TOWARD PRECISION MEDICINE: EFFECTS OF THE COMMON VAL66MET BDNF VARIANT IN THE AGING BRAIN AND IMPLICATIONS FOR THE FUTURE OF PARKINSON'S DISEASE THERAPEUTICS

By

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ABSTRACT

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The rs6265 (Val66Met) single nucleotide polymorphism in the gene for brain-derived neurotrophic factor (*BDNF*) is a common genetic variant that has been shown to alter therapeutic responses in patients with Parkinson's disease (PD). Possession of the variant Met allele results in decreased activity-dependent release of BDNF by disrupting BDNF transport and sorting into synaptic vesicles. In the experiments detailed in this thesis, I examine the effects of the Val66Met SNP, and its interaction with aging, on therapeutic efficacy and the development of aberrant side-effects following primary dopamine (DA) neuron transplantation, a restorative experimental therapeutic approach for PD that is currently experiencing a robust revitalization following a decade-long worldwide moratorium. In particular, I hypothesized that rs6265-mediated dysfunctional BDNF signaling is an unrecognized contributor to the limited clinical benefit observed in a subpopulation of individuals with PD despite robust survival of grafted DA neurons and extensive integration into the host brain. I also hypothesized that this genetic variant contributes to the development of graft-induced dyskinesias (GID). To test these hypotheses, we generated a novel CRISPR knock-in rat model of the rs6265 *BDNF* SNP to investigate for the first time the influence of a common genetic polymorphism on graft survival, functional efficacy, and side-effect burden in subjects grafted with embryonic ventral mesencephalic DA neurons. In two sister studies, I compared these

primary endpoints between wild-type (Val/Val) rats and those homozygous for the variant Met allele (Met/Met), in both young adult (8 m.o. at grafting) and middle-aged (15 m.o. at grafting) cohorts. In each study, rats were rendered unilaterally parkinsonian with intranigral 6-hydroxydopamine and primed with levodopa (12 mg/kg M-Fr) to induce stable expression of levodopa-induced dyskinesias (LID), the primary behavioral endpoint for assessing graft function. After levodopa priming, rats received an intrastriatal graft of embryonic ventral mesencephalic neurons (200,000 cells in young adult rats, 400,000 cells in middle-aged rats; E14 wild-type donors) or a sham graft. LID were evaluated for 9-10 weeks post-engraftment, and GID were assessed 24-48 hr prior to sacrifice. In young adult graft recipients, this research demonstrates that: 1) Met/Met rats display enhanced graft efficacy and paradoxically enriched graft-derived neurite outgrowth compared to Val/Val rats, and 2) the Met allele is strongly linked to GID development and this behavioral phenotype is correlated with neurochemical signatures of glutamatergic neurotransmission by grafted DA neurons. In middle-aged graft recipients, this research indicates that: 1) behavioral enhancement associated with the Met allele is maintained with advancing age, and 2) advanced age is associated with the induction of GID in rats of both genotypes despite the presence of widespread intrastriatal grafts. In this rapidly evolving era of precision medicine, understanding mechanisms underlying the beneficial versus detrimental impact of the Val66Met polymorphism, and/or its interaction with aging, will aid in the development of safe and optimized therapeutic approaches for remodeling the parkinsonian striatum.

For those who showed me that I can be more than my circumstances.

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As a first-generation college student, the journey to the place I find myself in today was far from easy. Armed with a strong sense of determination and a desire to do something meaningful with my career and my life, I have wrestled my way through countless obstacles and disappointments over the years. I could say that it was sheer determination that propelled me down this path, but that would not be the whole truth. Throughout the course of this journey, I have been lucky to meet countless generous souls along the way who provided refuge and guidance.

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As I close this chapter of my life and transition into the next stage of my career, I must emphasize once more that I am forever grateful for the collective friendship, expertise, guidance, and support that the individuals listed here have provided for me. I hope that I can continue to make you proud.

PREFACE

Upon completion of this dissertation, one chapter has been published and one chapter is currently being prepared for submission. Specifically, Chapter 3 is modified from the manuscript that was published online ahead of print in *Neurobiology of Disease* in November 2020 (PMID: 33188920) and is reproduced here with permission from the publisher. Chapter 4 is in preparation for submission in 2021.

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KEY TO ABBREVIATIONS

3D	Three-dimensional
5-HT	5-hydroxytryptamine (serotonin)
6-OHDA	6-hydroxydopamine
7,8-DHF	7,8-dihydroxyflavone
AADC	Aromatic L-amino acid decarboxylase
AAV	Adeno-associated virus
AD	Alzheimer's disease
AIM	Abnormal involuntary movement
ARTN	Artemin
BDNF	Brain-derived neurotrophic factor
CDNF	Cerebral dopamine neurotrophic factor
CMF	Calcium magnesium free
CNS	Central nervous system
COMTI	Catechol-O-methyltransferase inhibitor
DA	Dopamine
DBS	Deep brain stimulation
dMSN	Direct pathway medium spiny neuron
DNA	Deoxyribonucleic acid
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRN	Dorsal raphe nucleus

DRT	Dopamine replacement therapy
ELLDOPA	Earlier vs Later Levodopa Therapy in Parkinson's disease
ERK	Extracellular signal-regulated kinase
ESCs	Embryonic stem cells
FDA	Food and Drug Administration
GABA	Gamma aminobutyric acid
GAD	Glutamic acid decarboxylase
GID	Graft-induced dyskinesias
GDNF	Glial cell line-derived neurotrophic factor
GFP	Green fluorescent protein
GPe	Globus pallidus, external segment
GPi	Globus pallidus, internal segment
IHC	Immunohistochemistry
iMSN	Indirect pathway medium spiny neuron
iPSCs	Induced pluripotent stem cells
ISH	*In situ* hybridization
i.p.	Intraperitoneal
LB	Lewy body
LN	Lewy neurite
LID	Levodopa-induced dyskinesias
LTD	Long-term depression
LTP	Long-term potentiation
M	Molar

MANF	Mesencephalic astrocyte-derived neurotrophic factor
MAOBIs	Monoamine oxidase type B inhibitors
MAPK	Mitogen-activated protein kinase
MDS	International Parkinson and Movement Disorder Society
MFB	Medial forebrain bundle
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSA	Multiple system atrophy
MSN	Medium spiny neuron
NGF	Nerve growth factor
NGS	Normal goat serum
NIH	National Institutes of Health
NINDS	National Institute of Neurological Disorders and Stroke
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
$p75^{NTR}$	p75 neurotrophin receptor
PD	Parkinson’s disease
PET	Positron emission tomography
PI3K	Phosphatidylinositol 3-kinase

PLCγ	Phospholipase Cγ
PPN	Pedunculopontine nucleus
PSCs	Pluripotent stem cells
PSD95	Postsynaptic density protein 95
PSP	Progressive supranuclear palsy
PSPN	Persephin
RBD	Rapid eye movement behavior disorder
RhoA	Ras homolog gene family member A
RNA	Ribonucleic acid
s.c.	Subcutaneous
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
SNP	Single nucleotide polymorphism
SorCS2	Sortilin-related Vps10p domain containing receptor 2
SP	Synaptopodin
SPECT	Single photon emission computed tomography
STN	Subthalamic nucleus
TBI	Traumatic brain injury
TBS	Tris-buffered saline
TGN	*Trans*-Golgi network
TH	Tyrosine hydroxylase
THir	Tyrosine hydroxylase-immunoreactive

TPH2	Tryptophan hydroxylase 2
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
VGLUT	Vesicular glutamate transporter
VM	Ventral mesencephalon
VTA	Ventral tegmental area

CHAPTER 1: INTRODUCTION TO PARKINSON'S DISEASE

History

Parkinson's disease (PD) was first described in 1817 by Dr. James Parkinson, an English physician and avid political activist [1]. In his pioneering work entitled *An Essay on the Shaking Palsy*, Parkinson described a condition which he called "shaking palsy" or *paralysis agitans* [2]. Following careful observation of six afflicted individuals, three of which he observed at a distance on the street, Parkinson described the slow progression of this neurodegenerative disease and the profound decline in quality of life for those afflicted with this condition. Remarkably, in addition to non-motor features such as sleep disturbance and constipation, he also astutely detailed the classical motor symptoms that are now widely recognized as primary clinical features of PD:

> *Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.* [2]

These initial observations were refined and expanded upon over 50 years later by Jean-Martin Charcot, a French neurologist and professor. In addition to renaming the condition "Parkinson's disease," Charcot differentiated PD from other tremulous disorders and distinguished bradykinesia as a key feature of the disease [3].

Significant advances in our understanding of the etiology of PD were not made until the mid-twentieth century. In the late 1950s, a multitude of landmark discoveries by researchers such as Oleh Hornykiewicz, Sir Henry Dale, Peter Holtz, and Arvid Carlsson led to the groundbreaking hypothesis that striatal dopamine (DA) loss is involved in the pathogenesis of PD (reviewed in detail in [3] and [4]). Soon thereafter, the nigrostriatal DA pathway was mapped using histofluorescence and injury to the

substantia nigra (SN) was shown to cause DA loss in the nigral projection target, the striatum (reviewed in [4]). More than 100 years following Parkinson's initial description of this disease, these discoveries revolutionized our understanding of the pathogenesis of PD and form the basis from which therapeutic strategies aimed at restoring lost DA have been developed.

Clinical Presentation and Diagnosis

In the United States (US), PD is the second most common neurodegenerative disease, affecting approximately one million Americans and their caregivers with an estimated economic burden of $51.9 billion annually [5, 6]. Furthermore, in accordance with the growing elderly population in the US, the incidence of PD is growing substantially [7]. According to recent estimates, the prevalence of PD in the US is expected to increase to 1.64 million cases with projected annual costs reaching $79.1 billion by 2037 [6]. However, it is important to note that these estimates do not account for undiagnosed cases of PD.

As a movement disorder, an accurate clinical diagnosis of PD is dependent on the presence of the widely recognized primary motor symptoms that appear as the disease progresses. However, PD also presents with an array of non-motor symptoms, as Parkinson alluded to in his landmark 1817 publication [2]. These non-motor symptoms often appear before the onset of noticeable motor dysfunction, a time at which PD cases often go undiagnosed or are misdiagnosed as other forms of parkinsonism [8]. In the following paragraphs, both motor and non-motor features of PD are discussed in the context of how they may influence diagnostic accuracy in the clinic.

Motor Symptoms and Challenges Associated with Clinical Diagnosis

The classical motor symptoms of PD include bradykinesia, resting tremor, muscular rigidity, and postural instability [9]. These motor features are often heterogenous among individuals with PD, prompting classification of subtypes of PD and other forms of parkinsonism, though in general a patient must have bradykinesia plus rest tremor and/or muscular rigidity to be diagnosed with PD [10].

Bradykinesia, or slowness of movement, is a hallmark of some basal ganglia disorders and is essential for a PD diagnosis. Patients with this cardinal symptom typically experience difficulties with planning, initiating, and executing movement, and with more complex motor challenges such as performing simultaneous, sequential, and/or repetitive tasks [11, 12]. Resting tremor is another prominent feature of PD. Tremors in PD occur at a frequency of 4 to 6 Hz and are most often noticeable in the distal extremities [11]. Importantly, resting tremor disappears with action and during sleep, a characteristic that helps differentiate PD from other tremulous disorders such as essential tremor [11]. The third primary motor feature of PD, rigidity, is characterized by stiffness and increased resistance to passive movement. Rigidity is sometimes associated with pain; indeed, shoulder pain is a frequent initial manifestation of PD that often leads to misdiagnosis early in the disease [11, 13]. Finally, postural instability often occurs in the late stages of disease progression, typically after the onset of other primary symptoms [11]. Postural instability is a primary contributor to falls in individuals with PD, though the late manifestation of this motor feature means that falls characteristically do not occur until much later in the disease. In fact, the longer latency to the onset of falls is a key differentiating factor between PD and other parkinsonian

disorders such as progressive supranuclear palsy (PSP) and multiple system atrophy (MSA) [11].

There is currently no guaranteed test for the definitive diagnosis of PD. Furthermore, differentiating PD from other forms of parkinsonism can be difficult, as clinical characteristics of these various disorders overlap significantly, especially in early stages of the disease [8]. Indeed, PD is commonly misdiagnosed as PSP, MSA, corticobasal degeneration, or essential tremor [8, 9]. In the last three decades, the use of standard clinical diagnostic criteria has improved the accuracy of clinical PD diagnosis. These criteria were initially proposed by the UK Parkinson's Disease Society Brain Bank in the late 1980s [14, 15], and were promptly followed by additional groups such as Gelb and colleagues at the National Institute of Neurological Disorders and Stroke (NINDS) ([16], but see also [17, 18]). More recently, the diagnostic criteria for PD were revised and expanded upon by the International Parkinson and Movement Disorder Society (MDS). Notably, this report features the inclusion of non-motor symptoms as additional diagnostic criteria and takes into account for the first time the prodromal stage of PD in which early disease characteristics are present but clinical diagnosis is not yet possible [14, 19] **(Figure 1.1)**.

In general, there are three steps involved in the clinical determination of PD. First, as noted, a patient must present with bradykinesia plus resting tremor and/or rigidity. These features represent the core symptomology of PD and are necessary for identifying the presence of parkinsonism. Second, the patient must not possess symptoms or history indicative of a non-PD form of parkinsonism. As such, exclusionary features that would prompt consideration of alternative diagnoses are presented across

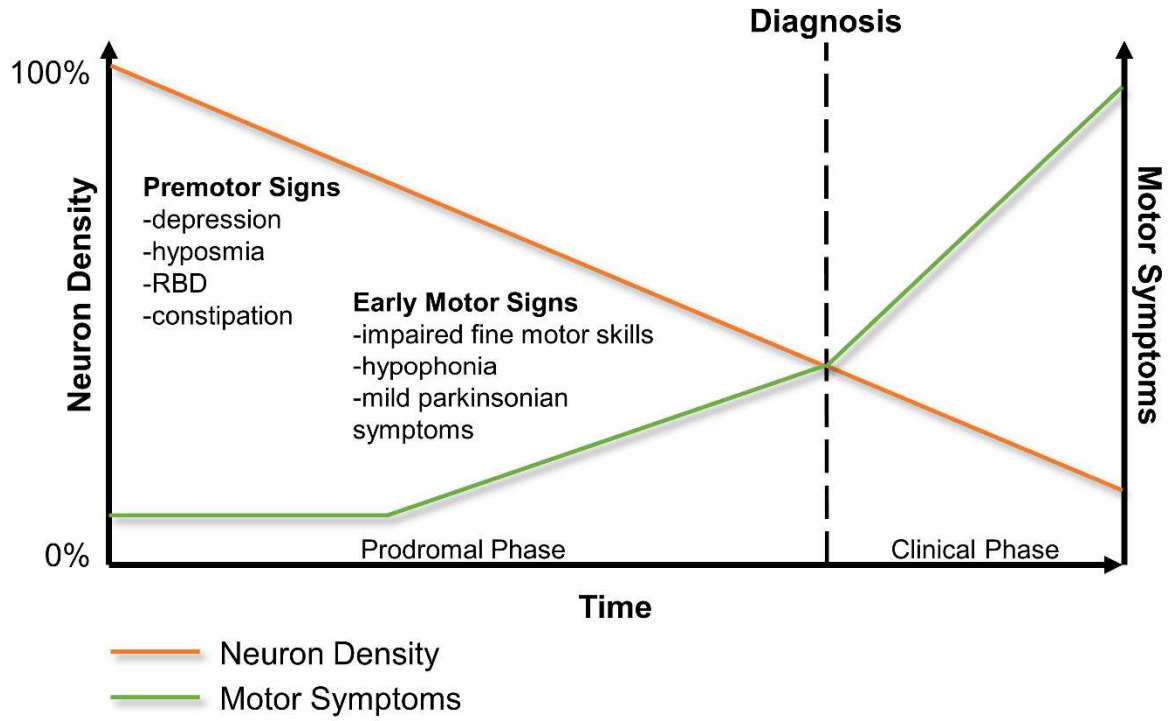

Figure 1.1 Typical time course of Parkinson's disease progression
Parkinson's disease consists of a preclinical/prodromal phase and a clinical phase. The preclinical phase is characterized by non-motor symptoms followed by noticeable, mild motor symptoms. Mild parkinsonian symptoms include bradykinesia, tremor, and rigidity. The clinical phase occurs following diagnosis. Extensive midbrain dopamine neuron loss has already occurred by the time diagnosis is confirmed. Adapted from [20]. Abbreviations: RBD, rapid eye movement behavior disorder

all sets of diagnostic criteria [15, 16, 19]. Third, the patient must possess at least two additional criteria supportive of a PD diagnosis [19]. In earlier descriptions of diagnostic criteria, these included features such as: unilateral disease onset, excellent response to levodopa (aka: L-3,4-dihydroxyphenylalanine), presence of levodopa-induced dyskinesias (LID), progressive decline with clinical course of 10 years or more, and persistent asymmetry primarily affecting the side of disease onset [9, 11, 16]. However, following scientific advancement and increased understanding of PD, the MDS has refined this list to include the following four features: (1) clear and dramatic beneficial response to dopaminergic therapy, (2) presence of LID, (3) rest tremor of a limb documented during clinical examination, and (4) presence of olfactory loss or cardiac sympathetic denervation [19].

To increase confidence in the differential diagnosis of PD, ancillary tests are often used in conjunction with clinical diagnostic criteria, and were included as a supportive element for the first time in the diagnostic criteria set forth by the MDS [19]. Specifically, drug challenges that test for dopaminergic responsiveness are key to differentiating PD from parkinsonian disorders such as PSP and MSA. Indeed, a clinical PD diagnosis may be substantiated by an “excellent and sustained response to levodopa” [8]. Additional methods that are used by clinicians to support the differential diagnosis of PD include – but are not limited to – olfactory testing (olfactory dysfunction is a typical early clinical sign of PD) and brain imaging [e.g., cranial CT, MRI, single photon emission computed tomography (SPECT), positron emission tomography (PET)] [8, 19].

Despite recent advances in the sensitivity and specificity of clinical diagnostic criteria for PD, they are not without issues [14], and diagnosis cannot be definitively confirmed until histopathological confirmation is provided upon postmortem examination [11, 16]. According to a recent report, postmortem studies have shown that the overall diagnostic accuracy for clinically diagnosed PD is 81% [10]. In other words, 1 out of 5 clinically determined PD cases is *not* actually PD. This brings attention to the need for additional methods to improve diagnostic accuracy, especially in the early stages of disease development when the potential benefits of slowing or stopping disease progression are highest. As such, there is much effort in the field to develop biomarkers for the accurate detection of PD at early stages of pathological development, even before motor symptoms appear (e.g., [9, 20-22]). Furthermore, as non-motor abnormalities often occur before onset of motor symptoms, additional attention is directed at identifying non-motor features during the prodromal phase of PD as predictors of future occurrence of clinically relevant PD-associated motor dysfunction [10] **(Figure 1.1)**.

Non-Motor Symptoms

In Parkinson's initial report of the "shaking palsy," he described sleep disturbance and severe constipation as non-motor side-effects experienced by the individuals he studied [2]. Today, we know that these are just two of a variety of non-motor complications experienced by those afflicted with PD. Though clinical attention is primarily given to the motor symptoms of PD, non-motor symptoms are an

underappreciated aspect of the disease that have a significant impact on quality of life and are often resistant to treatments directed at motor symptoms [23, 24].

Non-motor symptoms associated with PD are generally classified into one of four groups of related symptoms: neuropsychiatric, autonomic, sensory, or sleep [23, 25]. Neuropsychiatric features often include depression, anxiety, fatigue, apathy, hallucinations, and in later stages of the disease, cognitive decline and dementia [10, 11, 23]. Autonomic abnormalities include features such as gastrointestinal dysfunction (e.g., constipation), orthostatic hypotension, excessive sweating, erectile dysfunction, and drooling [11, 23]. Sensory abnormalities primarily include olfactory dysfunction (i.e., hyposmia), pain, and vision problems [11, 23, 25]. Finally, sleep disturbances include insomnia, excessive daytime sleepiness, and rapid eye movement behavior disorder (RBD), a condition that occurs in approximately one-third of individuals with PD and is characterized by the occurrence of violent dreams accompanied by yelling, punching, kicking, and other violent activity during sleep [11, 23].

Non-motor symptoms are extremely common in individuals with PD. In fact, recent reports indicate that nearly 100% of surveyed patients experienced at least one non-motor symptom, and 100% of those experiencing motor fluctuations also possessed non-motor abnormalities (reviewed in [25]). Despite their prevalence, non-motor symptoms are infrequently treated in routine clinical care of individuals with PD. For example, in a recent report by Baig and colleagues [26], treatment of non-motor abnormalities was provided in only 28% of individuals with PD with moderate to severe depression, 13% of individuals with urinary complications, 3% of cases with erectile dysfunction, and 2% of patients with RBD.

In the ongoing search for effective strategies to provide accurate and early diagnosis of PD, paying attention to non-motor characteristics of the disease is paramount. Non-motor symptoms typically occur years before the characteristic motor abnormalities appear and a clinical diagnosis can be made, and are frequently the presenting clinical feature of PD [25, 27]. Therefore, the presence of non-motor features during the prodromal stage of PD may reflect early pathogenic processes and could have significant implications for understanding the etiology of PD and advancing methods of earlier diagnosis [28].

Neuropathology

Histological pathology in PD is similar to, but distinct from, other forms of parkinsonism. Indeed, though the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) is common among parkinsonian conditions, each individual disorder has its own distinct “fingerprint” of neuropathology that can facilitate the differential diagnosis of PD when assessed postmortem [29]. Interestingly, the underlying pathology of PD itself is heterogeneous and can vary between individuals (e.g., [30]). While there are various important pathological features in PD that may contribute to individual differences in the clinical presentation of the disease, the present discussion is focused on the hallmark neuropathological characteristics of PD, namely the marked loss of SNc DA neurons and Lewy pathology.

The Basal Ganglia: Basic Structure and Function

As a movement disorder, PD involves dysregulation of the basal ganglia system, the normal functioning of which is essential for normal motor learning and function. The basal ganglia are a collection of subcortical nuclei that facilitate the translation of activity in the cortex to directed behavior such as voluntary motor behavior, motor learning, habit learning, and action selection [31, 32]. These nuclei, which have been evolutionarily conserved among vertebrate species for more than 500 million years [33-35], include the following deep brain structures: striatum, globus pallidus, subthalamic nucleus (STN), and SN **(Figure 1.2)**. Briefly, excitatory projections from the overlying cortex serve as the primary input to the basal ganglia system, while the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr) are the primary output nuclei. Additional input to the basal ganglia comes from the thalamus, amygdala, dorsal raphe nucleus (DRN), locus coeruleus, and hippocampus, while the basal ganglia output nuclei send inhibitory projections to brain systems involved in the generation of motor behavior, namely the thalamus, superior colliculus, and pedunculopontine nucleus (PPN) [31]. Under normal resting conditions, the basal ganglia system exerts tonic, inhibitory activity on downstream motor systems. According to the canonical model of basal ganglia function, when this inhibition is diminished via activation of the direct pathway, motor systems are permitted to activate (as described in detail below) [36, 37].

Striatal medium spiny neurons (MSNs) are core components of basal ganglia function. Comprising >95% of the striatal cytoarchitecture [38], MSNs are named for their appearance: they possess medium-sized cell bodies of 12-20 μm in diameter and

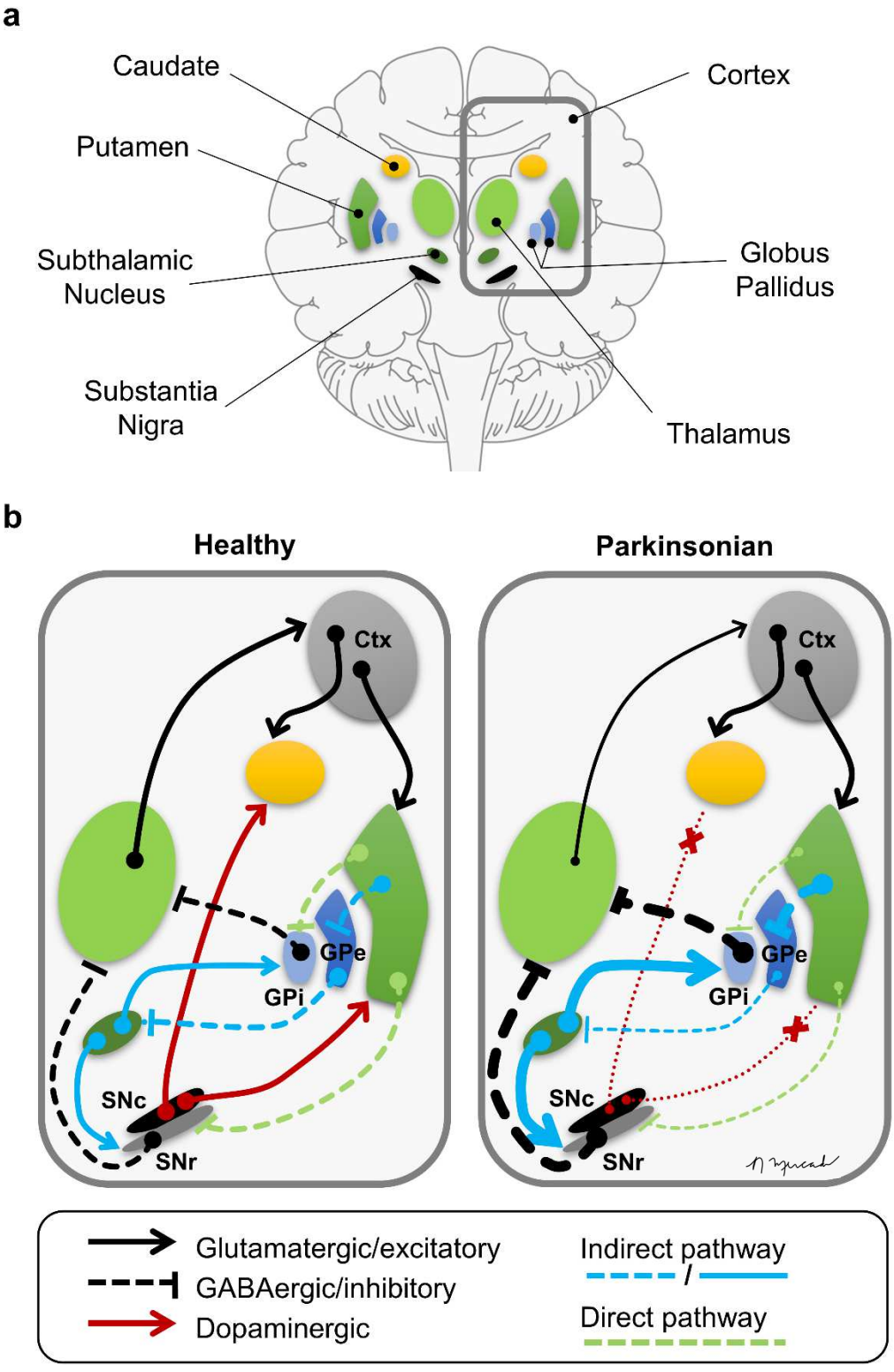

Figure 1.2 Basal ganglia circuitry in health and disease
(a) Coronal view of the basal ganglia in human brain. For simplicity, the two substantia nigra nuclei are combined into one structure. **(b)** Larger schematic depicting basal ganglia shown in panel (a). Basal ganglia circuitry is shown in the normal, healthy state and in

Figure 1.2 (cont'd)
the parkinsonian state. Note that this is a simplified representation of basal ganglia connectivity. Excitatory projections are shown with solid lines and inhibitory projections are shown with dashed lines. Dopaminergic projections are shown in red. The indirect and direct pathways are shown in blue and green, respectively. Loss of dopamine neurons in the parkinsonian state is represented by a large, red "X" and a change from solid to dotted lines. Abbreviations: Ctx, cortex; GPe, globus pallidus external; GPi, globus pallidus internal; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata

dendrites that are densely laden with dendritic spines [31]. Moreover, MSNs receive direct excitatory input from the cortex and thalamus and are the main output neurons of the striatum [31]. As the primary source of striatal output, MSNs are subdivided into two classes based on their efferent projections: those of the direct pathway (dMSNs) and those of the indirect pathway (iMSNs). Though they are largely indistinguishable based on morphology alone, these divergent cell populations express different proteins, project to different basal ganglia nuclei, and have different primary functions [39, 40]. When activated, MSNs of both subtypes release the inhibitory neurotransmitter gamma aminobutyric acid (GABA). As depicted in **(Figure 1.2)**, this induces an inhibitory influence on downstream projection targets. Upon activation of dMSNs, which project directly to GABAergic neurons in the output nuclei (GPi and SNr), the direct pathway stimulates an overall *disinhibition* of tonic inhibitory basal ganglia output, thus facilitating motor behaviors. Conversely, axonal projections from iMSNs terminate in the external segment of the globus pallidus (GPe), which connects iMSNs indirectly to the output nuclei of the basal ganglia **(Figure 1.2)**. Thus, activation of the indirect pathway *strengthens* the inhibitory output of the basal ganglia by reducing the inhibitory influence of downstream nuclei on basal ganglia output. All in all, these opposing systems are thought to provide “counterbalanced” regulation of basal ganglia output whereby they work in concert to select particular motor programs while inhibiting other motor programs during an ongoing motor behavior [31]; however, more recent findings suggest that the direct and indirect pathways are even more interconnected both structurally and functionally than was initially thought [39, 41].

The striatum is the main input target for excitatory projections to the basal ganglia system. Structurally, afferent terminals from the cortex and thalamus form asymmetric, glutamatergic synapses onto striatal MSNs. While corticostriatal synapses are preferentially formed with the heads of dendritic spines located on MSNs (e.g., [42-44]), thalamostriatal terminals synapse onto both dendritic spines and the dendritic shaft of MSNs [45-47]. In addition, dopaminergic afferents from the SNc are a vital source of modulatory input to the striatum. Nigral DA neurons make *en passant* synaptic appositions onto the necks of dendritic spines, where they modulate excitatory input onto the heads of the same dendritic spines (e.g., [42, 48-52]). This integration of excitatory glutamatergic and modulatory dopaminergic input onto striatal dendritic spines is critical for normal motor behavior.

Considering the conservative evolutionary history of the vertebrate basal ganglia, it stands to reason that the coordinated function of this collection of deep brain nuclei is essential for normal motor behavior. Indeed, movement disorders often occur as a consequence of dysregulation of basal ganglia pathways. For example, in PD, striatal DA depletion results in reduced inhibition of the indirect pathway and decreased activation of the direct pathway [53]. Put simply, this leads to a net increase in the inhibitory activity produced by the output nuclei of the basal ganglia, thus resulting in a hypokinetic state (reviewed in [31]).

Nigrostriatal Degeneration and Striatal DA Depletion

Degeneration in the SNc was linked to PD for the first time by Konstantin Nikolaevich Tretiakoff in his doctoral thesis published in 1919 [54], and was confirmed

by the German pathologist, Rolf Hassler, in 1938 [55]. Today, the selective degeneration of midbrain DA neurons located in the SNc, particularly in the ventrolateral tier, is widely recognized as the defining pathological characteristic of PD [56-58]. However, while the canonical view emphasizes the loss of DA cell bodies in the SNc, recent discussion in the field points to the degeneration of nigrostriatal axons as the primary determinant of PD progression and an underappreciated target for new therapeutic approaches [59].

Nigral DA neurons send axon collaterals to all nuclei of the basal ganglia [60-62], but the principal innervation target is the dorsal striatum. In the striatum, DA is released in a tonic manner with occasional transient bursts of phasic activation [63, 64] that are thought to play a role in reinforcement learning and action selection [65]. In normal striatum, tonic release of DA maintains constant striatal DA levels and continuous DA receptor stimulation that is necessary for normal basal ganglia function [66-68]. Accordingly, when SNc DA neurons degenerate in PD, this leads to a significant depletion of physiological striatal DA levels, which in turn introduces pathological alterations to the striatal architecture and significantly alters normal motor function.

The effects of nigral DA depletion on the structure and function of the striatum have been extensively studied. Indeed, over the last several decades, the complex morphological and functional changes that occur in the striatum following DA depletion have been documented in rodent and non-human primate models of PD and in individuals with PD (e.g., [69]). In particular, striatal DA depletion results in dramatic deterioration of dendritic spines and retraction of dendritic processes on MSNs (e.g., [70-77] but see [69] for a comprehensive review). Further, reorganization of

glutamatergic synapses (e.g., [78-80]) and aberrant increases in glutamatergic signaling and MSN excitability (e.g., [78, 81, 82]) have also been observed in the DA-denervated striatum. This pathological influx of glutamatergic neurotransmission is thought to contribute to MSN spine loss as evidence from both *in vitro* and *in vivo* studies indicates that removal of the cortex completely prevents MSN spine loss due to DA depletion [76, 83]. Additional evidence points to a strong causative role for intraspinous L-type CaV1.3 calcium channels, situated near glutamatergic postsynaptic densities in MSN spine heads, in DA-depletion-mediated MSN spine loss [80] and the pathophysiology of LID [84].

Lewy Pathology

Another hallmark pathophysiological feature of PD is the abnormal accumulation of misfolded alpha-synuclein (α-syn) in intracellular inclusions which are collectively referred to as Lewy pathology. This feature is not unique to PD. In fact, aggregation of misfolded proteins (e.g., α-syn, tau, amyloid beta) is now recognized as a common characteristic among neurodegenerative disorders [85]. In particular, PD is one of a subset of neurodegenerative diseases that are characterized by the presence of abnormal α-syn inclusions. Known as synucleinopathies, these conditions primarily include PD, dementia with Lewy bodies, and MSA [86].

In 1997, several landmark findings dramatically changed the landscape of PD research [87]. In the first of these studies, Mihael Polymeropoulos and colleagues provided the first evidence of a direct link between the gene encoding α-syn (*SNCA*) and a familial form of PD with autosomal-dominant inheritance; specifically, that an

alanine-to-threonine substitution (A53T) in *SNCA* was present in a large Italian family and in three unrelated Greek families with inherited PD [88]. Soon thereafter, Maria Grazia Spillantini and colleagues discovered that α-syn is the most abundant protein found in Lewy bodies (LBs) [89]. In the years following these pivotal discoveries, the field has erupted with a multitude of innovative findings about α-syn pathology in PD. Indeed, at the time of this writing, a PubMed search for “alpha-synuclein” and “Parkinson’s disease” since the year 1997 yielded 9,219 results with only sparse publications predating the landmark discoveries by Polymeropoulos, Spillantini, and their teams.

Despite this abundance of new literature, the physiological function of α-syn is not well understood. α-Syn is a 14 kDa protein that is thought to exist predominantly as a stable, unfolded monomer [90]. Under physiological conditions, α-syn is soluble and localized to presynaptic terminals [91]. Based on the presynaptic localization of α-syn (e.g., [92-94]) combined with evidence indicating that α-syn is closely associated with synaptic vesicles (e.g., [94, 95]) and synaptic transmission is impaired when α-syn is overexpressed (e.g., [96-98]) or knocked down (e.g., [99, 100]), this small protein is thought to play a role in maintenance of synaptic function and neurotransmitter release [90, 91]. Interestingly, α-syn was also recently shown to facilitate deoxyribonucleic acid (DNA) repair in the nucleus [101].

While α-syn was confirmed as the main component of Lewy inclusions just over 20 years ago, Lewy pathology itself has been observed in relation to PD for over 100 years [102]. Lewy pathology is a universal term for the abnormal intracytoplasmic inclusions known as LBs and Lewy neurites (LNs) that are now known to be formed by

insoluble multimeric forms of α-syn. Localized in neuronal cell bodies, LBs are composed of fibrillar α-syn and, in the SNc, have been shown by immunoelectron microscopy to present as a dense core of aggregated protein surrounded by a halo of radiating fibrils [103-105]. Interestingly, more recent evidence obtained using cutting-edge microscopy techniques has advanced this classical view by showing that, in addition to α-syn filaments, most LBs are filled with undigested lipid membrane fragments and damaged organelles [106]. Finally, LNs are dystrophic neuronal processes containing fibrillar α-syn and dysmorphic organelles similar to that which is found in LBs [89, 105, 106], though LNs often appear earlier in pathological development than LBs [107-110].

The predominant pathophysiological species of α-syn remains unknown. However, under pathological conditions, α-syn can be influenced by factors such as phosphorylation at serine 129 [111, 112] and oxidative stress [113] to convert from its native unfolded monomeric conformation to oligomeric forms, which are considered a highly toxic intermediate species in the process of α-syn fibrillization and LB formation [90]. These toxic α-syn oligomers can then be released from affected neurons and spread to other neurons [114] where they may nucleate polymerization of endogenous α-syn leading to aggregation and, ultimately, intercellular spread of Lewy pathology [90, 115-120]. This process is considered to be the primary mechanism underlying the spread of Lewy pathology in the brain [90].

In PD, Lewy pathology can be found within surviving nigral DA neurons and in other cell populations throughout the brain [91, 121] and body [9, 122]. Along these lines, Lewy pathology has been hypothesized to progress in a sequential pattern in

sporadic PD, beginning in the peripheral nervous system and gradually spreading through the central nervous system (CNS) in a caudal-to-rostral direction [109]. This model was established in 2003 when Braak and colleagues proposed a novel staging mechanism for monitoring the progression of α-syn deposition with disease progression [109]. In this model, the spread of Lewy pathology was described in six stages, moving from the dorsal motor nucleus of the glossopharyngeal and vagal nerves (stage 1) to the medulla oblongata and the pontine tegmentum (stage 2), then to the amygdala and the SNc (stage 3) before spreading to cortical regions in advanced PD (stages 4-6) [109]. While this staging scheme has been supported by additional groups [123-125], it certainly does not apply to all cases of α-syn pathology [30, 126-129].

Ultimately, the past several decades of research into the pathophysiology of PD have indicated that this progressive disorder is not simply a “single system” disease affecting only the nigrostriatal DA system, but rather a multisystem entity affecting various parts of the nervous system and the body. Therefore, as the field progresses toward better understanding of the pathogenesis of PD and development of new therapeutic targets, it will be crucial to recognize the inherent complexity of this disease as a heterogeneous multisystem malady.

Risk Factors and Etiology

While the etiology of PD is unknown, it is widely acknowledged as a heterogeneous, multifaceted neurodegenerative process involving interactions between genetic susceptibilities and environmental exposures **(Figure 1.3)**. Various genetic and environmental risk factors that are associated with an increased risk of developing PD

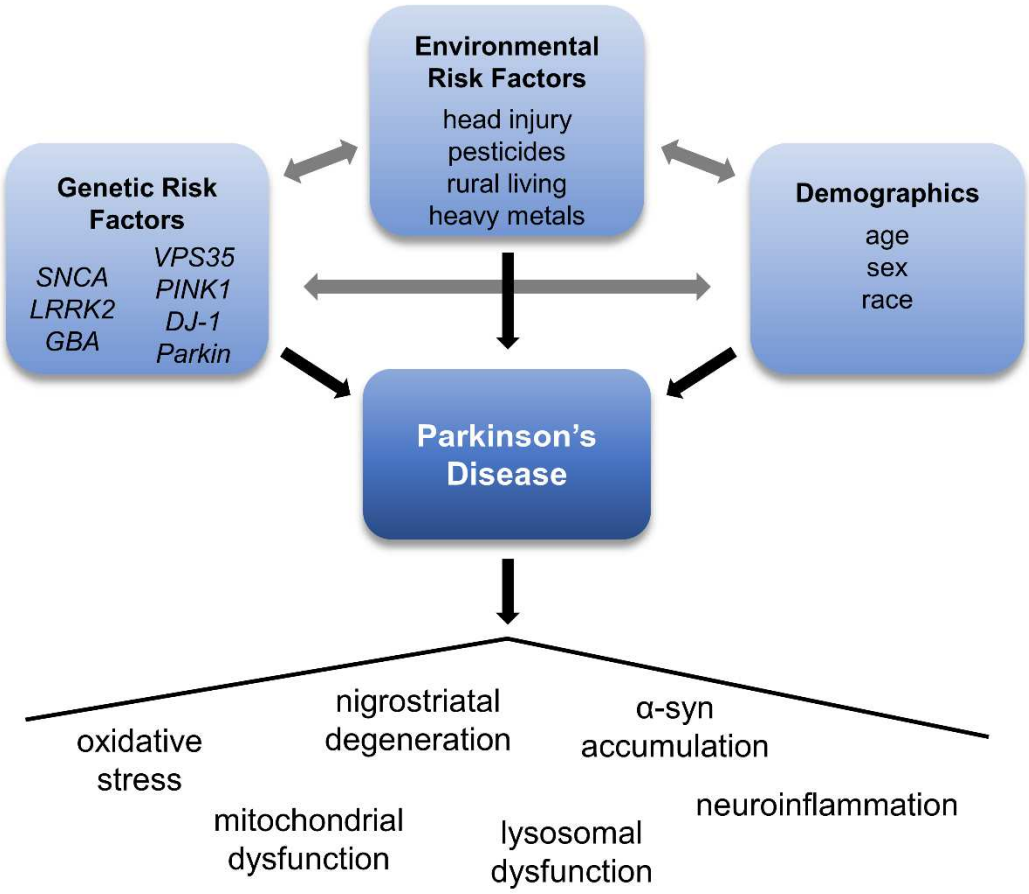

Figure 1.3 Risk factors and etiology of Parkinson's disease
Schematic representation of risk factors that contribute to Parkinson's disease incidence and the primary pathological changes that occur. While the etiology of Parkinson's disease is currently unknown, it is commonly thought to occur as a result of interactions between genetic, environmental, and demographic risk factors. Note: The selection of genetic risk factors provided here is non-exhaustive. Adapted from [130]. Abbreviations: α-syn, α-synuclein

have been identified in large part by genome-wide association studies and longitudinal studies, respectively. Furthermore, advanced age is recognized as the primary risk factor for developing PD and, as discussed below, recent scientific advances have shown that degenerative processes in both aging and PD share common features and may be more interconnected than was previously understood.

Genetic Risk Factors

In the over two decades since the identification of *SNCA* as the first gene linked to the development of PD [88], an assortment of additional causative genes and genetic risk factors have been identified. These mutations are characterized according to genetic penetrance and frequency within the general population, ranging from rare but highly penetrant causative mutations to common mutations with reduced penetrance and lower risk for developing PD [131, 132] **(Figure 1.4)**. Approximately 5-10% of PD cases are of the highly penetrant monogenic form, meaning they are caused by a mutation in a single gene [132]. These include autosomal dominant mutations in genes such as *SNCA*, *LRRK2*, and *VPS35,* and autosomal recessive mutations in genes such as *PINK1*, *DJ-1*, and *Parkin* that are involved in cellular functions including mitochondrial quality control and function, protection against oxidative stress, lysosomal and autophagy regulation, and synaptic vesicle formation and trafficking [132, 133]. In contrast to monogenic forms of PD, most PD cases (i.e., idiopathic PD cases) are considered genetically complex and are thought to result from the combined effects of genetic susceptibility and environmental factors. In total, 26 genetic risk loci have been identified to date by genome-wide association studies [132]. Of these, the greatest

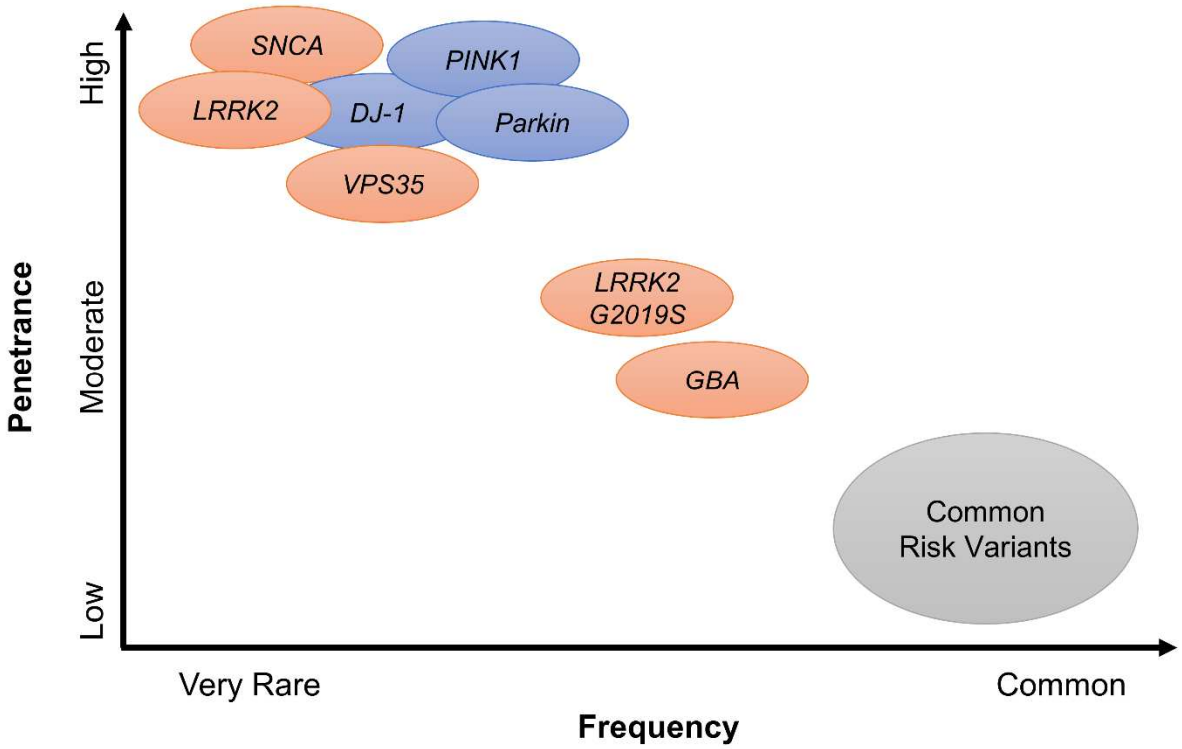

Figure 1.4 Genetic architecture of Parkinson's disease
Illustration of the continuum of genetic variants that contribute to genetic forms of Parkinson's disease. Causative mutations are rare, while variants with reduced penetrance are more common. Colors represent mode of inheritance: dominant (orange) and recessive (blue). Adapted from [131] and [132].

genetic risk factor for PD is mutation in the *GBA* gene, which encodes the lysosomal enzyme glucocerebrosidase and causes the lysosomal storage disorder known as Gaucher disease [9, 132, 133].

