression of two common immune markers including ionized calcium-binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) to provide a cursory examination in this study.

Microglial (Iba1) is an immune marker involved in generation and elimination of synaptic connections (Tremblay et al., 2011). In this study, Iba1 was used as an indication of inflammation and quantified in the striatum of all host/donor subjects. As a marker for astrocytes, which, upon immune activation, can release proinflammatory cytokines and chemokines (Giovannoni & Quintana, 2020), GFAP was analyzed as another indicator of inflammation. I hypothesized that Iba1 and GFAP expression would be increased in M/W host/donors, and that this would correlate to GID behavior. Iba1 and GFAP expression were reported as Iba1 volume (μm^3)/TH+ neuron and GFAP volume (μm^3)/TH+ neuron, respectively.

Contrary to this hypothesis, Iba1 expression per TH+ neuron was not significantly different between genotypic host/donor combinations, even when M/M, W/M, and W/W (GID-) groups are combined (**Figure 3.7bc**; M/W vs. M/M, W/M, W/W $p = 0.2894$).

Moreover, Iba1 expression was also not correlated to GID behavior (**Figure 3.7d**; M/W $r = 0.2674$, $p = 0.2834$). Likewise, the same outcome was demonstrated for GFAP expression (**Figure 3.7ef**; M/W vs. M/M, W/M, W/W $p = 0.5260$). While there is a slight positive trend between GFAP expression and total GID severity 10 weeks post-engraftment, statistical significance was not apparent (**Figure 3.7g**; M/W $r = 0.1786$, $p = 0.7131$).

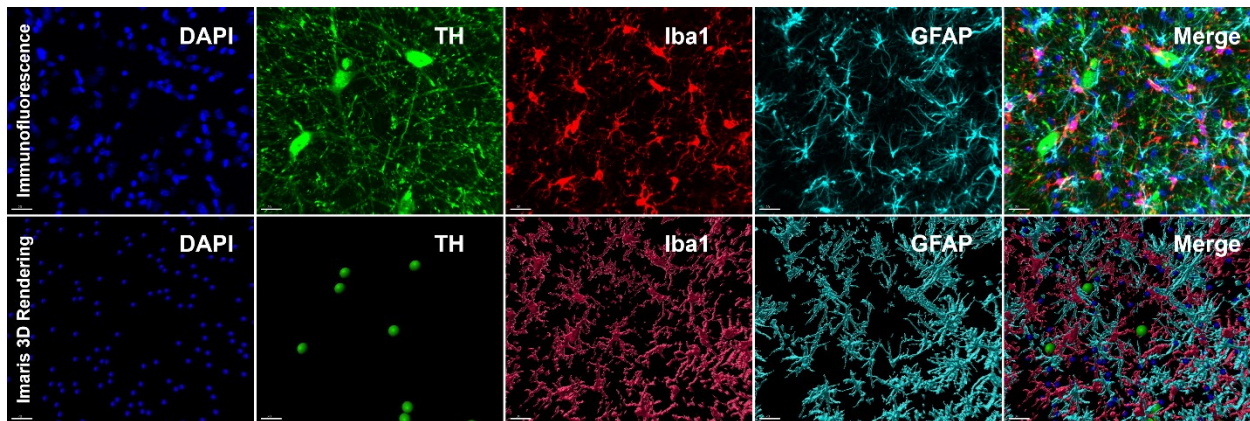
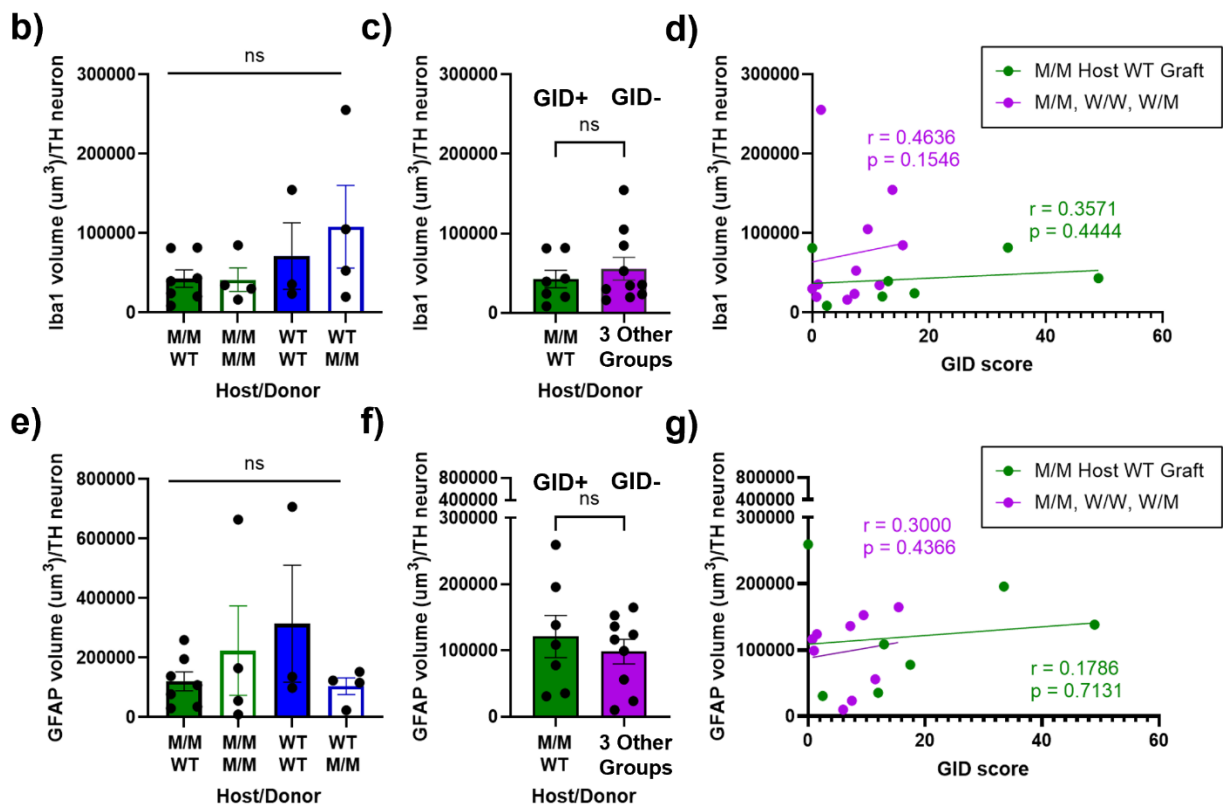


Figure 3.7: Impact of host/donor genotype on immune marker (Iba1 and GFAP) expression in parkinsonian rats.

(a) Representative confocal fluorescent micrograph illustrating the presence of Iba1+ (red) and GFAP+ (cyan) cells in the grafted parkinsonian striatum. Scale bar = 20 μm . **(b)** Quantity of Iba1+ cells normalized to the number of TH+ grafted DA neurons, expressed as Iba1 volume (μm^3)/TH neuron in each host/donor combination. Statistics: Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons, $p \geq 0.3433$ in all host/donor groups. **(c)** Quantity of Iba1 volume (μm^3)/TH neuron between the M/W host/donor group and the 3 other host/donor combinations combined. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, no significance ($p = 0.2894$). **(d)** Spearman correlation between quantity of Iba1

Figure 3.7 (cont'd)

volume (μm^3)/TH neuron and total GID severity at 10 weeks post-engraftment. Statistics: Spearman correlation, no significance. **(e)** Quantity of GFAP+ cells normalized to the number of TH+ grafted DA neurons, expressed as Iba1 volume (μm^3)/TH neuron in each host/donor combination. Statistics: Mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparisons, $p \geq 0.5161$ in all host/donor groups. **(f)** Quantity of GFAP volume (μm^3)/TH neuron between the M/W host/donor group and the 3 other host/donor combinations combined. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, no significance ($p = 0.5260$). **(g)** Spearman correlation between quantity of GFAP volume (μm^3)/TH neuron and total GID severity at 10 weeks post-engraftment. Statistics: Spearman correlation, no significance. Abbreviations: Iba1 = Ionized calcium binding adaptor molecule 1, GFAP = glial fibrillary acidic protein, 3 other host/donor groups combined, GID = graft-induced dyskinesia.



DISCUSSION

The primary objective of DA neuron transplantation therapy is to provide a safe and effective additional or alternative treatment option to the current therapies (e.g., DA replacement therapy) used to treat PD. However, while substantial progress has been made in neural grafting over the past two decades (Barker et al., 2024), mechanisms underlying heterogeneity in clinical responsiveness remains unknown with GID remaining a significant, aberrant side effect. Despite reinvigorated interest, with several clinical trials planned or ongoing ((Barker et al., 2019); clinical trial identifier examples NCT04802733, NCT01898390, NCT03309514, NCT03119636, NCT04146519), the question remains whether we understand the mechanisms underlying regenerative cell therapy enough for its safe incorporation into clinical practice. Until we can achieve optimal benefit while preventing side effect liability, neural transplantation will not be considered a viable, effective alternative therapeutic option for individuals with PD.

As discussed previously, our laboratory became interested in the common human SNP, rs6265, as a potential risk factor underlying the variability of clinical outcomes in DA neuron transplantation. Using a CRISPR knock-in parkinsonian rat model of the rs6265 SNP, we have demonstrated that homozygous rs6265 (i.e., Met/Met) parkinsonian rats engrafted with WT DA neurons exhibited enhanced therapeutic efficacy evidenced by earlier and more robust amelioration of LID behavior post-engraftment in comparison to grafted WT subjects. Moreover, a paradoxical enhancement of graft-derived neurite outgrowth was reported in these Met/Met animals (Mercado et al., 2021). This finding was contrary to our hypothesis since BDNF normally promotes dendritic spine and synapse formation within the striatum (Gonzalez et al.,

2016; Kowiański et al., 2018; Park & Poo, 2013; Sasi et al., 2017; Zagrebelsky et al., 2020) and increases graft-derived innervation in parkinsonian rats (Yurek, 1998; Yurek et al., 1996). Because the Met/Met genotype has decreased activity-dependent release of BDNF, we had theorized that grafted Met/Met parkinsonian rats would demonstrate diminished neurite outgrowth instead.

While seemingly a paradoxical phenomenon, research groups of other disease models have also highlighted a benefit of the rs6265 Met allele. For example, Met-allele carriers expressed enhanced recovery and axon regeneration following TBI in combat veterans (Barbey et al., 2014; Finan et al., 2018; Krueger et al., 2011). Remarkably, Met-allele carriers with MS (Zivadinov et al., 2007) or late-stage AD (Voineskos et al., 2011) have reported a reduction in cognitive decline compared to WT patients. Other preclinical studies in rodents have also reported similar findings (McGregor et al., 2019; McGregor & English, 2019). Collectively, this evidence supports the notion that the rs6265 SNP may confer protective, or neuroregenerative, effects in disease and likely has an evolutionary benefit (Di Pino et al., 2016).

Intriguingly, the BDNF prodomain/pro-peptide has been recently speculated as being responsible for the potential neuroregenerative effect of the Met allele. Because the rs6265 SNP is found within the BDNF prodomain/pro-peptide region, and because the pro-peptide has recently been discovered to function as an independent ligand similar to that of proBDNF and mature BDNF (Anastasia et al., 2013), it is reasonable to suggest that the Met BDNF pro-peptide could have an unexpected benefit of growth-enhancing properties in neural grafting. While some evidence shows differential functions of the Val- and Met-type BDNF pro-peptide (e.g., (Anastasia et al., 2013),

findings are limited to the hippocampus, and further research will be required to fully elucidate their function in the grafted parkinsonian striatum (see (Szarowicz et al., 2022) for a comprehensive discussion of the BDNF pro-peptide).

With our current study, I endeavored to investigate rs6265 in both host and donor neurons on functional outcomes of neural transplantation to understand fully the impact of the Met allele in the host and donor. Due to the high prevalence of rs6265 in the general population (i.e., 20%) (Petryshen et al., 2010; Tsai, 2018), the odds of a PD patient receiving a graft containing a Met allele is inevitable. Studies which precede this (Mercado et al., 2021, 2024) only engrafted WT DA neurons, and to our knowledge, this is the first experiment of its kind to examine rs6265 in both host and donor in a parkinsonian rat model. Thus, we engrafted WT and Met/Met parkinsonian host rats with either WT or Met/Met donor neurons, generating six different host/donor combinations including sham-grafted subjects. My goal was to determine the optimal host/donor combination that retained graft-derived functional benefit but had diminished side effect liability (i.e., GID).

As the primary behavioral readout of graft function, amelioration of LID was employed. In our previous study, Met/Met parkinsonian host rats engrafted with WT DA neurons demonstrated earlier and more robust amelioration of LID behavior over the entire 10-week time course compared to their WT counterparts (Mercado et al., 2021). In the current study, I hypothesized that host and/or donors with the Met/Met genotype would retain behavioral benefit and exhibit earlier amelioration of LID, based on our previous research and other evidence of the Met-allele benefit as discussed above (Barbey et al., 2014; Finan et al., 2018; Krueger et al., 2011; McGregor et al., 2019;

McGregor & English, 2019). In line with this hypothesis, the Met/Met genotype permitted a slightly earlier functional recovery (i.e., lower LID behavior), regardless of host or donor, when compared to WT hosts engrafted with WT DA neurons. All grafted groups in which the Met/Met genotype was present demonstrated a significant functional benefit (i.e., decrease in LID) starting at week four post-engraftment, whereas the WT/WT (host/donor) group did not demonstrate statistically significant recovery until week eight post-engraftment. Interestingly, at the completion of the study (10 week post-engraftment), the WT/WT host/donor rats lost statistically significant functional benefit while the LID behavior of the other three Met/Met host/donor groups remained significantly lower compared to sham-grafted animals.

I also employed amphetamine-mediated rotational behavior as a secondary readout of graft function (Collier et al., 1999, 2015; Dunnett & Torres, 2011; Soderstrom et al., 2008). Not only does the Met/Met genotype permit behavioral recovery through amelioration of LID behavior, these animals also exhibited a lower number of ipsilateral amphetamine-induced rotations per minute at 10 weeks post-engraftment. For example, similar to LID analysis, the groups containing a Met/Met genotype (i.e., M/W, M/M, W/M host/donors) collectively demonstrated a statistically significant reduction in amphetamine-mediated rotations compared to sham-grafted rats at 10 weeks post-engraftment. This difference was not apparent in the WT/WT host/donor subjects. Results collected from LID ratings and amphetamine rotational analysis further confirm that the Met-allele indeed retains functional benefit.

Although a significant increase in neurite outgrowth was prevalent in Met/Met hosts engrafted with DA neurons in our previous study (Mercado et al., 2021), this

difference was not apparent in this current experiment 10 weeks post-engraftment. Estimated total grafted DA neurons and graft volume (μm^3) were the same across host/donor combinations. Likewise, neurite density measurements per grafted DA neuron were not different among host/donor combinations, either proximal or distal distances from the graft. Because a notable difference is no longer detected, it is possible that the Met-allele-containing groups showed earlier enhanced outgrowth supported by functional data that was lost to detection over the 10 week time span, or it can be speculated that these animals have similar neurite densities because of more advanced host age: these rats are slightly older than those in our previous study (Mercado et al., 2021) by 2-3 months. Notably, in our middle-aged cohort (Mercado et al., 2024), enhanced neurite outgrowth between Met/Met and WT hosts was also no longer evident at 10 weeks post-engraftment. Therefore, the modest increase in age could have had an effect on neurite outgrowth in the animals of this current study, reaching a threshold and no longer presenting as an enhancement in the Met/Met genotype. As this is only speculation, an age-matched experiment would be necessary in the future.

Because our overarching hypothesis was based on the idea that a decrease in activity-dependent BDNF release (i.e., rs6265) could underlie the variability in clinical responsiveness to neural grafting (i.e., GID induction), we postulated that Met/Met hosts engrafted with Met/Met donor neurons would display the greatest severity of GID behavior since both host and donor have a deficit in released BDNF. Unexpectedly, however, Met/Met hosts engrafted with WT DA neurons were the only group to develop significant GIDs compared to all other host/donor combinations. Although unexpected,

this finding does corroborate our findings in (Mercado et al., 2021), which demonstrated that Met/Met hosts engrafted with WT DA neurons uniquely developed GID compared to WT hosts. Nevertheless, a possible explanation as to why GID only develop in the M/W host/donor animals remains unknown.

An earlier publication by our group ultrastructurally demonstrated that grafted DA neurons make asymmetric, atypical (presumed glutamatergic) synapses onto host MSNs in the striatum, and that the presence of these synapses positively correlated to increased GID behavior (Soderstrom et al., 2008). Consistent with these findings, Mercado and colleagues later reported that GID behavior in Met/Met host rats was strongly correlated to expression of VGLUT2 protein in the grafted DA neurons (Mercado et al., 2021). This is indicative that the grafts are maintaining an immature phenotype following transplantation, forming glutamatergic (asymmetric) synaptic connections onto striatal MSNs (El Mestikawy et al., 2011). Therefore, in my study, I also investigated the expression, and potential correlation, between VGLUT2 and GID behavior in the M/W host/donor group to ascertain whether this phenomenon was preserved. No longer was a statistical correlation found between VGLUT2 expression and GID behavior in these animals; however, a similar trend still existed. Due to the expression of VGLUT2 in the grafted neurons, it is still apparent that these grafts are maintaining an immature phenotype, yet this phenotype alone may not be sufficient to underlie GID behavior.

As discussed above, it has been reported that an imbalance between TrkB/p75^{NTR} proteins is implicated in neurodegenerative rodent models such as HD (e.g., (Suelves et al., 2019)). Specifically, Suelves and colleagues investigated the impact of

the p75^{NTR}/TrkB imbalance on motor behavior and striatal neuropathology in a HD mouse model (Suelves et al., 2019). Their results demonstrated increased levels of p75^{NTR} in the striatum of HD mice associated with the manifestation of motor abnormalities and a decrease in dendritic spine density. Once p75^{NTR} levels were genetically normalized, dendritic spine density was rescued, and motor deficits were delayed (Suelves et al., 2019). Other studies have similarly confirmed these findings (see (Brito et al., 2013; Zagrebelsky et al., 2020; Zuccato et al., 2008)). Of note, p75^{NTR} can also play a critical role in glutamate synaptogenesis where its activation can influence synapse development and glutamate release (Numakawa et al., 2003; Wang et al., 2022).

Because of the aberrant nature of grafted DA synapses onto MSN dendrites in the presence of decreased spine densities in the parkinsonian striatum (e.g., (Soderstrom et al., 2008)), I hypothesized that an imbalance between TrkB and p75^{NTR} transcript and receptor expression, with a propensity toward p75^{NTR} upregulation, would correlate with GID behavioral development in Met/Met parkinsonian rats engrafted with WT DA neurons. Since upregulation of p75^{NTR} has been associated with a decrease in dendritic spine density (Reichardt, 2006; Zagrebelsky et al., 2005), it is reasonable to suggest that, if the M/W host/donor group presents with an increase in p75^{NTR}, they may also demonstrate decreased spine density, which could impact synaptic circuitry between the host MSNs and the grafted DA neurons, ultimately leading to GID development. Moreover, if an upregulation of p75^{NTR} is found within the grafted DA neurons, it could suggest an activation of glutamate release from the DA neurons onto host MSNs.

Expectedly, in the intact striatum, there was an upregulation of TrkB mRNA expression in the Met/Met parkinsonian hosts engrafted with Met/Met DA neurons. Mercado and colleagues previously noted a similar finding where the intact side of the Met/Met parkinsonian hosts demonstrated an upregulation of TrkB mRNA compared to WT hosts (Mercado et al., 2021). Biologically, an upregulation of TrkB is expected in the homozygous rs6265 Met/Met genotype as there is a decrease in BDNF in the brain microenvironment (Egan et al., 2003). Additionally, we report no differences in TrkB mRNA expression in grafted DA neurons of all host/donor combinations, which is also in confirmation of the findings reported by Mercado and colleagues where Met/Met and WT DA-grafted parkinsonian animals expressed similar levels of TrkB mRNA (Mercado et al., 2021). Likewise, expression of p75^{NTR} mRNA was not statistically different between groups in the intact striatum or grafted DA neurons; however, there appeared to be a slight increase in p75^{NTR} transcripts inside the graft in M/W host/donor animals. Variability within this group was substantial and likely accounts for the lack of statistical significance of this increase.

Most importantly, when reported as a ratio (i.e., TrkB:p75^{NTR}) within TH+ neurons, GID+ M/W host/donor rats exhibit a significantly lower ratio compared to the three other GID- host/donor groups combined, suggesting that there are more p75^{NTR} transcripts per grafted DA neuron than TrkB transcripts in this host/donor combination. Based on the mechanism of action known for p75^{NTR} (Friedman, 2000; Teng et al., 2005; Woo et al., 2005), we can infer that an increased presence of p75^{NTR} may prevent proper formation of typical, symmetric DA neuron circuitry, potentially causing GID. Additionally, p75^{NTR} activation on the grafted DA neurons could lead to glutamate release onto host

MSNs, also potentially resulting in GID (see DA/glutamate co-transmission below). Nevertheless, total GID severity at 10 weeks post-engraftment was not statistically correlated with the TrkB:p75^{NTR} ratio, indicating that this imbalance may still be necessary but not a sufficient sole contributor of GID. Since the quantity of mRNA transcripts does not always coincide 1:1 to protein expression, further studies that examine both TrkB and p75^{NTR} protein levels are necessary to definitively determine the role of these receptors in this animal model. Furthermore, investigating activation state of BDNF receptors (e.g., phosphorylated) could provide additional insight into any potential changes in activity that could correlate to the expression of aberrant GID behaviors.

A consistent mechanism that has been posited as underlying GID behavior is uneven and/or excess DA release. Specifically, excess DA was first reported in PD patients who developed aberrant GID in the first double-blind clinical trials (for review (Piccini et al., 1999; Politis, 2010a; Politis et al., 2011)). Supporting the role of DA and its receptors in GID, Shin and colleagues demonstrated confirmatory evidence in DA-grafted parkinsonian rats, demonstrating that pharmacological blockade of D2 (i.e., eticlopride, buspirone) and D1 (i.e., SCH23390) receptors resulted in almost complete amelioration of GID behavior. Moreover, although buspirone is also primarily considered a partial 5-HT receptor agonist, blockade of D2 was found to be independent from activation of 5-HT because its effect was not prevented by a 5-HT antagonist (Shin et al., 2012). This preclinical model ultimately supports the action of buspirone in D2 receptor antagonism and points to a promising role of DA release in GID behavior.