Environmental Risk Factors

In 1983, the potent neurotoxic effects of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) metabolite, 1-methyl-4-phenylpyridinium (MPP+), were discovered when a group of young adults developed parkinsonism after intravenous use of heroin contaminated with MPTP [134]. As MPP+ induces mitochondrial toxicity in SNc DA neurons similar to that produced by the pesticides paraquat and rotenone [135], this discovery suggested that exposure to pesticides and other environmental toxins may contribute to the pathogenesis of idiopathic PD. Indeed, environmental and lifestyle exposures have been implicated in the etiology of PD in numerous longitudinal studies examining well-defined cohorts of healthy individuals who were monitored prospectively for the occurrence of new cases of PD [136]. In these studies, the pesticides rotenone and paraquat, which are known to disrupt mitochondrial function and cause oxidative stress, respectively, were positively associated with PD occurrence [136, 137]. Longitudinal studies also provide evidence of increased PD risk with factors such as high consumption of dairy products, melanoma diagnosis, and the use of postmenopausal hormones (reviewed in [136]), while further evidence implicates heavy metal exposure and illicit drug use [138] and exposure to the organochlorine pesticide known as dieldrin [139, 140] as additional risk factors linked to the development of PD. Furthermore, a meta-analysis conducted in 2012 [141] identified additional

environmental risk factors including: prior head injury, rural living, use of beta-blockers, agricultural occupation, and well-water drinking. This study also identified protective factors that are negatively associated with PD risk such as tobacco smoking, coffee consumption, hypertension, use of nonsteroidal anti-inflammatory drugs, use of calcium channel blockers, and alcohol consumption [141].

While it is understood that the etiology of idiopathic PD is likely the result of complex interactions between genetic and environmental risk factors, the specific interactions that affect PD risk are just beginning to be elucidated. For example, thus far, studies have shown that the reduction in PD risk associated with coffee consumption is affected by various single nucleotide polymorphisms (SNPs) [142-144] and that a polymorphic dinucleotide repeat sequence (REP1) in the promoter region of *SNCA* modifies PD risk associated with head injury [145]. Ultimately, a detailed understanding of the various interactions between environmental exposures and genetic susceptibilities that modify risk for PD will benefit efforts to elucidate mechanisms underlying PD pathogenesis, identify potential biomarkers, and develop personalized therapies for PD [9].

Aging: The Primary Risk Factor

In addition to genetic and environmental risk factors, several demographic characteristics are also associated with risk for PD. These include gender, ethnicity, and advanced age [9, 146]. Of particular importance, advanced age is widely recognized as the primary risk factor for PD [147, 148].

Aging is an inevitable and irreversible process characterized by the progressive deterioration of normal physiological states and a concomitant increase in vulnerability to disease and death. As a risk factor, advanced age is not specific to PD; in fact, aging is considered to be the leading risk factor for developing most neurodegenerative diseases including Alzheimer's disease (AD) [148]. On the molecular level, there are multiple "hallmarks of aging" that are widely used in aging research [148]. These hallmark characteristics are grouped as follows based on the order in which they proceed throughout the aging process: (1) *primary hallmarks* such as genomic instability and loss of proteostasis, which are considered the main drivers of aging; (2) *antagonistic hallmarks* such as mitochondrial dysfunction/ mitophagy and cellular senescence, which are meant to be compensatory or antagonistic responses to the primary insult that initially mitigate damage resulting from the primary hallmarks, but often become deleterious; and (3) *integrative hallmarks* including stem cell exhaustion and altered intercellular signaling, which arise with the cumulative deterioration brought on by the primary and antagonistic hallmarks [148, 149]. Importantly, various therapeutic approaches targeting these molecular mechanisms of aging are currently under investigation and being tested clinically for the treatment of neurodegenerative disease [148].

The exact relationship between aging-related degeneration and PD-related degeneration in DA neurons is elusive; in fact, the classical view in the field contends that the two processes are distinct [56, 150-152]. However, recent scientific advances have allowed for more detailed comparison between aging-associated and PD-associated degenerative effects on DA neurons in the SNc, thus altering this classical

view. In PD, the degeneration that occurs in ventrolateral tier SNc DA neurons has recently been postulated to involve the same cellular mechanisms as normal aging, but at an accelerated rate due to interactions between genetic predispositions and environmental exposures (i.e., “stochastic acceleration” hypothesis) [147]. In support of this view, studies using *Caenorhabditis elegans* (*C. elegans*) [153] and human induced pluripotent stem cell (iPSC) [154] models of PD have shown that degenerative changes in DA neurons are not fully expressed without aging (reviewed in detail in [155]), while more recent evidence from postmortem PD brain tissue and a mouse model of PD suggests that cellular senescence may contribute to the development of idiopathic PD [156]. Moreover, degenerative processes in both aging and PD share a number of important biological features such as oxidative damage, inflammation, mitochondrial dysfunction, and impaired proteostasis, among others [147, 155, 157]. Finally, the selective degeneration of DA neurons in the ventrolateral tier of the SNc in PD is thought to result from the inherent vulnerability of this cell population that is exacerbated with advancing age [147] and with gene-environment interactions [147, 155, 158]. For example, evidence from non-human primate studies suggests that in normal aging, ventrolateral tier SNc DA neurons are more prone to ubiquitin-proteasome system dysfunction, α-syn mislocalization, inflammation, and oxidative stress compared to midbrain DA neurons of the VTA and the dorsolateral tier of the SNc [147], thus rendering ventral SNc DA neurons more vulnerable to PD pathogenesis.

Current Therapeutic Strategies

Presently available treatments for the clinical management of PD include pharmacological DA replacement therapies (DRTs) and advanced therapeutic options that are typically considered when dopaminergic medications fall short. Unfortunately, these clinical strategies are used solely for the symptomatic treatment of motor dysfunction associated with PD; they do not slow or reverse underlying disease progression [159, 160] and they do not address the non-motor features of PD, which often have the most significant impact on quality of life for patients [23, 24, 161]. While the urgent need for new and disease-modifying therapies must not be understated, the current landscape of therapeutics for PD has itself undergone substantial growth and improvement in the last half century. Indeed, before the advent of levodopa therapy in the 1960s, antimuscarinics were used with limited success as the standard treatment option for PD [162].

Pharmacotherapies: First-Line Treatment

Current pharmacological therapies for the management of PD are aimed at restoring lost DA tone in the striatum. Of these, levodopa is considered the “gold standard” therapeutic option for persons with PD. As a DA precursor that can cross the blood-brain barrier, the beneficial role of levodopa in the management of PD symptoms was first described in the 1950s and 1960s. In 1957, Swedish neuropharmacologist Arvid Carlsson showed that levodopa ameliorated parkinsonian symptoms in rabbits treated with reserpine (i.e., an alkaloid that depletes stores of catecholamines and serotonin (i.e., 5-hydroxytryptamine or 5-HT) by blocking monoamine transport and

storage in synaptic vesicles) [163]. At the time, Carlsson et al. were unable to determine which catecholamine was responsible for the dramatic recovery of the reserpinized rabbits. However, the following year, the same group showed that DA is a monoamine that is present in the brain and is depleted with reserpine and restored with levodopa treatment ([164] but see also [4] for a detailed review). Two years later, in 1960, Herbert Ehringer and Oleh Hornykiewicz at the University of Vienna published their landmark findings in which they measured DA in postmortem brain tissue from persons with PD and other disorders, and showed for the first time that DA is depleted in the caudate and putamen of PD and postencephalitic parkinsonian brains [4, 165]. Hornykiewicz posited that levodopa might be a useful treatment for PD and went on to examine, with clinical neurologist Walther Birkmayer, the effects of intravenous injections of levodopa in individuals with PD [166]. In this work, published in 1961, Birkmayer and Hornykiewicz showed that low doses of levodopa could transiently alleviate motor symptoms in persons with PD [4, 166], though the troublesome side-effects of nausea and vomiting limited the use of more effective, higher doses. This concern was put to rest in 1967 when Greek neuropharmacologist George Cotzias showed that individuals with PD could tolerate clinically useful high doses of levodopa when started initially on low-dose levodopa that was then gradually increased over time [167]. This landmark discovery revolutionized the treatment of PD, and levodopa is to this day considered the most efficacious therapeutic option for persons with PD.

When compared to other DRTs, levodopa promotes superior motor function recovery in persons with PD as assessed with the Unified Parkinson's Disease Rating Scale (UPDRS) [168]. In standard clinical practice, levodopa is administered with DOPA

decarboxylase inhibitors, usually carbidopa or benserazide, to prevent conversion to DA in the periphery and to minimize adverse side-effects [23, 169]. Levodopa is now available in several formulations, the most well-known of which is carbidopa/levodopa immediate release tablets (Sinemet® in the US) which are taken by mouth 3-4 times per day [170]. Traditionally, clinicians have delayed levodopa therapy in order to postpone the development of motor complications, instead starting new patients on a long-acting DA agonist regimen and introducing levodopa only when DA agonist therapy is no longer sufficient [68]. However, more recent evidence indicates that LID development is a function of disease duration rather than cumulative levodopa exposure, and thus, delaying levodopa treatment is no longer recommended [171].

Additional pharmacological agents that are used in the clinical management of PD motor symptoms include DA agonists and monoamine oxidase type B inhibitors (MAOBIs), while drugs such as catechol-O-methyltransferase inhibitors (COMTIs), anticholinergics, and the N-methyl-D-aspartate (NMDA) receptor antagonist amantadine are used to treat motor fluctuations, tremor, and dyskinesia, respectively [9, 169] **(Figure 1.5)**. While these pharmacological options are clinically useful for the management of PD, they are not without side-effects. DA agonists and levodopa are associated with nausea, daytime somnolence, orthostatic hypotension, hallucinations, and edema [40, 169]. DA agonists are also associated with impulse control disorders such as compulsive spending and binge eating, while levodopa is associated with motor complications (i.e., dyskinesia and motor fluctuations) [9, 40, 169].

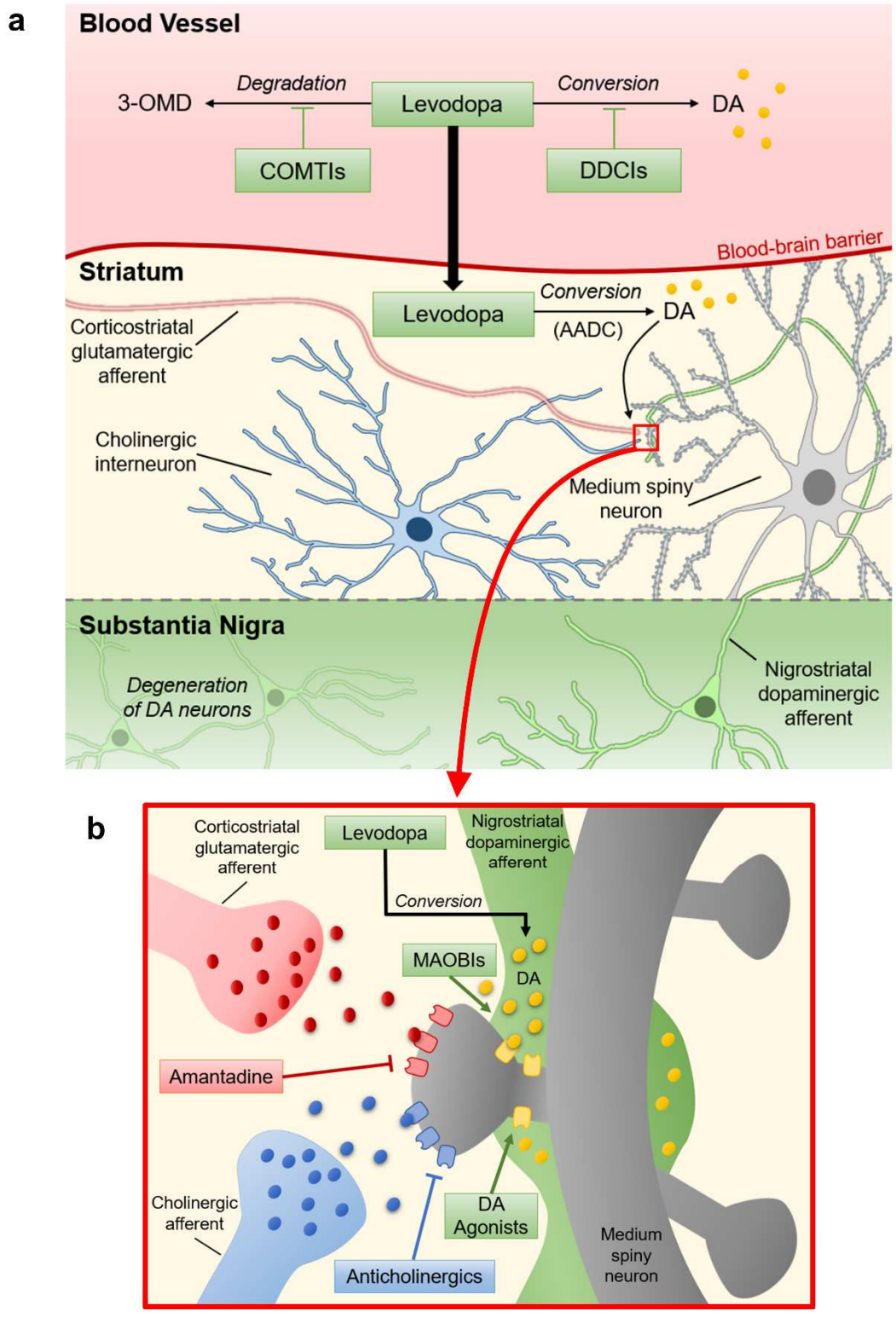

Figure 1.5 Sites of action for Parkinson's disease pharmacotherapies
Illustration of sites of action for medications used clinically for the treatment of motor symptoms in Parkinson's disease. **(a)** Nigrostriatal dopaminergic afferents, corticostriatal

Figure 1.5 (cont'd)
and thalamostriatal glutamatergic afferents, and cholinergic striatal interneurons converge to modulate the activity of striatal medium spiny neurons. Levodopa is transported across the blood-brain barrier and then converted to dopamine by AADC in monoaminergic neurons. In the peripheral circulation, COMTIs and DDCIs block the degradation and conversion of levodopa, respectively. **(b)** In the striatum, levodopa, MAOBIs, and dopamine agonists act on the dopamine system to restore motor function. Anticholinergics are used to treat tremor by blocking acetylcholine receptors, and amantadine is used to treat dyskinesia by blocking N-methyl-D-aspartate (NMDA) receptors. Red receptors represent NMDA glutamate receptors, blue receptors represent acetylcholine receptors, and yellow receptors represent dopamine receptors. Adapted from [169]. Abbreviations: 3-OMD, 3-O-methyldopa; AADC, aromatic amino acid decarboxylase; COMTIs, catechol-O-methyltransferase inhibitors; DA, dopamine; DDCIs, DOPA decarboxylase inhibitors; MAOBIs, monoamine oxidase type B inhibitors

Complications of Long-Term DA Replacement Therapy

Chronic DRT is associated with numerous complications such as motor and non-motor fluctuations, psychosis, and LID [9]. When individuals with PD first begin DRT, they generally experience a "honeymoon period" often lasting 3-5 years and characterized by significant amelioration of motor symptoms and improved quality of life [172]. However, as the disease progresses, the therapeutic window of levodopa efficacy is gradually shortened, while motor symptoms become increasingly resistant to treatment and side-effects associated with chronic levodopa administration such as motor fluctuations and dyskinesia begin to appear. Motor fluctuations involve transition from periods of good antiparkinsonian effect when plasma levodopa concentrations are maximal ("ON-time") to periods of reduced therapeutic efficacy and suboptimal motor control when plasma levodopa concentrations are low ("OFF-time") [9]. Dyskinesias (i.e., LID) are abnormal involuntary movements that occur with chronic levodopa treatment. The vast majority of individuals with PD who receive levodopa treatment eventually develop LID. Specifically, approximately 33-45% of patients develop LID after 4-6 years of levodopa administration [173-175], while after 15 years, this troublesome side-effect may be experienced by as many as 95% of patients (e.g., [176]).

Dyskinesias are generally classified into two categories: (1) *peak-dose dyskinesia*, which occurs during peak plasma levodopa concentrations and typically involves chorea in the upper limbs, neck, and trunk (though dystonia and other motor features may also be present), and (2) *diphasic dyskinesia*, which involves stereotypic leg movements and appears as the levodopa dose-cycle begins and later as the drug wears off [68, 171, 177] **(Figure 1.6)**. Diphasic dyskinesia is less common than

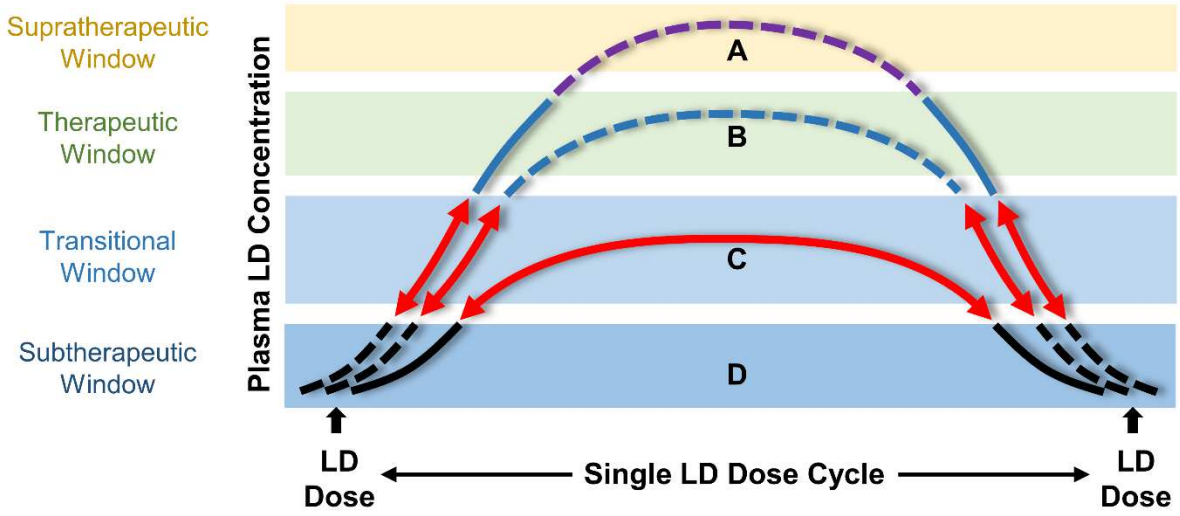

Figure 1.6 Time course of levodopa-induced dyskinesias
Illustration of four different theoretical plasma levodopa levels over the course of a single levodopa dose cycle, and associations with dyskinetic phenotypes. The supratherapeutic window is characterized by peak-dose dyskinesia (A; purple dotted line). The therapeutic window, or the ON-state, is characterized by optimal clinical benefit and no dyskinetic behavior (B; blue lines). During the transitional window, diphasic dyskinesias (red arrows) may appear at the beginning and the end of the levodopa dose cycle. They may also present as longer periods of dyskinetic behavior when the therapeutic window is not reached (C), which are sometimes mistaken for peak-dose dyskinesia. In the subtherapeutic window (D), or the OFF-state, clinical benefits are lost and parkinsonian features reemerge. Adapted from [171]. Abbreviations: LD, levodopa

peak- dose dyskinesia [68]. In fact, diphasic dyskinesia is underrecognized in the field – often being clinically mismanaged as peak-dose dyskinesia – and no clinical trials investigating therapeutic targets for this particular side-effect have been reported to date [171].

In an effort to understand how LID develop, various pathogenic mechanisms have been proposed. These include, for example, pulsatile stimulation of DA receptors, non-physiological release of DA by 5-HT neurons, hyperactive corticostriatal glutamatergic neurotransmission, overstimulation of nicotinic acetylcholine receptors on nigrostriatal DA terminals, and postsynaptic changes in striatal MSNs [40, 171]. In particular, the 90-minute half-life of levodopa is thought to be a key contributor to the development of drug-induced motor complications [168]. Indeed, with current clinical dosing parameters of 3-4x daily, the short half-life of levodopa produces abnormal, large fluctuations in plasma levodopa levels between doses. Brain DA levels mirror these fluctuations in advanced stages of PD, thus leading to non-physiological pulsatile stimulation of DA receptors [68, 168]. Based on these observations, it was hypothesized that continuous DA receptor stimulation might provide more physiological striatal DA tone and therefore reduce the risk of drug-induced motor complications [68]. In support of this hypothesis, substantial evidence from primate animal models and clinical studies has demonstrated that long-acting dopaminergic drugs (e.g., [175, 178, 179]), continuous delivery of DA agonists (e.g., [180-183]), or more frequent dosing of levodopa (e.g., 6x daily [184]) can provide continuous DA stimulation and reduce the occurrence of motor complications.

Additional efforts have been made to lengthen the half-life of levodopa. For example, given that COMT inhibitors block the peripheral metabolism of levodopa (effectively doubling its elimination half-life), a phase 3 clinical trial was initiated in 2004 to investigate the effects of co-administration of the COMT inhibitor, entacapone, and levodopa/carbidopa on the development of dyskinesia in individuals with PD (the STRIDE-PD study: NCT00099268; [68, 174]). In this study, levodopa/carbidopa/entacapone tablets (brand name Stalevo®) were administered 4x daily at 3.5-hour intervals for 134 weeks. Unexpectedly, the STRIDE-PD study failed to meet its primary and secondary endpoints as patients receiving the experimental therapy experienced a shorter time to onset of dyskinesia and higher dyskinesia frequency by the end of the study compared to those who received typical levodopa treatment [174]. Similarly, controlled-release formulations of levodopa did not reduce the risk of motor complications when compared to standard levodopa treatment in a double-blind clinical trial [185]. Interpretation of these unanticipated results suggests that continuous dopaminergic stimulation was not achieved in either study due to suboptimal dosing parameters [68, 186]. For a more detailed description of studies investigating continuous DA stimulation and the current state of the field, the reader is referred to a recently published review [186].

In addition to continuous dopaminergic therapy, various pharmacological and surgical approaches have been used clinically to control levodopa-induced motor complications. However, while there are numerous pharmacological options for minimizing motor fluctuation complications, treatments for dyskinesia are much more limited [9, 159, 187]. Primarily, amantadine and clozapine have been deemed clinically

useful in the management of dyskinesia [159] while a new drug, AV-101, is currently in clinical trials (NCT04147949; [188]). Several surgical options are also clinically useful (discussed in “Advanced Therapies” section below), while additional interventions such as the anticonvulsants levetiracetam and zonisamide [159] and gene therapy [84, 189] are currently under investigation. Finally, long-acting oral levodopa formulations are also under development [186].

Advanced Therapies

In late stages of PD, the appearance of motor complication side-effects associated with pharmacological DRTs introduces an added layer of difficulty to an afflicted individual’s ability to perform simple acts of daily living. When these side-effects become unmanageable and/or significantly debilitative, advanced therapeutic options may be considered. Deep brain stimulation (DBS) emerged in the late 1980s as a surgical option to treat PD motor symptoms while avoiding motor complication side-effects associated with DRTs [190]. The DBS system is composed of surgically implanted electrodes connected via a subcutaneous wire to a pulse generator that is typically placed against the anterior chest wall below the clavicle [190, 191]. The mechanism by which DBS exerts its beneficial effects is not fully understood, though it is generally agreed that therapeutic high frequency stimulation of deep brain structures serves to disrupt or “normalize” pathological neural firing patterns leading to improved motor function [191, 192].

The ventral intermediate nucleus of the thalamus was the first anatomical target for DBS in the management of PD symptoms. Though stimulation of this nucleus was

shown to be clinically useful for controlling tremor, it did not improve other cardinal motor symptoms [190]. Next, the STN and GPi were targeted in an effort to mimic earlier surgical pallidotomy techniques [193] that were known to improve motor function and dyskinesia. To this end, electrical stimulation of the STN and GPi was shown across multiple studies to be successful in ameliorating PD motor symptoms and motor complication side-effects [194-198]. Consequently, bilateral DBS in these basal ganglia structures was approved for the symptomatic treatment of PD by the US Food and Drug Administration (FDA) in 2002 [190]. As of 2019, over 160,000 patients worldwide have undergone DBS for a variety of neurological and non-neurological conditions [192]. In PD, most patients who undergo DBS experience improved motor function and decreased motor complication side-effects (possibly associated with the concurrent decrease in dosage of DA replacement medications) [190, 199, 200].

When matching patients to the right advanced therapy for PD, candidate recipients must be carefully selected based on expectations regarding individualized efficacy (i.e., which therapeutic strategy will benefit this individual the most?). Typically, patients are selected for DBS in advanced disease stages when motor fluctuations and dyskinesias associated with DRT become debilitating and/or unmanageable. In general, the average time to surgery is 10-13 years following PD diagnosis [9], though findings from the EARLYSTIM trial [201] demonstrated that administration of DBS early in the disease course is associated with improved quality of life and substantial clinical benefit. Interestingly, earlier DBS may lead to better long-term symptomatic outcomes [202]. In addition, candidate recipients must be diagnosed with idiopathic PD, as atypical parkinsonian disorders do not typically respond well to advanced therapies [190, 203].

Patients must also be free of dementia or psychosis, and must display a good response to levodopa [190, 203]. Ultimately, a “good” candidate for DBS is generally considered to be an individual with good response to levodopa but with refractory motor complication side-effects or medication-resistant tremor [190].

As DBS is an invasive surgical procedure, there are various adverse side-effects and limitations that must be carefully deliberated by clinicians in conjunction with their patients when considering DBS as a therapeutic strategy. These include risks associated with the surgical procedure (e.g., infection, hemorrhage), the implanted hardware (e.g., wire breakage, device failure), and/or stimulation parameters (e.g., dysarthria, visual disturbances, pain) [190, 203]. An additional limitation lies in the fact that DBS is a resource intensive procedure, the use of which has historically been limited to high-income countries [192]. Furthermore, the battery (if not a rechargeable model) must be surgically replaced every 5-9 years [199] and stimulation of the STN and GPi does not improve gait disturbances and other axial symptoms [190, 192]. In fact, chronic DBS has been described as creating a “new phenotype of PD”: patients who experience improvement in cardinal motor symptoms and motor complication side-effects, but continue to present with gait, speech, and cognitive problems [192].

While there is no doubt that DBS has been unequivocally shown to improve quality of life for persons with PD, it is not the be-all and end-all of therapeutic options for PD and there is much room for improvement. Considering the limitations discussed above, future DBS applications include closed-loop stimulation such as adaptive DBS in which stimulation is delivered and adjusted in response to physiological signals in the host [204], and stimulation of the PPN to target axial motor deficits in patients with

refractory gait freezing [205]. Both applications are still in development with much to be resolved, though a recent meta-analysis has indicated that low-frequency PPN-DBS shows promise for treating gait disorders [206].

Finally, currently available advanced therapies other than DBS that are used in the clinical management of PD include continuous levodopa-carbidopa intestinal gel and continuous subcutaneous apomorphine infusion [186, 199, 203]. When considering advanced therapies, physicians should balance the pros and cons of each approach and should endeavor to choose the approach that best suits the individual patient [199].

Physical Exercise: An Underappreciated Therapeutic Approach?

High-intensity aerobic exercise is emerging as a promising non-pharmacological and non-surgical treatment option for PD [207]. Indeed, regular exercise is widely recognized as an integral component of a healthy lifestyle, and the World Health Organization recommends at least 150 minutes of moderate-intensity aerobic exercise or at least 75 minutes of vigorous-intensity exercise each week for healthy adults [208]. The underlying mechanisms of brain health benefits associated with regular exercise are incompletely understood, though they are thought to involve neurogenesis, angiogenesis, increases in neurotrophic factor signaling, immune system activation, and improved mitochondrial function [209]. In PD, a growing collection of evidence from preclinical animal models [210-214] and clinical studies (e.g., [215, 216] but see also [217] for recent detailed discussion) suggests that high-intensity exercise improves motor symptoms and may even slow disease progression. In addition, epidemiological

studies have indicated that regular exercise of moderate- to vigorous-intensity is associated with a reduced risk of developing PD [218, 219].

Though much research in this regard is considered to be of low methodological quality (discussed in [216, 220]), two recent randomized controlled clinical trials, SPARX (NCT01506479; [215]) and Park-in-Shape (Dutch trial register NTR4743; [216, 221]), have increased confidence in the beneficial effects of exercise for PD. In these studies, researchers examined the effects of a 6-month high-intensity aerobic exercise intervention in *de novo* unmedicated individuals with PD (SPARX) and individuals with mild disease severity who were on DRT and/or receiving DBS (Park-in-Shape). In the SPARX study, which was completed in 2017, the authors concluded that high-intensity treadmill exercise may be a safe and feasible treatment for persons with PD [215]. This finding was confirmed and extended by the Park-in-Shape study in which it was shown that the clinically relevant beneficial effects of high-intensity aerobic exercise extend to patients treated with DRT and/or DBS when placed on a home-based cycling program. However, improvements in the primary outcome measure (changes in UPDRS motor scores) were observed only in the off-state, indicating that levodopa-induced motor benefit may “override” exercise-mediated benefit during the on-state [216]. Ongoing clinical trials (SPARX3: NCT04284436; CYCLE-II: NCT04000360) will extend these promising initial clinical findings by assessing exercise-mediated motor benefit following a 12-month high-intensity aerobic exercise program. Importantly, if positive, these studies will be the first to identify an effective approach for slowing the progression of PD [217]. Whether exercise interventions will also help individuals with more advanced disease must be further investigated.

Despite the consensus regarding the clear benefits of regular exercise for persons with PD, exercise recommendations often lack specificity in terms of frequency, duration, and intensity. Knowing this limitation, a recent review [217] has put forth recommendations based on clinical findings. This “exercise prescription” for PD states that persons with PD should perform aerobic exercise 3x/week in sessions that are 30-40 minutes in duration using 70-85% of maximum heart rate, while “dose” adjustments should be tailored to the individual patient’s needs [217].

A major limitation of prescribing an exercise program is patient adherence. Indeed, older adults often do not meet minimum activity level requirements [222] and persons with PD tend to lead an increasingly sedentary lifestyle hindered by disease-associated barriers [223, 224]. To address this concern and increase patient compliance, van der Kolk et al. made their Park-in-Shape exercise system home-based, coupled with remote supervision and a “gamified” exercise experience enhanced by virtual reality software [216]. Toward this end, a recent review [225] has established an extensive list of evidence-based barriers and motivators to engage in exercise among persons with PD. Importantly, the authors stressed that healthcare professionals play an important role in motivating patients to exercise and that further work is needed to determine the optimal “dose” for maximal therapeutic efficacy.

While a multitude of therapeutic options exist for the clinical management of PD, it must be emphasized that the currently available therapies are principally symptomatic treatments. Indeed, despite decades of rapid advances in our understanding of PD as a complex and heterogeneous neurodegenerative disorder, there are currently no clinically available treatments that have been definitively shown to be capable of slowing

or stopping disease progression. A significant barrier to the development of disease-modifying therapies is the fact that a large portion of SNc DA neurons are lost (estimates range from 30% to 70%) by the time clinically recognizable motor symptoms manifest [59], indicating that the optimal therapeutic window for disease modification may have already passed. This highlights the urgent need for the development of valid and reliable biomarkers for earlier detection of PD so that therapeutic strategies may be applied earlier in the degenerative process. Interestingly, the significance of this dilemma was recognized by Parkinson himself:

> *…there appears to be sufficient reason for hoping that some remedial process may ere long be discovered, by which, at least, the progress of the disease may be stopped... In this period, it is very probable, that remedial means might be employed with success: and even, if unfortunately deferred to a later period, they might then arrest the farther progress of the disease, although the removing of the effects already produced, might be hardly to be expected.* [2]

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CHAPTER 2: THE FUTURE OF PARKINSON'S DISEASE THERAPEUTICS

Experimental Therapies for PD

Currently available therapeutics for PD are reliable and effective clinical tools for alleviating motor abnormalities associated with striatal DA depletion, at least in earlier disease stages. However, despite providing excellent recovery of motor function, these therapies come with multiple notable limitations. Namely, they do not address α-syn pathology or levodopa-resistant non-motor features of PD. Furthermore, they are associated with adverse side-effects and – importantly – they do not slow, stop, or reverse disease progression. Therefore, novel therapeutics for PD should ideally address these unmet clinical needs.

Looking into the future of PD therapeutics, numerous exciting experimental strategies are in various stages of preclinical and clinical development. Emerging therapies currently under investigation are focused on (1) disease modification through modulation of α-syn and other PD-associated pathology, neuroprotection, and/or regeneration/replacement of nigral DA neurons, and (2) treatment of motor symptoms without bothersome side-effects [1]. While numerous novel therapies for PD have been described in detail elsewhere (e.g., [1-4]), descriptions of each experimental approach currently under development for the treatment of PD are beyond the scope of the present discussion. However, several interesting approaches are worth mentioning briefly. First, there is substantial interest in the field for repurposing drugs as potential disease-modifying treatments for PD. As such, various drugs that may prevent pathogenic mechanisms implicated in PD (e.g., α-syn accumulation, mitochondrial dysfunction, neuroinflammation) are under investigation in ongoing clinical trials [1, 3]. A major advantage to this approach is the potential for an expedited path to the clinic, as

repurposed drugs will have already available safety and pharmacokinetic data. Furthermore, there is growing interest in turning attention to non-dopaminergic neurotransmitter systems such as acetylcholine, 5-HT, and glutamate, which are integral to PD symptomatology and the development of motor fluctuations and dyskinesia [1, 3]. Lastly, non-invasive temporal interference and optogenetic approaches to DBS have recently emerged, though they are currently in early stages of development [3].

Targeting α-syn Pathogenicity

Disease-modifying therapies for PD include those designed to target genes and/or proteins associated with α-syn pathology or other underlying pathological processes [2-4]. In particular, experimental approaches that target α-syn pathology are focused on reducing the synthesis and aggregation of α-syn, increasing its clearance, and preventing intercellular spread by enhancing extracellular degradation [5] **(Figure 2.1)**. One approach to decreasing α-syn protein levels is through gene-silencing mechanisms that target α-syn messenger ribonucleic acid (mRNA). To this end, antisense oligonucleotides and RNA interference techniques are in experimental development for the reduction of α-syn synthesis [4, 5]. These therapeutic strategies are still in the preclinical phase for PD, but show promise for reducing α-syn burden (e.g., [6-9]). Next, therapeutic interventions that inhibit α-syn aggregation are also of interest in the field. In this regard, heat shock proteins have shown promise acting as molecular chaperones to assist in the proper folding of α-syn and thus preventing aggregation [10]. In addition, numerous small molecule inhibitors of α-syn assembly have been reported

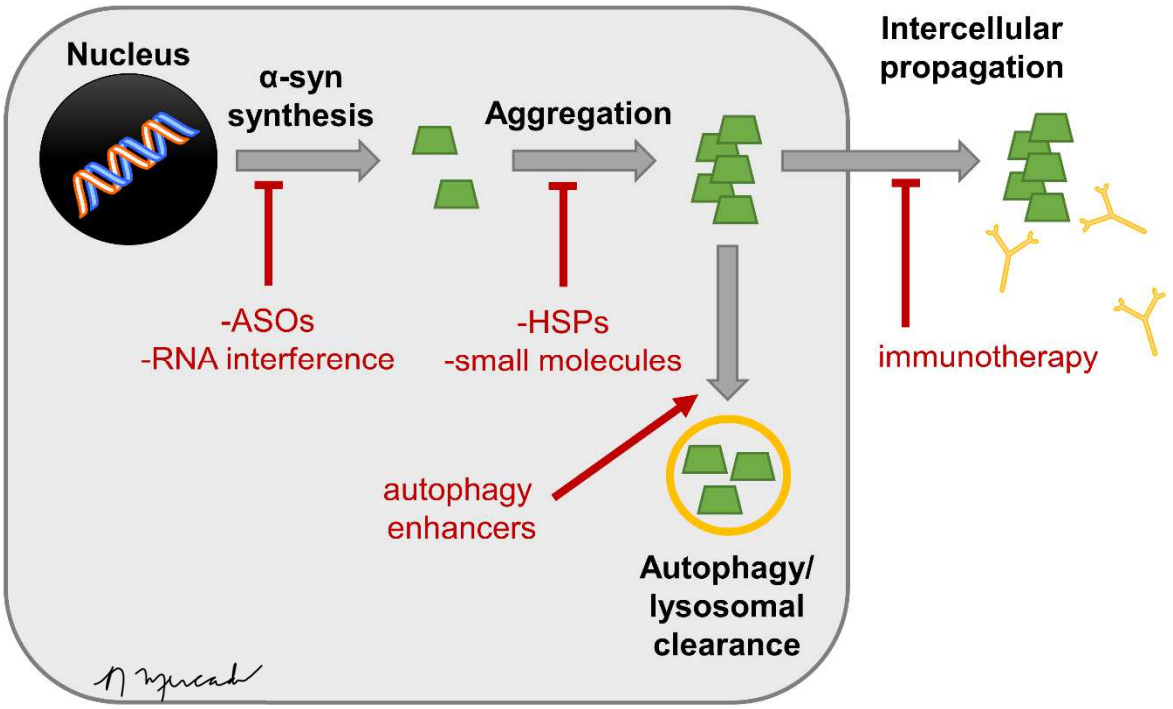

Figure 2.1 Prospective disease-modifying therapies targeting α-synuclein
Illustration of various therapeutic strategies currently in development for Parkinson's disease modification by targeting α-synuclein pathology. Current experimental strategies are aimed at reducing α-synuclein synthesis, inhibiting α-synuclein aggregation, enhancing degradation and clearance of α-synuclein aggregates, and preventing intercellular propagation of α-synuclein. Abbreviations: α-syn, α-synuclein; ASOs, antisense oligonucleotides; HSPs, heat shock proteins; RNA, ribonucleic acid

to reduce the formation of α-syn oligomers and are described elsewhere [5]. Further, to increase degradation and clearance of α-syn aggregates, multiple drugs are being investigated [4, 5]. For example, the drug MSDC-0160 has been shown to enhance autophagy in neurons and to protect DA neurons from MPP+ toxicity, though the effects of this drug on α-syn aggregation remain to be investigated in mammalian models [4].

Finally, immunotherapy is an emerging therapeutic approach that shows promise for reducing intercellular α-syn propagation in PD. Conceptually, immunotherapy involves using antibodies to target and degrade extracellular α-syn to prevent spread of pathological α-syn to neighboring cells. Immunotherapy approaches to neutralize α-syn include passive and active techniques. *Passive immunotherapy* involves parenteral administration of monoclonal antibodies against α-syn, while *active immunotherapy* refers to immunization with protein fragments and/or synthetically-produced peptides to stimulate the host immune system to produce antibodies against α-syn [4]. These techniques have been shown to be effective in animal models (e.g., [11-17]) and early clinical trials in humans are ongoing [2, 5, 18]. Two promising monoclonal antibodies targeted against α-syn that are currently in phase 2 clinical trials are PRX002 (Prothena; NCT03100149) and BIIB054 (Biogen; NCT03318523). A third immunotherapy approach which completed phase 1 clinical trials involves subcutaneous administration of α-syn fragments and α-syn-mimicking-epitopes to induce an active immune response (Affiris; NCT01568099, NCT02267434; [19]). For further discussion of immunotherapies currently in development for PD, the reader is referred to two recent reviews [2, 20].

A major concern associated with these approaches lies in the potentially negative consequences resulting from suppressing the physiological function of α-syn, which

remains incompletely understood [1, 3]. In support of this view, significantly reducing nigral α-syn expression in rat and non-human primate models has been shown to be associated with degeneration of the nigrostriatal system [21, 22]. In addition, there is concern that the antibodies used for immunotherapy might not efficiently penetrate the blood-brain barrier [23]. Perhaps most importantly, it remains to be seen whether immunotherapy approaches will prove to be effective in individuals with PD whose intracellular clearance mechanisms might be too far deteriorated by the time clinically recognizable symptoms begin to appear [23].

Neuroprotective and Regenerative Effects of Neurotrophic Factors

Neurotrophic factors are secreted proteins that support growth, survival, and differentiation of both developing and mature neurons [24]. Based on these well-established functions, there is considerable interest in using neurotrophic factors to promote survival and regeneration of nigral DA neurons that degenerate in PD. To this end, the neuroprotective and regenerative effects of neurotrophic factors on nigral DA neurons have been extensively investigated. The best-studied neurotrophic factors in the context of PD are glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), brain-derived neurotrophic factor (BDNF), cerebral dopamine neurotrophic factor (CDNF), and mesencephalic astrocyte-derived neurotrophic factor (MANF) [25].

GDNF and NRTN

The GDNF family ligands include GDNF, NRTN, artemin (ARTN), and persephin (PSPN) [26]. Of these, GDNF and NRTN have been intensively investigated as potential neuroprotective agents for nigral DA neurons. Indeed, GDNF was isolated for the first

time from cultured rat glial cell lines in 1993 as a factor that enhanced survival of midbrain DA neurons [27]. In toxin-induced animal models of PD, both GDNF and NRTN consistently demonstrated successful neuroprotection and regeneration of nigral DA neurons following intraventricular, intranigral, and/or intrastriatal injection (e.g., [28-36]). As there was much excitement surrounding the neuroprotective potential of GDNF, the first clinical trial investigating the effects of GDNF in individuals with moderately advanced idiopathic PD was initiated in 1996 [37] – only three years following its discovery [38]. However, in this initial double-blind randomized control trial, intraventricular administration of GDNF did not improve parkinsonian motor symptoms compared to placebo (as assessed using UPDRS) [37, 38]. Furthermore, this method of administration was associated with adverse side-effects such as anorexia, weight loss, nausea, and hyponatremia [37, 38]. Subsequent postmortem evaluation demonstrated that GDNF failed to diffuse from the ventricular cavity and, as such, there was no evidence of significant regeneration of nigral DA neurons [38, 39]. Based on these findings, subsequent open-label clinical trials evaluated continuous administration of GDNF directly to the putamen via subcutaneous pumps, and were met with marked success (e.g., improvement in UPDRS motor sub-scores and activities of daily living sub-scores, reduced LID, increased [(18)F]dopamine uptake) [40, 41]. The encouraging findings from these studies led to the initiation of double blind, placebo-controlled clinical trials investigating continuous [42] and intermittent [43, 44] GDNF infusion into the putamen. Unfortunately, these double-blind studies failed to meet their primary and secondary clinical endpoints, though they did demonstrate that GDNF infusion is clinically well-tolerated and safe [25, 38]. Finally, an open-label gene therapy study

evaluating viral vector-mediated delivery of the GDNF transgene to the putamen of individuals with PD is ongoing (NCT01621581; [45]).