Therefore, I analyzed immunohistochemical expression of DAT as a surrogate marker of DA release in postmortem tissue. Confirming my hypothesis, GID+ M/W host/donor animals demonstrated a significant increase in DAT expression (DAT sum intensity/ μm^3) compared to the three other GID- host/donor groups (i.e., M/M, W/M, W/W), indirectly indicating that more DA is being released in the M/W host/donor parkinsonian rats. Because Mercado and colleagues illustrated that there was an upregulation of *Drd2* mRNA (DA D2 receptor) in Met/Met hosts (Mercado et al., 2024) compared to WT hosts, it is further reasonable to speculate that increased DA release from the WT graft in an environment with (presumably) upregulated D2 receptors could increase activation of host MSNs, subsequently permitting GID behavior. Despite this logical postulation, there was no statistical correlation between GID severity and DAT expression, again suggesting that, while an increase in DAT expression may be necessary, DAT expression alone may not be sufficient to induce GID behavior. Additional studies that examine *direct* release of DA will be necessary.

Immune system activation is another promising mechanism that could potentially underlie GID behavior. As stated above, our group has previously shown that DA-grafted parkinsonian rats exhibited increased GID severity following immune challenge (Soderstrom et al., 2008). In grafted patients with PD, GID developed upon cessation of immune suppression (Hagell & Cenci, 2005; Olanow et al., 2003). Therefore, I investigated two common markers of the immune system including Iba1 (microglia) and GFAP (astrocytes) to provide cursory insight into whether the immune system impacted GID expression in my studies. However, no obvious differences were found between host/donor combinations in either Iba1 or GFAP expression. Moreover, no correlation

was exhibited between Iba1 or GFAP expression and total GID severity at the conclusion of the study. Although no correlation was evident with these specific markers, a role for immune activation should not be excluded as a potential GID mechanism based on historical data. Here, only pan markers for microglia and astrocytes that stain nearly all Iba1+ and GFAP+ cells in the brain were employed. Markers for activated immune factors such as major histocompatibility complex 2 (MHC-II) should be utilized for greater specificity in the future. Additionally, directly assessing the connection between immune suppression and GID induction in association with the rs6265 SNP in this parkinsonian rat model is warranted.

It is highlighted here that graft-derived functional benefit of the rs6265 (Met/Met) genotype is retained in parkinsonian rats whether it is found in the host or donor, and that the Met/Met hosts engrafted with WT DA neurons remain the only host/donor combination to develop aberrant GIDs. While we are aware that advances in the clinical grafting field have been made, with several clinical trials planned or ongoing ((Barker et al., 2019); clinical trial identifier examples NCT04802733, NCT01898390, NCT03309514, NCT03119636, NCT04146519), we recognize that a gap in our understanding regarding the underlying mechanism of GID still exists. In our continuing investigation of GID, we have established a probable role of excess DA release in this aberrant behavior—a finding that has been also supported in grafted PD patients who received buspirone (a drug with DA antagonist properties) that successfully reduced their GID (Politis, 2010a; Politis et al., 2011; Steece-Collier et al., 2012). The exact mechanism(s) that result in the association between GID and DA release warrants further investigation in preclinical models and clinical trials. Moreover, because it is not

common practice in clinical grafting trials, our strong cumulative data suggests that both participants and donor neurons are genotyped for the rs6265 SNP prior to transplantation. Once the field can harness the benefit, while preventing the detriment, of the rs6265 SNP, regenerative cell therapy has the potential to be a fully optimized therapeutic option to treat not only PD, but also other neurodegenerative and neurological disorders.

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**CHAPTER 4: EXOGENOUS BDNF TREATMENT EXACERBATES GRAFT-INDUCED
DYSKINESIA IN HOMOZYGOUS rs6265 (MET/MET) PARKINSONIAN RATS**

ABSTRACT

Dopamine (DA) neuron transplantation remains a promising therapeutic approach to restore lost DA in the parkinsonian striatum; however, a significant side effect of is graft-induced dyskinesia (GID). While several theories of GID have been posited, its underlying mechanisms remain unclear and controversial. Our investigations, aimed at understanding potential genetic contributions to GID, have focused on a common single nucleotide polymorphism (SNP), rs6265, found in the gene for brain-derived neurotrophic factor (BDNF), which results in decreased BDNF release. Using a CRISPR knock-in rat model, we first reported that parkinsonian rats homozygous for rs6265 (aka Met/Met) engrafted with wild-type (WT) primary DA neurons uniquely developed GID compared to their WT counterparts. Because rs6265 causes decreased BDNF release, we hypothesized that “replenishing” BDNF would ameliorate GID behavior. To evaluate this, exogenous intracerebral BDNF was infused into parkinsonian Met/Met rats engrafted with WT DA neurons using osmotic minipumps. Unexpectedly, BDNF infusion exacerbated GID in grafted Met/Met animals compared to vehicle-infused controls, and evidence suggests that dysregulated DA/glutamate co-transmission and/or excess DA release contributes to GID expression. While our findings are supported by clinical data, they reveal novel mechanisms that are related to an individual’s genetic profile that may be important to consider as cell transplantation therapies advance in ongoing clinical trials.

INTRODUCTION

PD is a complex, heterogeneous neurodegenerative disorder that affects over 9.3 million people worldwide (Espay et al., 2017; Maserejian et al., 2020; Schalkamp et al., 2022). While pharmacological interventions traditionally used to treat PD (e.g., levodopa) alleviate a majority of motor symptoms, there is significant heterogeneity in clinical responsiveness (Fischer et al., 2018, 2020; Hauser et al., 2009; Sortwell et al., 2022), and most individuals eventually experience waning efficacy and side effect development (e.g., levodopa-induced dyskinesia (LID)) as their disease progresses (Hauser et al., 2009). Based on the unmet need in clinical management of PD, additional/alternative therapies continue to be investigated (e.g., (Barker et al., 2024)). A promising regenerative medicine alternative involves DA neuron transplantation aimed at restoring DA terminals within the striatum to replace those that die off in PD. The transplantation method that has had most clinical success is grafting primary embryonic ventral mesencephalic (eVM) DA neurons into the caudate/putamen, demonstrating clear efficacy in a subpopulation of recipients with PD (Olanow et al., 2009; Steece-Collier et al., 2012; Stoker et al., 2017). Despite distinct, yet heterogeneous, success, clinical trials have also demonstrated heterogeneity in side effect development. Specifically, a subpopulation of patients exhibited an aberrant side effect known as GID in response to receiving DA neuron grafts (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). It was the occurrence of GID behavior that led to a worldwide moratorium on all clinical grafting trials in 2003 (Barker et al., 2019; Collier et al., 2019; Parmar et al., 2020; Stoker & Barker, 2020). After decades of rigorous preclinical research and retrospective analyses of clinical trials, several clinical grafting trials are planned or

ongoing (example clinical trial identifier examples NCT04802733, NCT01898390, NCT03309514, NCT03119636, NCT04146519), yet the underlying mechanism of GID behavior remains unclear and controversial.

GID are abnormal involuntary movements that, to date, have been observed to manifest only in individuals who received primary DA grafts (for review (Steece-Collier et al., 2012)). Several proposed underlying mechanisms of GID have been posited. These include, but are not limited to, uneven DA reinnervation/excess DA release, donor cell source and preparation, presence of non-DA cells (e.g., serotonin neurons), age of recipient, pre-graft levodopa history, the immune response, and abnormal/asymmetric synaptic connections (Ma et al., 2002; Mercado et al., 2021, 2024; Pagano et al., 2018; Soderstrom et al., 2008). Previously, our laboratory demonstrated that there was a significant association between GID behavior and the presence of excitatory asymmetric synaptic connections made by and onto grafted DA neurons in parkinsonian rats (Mercado et al., 2021; Soderstrom et al., 2008). Normally, mature DA neurons make *en passant*, symmetric appositions onto the dendritic spines of striatal medium spiny neurons (MSNs) (Gerfen & Surmeier, 2011; W. Shen et al., 2016). However, the grafted neurons in the GID-expressing parkinsonian rats have been reported to make atypical asymmetric synapses directly onto the dendrite or onto the cell soma (Soderstrom et al., 2008). These atypical synaptic profiles have also been observed in PD patients engrafted with embryonic DA neurons (Kordower et al., 1996), suggestive of impaired and/or delayed maturation in which a DA-glutamate co-transmission phenotype is common (El Mestikawy et al., 2011).

Given the necessity of BDNF for midbrain DA neuron maturation and synapse formation (Adachi et al., 2005; Baquet et al., 2005; Hyman et al., 1991; Yurek, 1998; Yurek et al., 1996), we began investigating the role of BDNF in GID behavior. We identified a common SNP, rs6265, found within the gene for BDNF which results in a decrease in activity-dependent BDNF release (Egan et al., 2003). The rs6265 SNP, also referred to as Val66Met, involves a valine to methionine substitution at codon 66 and occurs in approximately 20% of the general population (Petryshen et al., 2010; Tsai, 2018). Both the heterozygous (Val/Met) and homozygous (Met/Met) genotype result in a significant dose-dependent decrease of activity-dependent release of BDNF by disrupting the packaging of BDNF into secretory vesicles (for review (Egan et al., 2003; Mercado et al., 2021)). Notably, rs6265 is not associated with PD incidence (Egan et al., 2003; Mariani et al., 2015) but has been shown to reduce therapeutic efficacy of oral levodopa in PD patients (Fischer et al., 2020). Due to the considerable prevalence of rs6265 in the general population and the critical role of BDNF in promoting dendrite spine growth, formation of synapses in DA neurons, and maturation of DA neurons, I hypothesized that this genetic risk factor underlies the variability (i.e., GID behavior) in clinical response to DA neuron grafting in individuals with PD. We theorized that the decrease in BDNF release in the extracellular environment caused by the homozygous SNP (Met/Met) prevents proper graft maturation and that “replacing” the deficient BDNF would allow for graft maturation and proper integration into the host, ultimately ameliorating GID.

Using a CRISPR knock-in parkinsonian rat model of the homozygous rs6265 SNP (Met/Met) developed by our colleagues Dr. Caryl Sortwell and Dr. Timothy Collier,

we recently demonstrated that only Met/Met rats engrafted with WT DA neurons uniquely exhibited induction of GID behavior compared to their WT counterparts engrafted also with WT DA neurons ((Mercado et al., 2021); see also Chapter 3)). In an attempt to mitigate GID behavior in an environment of decreased extracellular BDNF (i.e., rs6265 Met/Met), in this current study, we infused exogenous mature BDNF into the striatum of Met/Met host rats engrafted with WT DA neurons. We achieved exogenous BDNF administration with a subcutaneous osmotic minipump attached to a cannula that was placed directly above the grafted DA neurons in the parkinsonian striatum.

We report here that, contrary to our hypothesis, exogenous BDNF infusion into DA-grafted homozygous Met/Met parkinsonian rats increased aberrant GID behavior compared to DA-grafted vehicle-infused controls. We also provide evidence that GID in these animals are correlated with indices of excess DA release demonstrated by an increase in DA transporter (DAT) expression and contralateral amphetamine-mediated rotational behavior. Importantly, these results corroborate findings in grafted PD patients where excess DA release was also found to be associated with GID (Piccini et al., 1999; Politis, 2010; Politis et al., 2011). Moreover, we provide evidence suggestive of an entirely novel mechanism associated with excess graft-derived DA signaling—a phenomenon known as vesicular synergy. Vesicular synergy, in this context, posits that the presence of vesicular glutamate transporter 2 (VGLUT2) on a vesicular monoamine transporter 2 (VMAT2)-positive synaptic vesicle within a DA neuron promotes increased DA loading into vesicles, resulting in excess DA release (El Mestikawy et al., 2011; Hnasko et al., 2010; H. Shen et al., 2021). Our supporting evidence illustrates high-

resolution confocal imaging of (presumed) co-localized VMAT2 and VGLUT2 protein in striatal DA-grafted neurites, which is remarkably correlated with GID behavior.

GIDs are a complex behavior in which my cumulative data suggest that several mechanisms appear to be necessary, but possibly not sufficient by themselves, to induce GID. Collectively, our research suggests that atypical DA/glutamate co-transmission and/or excess DA release are promising factors underlying GID induction, influenced by genetic characteristics of the host and donor. However, additional research is necessary to directly confirm that excess DA release underlies GID and to fully understand GID pathogenesis. Our findings reinforce the notion that a personalized medicine approach will be imperative to optimize clinical outcomes of cell transplantation for individuals with PD. Once we understand the mechanisms of GID, we may be able to provide a solution to prevent its occurrence in this host/donor combination.

METHODS

Experimental Animals

Male Sprague-Dawley rats homozygous for rs6265 (Met/Met) (6-7 months at lesioning; 11-12 months at sacrifice) were obtained from our in-house colony derived from CRISPR knock-in rats carrying the valine to methionine polymorphism in the rat BDNF gene (Val68Met, Val/Met). Using CRISPR/Cas-mediated homologous recombination, these rats were generated by Cyagen Biosciences (Santa Clara, CA). In this study, only homozygous Met/Met rats were used based on findings from our previous experiments which demonstrated that Met/Met host rats engrafted with wild-type (WT) DA neurons uniquely developed GID behavior (Mercado et al., 2021). Of note, the rat Val68Met SNP is equivalent to the human Val66Met SNP because rats have two additional threonine amino acids at positions 57 and 58. The BDNF gene in rats has approximately a 96.8% sequence homology with the human BDNF gene (BLAST queries: P23560 and P23363). The Michigan State University Institutional Animal Care and Use Committee approved all experimental procedures.

Two rats were removed from experimental evaluation due to spontaneous death during or following neural transplantation surgery. Other animals (N=18) were excluded a priori (i.e., prior to grafting) because they failed to develop sufficient LID, as well as to keep the N of each group to approximately 10. A small number of (N=4) were excluded from postmortem analyses due to having too few surviving cells in the graft (<100) or misplaced grafts (e.g., cortically placed grafts). Final experimental cohorts were N=9 BDNF (non-grafted), N=9 PBS (non-grafted), N=9 BDNF-infused (grafted), N=9 PBS-infused (grafted).

Experimental Timeline

Illustrated in **Figure 4.1**, rats were first unilaterally rendered parkinsonian via a stereotaxic injection of 6-hydroxydopamine (6-OHDA) in the SN and medial forebrain bundle (MFB). Lesion status was then confirmed two weeks later with amphetamine-mediated rotational behavior as described in Chapter 3. Two weeks following, rats were primed with daily levodopa to induce significant, stable LID, which was our primary behavioral readout of graft function. After four weeks of priming with levodopa, rats received intrastriatal grafts of embryonic ventral mesencephalic (VM) DA neurons from wild-type (WT; Val/Val) rats or no grafts as the control. Immediately following grafting surgery, subcutaneous osmotic minipumps containing either mature BDNF (R&D Systems, Inc. Bio-Techne Corporation catalog # 11166-BD) or vehicle-control phosphate-buffered saline (PBS) were implanted under the skin. The minipumps were attached to a cannula that was placed directly above the grafted cells. Following grafting surgery, levodopa was withdrawn for one week and then reinitiated for the remainder of the study. Parkinsonian rats were evaluated for amelioration of LID behavior 10 weeks following engraftment. At five and 10 weeks post-engraftment, amphetamine-induced rotational behavior was assessed as a secondary measure of graft function. Lastly, as an indicator of graft dysfunction, GID were evaluated at five and 10 weeks following LID assessment.

Nigrostriatal Lesioning with 6-OHDA

Anesthetized (2% isoflurane, Sigma St. Louis, MO, USA) rats, after being placed in a stereotaxic frame, received two μL of 6-OHDA (flow rate of 0.5 $\mu\text{L}/\text{minute}$) to the SNpc (4.8 mm posterior, 2.0 mm lateral, 8.0 ventral relative to bregma) and the MFB

(4.3 mm posterior, 1.6 mm lateral, 8.4 mm ventral relative to bregma). After surgery completion, rats received intraperitoneal (i.p.) injections of 5 mg/kg carprofen (Rimadyl) as an analgesic. For histological postmortem confirmation of successful nigral lesions, medial terminal nucleus (MTN) DA cell enumeration methods were used (Gombash et al., 2014).

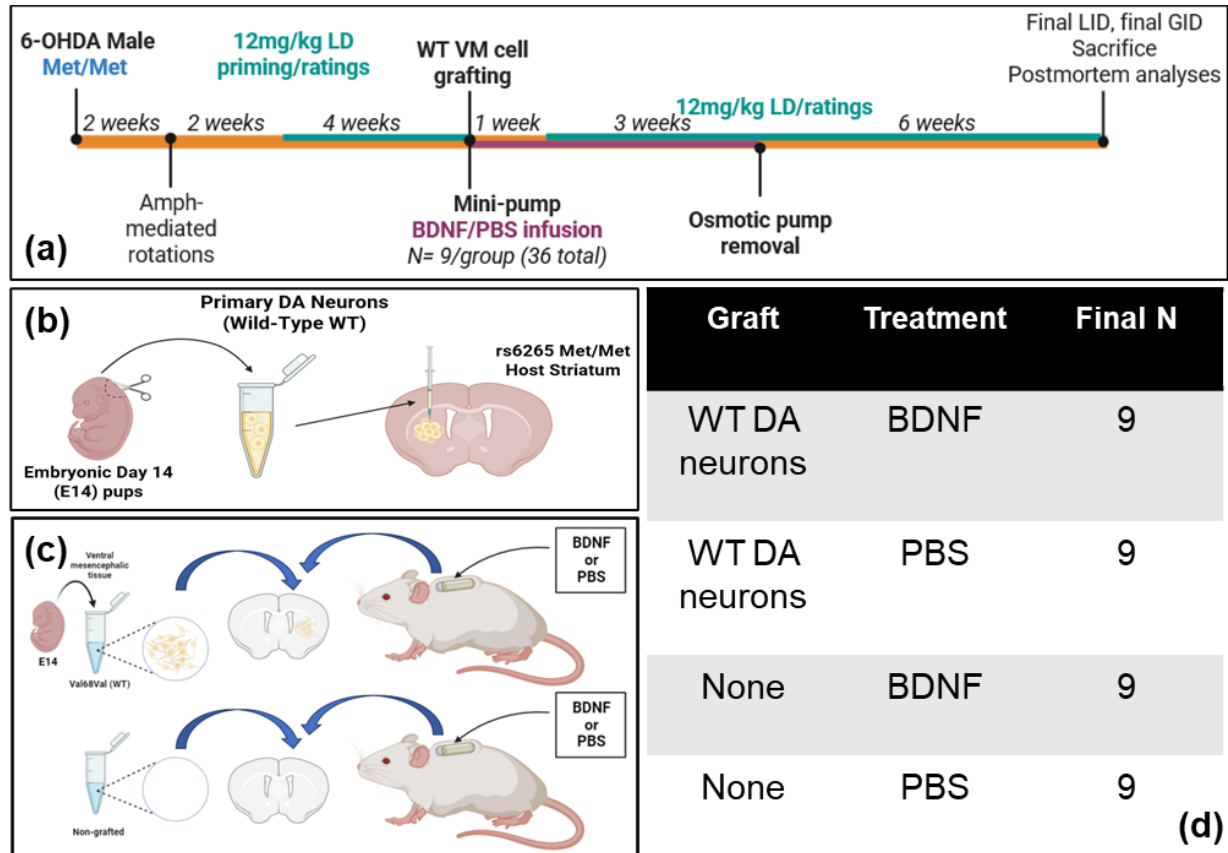


Figure 4.1: Experimental Design and Timeline.

(a) Experimental timeline of surgeries, behavioral evaluation, and drug administration. **(b)** Experimental schematic illustrating cell transplantation. E14 ventral mesencephalic tissue from WT (Val/Val) Sprague-Dawley rats was dissected and transplanted into homozygous rs6265 Met/Met host rats. **(c)** Following cell transplantation, subcutaneous osmotic minipumps containing either mature BDNF or PBS were implanted under the skin and attached to cannulas that were placed above the grafted cells. **(d)** Table including subsequent treatment of experimental groups and final group sizes. Abbreviations: 6-OHDA = 6-hydroxydopamine, amph-induced = amphetamine-induced, LD = levodopa, VM = ventral mesencephalic, GID = graft-induced dyskinesia.

Amphetamine-Induced Rotational Behavior

Amphetamine-mediated rotational behavior was utilized as a method to assess both lesion status following 6-OHDA surgeries and graft function and dysfunction (i.e., GID described below) following transplantation surgeries because it is a reliable measure of nigrostriatal DA depletion and graft-derived DA release (Collier et al., 1999, 2015; Dunnett & Torres, 2011; Soderstrom et al., 2008). Two weeks after lesion surgery, amphetamine-mediated rotational behavior was first assessed to confirm lesion status. Amphetamine sulfate (2.5 mg/kg) was injected (i.p.) into each subject. Rotational behavior was then monitored for a total of 90 minutes using the automated Rotameter System (TSE-Systems, Chesterfield, MO, USA). Rats that rotated 5 ipsilateral turns per minute or more over the 90 minute time course were included for the continuation of the study. Amphetamine rotations were also quantified manually at one-minute intervals in the rat's home cage at five and 10 weeks post-engraftment as a secondary measurement of graft function.

Levodopa Administration and LID ratings

Four weeks after lesion surgeries, rats were primed with daily (M-F) levodopa (12 mg/kg, 1:1 levodopa/benserazide, subcutaneous (s.c.) administration) for a total of four weeks before grafting surgeries. Levodopa was withdrawn one week following transplantation surgeries to prevent any potential toxic interactions between the grafted cells and levodopa (Collier et al., 2015; Steece-Collier et al., 1990). Levodopa was introduced again after the one-week hiatus and continued daily throughout the remainder of the experiment.

The well-established rat model of LID was employed as an indicator of graft function as this behavioral side effect can be ameliorated by DA neurons grafts in parkinsonian rats (Lane et al., 2006; Lee et al., 2000; Maries et al., 2006; Mercado et al., 2021; Soderstrom et al., 2008, 2010) and individuals with PD (Hagell & Cenci, 2005). LID were evaluated on pre-graft days 1, 7, 14, and 21, and at five post-graft timepoints including week 2, 4, 6, 8, and 10. The rating scale employed for LID severity was developed by our laboratory based on specific criteria comparable to attributes of dyskinesia (see (Caulfield et al., 2021; Maries et al., 2006) for details). A blinded investigator evaluated LID behavior at one-minute intervals 20, 70, 120, 170, and 220 minutes following levodopa injection as previously detailed (Mercado et al., 2021). A total LID severity score was calculated for each animal at each rating session as previously detailed in (Mercado et al., 2021).