Clinical trials involving NRTN treatment have involved a gene therapy approach rather than intracerebral protein infusion. While the initial open-label phase 1 trial offered promising results following intraputaminal delivery of the NRTN transgene [46], a subsequent double-blind clinical trial demonstrated that viral vector-mediated delivery of NRTN to the putamen did not provide significant clinical improvement over control groups, despite a modest, focalized induction of tyrosine hydroxylase expression (TH; rate-limiting enzyme in DA synthesis) in patients examined postmortem [47, 48]. In addition, postmortem evidence indicated that retrograde transport of NRTN to the SNc was suboptimal (likely due to the severity of nigrostriatal axonopathy in advanced PD stages), with only ~5% of SNc DA neurons expressing NRTN at a time point four years post-surgery [48]. Consequently, the next double-blind clinical trial investigated the effects of bilateral NRTN transgene delivery to both the putamen and SNc, though this study also found no differences between treatment groups in the primary clinical endpoint (OFF-time UPDRS motor scores) and most secondary endpoints [49]. Together, the evidence from these clinical studies is a far cry from the positive results observed in preclinical animal models, and ultimately, the future of intracerebral GDNF delivery as a treatment for PD is uncertain. Considerations for future clinical trials involving GDNF and NRTN were discussed in a recently published workshop summary [38].

BDNF

BDNF is well-known for its function both as a pro-survival factor and a potent modulator of neurotransmission and synaptic plasticity. BDNF promotes survival and function of nigral DA neurons both *in vitro* and in animal models of PD (e.g., [50-53]). Further, BDNF mRNA and protein are reduced in the SNc in individuals with PD [54, 55]. Taken together, this evidence suggests that BDNF may be useful for the development of neuroprotective strategies for PD. However, while BDNF administered *prior to* nigrostriatal lesioning has indeed been shown to prevent nigral cell loss in animal models of PD, this was generally not the case when BDNF was administered *after* inducing parkinsonism [56]. As such, administering BDNF to persons with late-stage PD is not supported by preclinical evidence, and thus, BDNF has not been clinically tested for the treatment of PD. For further discussion of BDNF biology and its role in aging and PD, please refer to the detailed BDNF section below.

CDNF and MANF

CDNF and MANF are members of a novel, evolutionarily conserved family of "unconventional" neurotrophic factors [2, 25, 57, 58]. In 2007, Lindholm et al. [58] described CDNF as a novel neurotrophic factor, and showed that it prevented degeneration of DA neurons in rats treated with the selective neurotoxin, 6-hydroxydopamine (6-OHDA). Additional preclinical studies in rodents and non-human primates produced supportive evidence indicating that CDNF promotes functional recovery while protecting nigral DA neurons from toxin-induced neurodegeneration [59-62]. A phase 1/2 clinical trial investigating the safety and tolerability (primary outcome measure) and therapeutic efficacy (secondary outcome measure) of monthly

intraputaminal CDNF infusions in 15 people with advanced PD was recently completed with encouraging preliminary results indicating that the treatment is safe and well-tolerated (NCT03295786; [63]). Finally, MANF was first described in 2003 and, like CDNF, has been shown to exert restorative and neuroprotective effects on DA neurons in *C. elegans* and rat models of PD [57, 64-66]. Clinical trials evaluating the effects of MANF in persons with PD have yet to be initiated.

While theoretically promising, there are various limitations associated with the use of neurotrophic factors as therapeutic strategies for PD. Most notably, neurotrophic factors do not cross the blood-brain barrier and thus must be intracerebrally infused, which is accomplished using invasive neurosurgery techniques. In addition, though GDNF has proven successful in toxin-induced animal models of PD, it was fairly ineffective in cases of severe lesions inducing >80% loss of DA neurons, and failed to protect DA neurons from cell death induced by α-syn overexpression [38, 67, 68].

Gene Therapy: A Multifaceted Approach

Gene therapy is a versatile experimental technique in which non-replicating viral vectors such as adeno-associated virus (AAV) or lentivirus are used to replace, modify, or silence targeted genes [3]. In PD, the utility of experimental gene therapies is threefold: clinical trials are underway for (1) DA restoration, (2) basal ganglia network modulation, and (3) delivery of neurotrophic factors.

To therapeutically restore lost DA function and improve PD motor symptoms, several experimental gene therapy approaches are currently in the clinical stages of development. First, an AAV therapy containing the gene for aromatic L-amino acid

decarboxylase (AADC), the enzyme that is responsible for converting levodopa to DA, is currently in phase 1 [69] and phase 2 (NCT03562494) clinical trials. This approach involves intraputaminal injection of the therapeutic vector to induce AADC expression in striatal MSNs, providing a resource for levodopa to DA conversion in the absence of nigral DA terminals. This experimental approach has yielded encouraging results in the phase 1 trial [1]. Second, a lentivirus vector therapy containing the genes for TH, AADC, and the enzyme GTP cyclohydrolase 1 (rate-limiting enzyme in tetrahydrobiopterin synthesis, which is required for DA and 5-HT synthesis) was well-tolerated in an open-label phase 1 clinical trial (OXB-101 or ProSavin®; [1, 70]). Though OXB-101 treatment produced improvements in OFF-time UPDRS motor scores at 12 months, the level of improvement was not sufficient to make this therapy competitive with already available treatments. An improved version of this lentivirus gene therapy approach, OXB-102, is currently being evaluated in a preliminary clinical trial (the AXO-Lenti-PD or SUNRISE-PD trial; NCT03720418).

Another symptomatic gene therapy that has reached clinical development targets basal ganglia circuit function [3]. This gene therapy involves AAV-mediated transfer of glutamic acid decarboxylase (GAD) to the STN to enhance GABAergic neurotransmission from the STN [3]. Following successful amelioration of bradykinesia, tremor, and gross motor skills in parkinsonian non-human primates [71], this therapy progressed to clinical trials for PD and has produced favorable outcomes, effectively reducing UPDRS motor scores and LID duration, and proving to be safe and well-tolerated [3, 72-74].

Maintaining its reputation as a versatile technique, gene therapy has also been used to target common side-effects of long-term DRT. Notably, recent preclinical studies have utilized viral vector-mediated technology to prevent and markedly reduce LID in a parkinsonian rat model, interestingly by targeting separate underlying pathogenic mechanisms [75, 76]. A gene therapy approach is also being investigated for targeted delivery of neurotrophic factors to the nigrostriatal system, as discussed in detail above. While being a promising and powerful approach, it is important to note that gene therapy is not without limitations. Specifically, it is generally irreversible and it can be difficult to restrict and regulate the amount of therapy that is delivered [3].

Restorative Potential of Cell-Based Therapies

The practice of neural grafting has a long and complex history spanning 130 years. In the 1980s, cell transplantation was attempted clinically for the first time in persons with PD and has since been incorporated into clinical trials for a variety of additional neurological disorders such as Huntington's disease, multiple sclerosis, and schizophrenia [77]. While the field has benefited from an abundance of new literature since the 1980s addressing numerous research objectives through the refinement and optimization of standard operating protocols and the introduction of cutting-edge techniques (e.g., novel transsynaptic tracing methods to study graft-host connectivity and circuit reconstruction with increased speed and precision [78]), it is important to note that this work was preceded by decades of research that paved the way for modern cell transplantation techniques. Indeed, the road to the first clinical trials for cell transplantation therapy was fraught with significant technical hurdles and a considerable

level of skepticism from the scientific community [77]. Therefore, an overarching discussion of neural grafting for PD is not complete without revisiting its humble, yet fascinating, origins.

History of Neural Transplantation

The concept of neural transplantation was experimentally investigated for the first time by W. Gilman Thompson, an American physician, in 1890 [79]. In this innovative work, Thompson attempted to exchange neocortical tissue between adult cats and dogs, citing inspiration from his work on cerebral localization of the occipital lobe in cats, dogs, and non-human primates [80]:

> *It occurred to me recently, while studying cerebral localization in the lower animals, that it would be interesting to graft a piece of brain tissue from one side of a dog's brain to the other, or from one animal's brain into another's, and study its vitality. Of course, I had no expectation of being able to restore abolished function by the operation, but the question of vitality of the brain tissue and the course of its degeneration is a subject which is of very wide interest.* [79]

In one experiment, Thompson transplanted occipital tissue from an adult cat into the corresponding brain region of a dog. The host dog was sacrificed seven weeks following the transplant procedure and the grafted tissue was examined microscopically. Though Thompson seemed rather optimistic in observing that the transplanted tissue appeared to be alive and a narrow band of connective tissue had formed at the graft-host interface, it is more likely that the transplanted tissue had died and left in its place neuron-free graft remnants, host-derived immune cells, and/or scar tissue [77, 81].

Thompson's work was the first of a collection of key experiments in the early history of cell transplantation studies in mammals **(Table 2.1)**. Notably, the first attempt to graft embryonic (non-neuronal) tissues into the brain was performed by Del Conte in

Year	Author(s)	Location	Main Contribution
1890	W. G. Thompson	New York, NY, USA	First attempt to graft adult CNS to brain
1907	G. Del Conte	Naples, Italy	First attempt to graft embryonic tissues to brain
1909	W. Ranson	Chicago, IL, USA	First successful grafting of spinal ganglia to brain
1911	F. Tello	Madrid, Spain	First successful grafting of peripheral nerve to brain
1917	E. Dunn	Chicago, IL, USA	First successful grafting of neonatal CNS to neonatal brain
1924	G. Faldino	Pisa, Italy	First successful grafting of fetal CNS to anterior eye chamber
1940	W. E. Le Gros Clark	Oxford, UK	First successful grafting of fetal CNS to neonatal brain
1971	G. Das & J. Altman	Lafayette, IN, USA	First demonstration of [^{3}H]thymidine-labeled grafted neonatal tissue migrating into host brain
1971	A. Björklund & U. Stenevi	Lund, Sweden	Demonstration of catecholamine neuron growth into smooth muscle grafts in mesencephalon
1972	L. Olson & Å. Seiger	Stockholm, Sweden	First report of reliable grafting to anterior eye chamber
1976	U. Stenevi et al.	Lund, Sweden	First description of conditions for reliable engraftment of embryonic CNS into adult brain
1979	M. Perlow et al.	Washington D.C., USA	First report of grafted DA neurons providing functional benefit in parkinsonian rats

Table 2.1 Early history of neural grafting in the mammalian CNS
Please note that this list is non-exhaustive. Adapted from [81]. Abbreviations: CNS, central nervous system; DA, dopamine

1907 [81, 82]. A decade later, in 1917, Elizabeth Dunn completed the first successful grafting of cortical tissue between neonatal rat pup littermates, though the survival rate was poor [81, 83]. Despite her low success rate, Dunn's work is generally credited as the first clear evidence of survival of CNS tissue transplanted into the brain [77]. This important milestone was followed by the work of W.E. Le Gros Clark, who was the first to successfully graft *embryonic* cortical tissue into the neonatal brain and showed that transplanted embryonic cortical tissue can survive and differentiate into mature neurons in the host brain [77, 84].

The middle decades of the twentieth century yielded little progress in neural transplantation research, owing in part to the persistent and widely-held belief among scientists at the time that neural regeneration cannot occur in the adult mammalian CNS [77]. The lack of progress was also partially due to insufficient technology to address the sophisticated experimental questions of this type [81]. However, following the development of new techniques such as electron microscopy, autoradiographic labeling of dividing cells, and improved histochemical methods, clear evidence of regenerative growth and neurogenesis in the adult mammalian CNS began to appear in the 1960s and 1970s [77].

Following these important challenges to the previous dogma, the field was reinvigorated, ushering in a new era characterized by a greater acceptance of neural grafting as a viable strategy for functional brain repair. Indeed, a pivotal study conducted around this time by Gopal Das and Joseph Altman [85, 86] is credited for launching the "modern era" of neural transplantation [77]. Using novel technology developed in their lab, Das and Altman injected neonatal rat pups with [^{3}H]thymidine to

label proliferating cells in the cerebellar cortex. Slabs of labeled tissue were then transplanted into the cerebella of unlabeled host rats of the same age. Remarkably, by 10-16 days following transplantation, labeled cells had migrated into the host cerebellum and differentiated into the appropriate neuronal phenotypes [85]. This impactful study was followed by a second defining experiment of the modern era of cell transplantation in which Lars Olson and Åke Seiger used fluorescent histochemical labeling methods to study the survival, differentiation, and fiber outgrowth of dopaminergic, serotonergic, and noradrenergic neurons grafted embryonically into the anterior eye chamber [77, 87]. These observations, combined with subsequent work by Olson and colleagues [88, 89], have allowed researchers to determine the optimal developmental ages for survival and growth of neural tissues following transplantation and form the foundation on which current cell transplantation strategies have been developed [77]. Other notable studies from this period include work by the Swedish group at the University of Lund demonstrating the growth capacity of central catecholamine neurons [90] and providing the first detailed description of conditions for reliable survival and growth of embryonic monoamine neurons transplanted into the adult brain [91] **(Table 2.1)**.

For a comprehensive discussion of the history of neural transplantation and the notable individuals who contributed to the development of the field as we know it, the reader is referred to two excellent reviews [77, 81].

Preclinical Experiments and Early Clinical Trials

The first evidence that nigral grafts could provide functional benefit in a rat model of PD were published by two independent groups in 1979 [92-94]. Interestingly, in a

recent commentary written by Anders Björklund and Olle Lindvall [95], the Swedish investigators recalled their surprise upon discovering that their report [93] was rejected by *Science* as it was too similar to a report by Mark Perlow and Richard Wyatt, in collaboration with the Swedish group in Stockholm (Karolinska Institute), that had already been published in the same journal earlier that year [92]. Despite their similarity, the two studies employed distinct methods to investigate the same question, ultimately suggesting entirely different functional mechanisms [95]. Specifically, Björklund and Stenevi [93] transplanted dissected fetal nigral tissue into a cavity created in the cerebral cortex overlying the striatum, whereas Perlow et al. [92] opted for transplantation into the lateral ventricle adjacent to the striatum. While evidence from the former suggested that grafted fetal DA neurons functioned through reestablishment of “nigrostriatal” synaptic connectivity, evidence from the latter suggested that the observed behavioral improvements resulted from diffusion of DA from the ventricle in the absence of graft-host connectivity [95]. Shortly thereafter, a transplantation technique involving the stereotaxic injection of dissociated cell suspensions directly to the striatum was developed and refined to achieve widespread reinnervation of the striatal target, and consequently, marked recovery of drug-induced and spontaneous sensorimotor behavior (e.g., [96-99]).

At this time, interest in translating neural grafting for clinical application was growing quickly. In the early 1980s, W.J. Freed et al. showed that grafts containing catecholamine-secreting adrenal chromaffin cells could reduce apomorphine-induced rotational behavior in a parkinsonian rat model [100]. Inspired by these encouraging findings, Backlund et al. initiated the first cell transplantation clinical trials for PD,

engrafting the caudate nucleus of two patients in 1982 and 1983 with tissue acquired from the patients' own adrenal glands [101]. In 1985, two additional individuals with PD received adrenal medullary autografts in the putamen [102]. However, the functional benefit experienced by these patients was transient and mild [102]. Furthermore, though a group in Mexico City reported dramatic clinical improvement in two clinical case studies using a different surgical approach involving adrenal medulla autografts into the medial wall of the caudate nucleus [103], subsequent systematic multicenter comparisons in the US were unable to replicate these findings and instead found that this procedure was associated with significant levels of mortality and morbidity [104].

Following the underwhelming outcome of adrenal autograft clinical trials, the Swedish research teams turned their attention to human fetal nigral tissue. Importantly, taking into account ethical concerns surrounding the use of cells from aborted human fetuses, ethical guidelines for fetal tissue transplantation were adopted by the Swedish Society of Medicine in 1986 [77, 95]. In collaboration with the Karolinska researchers, the Lund group carried out the first preclinical transplantation experiments using human fetal tissue from the developing ventral mesencephalon (VM), which was grafted into a parkinsonian rat model, in the late 1980s [95, 105-107]. Encouragingly, these pioneering preclinical experiments demonstrated that DA neurons from 6- to 9-week-old aborted human fetal tissue can not only survive, but also reinnervate the striatum and provide functional benefit when transplanted into the DA-depleted striatum of parkinsonian rats.

Human fetal tissue was transplanted into patients with severe PD for the first time in 1987 [95, 108], less than a decade following the first evidence that nigral grafts could

provide functional benefit in a rat model of PD. In this open-label study, the two patients received tissue from four fetal donors, deposited unilaterally into both the caudate and the putamen. Though the surgeries were completed without issue, at a 6-month follow-up assessment, it was determined that the patients experienced only minimal functional benefit and displayed insufficient evidence of graft survival as assessed with PET scanning of striatal ^{18}F-fluorodopa uptake [95, 108].

Determined to obtain a better outcome from following studies, researchers set out to improve the transplantation procedure. The next two individuals underwent transplantation surgery in 1989, this time with a thinner implantation cannula and an increased number of unilateral transplantation sites in the putamen [95]. Both patients experienced significant functional benefit within three months post-surgery and PET scanning indicated that the grafted cells had survived and restored DA synthesis [109]. At a 1-year follow-up, it was noted that the patients experienced sustained clinical benefit, namely reduced OFF-time duration and fewer daily OFF periods, with improved OFF-time motor function [110]. At 10 years post-transplantation, PET imaging demonstrated that DA release in the putamen was restored to normal levels when examined in one of the patients [111].

Following the marked success of the second clinical study, 18 additional individuals with PD were transplanted in the Lund program (i.e., open-label clinical trials) over the course of a decade [95]. The functional benefit experienced by the majority of transplanted patients was remarkable [112]. Similar results were also reported in open-label clinical trials from other centers [113-117].

In the mid-1990s, the National Institutes of Health (NIH) funded two double-blind placebo-controlled studies investigating neural grafting for PD in the US [118, 119]. Unfortunately, neither study demonstrated sufficient evidence of long-term graft survival or significant functional benefit. However, when data from these clinical studies were sorted by patient age or disease severity, younger patients [118] and those with milder PD pathology [119] showed significant clinical improvement compared to the sham group. Perhaps the most important outcome from these placebo-controlled clinical trials is the unexpected occurrence of a novel dyskinetic behavior that appeared during OFF periods (e.g., when LID behavior is absent) in as many as 56.5% [119] of patients. Due to the overall lack of clinical benefit and the troublesome occurrence of new graft-induced dyskinesias (GID) that persisted even when levodopa was withdrawn, neural grafting clinical trials for PD were suspended worldwide. The resulting disappointment experienced by the field at this time was conveyed by Björklund and Lindvall in their recent commentary:

> *The negative outcome of these two trials was a major set-back for the whole field and convinced many of our colleagues, particularly in the USA, that the cell transplantation approach had reached a dead-end. The wide media attention and the fact that these placebo-controlled trials were initiated and sponsored by the NIH helped to convey the impression that the outcome provided an authoritative and definitive answer: cell transplantation in PD does not work.* [95]

An Unexpected Side-Effect: Addressing GID

The appearance of GID in a significant subset of grafted individuals with PD warranted considerable concern from the scientific and medical communities. The resulting worldwide moratorium was defined by an international effort to understand and resolve this unexpected and adverse side-effect. The occurrence of GID was not unique

to the placebo-controlled trials; one retrospective analysis of 14 patients from open-label studies who were followed long-term after transplantation demonstrated that mild-to-moderate OFF-time dyskinesias appeared in these patients and persisted even after levodopa was withdrawn [120].

OFF-time dyskinesias (i.e., GID) vary in clinical appearance and severity. In some patients, GID appear similar to peak-dose LID involving choreic movements in the upper body [118], while in others, GID manifest as repetitive, stereotypic movements in the lower extremities, similar to diphasic LID [119-121]. Furthermore, while many cases of GID are mild, several patients involved in the NIH-sponsored clinical trials experienced GID so severe that subthalamic DBS was required to alleviate this side-effect (reply by Freed et al. in [122]; [123]). Interestingly, patients who developed GID had also been burdened with LID prior to transplantation surgery [123].

The pathogenesis of GID remains unclear, though several mechanisms have been proposed. Initially, Freed et al. [118] suggested that fiber overgrowth from the graft may cause an excess of DA (i.e., “hot spots”), leading to GID development. Consistent with this hypothesis, a subsequent imaging study showed that ^{18}F-fluorodopa uptake was significantly increased in patients who experienced GID compared to grafted patients who did not develop GID [124]. However, the observed increases in ^{18}F-fluorodopa uptake were localized to specific regions within the putamen, leading the authors of this follow-up study to suggest that uneven DA reinnervation may instead play a key role in the development of GID. This hypothesis is supported by evidence from a rat model of GID indicating that focal “hot spot” but not widespread grafts are associated with GID development [125]. In contrast to these findings, Hagell et al. [120]

reported no differences in striatal DA reinnervation between grafted patients who developed GID and those who did not, and Olanow et al. [119] similarly found no correlation between GID occurrence and striatal ^{18}F-fluorodopa uptake (i.e., DA reinnervation). All in all, evidence in support of the “fiber overgrowth” theory is minimal [126], while evidence of a pathogenic role for uneven DA reinnervation is supported by both preclinical and clinical studies.

As patients who developed GID had experienced significant LID preoperatively, it has also been proposed that the extent of preoperative LID may contribute to GID development [127]. However, Hagell and colleagues [120] found no correlation between preoperative LID severity and the likelihood of the patient developing GID postoperatively. Furthermore, subsequent attempts to replicate this phenomenon in animal models have provided mixed results. While one group showed that the induction of GID in a parkinsonian rat model was associated with chronic levodopa administration and the development of LID prior to grafting [128, 129], another group using a separate rodent GID model demonstrated that GID occurred in grafted, parkinsonian rats regardless of prior levodopa exposure [130]. Notably, rodent models of GID have been criticized for their requirement for pharmacological stimulation to elicit abnormal graft-derived behaviors, which suggests that these models are fundamentally different from what has been observed clinically [131, 132]. Thus, a recent study by Kordower et al. [131] sought to investigate GID using (levodopa-induced) dyskinetic non-human primates grafted with fetal nigral tissue. Interestingly, the monkeys did not develop OFF-time dyskinesias at any time during the 18-month observation period [131]. Whether this finding is a reflection of more controlled grafting methods, limitations of the animal

model in replicating the human condition, the intentional absence of non-DA neurons in the grafts, and/or a subdued immune response in comparison to clinical studies remains unclear.

A third hypothesis contends that abnormal patterns of graft-host synaptic connectivity might contribute to GID pathogenesis [119, 126, 132]. Indeed, collective ultrastructural evidence from postmortem clinical evaluations [133] and grafted, parkinsonian animal models [134-136] has demonstrated that grafted DA neurons establish abnormal synaptic connections with the host. For example, Freund and colleagues [134] used ultrastructural and immunostaining methods in a 6-OHDA rat model to show that grafted DA neurons make unusual connections with large cell bodies (presumed to be striatal cholinergic interneurons), forming “dense pericellular ‘baskets’” that were not observed in the normal striatum or the intact contralateral striatum of grafted rodents. In addition, Soderstrom et al. [135] identified alterations in the distribution of synaptic contacts made by/onto grafted DA neurons in the lesioned hemisphere of parkinsonian rats. Specifically, they observed a shift in synaptic targets of grafted DA neurons (i.e., higher proportion of axodendritic and axosomatic connections compared to the axospinous connections typical of nigral DA neurons) and a notable increase in atypical asymmetric synaptic specializations made *by* grafted DA neurons, as well as unlabeled asymmetric inputs *onto* grafted DA neurons. Importantly, these asymmetric synapses onto grafted DA neurons were found to be positively correlated with GID, while a positive trend was observed between GID and asymmetric specializations made by grafted DA neurons [135]. These findings are reminiscent of observations made not only in parkinsonian non-human primates [136], but also in

postmortem tissue from a grafted PD patient in which unlabeled asymmetric contacts *onto* grafted DA neurons and asymmetric synaptic contacts made *by* grafted DA neurons were observed ultrastructurally. The presence of OFF-time dyskinesias was not reported in this patient [116, 133, 137], though this may be due to the timing of the study as it occurred prior to the placebo-controlled studies that brought international attention to the presence of GID in grafted subjects, and thus, clinicians may not have thought to look for this troublesome side-effect, especially if it was of mild severity.

Additional proposed mechanisms for the development of GID suggest that this troublesome phenomenon might occur as a result of damage to the striatum, the presence of non-nigral DA neurons in the graft, preoperational disease severity, storage of the fetal tissue before grafting, and/or host immune response (e.g., [119, 123, 126, 127, 129, 138]). Perhaps most significantly, one group has posited that the unintended presence of 5-HT neurons in the grafted tissue may play a strong causative role in GID pathogenesis [139-141]. This (somewhat controversial) contention is supported by evidence of extensive graft-derived serotonergic hyperinnervation in three grafted individuals with PD who developed GID, and the marked reduction of this side-effect when the patients were treated with buspirone, a 5-HT_{1A} partial agonist that also displays DA D2 receptor (DRD2) antagonistic properties [140-142]. However, while 5-HT neurons are understood to play a significant role in LID (e.g., [75, 143]), the evidence supporting a causative role for 5-HT neurons in GID is minimal; rather, the majority of available evidence suggests a smaller, modulatory role for 5-HT neurons in GID development, while the DA system is thought to provide a more significant contribution [132, 144-146]. For a more complete discussion of 5-HT neurons and their

theorized roles in LID and GID, please see the discussion section in Chapter 3 of this document below.

Overall, the unexpected appearance of GID in a subpopulation of individuals with PD had a significant, negative impact on the field that led many to believe that neural grafting was not a viable therapeutic option for PD. Consequently, much of the research in the field has since been directed toward understanding mechanisms underlying the pathogenesis of this bothersome side-effect. However, while many contributing factors have been proposed, the exact mechanisms underlying GID pathogenesis remain unclear, and there are currently no simple clinical solutions for treating GID. In recent years, there has been a shift of interest in the field to the development of stem cell-based transplantation techniques, leaving much yet to be clarified regarding GID pathogenesis and treatment.

Alternative Cell Sources

The second defining feature of this period is the ongoing pursuit of a worthy alternative cell source. While early proof-of-principle clinical studies have clearly demonstrated that fetal nigral DA neurons are capable of surviving long-term, functionally integrating, and producing long-lasting motor benefit in the host, the use of fetal tissue is inherently limited by significant ethical concerns and practical issues. Therefore, in recent years the field has been predominantly characterized by international efforts to identify a better source of cells for neural grafting in PD. Ideally, the potential cell source must be more readily available in large quantities, be produced using methods that can be standardized across research centers, be safe and well-tolerated while minimizing the risks of adverse effects such as GID, and provide

functional benefit comparable to that which has been observed using fetal cell transplants. To this end, recent advances in developmental and stem cell biology have provided the means for researchers to develop authentic DA neurons from human pluripotent stem cells (PSCs), which possess several advantages over fetal cells including increased availability to near-unlimited numbers and the ability to standardize the manufacturing process (for review [147]). To date, various cell populations have been investigated as potential cell sources for grafting in PD, including neural stem cells, bone marrow mesenchymal stem cells, and fetal pig VM DA neurons [148]. However, the present discussion is focused on the two stem cell populations that have received the most attention in the field: human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs), which are collectively referred to here as PSCs **(Figure 2.2)**.

The first of these stem cell populations, ESCs, were derived from human blastocysts for the first time in 1998 [149]. In 2006, Roy and colleagues [150] provided the first evidence that DA neurons derived from human ESCs can survive and provide functional benefit when intrastriatally grafted in a parkinsonian rat model, though a major concern in this study was that the grafts also contained undifferentiated, mitotic neuroepithelial cells that were potentially tumorigenic. Subsequently, two groups developed refined methodology for converting human ESCs to mesencephalic DA neurons, demonstrating that DA neurons produced using these protocols could survive and provide functional benefit in mouse, rat, and non-human primate models of PD without risk of neuronal overgrowth or tumorigenicity [151, 152]. Next, in 2014, Grealish et al. showed that human ESC-derived DA neurons, when transplanted in a rat model of

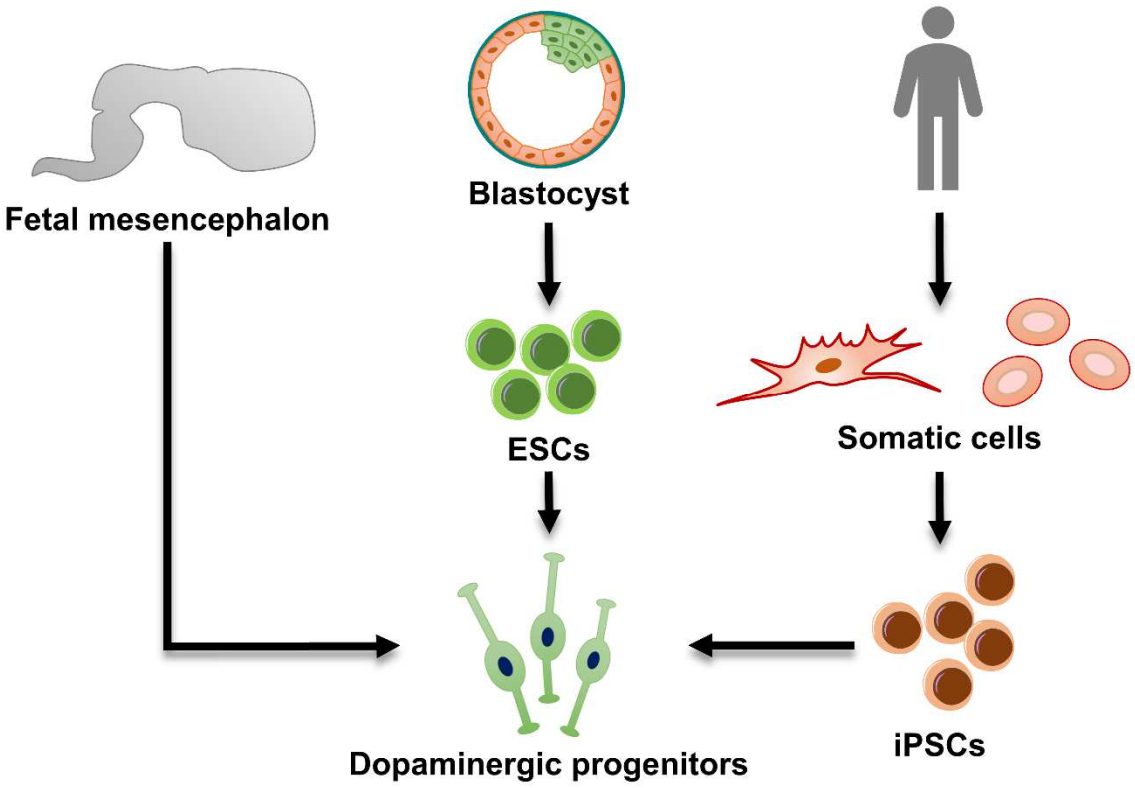

Figure 2.2 Cell sources for cell replacement therapy in Parkinson's disease
Schematic depicting sources of transplantable dopaminergic progenitors currently used in experimental cell replacement strategies for Parkinson's disease. Fetal cells are derived from human fetal ventral mesencephalon. Embryonic stem cells are harvested from preimplantation human blastocysts. Induced pluripotent stem cells are reprogrammed from somatic cells derived from adult donors, commonly skin cells or blood cells. Adapted from [147]. Abbreviations: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells

PD, were essentially identical to human fetal VM DA neurons with respect to molecular identity, *in vivo* function, and target-specific axonal outgrowth [147, 153]. Importantly, these crucial and encouraging findings set the stage for fast-tracking stem cell technology to the clinic.

The second stem cell population pertinent to the present discussion, iPSCs, can be generated by reprogramming fully differentiated cells (e.g., fibroblasts) into an embryonic state, as described by Takahashi and colleagues [154, 155]. The reprogrammed cells can then be differentiated into DA neurons (e.g., [156-160]). Alternatively, adult differentiated cells can be reprogrammed directly into DA neurons, skipping the pluripotent stage [161-163]. Importantly, iPSC technology allows for the development of transplantable cells specific to the patient, thus reducing the risk of immune reaction and avoiding ethical concerns associated with the use of embryonic cells. In preclinical animal models of PD, human iPSC-derived DA neurons have been shown to survive long-term and mediate motor benefit in the host (e.g., [156-159, 164]). Despite initial concern that evidence of sufficient fiber outgrowth from grafted human iPSC-derived DA neurons has not been convincingly demonstrated and the capacity of these cells to produce functional benefit in PD models is incompletely understood [157, 161, 165], the field is generally optimistic and clinically oriented. Indeed, recently-published methods for the generation of dopaminergic progenitors from human PSCs were designed to increase the translational potential of stem cell therapy for PD [166]. Moreover, a recent study demonstrated that grafted human iPSC-derived DA neurons can survive long-term, provide motor benefit, and extend dense neurites into the host striatum of MPTP-treated monkeys in the absence tumor formation [167]. The

encouraging results from this study facilitated a rapid transition to the first clinical trial investigating human iPSC-derived DA neurons in persons with PD in 2018 [3, 168].

In summary, stem cell therapy is a promising experimental approach that carries potential as a therapeutic option for persons with PD. Years of successful preclinical studies have demonstrated the capacity of human PSC-derived DA neurons to replicate the functional effects observed in earlier studies using fetal VM DA neurons, which has in turn facilitated a rapid translation to clinical studies. The field is overall optimistic, despite concerns regarding limited neurite outgrowth from stem cell-derived neurons and incomplete understanding of the capacity of these cells to produce functional benefit in animal models of PD [161]. Clinical trials investigating the tolerability and feasibility of stem cell therapy in PD are ongoing and are discussed below.

Additional Limitations of Cell Replacement Therapy

In stark contrast to the earliest years of neural grafting research in which progress was slowed by technological limitations and pervasive skepticism among scientific communities, cell replacement therapy has grown substantially in recent decades as a viable therapeutic strategy for persons with PD. However, as is the case with any clinically translatable therapeutic approach, there remains a need for researchers and clinicians to carefully consider the remaining limitations and potentially negative consequences associated with cell transplantation therapy and proceed accordingly. Thus, in addition to the shortcomings discussed above, the goal of the following paragraphs is to bring attention to several additional limiting factors that have influenced the field and warrant further discussion.

Perhaps the most notable shortcoming of cell transplantation therapy is the incredible heterogeneity in clinical efficacy among persons with PD. Indeed, while some patients have experienced marked and lasting improvement of motor symptoms, others have experienced little or no clinical benefit and a significant subset of patients developed GID. This variability is apparent not only between different trials, but also within groups of patients who were transplanted at the same center [138, 169]. Interestingly, the heterogeneity in clinical efficacy observed in grafted individuals is similar to the variable response to levodopa that has also been observed clinically. For example, a retrospective analysis of the Earlier vs Later Levodopa Therapy in Parkinson's disease (ELLDOPA) study showed that persons with early-stage PD receiving equivalent levodopa doses experienced variable levels of motor improvement ranging from a 100% improvement to a 242% worsening [170]. With regard to neural grafting, the variability in clinical outcomes is thought to be due at least in part to methodological differences between studies and patient selection [138, 169]. Accordingly, transplantation experts conducted a critical reappraisal of data from the early clinical trials in an effort to standardize transplantation methodology and improve patient selection for future trials. These renewed criteria form the basis of currently ongoing clinical trials which are discussed in more detail below.

An additional limitation of cell transplantation therapy for PD is the troubling observation that robust survival of grafted cells and extensive graft-derived neurite outgrowth does not always imply functional recovery, particularly in older subjects. This was demonstrated in a recent preclinical study aimed at understanding whether decreased viability of grafted DA neurons in the aged brain accounted for decreased

graft efficacy [171]. In this study, parkinsonian rats of varying ages (young (3 m.o.), middle-aged (15 m.o.), and aged (22 m.o.)) were engrafted with VM tissue dissected from embryonic day 14 rat pups. Based on an earlier experiment by the same group that showed a marked decrease in survival of grafted DA neurons in aged, parkinsonian rats [172], the number of grafted cells in this follow-up study was proportionately increased across age groups, with middle-aged rats receiving twice as many cells as young rats and aged rats receiving 5x as many cells as young rats [171]. Surprisingly, despite a five-fold increase in the number of surviving DA neurons and twice the degree of graft-derived neurite outgrowth, the functional benefit exhibited by aged rats was delayed and inferior compared to young rats [171]. These data suggest that factors beyond reduced cell viability prevent meaningful functional benefit following engraftment of new DA neurons in the aged brain.

Two recently reported case studies of individuals with PD who received DA neuron grafts support the validity of these preclinical findings [173, 174]. In the first report [173], Li et al. examined postmortem brain tissue from a PD patient who had received an intraputaminal embryonic DA neuron graft 24 years earlier. The patient experienced a dramatic recovery, displaying only very mild, “nontroublesome” OFF-time dyskinesia that appeared three years postoperatively. This period of marked recovery lasted for 12 years, after which the patient experienced progressively worsening rigidity and hypokinesia and a gradual loss of levodopa efficacy. By 18 years post-transplantation, the patient no longer showed signs of graft-derived motor benefit. Following the patient’s death, postmortem analysis revealed robust survival of transplanted DA neurons that provided extensive reinnervation of the putamen [173]. In

the second report [174], Kordower et al. described postmortem clinical findings from a PD patient who had died 16 years following transplantation. This patient, who had been randomized to the treatment group in one of the NIH-sponsored placebo-controlled studies [119], never experienced graft-related clinical benefit. In fact, eight years following the transplant procedure, the patient received bilateral subthalamic DBS due to worsening parkinsonism and the development of GID. During postmortem evaluation, it was discovered that this patient possessed what was determined to be the largest number of surviving grafted DA neurons and the densest and most widespread reinnervation of the striatum that has been reported to date [174]. Importantly, the mechanism(s) underlying the discordant finding of limited or no clinical benefit despite robust graft survival and extensive striatal reinnervation is currently unknown and warrants understanding if this experimental approach is to provide widespread utility for individuals with PD.

An additional drawback is the noteworthy lack of preclinical studies in non-human primates prior to the initiation of the first clinical trial in 1987 and the pervasive underappreciation for this important preclinical model in subsequent years. To the best of my knowledge, only two fetal DA neuron transplantation studies had been completed in parkinsonian non-human primate models prior to 1987 [175-177]. The majority of non-human primate studies were published after the fact (e.g., [136, 178-196]), during a time at which clinical grafting studies were already occurring around the world. This meant that persons with PD were undergoing invasive experimental surgery that, at the time, was based almost entirely on evidence obtained from rodent PD models that had only just begun to provide answers to important investigational questions regarding this

novel technique while also leaving many avenues unexplored. For example, while all individuals who had received fetal nigral transplants in the early clinical studies had been on antiparkinsonian medication and the vast majority of these patients had experienced LID preoperatively, it was not until 2016 that neural grafting was investigated in non-human primates that had been rendered dyskinetic with levodopa preoperatively [131]. In the same vein, it is also noteworthy that the parkinsonian rodents employed in early preclinical work were almost exclusively young, in contrast to individuals with PD, who are in most cases classified as elderly (e.g., [197]).

In addition, the NIH-sponsored clinical grafting trials did not emulate the experimental designs used in the most successful non-human primate studies at the time, which demonstrated that grafting into the caudate nucleus provided the most functional benefit [198]. For example, Taylor and colleagues [184] reported in a 1995 study that grafting fetal nigral tissue into the caudate nucleus of parkinsonian non-human primates produced robust antiparkinsonian effects, while grafting into the putamen or using a sham procedure did not. Despite this noteworthy finding, individuals in the NIH-sponsored clinical trials received transplants of fetal tissue in the putamen. This was not without sound reason, however; in individuals with PD, striatal DA loss is greatest in the postcommissural putamen (e.g., [199]), suggesting that transplants into this particular nucleus might provide the most benefit in humans.

In keeping with the much debated notion that crucial preclinical work was lacking at this time, it is worth noting that the push for translating neural grafting to the clinic was met with some concern among scientists who thought the field was not ready [200, 201]. In a written correspondence to the Editor of The New England Journal of Medicine

(reply by Redmond et al. in [122]) regarding the double-blind clinical trial by Freed et al. [118], D.E. Redmond, Jr. stated that the failure of this clinical trial was predicted by leading transplantation experts in 1994 [201]. He also cited the work by Taylor et al. [184], emphasizing the discrepancy between non-human primate studies and clinical trials. However, Redmond later went on to show in a controlled non-human primate study that grafting into either the caudate or the putamen produced good functional benefit [202], suggesting that the putaminal placement of fetal tissue in the NIH-sponsored clinical trials may not have been the reason why the studies failed to meet their primary endpoints. Nevertheless, had this important non-human primate study been completed just a decade earlier, it might have strengthened the rationale to begin large, controlled clinical trials and increased confidence in the chosen transplantation techniques.

Another important limitation in the field of cell replacement therapy for PD was brought to light in 2008 when two research groups demonstrated that α-syn Lewy pathology was present in grafted fetal VM DA neurons that had survived long-term (>10 years) in individuals with PD who had come to postmortem [203, 204]. These findings were corroborated in subsequent case reports showing that α-syn-positive protein inclusions are present in grafts as old as 24 years, with as many as 27% of transplanted DA neurons containing Lewy pathology [173, 174, 205-208]. The transfer of α-syn pathology from host to graft has also been demonstrated in mouse and rat models of PD [209, 210]. Importantly, however, the presence of Lewy pathology in grafted DA neurons has not been conclusively linked to reduced graft function and additional reports have found no Lewy pathology in transplanted DA neurons in long-term grafts

up to 14 years old [211, 212]. Collectively, these reports prompted researchers to hypothesize that Lewy pathology is spread from affected host neurons to healthy grafted neurons in a prion-like manner [204, 213-218], a notion that has fundamentally changed our understanding of PD pathogenesis and has inspired the development of novel therapeutic strategies that aim to reduce pathological α-syn propagation (e.g., immunotherapy) [219]. This hypothesis also poses implications for the use of autologous stem cell transplants to treat PD, as there is concern that cells derived from individuals with PD might be more vulnerable to the spread of pathology from the host than cells from healthy donors [147].

Finally, a discussion of the limitations of neural grafting for PD is not complete without touching on the fact that, as promising as this experimental therapeutic approach might be, it remains a symptomatic therapy meant to improve motor symptoms associated with striatal DA depletion and does not alter, slow, or stop the progression of this relentless disease. Indeed, cell transplantation therapy will only ever work as well as the best DRT (e.g., levodopa) [169] and is not meant to treat non-motor dysfunction or provide a cure for PD [220]. However, a therapeutic approach such as neural grafting that has the potential to provide long-lasting and marked recovery of PD-related motor dysfunction would be a valuable and clinically competitive addition to the collection of available treatment options for PD.

Reemergence of Clinical Trials: Are We Ready?

Less than 20 years following the controlled clinical trials that resulted in a worldwide moratorium of neural grafting for PD, clinical trials are emerging once again. In 2010, a European Union-funded multicenter trial for human fetal VM transplants

began recruiting in Europe (NCT01898390) [221]. Termed TRANSEURO, this open-label study was initiated on the basis of a critical reappraisal of data from earlier clinical trials [169]. Upon assessing the raw data compiled from all major fetal VM transplantation clinical trials, the TRANSEURO consortium identified a number of factors that were thought to contribute to the variability in clinical outcomes and used this knowledge to design the new trial [169, 221]. For instance, researchers determined that preoperative disease severity was critical for predicting therapeutic success, hypothesizing that patients with milder disease and negligible dyskinesia might benefit the most from cell transplantation therapy while also being less likely to develop GID [221]. Based on this reasoning, 11 individuals with early-stage PD that had been selected from an observational cohort received grafts of human fetal VM between 2015 and 2018. Originally, 20 patients had been selected for transplantation, but due to technical issues associated with procuring human fetal tissue, only 11 patients were grafted during the 3-year transplant period. Each patient received bilateral transplants in the putamen. The outcome of the transplant procedures will be assessed in 2021 using change in UPDRS motor scores as the primary endpoint [221].