Preparation of Donor Tissue and Cell Transplantation

Following levodopa priming, rats were assigned to either DA-grafted or non-grafted BDNF- or PBS-infused groups based on their mean pre-grafted LID severity scores, ensuring that pre-graft LID mean scores were statistically similar between all four treatment groups. Rats in the DA-grafted groups received an intrastriatal transplantation of 200,000 VM cells from embryonic day 14 (E14) timed-pregnant WT donors. Prior to surgery, the VM tissue was collected in cold calcium-magnesium free (CMF) buffer; cells were then dissociated as previously detailed in (Collier et al., 2015; Mercado et al., 2021). Briefly, the dissected tissue was incubated for 10 minutes at 37°C in CMF buffer containing 0.125% trypsin. Cells were then triturated with 0.005% DNase using a 2.0 mm tip Pasteur pipette and further triturated with a sterile 3cc, 22-

gauge syringe. The resulting cell suspension was layered onto sterile fetal bovine serum (FBS) and centrifuged at 1,000 rpm for 10 minutes at 4°C, then resuspended in 1.0 mL Neurobasal medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cell number and viability were evaluated with the trypan blue exclusion test, and the final cell suspension concentration was adjusted to 33,333 cells/ μ L. Cells were kept on ice during surgery and transplanted within five hours of preparation. The cells were injected at a single rostral-caudal striatal site (0.2 mm anterior, 3.0 mm lateral to bregma), with injections at three dorsal-ventral coordinates corresponding to 5.7, 5.0, and 4.3 mm ventral to the skull base (Collier et al., 2015; Mercado et al., 2021). At each coordinate, 2 μ L (injected at 0.5 μ L/min) of the VM cell suspension was delivered, for a total of 6 μ L per rat. Rats in the non-grafted group did not receive any cell suspension based on the logistics of transplanting cells within the 5-hour post-preparation period restraint, along with the necessity of implanting cannulas and minipumps.

Intrastriatal BDNF Infusions

In the same grafting surgical session described above, an infusion cannula was stereotaxically inserted to 0.3 mm dorsal of the transplanted cells (per Yurek et al., 1996/98). The cannula (Alzet® Brain Infusion Kit 2) was attached with tubing to a primed 28-day Alzet® minipump (model 2004; flow rate of 0.25 μ L/hour) that was then implanted into the subdermal intrascapular space. Prior to implantation, minipumps were primed for 48 hours in sterile 0.9% saline before being filled with either sterile PBS or 1.25 μ g/ μ L of recombinant human BDNF (R&D Systems, Inc. Bio-Techne Corporation catalog # 11166-BD) dissolved in sterile PBS, similar to what has been previously described in (Yurek, 1998; Yurek et al., 1996). Cannulas were then permanently fixed to

the skull using dental cement and anchor screws that were placed into the skull earlier in the surgery. Minipumps were surgically removed in a sterile environment following completion of BDNF or PBS infusion for a total infusion exposure of four weeks. Effective delivery of recombinant BDNF from osmotic minipumps into a rat model has been demonstrated successfully (Yurek, 1998; Yurek et al., 1996) and (Altar et al., 1994).

Graft-induced Dyskinesia (GID) Ratings

Amphetamine was utilized to assess graft-induced dyskinesia (GID); rats received a single 2 mg/kg i.p. dose of amphetamine sulfate. This method of amphetamine-mediated GID behavior is based on evidence that DA-grafted, but not sham-grafted, animals demonstrate dyskinetic behavior in response to low-dose amphetamine administration (Lane et al., 2009; Shin et al., 2012; Smith et al., 2012). This behavior, which appears phenotypically similar to LID, was rated by a blinded investigator using the same method and rating scale as was described for LID. GID were evaluated one week following minipump removal (i.e., week five post-engraftment) to prevent any acute effects of BDNF infusion on GID behavior. At 10 weeks post-engraftment, GID were evaluated again; this time 24 hours prior to sacrifice.

Necropsy

Rats were sacrificed as detailed in Mercado et al., 2021. Briefly, phenytoin/pentobarbital euthanasia solution (250 mg/kg; i.p., VetOne, Boise, ID, USA) was used to deeply anesthetize the rats. Rats then underwent intracardiac perfusions of room temperature heparinized 0.9% saline followed by cold 4% paraformaldehyde. After perfusion was completed, brains were removed and placed in 4% paraformaldehyde for

a total of 24 hours at 4°C. Brains were then submersed in 30% sucrose at 4°C until sectioning. For sectioning, brains were cut coronally using a sliding microtome at a thickness of 40 µm. Brain sections were stored in cryoprotectant solution at -20°C.

Histology

TH graft Cell Number and Volume

Briefly, tissue sections were rinsed in Tris-buffered saline containing 0.3% Triton-X (TBS-Tx). Sections were incubated in 0.3% hydrogen peroxide, then blocked in 10% normal goat serum (NGS) for 90 minutes. For primary antibody incubation, tissue sections were incubated overnight at room temperature with rabbit anti-TH (see **Table 4.1**). Following primary incubation, sections were incubated in biotinylated goat anti-rabbit secondary antibody (**Table 4.1**), then developed using avidin/biotin enzyme complex.

A blinded investigator used the Stereo Investigator® Optical Fractionator method (MBF Bioscience, Williston, VT, USA) to quantify TH-positive (TH+) cells within the grafted striatum. The 20x objective (numerical aperture 0.75) was used to count cells on a Nikon Eclipse 80i microscope with a 200 µm x 200 µm counting frame. The optical dissector height was set to 20 µm, and the guard zone was set to 2.0 µm. This method was completed in 4-12 serial (1:6) TH+ section in which the number of sections varied depending on the rostral-caudal extent of the graft.

Using the same tissue sections for total enumeration, a blinded investigator employed the Stereo Investigator® Cavalieri Estimator to quantify graft volume. Contours were traced around the central region of the graft, and then a grid with random

sampling sites (50- μm spacing) was superimposed over the contours. Collected data were expressed as total estimated graft volume (mm^3).

Neurite Outgrowth

Two-dimensional (2D) images fluorescently labeled for TH, at 4x magnification, were saved as .tiff files and imported into Fiji image processing package. Eight total regions of interest (ROIs) were created measuring $600\ \mu\text{m}^2$. Four ROIs were first placed around the edge of the perimeter of the grafted TH+ cells. This was considered the proximal region, including proximal dorsolateral, dorsomedial, ventrolateral, and ventromedial. The ROIs were placed in this way around the graft in order to avoid the cannula injection site located immediately dorsal from the graft. An additional four ROIs were placed $625\ \mu\text{m}$ from the edge of the grafted TH+ cell bodies. This was considered the distal regions, including distal dorsolateral, dorsomedial, ventrolateral, and ventromedial. ROIs were then added into the ROI manager, converted to 8-bit, and inverted from the original fluorescent color. The background of each image was removed and the contrast was enhanced for optimal analysis. Each image is then made into a binary, and the threshold function is applied. All white areas that were TH+ were measured for threshold amount and recorded. Data are reported as average neurite density in pixels^2 for proximal and distal regions, and distal neurite density in pixels^2 for each region surrounding the graft (i.e., dorsolateral, dorsomedial, ventrolateral, and ventromedial). These orientations were used to avoid the area of the striatum that was compromised by the overlying cannula (**Figure 4.2f**). This procedure was adapted from (Quintino et al., 2022).

Immunofluorescence

DAB-chromogenic TH-labeled sections as described above were used as a guide when choosing one representative grafted striatal section for each immunofluorescent assay. For all immunohistochemical procedures, tissue sections were rinsed in TBS-Tx, blocked in 10% NGS/0.3% TBS-Tx, and then incubated overnight at 4°C. Tissue sections were then labeled with their respective Alexa Fluor™ secondary antibodies (1:500 dilution; see **Table 4.1**) for 90 minutes at room temperature and protected from light. Sections were mounted and coverslipped with Vectashield® anti-fade mounting medium with DAPI (H-1500; Vector Laboratories, Inc. Burlingame, CA, USA).

Fluorescent Image Acquisition

Using a Nikon A1 laser scanning confocal system equipped with a Nikon Eclipse Ti microscope and Nikon NIS-Elements AR software, all confocal images of (1024 x 1024) immunofluorescent stained tissue sections were acquired. For DAT/TH IHC experiments, the 4x objective was used to collect a full image of the entire graft from each tissue section. One image of the contralateral intact striatum was also taken for comparison. Z-stacks of 2 µm and a scan speed of 1/8 frame/second were used. For VGLUT2/VMAT2 colocalization experiments, z-stacks were acquired through the entire thickness of the mounted tissue sections using the 60x oil-immersion objective (numerical aperture 1.40). A z-step of 1.5 µm was used with a scan speed of 1/8 frame/sec. Two images of each section were taken of the dorsolateral area of the graft because the dorsolateral striatum is a major input region of the basal ganglia and functions predominantly in motor control. Again, one image of the intact side was also taken for comparison. Lastly, for Iba1/GFAP/TH IHC experiments, z-stacks were

acquired using the 10x objective in which multiple images were taken in order to capture the entire graft region in the striatum. Z-steps were 2 um and the scan speed was 1/8 frame/sec. Additional 10x images of the intact striatum was also taken for comparison.

Antigen	Assay	Primary ab	Vendor	Catalog #	Dilution	2° Ab
Tyrosine hydroxylase	TH-VGLUT2	Rabbit anti-TH	Millipore	AB152	1:4000	A-11008
Tyrosine hydroxylase	TH-DAT	Mouse anti-TH	Millipore	MAB318	1:1000	A-11032
Tyrosine hydroxylase	TH-Iba1-GFAP	Sheep anti-TH	Novus Biologicals	NB300-110	1:1000	A-11015
VGLUT2	TH-VGLUT2	Mouse anti-VGLUT2	Abcam	Ab79157	1:1000	A-21124
DAT	TH-DAT	Rabbit anti-DAT	Millipore	D6944	1:1000	A-32733
Iba1	TH-Iba1-GFAP	Rabbit anti-Iba1	FUJIFILM Wako Chemicals	019-19741	1:1000	A-32740
GFAP	TH-Iba1-GFAP	Mouse anti-GFAP	Millipore	MAB360	1:1000	A-21240
VMAT2	TH-VMAT2-VGLUT2	Rabbit anti-VMAT2	Alomone Labs	AMT-006	1:1000	A-1008

Table 4.1: Targeted Antigens and corresponding antibodies.

Secondary antibody catalog numbers are Alexa Fluor®-conjugated, purchased from Invitrogen®.

Imaris Fluorescent Image Quantification

Dual-label protein analysis of VGLUT2 and TH

3-dimensional (3D) z-stacks of grafted tissue fluorescently labeled for VGLUT2 and TH proteins were imported into Imaris®, converted to the native Imaris® file format, and subtracted of any background fluorescence. The surface function was used to generate a 3D reconstruction of the TH+ neuron fibers in the graft (μm^3). The spots function then was used for VGLUT2 protein puncta, taking care to maintain the same parameters across all images. VGLUT2 puncta inside the TH+ grafted surface were filtered through Object-Object statistics using the “Shortest Distance to Surface” function to select only those located within the TH surface. Data are presented as the number of VGLUT2 protein puncta inside the grafted TH surface (μm^3).

Dual-label immunohistochemical DAT and TH protein analysis

Two-dimensional (2D) confocal images of tissue immunolabeled for TH and the dopamine transporter (DA) proteins were imported into Imaris® and converted into the native Imaris® file format. Background subtraction of each image was conducted in order to minimize any background fluorescence in each fluorescent channel. The surface function was used to create an accurate reconstruction of both TH and DAT fibers within the graft. Data are represented as the ratio of DAT fluorescent intensity sum/DAT surface area (μm^2) to TH fluorescent intensity sum/TH surface area (μm^2).

Triple-label immunohistochemical Iba1/GFAP/TH protein analysis

3D z-stacks of grafted brain sections immunolabeled for TH, Iba1, and GFAP proteins were imported into Imaris® and converted to the native Imaris® file format. Background subtraction of each image was conducted to minimize any background

fluorescence. 3D surface objects of TH, Iba1, and GFAP were created using semi-automatic thresholding and the surface function plugin. Data are reported as Iba1 surface volume (μm^3) normalized to the surface volume of the graft (TH; μm^3). GFAP is reported in a comparable manner.

Triple-label immunohistochemical VMAT2/VGLUT2/TH protein analysis

3D z-stacks of grafted tissue labeled for VMAT2, VGLUT2, and TH proteins were imported into Imaris® and converted to the native Imaris® file format. Background subtraction of each image was conducted to minimize any background fluorescence. As before, the surface function was used to generate an accurate 3D reconstruction of the TH+ neuron fibers in the graft (μm^3). The spots function was then used for both VMAT2 and VGLUT2 protein puncta, maintaining the same parameters across all images. The MATLAB “Colocalization” plugin was used to find VMAT2 and VGLUT2 protein puncta that were “co-localized” within 0.5 μm of each other. The co-localized VMAT2/VGLUT2 puncta were then filtered using the Object-Object statistics “Shortest distance to Surface” to include only co-localized puncta that were within the TH surface. Data are represented as the number of co-localized VMAT2/VGLUT2 protein puncta inside the grafted TH surface (μm^3).

ELISA Assay for Interleukin-6 (IL-6)

The Rat IL-6 ELISA kit from Invitrogen (catalog number BMS625) was used for IL-6 analysis. During perfusions at the conclusion of the study (10 weeks post-engraftment), cardiac punctures from the right atrium of the heart were performed to collect blood samples from each rat subject. Blood samples were subsequently spun down using a centrifuge at 2200 rpm for 10 minutes at 4°C. The plasma serum was

collected and stored at -80°C until processing. Plasma samples were prepared and diluted prior to ELISA according to the manufacturer's instructions.

Statistical Analysis

All behavioral data (i.e., GID and LID) were analyzed using non-parametric statistics including the Kruskal-Wallis test with Dunn's multiple comparisons or Mann-Whitney U tests with Dunn's multiple comparisons (between subject comparisons) as LID and GID behavioral data are created using an ordinal rating scale. This statistical test was also employed for the results collected from the IL-6 ELISA as the standard deviation (SD) was significantly different between groups. Amphetamine-mediated rotations were analyzed using a one-way ANOVA with Tukey's multiple comparisons test.

Unpaired two-tailed t-tests were used to compare average neurite outgrowth (proximal and distal) surrounding the DA grafts between treatment groups (BDNF- vs. PBS-infused animals). A two-way ANOVA with Tukey's multiple comparisons was used to analyze distal neurite outgrowth in each region from the graft (i.e., DL, DM, VL, VM). Unpaired two-tailed t-tests were employed for DAT:TH intensity sum/ μm^3 expression and VMAT2/VGLUT2 colocalization in TH+ neurons. This statistical test was also used to compare Iba1 volume (μm^3)/number of TH+ neurons and GFAP volume (μm^3)/number of TH+ neurons. A one-way ANOVA with Sidak's multiple comparisons was used for Iba1 or GFAP surface volume (μm^3) alone.

Non-parametric Spearman correlation tests were applied for all correlations with GID behavior. Amphetamine rotation correlations were analyzed using Pearson correlation. Statistical outliers, while rare, were identified using ROUT and Grubb's

outlier tests. Parametric statistical tests were chosen for analysis only when data met assumptions for normality and homogeneity of variances. All statistical analysis were completed using GraphPad Prism software for Windows (v. 10.4.1).

RESULTS

Exogenous BDNF infusion into DA-grafted animals enhances functional graft efficacy (i.e., amelioration of LID) and neurite outgrowth

We hypothesized that exogenous BDNF infusion into DA-grafted homozygous rs6265 (i.e., Met/Met) animals would generate enhanced behavioral recovery from LID earlier than the other grafted and non-grafted groups. As expected, in comparison to the non-grafted BDNF-infused subjects, BDNF administration to DA graft recipients led to faster amelioration of LID behavior compared to the DA-grafted PBS-infused parkinsonian rats. Specifically, grafted BDNF-infused rats demonstrated a significant reduction in LID behavior compared to non-grafted BDNF-infused animals by four weeks post-engraftment. In contrast, grafted PBS-infused animals did not exhibit a significant amelioration of LID severity until considerably later at week 10 post-engraftment (**Figure 4.2a**. Week 4: $p = 0.0421$ gBDNF vs. non-grafted BDNF; Week 10: $p = 0.0032$ gBDNF vs. non-grafted BDNF, $p = 0.0176$ gPBS vs. non-grafted PBS). At the final week (week 10), grafted BDNF- and PBS-infused animals exhibited approximately the same LID severity scores (**Figure 4.2a**; $p \geq 0.9999$ gBDNF vs. gPBS at week 10).

Because BDNF is a protein critical for neuronal survival, maturation, and function (Gonzalez et al., 2016; Hyman et al., 1991; Kowiański et al., 2018; Lai & Ip, 2013; Park & Poo, 2013; Sasi et al., 2017; Zagrebelsky et al., 2020), we investigated whether BDNF infusion impacted the size of the graft or the number of surviving grafted neurons. While no statistical significance was apparent (**Figure 4.2d**; $p = 0.1449$ gBDNF vs. gPBS), there was an inclination toward an increase in number of surviving DA neurons in the grafted BDNF-infused animals (**Figure 4.2d**). Likewise, a similar, but even

slighter, trend was demonstrated in graft size/volume with the grafted BDNF-infused subjects having a slightly larger graft volume, albeit not statistically significant (**Figure 4.2e**, $p = 0.3347$ gBDNF vs. gPBS, not significant). In addition to graft volume and number of grafted neurons, I also analyzed average TH+ neurite area as an indication of graft-derived outgrowth. Again, we had hypothesized that BDNF administration would enhance neurite outgrowth in the DA-grafted parkinsonian rats due to the known function of BDNF and based on previous findings (see (Yurek, 1998; Yurek et al., 1996)). Data revealed a significant increase in average neurite outgrowth in the DA-grafted BDNF-infused compared to the DA-grafted PBS-infused animals, both proximal and distal to the graft (**Figure 4.2g**, Proximal: $p = 0.0367$ gBDNF vs. gPBS; Distal: $p = 0.0175$ gBDNF vs. gPBS). Additionally, when reported as distal neurite density alone, the dorsolateral region of the graft in the DA-grafted BDNF-infused animals demonstrated significantly increased neurite density compared to the other regions (**Figure 4.2h**, DL vs. DM: $p = 0.0391$, DL vs. VL: $p = 0.0116$, DL vs. VM: $p = 0.0034$ in gBDNF) and compared to DA-grafted PBS-infused animals ($p = 0.0006$ DL gBDNF vs. DL gPBS).

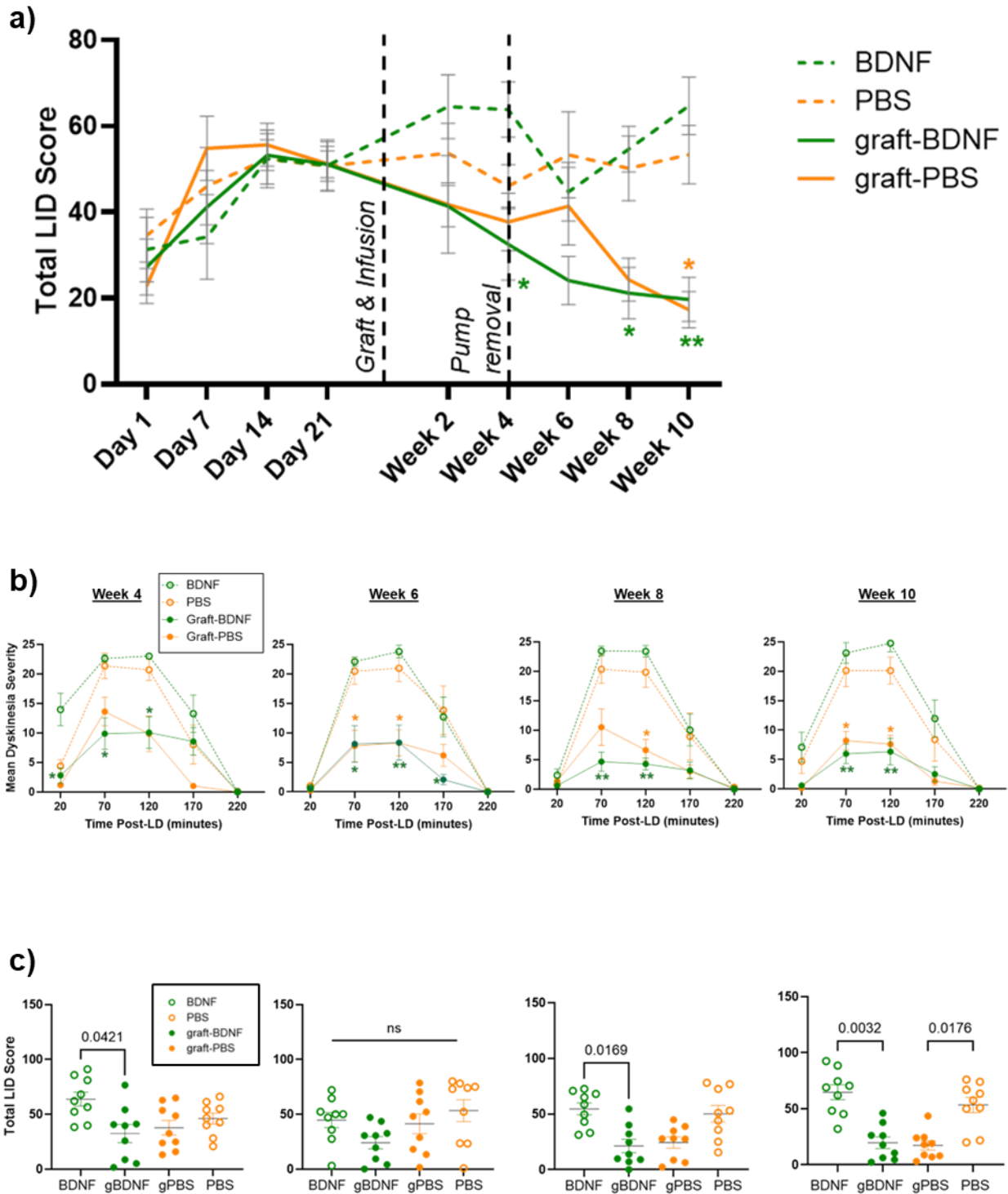


Figure 4.2: Impact of BDNF supplementation on LID behavior and neurite outgrowth.