New clinical trials investigating tolerability and feasibility of human PSC-derived DA neurons are also emerging **(Table 2.2)** [168]. In 2014, researchers from Europe, Japan, and the US joined forces to establish a new clinical initiative for human PSC-derived transplants, called GForce-PD [168]. The first of the GForce-PD trials, in which seven patients will receive allogenic grafts of iPSC-derived cells, was initiated in Japan in 2018 [147, 222]. Trials investigating human ESC-derived cells in Europe (STEM-PD) and the US (NYSTEM-PD) are currently in preparation to commence [1, 223, 224],

Trial	Country	Cell Source	No. of Patients	Status
Center for iPSC Research and Application	Japan	Allogenic iPSCs	7	Started
NYSTEM-PD	USA	ESCs	10	Pending FDA decision
European STEM-PD	UK and Sweden	ESCs	TBC	In set-up
Chinese Academy of Sciences	China	ESCs	50	Ongoing
Fujifilm cellular dynamics international	USA	Autologous iPSCs	TBC	In set-up
Allife Medical Science and Technology Co., Ltd.	China	Autologous iPS-neural stem cells	10	In set-up
Aspen Neuroscience	USA	Autologous iPSCs	TBC	In development
International Stem Cell Corporation	Australia	Parthenogenetic ESC-derived neural stem cells	12	Ongoing
TRANSEURO	UK and Sweden	Human fetal VM tissue	11	Completed

Table 2.2 Current and planned cell transplantation clinical trials in PD
The TRANSEURO clinical trial has completed transplanting patients with human fetal tissue and the final grafted patient will reach the 36-month post-graft clinical endpoint by 2021. Numerous stem cell-based clinical transplantation studies are currently in the pipeline, and the group in Japan transplanted its first patient with iPSCs in 2018. Adapted from [1] and [147]. Abbreviations: ESCs, embryonic stem cells; FDA, Food and Drug Administration; iPSCs, induced pluripotent stem cells; TBC, to be confirmed; UK, United Kingdom; USA, United State of America; VM, ventral mesencephalon

while another trial has been initiated in China by commercial groups outside of GForce-PD (NCT03119636) [1, 168]. Several more trials are expected to begin in the next 2-3 years [1] **(Table 2.2)**.

The recent enthusiasm to begin clinical trials anew is reminiscent of the early neural grafting clinical trials that were initiated less than 10 years following the first evidence of functional benefit from fetal DA neuron transplants in animal models of PD. Indeed, only 12 years had passed between the first functional evidence that grafted PSC-derived cells produce motor benefit in a parkinsonian animal model [150] and the initiation of stem cell-based clinical trials for PD in 2018 [147]. Importantly, several issues have thus far received limited attention, namely the mechanisms underlying GID pathogenesis, the discordant finding of limited therapeutic benefit despite robust graft survival in some patients, and concerns regarding insufficient growth of transplanted human PSCs, as discussed above. For example, despite evidence suggesting that GID is a multifaceted side-effect of yet unknown pathological origin, ongoing clinical trials have indicated that the only actions that were taken to reduce GID occurrence were better patient selection and the exclusion of 5-HT neurons from grafts [147, 168, 221]. As 5-HT neurons are thought to play only a small, modulatory role in GID development, if at all [144-146], one might expect that the exclusion of 5-HT neurons from transplants may not be sufficient for complete prevention of GID in grafted individuals. Ultimately, until these imperative issues have been fully addressed, the risk of failure in future clinical grafting trials for PD remains unacceptably high. Thus, it stands to reason that the field may not be ready to reinitiate trials with human patients. Indeed, the contention that the field of neural grafting for PD might be prematurely entering the clinic is not new

[200, 201, 225-227]. In a slightly prophetic perspective piece that was published in 1988, leading neural grafting expert Dr. John Sladek advised rather eloquently that the field practice patience in the pursuit of a clinical initiative:

> *The issues encompassed by fetal grafting research and its application to humans deserve our dispassionate and timely attention. As a society we have not yet had sufficient time to fully explore and understand the many issues attendant to embryonic cell grafting for neurodegenerative and other disorders… the scientific rationale continues to build for neural grafting as a therapy for neurological disease. Now, however, we could benefit from more* ***patience*** *rather than more* ***patients****.* [200]

Following decades of trial and error, the field of regenerative therapy has reached a new and exciting era. Looking to the future, several interesting approaches are on the horizon. In particular, there has been considerable interest in using cell replacement to provide complete nigrostriatal circuit reconstruction and combining transplants with growth factors and/or biomaterial scaffolds to enhance survival and growth of grafted cells [228, 229]. It has also been suggested that co-grafting DA neurons with other neuron types (e.g., cholinergic) might be useful in targeting non-motor PD symptoms that are mediated by degeneration of brain regions outside of the nigrostriatal pathway [147]. Perhaps most significantly, recognizing that PD is an impressively complex and heterogenous disorder, the use of precision medicine will be instrumental in generating new and effective therapies that can be tailored to the individual patient for maximal benefit.

Using Precision Medicine to Deconstruct the Complexity of Patient Response to Therapy

Introduction to Precision Medicine

As adopted by the National Research Council in 2011, *precision medicine* refers to "the tailoring of medical treatment to the individual characteristics of each patient… to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment" [230] **(Figure 2.3)**. Also referred to as personalized medicine, this novel healthcare approach has the potential to improve quality of care while reducing need for unnecessary diagnostic tests and therapies [231]. One primary long-term goal of precision medicine is to identify disease in presymptomatic individuals and significantly delay, or even prevent, disease onset [232]. As one might expect, this would prompt a gradual shift from *treating* disease to *preventing* disease in standard healthcare practice.

At the core of precision medicine is the generation and sharing of large datasets entailing various aspects of individual patients' lives such as family history, genetics, lifestyle, environment, and biological data obtained from clinical biospecimens. These large, complex datasets are collectively termed the "digital phenotype", and the ultimate goal is to translate this information into clinical benefits specific to the individual patient [231, 232]. To this end, the decreasing cost of next-generation sequencing technology has stimulated interest in translating whole genome sequencing to the clinical setting to perform genome-based diagnostics and improve therapeutic recommendations, though

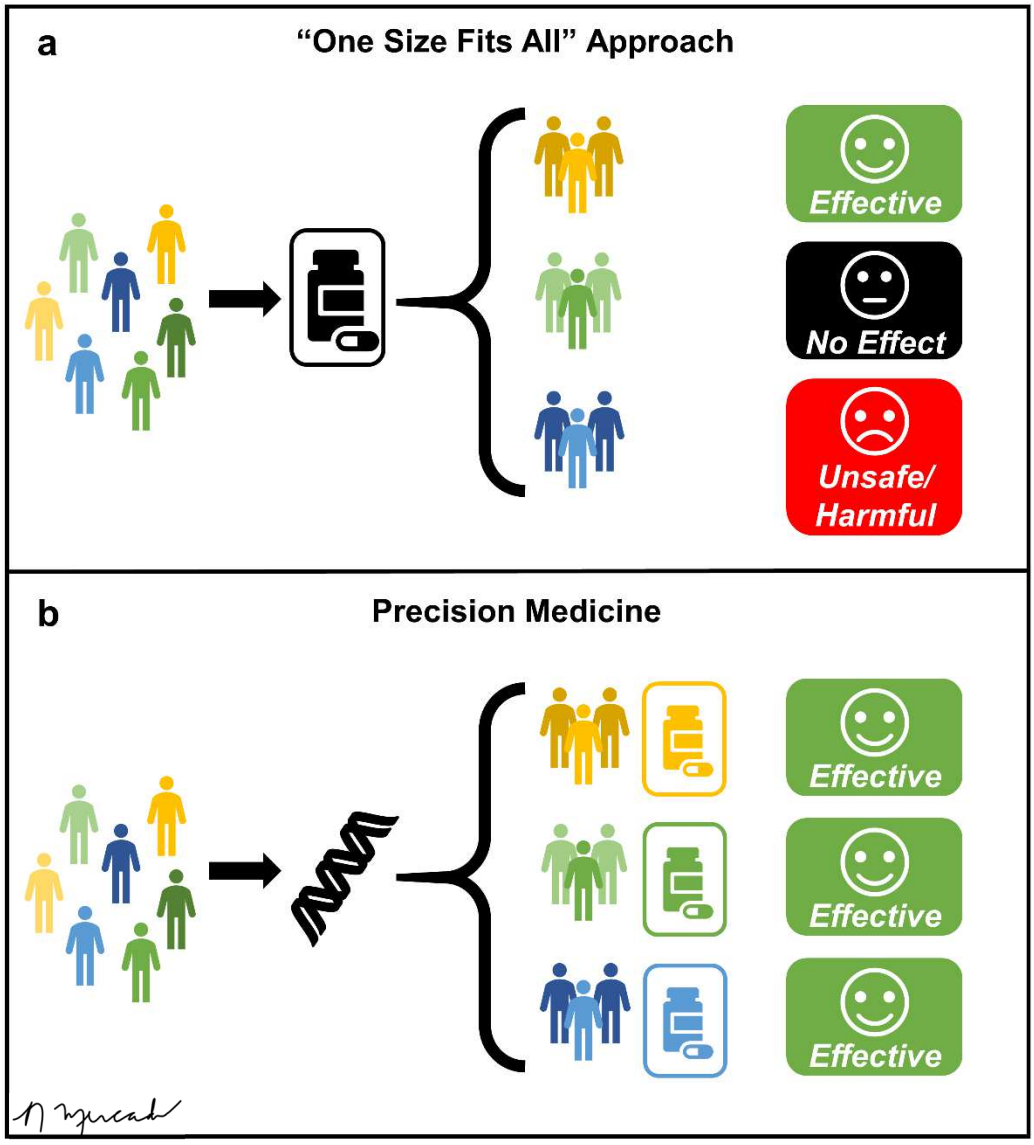

Figure 2.3 Using precision medicine to improve clinical outcomes
(a) The classic approach to medicine can be described as a "one size fits all" approach in which all patients afflicted with a given condition are administered the same treatment(s) regardless of individual characteristics such as age, sex, lifestyle, and/or genetics. Using this approach, a subpopulation of patients may experience significant clinical benefit whereas other subpopulations of patients receiving the same treatment may experience suboptimal or delayed benefit or no benefit at all, or may experience unsafe or harmful side-effects. **(b)** A targeted precision medicine approach differs from the classic approach in that individual characteristics are considered when determining best treatment for the individual patient. Attributes such as age, sex, lifestyle, comorbidities, and genetics are used to tailor treatments to individuals with the main objective of providing safe and effective treatment for as many patients as possible.

there are numerous barriers including ethical issues that must be overcome before widespread clinical implementation can be achieved [231-233].

Decreasing genome sequencing costs and widespread implementation of electronic medical records also influenced the government-funded precision medicine initiative that was introduced by President Barack Obama in 2015, called the *All of Us* Research Program [231, 234]. The main objective of the *All of Us* program is to build a database composed of participant-provided information from at least 1 million Americans. This database can then be accessed by researchers to explore "biological, social, and environmental determinants of health and disease" [234]. As the world of healthcare moves toward a new era of precision medicine, meeting this goal will be crucial in developing a detailed understanding of the sources of variability in clinical response to therapeutics.

Precision Medicine and PD

In individuals with PD, there is incredible variability in not only the severity and progression of the disease, but also the response to therapy. This is clearly evidenced, for example, by the heterogeneity observed in clinical outcomes for cell replacement and levodopa therapy in PD, as discussed above. Therefore, it is logical to assume that a "one size fits all" approach to PD treatment is unlikely to succeed. Fortunately, the field has recently begun to move toward a precision medicine approach (e.g., [235-238]). For instance, several therapies targeting genetic forms of PD (i.e., specifically involving patients with known PD-associated mutations) are currently being tested in clinical trials with a focus on the underlying disease mechanism rather than symptoms

[235]. However, it is important to note that precision medicine extends to factors beyond genetics; individual characteristics such as lifestyle, age, sex, body weight, comorbidities, medical history, and environment must also be taken into account in order to provide a high-resolution, detailed understanding from which effective precision medicine-based treatments may be constructed (e.g., [232, 239]).

While precision medicine holds promise for the future of PD therapeutics, there are several barriers to overcome before this revolutionary approach to healthcare can become mainstream in the PD community. For example, it will be important to boost public interest in taking an active role in research while also securing their trust in sharing personal data [237]. In addition, steps must be taken to make “digital phenotype” data more accessible, to facilitate the transfer of information from research studies to clinical practice, to advance biomarker discovery efforts to segregate patients into disease subtypes, to obtain approval for routine use of precision medicine approaches from regulatory agencies, and to increase acceptance among physicians, insurance companies, patients, and the general public [233, 236]. Meeting these challenges will require considerable and collaborative effort from clinicians, researchers, and patients. Despite the challenges ahead, the ongoing pursuit of a personalized approach to PD treatment is a worthy endeavor that will undoubtedly open countless doors for the advancement of experimental therapeutics in PD. Future therapies will likely not be widely applicable to large populations of persons with PD unless they address the inherent heterogeneity among individuals. Of course, it may never be possible for any single therapeutic strategy to effectively treat the majority of patients;

instead, it would be most beneficial to develop a collection of various therapies targeted to specific subpopulations of patients based on individual characteristics.

As mentioned, efforts to implement precision medicine initiatives into PD therapeutics have already begun. Thus far, precision medicine in PD has focused on genetics as an important factor underlying heterogeneity in disease phenotype and clinical outcomes. While some genetics-based treatments for PD have already reached the clinical trial phase [235], the field of pharmacogenetics (the study of how genes affect an individual's response to medicine) has also gained traction in recent years [237, 239, 240]. For example, in a recent notable study, Fischer et al. [241] demonstrated that a common SNP in the gene for BDNF is associated with reduced efficacy of oral levodopa in persons with early-stage PD carrying this *BDNF* mutation. The effects of this particular SNP on clinical response to other forms of therapy for PD, namely cell replacement therapy, have not yet been investigated and form the basis of the present work (as discussed in detail below).

BDNF: An Underrecognized Contributor to Heterogeneity in Clinical Outcomes for PD

Investigating the effects of genetic variants on disease phenotype and response to therapy is an important first step in the journey toward a personalized approach to PD treatment. Specifically, there has been much interest in studying *BDNF* SNPs in the context of PD (e.g., [241-249]). BDNF is of particular interest due to its well-established role as a potent modulator of synaptic plasticity (e.g., [250]) and its neuroprotective effects on nigral DA neurons (e.g., [50]). Furthermore, BDNF is well-studied in the

context of neurodegenerative disease (e.g., [249, 251-255]) and has been studied in relation to PD for the past several decades following the initial discovery that BDNF promotes survival of nigral DA neurons [50]. Given these facts, the aim of the present discussion is to briefly review the biology of BDNF and its significance in aging and PD while also touching on the relevance of a common *BDNF* SNP.

Introduction to Neurotrophins

Broadly speaking, neurotrophic factors are growth factors that support survival, growth, and differentiation of neurons. Neurotrophic factors are classified according to structural homology into distinct families such as: (1) *neurotrophins* (nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6), and neurotrophin-7 (NT-7)) **(Figure 2.4)**, (2) *GDNF family ligands* (GDNF, NRTN, ARTN, and PSPN), and the recently characterized (3) *CDNF/MANF family* [256]. Within the neurotrophin family, NGF was the first to be discovered. It was described for the first time by Rita Levi-Montalcini, Stanley Cohen, and Viktor Hamburger in the 1950s [257-264]. In the initial work performed by Levi-Montalcini and Hamburger [257], they reported that mouse sarcoma transplanted into chick embryos stimulated growth of sensory and sympathetic nerve fibers located in adjacent dorsal root and sympathetic ganglia. In subsequent work, they used extraembryonic transplants of tumor tissues (i.e., positioned in the chorioallantoic membrane so as not to be in direct contact with the chick embryo but still sharing circulatory blood supply) to confirm that the tumors were releasing a diffusible growth factor that stimulated growth of sensory and sympathetic nerve fibers [258]. Perhaps most strikingly, Levi-Montalcini et al. then

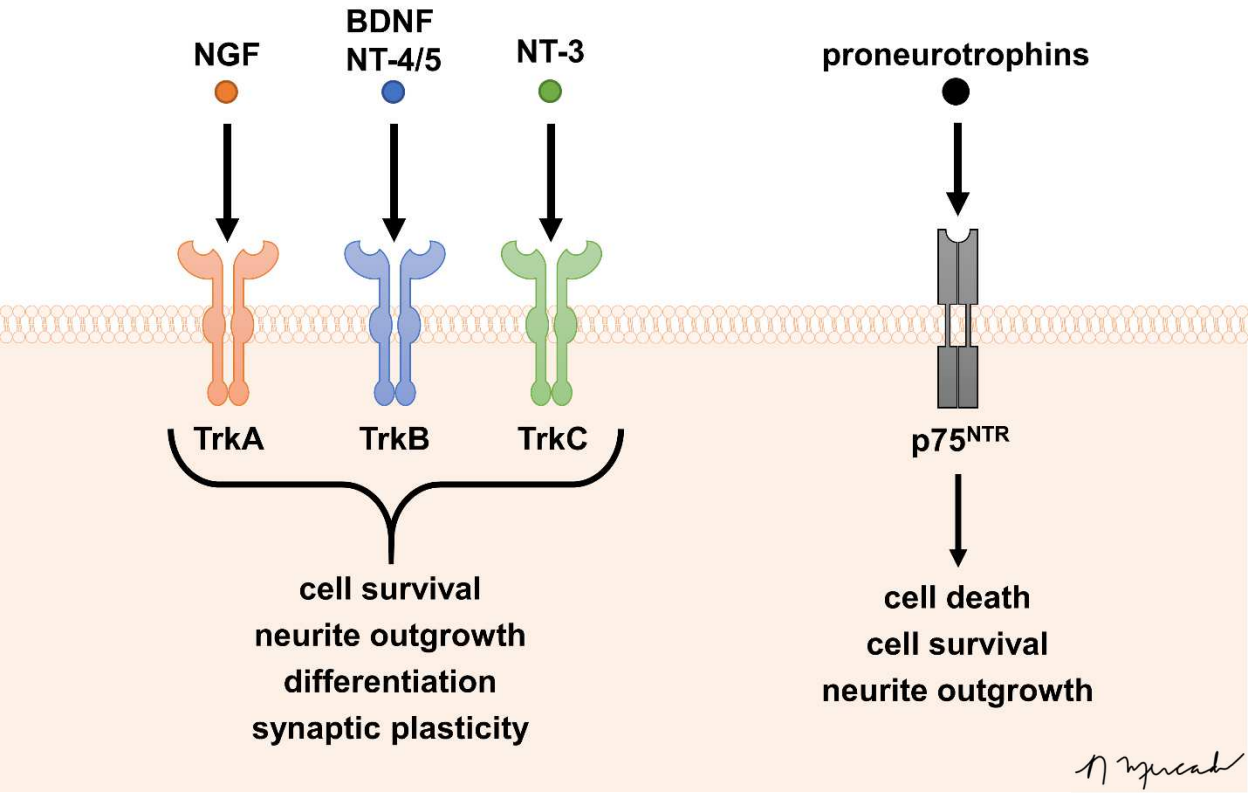

Figure 2.4 Mammalian family of neurotrophins and their receptors
Simplified representation of the mammalian family of neurotrophins which consists of NGF, BDNF, NT-3, and NT-4/5. All neurotrophins are synthesized as pre-pro-neurotrophins. The pre-sequence is cleaved off in the endoplasmic reticulum, while the resulting proneurotrophins and mature neurotrophins (after cleaving off the pro-sequence) go on to be secreted from the cell. Proneurotrophins bind with high affinity to $p75^{NTR}$, while mature neurotrophins bind with high affinity to members of the tyrosine receptor kinase family. Proneurotrophin-$p75^{NTR}$ binding activates intracellular signaling cascades that regulate programmed cell death, cell survival, and neurite outgrowth. Mature neurotrophin-Trk binding stimulates signaling pathways involved in cell survival and differentiation, neurite outgrowth, and synaptic plasticity. NGF preferentially binds to TrkA, NT-3 preferentially binds to TrkC, and BDNF and NT-4/5 bind preferentially to TrkB. Note that this illustration is a simplified representation. Factors such as co-receptor binding and weak affinity to non-preferential binding partners introduce complexity that is not illustrated here for simplicity. For example, NT-3 can also weakly bind to TrkA and TrkB. Abbreviations: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin 4/5; $p75^{NTR}$, p75 neurotrophin receptor; TrkA, -B, -C, tyrosine receptor kinase A, B, C

showed that explants of sensory and sympathetic ganglia from chick embryos, when cultured *in vitro* in proximity to (but not touching) mouse sarcomas, produced robust outgrowth of nerve fibers on the side of the explant that was facing the tumor tissue [259]. In the same year, Cohen isolated the nerve growth-promoting factor from mouse sarcomas [260], and by way of a fortunate accident, also isolated the growth factor from snake venom that produced the same nerve growth-promoting effects as those elicited by mouse sarcomas [261-263, 265]. Following this discovery, Cohen next looked to the mouse salivary gland as a homolog of the snake venom gland, and was able to successfully purify NGF from the salivary gland [264]. Cumulatively, these groundbreaking studies inspired decades of research into the chemical structure and biological function of NGF, which is described in detail elsewhere (e.g., [265]).

In 1982, Yves-Alain Barde and colleagues successfully isolated a second neurotrophic factor, BDNF, for the first time from pig brain [266]. Similar to NGF, Barde et al. showed that this novel growth factor induced fiber outgrowth from, and promoted survival of, cultured embryonic chick sensory neurons. However, the survival-promoting effects of the new growth factor were not diminished by the addition of antibodies targeted to NGF [266], suggesting that the isolated growth factor was distinct from NGF. Subsequent molecular cloning of BDNF revealed that BDNF protein is structurally similar to NGF, sharing approximately 50% amino acid identity [267]. In fact, all members of the neurotrophin family share considerable sequence homology and are highly conserved between species (e.g., [268, 269]). The remaining neurotrophin family members were discovered in the 1990s (NT-3: [270-273]; NT-4/5: [268, 274, 275]; NT-6 and NT-7: [276, 277]). While NT-6 and NT-7 are members of the complete neurotrophin

family, neither is expressed in mammals and thus will not be further discussed in this review.

BDNF Biology

The BDNF Gene

The *BDNF* gene was cloned from pig brain and sequenced for the first time in 1989 [267] and described in the rat in the early 1990s [278, 279] **(Figure 2.5a)**. In one of the first descriptions of the rodent *Bdnf* gene, Timmusk et al. [278] demonstrated that the *Bdnf* gene consists of four 5' exons and one 3' exon encoding the final BDNF protein. They also showed that four mRNA transcripts are produced from the rodent *Bdnf* gene as a result of differential splicing and the presence of distinct promoters at each exon, while an additional four mRNA transcripts are produced through the use of two polyadenylation sites at the 3' end of the protein coding exon [278]. This work was expanded upon by later studies that identified novel exons in the rodent *Bdnf* gene [280, 281] **(Figure 2.5a)**. In the most recent description by Aid et al. [281], it was determined that the rodent *Bdnf* gene consists of eight untranslated 5' exons (I-VIII) driven by eight separate promoters and spliced to a common protein coding exon (IX). The various 5' exons, in turn, produce eight distinct mRNA transcripts. This report also identified a novel transcript consisting of the protein coding exon with an extended 5' region. Finally, increasing the complexity among rodent *Bdnf* mRNA transcripts, the presence of two polyadenylation sites in the 3' exon and three splice variants of exon II were also described, producing a putative total of 22 distinct transcript variants in the rodent [281].

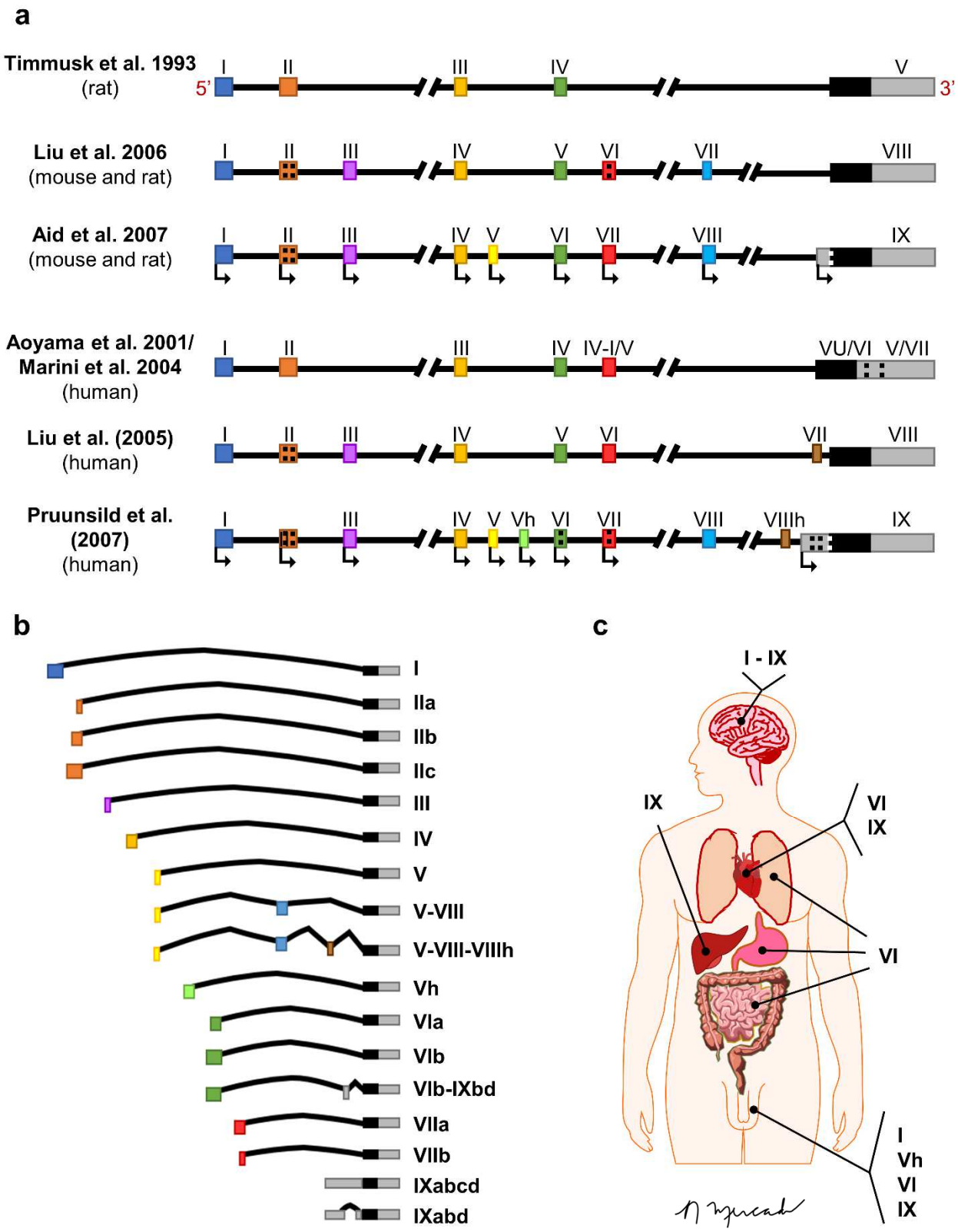

Figure 2.5 The *BDNF* gene and mRNA transcript variants
(a) Representative illustration of the *BDNF* gene exon/intron structure in rodents

Figure 2.5 (cont'd)
(top three gene schematics) and humans (bottom three gene schematics) as they were expanded upon over time. The earliest description in rat contained five exons, one of which (exon V) encoded the final protein product. It is now widely accepted that the rodent *Bdnf* gene consists of nine exons driven by distinct promoters. The human gene is more complex, containing 11 exons driven by nine distinct promoters. Vertical dotted lines indicate sites for alternative splicing. Colored boxes indicate exons and solid black lines indicate introns. Long introns are represented by double slashes. The untranslated region of the protein coding exon is shown in gray, while the protein coding region is shown in black. Transcription start sites are indicated by arrows. Exons of the same color indicate homology between studies and between species. For simplicity, polyadenylation sites are not shown. Note: the "h" identifier on exons V and VIII indicates exons that are present only in the human *BDNF* gene. **(b)** Simplified schematic illustrating the large variety of *BDNF* transcript variants present in humans that direct stimulus-, tissue-, and development-specific expression of the final BDNF protein. Each transcript may be further modified by polyadenylation. **(c)** Illustration depicting examples of tissue-specific expression of various *BDNF* transcripts in adult human tissues. The majority of *BDNF* transcripts are present in the brain at high levels. In addition to the brain, transcript VI is present at high levels in the lungs, heart, prostate, testes, and placenta (not shown), and in low to moderate levels in the stomach, small intestine, and muscle (not shown). Transcript IX is present in moderate to high levels in the heart, liver, prostate, testes, and placenta (not shown). Adapted in part from [282].

The human *BDNF* gene is even more complex, containing 11 exons driven by nine promoters, with two alternative polyadenylation sites in the protein coding exon and the added production of antisense *BDNF* transcripts that are specific to humans and non-human primates ([282] but see also [283-285]), thus producing a large variety of mRNA transcripts **(Figure 2.5a,b)**. Remarkably, each transcript variant produces the same final BDNF protein [281, 286], and the structure of the *BDNF* gene is well-conserved between humans and rodents (96.8% homology between human and either rat or mouse; BLAST queries P23560, P23363, P21237).

Importantly, the production of multiple distinct transcripts driven by separate promoters allows for tissue-, development-, and stimulus-specific expression of BDNF protein [278, 281, 282, 287, 288]. For instance, in humans, *BDNF* transcript distribution was shown to be tissue specific, with expression of several transcripts present in nonneuronal adult tissues such as the lung and heart, while other transcript variants were exclusively present in the brain [282, 288] **(Figure 2.5c)**. Specific distribution patterns were also identified within the brain [282].

BDNF Synthesis and Signaling Mechanisms

BDNF is a ~27 kDa protein that, similar to the other neurotrophins, exists primarily as a stable homodimer [289]. To obtain this final protein structure, neurotrophins are first synthesized as pre-pro-neurotrophin precursors [290]. In general, pre-pro-BDNF protein is synthesized from mRNA by ribosomes in the rough endoplasmic reticulum. The signal peptide (i.e., pre-sequence), which directs neurotrophin synthesis to the endoplasmic reticulum, is then cleaved off and the resulting proBDNF is transported through the Golgi apparatus to the *trans*-Golgi network

(TGN) [290-292]. Inside the TGN, proBDNF is sorted into two types of secretory vesicles: those of the *constitutive* pathway and those of the *regulated* pathway **(Figure 2.6)**. When sorted to the constitutive pathway, resident protein convertases in the TGN (e.g., furin) cleave the pro-sequence from the final mature protein, which is then sorted into constitutive vesicles and transported to the cell periphery where the vesicles fuse with the plasma membrane and release BDNF in a stimulus-independent manner [286, 290]. Alternatively, proBDNF may be sorted into large dense core vesicles of the regulated pathway by intracellular chaperone proteins such as sortilin [293] and carboxypeptidase E [294], where it can be cleaved into mature BDNF by intravesicular protein convertases [290, 291]. In contrast to the constitutive pathway, regulated secretory vesicles fuse with the plasma membrane and release BDNF in a strictly calcium-dependent manner (i.e., triggered by neuronal activity) ([290]; see also [295-299]) **(Figure 2.6)**. In most cases, BDNF is preferentially targeted to the regulated pathway in neurons [300, 301]. *BDNF* mRNA is also shuttled to the dendrites where it is locally translated and plays a significant role in synaptic plasticity and spine morphology [302].

Following release into the synapse, mature BDNF preferentially binds with high affinity to tyrosine receptor kinase B (TrkB). TrkB is a member of a subfamily of receptor tyrosine kinases which also includes TrkA and TrkC (which preferentially bind NGF and NT-3, respectively). Trk receptors consist of extracellular immunoglobulin-like domains, a transmembrane region, and cytoplasmic tyrosine kinase domains [24]. TrkB is present in the brain primarily in two isoforms: full-length TrkB (contains tyrosine kinase domain) and truncated TrkB (no tyrosine kinase domain). While the full-length isoform mediates

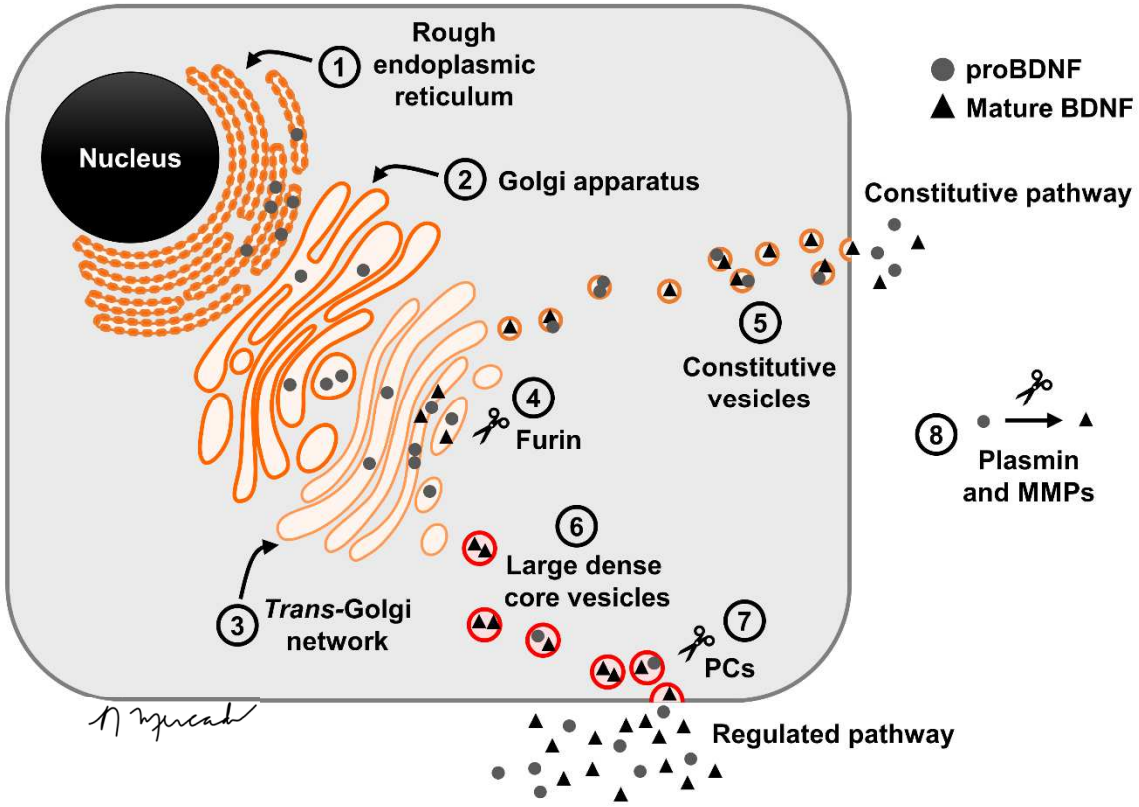

Figure 2.6 Neuronal synthesis and secretion of BDNF
Schematic representation of the path from initial BDNF synthesis to secretion via the constitutive or regulated pathway. The mRNA transcript encoding pre-pro-BDNF is translated by ribosomes in the endoplasmic reticulum **(1)**. After cleavage of the pre-sequence, proBDNF is transported through the Golgi apparatus **(2)** to the *trans*-Golgi network **(3)**. Within the *trans*-Golgi network, proBDNF may be cleaved by resident protein convertases such as furin **(4)** before being sorted to secretory vesicles of the constitutive release pathway **(5)**. Alternatively, proBDNF may be sorted into large dense core vesicles of the regulated pathway **(6)** where it can be cleaved into mature BDNF by intravesicular protein convertases **(7)**. Whereas the constitutive pathway operates independently of neuronal depolarization, the regulated pathway releases BDNF in an activity-dependent manner. Unprocessed proBDNF released into the synapse can be cleaved into mature BDNF by extracellular proteases such as plasmin and matrix metalloproteases **(8)**. Abbreviations: BDNF, brain-derived neurotrophic factor; MMPs, matrix metalloproteases; PCs, protein convertases

canonical BDNF-TrkB signaling mechanisms, the truncated isoform is known to act as a dominant negative receptor, sequester and store extracellular BDNF, regulate filopodia and neurite outgrowth in a BDNF-independent manner, and activate intracellular signaling cascades [303].

When BDNF binds to the extracellular domain of full-length TrkB, it initiates dimerization of the receptor and autophosphorylation of the intracellular tyrosine residues [24, 304]. Phosphorylated TrkB then activates three canonical downstream signaling pathways, namely the phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways (reviewed in [304-306]) **(Figure 2.7)**. These signaling cascades regulate gene expression and protein translation to promote cell survival, growth, and differentiation [291, 306]. BDNF-TrkB signaling also regulates synaptic plasticity and structural remodeling via activation of separate kinase-mediated pathways including Tiam1/Rac1 and ROCK/LIMK1 pathways [307, 308].

It was initially thought that proBDNF is an inactive precursor that is exclusively localized intracellularly. However, it is now known that unprocessed proBDNF is released into the synaptic cleft where it binds preferentially to the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor family [309]. Extracellular proBDNF may also be cleaved by extracellular proteases (e.g., plasmin and matrix metalloproteases) to produce mature BDNF [292, 309-311], a mechanism that is critical for regulating the opposing functions of mature BDNF and proBDNF in the synapse [312]. Upon proneurotrophin binding, $p75^{NTR}$ initiates downstream signaling pathways involving nuclear factor-κB, Jun kinase, and Ras homolog gene family

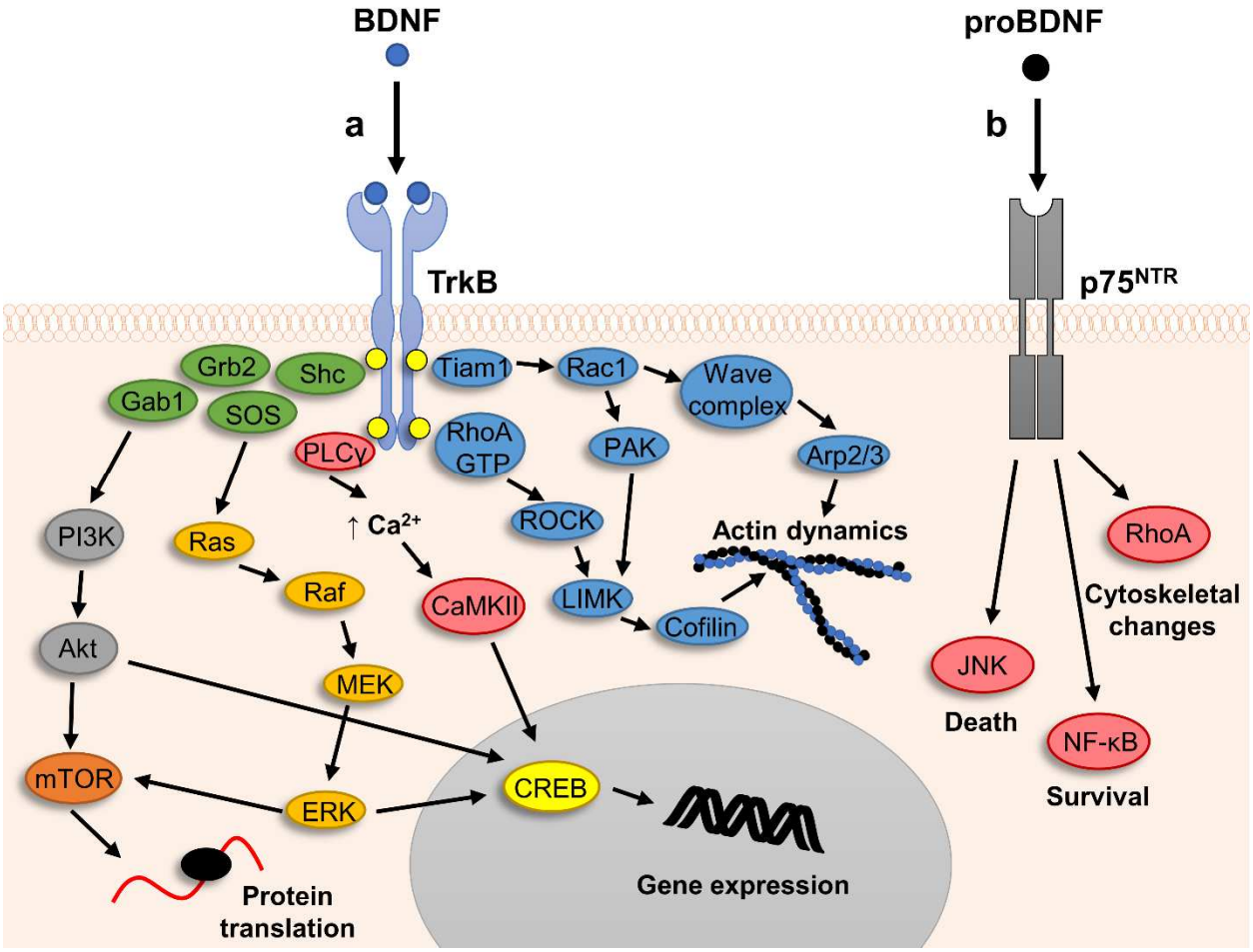

Figure 2.7 BDNF-TrkB and proBDNF-p75^{NTR} signaling pathways
Simplified representation of downstream signaling cascades activated by binding of mature BDNF and proBDNF to their respective receptors. **(a)** Upon binding with BDNF, TrkB dimerizes and its intracellular tyrosine kinase domains are autophosphorylated, triggering activation of the three main signaling pathways: (1) PI3K is activated by the Shc/Grb2/SOS complex through Gab1. Activated PI3K generates lipid products (i.e., phosphatidylinositides) that bind and activate Akt, a protein kinase that activates the transcription factor, CREB, thus mediating transcription of genes involved in the survival and differentiation of neurons. (2) PLCγ recruitment by phosphorylated TrkB increases intracellular calcium stores which, in turn, stimulates CaMKII, another protein kinase that phosphorylates (i.e., activates) CREB. (3) The MAPK/ERK kinase signaling cascade is activated by the Shc/Grb2/SOS complex. The final kinase in this pathway, ERK, directly activates CREB. In addition, Akt and ERK can also activate mTOR, which regulates protein translation initiation. A final set of pathways responsible for actin dynamics and structural remodeling are also activated by TrkB autophosphorylation. These pathways ultimately stimulate Arp2/3 (actin nucleator that stimulates actin branch formation) and inhibit cofilin (depolymerizes actin), thus influencing actin filament assembly and stabilization. There is also extensive crosstalk between the pathways discussed above (not pictured). **(b)** All proneurotrophins including proBDNF bind to and activate P75^{NTR}, which stimulates signaling cascades that mediate cell death (JNK), cell survival (NF-κB),

Figure 2.7 (cont'd)
and neurite outgrowth (RhoA). Adapted from [313] and [308]. Abbreviations: Akt, protein kinase B; Arp2/3, actin Related Protein 2/3 complex; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium; CaMKII, calcium-calmodulin dependent kinase; CREB, cAMP-calcium response element binding protein; ERK, extracellular signal regulated kinase; Gab1, Grb-associated binder 1; Grb2, growth factor receptor-bound protein 2; JNK, c-Jun N-terminal kinase; LIMK, LIM kinase-1; MEK, MAP/Erk kinase; mTOR, mammalian target of rapamycin; NF-kB, nuclear factor kB; $p75^{NTR}$, p75 neurotrophin receptor; PAK, p21-activated kinase; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; Rac1, Ras-related C3 botulinum toxin substrate 1; Raf, Ras associated factor; Ras, GTP binding protein; RhoA, Ras homolog gene family member A; ROCK, Rho-associated protein kinase; Shc, src homology domain containing; SOS, son of sevenless; Tiam1, T-cell lymphoma invasion and metastasis-inducing protein 1; TrkB, tyrosine receptor kinase B

member A (RhoA), which mediate cell survival, apoptosis, and regulation of neurite outgrowth, respectively [291, 304, 306, 314] **(Figure 2.7)**.