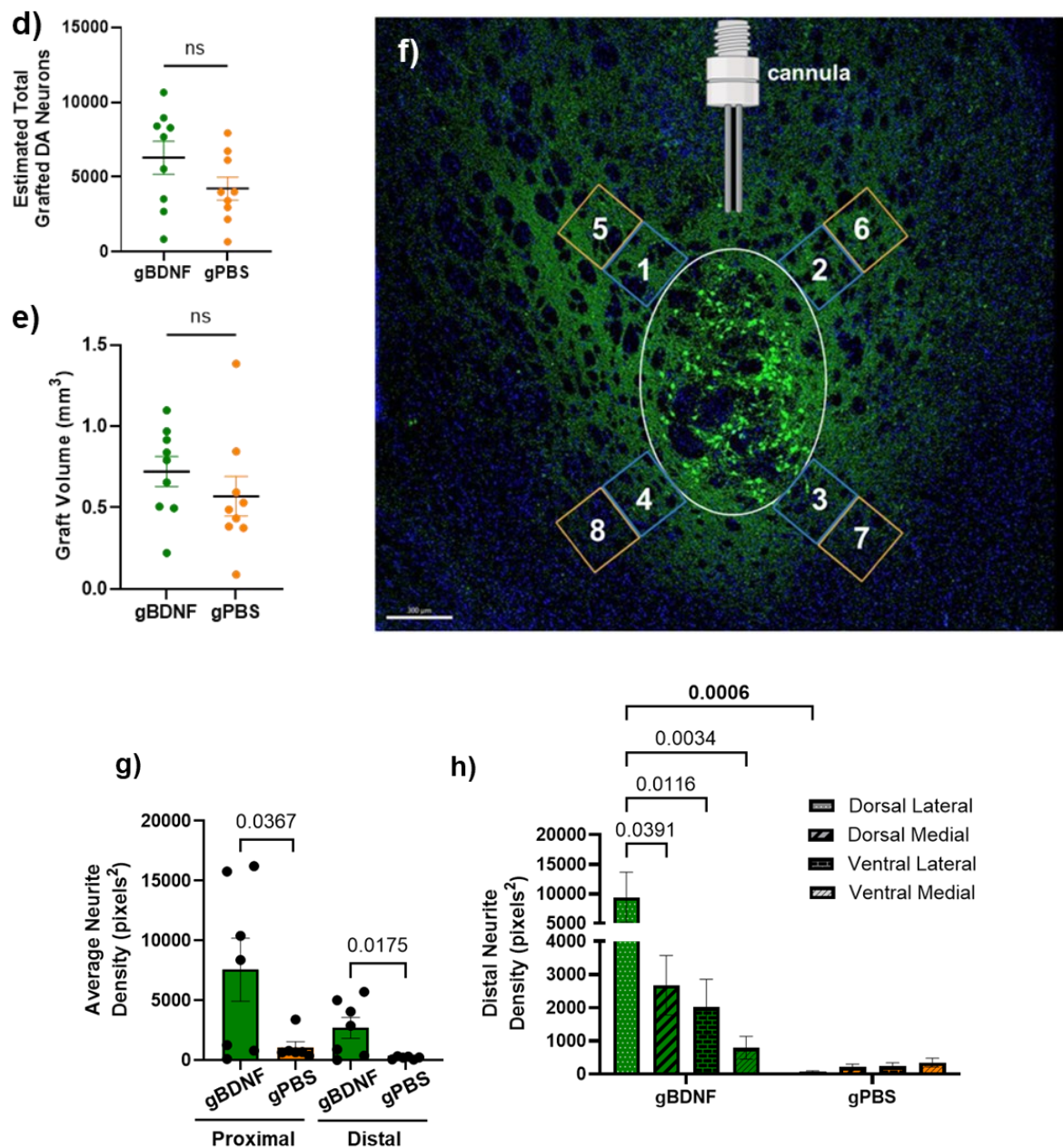
(a) Total LID severity scores for grafted and non-grafted treatment groups throughout the pre-graft period and for 10 weeks post-graft behavioral recovery. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test at each time point.

Figure 4.2 (cont'd)

Week 4: *p = 0.0421 gBDNF vs. non-grafted BDNF. **Week 8:** *p = 0.0169 gBDNF vs. non-grafted BDNF. **Week 10:** **p = 0.0032 gBDNF vs. non-grafted BDNF; *p = 0.0176 gPBS vs. non-grafted PBS. Non-grafted groups (BDNF vs. PBS) were not significantly different at any post-graft time points ($p \geq 0.9324$ for all time points). Grafted groups (BDNF- and PBS-infused) were not significantly different at any post-graft time points ($p \geq 0.9319$ for all time points). **(b)** LID severity over the 220-minute time course for each animal response at week 4, 6, 8, and 10 post-engraftment. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons tests at each time point. **Week 4:** (20 minutes): *p = 0.0129 gBDNF vs. non-grafted BDNF; (70 minutes) *p = 0.0130 gBDNF vs. non-grafted BDNF; (120 minutes) *p = 0.0184 gBDNF vs. non-grafted BDNF; **Week 6:** (70 minutes) *p = 0.0314 gBDNF vs. non-grafted BDNF, *p = 0.0189 gPBS vs. non-grafted PBS; (120 minutes) **p = 0.0047 gBDNF vs. non-grafted BDNF, *p = 0.0307 gPBS vs. non-grafted PBS; (170 minutes) *p = 0.0368 gBDNF vs. non-grafted BDNF; **Week 8:** (70 minutes) **p = 0.0016 gBDNF vs. non-grafted BDNF; (120 minutes) **p = 0.0015 gBDNF vs. non-grafted BDNF, *p = 0.0259 gPBS vs. non-grafted PBS; **Week 10:** (70 minutes) **p = 0.0018 gBDNF vs. non-grafted BDNF, *p = 0.0390 gPBS vs. PBS; (120 minutes) **p = gBDNF vs. non-grafted BDNF, *p = 0.0376 gPBS vs. non-grafted PBS **(c)** Total LID score for each treatment group showing each individual animal response at weeks 4, 6, 8, and 10. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test at each time point. **(d)** Stereologically estimated total number of grafted DA neurons. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, not significant. **(e)** Stereologically estimated total grafted volume. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-test, not significant. **(f)** Representative confocal fluorescent micrograph of the DA-grafted striatum in the Met/Met host parkinsonian rats. Magnification at 4x, scale bar = 300 μ m. The cannula placement is depicted in the dorsal region of the striatum above the grafted DA neurons. Each numbered box represents the analysis region for neurite outgrowth of TH+ DA fibers. 1 = Proximal dorsomedial, 2 = Proximal dorsolateral, 3 = Proximal ventrolateral, 4 = Proximal ventromedial, 5 = Distal dorsomedial, 6 = Distal dorsolateral, 7 = Distal ventrolateral, 8 = Distal ventromedial.

Figure 4.2 (cont'd)

(g) Average neurite density of total fibers surrounding cell bodies of DA graft, both proximal and distal to the graft. Data are reported as pixels². Statistics: Mean ± SEM. Unpaired two-tailed t-test between proximal and distal. **(h)** Distal neurite density (pixels²) of each region (DL, DM, VL, VM) surrounding the graft. Statistics: Mean ± SEM. Two-way ANOVA with Tukey's multiple comparisons. Abbreviations: LID = levodopa-induced dyskinesia, BDNF = brain-derived neurotrophic factor, LD = levodopa, DA = dopamine, DL = dorsolateral, DM = dorsomedial, VL = ventrolateral, VM = ventromedial.



Exogenous BDNF administration increased the severity and incidence of GID in DA-grafted homozygous rs6265 (Met/Met) rats

In contrast to our hypothesis, DA-grafted BDNF-infused animals exhibited significantly higher GID severity when compared to DA-grafted PBS-infused animals at week 5 post-engraftment (**Figure 4.3a**, $p = 0.0193$ gBDNF vs. gPBS). While this significant difference was lost at 10 weeks post-engraftment, a similar trend was retained with a slightly higher GID severity in the DA-grafted BDNF-infused rats (Figure 4.3a, $p = 0.0991$ gBDNF vs. gPBS). To complement the GID severity results, I also examined the incidence of GID behavior in both grafted groups. Total GID incidence was defined as the number of animals in each group that demonstrated a total GID rating score of 4 or higher. I have additionally included the incidence of peak amphetamine-mediated GID behavior which we defined as the number of animals in each group with a peak (70 minute timepoint) GID score as 2 or higher. The incidence scores of GID (total and peak) were determined accordingly because a total score of <4 and a peak score <2 are reflective of stereotypic behaviors that can occur in non-grafted/non-lesioned rats (e.g., intermittent licking and chewing).

At 5 weeks post-engraftment, DA-grafted BDNF-infused animals had a much greater percent incidence of both total and peak GID behavior in comparison to the DA-grafted PBS-infused animals (**Figure 4.3b**; 55.6% compared to 11.1% in total GID, and 66.7% to 22.2% in peak GID incidence). Likewise, at 10 weeks post-engraftment, percent incidence of GID behavior in the grafted BDNF-infused animals was 44.4% total GID compared to 22.2% total in grafted PBS-infused animals, and 33.3% peak GID compared to 22.2% peak in grafted PBS-infused animals (**Figure 4.3c**).

Mercado and colleagues demonstrated a statistically positive correlation between total GID severity and the expression of VGLUT2 within grafted DA neurons only in the homozygous rs6265 (Met/Met) animals engrafted with WT eVM cells (Mercado et al., 2021). VGLUT2 inside DA neurons is atypical and indicative that the grafted DA neurons are co-releasing glutamate (El Mestikawy et al., 2011). In embryonic stages, DA neurons co-express VGLUT2; however, as the neurons mature, their immature phenotype of VGLUT2 co-expression is lost (El Mestikawy et al., 2011; Kordower et al., 1996) for the most part (Kawano et al., 2006; Morales & Root, 2014; Yamaguchi et al., 2015). In our study, we hypothesized that administering exogenous BDNF would induce the maturation of the WT DA neurons grafted into homozygous Met/Met rats, therefore decreasing the expression of VGLUT2 and ameliorating GID behavior correlated with this marker (Mercado et al., 2021). However, our results showed that VGLUT2 expression is maintained even after exogenous administration of BDNF in the DA-grafted animals. Indeed, no statistical differences were found between grafted BDNF- and grafted PBS-infused animals (**Figure 4.3e**, $p = 0.7422$ gBDNF vs. gPBS). Unexpectedly, VGLUT2 expression was also no longer correlated with GID behavior (**Figure 4.3f**, $r = 0.6303$, $p = 0.0751$ gBDNF; $r = -0.07207$, $p = 0.8889$ gPBS), although there was a positive trend in the DA-grafted BDNF-infused group.

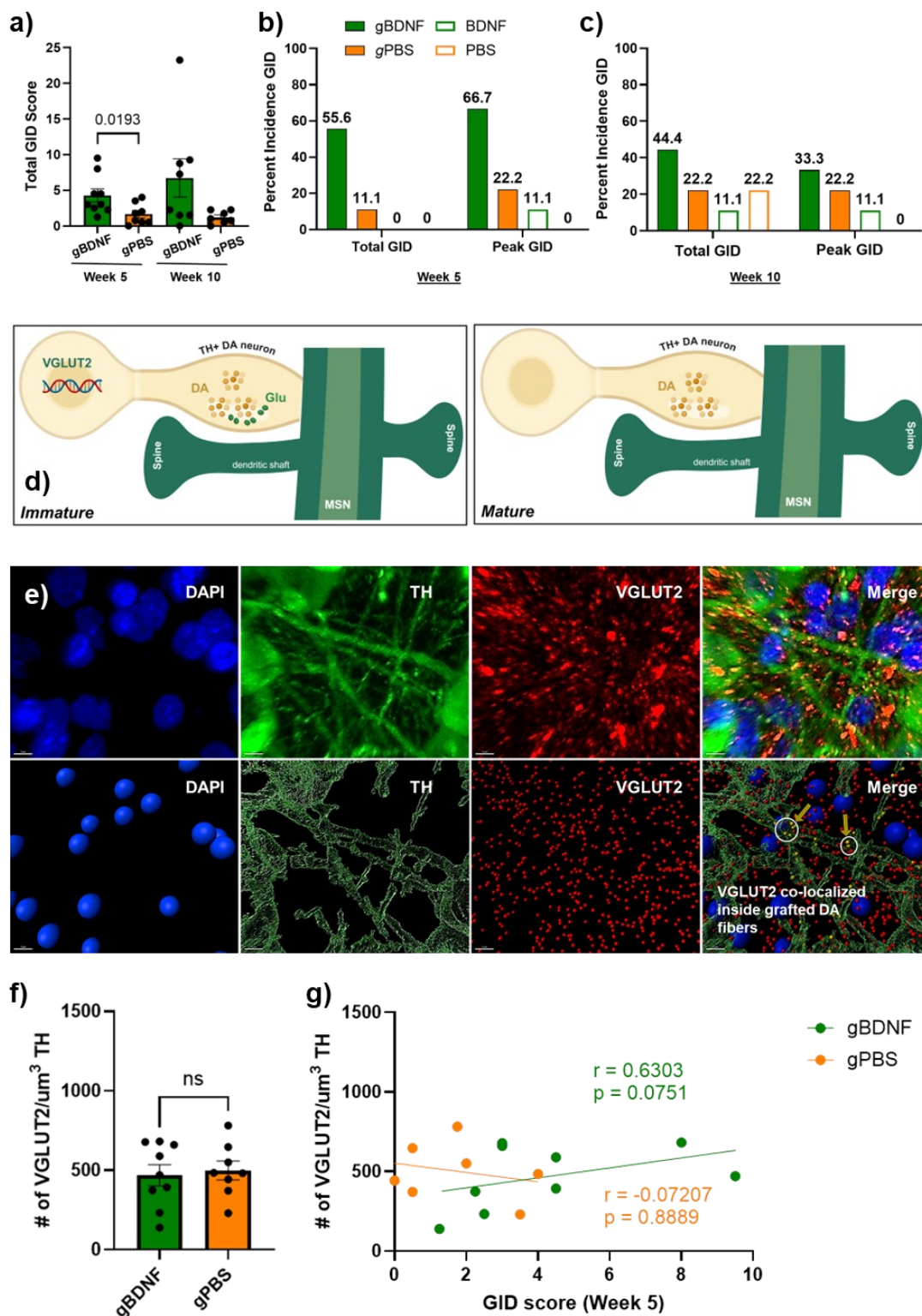


Figure 4.3: Impact of BDNF supplementation of GID behavior.

(a) Total amphetamine-induced GID severity scores at week 5 and week 10 post-engraftment. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-tests between gBDNF and

Figure 4.3 (cont'd)

gPBS groups. **Week 5:** $p = 0.0193$ gBDNF vs. gPBS. **Week 10:** $p = 0.0991$. **(b)** Percent incidence of total GID severity score of ≥ 4 in all four treatment groups at week 5 and week 10 post-engraftment. Data expressed as Mean $\pm$ SEM. **(c)** Percent incidence of peak (70 minutes post-amphetamine administration) GID severity score of ≥ 2 in all four treatment groups at week 5 and week 10 post-engraftment. **(d)** Schematic diagram depicting synaptic connectivity and VGLUT2 expression in immature (embryonic) and mature dopaminergic neurons. Immature DA neurons express VGLUT2 and form asymmetric, atypical connections directly onto the dendritic head of MSNs. As the neurons mature, they lose the VGLUT2 phenotype and form typical *en passant* associations onto the shaft of the dendritic spine of MSNs (El Mestikawy et al., 2011). **(e)** Fluorescent micrograph and subsequent Imaris 3D reconstruction of DA (THir) fibers containing VGLUT2 protein. Scale bar = 5 μm . **(f)** Quantification of the number of VGLUT2 protein found within TH+ DA fibers, normalized to the TH surface volume (μm^3). Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-tests between gBDNF and gPBS treatment groups. **(g)** Spearman correlation between quantify of VGLUT2 protein located inside TH+ neurons and total amphetamine-mediated GID score at week 5 post-engraftment. No significance. Data for week 10 post-engraftment was also not significant: data not shown. Abbreviations: GID = graft-induced dyskinesia, VGLUT2 = vesicular glutamate transporter 2, DA = dopamine, MSNs = medium spiny neurons, TH = tyrosine hydroxylase.

GID behavior is associated with behavioral and morphological indices of excess

DA release in DA-grafted BDNF-infused animals

In a 6-OHDA-lesioned parkinsonian rat, the subject normally rotates ipsilateral, or in the same direction, toward the lesioned hemisphere upon administration of amphetamine which causes DA release from intact DA terminals in the intact contralateral striatum (**Figure 4.4a**, see (Dunnett & Torres, 2011)). Amphetamine, an indirect DA agonist, will bind to monoamine transporters, thereby increasing the release of DA into the synapse from intact nigrostriatal DA terminals (or grafted DA neurons). Since one hemisphere is lesioned in our unilaterally lesioned rat model, amphetamine will only activate increased DA release from the intact hemisphere, causing the animal to rotate ipsilaterally (Dunnett & Torres, 2011). The DA graft should mitigate rotation behavior if equal release of DA occurs between the two striatal hemispheres. However,

if the graft is releasing more DA than the intact striatum, the rat will rotate contralaterally, or away from, the lesioned side after amphetamine administration.

As expected, there were no differences in net ipsilateral amphetamine rotations per minute (**Figure 4.4b**; $p = 0.4297$ Week 5, $p = 0.9842$ Week 10 non-grafted BDNF vs. non-grafted PBS) or in total ipsilateral rotations over 220 minutes post-amphetamine (**Figure 4.4cd**; $p = 0.9764$ Week 5, $p > 0.9999$ Week 10 non-grafted BDNF vs. non-grafted PBS) found between the sham-grafted (BDNF- and PBS-infused) animals at either week 5 or week 10 of the study. Both grafted BDNF-infused and grafted PBS-infused parkinsonian rats demonstrated recovery of amphetamine-mediated rotational behavioral following engraftment at week 5 (**Figure 4.4b**, $p < 0.0001$ gBDNF vs. non-grafted BDNF; $p = 0.0071$ gPBS vs. non-grafted PBS) and week 10 ($p < 0.0001$ gBDNF vs. non-grafted BDNF, $p < 0.0001$ gPBS vs. non-grafted PBS). When comparing DA-grafted BDNF- and PBS-infused animals, there was a significant increase in the number of ipsilateral rotations in the PBS-infused animals (i.e., increased contralateral rotations in the BDNF-infused animals) at week 5 (**Figure 4.4b** per minute: $p = 0.0159$; **Figure 4.4c** total: $p = 0.0307$ gBDNF vs. gPBS). Although significance is lost at week 10 between these groups, there remains a similar trend between the grafted BDNF- and grafted PBS-infused animals in which the BDNF-infused animals have a greater number of contralateral rotations, suggesting that excess DA is being released from the DA-grafted rats there were exposed to exogenous BDNF infusion (**Figure 4.4d**).

In order to further assess whether the DA-grafted BDNF-infused animals have a propensity for increased DA release, immunohistochemical postmortem expression of DAT was examined. DAT is a transmembrane protein responsible for clearing DA from

the extracellular space; an increase in DAT has been linked to an increase in DA release since DAT upregulation is required in order to clear higher concentrations of DA from the synapse (Lohr et al., 2017; Zhu & Reith, 2008). Affirmatively, there was a significant increase in DAT expression per TH+ neuron (i.e., DAT:TH Intensity/ μm^2) in the grafted BDNF-infused animals (**Figure 4.4f**, $p = 0.0174$ gBDNF vs. gPBS). Fluorescent intensity of DAT immunohistochemical staining here is synonymous with expression of the DAT protein as the staining pattern of DAT fills the entire TH+ neuron. Strikingly, expression levels of DAT:TH were positively and robustly, correlated with GID behavior at week 5 post-engraftment (**Figure 4.4g**; $r = 0.8320$, $p = 0.00716$ gBDNF). This correlation was no longer significant at week 10 (data not shown), although GID behavior was also not significant at this timepoint.

DAT:TH expression and ipsilateral rotations were investigated to determine any correlation of these measures. While not statistically significant, DAT:TH expression seemed to have a negative trend with net ipsilateral rotations at week 5 post-engraftment: the animal with the highest DAT:TH expression had the lowest number of ipsilateral rotations (or highest contralateral rotations) only in the grafted BDNF-infused group (**Figure 4.4hi**). When DAT and TH expression were examined separately and then correlated to net ipsilateral rotations, a similar negative trend was apparent in the grafted BDNF-infused animals in which a higher DAT expression denoted a lower number of ipsilateral rotations (**Figure 4.4hii**). In contrast, with TH+ expression alone, no significant correlation or trend existed between TH and the number of rotations (**Figure 4.4hiii**), suggesting that DAT expression alone is more likely associated with

the number of rotations a unilaterally lesioned parkinsonian rat makes in response to amphetamine administration.

Recent evidence has established that VMAT2 is co-expressed with VGLUT2 in a subpopulation of midbrain DA neurons in the ventral tegmental area (VTA) and the SNpc (Hnasko et al., 2010; H. Shen et al., 2021). Therefore, this indicates that a subpopulation of DA neurons can co-release DA and glutamate from terminals in the striatum (Hnasko et al., 2010; H. Shen et al., 2018). Furthermore, this lends to the theory of vesicular synergy, as introduced above, which posits that, if VMAT2 and VGLUT2 are co-localized on the same synaptic vesicle, the presence of VGLUT2 (glutamate) will increase the pH gradient by acidifying the inside of the synaptic vesicle, allowing for more loading of DA (Buck et al., 2021; Hnasko et al., 2010; H. Shen et al., 2018).

In the context of our studies based on this theory, I hypothesized that VMAT2 and VGLUT2 are co-localized on the same synaptic vesicles, increasing the amount of DA that is loaded, thereby increasing the amount of vesicular DA release and GID behavior in the grafted BDNF-infused animals. As such, I have examined whether the (presumed) co-localization of VMAT2 and VGLUT2 exists inside TH+ DA fibers. Although no significant difference were found between the DA-grafted BDNF-infused and DA-grafted PBS-infused animals, there was a slight trend of increased VMAT2/VGLUT2 presumed colocalization in the grafted BDNF-infused animals (**Figure 4.4j**, $p = 0.1758$). More importantly, however, the number of (presumed) co-localized VMAT2/VGLUT2 inside TH+ fibers was significantly correlated with GID behavior in the grafted BDNF-infused animals. Specifically, an increase of GID behavior correlated to an increase in

(presumed) VMAT2/VGLUT2 colocalization inside TH+ fibers (**Figure 4.4k**; $r = 0.7647$, $p = 0.02050$ gBDNF). To my knowledge, this is the first evidence suggestive that VMAT2 and VGLUT2 are co-localized in the same vesicle in grafted eVM DA neurons.