Neurotrophin-receptor interactions are made more complex by co-receptor binding. For example, $p75^{NTR}$ association with sortilin (a Vps10p domain sorting receptor) is necessary for the pro-apoptotic actions of $p75^{NTR}$-Jun kinase activation [306, 314], and neurotrophin-Trk signaling can be potentiated by $p75^{NTR}$ association with Trk receptors [304, 315, 316]. Furthermore, a second Vps10p family member, sortilin-related Vps10p domain containing receptor 2 (SorCS2), has been shown to mediate BDNF-dependent synaptic plasticity in hippocampal neurons upon associating with $p75^{NTR}$ and TrkB [317]. Trk receptors are also known to exert specific effects on neighboring ion channels and can be transactivated by G-protein-coupled receptors independent of neurotrophin activity [304, 313, 318].

BDNF Function

According to the "neurotrophic hypothesis" originally developed by Levi-Montalcini and Hamburger, the primary function of neurotrophins is to provide neurotrophic support to various neuronal populations [319]. However, it is now understood that the neurotrophins are involved in a remarkable array of functions in addition to this primary role. For example, BDNF is widely recognized for its unique role as a powerful modulator of synaptic plasticity (e.g., [250]) and has also been extensively studied in relation to energy balance and weight management [320]. While the biological functions of mammalian neurotrophins have been investigated in various contexts, the present review is focused on the well-established roles of BDNF.

In accordance with the original neurotrophic hypothesis, BDNF is a known regulator of cell survival and death. All four mammalian neurotrophins are crucially involved in the regulation of peripheral neuron survival during development, but interestingly have only a modest impact on the survival of developing central neurons [316]. For example, evidence from animal models of conditional BDNF depletion demonstrated that BDNF is crucial for the postnatal growth, but not necessarily the survival, of striatal MSNs [321, 322]. However, BDNF is known to exhibit pro-survival effects on specific subpopulations of neurons including mesencephalic DA neurons [50], cerebellar granule cells [323], and retinal ganglion cells [324, 325]. BDNF may also play an important role in the survival of CNS neurons following injury (e.g., [326] but see also [316]). In contrast, neurotrophins can also induce apoptosis through binding with the $p75^{NTR}$ receptor (e.g., [327]), which is important for the normal developmental elimination of inessential neurons [316, 328].

As mentioned above, BDNF also has a well-characterized role as a significant modulator of neuroplasticity and spine structure. Specifically, BDNF is instrumental in promoting long-term potentiation (LTP; i.e., strengthening of synaptic connections) and has been shown to be necessary for normal hippocampal LTP in BDNF knockout mice [329, 330]. Furthermore, it is now known that BDNF acts both pre- and postsynaptically to enhance LTP and synaptic transmission. Indeed, BDNF increases the frequency and amplitude of excitatory postsynaptic currents by acting on both pre- and postsynaptic neurons. It also promotes presynaptic glutamate release, enhances the open probability of NMDA receptors, and increases the density of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the synapse (reviewed in [331]).

BDNF also has notable effects on dendritic spine density and structure. For example, BDNF is important for controlling spine density in the striatum (e.g., [321, 322]) with additional modulatory effects on spine structure and phenotype in the hippocampus [322, 332]. Importantly, BDNF and proBDNF exhibit bidirectional regulation of activity-dependent synaptic plasticity and structure [250, 333, 334]. Whereas BDNF-TrkB signaling promotes LTP and dendritic spine formation and growth [322, 330, 335], proBDNF-$p75^{NTR}$ signaling potentiates long-term depression (LTD) and induces growth cone retraction and spine shrinkage [336-339]. These complementary and tightly regulated alterations in synapse structure and function are integral to normal brain function such as hippocampal-dependent learning and memory and motor learning [331, 340].

BDNF has also been implicated in energy homeostasis and body weight control. Initial evidence of a role for BDNF in feeding behavior and weight management was demonstrated in rodent studies in which chronic intracerebroventricular delivery of BDNF attenuated weight gain (e.g., [341]). In subsequent studies it was shown that BDNF+/- mutant mice are hyperphagic and obese [342-344]. These studies are supported by evidence of abnormal feeding behavior and obesity in humans with genetic mutations in *BDNF* and *NTRK2* (TrkB gene) [320]. In recent years considerable work has been devoted to elucidating the neural substrates underlying the appetite-suppressing effects of BDNF. While it is beyond the scope of the present discussion to describe these studies in detail, it is worthwhile to note that the evidence supporting a significant role for BDNF in energy homeostasis is abundant. Collectively, this work has demonstrated that BDNF is a fundamental regulator of feeding behavior and body

weight, acting through energy balance centers in the hypothalamus and the dorsal vagal complex to modulate homeostatic feeding behavior, and through the mesolimbic DA pathway to control hedonic feeding behavior (reviewed in [320]).

BDNF and Aging

In the context of aging, BDNF has been studied surprisingly little. Furthermore, investigations into aging-related changes in BDNF and TrkB expression in rodents and postmortem human brain tissue have yielded varying results [345] **(Table 2.3)**. For example, in the hippocampus of aging rats, some groups have demonstrated that *Bdnf* mRNA decreases with age [346-348] while others have shown that it increases [349] or remains unchanged [350, 351]. Evidence of aging-related change in TrkB expression in the brain has been more consistent; in both humans and rodents, TrkB protein and mRNA levels diminish with aging (e.g., [347, 350, 352-354]). Additional studies have revealed aging-related impairments in compensatory BDNF upregulation in response to neurotoxic lesion in animal models of PD [355, 356].

More recent work has implicated BDNF in a variety of aging-associated contexts. In a report published in 2017, Suire et al. [357] measured BDNF and proBDNF levels in plasma and extracellular vesicles harvested from peripheral blood from 150 elderly human participants with and without aging-associated motor decline (as indicated by decreased walking speed). They discovered that proBDNF levels are increased in extracellular vesicles of neuronal origin from participants who experienced motor decline, and using regression analysis, they showed that increased proBDNF in extracellular vesicles of neuronal origin is associated with increased risk for aging-

Study	Subjects	Ages	Brain Region(s)	TrkB	BDNF	Change with Age
Lapchak et al. (1993) **[351]**	Male SD & F344 rats	SD: 3, 6, 12, 24 mos; F344: 7, 16, 24 mos	Hippocampus	mRNA	mRNA	No change
Narisawa-Saito et al. (1996) **[349]**	Male F344 rats	2, 6, & 18 mos	Hippocampus Frontal cortex	*N/A*	mRNA & protein	↑ BDNF mRNA in hippocampus No change in BDNF protein
Croll et al. (1998) **[352]**	Male SD rats	3-4, 12-13, & 22-26 mos	20 regions throughout brain including cortical, subcortical, midbrain, & hindbrain regions	mRNA	mRNA & protein	↓ BDNF protein in midbrain ↓ BDNF mRNA in pons ↓ TrkB mRNA in thalamus, hypothalamus, hippocampus, CA3, dentate gyrus, retrosplenial cortex
Romanczyk et al. (2002) **[353]**	Postmortem human brain	Infants (1.25-12 mos), adolescents (14-18 yrs), young adults (20-24 yrs), adults (34-43 yrs), elderly (68-86 yrs)	Prefrontal cortex	mRNA	*N/A*	↓ FL.TrkB mRNA in all cortical layers No change in T.TrkB mRNA
Webster et al. (2002) **[358]**	Postmortem human brain	Infants (2-12 mos), adolescents (14-18 yrs), young adults (20-24 yrs), adults (34-43 yrs), elderly (73-86 yrs)	Prefrontal cortex	*N/A*	mRNA	↑ BDNF mRNA in adulthood compared to infants and adolescents; no change across adult groups
Silhol et al. (2005) **[350]**	Male SD rats	Neonatal & postnatal (P1, P7, P14, P30); 2-4, 12-16, 22-24 mos	Hippocampus Hypothalamus	mRNA & protein	mRNA & protein	↓ FL.TrkB protein in both regions ↑ T.TrkB protein in both regions No change in TrkB mRNA No change in BDNF protein or mRNA

Table 2.3 Changes in BDNF and TrkB expression across the lifespan
Studies are listed in chronological order. Modified from [345]. Abbreviations: BDNF, brain-derived-neurotrophic factor; F344, Fischer 344; FL.TrkB, full-length tyrosine receptor kinase B; N/A, not applicable; SD, Sprague-Dawley; T.TrkB, truncated tyrosine receptor kinase B

Table 2.3 (cont'd)

Study	Subjects	Ages	Brain Region(s)	TrkB	BDNF	Change with Age
Webster et al. (2006) **[354]**	Postmortem human brain	Neonates (1-3 mos), infants (4-12 mos), adolescents (14-18 yrs), young adults (20-24 yrs), adults (34-43 yrs), elderly (68-86 yrs)	Hippocampus Temporal cortex	mRNA	mRNA	↓ FL.TrkB mRNA in hippocampus & cortex ↓ T.TrkB mRNA in hippocampus No change in T.TrkB in cortex ↓ BDNF mRNA in cortex No change in BDNF mRNA in hippocampus
Rage et al. (2007) **[359]**	Male SD rats	Neonatal & postnatal P0, P7 P14, P21, P30; 2-4, 10, & 22-24 mos	Pituitary	mRNA & protein	mRNA & protein	↓ FL.TrkB and T.TrkB protein ↑ BDNF mRNA No change in FL.TrkB or T.TrkB mRNA No change in BDNF protein
Chapman et al. (2012) **[346]**	Male F344/Brown Norway F1 hybrid rats	3 & 24 mos	Hippocampus	*N/A*	mRNA	↓ BDNF mRNA in CA1 and CA3 No change in dentate gyrus
Calabrese et al. (2013) **[347]**	Male Wistar Han rats	3, 12, & 18 mos	Hippocampus Prefrontal cortex	Protein	mRNA & protein	↓ BDNF mRNA & protein in both regions ↓ TrkB protein in both regions
Perovic et al. (2013) **[348]**	Wistar rats	6, 12, 18, & 24 mos	Hippocampus Cortex	Protein	mRNA & protein	↑ BDNF mRNA in cortex ↓ BDNF mRNA in hippocampus ↓ FL.TrkB protein in both regions No change in mature BDNF protein ↓ proBDNF protein in cortex ↑ proBDNF protein in hippocampus
Tong et al. (2015) **[360]**	Adult cats	Young (1-3 yrs), old (10-13 yrs)	Lateral geniculate nucleus	Protein	Protein	↓ BDNF protein ↓ TrkB protein

related motor decline [357]. The authors concluded that these findings could inform biomarker discovery efforts for neurological conditions involving BDNF dysfunction. In a separate study published in 2018, Ihara and colleagues [361] assessed the methylation status of the *BDNF* gene in peripheral blood from healthy adult women and discovered that *BDNF* DNA methylation is changed significantly with age, which is in agreement with evidence obtained from human postmortem frontal cortex. In discussing the relevance of this work, the authors reasoned that DNA methylation of the *BDNF* gene in peripheral blood may be a useful predictor of aging-related neurological disease. In another recent investigation using an *in vitro* model of aging in basal forebrain cholinergic neurons, Shekari and Fahnestock [362] showed that retrograde axonal transport of BDNF and proNGF is diminished in cultured neurons expressing an aging phenotype. Interestingly, expression of TrkA and TrkB receptors, but not $p57^{NTR}$, is also downregulated in this *in vitro* model. Ultimately, the authors reasoned that these changes could explain the unique vulnerability of this neuronal population in aging-related neurodegenerative diseases such as AD. Lastly, in a study examining the effects of viral vector-mediated BDNF delivery to the hypothalamus in middle-aged mice, McMurphy and colleagues [363] demonstrated that BDNF treatment prevented aging-associated metabolic decline and reduced anxiety-like and depression-like behaviors, indicating that hypothalamic BDNF delivery may be a useful target for promoting healthy aging. Collectively, these studies highlight the translational significance of studying BDNF as it relates to aging. Therefore, continued investigation of the potential interactions between aging and BDNF function/dysfunction is highly warranted.

BDNF and PD

Changes in BDNF expression and function may also offer clues regarding PD pathogenesis. For instance, BDNF protein and mRNA are decreased in the SNc and serum of Individuals with PD compared to healthy controls, and serum BDNF levels are correlated with the severity of PD symptoms [54, 55, 364, 365]. Furthermore, inhibiting nigral BDNF production with antisense oligonucleotides causes loss of SNc DA neurons in a rat model, resulting in a parkinsonian phenotype [366], and BDNF overexpression in the SNc recovers motor behavior in an aged, parkinsonian rat model [367]. In addition, pathogenic α-syn mutations linked with familial PD are associated with the loss of BDNF production in glioma cell lines [368].

BDNF also plays a crucial role in controlling dendritic spine density and synaptic plasticity in striatal MSNs, the principal cell population in the striatum [321, 322, 369, 370], as well as promoting the survival, maturation, and maintenance of postnatal MSNs [371]. Importantly, the striatum does not manufacture BDNF and instead relies heavily on anterograde transport of BDNF primarily from the cortex and the SNc [372, 373] **(Figure 2.8)**, in contrast to the classical retrograde model of neurotrophin transport (reviewed in [374]). In PD, it is well-known that DA depletion causes significant structural alterations and spine loss in MSNs, resulting in significant pathological consequences [375]. However, it remains unclear how aging- and PD-associated changes in BDNF activity might further impact MSN spine density, synaptic plasticity, and overall function of the basal ganglia.

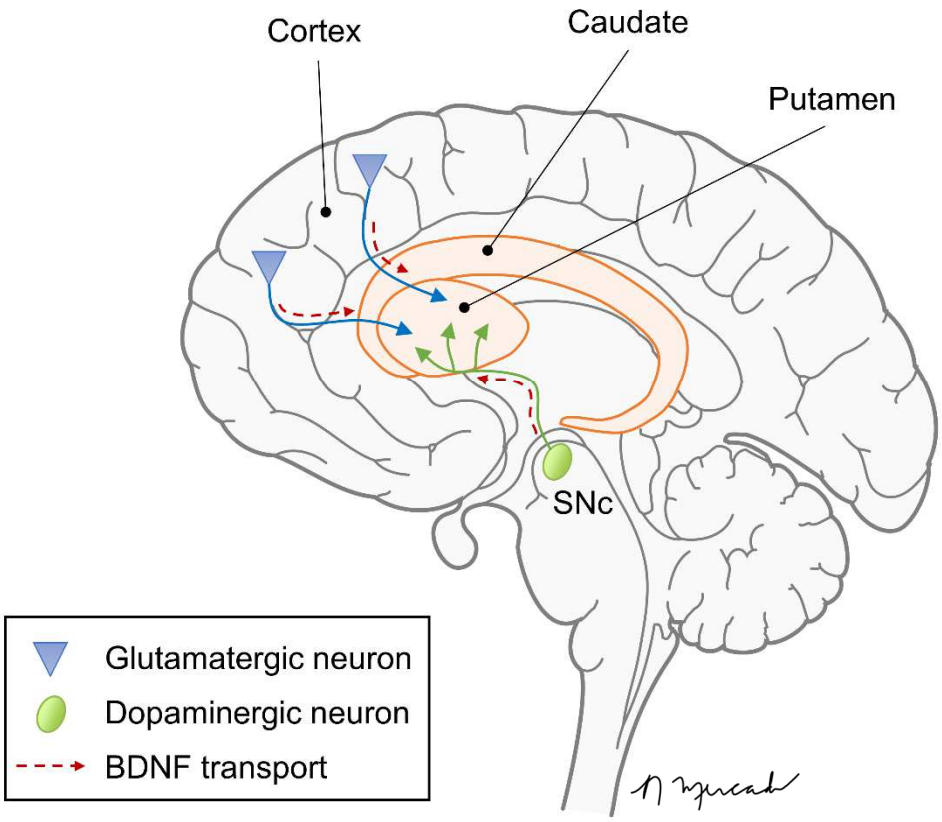

Figure 2.8 Anterograde BDNF transport to the striatum
Simplified sagittal view of anterograde BDNF transport to the dorsal striatum in the human brain. According to the classical view of neurotrophin transport, neurotrophins are produced and secreted by postsynaptic cells, then endocytosed at the axon terminal of the presynaptic neuron and retrogradely transported to the neuronal cell body. In the striatum, however, BDNF is anterogradely transported primarily from corticostriatal (glutamatergic) and nigrostriatal (dopaminergic) afferents. The proper function of this afferent supply of BDNF is crucial for survival, maturation, and maintenance of striatal medium spiny neurons, as BDNF is not synthesized in the striatum. Anterogradely transported BDNF is also a key player in striatal dendritic spine density and synapse maturation. BDNF transport is indicated by red dashed arrows. Abbreviations: BDNF, brain-derived neurotrophic factor; SNc, substantia nigra pars compacta

The Val66Met *BDNF* Variant

Considering the impressive array of BDNF function in neuronal survival and maintenance, synaptic plasticity, and energy homeostasis, as well as its association with PD, it is logical to assume that mutations in the *BDNF* gene would have far-reaching consequences and could have the potential to impact disease progression and response to therapeutics in persons with PD. To this end, one of the most frequently studied *BDNF* variants is the Val66Met SNP (reference SNP accession #: rs6265). The Val66Met SNP consists of a valine to methionine amino acid substitution at codon 66 (G196A) in the prodomain region of the protein coding exon in the *BDNF* gene **(Figure 2.9)**. It is common in the human population, with a global prevalence of approximately 15-20%, though this estimate varies considerably between populations (e.g., minor allele frequency of <5% in African populations and as high as 72% in East Asian populations [376, 377]).

The functional consequences of the Val66Met *BDNF* variant were partially elucidated for the first time in 2003. Using cultured rat hippocampal neurons transfected with a Val66 or Met66 *BDNF* construct fused to green fluorescent protein (GFP), Egan and colleagues [378] demonstrated that the Val66Met SNP reduces depolarization-dependent release of BDNF protein, while BDNF expression levels and constitutive BDNF release remain unaffected. Furthermore, Egan et al. showed that Met66-BDNF fails to localize to dendrites and synapses, suggesting that the intracellular sorting of Met66-BDNF to secretory vesicles of the regulated pathway is impaired [378]. Subsequently, in an elegant series of experiments, Chen et al. [293, 379, 380] demonstrated that: (1) cultured neurons, but not nonneuronal cells, exhibit abnormal

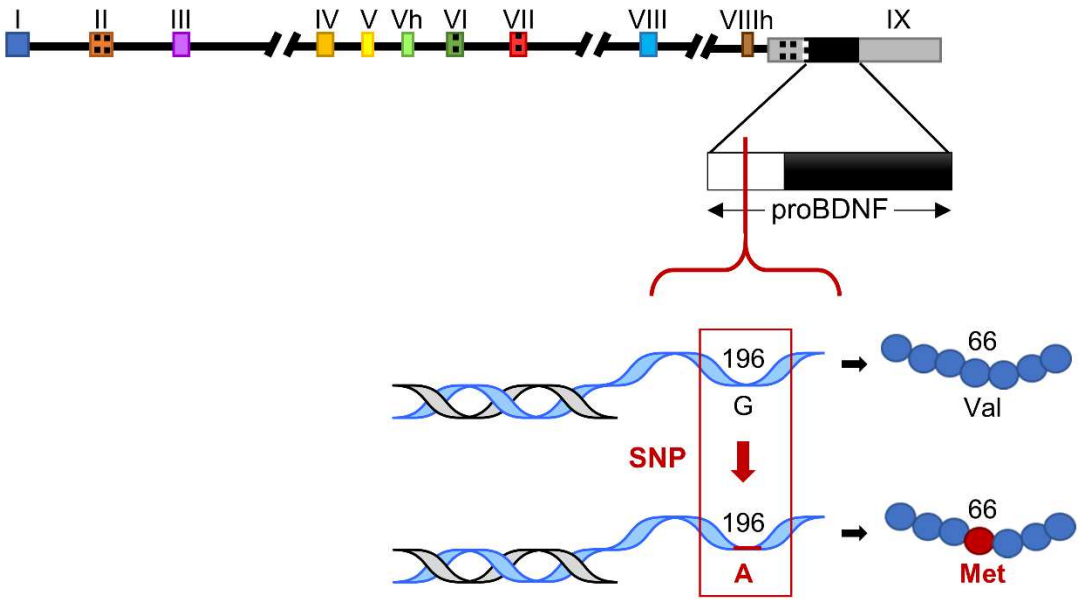

Figure 2.9 The Val66Met single nucleotide polymorphism in the *BDNF* gene
The Val66Met SNP consists of a valine to methionine substitution at codon 66 (G196A) in the prodomain region within the protein coding exon of the *BDNF* gene. This SNP impairs proBDNF sorting into synaptic vesicles of the regulated pathway, effectively decreasing activity-dependent secretion of BDNF. Val66Met is also associated with a variety of clinical phenotypes and has been implicated in patient response to levodopa therapy for Parkinson's disease. The human *BDNF* gene is shown. The portions of the gene corresponding to the prodomain and mature BDNF are represented by the white box and black box, respectively. Adapted from [381]. Abbreviations: A, adenine; BDNF, brain-derived neurotrophic factor; G, guanine; Met, methionine; SNP, single nucleotide polymorphism; Val, valine

Met66-BDNF trafficking, (2) Met66-BDNF forms heterodimers with Val66-BDNF in cultured heterozygous cortical neurons, thus impairing intracellular trafficking of the wild-type protein, (3) sortilin is responsible for intracellular sorting of BDNF to the regulated pathway by binding to the BDNF prodomain, and (4) the Val66Met SNP is associated with reduced hippocampal volume and reduced dendritic arbor complexity in a Val66Met mouse model. Furthermore, using cultured hippocampal and cortical neurons from the Val66Met mouse, Chen and colleagues confirmed that activity-dependent, but not constitutive, BDNF release is reduced in both heterozygous and homozygous Met allele carriers [380]. They speculated that this diminution of BDNF secretion was due to inefficient sortilin-mediated binding to the Met66 BDNF prodomain, thus impairing sorting of BDNF to regulated secretory vesicles. In accordance with the reduction in available BDNF in the synapse, this SNP was also shown to impair NMDA receptor-mediated synaptic plasticity in the medial prefrontal cortex, hippocampus, central amygdala, and dorsal striatum [382-386]. Moreover, a transcriptome profiling analysis of the Val66Met mouse model revealed that the Val66Met variant is also associated with an array of region-specific and gene-dose dependent gene expression changes in structures such as the hippocampus, prefrontal cortex, and amygdala [387].

Clinically, Val66Met is associated with anxiety and depression [380, 388, 389], obesity [390-392], eating disorders [393-395], bipolar disorder [396-398], schizophrenia [399], and episodic memory impairment [378, 400], and has also been implicated in aging-associated memory impairment [401] and cognitive decline in PD [249, 402]. However, the clinical association with some of these phenotypes has been challenged in several meta-analysis studies [403, 404]. Interestingly, the Val66Met SNP has also

been implicated in improved recovery from stroke and traumatic brain injury (TBI) [405-407], preservation of gray matter volume and cognitive function in multiple sclerosis patients [408], and protection against the decline of motor and psychomotor cognitive functions in individuals with the autoimmune disease, systemic lupus erythematosus [409]. The protective effects of the Val66Met *BDNF* SNP are incompletely understood; however, the growing collection of evidence indicating a protective role for the Val66Met SNP in various neurological conditions could explain the significant prevalence of this genetic mutation in the human population.

Val66Met has also been investigated in the context of pharmacogenetics. For example, the effects of the variant Met allele on patient response to antidepressants and antipsychotics have been investigated in recent studies with mixed results [410-416]. In PD, Val66Met is associated with milder clinical symptoms in early-stage, unmedicated individuals [247] and later disease onset in cases of familial PD [242], and may also be associated with earlier onset of LID in medicated patients [243, 417] (though this finding has not been consistent among studies [246]). Perhaps most significantly, this SNP has also been shown to diminish the therapeutic efficacy of “gold standard” oral levodopa therapy in persons with early-stage PD [241].

Notably, the current dogma dictates that the Val66Met *BDNF* variant is not associated with risk for developing PD [418-420]. However, the evidence supporting this contention is mixed, and a recently published analysis investigating the risk of developing levodopa-induced motor complications in association with several genetic polymorphisms revealed that heterozygous rs6265 status was associated with a 6-fold higher risk of developing PD in a cohort of individuals with idiopathic PD compared to

controls [248]. Overall, given the conflicting evidence among these studies and the significant variability in frequencies of the variant rs6265 Met allele between populations [376, 377], it will be important to consider individual patient characteristics such as ethnicity and the presence of other risk factors when interpreting results of risk association studies between this SNP and PD. For instance, one meta-analysis found no association between the Val66Met variant and PD risk in all study subjects, but a significant association was revealed when participant ethnicity was taken into account [421]. Ethnicity-specific analysis did not present significant associations in all studies, however [420], thus highlighting the importance of considering interactions with additional individual characteristics among study participants.

While considerable effort has been devoted to determining PD risk associated with the Val66Met SNP, and the pharmacogenetic effects of Val66Met on levodopa efficacy and disease progression in individuals with PD are just beginning to be elucidated, the impact of this *BDNF* variant on determining clinical outcomes for other PD therapeutics remains unexplored. Indeed, while variability in clinical outcomes has been observed for levodopa treatment, it has also been observed in clinical response to experimental DA cell replacement therapy. As discussed above, the efficacy of DA cell replacement in early clinical trials for PD was highly variable; while some patients experienced significant motor improvement that lasted for years, others experienced limited, or even no, motor benefit, and a significant subpopulation of patients developed the deleterious side-effect known as GID. Accordingly, given that the Val66Met SNP has been shown to impact clinical response to levodopa, I hypothesized that this *BDNF* variant also underlies the variability in clinical response to DA neuron grafting in clinical

trials for PD. Furthermore, considering that (1) a significant portion of the human population carries the Val66Met SNP, (2) a significant portion of grafted individuals with PD developed GID, and (3) BDNF plays a crucial role in regulating neuroplasticity (the aberrant alteration of which has been implicated in GID development), I also hypothesized that this SNP underlies the development of GID in grafted individuals with PD.

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CHAPTER 3: IMPACT OF THE VAL66MET *BDNF* SNP ON THERAPEUTIC EFFICACY OF DOPAMINE NEURON GRAFTING AND MECHANISMS OF BRAIN REPAIR IN MATURE YOUNG ADULT PARKINSONIAN RATS

Abstract

Prevalent in approximately 20% of the worldwide human population, the rs6265 (Val66Met) single nucleotide polymorphism (SNP) in the gene for brain-derived neurotrophic factor (BDNF) is a common genetic variant that can alter therapeutic responses in individuals with Parkinson's disease (PD). Possession of the variant Met allele results in decreased activity-dependent release of BDNF. Given the resurgent worldwide interest in neural transplantation for PD and the biological relevance of BDNF, the current studies examined the effects of the rs6265 SNP on therapeutic efficacy and side-effect development following primary dopamine (DA) neuron transplantation. Considering the significant reduction in BDNF release associated with rs6265, we hypothesized that rs6265-mediated dysfunctional BDNF signaling contributes to the limited clinical benefit observed in a subpopulation of individuals with PD despite robust survival of grafted DA neurons, and further, that this mutation contributes to the development of aberrant graft-induced dyskinesias (GID). To this end, we generated a CRISPR knock-in rat model of the rs6265 *BDNF* SNP to examine for the first time the influence of a common genetic polymorphism on graft survival, functional efficacy, and side-effect liability, comparing these parameters between wild-type (Val/Val) rats and those homozygous for the variant Met allele (Met/Met). Counter to our hypothesis, the current research indicates that Met/Met rats show enhanced graft-associated therapeutic efficacy and a paradoxical enhancement of graft-derived neurite outgrowth compared to wild-type rats. However, consistent with our hypothesis, we demonstrate that the rs6265 genotype in the host rat is strongly linked to development of GID, and that this behavioral phenotype is significantly correlated with

neurochemical signatures of atypical glutamatergic neurotransmission by grafted DA neurons. This study was published online in *Neurobiology of Disease* in November 2020 [1] and is used here in a modified format with permission from the editor.

Introduction

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While there are various therapeutic options for individuals with Parkinson's disease (PD), these therapies do not work uniformly well in all patients and eventually most are plagued with waning efficacy and significant side-effects as the disease progresses. Indeed, there is growing consensus surrounding the contention that PD is a complex and heterogeneous neurodegenerative process, the molecular underpinnings and clinical presentation of which vary greatly among individuals. For example, while the mainstay pharmacotherapy for PD, oral levodopa, is generally effective in treating the motor symptoms of PD, the clinical response can be variable. In accordance, a retrospective analysis of the Earlier vs Later Levodopa Therapy in Parkinson's disease (ELLDOPA) study reported that early-stage PD subjects receiving equivalent levodopa doses experienced a magnitude of response ranging from a 100% improvement to a 242% worsening as assessed with the United Parkinson's Disease Rating Scale part III (UPDRS-III, motor sub-score) [2]. This inherent heterogeneity is a significant hindrance to the overall therapeutic goal of "implementing safe, effective, and individually tailored interventions with minimal complications" [3] for those afflicted with PD.

In an effort to increase the number of therapeutic options for persons afflicted with PD, for more than three decades experimental therapies in the field of regenerative

medicine have examined means of restoring lost dopamine (DA) terminals within the striatum, whether through grafting replacement neurons (primary DA neurons or stem cells) [4-6] or vector-mediated delivery of trophic factors to induce terminal sprouting from remaining DA neurons [7, 8]. The approach that has had most success clinically is embryonic ventral mesencephalic (VM) DA neuron engraftment into the caudate/putamen, which clearly shows efficacy in a subpopulation of individuals with PD (e.g., [4, 9, 10]). As recently reviewed [4, 11], despite strong biological rationale, a lack of consistent benefit and the occurrence of significant graft-derived side-effects [12-17] have tempered enthusiasm regarding the clinical utility of DA neuron grafting for PD. However, after more than a decade of refinement, more rigorously designed grafting studies are ongoing or planned for the near future (e.g., [6], Clinical Trial Identifiers NCT01898390, NCT03309514, NCT03119636, NCT04146519).

While the field has gained an understanding of the role of *global* risk factors (e.g., disease severity, host age, etc.), the role that specific *genetic* risk factors might play in neural transplantation studies remains entirely unexplored. With this in mind, one approach to deconstructing the complexity of PD and response to therapy is the identification of common genetic variants that influence these variables in order to predict disease characteristics and tailor treatments to those most effective for subpopulations of patients. We recently identified one genetic variant which may prove useful in this regard. Specifically, we have found that the single nucleotide polymorphism (SNP) rs6265 in the brain-derived neurotrophic factor gene (*BDNF*) reduces the therapeutic efficacy of oral levodopa in two distinct cohorts of individuals with PD [18]. The rs6265 SNP results in a methionine (Met) amino acid substitution for

valine (Val) at codon 66 (Val66Met). The rs6265 Met allele has a prevalence of 15-20% in the general worldwide population, though estimates vary between studies and between populations (e.g., the estimated prevalence of the variant allele in African communities is <5%, whereas in East Asian populations, this estimate is as high as 72%) [19, 20]. Both the heterozygous major allele (Val/Met) and homozygous minor allele (Met/Met) of the *BDNF* SNP result in decreased activity-dependent release of BDNF by disrupting BDNF transport and packaging into secretory vesicles, whereas constitutive levels of BDNF secretion remain unaffected [21-25]. Importantly, the majority of BDNF in the adult brain is released from neurons via the regulated secretory pathway; therefore, the impact of the rs6265 SNP leads to a significant decrease in available BDNF [21, 22] in approximately 15-20% of the general human population.

The rs6265 SNP is not associated with PD incidence [26]. However, based on the prevalence of this SNP, the known influential role of BDNF on embryonic VM grafts [27-30], and the critical role that BDNF plays in promoting dendritic spine growth and formation of synapses in the central nervous system [31], we hypothesized that this genetic risk factor underlies the variability in clinical response to DA neuron grafting in individuals with PD. To examine this hypothesis, we generated a knock-in rat model of the human rs6265 *BDNF* variant and used this novel tool to characterize, for the first time, the effects of this polymorphism on the function and synaptic integration of new DA terminals into the parkinsonian striatum using neural grafting as a model system. Specifically, we were interested in testing the overarching hypothesis that the rs6265 *BDNF* variant expressed by the graft recipient is an unrecognized contributor to the lack of behavioral efficacy despite robust survival of grafted neurons and/or induction of

graft-induced dyskinesias (GID) reported in a subpopulation of individuals with PD [12-17].

In the current studies, we shifted focus from the grafted cells to the environment into which the cells are grafted. In this context, we provide novel and compelling evidence that, contrary to one aspect of our hypothesis, parkinsonian rats homozygous for the Met allele show a paradoxical enhancement of grafted DA neurite outgrowth and graft-derived efficacy despite equivalent survival of grafted DA neurons compared to wild-type rats. This enigmatic finding of enhanced functional recovery in Met allele carriers is corroborated by previous clinical and preclinical evidence [32-34]. However, consistent with our hypothesis, only Met68Met subjects exhibited an induction of GID behavior demonstrating for the first time that an individual's genetic profile, specifically the rs6265 SNP, is uniquely linked to development of aberrant behavioral side-effects following DA neuron grafting. While the mechanism(s) underlying these findings associated with the Met/Met genotype remains uncertain, the current investigation suggests that an atypical neurochemical phenotype of the grafted neurons and atypical graft-host circuitry may contribute to GID expression.

Methods

(Reproduced with permission from Mercado et al [1])

Animals

Rats were derived from a heterozygous female Sprague-Dawley rat (CD® International Genetic Standardization Program, Charles River Laboratories, Wilmington,

MA, USA) carrying the valine to methionine polymorphism (Val68Met) in the rat *Bdnf* gene (GenBank: NM_001270630; Ensembl: ENSRNOG00000047466). Note that rats have two additional threonine amino acids at positions 57 and 58, making Val68Met equivalent to the human Val66Met SNP, and that the rat *Bdnf* gene is 96.8% homologous with the human *BDNF* gene (BLAST queries: P23560 and P23363). This *Bdnf* knock-in rat model was generated, under contract and guidance by our group, by Cyagen Biosciences (Santa Clara, CA, USA) using CRISPR/Cas-mediated homologous recombination **(Supplementary Figure 3.11)**. Cas9, guide RNA (gRNA) targeting vector (target sequence: 5'-GCACGTGATCGAAGAGCTGCTGGATG-3'; gRNA sequence: 3'-GCAGGGACCGACTGTGAAAACTCGTGCAC-5'), and a Val68Met template donor (template sequence: 5'-ACGTCCCTGGCTGACACTTTTGAGCACATGATCGAAGAGCTGCTGGATGA-3') were injected into zygotes to generate the Val68Met Sprague-Dawley rat **(Supplementary** Figure 3.11**a,b)**. Polymerase chain reaction followed by DNA sequencing (DNA sequencing primer: 5'-AGGTCTGAAATTACAAGCAGATGG-3') were performed to confirm that the founder female rat was carrying the valine to methionine polymorphism **(Supplementary Figure 3.11c,d)**. Next, the founder female was bred once with a wild-type Val68Val male Sprague-Dawley rat to generate heterozygous Val68Met offspring of both sexes. Subsequent breeding of heterozygous Val68Met rats produced offspring of all genotypes (wild-type Val68Val, heterozygous Val68Met, and homozygous Met68Met) for colony maintenance and experimentation. Offspring were genotyped using a custom Taqman® SNP genotyping assay **(Supplementary Figure 3.11e)**. Furthermore, we confirmed that *in vitro* BDNF release was reduced in cultured neurons

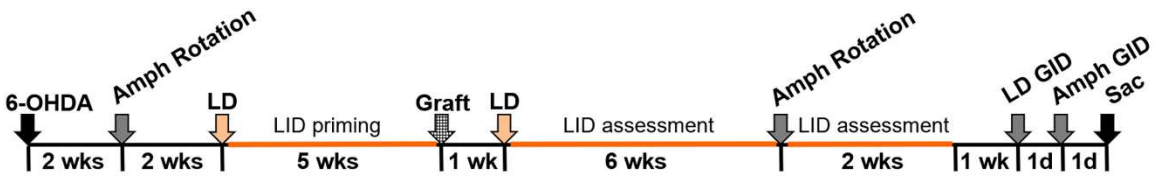
from Met68Met rats without altering total BDNF brain tissue content **(Supplementary Figure 3.12)**. Colonies of Val68Val, Val68Met, and Met68Met rats were established with no breeding issues. Both heterozygous Val68Met and homozygous Met68Met rats are viable for at least 22 months, the longest we have aged them.

For the current experiments, mature young adult male rats (6 m.o. at time of lesion and hereafter referred to as “young”; *N* = 24 rs6265 rats, *N* = 23 wild-type rats) were produced in-house within our Val68Met colony as described above. In these initial proof-of-principle studies, wild-type (Val68Val) and homozygous SNP (Met68Met) rats were used to maximize the probability of observing an effect associated with the variant allele. Rats were housed on a 12-hour light/dark cycle (lights on at 06:00) and given access to food and fresh water *ad libitum.* Rats were housed two per cage until initiation of levodopa treatment and dyskinesia rating when they were then individually housed with environmental enrichment to allow accurate behavioral assessment in their home cage. All experimental procedures were approved by the Michigan State University Institutional Animal Care & Use Committee. Further, "principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed, as well as specific national and international laws in accordance with the ethical standards established by the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Experimental Design Overview

Briefly, as shown in the experimental timeline **(Figure 3.1)** and detailed in the paragraphs below, rats were rendered unilaterally parkinsonian via stereotaxic injection

a



b

HOST Genotype	GRAFT Genotype	Final *N*
WT	WT	5
WT	Sham	6
Met68Met	WT	9
Met68Met	Sham	8

c

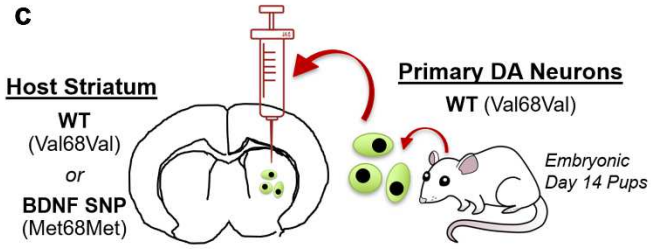


Figure 3.1 Experimental design
(a) Experimental timeline of surgical procedures, behavioral evaluation, and drug treatment. **(b)** Experimental groups and final group size "*N*" upon completion of the study. **(c)** Schematic depicting the design of experiments. Ventral mesencephalic tissue was dissected from wild-type embryonic day 14 Sprague-Dawley rat pups, dissociated, then transplanted into wild-type Val68Val and homozygous Met68Met rats. Figure reproduced with permission from Mercado et al [1]. Abbreviations: 6-OHDA, 6-hydroxydopamine; Amph GID, amphetamine-mediated GID assessment; Amph rotation, amphetamine rotation behavioral assessment; BDNF, brain-derived neurotrophic factor; DA, dopamine; GID, graft-induced dyskinesias; LD, levodopa; LD GID, levodopa-mediated GID assessment; LID, levodopa-induced dyskinesias; sac, sacrifice; SNP, single nucleotide polymorphism; WT, wild-type

of 6-hydroxydopamine (6-OHDA). Two weeks following 6-OHDA surgery, lesion success was verified with amphetamine-induced rotational behavior. Two weeks later, rats were primed with daily levodopa to induce stable levodopa-induced dyskinesias (LID). After 5 weeks of levodopa priming, all rats received an intrastriatal graft of embryonic VM DA neurons from wild-type (Val68Val) rats or a cell-free sham graft. Levodopa was withdrawn for one week following graft surgery, after which levodopa treatment was reinitiated. Parkinsonian rats were evaluated for amelioration of LID behavior for 9 weeks following engraftment. At 7 weeks post-engraftment, amphetamine-induced rotational behavior was assessed once again as a secondary measure of graft function. As an indicator of graft dysfunction, GID were evaluated following the last week of LID assessment (i.e., 10 weeks post-grafting). Following the conclusion of the study, all rats were genotyped to confirm Val68Met SNP genotype.

Nigrostriatal 6-OHDA Lesion Surgery

Rats were anesthetized with inhalant isoflurane (2-3%; Sigma, St. Louis, MO, USA) and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Each rat received 4 µl of 6-OHDA (5mg/ml; 2 µl at each site) using a 5 µl Hamilton syringe with a 26-gauge needle. 6-OHDA was administered at a flow rate of 0.5 µl/min into the substantia nigra pars compacta (SNc; 4.8 mm posterior, 1.7 mm lateral, 8.0 mm ventral, relative to bregma) and the medial forebrain bundle (MFB; 4.3 mm posterior, 1.6 mm lateral, 8.4 mm ventral, relative to bregma). These lesioning parameters result in greater than 90% SNc DA neuron death, which is required in this model to produce reliable LID and significant parkinsonian motor deficits [35-38]. After surgery completion,

rats received carprofen (as Rimadyl®; 5 mg/kg) as analgesic treatment. Nigral lesion status was confirmed histologically postmortem with medial terminal nucleus DA cell enumeration, as described previously [39].

Amphetamine-Induced Rotational Behavior

Amphetamine-induced rotational behavior is a reliable measure of nigrostriatal DA depletion and graft function (e.g., [16, 40-42]). Accordingly, amphetamine-induced rotational asymmetry was assessed 2 weeks following lesion surgery to confirm successful lesion status, and again at 7 weeks post-engraftment as a secondary endpoint to assess DA neuron graft function per [40]. Rats were injected with amphetamine sulfate [5 mg/kg, intraperitoneal (i.p.)] and rotational behavior was monitored using an automated Rotometer System (TSE-Systems, Chesterfield, MO, USA) for 90 min. Rats rotating at a rate of ≥7 ipsilateral turns per minute over 90 min 2 weeks post-lesion were included for further study.

Levodopa Administration

Beginning 4 weeks after 6-OHDA surgery, rats were primed for 5 weeks with once daily (M-Fr) injections of levodopa plus benserazide [12 mg/kg, 1:1; subcutaneous (s.c.)]. Levodopa was withdrawn for one week following graft surgeries to prevent any potential toxic interaction of the drug with grafted cells [40, 43] and was then reinitiated and continued daily (M-Fr) throughout the remainder of the experiment **(Figure 3.1)**.

Levodopa-Induced Dyskinesia Rating

LID are abnormal involuntary movement (AIM) side-effects of levodopa therapy (e.g., [44]). In the current studies we have used the well-validated rat model of LID as our primary indicator of graft function because this complex behavioral malady can be ameliorated by DA neuron grafts in parkinsonian rats [16, 45-48] and individuals with PD [49]. To date, all individuals that have received a DA cell graft have been on long-term daily levodopa replacement therapy. For these reasons, behavioral assessment of LID was the primary behavioral endpoint used in our recent study demonstrating discordance between DA graft survival and behavioral efficacy in aged parkinsonian rats [40].

In the current study, LID were evaluated on pre-graft days 1, 6, 12, 20, and 25, and at five post-graft timepoints (weeks 3, 5, 6, 8, and 9 post-engraftment). AIMs induced by levodopa were rated according to a LID severity rating scale for rats developed in our laboratory based on specific criteria reflective of the nature and occurrence of multiple behavioral attributes of dyskinesia as previously detailed (e.g., [46, 50, 51]). On behavioral rating days, food and water were removed from the home cage to prevent interference or distractions from these sources during behavior evaluation. LID behavior was evaluated in one-minute intervals at 20, 70, 120, 170, and 220 minutes following s.c. levodopa/benserazide (12 mg/kg, 1:1). All rats were rated by the same blinded investigator throughout the duration of the study.

Preparation of Donor Tissue

VM tissue containing developing A8–A10 DA cell groups was dissected from wild-type (Val68Val) embryonic day 14 (E14) Sprague-Dawley dams. The VM tissue was collected in cold calcium–magnesium free (CMF) buffer, then immediately dissociated into a homogenous cell suspension, as described previously [40]. Briefly, dissected tissue was incubated in CMF buffer containing 0.125% trypsin in a bead warmer set to 37°C for 10 min. Next, the cells were triturated in 0.005% DNase using a Pasteur pipette of 2.0 mm tip diameter, followed by a sterile 3cc 22-gauge syringe. The resulting suspension was carefully layered into a conical tube containing 5 ml sterile fetal bovine serum, then pelleted by centrifugation at 190 × g for 10 min at 4°C. The pellet was resuspended in 1.0 ml of Neurobasal™ medium (Gibco®; Thermo Fisher Scientific, Waltham, MA, USA). The trypan blue exclusion test was used to estimate cell number and viability. Final suspensions were prepared at a density of 33,333 cells/µl. Cells were kept on wet ice during transplantation surgery and used within 4 h of preparation. Cell-free Neurobasal™ medium was used for sham grafts.