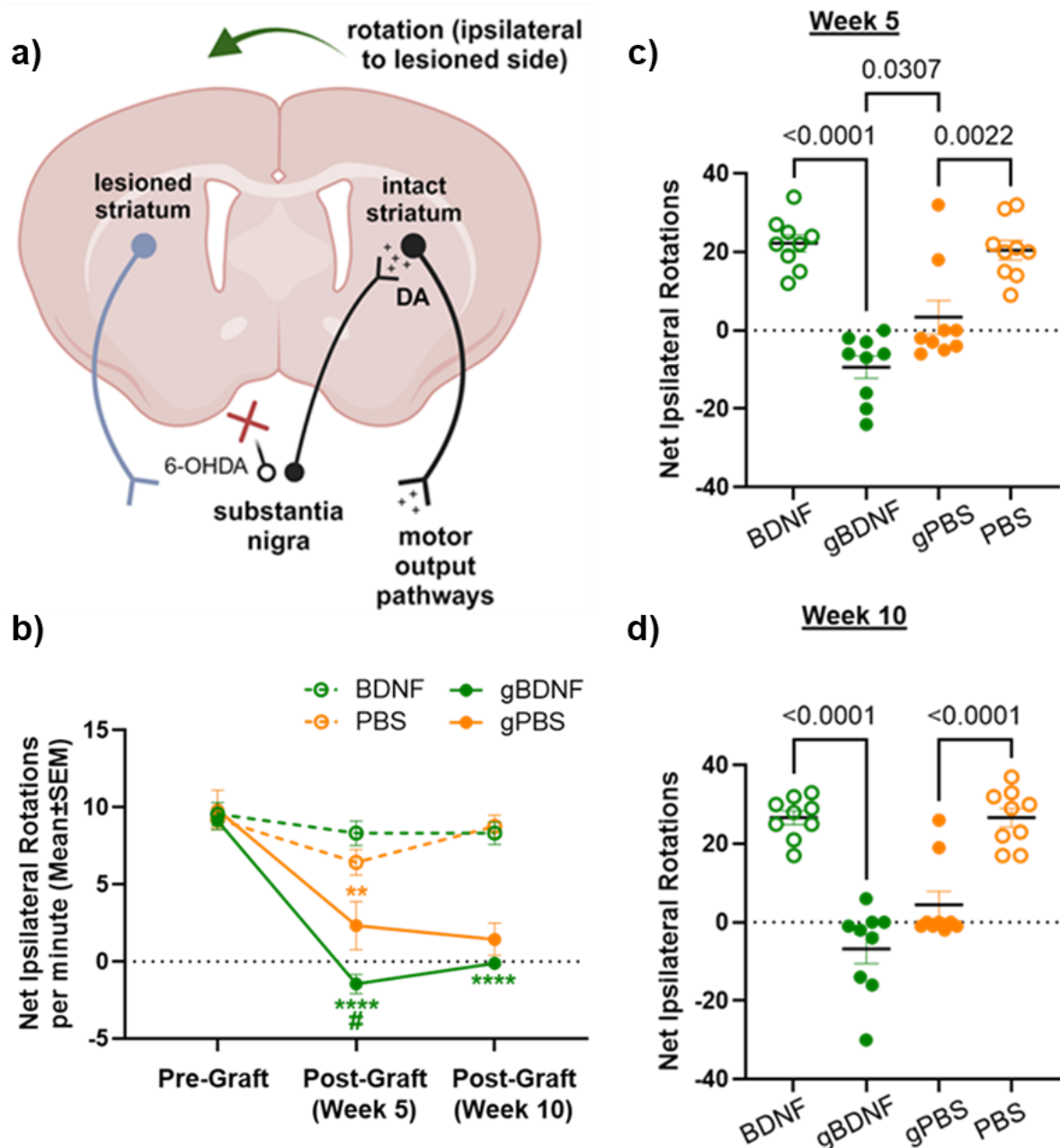


Figure 4.4: Exogenous BDNF administration is associated with indices of excess DA release.

(a) Schematic depicting the amphetamine-mediated rotational behavior of a unilaterally 6-OHDA-lesioned animal. Upon amphetamine administration, a lesioned animal will

Figure 4.4 (cont'd)

rotate ipsilateral (same side) toward the lesioned striatum. Modified from (Dunnett & Torres, 2011). **(b)** Net ipsilateral rotations per minute, manually counted at the 70-minute post-amphetamine injection timepoint. Rotations are reported for both week 5 and week 10 post engraftment. Statistics: Mean $\pm$ SEM. Two-way ANOVA with Tukey's multiple comparisons. **Week 5 post-graft:** ****p = <0.0001 gBDNF vs. non-grafted BDNF, **p = 0.0071 gPBS vs. non-grafted PBS. #p = 0.0159 gBDNF vs. gPBS. **Week 10 post-graft:** ****p < 0.0001 gBDNF vs. non-grafted BDNF, ****p < 0.0001 gPBS vs. non-grafted PBS. **(c)** Amphetamine rotational behavior expressed as net ipsilateral rotations at week 5 and 10 **(d)** post-engraftment. Ordinary one-way ANOVA with Tukey's multiple comparisons tests. **Week 5:** p = 0.0307 gBDNF vs. gPBS. p = 0.0022 gPBS vs. non-grafted PBS. p = <0.0001 gBDNF vs. non-grafted BDNF. **Week 10:** p = 0.0514 gBDNF vs. gPBS. p = <0.0001 gPBS vs. non-grafted PBS. p = <0.0001 gBDNF vs. non-grafted BDNF.

Figure 4.4 (cont'd)

(e) (i) Representative confocal fluorescent micrograph demonstrating differing staining patterns of DAT and TH expression in the grafted parkinsonian rat striatum. (ii) Fluorescent micrographs depicting an increase in DAT staining (cyan) in the grafted parkinsonian striatum treated with BDNF administration compared to PBS treatment (iii). Scale bar = 300 μm . **(f)** Quantification of DAT expression in the grafted DA neurons. Data are expressed as the ratio of the sum fluorescent intensity of DAT to the sum fluorescent intensity of TH, both normalized to their respective surface areas (μm^2). Statistics: Unpaired two-tailed t-tests. $p = 0.0174$ gBDNF vs. gPBS. **(g)** Spearman correlation between DAT:TH intensity/ μm^2 and total amphetamine-mediated GID severity score at week 5 and week 10 post-engraftment. Only DAT:TH intensity/ μm^2 was significantly correlated in the grafted BDNF-infused animals at week 5. $p = 0.007716$. Correlation for week 10 not shown, not significant ($p = 0.1967$).

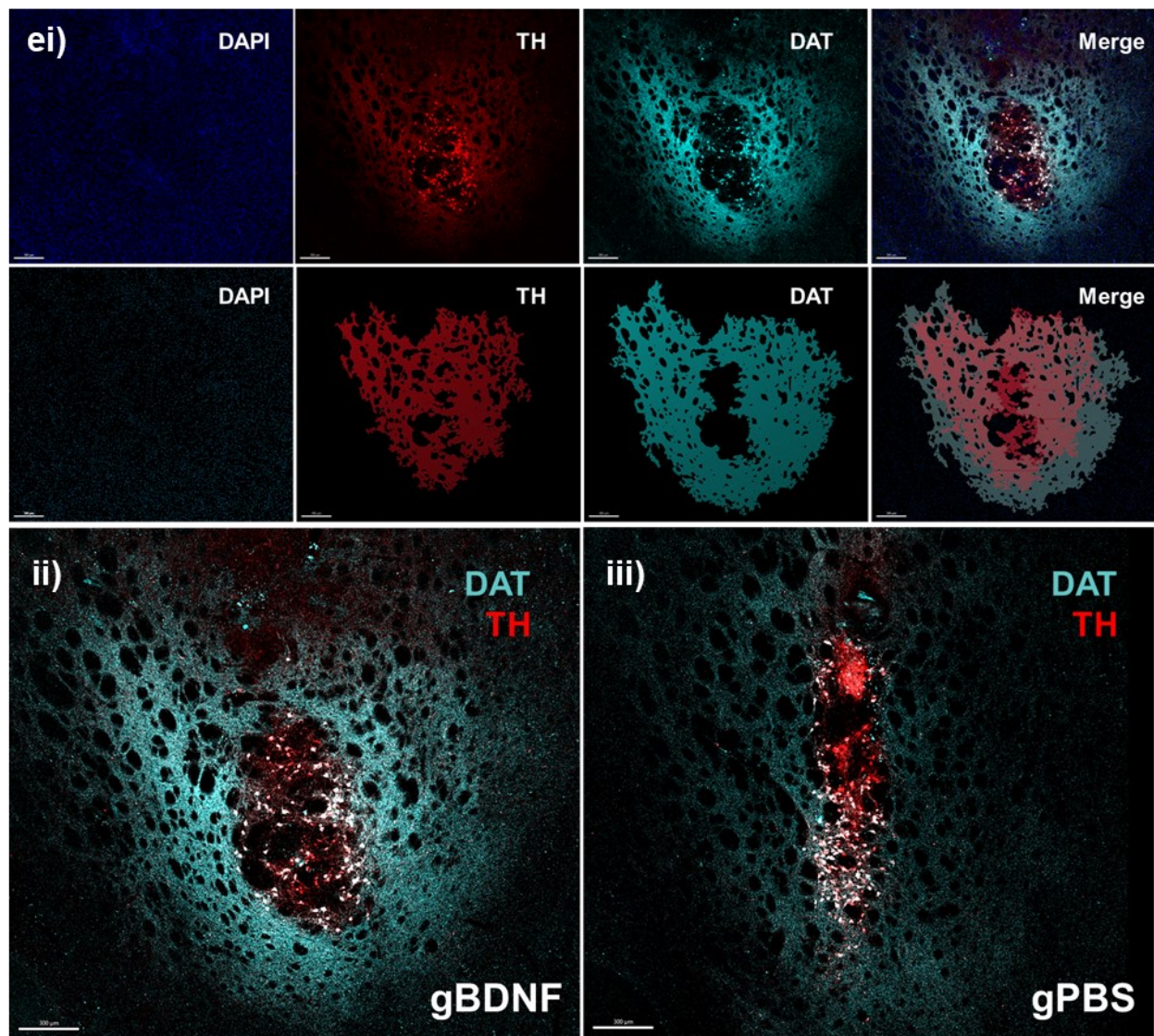


Figure 4.4 (cont'd)

(hi) Spearman correlation between DAT:TH intensity/ μm^2 and net ipsilateral amphetamine-mediated rotations. **(hii)** Spearman correlation between DAT intensity/ μm^2 alone, and **(hiii)** TH intensity/ μm^2 alone. **(i)** Confocal fluorescent micrograph depicting (presumed) co-localization of VMAT2 and VGLUT2 protein located inside TH+ DA neuron fibers. Scale bar = 2 μm

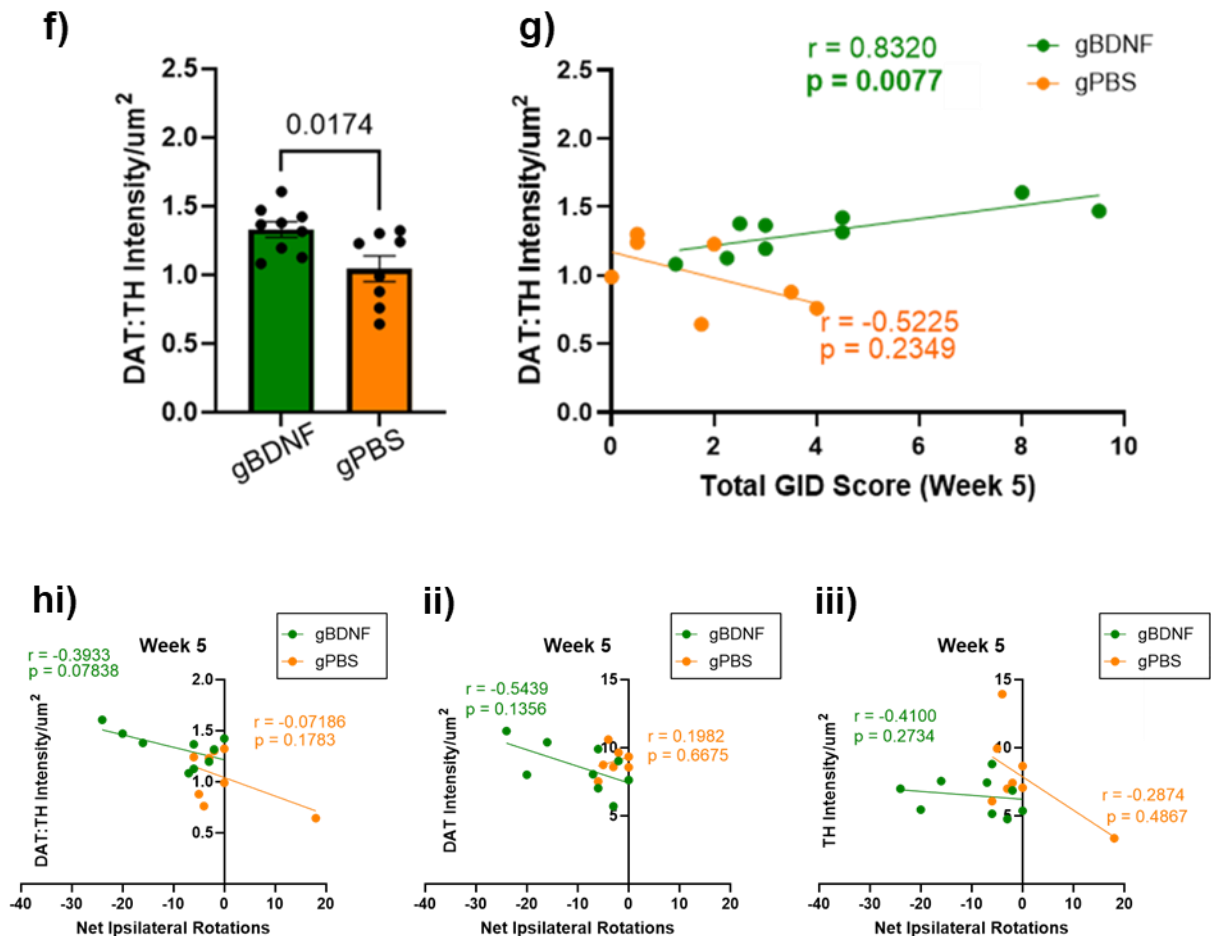
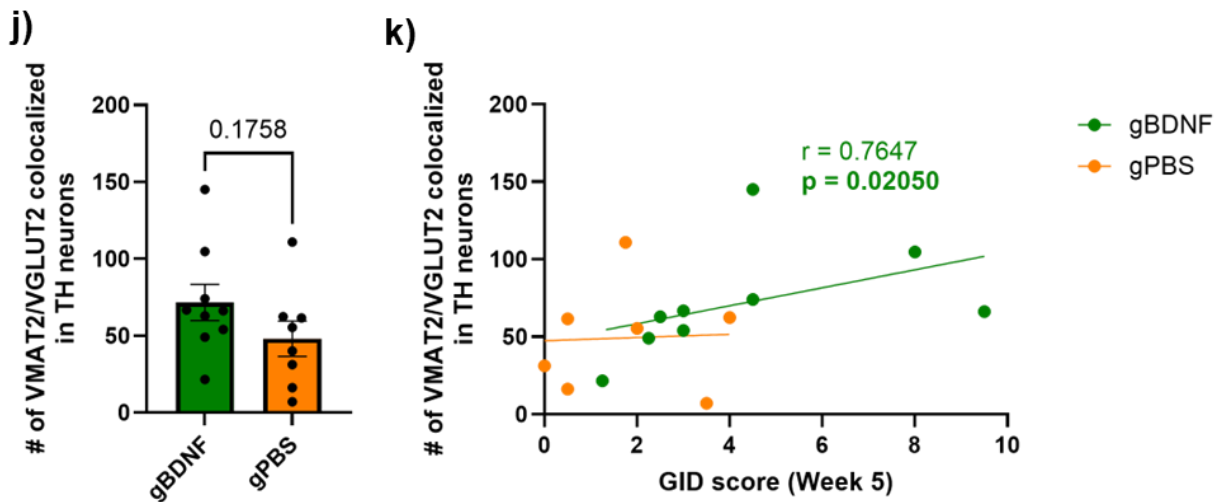
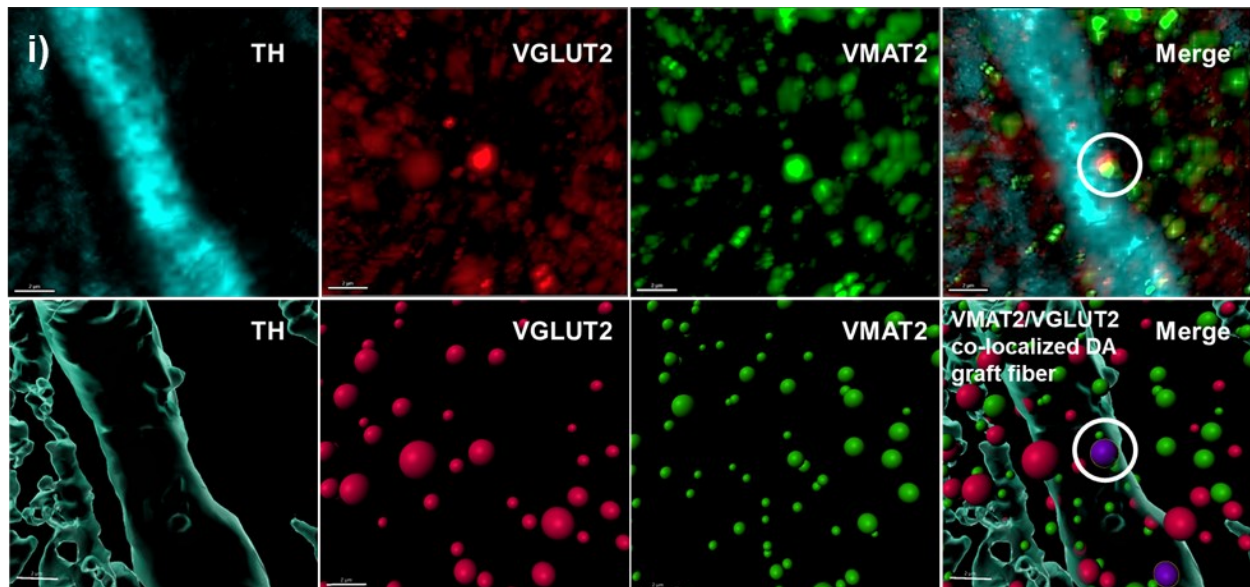


Figure 4.4 (cont'd)

(j) Quantity of number of (presumed) co-localized VMAT2/VGLUT2 protein located inside TH DA neuron fibers. Statistics: Mean $\pm$ SEM. Unpaired two-tailed t-tests. $p = 0.1758$ gBDNF vs. gPBS. **(k)** Spearman correlation between the quantity of (presumed) co-localized VMAT2/VGLUT2 protein located inside TH DA neuron fibers and total GID severity scores at week 5 post-grafting. $p = 0.02050$ in the grafted BDNF-infused animals.



Exogenous BDNF infusion increases microglial (Iba1) expression in DA-grafted animals

As introduced in Chapter 3, we demonstrated previously that engrafted parkinsonian rats exhibited higher percentages of asymmetric synapses following immune activation, and that this correlated significantly with increased GID (Soderstrom et al., 2008). Similarly, in clinical trials, patients developed GID behavior after withdrawal of immune suppression (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). Because this evidence points to a possible influential role of the immune response in the induction of GID behavior, we investigated two well-known immune markers, Iba1 and GFAP.

Iba1, which is a factor that can be involved in the creation and elimination of synapses (Tremblay et al., 2011), was used as an indicator of inflammation and quantified in the striatum of all rat subjects. To label astrocytes, GFAP was employed and served as an additional inflammatory marker. Injury, such as grafting, can activate astrocytes, leading to the release of proinflammatory chemokines and cytokines (Alhadidi et al., 2024; Giovannoni & Quintana, 2020). Thus, along with Iba1 expression, elevated GFAP is also associated with inflammation.

BDNF-infused DA-grafted animals demonstrated a slight, though not statistically significant, increase in overall Iba1 expression compared to the vehicle (PBS)-infused DA-grafted subjects (**Figure 4.5b**; $p = 0.3806$ gBDNF vs. gPBS). Similarly, in the non-grafted animals, Iba1 was slightly increased in the BDNF-infused animals than in the PBS-infused group, but this difference was not statistically significant (**Figure 4.5b**, $p = 0.5645$ gBDNF vs. non-grafted BDNF). When normalized to the number of grafted TH+

neurons, Iba1 expression was significantly increased in the DA-grafted BDNF-infused rats compared to the grafted PBS-infused treatment (**Figure 4.5c**, $p = 0.0232$ gBDNF vs. gPBS). Although statistical significance was noted, there was no significant correlation found between Iba1 expression and GID behavior at week 5 (**Figure 4.5d**; $r = -0.06723$, $p = 0.8685$) or week 10 (data not shown).

GFAP expression was significantly greater in the DA-grafted, compared to the non-grafted, BDNF-infused animals, suggesting that grafting stimulates astrocyte upregulation (**Figure 4.5e**, $p = 0.0168$ gBDNF vs. non-grafted BDNF). Following normalization to the number of grafted TH+ neurons, no significant differences were observed between the grafted treatment groups (**Figure 4.5f**, $p = 0.3510$ gBDNF vs. gPBS). Furthermore, similar to Iba1 expression, GFAP expression did not significantly correlate with GID behavior at either week 5 (**Figure 4.5g**; $r = -0.03361$, $p = 0.9397$ gBDNF) or 10 post-engraftment (data not shown).

To complement the immunohistochemical findings, an interleukin-6 (IL-6) sandwich enzyme-linked immunosorbent assay (ELISA) was conducted on serum collected from cardiac punctures at the conclusion of the study (week 10). IL-6 is a proinflammatory cytokine often elevated in response to inflammation or injury (Tanaka et al., 2014). The results indicated no significant differences in IL-6 concentrations among the non-grafted or grafted treatment groups (**Figure 4.5h**, $p > 0.9999$ for all groups). However, a subtle increase in IL-6 was observed in the grafted BDNF-infused rats, consistent with the trends seen in Iba1 and GFAP expression (**Figure 4.5h**). Despite this, no significant correlation was found between IL-6 levels and GID behavior at weeks

5 (**Figure 4.5i**; $r = -0.1907$, $p = 0.6189$ gBDNF; $r = -0.3554$, $p = 0.3806$ gPBS) or 10 in either the grafted BDNF- or grafted PBS-infused animals (week 10 data not shown).