Cell Transplantation

After 5 weeks of levodopa priming, rats were assigned to DA graft and sham graft groups such that the mean pre-graft LID severity scores were statistically similar between groups. Rats designated to the DA graft group received an intrastriatal transplantation of 200,000 VM cells from E14 timed pregnant wild-type donors. This number of cells is known to result in robust behavioral improvement in amphetamine rotational asymmetry and amelioration of LID severity in young, wild-type parkinsonian

rats [40, 46]. Cells were deposited at a single rostral-caudal striatal site (0.2 mm anterior, 3.0 mm lateral, relative to bregma), distributed along three dorsal-ventral coordinates at this site corresponding to 5.7, 5.0, and 4.3 mm ventral to the bottom edge of the skull [40]. Each dorsal-ventral coordinate was injected with 2 µl (0.5 µl/min) of the VM cell suspension for a total volume of 6 µl. Sham grafted rats received a total of 6 µl of cell-free vehicle using the same stereotaxic coordinates. The needle was left in place for 4 min following the last injection of cells or cell-free media before being retracted. Levodopa treatment was discontinued for 1 week post-grafting to prevent any potential toxic interaction of the drug with grafted cells [40], after which levodopa treatment was reinitiated.

Graft-Induced Dyskinetic Behavior

To provide a comprehensive assessment of GID behavior, which is an indicator of graft *dysfunction* in both individuals with PD and parkinsonian animal models, we employed two previously established approaches for modeling this malady: levodopa- and amphetamine-mediated GID. In rats, levodopa-mediated GID behaviors, similar to GID in individuals with PD, are novel (not present prior to grafting) and focal (generally localized as orolingual and forelimb stereotypy) dyskinetic behaviors that develop with graft maturation as detailed previously [16, 46, 47, 50].

The alternative approach of amphetamine-mediated GID capitalizes on the finding that DA grafted, but not sham grafted, rats display dyskinetic behavior in response to low-dose amphetamine, which appears phenotypically similar in appearance to LID [52-56]. To assess amphetamine-induced GID, rats received a single

dose of amphetamine sulfate (2 mg/kg, i.p.) and were then returned to the home cage for behavioral assessment. The resulting dyskinetic behavior was evaluated by a blinded investigator using the same method and rating scale described above for LID rating.

Both levodopa- and amphetamine-mediated GID were examined after the final week 9 post-grafting LID assessment (i.e., week 10 post-grafting), with amphetamine-mediated GID rated 24 hours after levodopa-mediated GID.

Necropsy

Rats were deeply anesthetized with phenytoin/pentobarbital euthanasia solution (250 mg/kg pentobarbital, i.p.; VetOne, Boise, ID, USA) then perfused intracardially with 200 ml room temperature heparinized 0.9% saline followed by 200 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and post-fixed in 4% paraformaldehyde for 24 hours at 4°C, submersed in 30% sucrose solution, then stored at 4°C until time of sectioning. Brains were sectioned coronally at 40-µm thickness using a sliding microtome and stored in a cryoprotectant solution at -20°C. Ear clippings were collected during necropsy and stored at -80°C for automated genotype confirmation (Transnetyx Inc., Cordova, TN, USA).

Tyrosine Hydroxylase Immunohistochemistry

A 1:6 series of 40-µm sections through the rostrocaudal extent of the brain was used for tyrosine hydroxylase (TH) immunolabeling. All steps were performed at room temperature. Sections were rinsed thoroughly in tris-buffered saline containing 0.3%

Triton X-100 (TBS-Tx) before incubating in 0.3% hydrogen peroxide for 15 min, then blocking in 10% normal goat serum (NGS) for 90 min. The sections were then incubated with rabbit anti-TH primary antibody **(Table 3.1)** for 24 hours. The next day, sections were incubated with biotinylated goat anti-rabbit secondary antibody with 1% NGS (Vector Laboratories, Inc., Burlingame, CA, USA; Cat No. BA-1000) for 90 min, then developed with avidin/biotin enzyme complex (Vector Laboratories, Inc., Burlingame, CA, USA; Cat No. PK6100) and 3,3'-diaminobenzidine (DAB; 0.5 mg/ml).

Stereological Quantification of Graft Cell Number & Graft Volume

A blinded investigator quantified TH-immunoreactive (THir) cells in the grafted striatum using the Stereo Investigator® Optical Fractionator workflow for total enumeration estimation (MBF Bioscience, Williston, VT, USA), similar to what we have previously reported [40]. Briefly, THir cells were systematically counted within a 200 µm × 200 µm counting frame superimposed on a 200 µm × 200 µm grid using a 20x objective (numerical aperture 0.75) on a Nikon Eclipse 80i microscope. This was completed in 5-7 serial (1:6) TH-immunolabeled sections throughout the striatum. The optical disector height was 22 µm, with guard zones of 2.5 µm.

The same sections used for graft cell counts were used for quantification of graft volume. Graft volumes were estimated using the Cavalieri Estimator probe from Stereo Investigator®. Contours were traced around the central portion of the graft containing THir cell bodies (**Figure 3.4e**, central black oval) in serial tissue sections by a blinded investigator. The Cavalieri probe was applied, superimposing a grid of randomly placed

Antigen	Assay	Primary Antibody	Vendor	Catalog Number	Dilution	Secondary Antibody [a]
Tyrosine Hydroxylase	TH-VGLUT2-PSD95	rabbit anti-TH	Millipore	AB152b	1:4000	SuperBoost™
Tyrosine Hydroxylase	TH-Synaptopodin and TH-VGLUT1-PSD95	mouse anti-TH	Millipore	Mab318	1:4000	SuperBoost™
Synaptopodin	TH-Synaptopodin	rabbit anti-synaptopodin	Synaptic Systems	163002	1:4000	A21207
PSD95	TH-VGLUT1/2-PSD95	mouse anti-PSD95 IgG2a	LSBio	C150376	1:1000	A21241
VGLUT2	TH-VGLUT2-PSD95	mouse anti-VGLUT2 IgG1	Abcam	Ab79157	1:300	2045303
VGLUT1	TH-VGLUT1-PSD95	mouse anti-VGLUT1 IgG1κ	BioLegend	821302	1:1000	2045303

Table 3.1 Targeted antigens and corresponding antibodies
[a] NOTE: Secondary antibody catalog numbers are Alexa Fluor®-conjugated, purchased from Invitrogen®. Table reproduced with permission from Mercado et al [1].

sampling sites (50-μm spacing) over the contours. Graft volume data are expressed as total estimated volume corrected for over-projection (mm^3).

Stereological Quantification of Neurite Outgrowth

Graft-derived innervation of the host striatum was measured stereologically via the Stereo Investigator® Spaceballs workflow. For each animal, the TH-immunolabeled tissue section containing the largest portion of the graft was chosen for analysis. Rectangular contours measuring 345 μm × 265 μm were drawn around regions of interest both proximal and distal to the graft, in all directions relative to the graft (i.e., medial, dorsal, lateral, ventral to the graft), for a total of eight contours. The “proximal” region was defined as a distance of 100-500 μm from the edge of the graft, with 0-100 μm from the graft serving as a “buffer zone.” Similarly, the “distal” zone was defined as 700-1100 μm from the edge of the graft, with 500-700 μm serving as an additional “middle buffer” zone. The Spaceballs workflow was applied to the contours, using systematic random sampling of sites within a grid superimposed over the contours. The probe was spherical with a radius of 5.0 μm and guard zones of 1.0 μm. Additional sites were sampled in the intact striatum using two contours of the same dimensions described above. All neurite density measurements were collected by a blinded investigator using the 60x oil immersion objective (numerical aperture 1.40) on a Nikon Eclipse 80i microscope. Data are expressed as estimated neurite length per probe volume ($\mu m/mm^3$).

Brightfield *In Situ* Hybridization

To examine the impact of rs6265 on host striatal mRNA for the BDNF receptor, tyrosine receptor kinase B (TrkB), we performed *in situ* hybridization (ISH) for *Trkb* mRNA on 1-2 tissue sections per animal at 40-µm thickness using the manual RNAscope® 2.5 HD assay (Advanced Cell Diagnostics Inc., Hayward, CA, USA) according to manufacturer instructions, then counterstained with cresyl violet. Images (2880 × 2048) were acquired in the dorsolateral striatum adjacent to the graft using the 20x objective on a Nikon Eclipse Ni microscope, maintaining identical light settings across all images. The images were imported into the image visualization and analysis software, Imaris® (v. 9.3.1, Oxford Instruments) using the ImageJ (FIJI) extension. The Imaris® spots function was used to reconstruct *Trkb* mRNA puncta in a two-dimensional field of view, using the same parameters for all images, and the resulting data were exported. Data are represented as the average of values collected from two to four images per striatal hemisphere.

Immunofluorescence

In each immunofluorescent assay that was performed, 1-2 representative tissue sections were used per animal. Full series tissue sections containing DAB-developed TH as described above were used as guides when choosing striatal tissue sections for immunofluorescence studies, as we endeavored to select sections that contained a central portion of the DA graft in each grafted animal. To fluorescently label mRNA targets **(Table 3.2)**, the manual RNAscope® Multiplex Fluorescent V2 assay was used with Opal dyes (Akoya Biosciences, Marlborough, MA, USA) according to manufacturer

RNA Target	Probe	Accession Number	Catalog Number
Vglut2	Rn-Slc17a6	NM_053427.1	317011
Tph2	Rn-Tph2	NM_173839.2	316411
Bdnf	Rn-Bdnf-CDS	NM_012513.4	409031

Table 3.2 mRNA targets and corresponding RNAscope® probes
Table reproduced with permission from Mercado et al [1].

instructions. With regard to *Bdnf* mRNA*,* the *Bdnf* gene is transcribed into a variety of mRNA transcripts containing different 5' untranslated regions that regulate regional-, stimulus-, and cell type-specific expression of the final protein product encoded by a single protein coding exon [57, 58]. Accordingly, to simplify *Bdnf* mRNA detection, the ISH probe used in the current study was designed to target only the protein coding sequence (RNAscope® Probe Rn-Bdnf-CDS, Cat No. 409031). Following RNAscope®, immunofluorescent staining for TH protein was completed using the Alexa Fluor™ 488 Tyramide SuperBoost™ kit (goat anti-mouse IgG; Invitrogen®; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions to enhance TH fluorescence and produce bright, distinct THir fibers.

For protein-only immunofluorescent assays (no mRNA), Alexa Fluor™ 488 Tyramide SuperBoost™ kits (goat anti-rabbit IgG and goat anti-mouse IgG) were used first with TH primary antibodies pertinent to each assay **(Table 3.1)**. Additional target antigens were labeled as follows after completion of the enhancement step. Briefly, TH-enhanced tissue was rinsed in TBS-Tx, then blocked in 10% serum for 1 hour at room temperature. Next, the tissue was incubated with primary antibodies pertinent to each assay (Table 1) for 24 hours at room temperature (except for synaptopodin (SP), which was incubated for 48 hours at 4°C). The tissue was then incubated with the corresponding Alexa Fluor® secondary antibodies (1:400 dilution; **Table 3.1**) for 90 min at room temperature in the dark, then mounted onto 2% subbed slides and coverslipped with Vectashield® anti-fade mounting medium (Vector Laboratories Inc., Burlingame, CA, USA).

Fluorescent Image Acquisition

Confocal images (1024 × 1024) were acquired on a Nikon A1 laser scanning confocal system equipped with a Nikon Eclipse Ti microscope and Nikon NIS-Elements AR software (v. 5.02). For synaptic characterization experiments, z-stacks were acquired through the entire thickness of the mounted tissue sections using the 60x oil-immersion objective (numerical aperture 1.40) with a digital zoom of 1.67x for a final magnification of 100x. A z-step of 0.3 µm was used per [59] with a scan speed of ⅛ frame/sec. In the synaptic characterization analyses using TH, postsynaptic density protein 95 (PSD95), and vesicular glutamate transporters 1 and 2 (VGLUT1 and VGLUT2) described below, two images (125 µm × 125 µm) were collected in the striatum proximal to the lateral edge of the graft. The “proximal” region was defined as described above. In the TH-SP analysis, two images were collected using these same parameters, except one image was acquired in the “proximal” zone while the second image was acquired in the “distal” zone lateral to the graft border, as described above. In all experiments, additional images were collected in the intact contralateral striatum using the same parameters. Images of intact striatum were captured in striatal regions comparable to those of images collected in the grafted striatum.

For general mRNA detection and cell counts, z-stacks of the entire graft region were acquired using a 10x or 20x objective (numerical aperture 0.45 or 0.75, respectively), with a z-step of 2 µm and a scan speed of ⅛ frame/sec. In most cases, multiple images were collected in order to capture the entire graft region present in the striatal tissue section.

Imaris® Fluorescent Image Quantification

All fluorescent images were analyzed with Imaris® software (v. 9.3.1, Oxford Instruments, Bitplane Inc., Concord, MA, USA).

Dual-Label Protein Analysis: TH & SP

Three-dimensional (3D) z-stacks of tissue immunolabeled for TH and SP proteins were imported into Imaris® and converted into native Imaris® file format. The surface function was used with semi-automatic thresholding to create an accurate 3D reconstruction of TH fibers within each image. Then, the spots function was used to reconstruct SP puncta, using the same parameters across all images. The MATLAB® "Find Spots Close to Surface" Imaris® XT plugin was then applied to the TH surface, setting the distance of putative TH-SP synapses to 0.6 μm per [59]. Data are represented as the number of SP puncta located ≤ 0.6 μm from TH fibers and normalized to TH surface volume (μm^3).

Dual-Label Protein & mRNA Analysis: TH Protein and Vglut2 mRNA

Z-stacks of tissue labeled for TH protein and *Vglut2* mRNA were imported into Imaris® and a TH surface was created manually using the marching cubes function so that all THir cell bodies were accurately reconstructed with a 3D surface object. Next, the spots function was used to reconstruct *Vglut2* mRNA puncta, using the same parameters across all images. A binary mask was applied to the TH surface. The *Vglut2* mRNA spots were then filtered using the masked channel so that only *Vglut2* mRNA puncta inside TH surface objects were included in the analysis. Finally, the MATLAB® "Split into Surface Objects" plugin was applied to the filtered spots and the resulting

data were exported. In cases where multiple images were acquired to include the entire graft region, the data are expressed as the sum of the values from all images collected per animal. Care was taken to ensure that the same cells were not counted twice in adjacent images. Only THir cells with ≥ 2 *Vglut2* mRNA puncta were included for analysis.

Triple-Label Protein Analysis: TH, VGLUT2, & PSD95 Proteins

Z-stacks of tissue immunolabeled for TH and VGLUT2 (presynaptic markers) combined with PSD95 (postsynaptic marker) were imported into Imaris®. The surface function was used with semi-automatic thresholding to create accurate 3D reconstructions of TH fibers and PSD95. The spots function was applied to reconstruct VGLUT2 protein puncta within each image, using the same parameters across all images. Next, a binary mask was applied to the TH surface object. PSD95 surfaces were filtered using the masked channel to obtain PSD95 surfaces located outside of the TH surface (PSD95(out)). Similarly, VGLUT2 spots were filtered using the masked channel to select VGLUT2 spots with centers located inside the TH surface (VGLUT2(in)). Then, the MATLAB® "Find Spots Close to Surface" plugin was used to find VGLUT2(in) ≤ 0.6 μm from PSD95(out). Finally, the MATLAB® "Distance Transformation" plugin was used to find PSD95(out) surfaces located ≤ 0.6 μm from the TH surface. Data are represented as the number of VGLUT2(in) puncta, number of VGLUT2(in)-PSD95(out) appositions, and total volume of PSD95(out) surfaces, all normalized to TH surface volume (μm^3).

Triple-Label Protein Analysis: TH, VGLUT1, & PSD95 Proteins

Z-stacks of tissue immunolabeled for TH, VGLUT1, and PSD95 proteins were imported into Imaris® and deconvolved using Imaris ClearView^{TM} Deconvolution to improve image clarity (iterative algorithm with 10 iterations, pinhole = 15.3 µm, specimen refractive index = 1.37, distance from coverslip = 7.97 µm). Next, 3D surface objects of TH and PSD95 were created using semi-automatic thresholding, as described above. The spots function was used to create VGLUT1 protein puncta, maintaining the same parameters across all images. As before, a binary mask was applied to the TH surface object, and VGLUT1 spots were filtered using the masked channel to find VGLUT1 puncta located outside of the TH surface (VGLUT1(out)). Similarly, the PSD95 surface objects were filtered by the masked channel to find PSD95 structures located inside the TH surface (PSD95(in)). Then, the MATLAB® "Find Spots Close to Surface" plugin was used to find VGLUT1(out) located ≤ 0.6 µm from the TH surface and VGLUT1(out) located ≤ 0.6 µm from PSD95(in). Data are represented as the number of VGLUT1(out) puncta near (≤ 0.6 µm) the TH surface and the number of VGLUT1(out)-PSD95(in) appositions, all normalized to TH surface volume (μm^3).

Dual-Label Protein & mRNA Analysis: TH Protein & Tryptophan Hydroxylase 2 (Tph2) mRNA

Z-stacks of tissue labeled for TH protein and *tryptophan hydroxylase 2* (*Tph2)* mRNA (i.e., an isozyme of tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of serotonin (5-HT)) were imported into Imaris®. Surface objects for TH protein and *Tph2* mRNA (which presented as a soma-filling, rather than punctate, stain) were created manually with the marching cubes function so that all cell bodies

containing *Tph2* mRNA or TH protein were accurately reconstructed in 3D. The number of surface objects for each cell type was recorded. Because of limited amount of striatal tissue containing grafted neurons and extensive analyses done in our study, data are expressed as the number of *Tph2* mRNA-containing cells (i.e., 5-HT neurons) relative to the number of THir cells (i.e., DA neurons) in each image (*Tph2*/TH ratio) in 2 striatal sections. Care was taken to ensure that the same grafted cells were not counted twice in adjacent images.

Dual-Label Protein & mRNA Analysis: TH Protein & Bdnf mRNA

Because of limited amount of striatal tissue containing grafted neurons and the extensive analyses done in our study, tissue from only 3 Val68Val subjects was available for examining *Bdnf* mRNA in grafted DA neurons, while grafted tissue from 9 Met68Met subjects was available for this assay. Z-stacks of tissue immunolabeled for TH protein and *Bdnf* mRNA were imported into Imaris®. Surface objects for TH were created manually with the marching cubes function so that all THir cells were accurately reconstructed in 3D. Next, the spots function was used to reconstruct *Bdnf* mRNA puncta, using the same parameters across all images. The MATLAB® "Split into Surface Objects" plugin was applied to the *Bdnf* mRNA spots and the resulting data were exported. As described above, in cases where multiple images were acquired to include the entire graft region, the data are expressed as the sum of the values from all images collected per animal. Care was taken to ensure that the same grafted cells were not counted twice in adjacent images. Only THir cells with ≥ 2 *Bdnf* mRNA puncta were included for analysis.

BDNF Release and Tissue Content in rs6265 Rats

BDNF Release in Hippocampal Cultures

Timed pregnant (embryonic day 18) female Val68Val or Met68Met rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.). Hippocampi were dissected using a Leica dissecting microscope and pooled in cold, sterile, CMF buffer (pH 7.3). Cell suspensions were prepared through a series of CMF rinses, incubation in 0.125% trypsin for 10 minutes at 37°C, rinsing in CMF again, and trituration in 0.004% DNase to disperse the cells into solution. Trypan blue was used to assess cell viability. Cell suspensions of $\geq$95% viability were plated at a density of 1,000,000 cells/well on poly-D-lysine coated 6-well plates in Neurobasal™ medium supplemented with B27.

For determining basal BDNF release, 72 hours after plating, all culture media was removed and replaced with artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose). Thirty minutes later aCSF was collected for analysis of BDNF via ELISA as per manufacturer's instructions (BDNF Emax ELISA, Promega, Madison, WI) using samples loaded in triplicate.

BDNF Tissue Content in rs6265 Rats

For determination of BDNF tissue content, tissue punches from the hippocampus, striatum, and M1 cortex were collected from 3 m.o. male Val68Val, Val68Met, and Met68Met rats. Samples were thawed on ice and 250 µl of RIPA Lysis Buffer System was added to each sample (sc-249-48, Santa Cruz Biotechnology, Dallas, TX). Samples were homogenized by sonication on ice, followed by a 30-minute incubation after which a portion of the sample was taken for total protein determination

via Pierce BCA Assay (ThermoFischer, Waltham, MA) per manufacturer's instructions. The remaining sample was centrifuged and the supernatant collected. A BDNF ELISA was completed on triplicate samples as per manufacturer's instructions. BDNF levels in tissue lysates (picogram (pg)) were calculated per milligram (mg) total protein.

Statistical Analysis

All LID and GID behavioral data were analyzed by non-parametric statistics including Kruskal-Wallis with Dunn's multiple comparison tests or Mann-Whitney *U* tests (between subject comparisons) and Friedman tests with Dunn's multiple comparison tests (within subjects comparisons). Amphetamine rotations were analyzed using an unpaired two-tailed *t*-test with Welch's correction to account for unequal variances and one-way ANOVA with Šídák's multiple comparisons test.

Unpaired two-tailed *t*-tests were used to compare the following data between genotypes: grafted DA neuron cell counts, graft volumes, 5-HT/DA neuron ratios, *Vglut2* mRNA expression in naïve SNc and ventral tegmental area (VTA) separately, and DA neuron *Bdnf* mRNA expression (expressed as percentage of total DA neurons). Unpaired one-tailed *t*-tests were used to compare neurite outgrowth in each direction (dorsal, ventral, medial, lateral) surrounding the DA graft between genotypes. An unpaired two-tailed *t*-test with Welch's correction was used to analyze the level of *Bdnf* transcript in grafted DA neurons.

Two-way ANOVAs were used to compare *Vglut2* mRNA expression between naïve and grafted animals of both genotypes (2 × 2: genotype × treatment) and VGLUT2 protein content in DA neuron fibers (2 × 3: genotype × treatment), combined

with Šídák's multiple comparisons test and Tukey's multiple comparisons test, respectively. Two-way repeated measures ANOVAs were used with Šídák's multiple comparisons test to analyze the following data: grafted neurite outgrowth (2 × 2: genotype × distance from graft), neurite density vs intact contralateral striatum (2 × 2: genotype × treatment), proximal and distal TH-SP contact densities within each genotype (2 × 2: genotype × distance from graft), PSD95 volume near DA fibers (2 × 2: genotype × treatment), VGLUT1-PSD95 appositions (2 × 2: genotype × treatment), VGLUT1 input onto DA neurons (2 × 2: genotype × treatment), total VGLUT1 innervation (2 × 2: genotype × treatment), and *Trkb* mRNA expression in host striatum (2 × 2: genotype × treatment).

A one-way mixed-effects model with repeated measures was used with the post-hoc Holm-Šídák's multiple comparisons test to analyze distal neurite density separated by region for each genotype. TH-SP contact density comparisons between lesioned and intact striatum, and between genotypes, were analyzed using a two-way mixed-effects model with repeated measures and Šídák's and Dunnett's multiple comparisons tests (2 × 3: genotype × treatment). VGLUT2(in)-PSD95(out) apposition data were analyzed using a two-way mixed-effects model with repeated measures (2 × 2: genotype × treatment) and Šídák's multiple comparisons test.

Non-parametric Spearman correlation tests were used for all correlations with LID and GID behavior. Correlations with amphetamine rotational behavior were analyzed using Pearson correlation. Statistical outliers, though uncommon, were identified using ROUT and Grubbs' outlier tests. Parametric statistical tests were chosen for analysis only

when data met assumptions for normality and homogeneity of variances. All statistical analyses were completed using GraphPad Prism® software for Windows (v. 8.4.2).

Results

(Reproduced with permission from Mercado et al [1])

Met68Met rats show enhanced behavioral efficacy compared to wild-type Val68Val rats

We hypothesized that as a consequence of reduced BDNF release in Met68Met rats, these subjects would experience reduced graft-mediated improvement of LID. In contrast to this hypothesis, Met68Met rats showed enhanced functional benefit from VM DA grafts compared to wild-type subjects as indicated by a more rapid and overall greater decrease in LID severity. Specifically, parkinsonian DA grafted Met68Met rats showed a significant reduction in LID severity compared to DA grafted Val68Val and sham grafted subjects beginning 5 weeks post-grafting (**Figure 3.2a**; Week 5: p = 0.0015 Met68Met-DA vs Sham, p = 0.0302 Met68Met-DA vs Val68Val-DA; Week 6: p = 0.0284 Met68Met-DA vs Sham, p = 0.0475 Met68Met-DA vs Val68Val-DA; Week 8: p = 0.0198 Met68Met-DA vs Sham; Week 9: p = 0.0003 Met68Met-DA vs Sham). In contrast, the DA grafted Val68Val rats required 8 weeks to display a similar level of reduction in LID severity; however, a statistically meaningful reduction was not apparent until 9 weeks post-engraftment (**Figure 3.2a,c**; p = 0.0312 Val68Val DA graft; p = 0.0078 Met68Met DA graft; p=0.0078 sham graft; vs pre-graft baseline, Wilcoxon matched-pairs signed rank test). At the final 9 week post-graft time point, the DA grafted

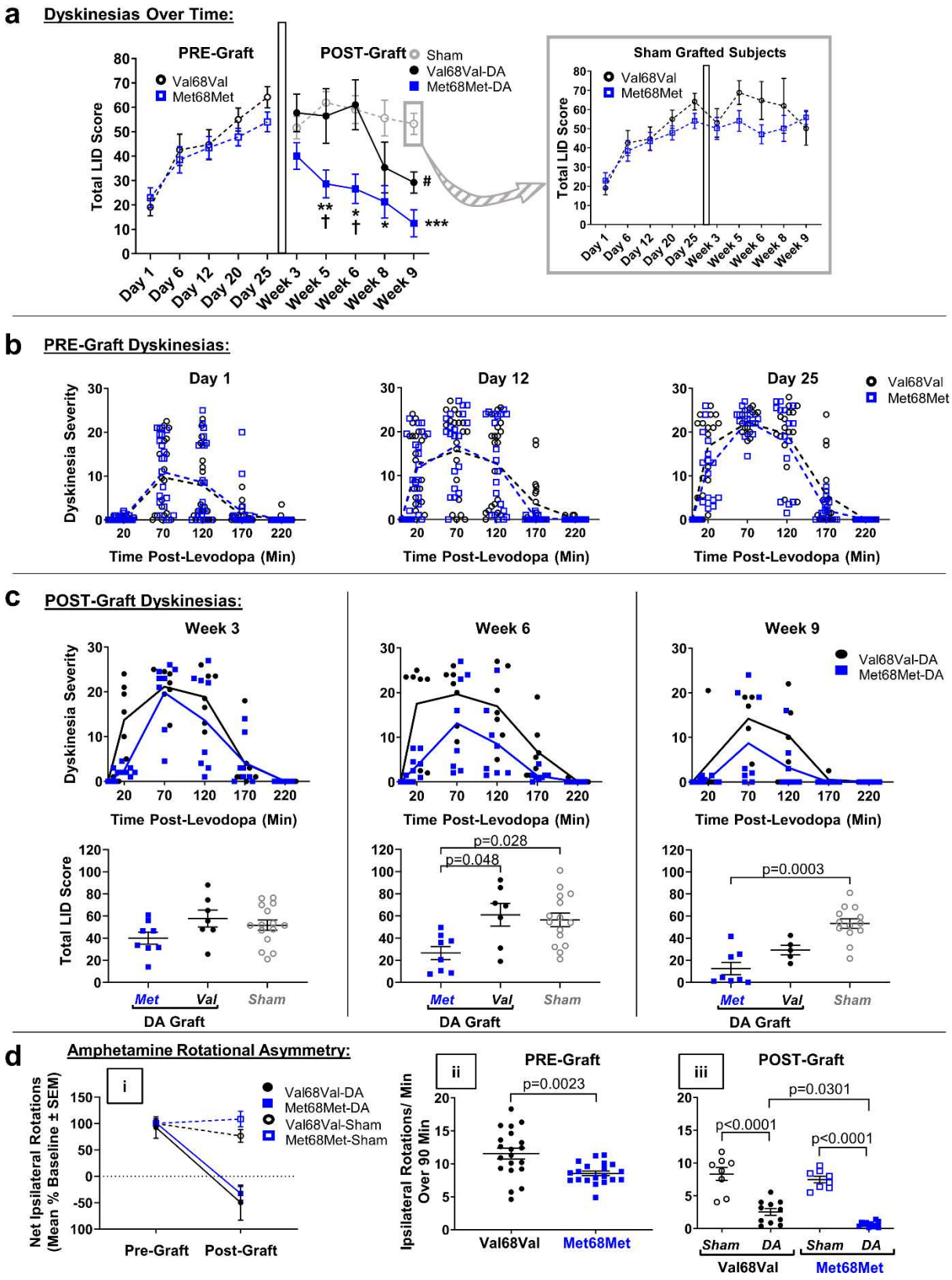


Figure 3.2 Behavioral measures of graft functional efficacy

Figure 3.2 (cont'd)
(a) Total LID score (primary behavioral endpoint) for Val68Val and Met68Met rats throughout levodopa priming (pre-graft period) and for 9 weeks post-engraftment. Dyskinesia severity scores were not significantly different between genotypes in sham grafted rats; thus, sham subjects were combined into one group post-graft. Inset graph depicts total dyskinesia score over time for sham grafted subjects, separated by genotype. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test at each time point. Week 5: **p = 0.0015 Met68Met-DA vs Sham, †p = 0.0302 Met68Met-DA vs Val68Val-DA; Week 6: *p = 0.0284 Met68Met-DA vs Sham, †p = 0.0475 Met68Met-DA vs Val68Val-DA; Week 8: *p = 0.0198 Met68Met-DA vs Sham; Week 9: ***p = 0.0003 Met68Met-DA vs Sham. Sham groups were not significantly different at any of the post-graft time points ($p \geq 0.12$ for all time points post-graft). **(b)** Pre-graft LID severity time course showing individual animal responses during levodopa priming days 1, 12, and 25. Rats were rated at 20, 70, 120, 170, and 220 mins post-levodopa each day. **(c)** LID severity time course and total LID scores showing individual animal responses at weeks 3, 6, and 9 post-engraftment. Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test at each time point. **(d)** Amphetamine rotational asymmetry (secondary behavioral endpoint), measured at 7 weeks pre-graft and 7 weeks post-graft. Data are expressed as net ipsilateral rotations (i) and ipsilateral rotations per min over 90 mins (ii-iii). Unpaired t-test with Welch's correction (ii) and one-way ANOVA with Šídák's post-hoc test (iii). Figure reproduced with permission from Mercado et al [1]. Abbreviations: LID, levodopa-induced dyskinesia; DA, dopamine graft; CCW, counterclockwise

Met68Met rats showed a 73.92 ± 12.51% (mean ± SEM) reduction and the DA grafted Val68Val rats a 55.21 ± 2.22% reduction in total LID severity **(Figure 3.2a,c)**.

While it has been reported that individuals with PD carrying the Met allele show a significantly higher risk of developing LID earlier in the time course of treatment [60], to the best of our knowledge no information is available on whether this results in enhanced severity over time. In contrast to this clinical report, we found no significant impact of genotype on LID in the sham grafted rats using high dose levodopa (Kruskal-Wallis, $p \geq 0.12$ for all time points post-engraftment). Accordingly, for the post-graft time period, the sham groups were combined.

Evidence of enhanced benefit in DA grafted Met68Met compared to Val68Val rats was also observed using amphetamine-induced rotational asymmetry examined at 7 weeks post-engraftment (one-way ANOVA with Šídák's post-hoc test, $p = 0.0301$; **Figure 3.2diii**). Interestingly, while there was a difference in pre-graft amphetamine rotational rate with Met68Met rats showing significantly fewer rotations per minute than Val68Val rats (**Figure 3.2dii**; $p = 0.0023$, Val68Val vs Met68Met, Unpaired *t*-test with Welch's correction), there was no significant difference between genotypes in the sham grafted rats at the 7 week post-graft timepoint **(Figure 3.2diii**; $p = 0.5620$, Val68Val vs Met68Met; two-way ANOVA with Šídák's multiple comparisons test). Of note was the homogeneity of response to amphetamine, both pre- and post-graft, in Met68Met subjects **(Figure 3.2dii, iii)**.

The Met allele is associated with the development of GID in response to levodopa and amphetamine

In keeping with our hypothesis that Met68Met rats would experience elevated graft-derived side-effects, GID behavior was manifest only in Met68Met rats under the current grafting protocol which resulted in wide-spread striatal reinnervation [46] **(Figure 3.3a-d)**. There was a single Val68Val DA grafted rat that displayed a low level of amphetamine-mediated GID and a moderate level of levodopa-mediated GID. While both of these behaviors have been reported to be uniquely associated with DA grafting in parkinsonian rats [16, 46, 47, 50, 52-56], we provide here the first direct comparison of these behavioral assays. While both methods of GID assessment support that the Met risk allele is associated with induction of aberrant GID-like behaviors, based on differences in their phenotype (see "Graft-Induced Dyskinetic Behavior" section above) it is not necessarily surprising that there is a lack of correlation of these behaviors with each other **(Figure 3.3e)**. Future investigations are warranted for understanding mechanistic differences between amphetamine- and levodopa-mediated GID behaviors in this rat model of neural grafting.

Despite equal numbers of surviving grafted DA neurons, graft-derived reinnervation is more extensive in Met68Met than in Val68Val striatum

Large, THir grafts of DA neurons were observed in all VM grafted subjects, extending neurites into the surrounding striatal parenchyma **(Figure 3.4a)**. TH-immunoreactivity in the lesioned striatum is presumed to be derived from grafted DA neurons based on the fact that there was a near-complete depletion of host nigral DA

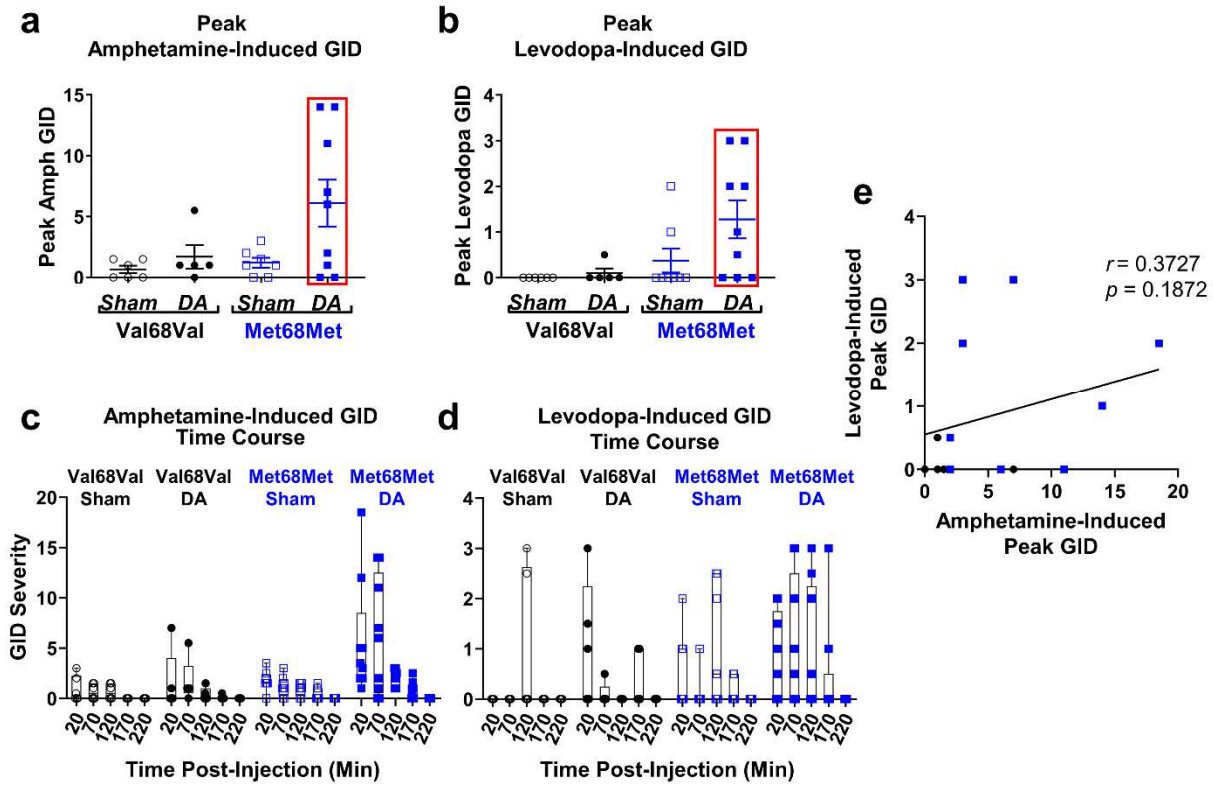


Figure 3.3 Behavioral measures of graft dysfunction
(a,b) Peak amphetamine-induced (a) and levodopa-induced (b) GID severity score at week 10 post-engraftment. Non-parametric Kruskal-Wallis test with Dunn's post-hoc comparisons. **(c,d)** Time course of amphetamine-mediated (c) and levodopa-mediated (d) GID behavior at week 10 post-engraftment. **(e)** Spearman correlation between amphetamine-mediated peak GID behavior and levodopa-mediated peak GID behavior. Figure reproduced with permission from Mercado et al [1]. Abbreviations: GID, graft-induced dyskinesia; Amph, amphetamine; DA, dopamine graft

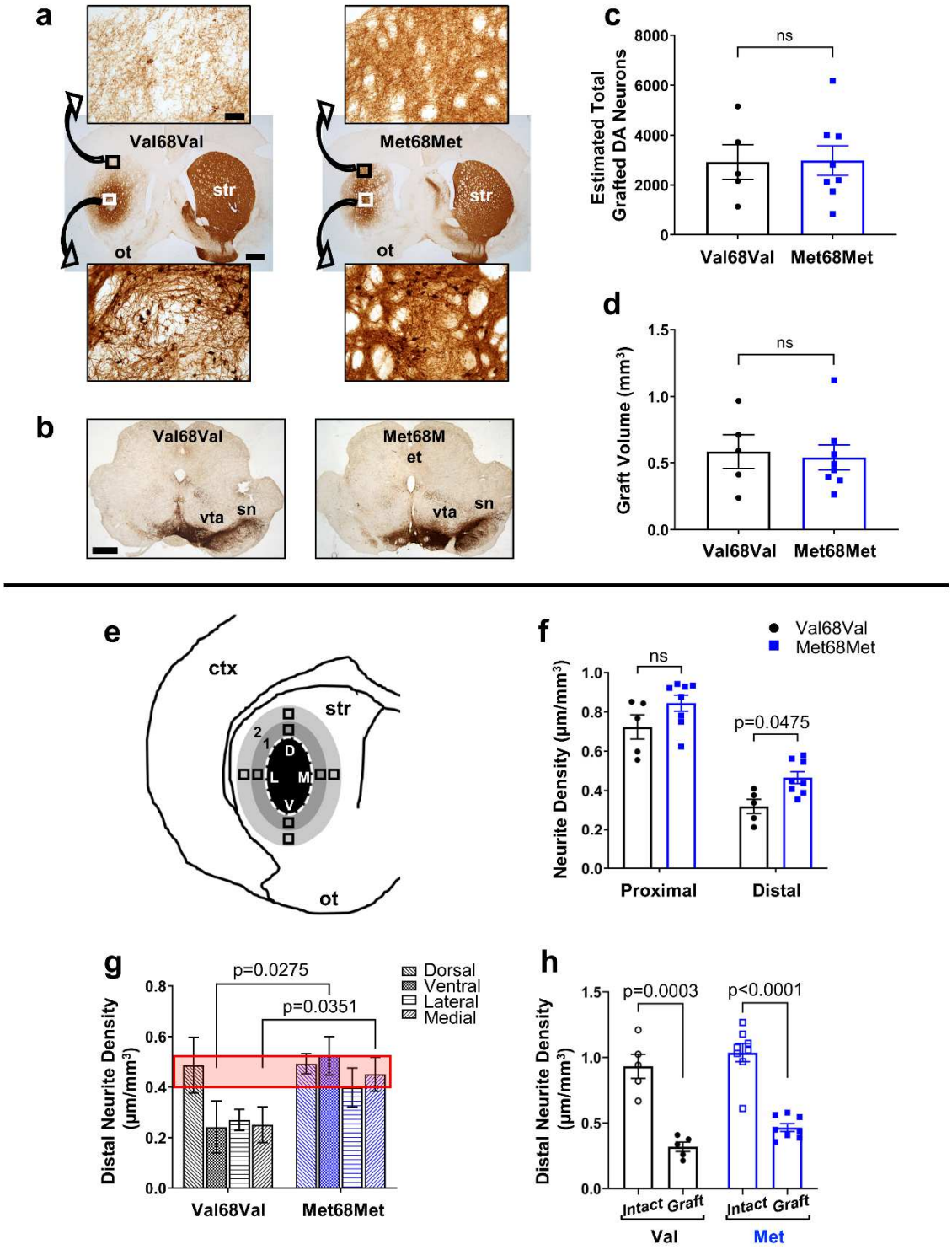


Figure 3.4 Graft histology in parkinsonian striatum
(a) Representative micrographs of THir striatum demonstrating more extensive grafted DA neurite outgrowth in Met68Met host striatum. Scale bars = 100 µm (10x micrographs), 1000 µm (1x micrographs). **(b)** Corresponding THir nigral tissue sections showing near-complete depletion of host SNc DA neurons in the lesioned hemisphere.

Figure 3.4 (cont'd)
Scale bar = 1000 μm. **(c)** Stereologically estimated total number of grafted DA neurons. Mean ± SEM. Unpaired t-test. **(d)** Stereologically estimated total graft volume. Mean ± SEM. Unpaired t-test. **(e)** Schematic illustrating fields of view used for analysis of grafted DA neurite outgrowth. Proximal and distal regions are denoted by "1" and "2," respectively. **(f)** Average grafted DA neurite density proximal and distal to the graft border. Mean ± SEM. Two-way repeated measures ANOVA with Šídák's post-hoc test. **(g)** Comparison of distal grafted DA neurite outgrowth separated into regions surrounding the graft. Mean ± SEM. Unpaired t-tests and mixed-effects model. Red bar indicates maximum and minimum neurite density means for Met68Met subjects. **(h)** Distal grafted DA neurite density compared with endogenous DA innervation of intact contralateral striatum. Mean ± SEM. Two-way repeated measures ANOVA with Šídák's post-hoc test. Figure reproduced with permission from Mercado et al [1]. Abbreviations: THir, tyrosine hydroxylase immunoreactive; str, striatum; ot, olfactory tubercle; vta, ventral tegmental area; sn, substantia nigra; ctx, cortex; D, dorsal; M, medial; V, ventral; L, lateral; 1, proximal zone; 2, distal zone; ns, not significant

neurons in animals of both genotypes (mean percent SNc DA neuron loss compared to intact hemisphere ± SEM: Val68Val: 96.31 ± 0.60%; Met68Met: 96.59 ± 0.32%; **Figure 3.4b** and **Supplementary Figure 3.13**). Stereological quantification indicated that the number of surviving transplanted DA neurons was not different between genotypes (mean estimated total number of grafted DA neurons ± SEM: Val68Val: 2922.63 ± 694.46; Met68Met: 2978.86 ± 592.43; $t(11) = 0.0604$, $p = 0.9529$, **Figure 3.4c**). Accordingly, graft volumes also did not differ significantly (mean volume ± SEM: Val68Val: 0.5835 ± 0.1254 mm^3; Met68Met: 0.5394 ± 0.0940 mm^3; $t(11) = 0.2858$, $p = 0.7803$; **Figure 3.4d**).