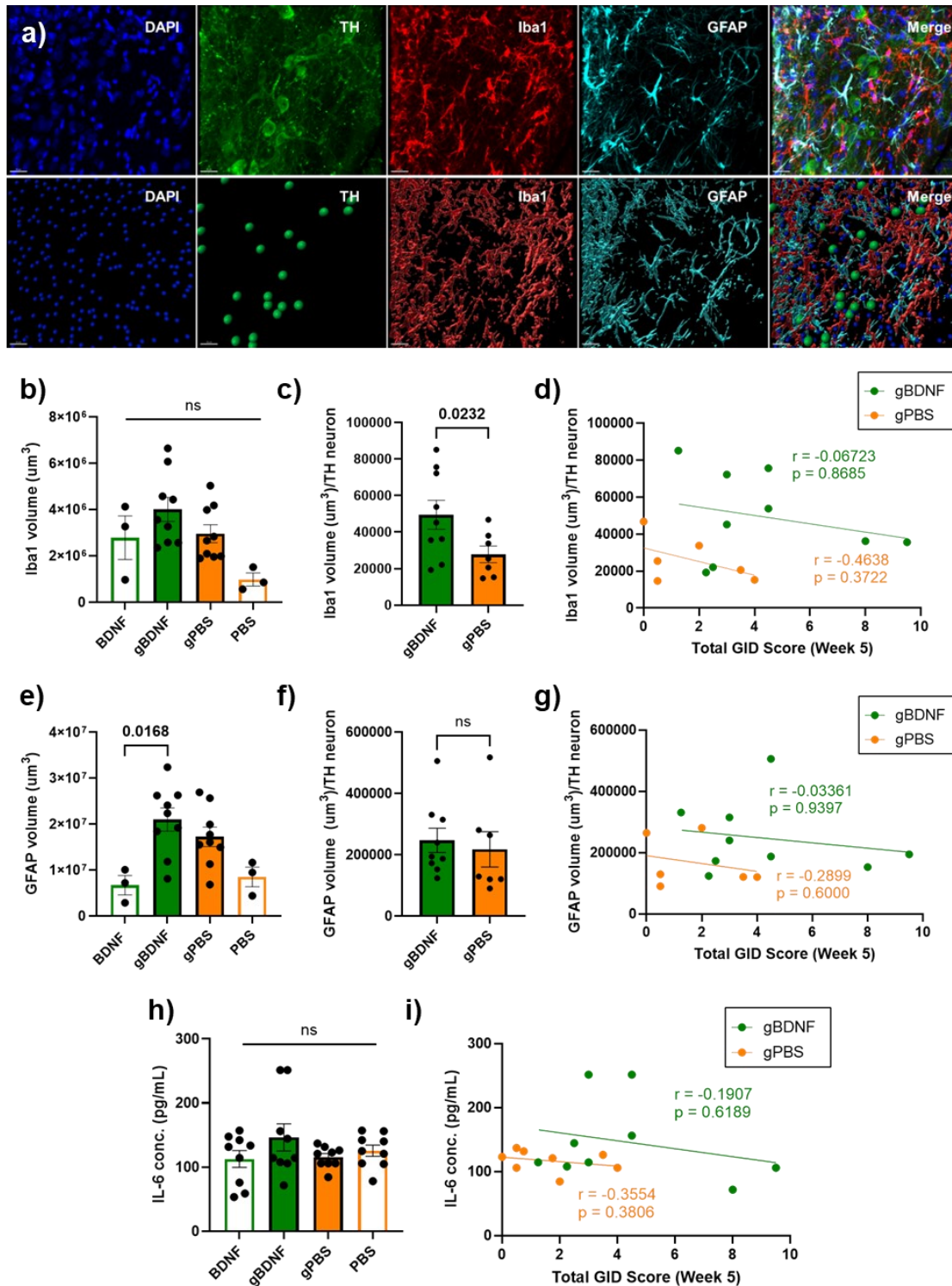


Figure 4.5: Exogenous BDNF infusion increases microglial (Iba1) expression in DA-grafted animals.

(a) Confocal fluorescent micrograph and ImarisTM 3D image rendering highlighting Iba1+ and GFAP+ cells. Scale bar = 20 μm . **(b)** Quantity of Iba1+ cells (volume μm^3) expressed

Figure 4.5 (cont'd)

in the lesioned/grafted striatum of each treatment group. Ordinary one-way ANOVA with Šídák's multiple comparisons test; no significance between groups. **(c)** Quantity of Iba1+ cells (volume μm^3) normalized to the number of grafted TH+ neurons. Mean $\pm$ SEM. Unpaired t-tests. $p = 0.0232$. **(d)** Spearman correlation between quantity of Iba1+ cells (volume μm^3) normalized to the number of grafted TH+ neurons and total GID severity scores at week 5 post-engraftment. Week 10 was not significant; data not shown. **(e)** Quantity of GFAP+ cells (volume μm^3) expressed in the lesioned/grafted striatum of each treatment group. Ordinary one-way ANOVA with Šídák's multiple comparisons test. $p = 0.0103$ gBDNF vs. non-grafted BDNF. **(f)** Quantity of GFAP+ cells (volume μm^3) normalized to the number of grafted TH+ neurons. Statistics: Mean $\pm$ SEM. No significance. **(g)** Spearman correlation between quantity of quantity of GFAP+ cells (volume μm^3) normalized to the number of grafted TH+ neurons and total GID severity scores at week 5 post-engraftment. Week 10 was not significant; data not shown. **(h)** Serum concentration of IL-6 (pg/mL) from each treatment group. Statistics: Mean $\pm$ SEM. Non-parametric Kruskal-Wallis with Dunn's multiple comparisons test. **(i)** Spearman correlation between serum concentration of IL-6 (pg/mL) and GID severity scores at week 5 post-engraftment. Week 10 was also not significant. Data not shown. Abbreviations: Iba1 = ionized calcium-binding adaptor molecule 1, GFAP = glial fibrillary acidic protein, IL-6 = interleukin-6.

DISCUSSION

While neural transplantation does not offer a “cure” for PD, it does offer a promising non-pharmacological alternative to the therapies currently prescribed for PD. In both preclinical and clinical settings, the past two decades have seen rigorous research in neural grafting, taking strides to optimize patient selection (e.g., age, disease severity) and transplantation methods (e.g., cell source, preparation) (Barker et al., 2024). Despite refinement, many obstacles still exist with GID continuing to be a prominent, detrimental side effect. The lack of underlying mechanisms responsible for GID has generated a large gap in our understanding of how to make cell therapy a viable therapeutic option. In order to move forward, it will be imperative to harness the benefit while preventing the side effect of GID to fully optimize cell transplantation as a therapeutic for PD.

One of the major focus areas in our laboratory involves striving to understand factors linked to GID in parkinsonian rats that receive eVM DA grafts. More recently, we began investigating GID in the context of the rs6265 SNP, testing the hypothesis that this SNP is an unrecognized contributor to the development of this side effect in a subpopulation of PD patients who received embryonic DA neuron grafts. Using the novel CRISPR knock-in rat model of the rs6265 BDNF SNP, we indeed demonstrated that parkinsonian rats homozygous for rs6265 (i.e., Met/Met) engrafted with WT DA neurons uniquely developed GID behavior compared to their WT counterparts engrafted with the same DA neurons (Mercado et al., 2021). We have also demonstrated, for the first time, that DA grafts exhibit neurochemical evidence of DA/glutamate co-transmission evidenced by the expression of VGLUT2mRNA and protein co-localized

inside TH⁺ neuronal fibers (Mercado et al., 2021). Compellingly, only in the Met/Met hosts was VGLUT2 expression significantly correlated to GID behavior. As previously suggested (Kordower et al., 1996), our continuing research indicates that grafted eVM DA neurons maintain an immature phenotype (i.e., VGLUT2; (El Mestikawy et al., 2011)), establishing asymmetric (presumed) glutamatergic synapses onto MSNs (Mercado et al., 2021; Soderstrom et al., 2008). These ultrastructurally-defined asymmetric synapses formed by grafted DA neurons positively correlated with an increase in GID (Soderstrom et al., 2008).

Based on these compelling data pointing to improper DA-glutamate circuitry/wiring as a potential underlying mechanism of GID, we hypothesized that the rs6265 (Met/Met) host environment, due to a decrease in BDNF release, prevents proper graft maturation and permits synaptic miswiring of the transplanted DA neurons, thus giving rise to GID induction. Without sufficient BDNF, a protein critical for synaptic formation and dendritic spine formation (Gonzalez et al., 2016; Hyman et al., 1991; Kowiański et al., 2018; Lai & Ip, 2013; Park & Poo, 2013; Sasi et al., 2017; Zagrebelsky et al., 2020), the grafted neurons may not be able to form proper connections with the host MSNs. Consequently, within this current study, we predicted that, if we could “replenish” the deficient BDNF, grafted DA neuron maturation and proper graft-host integration would occur, preventing aberrant miswiring and behavior side effects (i.e., GID development).

Strikingly, BDNF infusion into Met/Met parkinsonian rats engrafted with WT DA neurons exhibited significantly higher GID behavior compared to DA-grafted vehicle PBS-infused control animals. Further, not only was this demonstrated behaviorally,

BDNF infusion was also unsuccessful in allowing for maturation of the grafted DA neurons, evidenced by remaining expression of VGLUT2 protein inside TH+ neurites. Although VGLUT2 expression was no longer correlated with GID behavior in these DA-grafted parkinsonian rats, it is probable that BDNF is not the sole factor required for full maturation of DA neurons transplanted into the mature adult parkinsonian striatum. Moreover, there is the possibility that four weeks of infusion was insufficient to induce full gestational maturation of these embryonic neurons placed into the adult striatum. These disparate findings collectively indicate that factors in addition to, or distinct from, BDNF and/or VGLUT2 expression contribute to GID induction in Met/Met parkinsonian animals.

Despite a significant difference in GID behavior between infusion groups in this last study, no statistical differences were found in graft-mediated reduction in LID behavior. Interestingly, while grafted rs6265 Met/Met animals seem to uniquely develop GID behavior, this genotype type has conversely demonstrated enhanced behavioral recovery (i.e., LID amelioration) compared to their grafted WT counterparts in our previous study (Mercado et al., 2021). In the current experiment, we hypothesized that the DA-grafted BDNF-infused animals would have an even greater/faster amelioration of LID behavior than the DA-grafted PBS-infused animals. While this hypothesis was somewhat accurate, at the conclusion of the study, reduction in LID scores of both grafted groups were statistically similar, ultimately indicating that BDNF treatment did not effectively impact functional recovery in the paradigm employed.

Because BDNF is known to induce neurite outgrowth in cultured neurons (Barde et al., 1982; Kellner et al., 2014) and in vivo ((Yurek et al., 1996; Yurek, 1998; J. Zhang

et al., 2011), we hypothesized that the grafts that received BDNF infusion would exhibit enhanced neurite outgrowth compared to grafts that received only PBS infusion. Indeed, the grafted BDNF-infused animals demonstrated significantly increased average neurite outgrowth, both in the proximal and distal regions of the graft, compared to the grafted PBS-infused animals. Moreover, in the distal region of the graft in these grafted BDNF-infused animals, the dorsolateral neurite outgrowth was remarkably higher than the neurite outgrowth in the same area of the grafted PBS-infused animals. Additionally, these findings also confirm what has been shown with exogenous BDNF infusion in grafted parkinsonian rats in (Yurek, 1998; Yurek et al., 1996). Because the cannula was placed directly dorsal to the graft in each animal subject to infused BDNF, it is understandable that the dorsolateral region in the grafted-BDNF exhibited the most neurite outgrowth compared to grafted PBS-infused animals. We can infer that this increase in neurite outgrowth may have had a positive impact on the slight enhancement of graft efficacy in the grafted BDNF-infused animals, but we cannot yet definitively ascertain whether this increase in neurite outgrowth influences GID behavior. It could be postulated that increased neurite outgrowth leads to increased asymmetric synaptic connections, and therefore GID behavior; however, further investigation is warranted.

To further explore potential mechanisms of GID in the Met/Met parkinsonian animals and to understand how BDNF infusion could induce more severe GID, I utilized amphetamine-mediated rotational behavior and postmortem immunohistochemical expression of the DAT, VMAT2, and VGLUT2 proteins to help define the mystery of GID induction.

Amphetamine-mediated rotations, my secondary readout of graft function, does not necessarily determine graft size or extent of reinnervation (Björklund & Lindvall, 2017); however, it can assess whether the transplanted graft is functioning properly. A unilaterally lesioned rat with no grafted DA neurons will rotate ipsilateral to the lesioned hemisphere upon amphetamine administration as detailed earlier (Dunnett & Torres, 2011). After receiving a DA graft, the parkinsonian rat should no longer rotate if the graft balances the amount of DA between the intact and lesioned striatal hemispheres. In this way, I used amphetamine-mediated rotations to indirectly assess DA release from the DA grafts in the presence or absence of BDNF supplementation. While the non-grafted BDNF- or PBS-infused parkinsonian rats maintained a high level of ipsilateral rotations indicative of their lesioned status, the DA-grafted PBS-infused rats showed a normalization of rotational asymmetry. Compellingly, DA-grafted BDNF-infused animals rotated contralaterally to the lesioned hemisphere, suggesting that these grafted neurons were producing excess DA upon amphetamine administration in comparison to their vehicle-control counterparts. Curiously, rotations were not correlated to GID behavior. This functional measure of DA release provides insight into the consequences of BDNF administration and how it relates to one underlying mechanism of GID behavior (i.e., excess DA release).

Due to the increase in contralateral rotations in the grafted BDNF-infused animals and the functional confirmation that these grafts are releasing more DA than observed in the DA-grafted PBS-infused group, I next analyzed the expression of DAT to confirm or refute the hypothesis that, if BDNF was promoting increased DA release, there would be increased DAT expression in the DA-grafted BDNF-infused animals

which would be significantly correlated to GID behavior. Indeed, the data demonstrate that DAT expression was significantly higher in the DA-grafted BDNF-infused animals compared to the DA-grafted PBS-infused animals. Importantly, the increase in DAT was also significantly correlated with GID scores in the DA-grafted BDNF-infused animals, supporting the association between GID behavior and DA release. Also at week 5, we compared DAT:TH intensity/ μm^2 with net ipsilateral amphetamine-induced rotations. Further examining the relationship between DAT fluorescent intensity or TH intensity, only DAT and net ipsilateral rotations were correlated, confirming that DAT expression likely is related to functional DA release mediated by amphetamine. Thus, DA release continues to be a promising mechanism responsible for GID. It is also important to note that DAT function, not just expression (Bosse et al., 2012), could be altered as well, but further research would be required to evaluate this.

It is not surprising that BDNF administration seemingly promotes DA release. Several groups have demonstrated a relationship between BDNF and DA, showing that BDNF plays a critical role in DA neurotransmission. For example, Blochl and colleagues demonstrated enhanced depolarization and basal DA release upon BDNF administration to E14 eVM cultured neurons (Blöchl & Sirrenberg, 1996). Similarly, BDNF stimulated DA uptake activity also in eVM cultured neurons ((Beck et al., 1993; Knüsel et al., 1991). Striatal in vivo infusions of BDNF increased electrical activity in rat midbrain DA neurons (Bosse et al., 2012; R. Y. Shen et al., 1994) and elevated activity-dependent release of DA (Goggi et al., 2002) in both rat brain striatal slices and in the hippocampus (Paredes et al., 2007). Altar and colleagues, in contrast, did not see a change in striatal DA levels after two-week BDNF infusion to the SNpc in adult rats but

saw an increase in DA metabolite concentrations, indicating increased DA turnover, more so after amphetamine administration (Altar et al., 1992). Based on the evidence that BDNF administration evokes DA release, and because our study is consistent with these findings, I explored how this could mechanistically be related to GID induction. This exploration led me to a possible connection between DA release and glutamate co-transmission.

For over 20 years, it has been known that DA neurons have the potential to co-transmit both DA and glutamate neurotransmitters. Yet, the functional significance and benefit behind this phenomenon remains uncertain. A number of laboratories have established that a subpopulation of DA neurons co-express/co-release DA and glutamate, marked by co-expression of either Vglut2 mRNA or VGLUT2 protein (Bérubé-Carrière et al., 2009; Buck et al., 2022; Dal Bo et al., 2004; Fortin et al., 2019; Mingote et al., 2019; Root et al., 2016; T. Shen et al., 2018; Sulzer et al., 1998; Trudeau et al., 2014). Most have demonstrated that this subset of DA+/glutamate+ neurons are localized to the VTA, and only a small subset of these neurons project from the SNpc to the dorsal striatum (Buck et al., 2022; Eskenazi et al., 2021; Kawano et al., 2006). Behaviorally, VGLUT2 knock-out (KO) in DA neurons diminished neurochemical responses of mice to methamphetamine (H. Shen et al., 2021) and reduced locomotor response to cocaine (Hnasko et al., 2010), both of which are DA-releasing pharmacological agents. Furthermore, Hnasko and colleagues also demonstrated both decreased glutamate and DA release from ventral striatum slice cultures of VGLUT2 KO DA neurons (Hnasko et al., 2010), expressive of an important function of dual-release of these two neurotransmitters.

The most prominent theory that has been increasingly recognized as a logical functional explanation of DA/glutamate co-transmission, and the findings above in VGLUT2 KO DA neurons, is vesicular synergy (**Figure 4.6**). Vesicular synergy is a process that leads to enhanced loading of a primary neurotransmitter into secretory vesicles (for review (El Mestikawy et al., 2011)). For instance, it is well known that, with VGLUT3 and vesicular acetylcholine transporter (VACHT) on the same vesicle in cholinergic neurons, enhanced packaging of acetylcholine occurs (Gras et al., 2008). Although well established in this system, other systems such as dopaminergic neurons, GABA neurons, etcetera, remain relatively unexplored. In the dopaminergic system, the hypothesis of vesicular synergy suggests that the presence of VGLUT2 on the same vesicle as VMAT2 enhances the loading of DA, leading to increased DA release (Aguilar et al., 2017; Hnasko et al., 2010; H. Shen et al., 2018). Particularly, glutamate would enter through VGLUT2, increase the chemical gradient (Trudeau et al., 2014), and acidify the inside of the synaptic vesicle. Aguilar and colleagues has confirmed this, showing the hyperacidification of DA vesicles in a VGLUT2-dependent manner in mice (Aguilar et al., 2017). The increase in the chemical gradient promotes increased loading of DA through VMAT2, increasing the concentration and release of DA (**Figure 4.6bc**) (Eskenazi et al., 2021). To date, whether VMAT2 and VGLUT2 are on the same vesicle remains controversial (Aguilar et al., 2017; S. Zhang et al., 2015). One study has shown that a population of TH+/VGLUT2+ neurons in the VTA contain VMAT2 using PCR (Li et al., 2013), and another has shown co-immunoprecipitation of VMAT2 and VGLUT2 in a population of striatal synaptic vesicles (Hnasko et al., 2010; H. Shen et al., 2021; Silm et al., 2019). In contrast, Zhang and colleagues established that VMAT2 and VGLUT2 tend

to segregate into separate vesicles using immunolabeling, co-immunoprecipitation, and ultrastructural analysis in the adult nucleus accumbens (S. Zhang et al., 2015).

Vesicular synergy and its potential for increased DA packaging and release would be an entirely novel explanation that takes into account both DA/glutamate co-transmission (i.e., VGLUT2) and the DA release correlation we have demonstrated in our previous study (Mercado et al., 2021) and in my thesis studies, respectively. For confirmation, I endeavored to investigate whether I could find any evidence of VMAT2 and VGLUT2 co-localization in the TH+ grafted neurons in the BDNF-infused Met/Met rats and whether any association with GID existed. I was able to demonstrate using triple-label immunohistochemistry, confocal microscopy, and the Imaris imaging software the existence of VMAT2/VGLUT2 (presumed) co-localizations in the TH+ neurons of DA-grafted Met/Met BDNF-infused rats that demonstrated a slight increase in number compared to DA-grafted PBS-infused rats. It is noteworthy that this presumed co-localization of VMAT2/VGLUT2 in this treatment group was strongly correlated with GID behavior, demonstrating additional favorable evidence that GID behavior may indeed be caused by increased DA release mediated by vesicular synergy within grafted DA neurons. Nevertheless, additional studies are required to determine undoubtedly that these proteins are within the same synaptic vesicle.

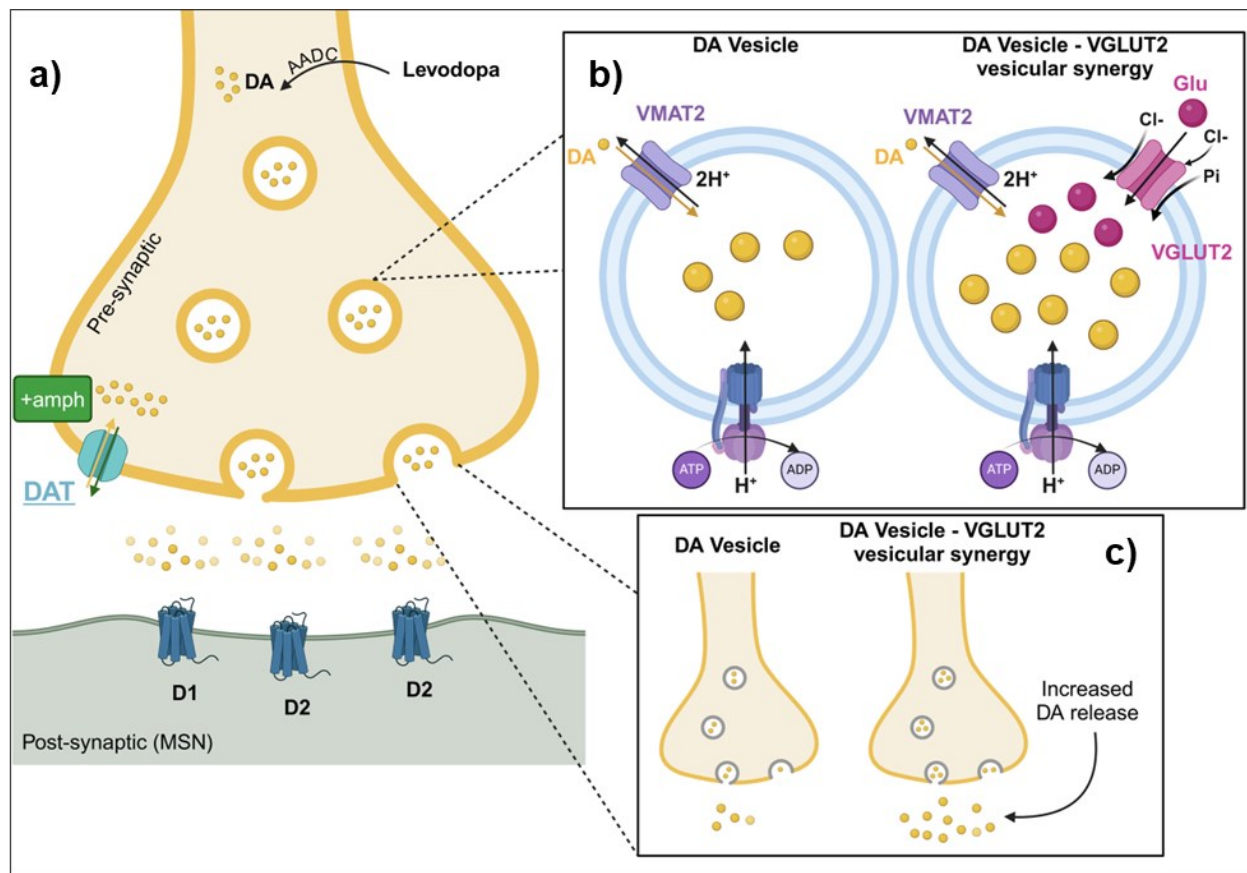


Figure 4.6: Schematic diagram depicting the proposed mechanism of vesicular synergy.

(a) Levodopa taken up into the dopaminergic neuron and converted to dopamine via aromatic amino acid decarboxylase (AADC). Normal packaging of dopamine occurs here; DA is released into the synapse and activates both D1 and D2 receptors on the post-synaptic membrane of MSNs. **(b)** At baseline, VMAT2 on a DA synaptic vesicle will exchange 2 hydrogen ions for one molecule of DA to achieve sufficient DA uptake and release. **(c)** In synaptic vesicles that co-express VMAT2 and VGLUT2, VGLUT2 will transport one chlorine and one phosphate ion, acidifying the inside of the vesicle, thereby increasing the concentration gradient, and ultimately resulting in the uptake of an increased amount of DA molecules via VMAT2. **(d)** Subsequently, the uptake of more dopamine will lead to the increased dopamine release from these VMAT2/VGLUT2 vesicles. Adapted from (Eskenazi et al., 2021).

Although I recognize that there has been conflicting evidence of both the co-localization of DA/glutamate co-release in the dorsal striatum (responsible for motor behavior) and the co-localization of VMAT2/VGLUT2 on the same vesicle, it is not yet possible to exclude the possibility that both exist in the context of neural grafting in our experiments. No other group has investigated these phenomena in a grafted rs6265 Met/Met parkinsonian rat, or in other models of DA neuron grafting, to the best of my knowledge. Our behavioral results and postmortem analyses are promising evidence in support of the theory of vesicular synergy, and vesicular synergy offers a logical mechanism responsible for GID behavior, at least in the context of this study. Future research further examining the potential for VMAT2/VGLUT2 co-localization are warranted and could offer new avenues for therapeutic development to prevent GID in patients.

In addition to evidence of excess DA release and DA/glutamate co-transmission, past experiments have also revealed a role of the immune system in GID behavior, including a study conducted by our group (see Soderstrom et al., 2008). Grafted parkinsonian animals exposed to immune activation exhibited increased GID severity compared to non-challenged rats (Soderstrom et al., 2008). In the clinic, patients who underwent withdrawal of immunosuppression (Hagell & Cenci, 2005; Olanow et al., 2003) or did not receive immunosuppression (Freed et al., 2001) developed GID behavior. Therefore, we considered the presence of the immune markers Iba1 (microglia) and GFAP (astrocytes). Grafted BDNF-infused parkinsonian rats showed an increase in expression of Iba1 in comparison to grafted PBS-infused animals but no differences in GFAP expression. Notably, there was no correlation between Iba1

expression and GID in these animals. Nevertheless, this does not mean the immune system does not play a role in GID development. Future studies warrant analysis of additional immune markers and could also take into account activated versus inactivated microglia and morphology. Furthermore, it would have been advantageous to examine postmortem tissue immediately following cessation of BDNF infusion instead of at the end of 10 weeks post-engraftment, although resources were not available to investigate this for my thesis studies. GID severity differences were more prominent between treatment groups at week five post-engraftment, and acute effects of BDNF on these immune markers could have been more apparent at this timepoint. Lastly, studies directly assessing the association between GID development and immune suppression are needed to definitively confirm the role of the immune system, and more importantly, how to abate these factors to allow neural grafting to become a more uniformly effective therapy option.

Our current study has demonstrated that exogenous BDNF treatment does not induce maturation of DA neuron transplants and would not be a safe and/or efficacious solution in the clinic to prevent GID as a side effect for grafted parkinsonian patients. In spite of this, we did, however, present evidence that confirms the clinical GID pharmacotherapy (i.e., buspirone) and offers great promise for the role of excess DA release and/or vesicular synergy underlying GID behavior. Collectively, these results provide a foundation for an abundance of future investigations. Furthermore, as the colocalization of VMAT2/VGLUT2 can only be presumed, additional methods (e.g., proximity ligation assays, ultrastructural analysis) will be necessary to prove, without a doubt, that these proteins are indeed co-localized together on the same synaptic

vesicle. Keeping the current precision-medicine climate in mind, these experiments, along with our other studies, continue to provide a convincing argument for genotyping patients prior to their participation in cell transplantation trials for PD.

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CHAPTER 5: FUTURE DIRECTIONS AND CONCLUDING REMARKS

Parkinson's disease (PD) is a relentlessly progressive neurodegenerative disorder that continues to negatively affect society. As we continue to study PD, the leading consensus is that PD is not a unitary disease entity; it is instead a multifaceted clinical syndrome with complex, heterogeneous etiologies. In this way, safe, efficacious treatments have been considerably difficult to develop. Indeed, as has been discussed at length in this thesis, several available therapeutic options are prescribed to treat PD, yet patient responsiveness is not uniform (Bove & Calabresi, 2022; Fabbri et al., 2016; Varanese et al., 2010), and even the best therapies have incomplete and/or limited lasting benefit. Most notably, with levodopa treatment, significant heterogeneity remains, both in clinical benefit and in the development of levodopa-induced dyskinesia (LID). As a beacon of hope, various experimental procedures are being examined as potential alternatives to the current dopamine (DA)-replacement strategies. Some examples include developing extended-release agents to achieve long-acting levodopa release and creating gene-therapy agents that target α -synuclein pathology. Arguably one of the most promising is the focus of my thesis work—regenerative neural cell replacement therapy. While cell therapy is not, nor will ever be, considered a “cure” for PD, scientists and clinicians have endeavored to optimize neural transplantation as a one-time procedure that will offer symptomatic relief for individuals with PD for decades to come (Barker et al., 2024).

In the following sections, I will discuss the key findings from my dissertation research and how these findings positively contribute to the field of neural transplantation for PD. Moreover, I will discuss limitations and potential caveats of my

studies, and I will share additional insight into how evidence collected here provides a strong foundation for the continuation of this research.

USING PRECISION MEDICINE TO GARNER MECHANISTIC INSIGHTS INTO GID BEHAVIOR

To date, the underlying cause of the aberrant side effect, graft-induced dyskinesia (GID), in response to neural transplantation of replacement DA neurons into the parkinsonian striatum remains elusive. Upon the conclusion of two clinical grafting trials funded by the NIH in the early 2000s, clinicians discovered this novel dyskinetic behavior that manifested only in subpopulation of individuals who received primary embryonic ventral mesencephalic (eVM) DA grafts (Freed et al., 2001; Olanow et al., 2003). Consequently, a worldwide moratorium was enacted following these trials as well as a clinical trial in Sweden (Hagell et al., 2002), halting all clinical grafting trials for PD (Hagell & Cenci, 2005). Now, after rigorous preclinical studies and re-evaluation of clinical studies, clinical grafting trials are scheduled or currently ongoing (see **Table 1.1** in Chapter 1 for a comprehensive list). Therefore, our research group has posited that, for cell transplantation to be an optimal therapeutic for patients with PD, we must investigate and elucidate the heterogeneity, namely GID development, in this subpopulation of patients.

As discussed in previous chapters, we have been studying the common human single nucleotide polymorphism (SNP), rs6265, as a potential underlying genetic risk factor for the development of GID behavior due to its resultant decrease in activity-dependent release of BDNF (Egan et al., 2003). Using a CRISPR knock-in parkinsonian rat model of the rs6265 SNP (Met/Met), my predecessor, Dr. Natosha Mercado,

demonstrated that Met/Met parkinsonian rats paradoxically exhibited enhanced functional recovery (i.e., earlier/more robust amelioration of LID behavior) and neurite outgrowth following primary eVM transplantation from wild-type (WT; Val/Val) donor neurons; however, these animals uniquely developed significant GID behavior compared to their WT counterparts (Mercado et al., 2021).

To complement her findings, I was able to demonstrate that, when additional host/donor combinations were studied (i.e., WT and Met/Met hosts engrafted with WT or Met/Met donor neurons), the homozygous rs6265 Met/Met genotype retained its beneficial functional action compared to the WT genotype, shown by an earlier amelioration of LID behavior (Chapter 3). Strikingly, however, I found that Met/Met rats engrafted with WT DA neurons were the only host/donor combination to exhibit significantly meaningful GID. Based on the similarities in GID induction in this genotypic host/donor combination between my study and Dr. Mercado's, I endeavored to investigate underlying mechanisms that may be responsible for this aberrant DA-graft-mediated behavior.

In both my in vivo studies, the data suggests that an increase in DA release (i.e., DAT expression) was prevalent and positively associated with GID behavior, which showed strong statistical significance in the exogenous BDNF supplementation study (see Chapter 4). I additionally demonstrated that, inside the grafted DA neurons of the BDNF-infused animals, there is (presumed) co-localization of vesicular monoamine transporter 2 (VMAT2) and vesicular glutamate transporter 2 (VGLUT2) which correlated strongly to GID behavior. This novel finding, again, is suggestive of vesicular synergy, which provides a logical explanation for how excess DA release and

DA/glutamate co-transmission could both be implicated in GID behavior. This evidence does not stand alone: other evidence in favor of excess DA release and/or DA/glutamate co-transmission in GID behavior has been collected clinically and preclinically. Indeed, fluorodopa positron emission topography (FDOPA PET) scans in graft recipients indicated that increases of DA were apparent in patients who exhibited GID compared to those who were GID-negative (Ma et al., 2002) (see Chapter 1: “Uneven DA reinnervation/DA release”). Shin and colleagues demonstrated that pharmacological blockade of D2 receptors using a DA antagonist, buspirone, resulted in significantly diminished GID behavior (Shin et al., 2012). Additionally, in the same preclinical rat model as my studies, Mercado and colleagues reported an upregulation of *Drd2* mRNA in the host MSNs of Met/Met rats engrafted with WT DA neurons which correlated with GID (Mercado et al., 2024). Lastly, for DA/glutamate co-transmission, Met/Met rats exhibited VGLUT2 expression that correlated strongly with GID behavior (Mercado et al., 2021). These previous analyses, along with the current evidence I have collected, strongly corroborate that there is a link between excess DA release and DA/glutamate co-transmission as an underlying mechanism of GID behavior. To the best of my knowledge, this is the first study to discover (presumed) co-localization of VMAT2 and VGLUT2 in grafted eVM neurons and the theory of vesicular synergy in the expression of GID, and therefore, this is an innovative avenue that should be examined further.

While we had hypothesized that BDNF-mediated maturation may be a way to prevent GID, it is also important to note that, because exogenous BDNF administration exacerbated GID behavior in grafted Met/Met parkinsonian rats, this does not appear to

be a safe or effective option to treat GID in grafted patients with PD. For years, BDNF administration has been considered a potential neuroprotective agent that could prevent the degeneration of nigrostriatal DA neurons (Nagahara & Tuszynski, 2011). Although not yet tested clinically in PD, preclinical studies of neurodegenerative and psychiatric disorders have utilized intrastriatal injections of viral vectors with the BDNF gene to increase BDNF protein production (Chen et al., 2020; Kells et al., 2004) (see Chapter 2). Engineered fibroblasts that produce BDNF have also been transplanted into the brain to study its therapeutic potential (Kells et al., 2004; Levivier et al., 1995). Despite some promising reports, it is difficult to tightly regulate gene therapy for BDNF, and overproduction can negatively affect host circuitry (Yeom et al., 2016; Zuccato & Cattaneo, 2009) (see (Szarowicz et al., 2022) for review). Therefore, until these BDNF administration therapies are optimized, and based on the results from my studies, supplementing with BDNF cannot be recommended, especially in this context of clinical grafting in PD.

After reviewing the clues assembled from my studies regarding GID behavior, along with several other comparable experiments, it is clear there is no “one-size-fits-all” approach to effectively treat PD. Ultimately, the conclusions regarding the rs6265 SNP provide a compelling argument for implementing a precision-medicine-based approach in neural transplantation for PD. Accordingly, **Figure 5.1** illustrates a proposed precision-based therapeutic approach to prevent and/or treat GID in the context of neural transplantation based on the findings discussed above. Specifically, it is reasonable to recommend that both the recipient and donor neurons are genotyped for the rs6265 SNP as a way of preventing GID development following DA neuron

transplantation (**Figure 5.1a**). Alternatively, in patients who have already received DA transplants, and subsequently developed GIDs, various tailored therapeutics, once discovered, could be administered to target the underlying mechanisms, therefore preventing GID occurrence (**Figure 5.1b**).

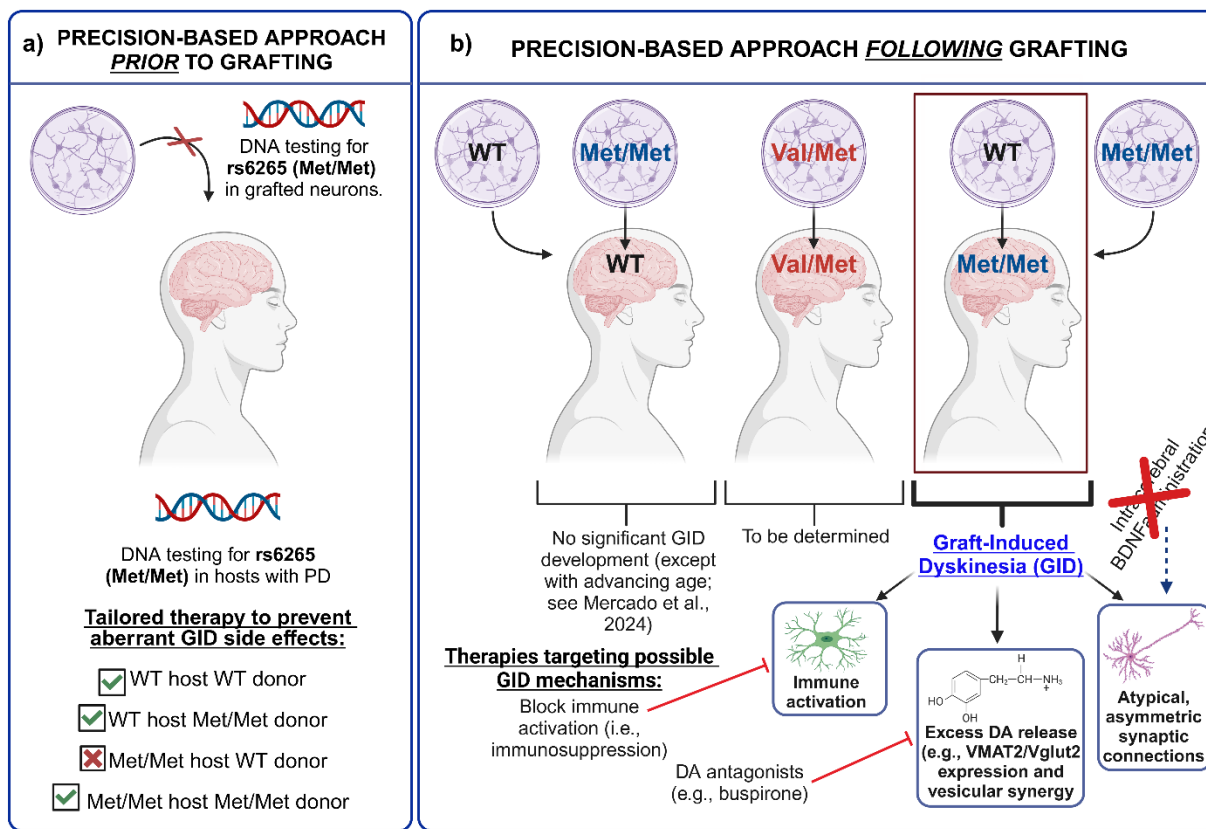


Figure 5.1: A possible precision-medicine-based therapeutic approach to prevent and/or treat GID behavior prior or following DA cell transplantation.

(a) Prior to grafting, genotyping the human patient and the donor neurons for the rs6265 SNP, is a recommended precision-based approach aimed at preventing the development of GID behavior. Since our previous (Mercado et al., 2021, 2024) and current studies have demonstrated that the homozygous Met/Met host parkinsonian rats engrafted with WT DA neurons uniquely develop GID behavior, preventing this host/donor transplantation combination would help avoid GID induction in grafted patients with PD. **(b)** Following cell transplantation, several mechanisms that could underlie GID behavior are proposed here as targets. For example, if excess DA release is responsible for GID behavior, DA antagonists such as buspirone can be administered to prevent potential aberrant GID behavior. With our studies, we have ruled out the administration of exogenous BDNF as it was shown to exacerbate GID behavior in Met/Met host rats engrafted with WT DA neurons (see Chapter 4).

THE FUNCTIONAL BENEFIT OF rs6265 AND A POTENTIAL ROLE FOR THE BDNF PRO-PEPTIDE

Although not at the forefront of my thesis studies, I did confirm that the homozygous rs6265 Met/Met genotype confers a degree of functional benefit and recovery following cell transplantation when compared to the WT genotype. Specifically, regardless of the presence of rs6265 in the host or donor neurons, animal groups with the Met/Met genotype demonstrated enhanced behavioral efficacy with earlier amelioration of LID behavior along with improvement of amphetamine-mediated rotational asymmetry compared to the WT hosts engrafted with WT DA neurons (see Chapter 3). Findings gained from my studies reinforce what was reported in (Mercado et al., 2021): Met/Met parkinsonian hosts exhibited enhanced graft-derived efficacy and increased neurite outgrowth in comparison to WT hosts (Mercado et al., 2021). Furthermore, a potential benefit of the Met allele (both heterozygous and homozygous) has been shown in other neurological conditions including traumatic brain injury (TBI) (Barbey et al., 2014; Finan et al., 2018; Krueger et al., 2011), stroke (Qin et al., 2014), multiple sclerosis (MS) (Zivadinov et al., 2007), Alzheimer's disease (AD) (Voineskos et al., 2011), and peripheral nerve injury (McGregor et al., 2019) (see Chapter 3: Discussion). Evidently, these reports contradict the historical paradigm that the Met allele is solely a “risk” allele. Instead, they indicate that the Met allele has, at least to some extent, some evolutionary benefit that explains its relatively high prevalence (approximately 20%) (Mercado et al., 2021; Petryshen et al., 2010; Tsai, 2018) in the general human population.

The rs6265 SNP is found within the prodomain/pro-peptide region of the BDNF gene, and therefore, this may suggest an important role for the BDNF Met pro-peptide in neuroregeneration based on the unexpected benefit addressed above.

Encouragingly, researchers have recently become aware that the BDNF pro-peptide appears to act an independent and functional ligand similar to that of mature and proBDNF (Anastasia et al., 2013; Kojima & Mizui, 2017; Mizui et al., 2017). For example, several research studies have investigated the expression levels and differential function of both the WT and Met BDNF pro-peptides, albeit mostly in the region of the hippocampus (see **Table 5.1** for a comprehensive list of experiments). In the context of our parkinsonian rat model, I hypothesized that the BDNF Met pro-peptide may be responsible for permitting enhanced neurite outgrowth of transplanted DA neurons demonstrated in (Mercado et al., 2021) and enhanced functional recovery ((Mercado et al., 2021); Chapter 3 of my studies).

Study	Subjects/Model	Region/Source	Effect
Dieni et al. (2012)	C57BL/6, Bdnf-Myc, cbdnf ko, Bsn mutant mice all 8 weeks of age.	Hippocampus	Mature BDNF and the BDNF pro-peptide are stored at equimolar ratios in large dense core vesicles in presynaptic terminals of excitatory neurons.
Anastasia et al. (2013)	Cultures prepared from E18 BDNF ^{Val/Val} and BDNF ^{Met/Met} knock-in mice	Hippocampal-cortical neurons	In hippocampal-cortical neurons, secreted levels of Met prodomain was significantly lower compared to Val prodomain secretion.
	Primary neurons were isolated from E15 C57BL/6 mouse embryos.	Hippocampus	In hippocampal neurons, growth cone retraction was induced by Met prodomain application in p75+ cells; Val prodomain was inactive. Met prodomain only interacted with SorCS2 receptor.
Lim et al. (2015)	SH-SY5Y neuroblastoma cells	Hippocampus	In culture, application of the Met prodomain negatively affected cell viability only in the presence of A β ; Val prodomain had no effect.
	Extracts from post-mortem tissue (AD patients)		Levels of pro-peptide were 16-fold higher in AD patients and correlated with A β accumulation.
Mizui et al. (2015)	Slices prepared from 3–4-week-old C57BL/6 and Bdnf KO mice.	Hippocampal tissue slices	Application of the Val pro-peptide facilitated LTD in hippocampal slices and required the activation of GluN2B-containing NMDA receptors.
	DIV21 cultures prepared from E18 Wistar rats.	Hippocampus	In cultured neurons, Val pro-peptide also induced endocytosis of AMPA receptors.
			In cultured neurons, the presence of the Val66Met SNP in the pro-peptide inhibited LTD.

Table 5.1: Evidence of varied BDNF pro-peptide activity associated with rs6265 SNP expression.

Abbreviations: HC: hippocampus, mPFC: medial pre-frontal cortex, CSF: cerebral spinal fluid, MDD: major depressive disorder, SCZ: schizophrenia, B6: C57BL/6, NAc: nucleus accumbens, BD: bipolar disorder, E: embryonic, DIV: Day in vitro, SD: Sprague Dawley, AD: Alzheimer's disease, CBM: cerebellum, PC: parietal cortex, P: postnatal. Adapted from (Szarowicz et al., 2022).