In contrast to what would be expected in an environment of reduced activity-dependent BDNF release, but in keeping with the functional evidence described above, stereological quantification **(Figure 3.4e)** demonstrated that neurite outgrowth was significantly more extensive in Met68Met than in Val68Val subjects (Fig. 4a, higher magnification images). While both genotypes exhibited similarly robust neurite density proximal to the graft (Fig. 4f), there was a significant impact of the Met68Met genotype on graft-derived neurite outgrowth observed at regions distal to the graft (**Figure 3.4a,f**; mean distal neurite density ± SEM: Val68Val: 0.3189 ± 0.0363 $\mu m/mm^3$; Met68Met: 0.4655 ± 0.0303 $\mu m/mm^3$; two-way repeated measures ANOVA $F(1,11) = 5.958$, $p = 0.0328$; Šídák's multiple comparisons test: Proximal: $t(22) = 2.011$, $p = 0.1103$; Distal: $t(22) = 2.424$, $p = 0.0475$). Notably, in Met68Met subjects, the more robust distal neurite outgrowth was evenly distributed in all regions surrounding the graft (i.e., dorsal, ventral, lateral, medial; mixed-effects model $F(1.168, 5.449) = 0.6657$, $p = 0.4723$; **Figure 3.4g**). This contrasts with that observed in the striatum of Val68Val subjects, where the

densest neurite growth was restricted to the dorsal striatum. While neurite density in the dorsal region of Val68Val striatum was not significantly different from other regions surrounding the graft (mixed-effects model $F(1.551, 7.756) = 1.970$, $p = 0.2037$), neurite outgrowth was significantly less in ventral and medial regions when compared to Met68Met subjects (Ventral: $t(8) = 2.244$, $p = 0.0275$; Medial: $t(9) = 2.054$, $p = 0.0351$).

In proximal regions, graft-derived reinnervation of the parkinsonian striatum was statistically similar to the intact striatum regardless of genotype (two-way repeated measures ANOVA $F(1,11) = 1.041$, $p = 0.3295$; Šídák's multiple comparisons test: Val68Val: $t(11) = 2.097$, $p = 0.1163$ vs intact; Met68Met: $t(11) = 1.007$, $p = 0.5586$ vs intact; **Supplementary Figure 3.14**). Specifically, graft-derived reinnervation proximal to the graft reached 80.98 ± 4.05% and 87.56 ± 4.49% of THir neurite density observed in the intact hemisphere for Val68Val and Met68Met rats, respectively. This was not observed distal to the graft, where THir neurite density was significantly less than that of the intact striatum for animals of both genotypes (two-way repeated measures ANOVA $F(1,11) = 73.22$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(11) = 5.650$, $p = 0.0003$ vs intact; Met68Met: $t(11) = 6.650$, $p < 0.0001$ vs intact; **Figure 3.4h**). Indeed, graft-derived reinnervation distal to the graft reached only 35.43 ± 5.56% and 48.21 ± 7.40% of that observed in the intact hemisphere in Val68Val and Met68Met rats, respectively. Notably, though distal reinnervation was significantly less than in the intact striatum for both genotypes, the percentage of reinnervation distal to the DA graft was significantly higher in Met68Met subjects ($t(11) = 3.058$, $p = 0.0109$).

Presumed graft-host synaptic connections are correlated with LID behavior in Val68Val but not Met68Met rats, despite similar contact densities

Synaptopodin/SP is an actin-associated structural protein found in mature dendritic spines [61, 62]. We used dual-label immunofluorescence (TH to label striatal DA fibers; SP to label host MSN dendritic spines) with confocal microscopy and Imaris® 3D reconstruction to quantify the number of presumed synaptic contacts formed between THir fibers and their preferential target, the dendritic spines of striatal MSNs **(Figure 3.5a,b)** per [40].

Val68Val and Met68Met rats had similar TH-SP synaptic contact densities, both proximal (TH-SP appositions per μm^3 TH ± SEM: Val68Val: 0.3532 ± 0.0840; Met68Met: 0.2731 ± 0.0230; mixed effects model $F(1,12) = 0.4762$, $p = 0.5033$; Šídák's multiple comparisons test: $t(4.606) = 0.9205$, $p = 0.7872$; **Figure 3.5c**) and distal to the graft (Val68Val: 0.4596 ± 0.1093; Met68Met: 0.3433 ± 0.0338; mixed effects model $F(1,12) = 0.4762$, $p = 0.5033$; Šídák's multiple comparisons test: $t(4.784) = 1.025$, $p = 0.7308$; **Figure 3.5c**). Moreover, the density of TH-SP appositions in the reinnervated striatum was statistically similar to that of the intact contralateral striatum, except for the proximal region in parkinsonian Met68Met striatum, which was significantly lower than that of the intact contralateral striatum (TH-SP contact density versus intact striatum; mixed-effects model $F(1.523, 16.00) = 3.649$, $p = 0.0596$; Dunnett's multiple comparisons test: Val Proximal: $q(4) = 2.464$; $p = 0.1148$; Val Distal: $q(4) = 0.0599$, $p = 0.9974$; Met Proximal: $q(6) = 3.234$, $p = 0.0313$; Met Distal: $q(6) = 1.134$, $p = 0.4642$).

As expected, TH-SP connectivity was negatively correlated with total LID severity on the final rating day in wild-type (Val68Val) rats **(Figure 3.5d)**. Specifically, Val68Val

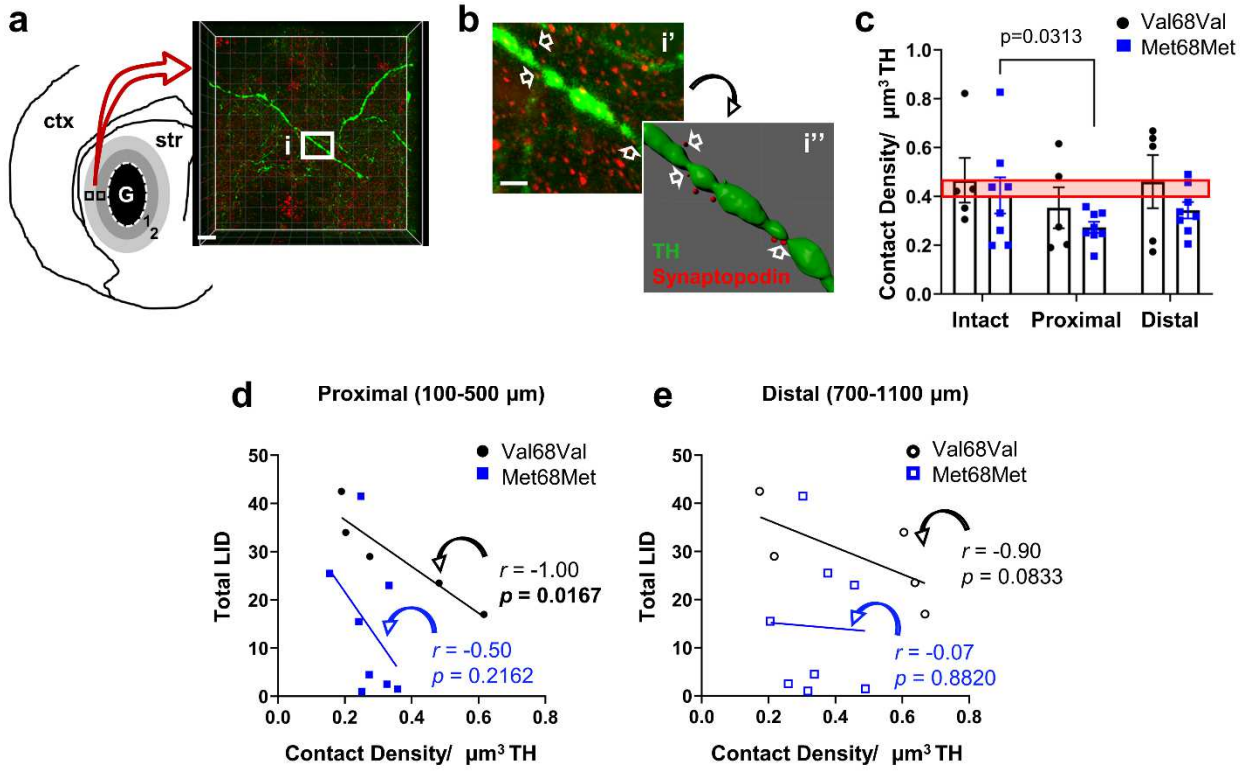


Figure 3.5 Impact of the Met allele on graft connectivity with host MSN spines
(a) Schematic representation of regions relative to the graft in which confocal images were acquired (left), and representative 3D confocal z-stack of grafted DA neurites in tissue stained for TH and synaptopodin (right). Scale bar = 10 µm. **(b)** (i') Increased magnification of micrograph in panel (a), and (i'') Imaris® 3D reconstruction of DA fiber denoted by (i) in panel (a). Scale bar = 1 µm. **(c)** Comparison of TH-synaptopodin contact density normalized to TH surface volume. Mean ± SEM. Two-way mixed-effects model with repeated measures, followed by Šídák's and Dunnett's post-hoc tests. *Red bar* indicates maximum and minimum contact density means in the intact striatum. **(d)** Spearman correlation between proximal TH-synaptopodin contact density and total LID severity score at 9 wks post-engraftment. **(e)** Spearman correlation between distal TH-synaptopodin contact density and total LID severity score at 9 wks post-engraftment. Figure reproduced with permission from Mercado et al [1]. Abbreviations: ctx, cortex; str, striatum. 1, proximal zone; 2, distal zone

subjects with more TH-SP synaptic appositions showed a greater reduction in the severity of LID behavior, consistent with previous data from our group [40]. This correlation was statistically significant for synaptic appositions located in the region with more dense reinnervation proximal to the graft (Proximal: Spearman r = -1.00, p = 0.0167; Distal: Spearman r = -0.90, p = 0.0833; **Figure 3.5d,e**). Surprisingly, there were no significant correlations of these appositions with total LID severity in Met68Met rats (Proximal: Spearman r = -0.50, p = 0.2162; Distal: Spearman r = -0.0714, p = 0.8820). Additionally, TH-SP synaptic contact density did not correlate with amphetamine-mediated GID, levodopa-mediated GID, or post-graft amphetamine-induced rotational behavior for either genotype (data not shown).

Grafted DA neurons maintain an immature phenotype, as evidenced by elevated *Vglut2* mRNA and corresponding protein expression compared to the naïve adult midbrain

Preclinical electron microscopic data from rat studies in our lab [16], together with evidence from grafted parkinsonian non-human primates [63] and postmortem clinical evidence in grafted individuals with PD [64], show that grafted DA neurons make asymmetric (presumed excitatory/glutamatergic) synapses onto unlabeled dendrites and dendritic spines in the host striatum. Accordingly, we sought to characterize the expression of vesicular glutamate transporter in embryonic DA neurons transplanted into the parkinsonian striatum by quantifying levels of *Vglut2* mRNA and protein in this cell population. We demonstrate here, to the best of our knowledge, the first evidence that grafted THir neurons show colocalization with *Vglut2* mRNA (TH+/*Vglut2*+; **Figure**

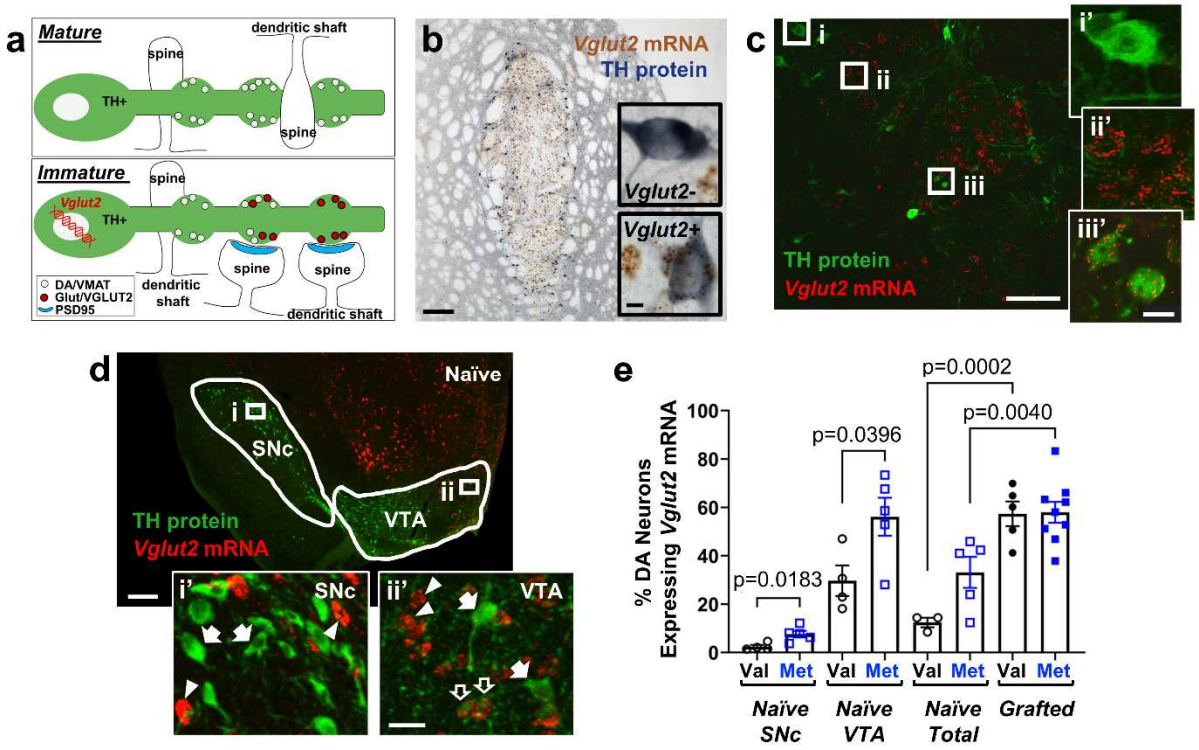


Figure 3.6 *Vglut2* mRNA expression in grafted DA neurons
(a) Schematic depicting normal mature vs immature DA neuron phenotype, based on data from [65]. **(b,c)** *Vglut2* mRNA expression in THir grafted DA neurons and unidentified grafted TH-negative cells. Cells denoted by (i-iii) in panel (c) are shown with increased magnification in (i'-iii'). Scale bars = 250 µm in panel (b) and 5 µm for insets in this panel; 100 µm in panel (c), or 10 µm for insets in this panel. **(d)** *Vglut2* mRNA in DA neurons of the naïve adult rat midbrain. DA neurons of the substantia nigra (i) and ventral tegmental area (ii) are shown with increased magnification in (i') and (ii'), respectively. *Vglut2*-negative DA neurons are indicated by solid arrows in (i') and (ii'), whereas solid arrowheads indicate cells containing *Vglut2* mRNA only, and unfilled arrows indicate cells containing colocalized TH-*Vglut2.* Scale bars = 300 µm for panel (d), and 30 µm for i' and ii'. **(e)** Percentage of DA neurons expressing *Vglut2* mRNA in naïve adult rat midbrain and grafted, parkinsonian striatum. NOTE: Grafted DA neurons are from wild-type donors. Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test (Naïve Total and Grafted); unpaired *t*-tests (naïve SNc and naïve VTA). Figure reproduced with permission from Mercado et al [1]. Abbreviations: DA, dopamine; VMAT, vesicular monoamine transporter; Glut, glutamate; VGLUT2, vesicular glutamate transporter 2; TH, tyrosine hydroxylase; SNc, substantia nigra pars compacta; VTA, ventral tegmental area

3.6b,c). We also demonstrate that VM-derived grafts contain TH-only (TH+/*Vglut2*-) neurons, and an abundance of centrally-located TH-/*Vglut2*+ cells **(Figure 3.6b,c)**. Overall, in VM grafts transplanted into animals of both genotypes, we observed TH-only, *Vglut2*-only, and combined TH+/*Vglut2*+ cells, similar to what has been observed in the naïve midbrain [66, 67] **(Figure 3.6d)**.

Remarkably, nearly 60% of grafted DA neurons contained *Vglut2* mRNA in host animals of both genotypes at 10 weeks post-engraftment (percent of *grafted* DA neurons containing *Vglut2* mRNA: Val68Val: 57.34 ± 5.13%; Met68Met: 57.96 ± 4.34%), a time at which midbrain DA neurons should be fully mature [68]. In contrast, we found that naïve (endogenous) midbrain DA neurons in both genotypes had significantly less *Vglut2* mRNA compared to grafted DA neurons (percent of *naïve midbrain* DA neurons expressing *Vglut2* mRNA: Val68Val: 12.4 ± 1.95%; Met68Met: 33.13 ± 6.42%; two-way ANOVA $F(1,18) = 38.00$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(18) = 4.996$, $p = 0.0002$, naïve vs grafted; Met68Met: $t(18) = 3.615$, $p = 0.0040$, naïve vs grafted; **Figure 3.6e**).

It is noteworthy that although *grafted* DA neurons expressed similar levels of *Vglut2* mRNA, this was not the case when comparing *endogenous* DA neurons located in the naïve midbrain. Specifically, SNc and VTA DA neurons in naïve adult Met68Met midbrain expressed significantly more *Vglut2* mRNA than their wild-type counterparts (SNc: $t(7) = 3.062$, $p = 0.0183$; VTA: $t(7) = 2.524$, $p = 0.0396$; **Figure 3.6e**).

To corroborate that grafted DA neurons maintained an immature DA/glutamate co-expression phenotype, we sought evidence of VGLUT2 protein in the grafted TH

neurites. VGLUT2 protein was indeed found to be colocalized within grafted THir neurites in the grafted parkinsonian striatum **(Figure 3.7a)**. This is in contrast to the naïve brain, where there was sparse evidence of TH-VGLUT2 colocalization in the striatum **(Figure 3.7b)**. Indeed, grafted THir neurites contained significantly more VGLUT2 protein than nigrostriatal THir fibers in the naïve striatum (two-way ANOVA $F(2,32) = 14.44$, $p < 0.0001$; Tukey's multiple comparisons test, naïve vs grafted striatum: Val68Val: $q(32) = 5.497$, $p = 0.0014$; Met68Met: $q(32) = 4.897$, $p = 0.0043$; **Figure 3.7b**). Unexpectedly, TH-VGLUT2 colocalization was also increased in the intact contralateral striatum of grafted parkinsonian rats compared to experimentally naïve animals (two-way ANOVA $F(2,32) = 14.44$, $p < 0.0001$; Tukey's multiple comparisons test, naïve vs intact striatum: Val68Val: $q(32) = 4.462$, $p = 0.0095$; Met68Met: $q(32) = 3.564$, $p = 0.0435$; **Figure 3.7b**). There were no significant differences in VGLUT2 protein content within striatal THir fibers between genotypes **(Figure 3.7b)**.

Interestingly, TH-VGLUT2 colocalization was significantly and positively correlated with amphetamine-mediated GID behavior in Met68Met subjects that displayed this aberrant graft-associated behavior (GID+ rats, i.e., total GID score >10; Spearman $r = 1.00$, $p = 0.0167$; Figure 3.7**c**). In contrast, significant correlations were not observed in Val68Val subjects that expressed very low levels of this GID behavior (Spearman $r = 0.70$, $p = 0.2333$; **Figure 3.7c**) or in the smaller subset of Met68Met subjects that did not display GID behavior (GID- rats, i.e., total GID score ≤10; Spearman $r = 0.80$, $p = 0.3333$). No significant correlations were found between TH-VGLUT2 colocalization and levodopa-mediated GID, LID, or post-graft amphetamine rotational behavior.

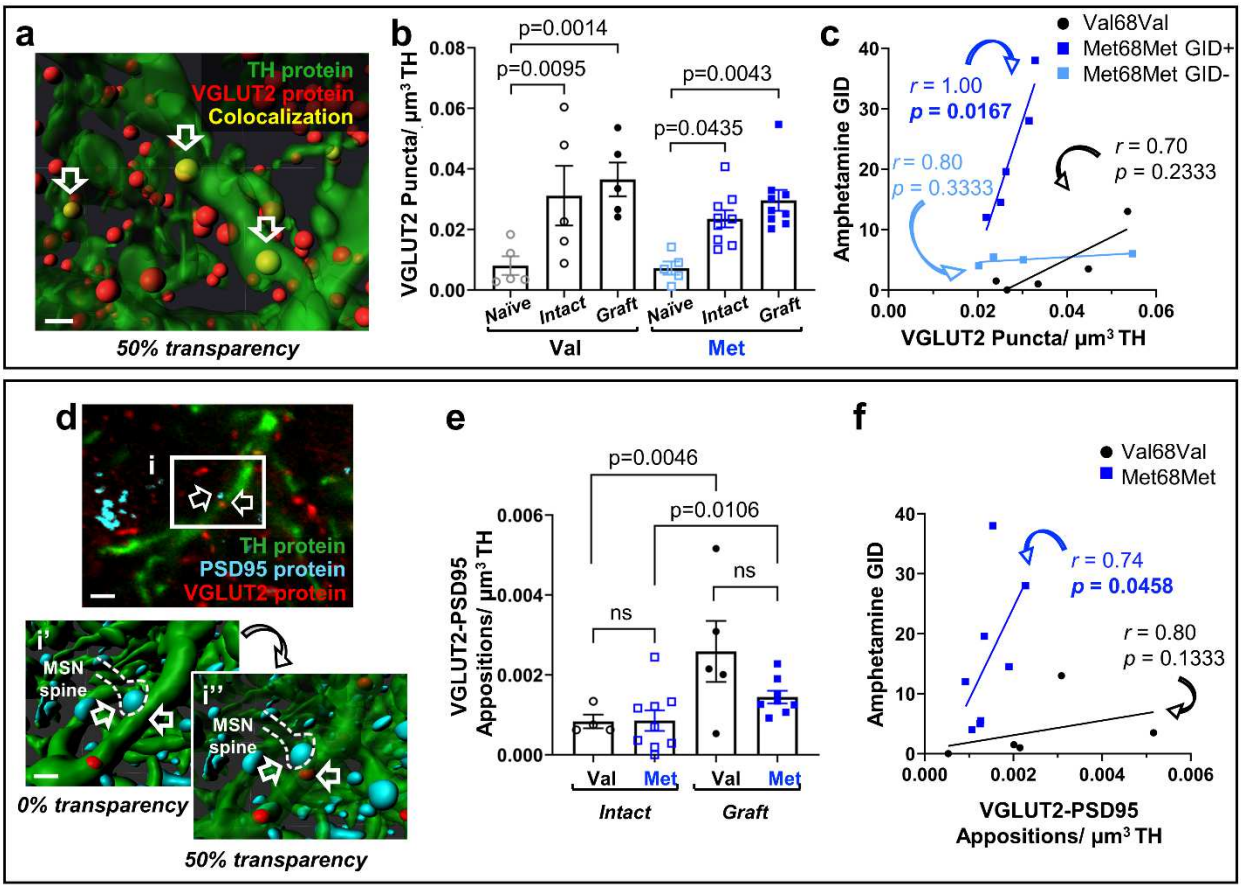


Figure 3.7 VGLUT2 protein expression in grafted THir DA fibers
(a) Computer generated Imaris® 3D reconstruction of confocal z-stack depicting VGLUT2 colocalization within grafted DA neurites. The advanced Imaris® algorithms allow visualization of fine structures (i.e., vesicles) inside cellular elements (i.e., neurites), and allows the cellular element to be visualized at varying levels of transparency (i.e., 50% shown here) to allow visualization of internal elements. Scale bar = 1 µm. **(b)** Quantification of VGLUT2 protein located within THir DA fibers, normalized to TH surface volume. TH-VGLUT2 colocalization in the striatum of naïve rats was compared with grafted DA fibers in the parkinsonian striatum and endogenous DA fibers in the intact striatum contralateral to the lesion. Mean ± SEM. Two-way ANOVAs with Tukey's post-hoc test. **(c)** Spearman correlation between TH-VGLUT2 colocalization in grafted DA neurites and total amphetamine-mediated GID score at 10 wks post-engraftment. **(d)** Confocal micrograph indicating synaptic apposition (≤ 0.6 µm) between VGLUT2 protein located inside a grafted DA neurite (indicated by open arrow on the right) and PSD95 located adjacent to the DA neurite (indicated by open arrow on the left). The presumed synapse (i) is shown with increased magnification using Imaris® 3D imaging in (i') and (i''). Scale bars = 2 µm (confocal micrograph), 1 µm (Imaris® 3D reconstructions). % transparency indicates that applied to TH fibers. **(e)** Quantification of presumed excitatory VGLUT2-PSD95 synapses made by DA neurites in the grafted striatum and intact contralateral striatum, normalized to TH surface volume. Mean ± SEM. Mixed-effects model with Šídák's post-hoc test. **(f)** Spearman correlation between presumed excitatory VGLUT2-PSD95 synapses made by DA neurites and total amphetamine-mediated GID

Figure 3.7 (cont'd)
score at 10 wks post-engraftment. Note that one outlier was removed from the Met68Met group. Figure reproduced with permission from Mercado et al [1]. Abbreviations: VGLUT2, vesicular glutamate transporter 2; PSD95, postsynaptic density protein 95; TH, tyrosine hydroxylase; MSN, medium spiny neuron; ns, not significant

Grafted DA neurons show neurochemical signatures of excitatory synapses in the parkinsonian striatum

Next, we examined whether grafted DA neurons containing VGLUT2 protein showed neurochemical evidence of atypical, presumed neurochemically active, excitatory synapses. To address this question, we used triple-label immunofluorescence for TH, VGLUT2, and PSD95, a postsynaptic scaffolding protein found in excitatory synapses and a potent regulator of synaptic strength (e.g., [69]). We define here a putative "neurochemically active" excitatory synapse as presynaptic VGLUT2 protein present *within* a THir neurite (VGLUT2(in); i.e., pre-synaptic) and in close proximity (≤ 0.6 μm) to PSD95 protein located outside of the THir fibers (PSD95(out); i.e., post-synaptic; **Figure 3.7d**) per [59]. Similar synaptic mapping approaches involving confocal microscopy and computational software paired with electrophysiology have shown correlation of these "putative" synapses with neuronal firing output [70]. In the present study, putative excitatory synapses made by DA neurons in the grafted striatum were observed in DA grafted animals of both genotypes, though the number of excitatory synapses did not differ significantly between Val68Val and Met68Met subjects (mixed-effects model $F(1,12) = 1.737$, $p = 0.2121$; **Figure 3.7e**). The number of VGLUT2(in)-PSD95(out) appositions, however, was increased in grafted striatum compared to the intact contralateral striatum in animals of both genotypes (mixed-effects model $F(1,10) = 28.70$, $p = 0.0003$; Šídák's multiple comparisons test: Val68Val: $t(10) = 4.054$, $p = 0.0046$; Met68Met: $t(10) = 3.544$, $p = 0.0106$; **Figure 3.7e**).

Our group has previously reported [16] a positive trend between atypical THir asymmetric contacts onto host striatal cells and *levodopa*-mediated GID behavior using

immunoelectron microscopy, though a statistically significant correlation was not found. In the present study, the number of THir asymmetric synaptic contacts (i.e., TH-normalized VGLUT2(in)-PSD95(out) synaptic appositions) was significantly, positively correlated with *amphetamine*-mediated GID in Met68Met subjects (Spearman $r = 0.74$, $p = 0.0458$), but not in Val68Val subjects that displayed very low levels of this behavior (Spearman $r = 0.80$, $p = 0.1333$; **Figure 3.7f**). Furthermore, in the current study, the number of THir asymmetric contacts showed a positive trend in Met68Met rats but did not significantly correlate with *levodopa*-mediated GID, similar to [16]. They also did not correlate with LID or post-graft amphetamine rotational behavior **(Supplementary Figure 3.15)**.

Postsynaptically, there was significantly more PSD95 in close proximity (≤ 0.6 μm) to THir fibers in the grafted striatum (vs intact striatum) only in Met68Met rats, indicating an increase in asymmetric synaptic contacts made by DA neurons in the grafted striatum in Met allele carriers (two-way repeated measures ANOVA $F(1,10) = 15.72$, $p = 0.0027$; Šídák's multiple comparisons test: Val68Val: $t(10) = 2.168$, $p = 0.1077$; Met68Met: $t(10) = 3.577$, $p = 0.0100$; **Supplementary Figure 3.16a**). This was found to be true despite similar total PSD95 volumes between intact and grafted Met68Met striatum (two-way repeated measures ANOVA $F(1,10) = 1.037$, $p = 0.3326$; Šídák's multiple comparisons test: $t(10) = 1.267$, $p = 0.4132$; **Supplementary Figure 3.16b**).

Atypical glutamatergic input onto striatal THir fibers is significantly increased in the grafted striatum only in Met68Met rats

Previous studies have identified unlabeled asymmetric synapses (presumed corticostriatal afferents) onto somas and dendrites of grafted primary DA neurons in the parkinsonian striatum (**Figure 3.8a**; [16, 64]). Importantly, previous evidence from our lab indicated that these atypical synaptic connections correlated with levodopa-mediated GID behavior in grafted parkinsonian rats [16]. To assess whether this phenomenon is associated with GID in Met allele-carrying subjects, we employed triple-label immunofluorescent staining for VGLUT1 (a marker for pre-synaptic corticostriatal afferents), PSD95, and TH **(Figure 3.8b,c)** and then quantified the number of these synaptic triads (VGLUT1+PSD95+TH; **Figure 3.8d**). Pre-synaptic VGLUT1 puncta (VGLUT1(out)) in close apposition (≤ 0.6 μm) to PSD95 positioned inside THir fibers (PSD95(in)), which we defined as putative excitatory synapses onto grafted DA neurons, were exceedingly rare. Though the number of VGLUT1(out)-PSD95(in) appositions appeared to increase in grafted striatum, this finding was not statistically significant for either genotype (two-way repeated measures ANOVA $F(1,10) = 5.317$, $p = 0.0438$; Šídák's multiple comparisons test: Val68Val: $t(10) = 1.346$, $p = 0.3728$; Met68Met: $t(10) = 1.980$, $p = 0.1460$; **Figure 3.8d**) or between genotypes (two-way repeated measures ANOVA $F(1,10) = 5.341$, $p = 0.0434$; Šídák's multiple comparisons test: Grafted: $t(20) = 0.9922$, $p = 0.5551$; Intact: $t(20) = 1.419$, $p = 0.3133$; **Figure 3.8d**). Moreover, the number of these synaptic triads did not correlate with GID, LID, or post-graft amphetamine rotational behavior (data not shown).

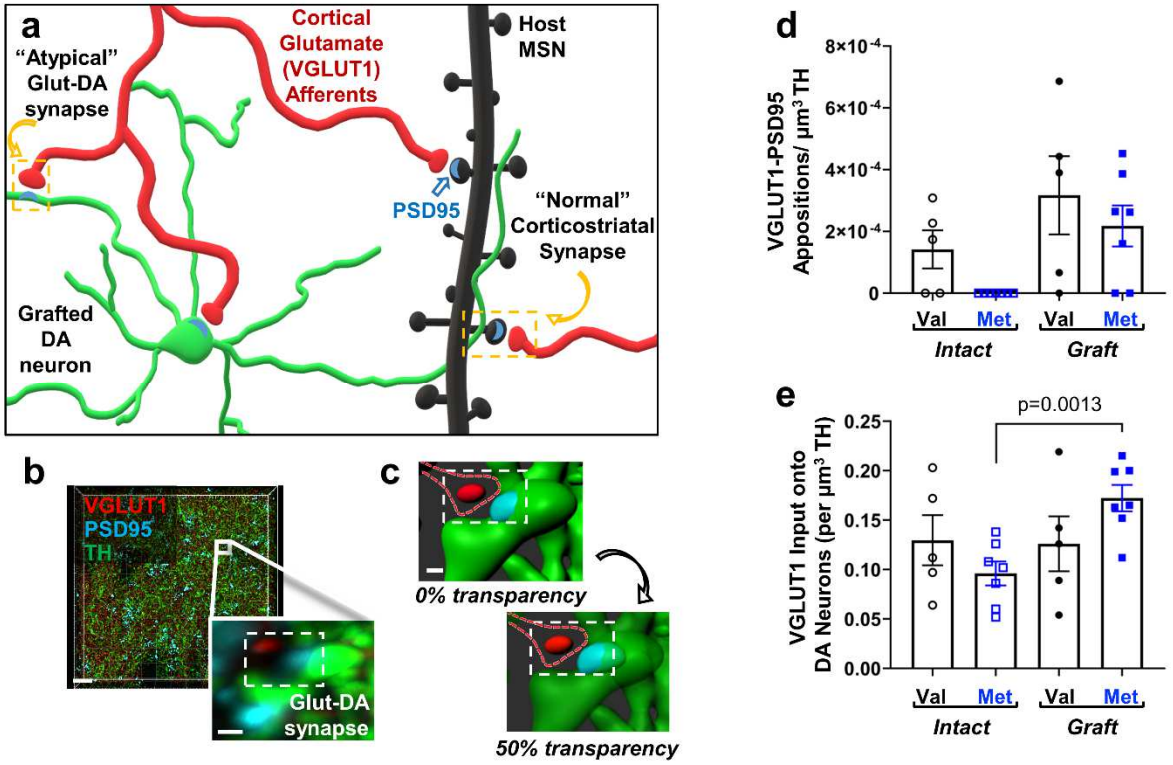


Figure 3.8 Excitatory corticostriatal synaptic input onto grafted DA neurons
(a) Schematic depicting "normal" corticostriatal synapse with modulatory DA input (right side of diagram), and "atypical" excitatory glutamatergic synapse onto grafted DA neurons (left side of diagram). **(b)** Representative confocal z-stack of tissue stained for VGLUT1, PSD95, and TH proteins. Scale bar = 10 µm. Inset: Increased magnification of a presumed glutamatergic (VGLUT1) synapse onto PSD95 protein located inside a grafted THir DA fiber (≤ 0.6 µm). Inset scale bar = 0.5 µm. **(c)** Imaris® 3D reconstruction of the inset image in panel (b). % transparency indicates that applied to TH fibers. **(d)** Quantification of presumed corticostriatal (VGLUT1) synapses with PSD95 located inside grafted DA fibers, normalized to TH surface volume. Mean ± SEM. Two-way repeated measures ANOVA with Šídák's post-hoc test. **(e)** Quantification of presumed corticostriatal (VGLUT1) synapses onto grafted DA fibers, regardless of PSD95 presence, normalized to TH surface volume. Mean ± SEM. Two-way repeated measures ANOVA with Šídák's post-hoc test. Figure modified with permission from Mercado et al [1]. Abbreviations: PSD95, postsynaptic density protein 95; VGLUT1, vesicular glutamate transporter 1; Glut, glutamate; DA, dopamine; TH, tyrosine hydroxylase

While these excitatory neurochemical triads were rare, there was, as expected, an abundance of VGLUT1 protein (indicating corticostriatal afferents) in the intact and grafted striatum. There also was a relative abundance of VGLUT1 making apparent appositions onto THir fibers, defined as VGLUT1 puncta located ≤ 0.6 μm from THir fibers [59] in both the intact and grafted striatum. While there was no difference in the total amount of VGLUT1 in the striatum between genotypes or between intact and grafted striata (two-way repeated measures ANOVA $F(1,10) = 2.862$, $p = 0.1215$), we did find that the VGLUT1 corticostriatal afferents showed increased apposition onto TH fibers in grafted striatum (vs intact striatum) in Met68Met but not Val69Val rats (two-way repeated measures ANOVA $F(1,10) = 8.929$, $p = 0.0136$; Šídák's multiple comparisons test: Val68Val: $t(10) = 0.1938$, $p = 0.9776$; Met68Met: $t(10) = 4.858$, $p = 0.0013$; **Figure 3.8e**).

While DA grafted Met68Met rats (a group displaying significant GID behavior) were found to have significantly higher levels of corticostriatal VGLUT1-TH appositions in grafted compared to the intact striatum, statistical correlations were not found between these appositions and amphetamine-mediated GID, levodopa-mediated GID, or post-graft amphetamine rotational behavior for either genotype.

VM grafts contain modestly, but significantly, more 5-HT neurons when transplanted into Met68Met striatum

Serotonergic neurons (i.e., cells containing *Tph2* mRNA) were observed in VM grafts in host subjects of both genotypes **(Figure 3.9a)**. However, these grafts in

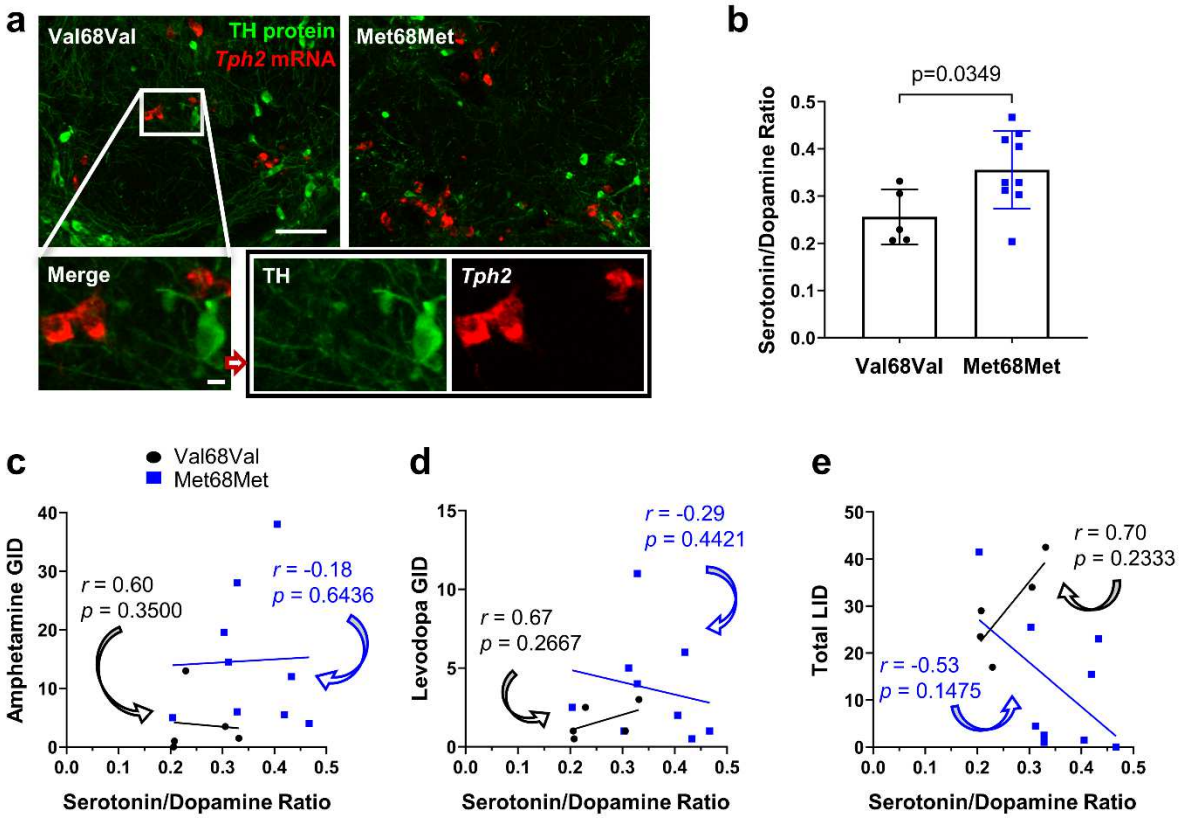


Figure 3.9 DA and 5-HT cell composition in VM grafts

(a) Confocal micrographs depicting serotonergic neurons in VM grafts from wild-type donors, in Val68Val and Met68Met host striatum. Inset: Increased magnification of DA neurons (THir) and serotonin neurons (*Tph2*-immunoreactive) present in grafted striatum. As shown in these representative images, there was no colocalization of *Tph2* mRNA and TH in VM grafts, and this finding was the same between genotypes. Scale bars = 100 µm in panel (a), 10 µm in inset panels. **(b)** Quantification of grafted serotonin neurons relative to the number of grafted DA neurons. Mean ± SEM. Unpaired *t*-test. **(c)** Spearman correlation between the proportion of serotonin neurons relative to DA neurons and total amphetamine-mediated GID score at 10 wks post-engraftment. **(d)** Spearman correlation between the proportion of serotonin (5-HT) neurons relative to DA neurons and total levodopa-mediated GID score at 10 wks post-engraftment. **(e)** Spearman correlation between the proportion of serotonin neurons relative to DA neurons and total LID score at 9 wks post-engraftment. Figure reproduced with permission from Mercado et al [1]. Abbreviations: TH, tyrosine hydroxylase; *Tph2,* tryptophan hydroxylase 2

Met68Met hosts contained a modest but significantly higher proportion of 5-HT neurons relative to the number of transplanted DA neurons (expressed here as the 5-HT/DA cell ratio) compared to Val68Val hosts (mean 5-HT/DA cell ratio ± SEM: Val68Val: 0.2559 ± 0.0262; Met68Met: 0.355 ± 0.0274; $t(12) = 2.378$, $p = 0.0349$; **Figure 3.9b**).

5-HT neurons in VM grafts are not associated with amphetamine-mediated or levodopa-mediated GID behavior

The presence of 5-HT neurons in VM grafts transplanted into parkinsonian striatum was not associated with GID behavior in the current study. Specifically, there was no correlation between the 5-HT/DA cell ratio and amphetamine-mediated GID (Val68Val: Spearman $r = 0.60$, $p = 0.3500$; Met68Met: Spearman $r = -0.18$, $p = 0.6436$; **Figure 3.9c**) or levodopa-mediated GID (Val68Val: Spearman $r = 0.67$, $p = 0.2667$; Met68Met: Spearman $r = -0.29$, $p = 0.4421$; **Figure 3.9d**). Furthermore, we found no significant correlation between the 5-HT/DA cell ratio and LID severity, though both groups exhibited moderately strong correlations in *opposite directions* (i.e., increasing 5-HT/DA ratio, increasing LID severity in Val68Val rats; increasing 5-HT/DA ratio, decreasing LID severity in Met68Met rats) (Val68Val: Spearman $r = 0.70$, $p = 0.2333$; Met68Met: Spearman $r = -0.53$, $p = 0.1475$; **Figure 3.9e**). There also was no correlation between post-graft amphetamine rotational behavior and the 5-HT/DA cell ratio in VM grafts for either genotype (data not shown).

Bdnf mRNA is abundant in DA grafts transplanted into both Val68Val and Met68Met hosts

As the present study emphasizes a shift in focus to the environment into which embryonic cells are transplanted, we sought to characterize the effects of host genotype on expression of *Bdnf* mRNA within wild-type grafted DA neurons. Under normal conditions, *Bdnf* mRNA is rarely observed in the striatum (e.g., [71-73]). Instead, BDNF protein is anterogradely transported to the striatum primarily from the motor cortex and SNc DA neurons [71]. As such, grafted DA neurons may be an important source of BDNF to the denervated, parkinsonian striatum **(Figure 3.10a)**. Thus, we examined whether the disparate levels of extracellular host striatal BDNF in Met68Met and Val8Val rats differentially impacted *Bdnf* mRNA expression in grafted DA neurons. We found that approximately 87% of transplanted DA neurons expressed *Bdnf* mRNA in host subjects of both genotypes **(Figure 3.10b,c)**. Indeed, there was no difference in the percentage of grafted DA neurons expressing *Bdnf* mRNA between genotypes (mean percentage of DA neurons containing *Bdnf* mRNA ± SEM: Val68Val: 87.55 ± 3.408%; Met68Met: 87.27 ± 1.751%; $t(10) = 0.07763$, $p = 0.9397$; **Figure 3.10c**). There also was no difference in the level of *Bdnf* mRNA transcript in grafted DA neurons when transplanted into either Met68Met or Val68Val hosts ($t(9) = 0.2020$, $p = 0.8444$).