Table 5.1 (cont'd)

Study	Subjects/Model	Region/Source	Effect
Guo et al. (2016)	DIV16 rat neuronal cultures electroporated with plasmid-expressing eGFP.	Hippocampus	Val prodomain application reduced spine density and increased spine length. Val prodomain increased caspase-3 activity and mitochondria elongation. *Met prodomain was not studied.
Yang et al. (2016)	7-week-old male Sprague Dawley rats of learned helplessness (LH) model of depression (WT and Bdnf KO).	Medial prefrontal cortex (mPFC), CA3 and dentate gyrus of hippocampus, nucleus accumbens.	Significantly higher expression of BDNF pro-peptide in mPFC and CA3 regions of LH rats compared to controls. Significantly lower expression of BDNF pro-peptide in nucleus accumbens and dentate gyrus compared to controls.
Uegaki et al. (2017)	BIAcore sensor chip and recombinant human BDNF protein Slices prepared from male C57BL/6J mice (3-4-weeks-old)	Hippocampus	Using BIAcore chip, the BDNF pro-peptide binds to mature BDNF with high affinity. Using BIAcore chip, The Met pro-peptide is more stable in acidic and neutral pH environments compared to Val pro-peptide. In hippocampal slices, pre-incubation of the Val pro-peptide reduced the ability of mBDNF to inhibit LTD.
Yang et al. (2017)	Patients with MDD, SCZ, and bipolar disorder (BD)	Postmortem samples of cerebellum, parietal cortex, liver, and spleen	BDNF pro-peptide levels were significantly lower in the cerebellum and the spleen of MDD, SCZ, and BD patients compared to control groups. BDNF pro-peptide levels were significantly higher in the parietal cortex of MDD, SCZ, and BD patients compared to control groups.

Table 5.1 (cont'd)

Study	Subjects/Model	Region/Source	Effect
Giza et al. (2018)	DIV21 primary neurons prepared from C57BL/6 mice.	Hippocampus (ventral CA1 neurons)	In culture, the Met prodomain decreased mushroom spines and reduced PSD95 density in p75+ and SorCS2+ cells; Val prodomain had no effect.
	BDNF ^{Val/Val} , BDNF ^{Val/Met} , BDNF ^{Met/Met} P23-P60 male mice.	Hippocampus	Increased freezing behavior/decreased fear extinction was demonstrated in Met-prodomain injected mice. Fewer spines were also found in Met-prodomain treated mice compared with the Val-prodomain injected mice.
Mizui et al. (2019)	Japanese patients with Major depressive disorder (MDD) or Schizophrenia (SCZ)	Cerebral spinal fluid	The ratio of BDNF pro-peptide to total protein in MDD patients was lower in males and not females compared to controls.
			The ratio of BDNF pro-peptide to total protein was lower in SCZ patients, but it was not statistically significant.

To test my hypothesis, I employed an in vitro cell culture method which involved plating WT E14 eVM DA neurons (same cell source as our grafts) and treating them with exogenous administration of the WT or Met pro-peptide (25 ng/mL based on (Anastasia et al., 2013)) once per hour for a total of 16 hours (DIV4-5). Cells were then fixed with 4% paraformaldehyde and fluorescently stained for tyrosine hydroxylase (TH) to identify DA-positive neurons. Strikingly, Met pro-peptide application increased both the number and volume of TH+ neurons (**Figure 5.2**), indicating that the BDNF Met pro-peptide may have a positive impact on embryonic DA neurons, at least within this primary cell source. Further trials of this experimental design are warranted in order to

optimize the timing of application (e.g., continuous vs. per hour administration) and pro-peptide concentration. Again, while this was an ancillary study of my thesis, these findings provide an exciting new path for future research on the paradoxical benefit of the Met-allele.

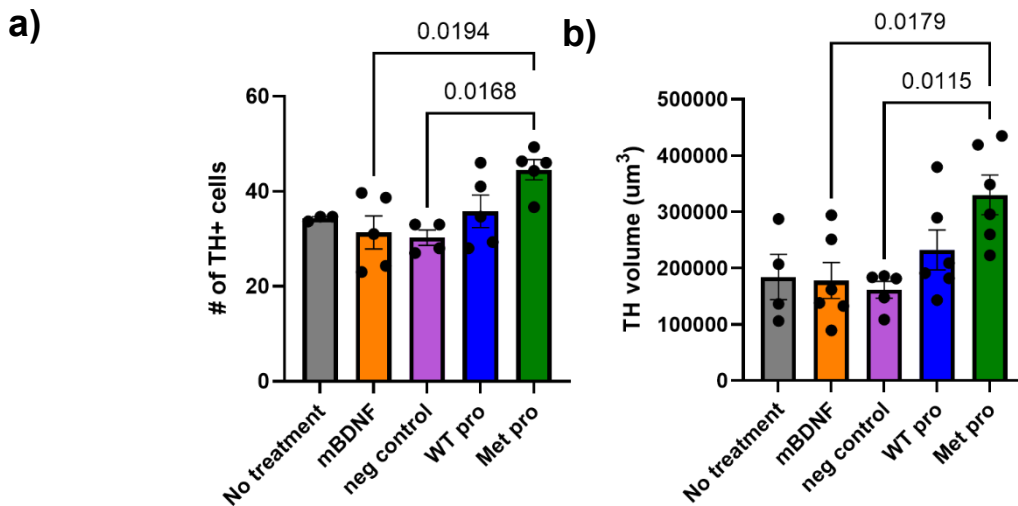


Figure 5.2: Impact of the BDNF Met and WT pro-peptides on survival and volume (μm³) of TH+ DA neurons in cell culture.

(a) Number of TH+ dopaminergic neurons following treatment of the BDNF Met or WT pro-peptide and their respective controls (i.e., mature BDNF or water as a negative control). Statistics: Mean ± SEM, Ordinary one-way ANOVA with Tukey's multiple comparisons, $p = 0.0194$ mBDNF vs. Met pro-peptide-treatment; $p = 0.0168$ negative control vs. Met pro-peptide-treatment. **(b)** Average volume (μm³) of TH+ dopaminergic neurons following treatment of BDNF Met or WT pro-peptide and their respective controls. Statistics: Mean ± SEM, Ordinary one-way ANOVA with Tukey's multiple comparisons, $p = 0.0179$ mBDNF vs. Met pro-peptide-treatment; $p = 0.0115$ negative control vs. Met pro-peptide-treatment.

LIMITATIONS AND ALTERNATIVE APPROACHES

Two limitations of my studies include the exclusion of heterozygous rs6265 (Val/Met) parkinsonian rats and the exclusion of female parkinsonian rats. These omissions were made for several practical reasons. In our first proof-of-concept experiment (Chapter 3), only WT and homozygous rs6265 (Met/Met) animals were studied in order to maximize the chances of observing any effect that might be

associated with the rs6265 SNP as the homozygous rs6265 genotype has the largest decrease in activity-dependent release of BDNF (Mercado et al., 2021). Additionally, because several host/donor combinations were being studied, including another genotypic profile would not be feasible to maintain as the total animals would be too numerous for proper behavioral analysis. The homozygous Met/Met genotype engrafted with WT DA neurons was employed for the second study (Chapter 4) as that was the only host/donor combination in our previous studies to develop significantly meaningful GID behavior. Similarly, only male rats were studied to maintain experimental feasibility. Because BDNF is well-known to interact with sex hormones (Chan & Ye, 2017; Wei et al., 2017), and rs6265 has been found to drive sex-specific susceptibility in various neurological disorders such as AD (Laing et al., 2012), MDD (Chagnon et al., 2015; Tsai, 2018), and schizophrenia (Chao et al., 2008; Suchanek et al., 2013; Yi et al., 2011) (see (Szarowicz et al., 2022)) for review), it would be highly relevant to conduct future studies repeated in females moving forward.

We had originally planned to include only two grafted cohorts for the second experimental study with BDNF infusion (i.e., DA-grafted BDNF-infused and DA-grafted PBS-infused animals) (Chapter 4). In one cohort of animals, we had explored the idea of sacrificing immediately following osmotic minipump removal at the end of the four week infusion, and the other cohort was to be extended an additional six weeks after pump removal. However, due to unforeseen obstacles, we had to re-design our experiment: after purchasing timed-pregnant female rats for cell collection, the rats were not pregnant. Because we had already begun priming the pumps with BDNF and PBS, we made the decision to re-design the experiment to instead include “non-grafted”

BDNF- and PBS-infused animals and extend all animals to 10 weeks post-engraftment. In addition to this, we could have alternatively extended BDNF supplementation for a longer period; however, we were restricted by the cost of the BDNF protein (i.e., \$50,000 for 8 mg). Despite these difficulties, the results obtained from these first proof-of-concept studies are meaningful and provide a foundation for additional future research.

For both in vivo experiments, I was only able to use indirect markers to determine whether there was evidence of differences in DA release (i.e., DAT expression) and immune activation (i.e., Iba1, GFAP). Although the markers I employed provide a sufficient starting point, neither directly demonstrate whether excess DA release or immune activation are underlying mechanisms of GID behavior. For instance, the DAT protein was immunohistochemically stained postmortem to indirectly assess DA release; however, DAT expression alone may not be affected in these animals. Accordingly, investigating the function of the receptor or other DA markers (e.g., D1/D2 receptors) would be a great addition to further assess whether DA release is indeed associated with GID in this model. Future experiments designed to directly measure DA release at the grafted DA neurons in the striatum would be important to collect definitive evidence. Such studies could involve in vivo voltammetry, which is further discussed below.

Moreover, for immune markers, Iba1 and GFAP were chosen because both are pan markers commonly utilized to identify microglia and astrocytes, respectively. However, because they are solely pan markers, I was limited in realizing whether these immune cells are active or inactive as their presence alone does not necessarily reflect an increased activation of the immune system. Alternatively, other markers such as

MHC-II could be used, or the morphology of Iba1+ and GFAP+ cells in my studies could be analyzed in the future as another indication of activated vs. inactivated microglia or astrocytes, respectively.

My data demonstrate the novel co-localization of VMAT2 and VGLUT2, which correlated significantly with GID behavior in DA-grafted BDNF-infused Met/Met parkinsonian rats. Again, to the best of my knowledge, this is the first time VMAT2 and VGLUT2 are shown to co-localize in grafted eVM neurons. However, as addressed in Chapter 4, the fluorescent immunohistochemistry approach I used for the analysis of VMAT2/VGLUT2 was limited as it could not visualize specific synaptic vesicles, which are only roughly 40 nm in diameter. Therefore, an additional approach to general immunohistochemical analysis is to employ electron microscopy or a proximity ligation assay (PLA), which is discussed in detail below.

Lastly, to expand the translatability of my studies to the current clinical trials that are planned or ongoing, using another cell source other than primary DA neurons would be beneficial. For example, due to several ethical concerns and difficulty in obtaining sufficient quantities of embryonic cells, induced pluripotent stem cells (iPSCs) are a cell source that clinicians and researchers are shifting toward utilizing (Barker et al., 2024). In my experiments, we employed primary embryonic DA neurons because this is the only cell source that is currently known to induce substantial GID behavior. Nevertheless, alternative tests that employ iPSCs transplants are warranted to achieve an experimental design that is more translatable to human trials.

FUTURE DIRECTIONS

The Benefit of the Met allele and the BDNF Met Pro-peptide

As discussed previously in Limitations and Alternative Approaches, I conducted an in vitro cell culture experiment aimed at studying the differential impact of the BDNF WT vs. Met pro-peptide on neurite outgrowth in primary eVM DA neurons. In order to focus on the detriment, rather than the benefit, of the rs6265 Met/Met genotype in parkinsonian rats, I have not included these data in my dissertation. It is important to note, however, that our group submitted these cells to NanoString to be analyzed for differences in transcriptomic profiles between the WT- and Met-pro-peptide-treated cells, and we are currently in the process of analyzing the results. If certain genes related to neurite outgrowth are upregulated in the Met-pro-peptide-treated cells, these genes could potentially be targeted therapeutically to enhance the beneficial outcomes of neural transplantation in rs6265-carrying subjects.

Directly measuring DA release

Previous clinical trials have demonstrated promising evidence in favor of excess DA release underlying GID behavior. For instance, FDOPA PET signals were significantly higher in grafted patients who developed GID compared to the patients who did not (Ma et al., 2002) (see Chapter 1: Uneven DA reinnervation/DA release). Now, with our preclinical parkinsonian model, I have demonstrated indices of excess DA release in WT-grafted homozygous rs6265 (Met/Met) parkinsonian rats (i.e., increased DAT expression) (Chapter 3 and 4). Regrettably, however, both PET scans and DAT expression are not direct measures of DA release, so we can only infer that increased DA is associated with GID behavior. Therefore, future studies that directly assess DA

release from the graft are warranted. Accordingly, it would be advantageous to employ in vivo voltammetry, a technique that is commonly used to measure neurotransmitter release concomitantly with behavioral assessment. With this technique, we could be more confident whether DA release underlies GID behavior in this parkinsonian rs6265 rat model.

Co-localization of VMAT2/VGLUT2 and Vesicular Synergy

It is likely that excess DA alone does not result in GID behavior, and thus, I investigated a possible connection between DA release and glutamate co-transmission based on previous findings that correlated VGLUT2 expression to GID in Met/Met hosts engrafted with WT DA neurons (Mercado et al., 2021). My results demonstrated that the number of (presumed) co-localized VMAT2/VGLUT2 was strongly, and significantly, correlated with GID behavior in WT DA-grafted BDNF-infused Met/Met parkinsonian rats (see Chapter 4), providing a compelling argument for the theory of vesicular synergy in these animals. However, VMAT2 and VGLUT2 are only presumed to be colocalized in the grafted DA neurons due to the limitations of magnification in my postmortem analyses. Thus, as a future direction, I propose employing a PLA assay that would aid in the definitive determination of whether VMAT2 and VGLUT2 are co-localized on the same synaptic vesicle. Using the PLA assay, the transporters will fluoresce if they are found within 40 nm of each other in the grafted DA neurons. Because there remains considerable contention as to whether VMAT2 and VGLUT2 are found on the same vesicle (e.g., (Aguilar et al., 2017; Zhang et al., 2015)), and as VMAT2/VGLUT2 have not been studied in eVM tissue or in the context of neural grafting, findings that demonstrate same vesicle colocalization could be groundbreaking.

A promising role for the immune system

Although no exhibition associating the immune response to GID behavior was found in my studies, this does not exclude immune activation from potentially underlying GIDs, especially due to the limitations of immune marker analysis addressed in Limitations and Alternative Approaches. Indeed, clinical trials (Freed et al., 2001; Olanow et al., 2003) have demonstrated that, only after immunosuppression was discontinued, GIDs developed in DA-grafted individuals with PD. Our group has additionally confirmed that, in preclinical parkinsonian rat studies, GID severity was increased following immune challenge (Soderstrom et al., 2008). Future studies should directly investigate the role of immune activation in our rs6265 parkinsonian rat model to determine whether immune function correlates to GID behavior. In this way, immunosuppression agents (**Figure 5.1b**) could be given to DA-grafted WT and rs6265 (Met/Met) parkinsonian rats; GID behavior and postmortem morphological changes of immune markers could then be assessed. Ultimately, this could be a promising potential therapeutic target aimed at the prevention of GID.

Graft Location

While not quantitative, I anecdotally observed that the location of the graft (e.g., dorsolateral vs. ventrolateral) influenced the development of GID behavior in both of my studies. For instance, in the host/donor combination study, I was able to qualitatively show that the homozygous rs6265 Met/Met hosts engrafted with WT DA neurons demonstrated a higher percentage of DA grafts placed in the dorsolateral region of the striatum and subsequently had a higher occurrence of significant GID induction compared to the other DA-grafted host/donor groups (see **Figure 5.3**). Likewise, in my

second experiment, based on the neurite outgrowth data (see Chapter 4), the higher neurite density of the graft was found in the dorsolateral region of the striatum in the DA-grafted BDNF-infused animals, the same group that exhibited significant GID behavior. Differential graft placement could also have been a contributor of GID in clinical trials. For example, in the Denver/Columbia trial (Freed et al., 2001), GID mainly affected the upper body, manifested primarily as dystonic movements, and increased FD uptake (PET) in the dorsal putamen. Conversely, in the Tampa/Mount Sinai trial (Olanow et al., 2003), GID developed largely in the lower extremities with more stereotypic movements, and FD uptake was increased in the ventral region of the putamen. These differences in GID suggest that variability is likely due to the differential placements of the DA graft (Steece-Collier et al., 2012).

Since the dorsal and ventral striatum have differential functions, these findings are not entirely unexpected. Indeed, the dorsal striatum generally controls motor and cognitive function (Cataldi et al., 2022; Corbit et al., 2017; Haith & Krakauer, 2018), while the ventral striatum regulates motivation and reward (Grueter et al., 2012; Nestler et al., 2002; Russo et al., 2010). While the graft location findings from my thesis are not quantitative and need to be studied further, other evidence from clinical grafting trials provide insights into the possibility of graft location influencing GID induction. Therefore, a small preliminary animal experiment could be designed in which eVM neurons are grafted into specific regional locations of the striatum (e.g., dorsolateral vs. ventrolateral) and assessed for GID behavior.

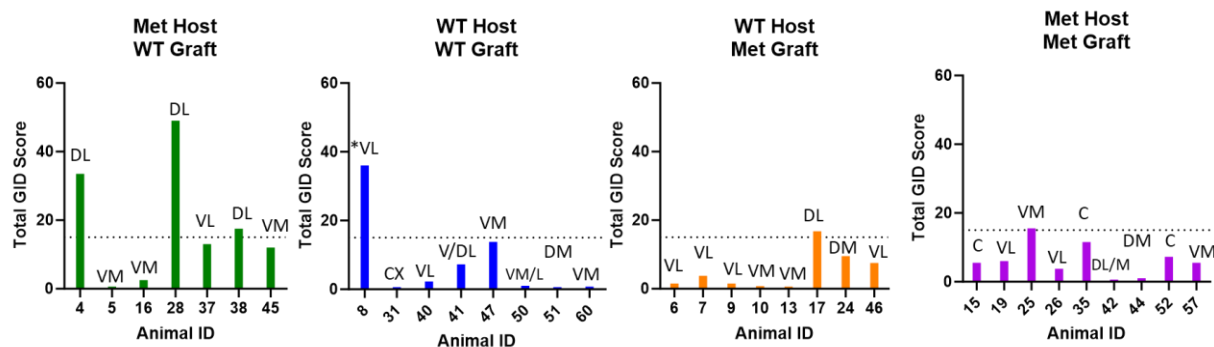


Figure 5.3: Qualitative comparison of graft location and GID scores in each host/donor combination.

Homozygous Met/Met parkinsonian rats engrafted with WT DA neurons (shown above in green) demonstrated a higher number of animals that exhibited increased GID behavior, and an increased number of these animals with GID behavior had grafts that were placed more dorsolateral in the striatum. The dotted line demarcates a total GID score of 15 or above at 10 weeks post-engraftment.

Transplanting iPSCs into our rs6265 Parkinsonian Rat Model

Previously discussed above, clinical trials are now shifting (see **Table 5.2**) toward utilizing iPSCs as a cell source for neural transplantation in PD due to the ethical concerns of using embryonic neurons and the obstacles of obtaining a sufficient amount of tissue (Barker et al., 2024). However, because VM transplants are currently the only cell source to induce GID behavior, the clinical outcomes of iPSC transplants remain unknown. Moreover, like past trials, iPSCs have not been genotyped for the rs6265 SNP to the best of my knowledge, and therefore, it is unknown whether the host/donor interactions we have demonstrated in our preclinical parkinsonian rats also apply to iPSC transplants. Regardless, an advantage of using iPSCs is that they could be programmed and/or genetically manipulated to produce less, or more, DA and/or express less or more VMAT2/VGLUT2, if these are indeed the underlying factors responsible for GID behavior. It could, however, be entirely possible that transplantation of iPSCs do not induce GID behavior in DA-grafted parkinsonian individuals. Therefore,

I would recommend that a similar experiment to my studies be conducted in the future using iPSCs as the cell source for transplantation to determine whether these cells have the potential to induce GID.

Clinical Trial ID	Location	Cell Source	Status
NCT06687837	Boston, MA, USA	Autologous iPSCs	Recruiting
NCT06482268	La Jolla, CA, USA	Human iPSCs	Recruiting
NCT06422208	Boston, MA, USA	Autologous iPSC-derived DA neurons	Enrolling my invitation
NCT06141317	San Jose, Costa Rica	PASCs	Active, not recruiting
NCT05901818	Beijing, China	Autologous induced neural stem cell-derived DA precursor cells	Recruiting
JMA-IIA00384	Kyoto, Japan	Allogenic human iPSCs	Completed
NCT06145711	Shanghai, Shanghai China	HiPSC-derived dopaminergic neural precursor cells	Recruiting
NCT06821529	Hangzhou, Zhejiang, China	iPSC-DAPs	Not yet recruiting

Table 5.2: Clinical Trials using iPSCs.

Current planned or ongoing clinical trials using iPSCs as a cell source for neuron transplantation in PD. Abbreviations: iPSCs = induced pluripotent stem cells; PASCs = PASCs = pluripotent stem cells isolated from adipose tissue; DA = dopamine; HiPSC = human induced pluripotent stem cells; iPSC-DAPs = induced pluripotent stem cells dopaminergic progenitor cells.

CONCLUDING REMARKS

The key findings presented in this thesis are three-fold: (1) the homozygous rs6265 Met/Met genotype, regardless of host or donor, confers a degree of functional graft-derived benefit in parkinsonian rats; (2) homozygous rs6265 Met/Met parkinsonian rats engrafted with WT DA neurons remain the only host/donor combination to develop aberrant GID behavior; (3) excess DA release and/or DA/glutamate co-transmission are promising factors that likely underlie GID behavior. These findings ultimately support the notion that PD remains a complex, heterogeneous disorder, making it almost impossible to develop a “one-size-fits-all” therapy that works uniformly for everyone. Instead, a precision-medicine-based approach, especially in regenerative cell therapy, is warranted to treat PD. Results obtained from this thesis have provided a solid foundation for future studies moving forward in this precision-medicine-based climate.

It is, again, highly recommended that PD patients and donors be genotyped for the rs6265 SNP prior to receiving cell transplants. Additionally, now that the field have shifted toward implementing iPSCs as a new cell source in clinical trials, we can now determine whether iPSC-engrafted patients develop GID. In the event that GID do develop, we now have at least some insight into the underlying GID mechanisms based on my thesis findings. While cell transplantation does not, and will not, offer a “cure” for PD, it does offer a promising non-pharmacological alternative to the therapies that are currently prescribed. However, until cell transplantation is completely optimized by harnessing the benefits while preventing the side effects (e.g., GID), neural transplantation will not be considered a fully safe and effective therapeutic alternative to treat PD.

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