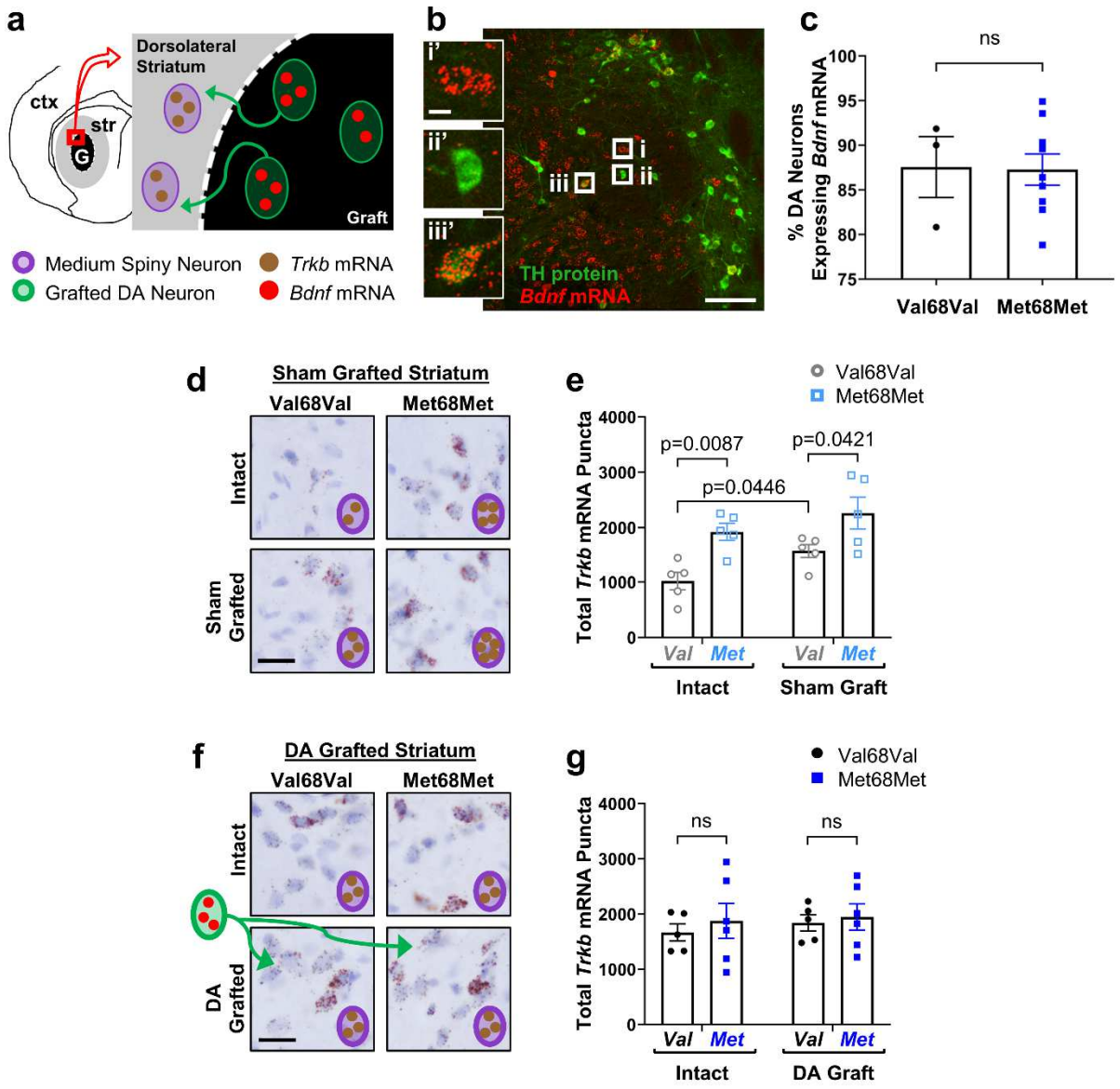


Figure 3.10 Grafted DA neuron *Bdnf* mRNA and host striatal *Trkb* mRNA
(a) Schematic illustrating *Trkb* expression in host dorsolateral striatum and *Bdnf* expression in grafted DA neurons. **(b)** Confocal micrograph of *Bdnf* mRNA in DA grafted tissue. Cells depicted in (i-iii) are shown at increased magnification in inset (i'-iii'). Scale bars = 100 µm for panel (b), 10 µm for inset images. **(c)** Percentage of grafted DA neurons expressing *Bdnf* mRNA in Val68Val and Met68Met grafted rats. Mean ± SEM. Unpaired *t*-test. NOTE: *Bdnf* mRNA data are available from only three Val68Val grafted subjects due to limited tissue sections containing grafts. **(d,f)** Micrographs of *Trkb* mRNA expression, presumed to be principally within medium spiny neurons, in grafted and intact dorsolateral striatum of sham grafted (d) and DA grafted (f) rats. Scale bars = 25 µm. **(e,g)** Quantification of *Trkb* mRNA in sham grafted (e) and DA grafted (g) rats depicted in panels (d) and (f), respectively. Mean ± SEM. Two-way repeated measures ANOVAs with Šídák's post-hoc test. Figure reproduced with permission from Mercado et al [1].

Figure 3.10 (cont'd)
Abbreviations: ctx, cortex; str, striatum; *Bdnf,* brain-derived neurotrophic factor; *Trkb,* tyrosine receptor kinase B; DA, dopamine; ns, not significant

Met68Met host striatal neurons contain more *Trkb* mRNA than Val68Val striatal neurons in sham grafted subjects, but not in DA grafted subjects

We next examined the impact of host genotype and the presence of grafted DA neurons on mRNA expression for the high affinity BDNF receptor, TrkB, in the host striatum. *Trkb* mRNA expression was quantified in the dorsolateral region of both the intact and lesioned striatum. In DA grafted subjects, *Trkb* mRNA expression was measured in the dorsolateral striatum adjacent to the grafts. As might be anticipated, in sham grafted subjects, striatal *Trkb* mRNA expression was significantly higher in Met68Met hosts than in their wild-type counterparts **(Figure 3.10d,e)**. This finding was consistent for intact and 6-OHDA lesioned striatum (two-way ANOVA $F(1,8) = 11.56$, $p = 0.0094$; Šídák's multiple comparisons test: Intact: $t(16) = 3.318$, $p = 0.0087$; Lesion: $t(16) = 2.553$, $p = 0.0421$). With the loss of nigral DA input to the striatum (a primary source of BDNF), there was an increase in *Trkb* mRNA expression with 6-OHDA lesion in Val68Val subjects (two-way ANOVA $F(1,8) = 10.42$, $p = 0.0121$; Šídák's multiple comparisons test: Val68Val: $t(8) = 2.818$, $p = 0.0446$; Met68Met: $t(8) = 1.746$, $p = 0.2238$). In the striatum of DA grafted rats there was no significant difference in *Trkb* mRNA expression between genotypes; curiously, this was also found to be true in the contralateral intact striatum (two-way ANOVA $F(1,9) = 0.2564$, $p = 0.6247$; Šídák's multiple comparisons test: Intact: $t(18) = 0.6221$, $p = 0.7899$; Lesion: $t(18) = 0.3204$, $p = 0.9387$); **Figure 3.10f,g**).

Discussion

(Reproduced with permission from Mercado et al [1])

Renewed Interest in Clinical Grafting Trials: Are We Ready?

Recent preclinical data from our laboratories [40] together with that from two milestone clinical reports [74, 75] provide compelling and sobering evidence demonstrating that despite robust survival and extensive neurite outgrowth from grafted DA neurons, obstacle(s) remain that interfere with functional circuit restoration within the aged, parkinsonian brain. Currently, clinical grafting trials, refined by decades of research, are ongoing or planned for the near future (e.g., [6], Clinical Trial Identifiers NCT01898390, NCT03309514, NCT03119636, NCT04146519). However, as the primary clinical objective is to provide an additional treatment option for individuals with PD that is safe and effective, the question remains whether our current understanding of this experimental regenerative therapy is sufficient for safe and informed clinical practice. While the field of regenerative medicine has gained an understanding of the role of *global* risk factors in cell transplantation for PD, the current study is the first indicating the importance of understanding the role of individual *genetic* risk factors for this therapeutic approach.

The Potential Role of Precision Medicine in Clinical Grafting Trials

The potential importance of the findings reported here related to the rs6265 SNP and the clinical regenerative approach of cell transplantation in PD, whether it be through primary embryonic or stem cells, lies in the fact that it is estimated that

approximately 20% of the worldwide human population possesses the Val66Met/rs6265 SNP in the *BDNF* gene, though there is much variability between populations [19]. For example, while this SNP is uncommon in African populations (<5% Met allele frequency), it is extremely common in East Asian populations (up to 72% Met allele frequency) [19, 20]. Important to the current topic, BDNF promotes dendritic spine integrity as well as synapse development and maturation within the striatum [76, 77]. It is also known to significantly impact graft-derived innervation following engraftment of embryonic VM neurons into parkinsonian rats [28, 29] and differentiation and maturation of embryonic and adult neural stem/progenitor cells [78].

Given the relevant biology of this trophic factor and the prominence of rs6265 in the human population, we asked the question: Since there is a subpopulation of individuals with PD that does not respond well to DA neuron transplantation, and there is a subpopulation of individuals with PD that carries this SNP, could the Met risk allele make the striatum a less hospitable environment for transplanted DA neurons to make normal/meaningful connections? As detailed above, our data using the rs6265 knock-in rat model to test the hypothesis that dysfunctional BDNF associated with this SNP is an unrecognized contributor to suboptimal clinical benefit and development of graft-derived side-effects suggests that this common human SNP may undoubtedly impact functional outcome in clinical grafting trials in PD. In the following discussion, we highlight how the current data integrate with the current understanding of the biology of this SNP and provide novel insight on how this SNP might impact cell replacement strategies in PD.

Paradoxical Enhancement of Neurite Outgrowth Associated with the Met Allele

Contrary to one aspect of our hypothesis, we found that parkinsonian Met68Met rats showed enhanced therapeutic efficacy evidenced by an earlier and more robust amelioration of LID behavior post-engraftment compared to wild-type Val68Val rats. In line with this, we discovered that rats with the Met allele displayed significantly enhanced neurite outgrowth derived from wild-type embryonic DA neurons compared to that seen in Val68Val hosts. While this discovery seems paradoxical to what would be expected in an environment of diminished BDNF availability, the rs6265 risk allele has previously been indicated in enhanced recovery following stroke and TBI [32, 33, 79]. Interestingly, in a study using a rs6265 mouse model, Qin and colleagues [33] found, contrary to their hypothesis, that mice homozygous for the variant Met allele displayed better motor recovery after receiving a transient middle cerebral artery occlusion when compared to their wild-type counterparts. Further, Krueger and colleagues [32] examined the recovery of executive functioning in Vietnam veterans who had sustained combat-related TBI. Though the authors initially hypothesized that the Val allele would promote recovery of executive functioning, it was the Met allele carriers who experienced better recovery of this behavioral measure. The rs6265 SNP was also found to predict mortality in a longitudinal study in patients with severe TBI [79]. In this study, Failla and colleagues examined patients receiving care for a closed-head injury, both acutely (0-7 days post-injury) and post-acutely (8-365 days post-injury). Unexpectedly, they found that Met allele carriers had greater survival probability at the acute timepoint compared to Val66Val subjects. Most recently, the Met allele has been found to be associated with enhanced neurite outgrowth of human iPSC-derived spinal

motor neurons (personal communication, Dr. Colin K. Franz, https://www.abstractsonline.com/pp8/#!/7883/presentation/69599) and excitatory cortical projection neurons (personal communication, Dr. Colin K. Franz, https://www.abstractsonline.com/pp8/#!/7883/presentation/68155) *in vitro*. In addition, neurite regeneration was enhanced following transection injury in human iPSC-derived motor neurons cultured in microfluidic chambers (personal communication, Dr. Colin K. Franz; https://www.abstractsonline.com/pp8/#!/7883/presentation/69599). In keeping with this observation in human iPSCs, enhanced peripheral axon regeneration has been observed in association with rs6265 both *in vivo* and in cultured dorsal root ganglion neurons in a mouse model of this SNP [80].

Ultimately, considering the abundance of the rs6265 Met allele in the human population, it would seem illogical that such a common genetic variant is entirely disadvantageous [81]. While an increasing collection of evidence suggests a beneficial role for the variant Met allele in axonal growth and regeneration, we show here that even wild-type neurons can be induced to develop this enhanced phenotype when transplanted ectopically into a Met allele-carrying host. In addition, despite the Met allele being associated with enhanced neurite outgrowth and enhanced functional benefit in our grafting study and in other conditions [32, 33, 79], we provide the first evidence that an individual's genotype may also underlie development of aberrant mechanisms associated with GID behavioral phenotype.

The BDNF Prodomain as a Biologically Active Ligand: Implications for Maturation of Neuronal Circuitry

Historically, BDNF has been shown to have a direct impact on the morphological development of neurons, typically by promoting growth and branching of axon terminals and facilitating the establishment of mature neuronal circuitry (reviewed in [82]). Why, then, a host environment with significantly reduced activity-dependent BDNF release would promote neurite outgrowth is a mystery. Indeed, the molecular mechanism underlying the paradoxical phenomena of increased neurite outgrowth and enhanced recovery from injury in association with the rs6265 Met allele is currently unknown. However, the extensive outgrowth that we observed from grafted DA neurons is reminiscent of the formation of extraneous neuronal processes and synapses during development that are later pruned back to facilitate the formation of functionally mature neuronal circuitry [83, 84]. Recently, it has been shown that dendritically translated BDNF and its precursor, proBDNF, are crucially involved in synaptic maturation and pruning in developing hippocampal neurons [85]. Furthermore, BDNF and proBDNF have been extensively studied as modulators of synapse structure, maturation, and plasticity and are known to have opposing roles in this regard (e.g., [76, 77, 86, 87]). Specifically, whereas mature BDNF promotes dendritic spine formation and LTP, proBDNF promotes growth cone retraction, spine shrinkage, and LTD (e.g., [87-94]). As BDNF signaling is tightly regulated, it is logical to assume that a disturbance in BDNF signaling may impact this intricate balance of synapse pruning and maturation in developing (grafted) neurons.

Recently, the BDNF prodomain, which is cleaved from proBDNF to produce the mature BDNF protein, has been implicated as another independent and biologically active ligand with modulatory effects at the synapse [95-98]. As reviewed in [99], the first of these studies [95] revealed that the BDNF prodomain containing the variant Met allele induced growth cone retraction and collapse in cultured hippocampal neurons. The same group later showed that the Met-prodomain eliminated mature mushroom spines and reduced axonal projection density in ventral CA1 hippocampal (vCA1) neurons during peri-adolescence, whereas the wild-type Val-prodomain had no effect [98]. Conversely, a separate group of researchers showed that the Val-prodomain facilitated LTD in the hippocampus, whereas the Met-prodomain completely prevented this effect [96]. Finally, in a report by a third group, the Val-prodomain reduced dendritic spine density in rat hippocampal neurons [97].

Much remains to be understood regarding the function of the BDNF prodomain as an independent ligand, especially in structures outside of the hippocampus, and how the rs6265 SNP impacts neuron and synapse function. However, these initial findings may still have important implications. Specifically, Giza and colleagues [98] concluded that the Met-prodomain, when applied to developing vCA1 neurons during peri-adolescence, rendered these neurons “underdeveloped,” thus preventing maturation of fear extinction circuitry in rs6265 Met allele carriers. Considering the compelling evidence that the prodomain may act as an independent and biologically active ligand with modulatory effects at the synapse, the current data could be hypothesized to suggest that the Met-prodomain prevents maturation and pruning of synaptic connectivity between grafted DA neurons and the host striatum.

Abnormal Target Plasticity: Evidence for Aberrant Graft-Host Synaptic Connectivity in rs6265 SNP Carriers

Structural changes mediated by DA depletion in the parkinsonian striatum have been postulated as potential contributors to GID development (reviewed in [10]). Specifically, it is known that striatal DA depletion causes morphological alterations to striatal MSNs (primary targets of grafted DA neurons) including dendritic spine loss, both in human individuals with PD and parkinsonian animal models [100-105]. Furthermore, preventing this loss of MSN spines with the calcium channel antagonist nimodipine reduces GID behavior in DA grafted rats [47].

As described above, BDNF signaling is a potent modulator of spine and synapse dynamics in the striatum [94, 106-110] and corticostriatal plasticity [111]. In a mouse model of the rs6265 *BDNF* SNP, Jing and colleagues [112] observed an increase in immature thin spines and a decrease in mature mushroom spines in the dorsolateral striatum of BDNF Met/Met mice, despite no change in total spine density. This phenomenon was also observed in cultured hippocampal neurons treated with the Met-prodomain [98]. Moreover, in addition to its known role in impairment of cortical and hippocampal plasticity [113-116], the rs6265 SNP is also associated with impaired striatal plasticity [112]. Based on the finding that total MSN spine density does not change with rs6265, the authors suggested that dendritic spine density *per se* may not contribute to the observed changes in striatal plasticity [112]. However, the shift in spine phenotype from mature to immature suggests that the development of functionally mature synapses may be impaired in rs6265 striatum.

In the current study, we quantified presumed synaptic connections between MSN dendritic spines (i.e., synaptopodin/SP) and grafted DA neurons. Interestingly, despite similar numbers of these graft-host synaptic appositions, only wild-type rats exhibited a significant decrease in LID behavior with increased density of synaptic contacts, as expected per [40]. This evidence suggests that grafted DA neurons in Met68Met rats may not be capable of establishing functionally appropriate/mature synaptic contacts with striatal dendritic spines, despite contact densities similar in number to that of wild-type rats. We propose that this may be due to structural differences in the MSN spines available for establishing connections and/or an inability to develop functionally mature connections with grafted cells, and that the enhanced amelioration of LID observed in Met68Met rats may occur through a separate underlying mechanism (e.g., autocrine release of DA from the extensive neurite network).

VGLUT2 Expression Indicative of Immature Phenotype in Transplanted DA Neurons: A Molecular Driver of GID?

In the normal adult striatum, nigrostriatal DA synapses exhibit ultrastructural features common to symmetric (Gray type-II) synapses [117-119]. Interestingly, atypical asymmetric (Gray type-I/excitatory) synapses formed by THir fibers have been documented postmortem in DA grafts from persons with PD [64], parkinsonian non-human primates [63], and parkinsonian rats [16]. In parkinsonian rats, we previously reported that asymmetric synapses made by grafted DA neurons are associated with GID [16]. In the current study, grafted Met68Met rats were the only group to develop significant GID, despite the presence of a widespread graft which does not typically

cause GID in wild-type rats [46]. Accordingly, we reasoned that if GID in Met68Met hosts are associated with asymmetric (presumed excitatory) DA synapses, these DA neurons should show neurochemical evidence of DA-glutamate co-transmission. As such, the grafted DA neurons should contain *Vglut2* mRNA and VGLUT2 protein.

During normal development, nigral DA neurons do indeed express *Vglut2* mRNA and VGLUT2 protein and show evidence of DA-glutamate co-transmission, a phenotype that typically disappears with maturation (for review [65]). We show here the first neurochemical evidence supporting the fact that grafted DA neurons maintain this immature phenotype well into a timeframe that should be associated with maturation and loss of DA-glutamate co-expression [68]. Indeed, in our study, approximately 60% of transplanted DA neurons contained *Vglut2* mRNA, and this occurred regardless of host genotype, suggesting this is inherent to the graft. This contrasts the <5% found in mature SN and ~25% in mature VTA DA neurons that we **(Figure 3.6)** and others (e.g., [66, 67]) have found.

As would be expected in DA neurons expressing *Vglut2* mRNA, we provide evidence of VGLUT2 protein found within transplanted DA fibers. Similar to *Vglut2* mRNA in mature SN and VTA, there was significantly less VGLUT2 in mature nigral DA fibers projecting to the striatum in naïve adult rats. While we did not perform ultrastructural analyses in the current study, we provide neurochemical evidence that grafted DA neurons create presumed excitatory (glutamatergic) synapses in the grafted striatum as evidenced by VGLUT2 *within* DA neurites making close (≤0.6 μm) appositions with PSD95 in the host striatum. While we appreciate that synapses are orders of magnitude smaller, we used a semi-automated approach that combines triple

label immunofluorescence and high-resolution confocal microscopy to provide the first evidence of neurochemical signatures of excitatory synapses made by DA neurons in the grafted parkinsonian striatum. In keeping with our previously reported data showing a positive trend between ultrastructurally identified asymmetric DA synapses and levodopa-mediated GID [16], in the present study, we show that amphetamine-mediated GID is significantly, positively associated with asymmetric DA synapses, but interestingly *only in Met68Met rats*.

Again, as wild-type rats do not typically develop GID with widespread DA grafts [46], the lack of correlation between asymmetric DA synapses and GID in grafted Val68Val subjects was not unexpected. However, it is notable that the lack of association between these two factors occurred in Val68Val rats despite similar levels of VGLUT2 expression between genotypes. We propose here that this collective evidence is suggestive of synaptic rewiring or “miswiring” between transplanted DA neurons and the host brain in Met68Met subjects – perhaps reflective of an inability to establish and/or maintain mature synaptic connectivity – which in turn promotes aberrant graft-induced side-effects.

Corticostriatal Connections with Grafted Cells

Previous evidence from our group [16] revealed a significant positive correlation between total levodopa-mediated GID severity and the proportion of aberrant asymmetric synapses onto grafted DA neurons in parkinsonian rats. These connections were presumed to be new corticostriatal inputs onto the grafted DA neurons, which were themselves making atypical excitatory (dopaminergic/ glutamatergic) contacts onto

host striatum neurons, and were hypothesized to create a nidus of aberrant excitatory drive leading to GID [16]. Accordingly, in the current study, we examined whether GID behavior in Met68Met rats correlated with excitatory VGLUT1-labeled corticostriatal input onto grafted DA neurons.

In contrast to our earlier findings, we did not find a correlation between GID and corticostriatal connections in grafted rats of either genotype. This may be due to the fact that in the present study we analyzed VGLUT1 input onto grafted DA fibers extending into the striatal parenchyma, while our previous electron microscopic evidence found that these connections occurred more frequently on grafted DA somas and proximal dendrites [16]. While the precise relationship of VGLUT1 to GID behaviors remains unclear, the current study did reveal that there was a significant increase in corticostriatal VGLUT1 input onto DA fibers in the grafted vs intact striatum in Met68Met rats that express this behavioral phenotype, but not in Val68Val rats. While the implications associated with this phenomenon of elevated corticostriatal input onto DA fibers in Met68Met GID-expressing rats are uncertain, it is known from the rs6265 mouse model that there is a basal elevation of glutamatergic neurotransmission in dorsolateral striatum of subjects with the Met risk allele [112]. A similar enhancement of glutamatergic neurotransmission in the striatum has been observed in animal models for Huntington's disease where BDNF availability is decreased [120]. The increased strength of glutamatergic synapses in the striatum has been suggested to play a role in aberrant plasticity involved with the enhancement of basal ganglia related behaviors such as anxiety and drug-seeking behaviors in Met allele carriers (for review [112]). Given the proposed similarities in aberrant striatal plasticity mechanisms between

addiction and dyskinesias (e.g., [121]), it is reasonable to hypothesize that excessive corticostriatal neurotransmission could be a mechanism contributing to the expression of GID behaviors in subjects with the Met allele.

Cell Composition in VM Grafts: 5-HT Neurons and Implications for Dyskinetic Behavior

5-HT Neurons and LID

In the parkinsonian striatum, levodopa is converted to DA by the enzyme AADC within remaining striatal DA terminals. However, as striatal DA terminals are depleted in advancing PD, this function is maintained instead by 5-HT neurons of the dorsal raphe nucleus (DRN) that sprout into the parkinsonian striatum. Specifically, it is known that serotonergic innervation of the striatum is markedly increased following DA depletion [122, 123] and levodopa treatment [124] and that DRN 5-HT neurons contain AADC and the vesicular monoamine transporter 2 (VMAT2). Therefore, endogenous striatal 5-HT terminals are capable of taking up exogenous levodopa and subsequently synthesizing, storing, and releasing DA as a “false neurotransmitter” after levodopa administration (reviewed in detail elsewhere, e.g., [10, 125, 126]). Unlike DA neurons, however, 5-HT neurons do not possess mechanisms of regulatory feedback (i.e., DA D2 autoreceptors and the DA transporter). Thus, the release of DA from 5-HT terminals following levodopa administration is unregulated and non-physiological, which is thought to contribute to dyskinesogenesis (e.g., [127]). Indeed, in support of this theory, it has been shown that viral vector-mediated expression of the DA D2 autoreceptor in DRN 5-HT neurons blocks LID in parkinsonian rats [128].

5-HT Neurons and GID

In addition to their role in contributing to LID, 5-HT neurons have been implicated, with some controversy, as a causative factor in the development of GID in grafted subjects [129, 130]. This contention is supported by evidence indicating that GID were markedly reduced in three grafted individuals with extensive graft-derived serotonergic hyperinnervation when treated with buspirone, a 5-HT_{1A} partial agonist that also displays DA receptor D2 (DRD2) antagonistic properties [10, 129, 130]. However, the role of 5-HT neurons – both graft-derived and endogenous – in GID development has yet to be conclusively defined. For instance, the presence (often in abundance) of 5-HT neurons in transplants has been observed in the absence of GID in VM grafted individuals [131, 132]. Furthermore, additional evidence strongly suggests that buspirone suppresses GID through its action as a DRD2 antagonist, rather than its interaction with the 5-HT_{1A} receptor [54, 133]. Indeed, buspirone was initially developed as an antipsychotic drug based on its interaction with the DRD2 [134] and is known to exhibit varying levels of antagonistic affinity for DA receptors D1-D4 [135-137]. Ultimately, though the topic remains a matter of debate, the majority of available evidence is supportive of a major role for the DA system in the development of GID, with a modulatory role for the 5-HT system [52, 138-140].

In keeping with the current dogma regarding 5-HT neurons and LID causation (e.g., [124, 127, 128]), we observed a positive trend between the 5-HT/DA cell ratio and LID in wild-type rats. However, data from our current study do *not* support a role of graft-derived 5-HT neurons in the expression of GID behaviors since there was a similar proportion of 5-HT/DA neurons in both Met68Met and Val68Val rats, as well as a lack of

correlation of this behavior with 5-HT/DA ratios in both genotypes. Despite this finding, it may be of interest in future studies to consider whether there are differences in 5-HT and VGLUT3 colocalization between genotypes and whether this parameter correlates with GID and/or LID. Similar to DA neurons colocalizing VGLUT2, 5-HT neurons, even in the mature brain, co-express vesicular glutamate transporter 3 (VGLUT3) which has been proposed to be involved in the phenomenon called “vesicular synergy” that results in increased extracellular 5-HT [65]. Thus, understanding whether such a mechanism correlates with GID and/or LID may be warranted [10].

Conclusion & Future Direction

In this era of personalized medicine, understanding both global (e.g., aging) and specific (e.g., rs6265 SNP) factors that might impact efficacy of clinical interventions such as cell replacement therapy could provide significant advances in the field of regenerative medicine. The current studies, performed in mature young adult (6 mos at time of lesion) rats homozygous for the rs6265 SNP, were undertaken as proof-of-principle studies to determine whether the variant Met allele would have an impact on graft function and/or dysfunction compared to wild-type rats. To this end, subjects homozygous for the variant allele were chosen to maximize the chances of observing a genotype-specific effect in these novel studies. These experiments provide clear evidence that the Met68Met genotype in the host significantly impacts DA graft efficacy in this model. While suggesting some novel mechanisms associated with DA graft function and/or dysfunction in association with the rs6265 SNP as discussed above, the current studies leave many questions unanswered. Nevertheless, these novel studies

provide an important foundation for an abundance of future investigations. For example, as the present study clearly demonstrates the significant impact of *host* genotype on wild-type donor cells, additional studies will be essential to examine the role of *graft* genotype. In addition, while the inclusion of heterozygous Val/Met subjects was beyond the scope of the present investigation, this is highly warranted for future studies. Finally, based on the known association of advanced age with PD and poor graft efficacy [13], we have an ongoing study in our rs6265 rats in which we are examining the interaction of advancing age with this SNP. We posit that a combination of data provided from our studies in aged (15 mos at time of grafting) parkinsonian Met68Met and wild-type rats together with that which might be obtained from grafted individuals with PD will be needed to provide the most comprehensive understanding of how these two factors, one global and one genetic, impact therapeutic outcome for this experimental therapy.

Indeed, to the best of our knowledge, transplant recipients and transplanted donor cells are not currently genotyped for this SNP. Notably, a large clinical grafting study is currently in the recruiting phase in China (Clinical Trial Identifier: NCT03119636). Considering that the rs6265 SNP is highly prevalent in East Asian countries, with some allelic frequency estimates as high as 72% [20], the findings of the current study suggest that it would be prudent to genotype the clinical participants so the impact of the rs6265 SNP may be considered during interpretation of the study results. While the current studies are most relevant to PD, data from these studies together with those from human TBI subjects with the variant Met genotype and the rs6265 mouse stroke model [32, 33, 79] suggest that understanding how to harness the “good” phenotype (i.e., enhanced neurite outgrowth and functional benefit) while

subverting the “bad” phenotype (i.e., motor dysfunction/GID; anxiety and depression [112]) associated with the Met allele could provide a means for optimizing not only the clinical regenerative medicine approach of cell transplantation for PD but also treatment for a variety of traumatic, degenerative, and/or developmental maladies.

APPENDIX

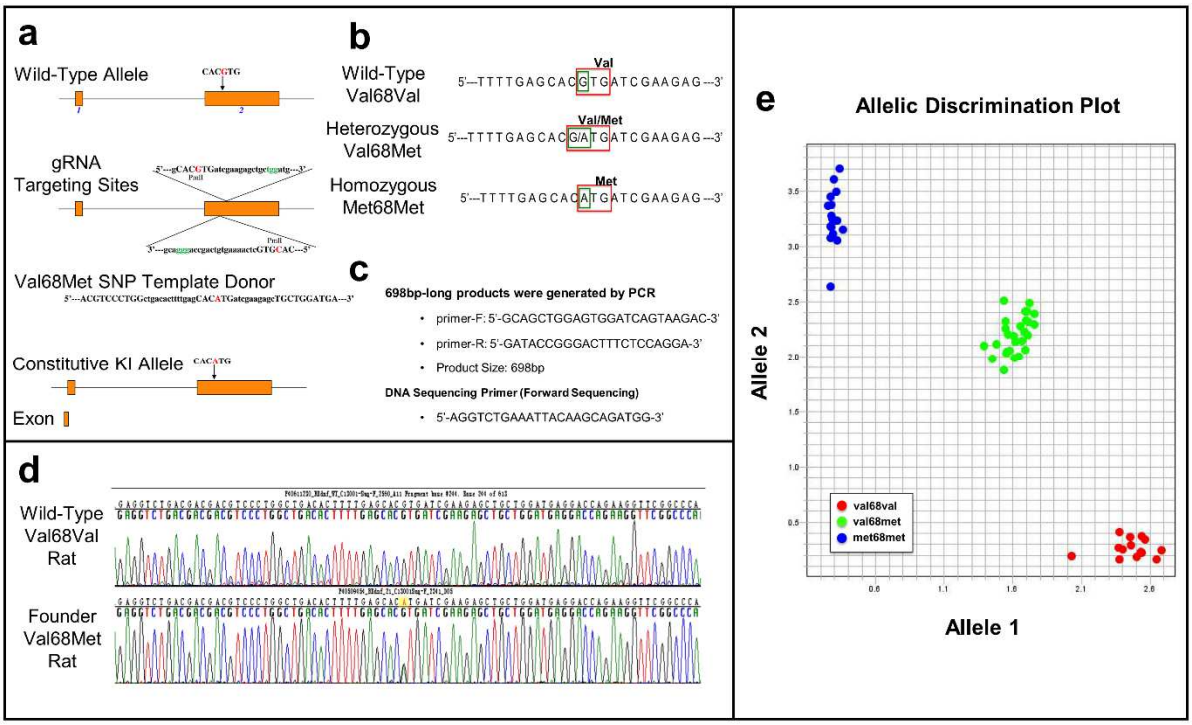


Figure 3.11 CRISPR-Cas9 generation of the *Bdnf* rs6265 knock-in rat model
(a) Schematic of guide RNA targeting vector and Val68Met template donor used for generation of the Val68Met founder female using CRISPR-Cas9-mediated homologous recombination by Cyagen Biosciences (Santa Clara, CA). Cas9, guide RNA targeting vector, and a Val68Met template donor were injected into zygotes to generate the germline transmitted Val68Met rat. **(b)** Schematic of nucleotide sequences for wildtype Val68Val, heterozygous Val68Met and homozygous Met68Met. Note that rats have two additional threonine amino acids at positions 57 and 58, making the rat Val68Met equivalent to the human Val66Met. **(c)** Products generated by PCR and DNA sequencing primers used. **(d)** Sequence of founder Sprague-Dawley Val68Met female rat confirmed to be carrying the valine to methionine polymorphism (Val68Met) in *Bdnf*. The founder female was bred once with a wild-type Val68Val male rat to generate heterozygous Val68Met offspring of both sexes. **(e)** Genotyping of the offspring was conducted using a custom Taqman® SNP genotyping assay allelic discrimination plot demonstrating three genotypes produced from mating of founder Val68Met female with Val68Met male showing resulting Met68Met, Val68Met, and Val68Val offspring. Figure reproduced with permission from Mercado et al [1]. Abbreviations: bp, base pair; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR associated protein 9; gRNA, guide RNA; KI, knock-in; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

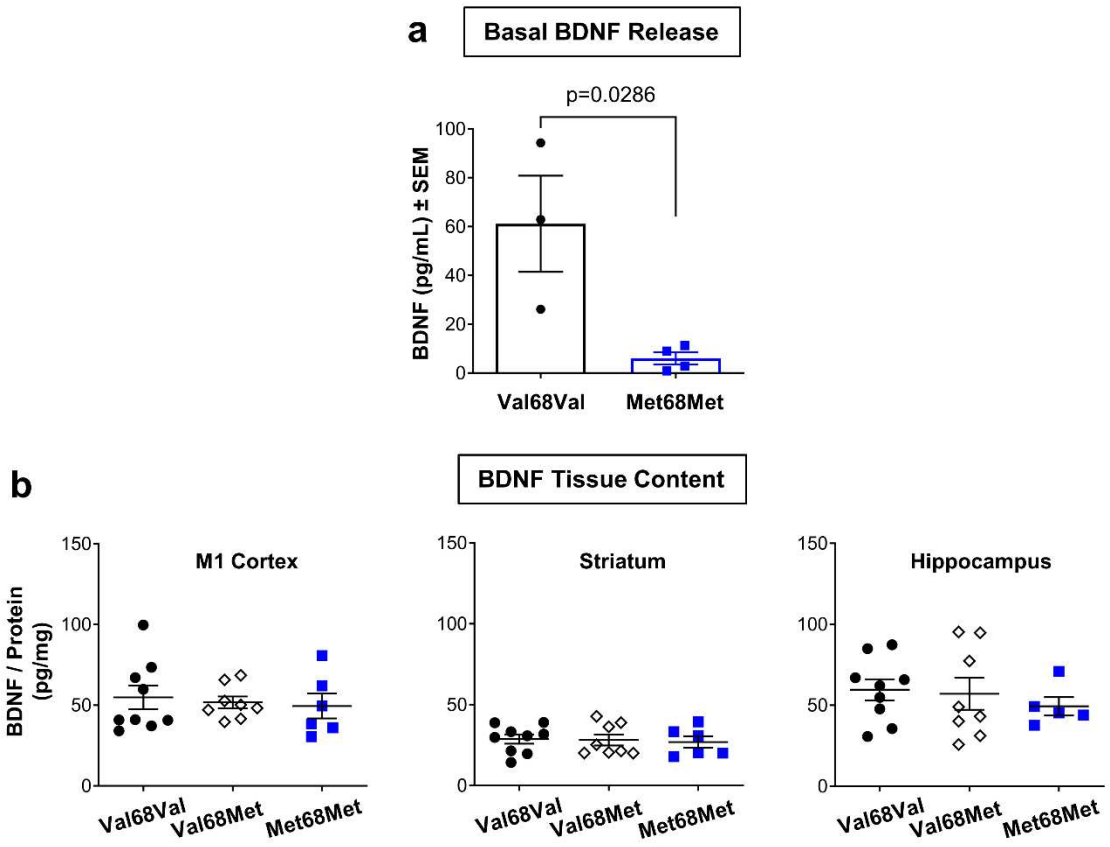


Figure 3.12 *In vitro* validation of diminished BDNF release in the absence of altered whole tissue BDNF content in rs6265 SNP-carrying rats
(a) Levels of basal BDNF release in culture media from embryonic day 18 hippocampal cultures determined by ELISA. **(b)** BDNF content obtained from tissue punches of hippocampus, striatum, or M1 cortex of 3 m.o. Val68Val, Val68Met, and Met68Met rats. Figure reproduced with permission from Mercado et al [1]. Abbreviations: BDNF, brain-derived neurotrophic factor

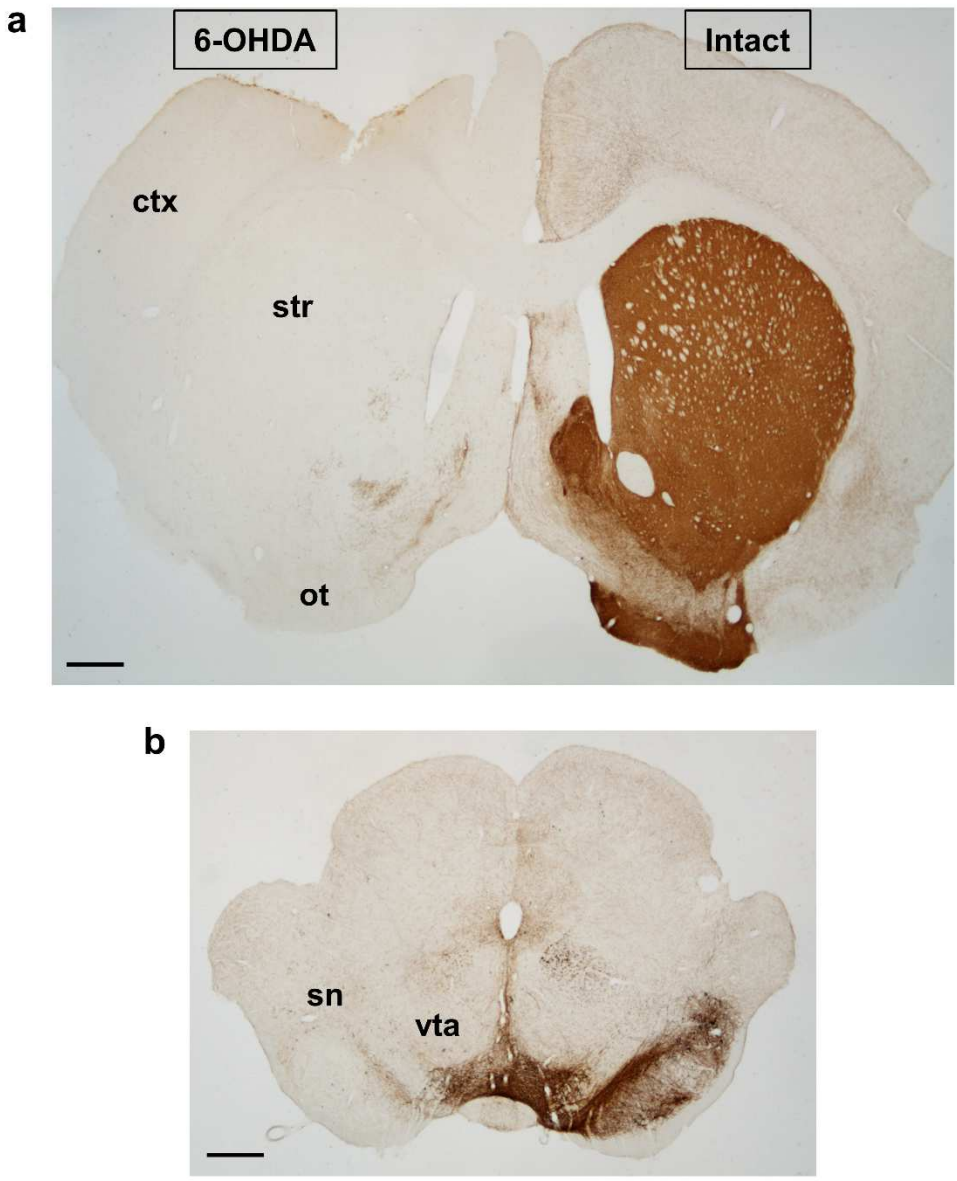


Figure 3.13 Depletion of the nigrostriatal DA system after 6-OHDA lesion
(a) Striatal tissue section from a representative unilaterally lesioned and sham grafted Met68Met rat, stained for tyrosine hydroxylase. **(b)** Tyrosine hydroxylase-labeled nigral tissue section from the same sham grafted rat. Both tissue sections were stained at the same time. Scale bar = 1000 µm. Figure reproduced with permission from Mercado et al [1]. Abbreviations: 6-OHDA, 6-hydroxydopamine; ctx, cortex; str, striatum; ot, olfactory tubercle; sn, substantia nigra; vta, ventral tegmental area

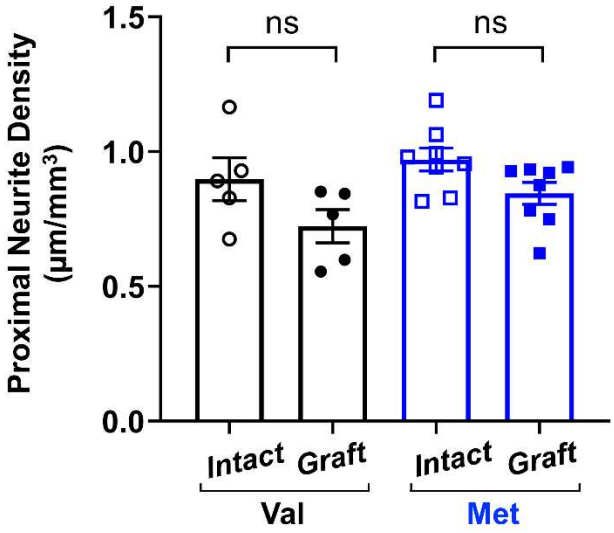


Figure 3.14 Comparison of proximal grafted DA neurite density with intact contralateral striatum

Graph depicting (presumed) graft-derived THir neurite density in regions proximal to the DA graft (100-500 µm from lateral graft edge) and in intact contralateral striatum. Graft-derived striatal reinnervation was compared to the intact contralateral striatum using two-way ANOVA (lesion status x genotype) with Šídák's multiple comparisons test. Figure reproduced with permission from Mercado et al [1]. Abbreviations: Met, Met68Met; ns, not significant; Val, Val68Val

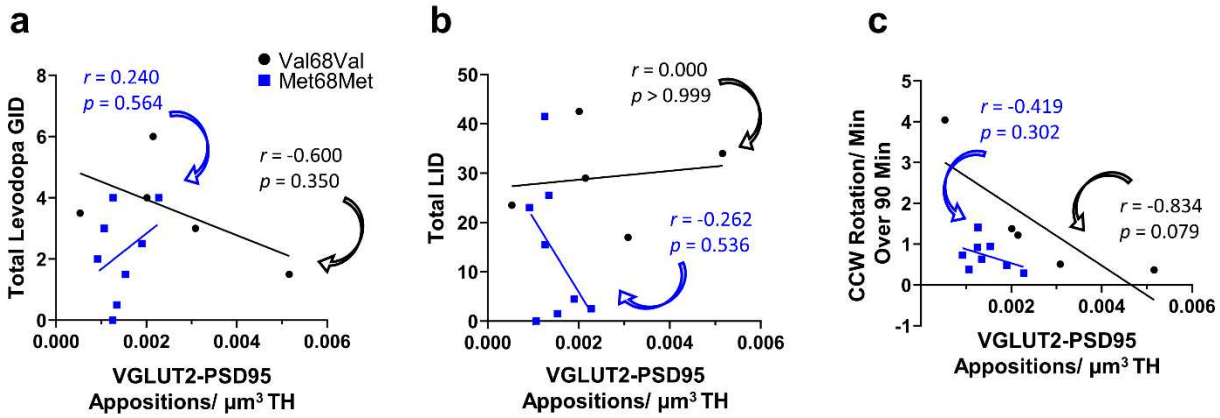


Figure 3.15 Correlations between VGLUT2-PSD95 appositions and behavior
(a) Spearman correlation between VGLUT2-PSD95 appositions and levodopa-mediated GID at 10 wks post-engraftment. **(b)** Spearman correlation between VGLUT2-PSD95 appositions and LID at 9 wks post-engraftment. **(c)** Pearson correlation between VGLUT2-PSD95 appositions and amphetamine rotational behavior at 7 wks post-engraftment. Figure reproduced with permission from Mercado et al [1]. Abbreviations: CCW, counterclockwise; GID, graft-induced dyskinesias; LID, levodopa-induced dyskinesias; PSD95, postsynaptic density protein 95; TH, tyrosine hydroxylase; VGLUT2, vesicular glutamate transporter 2

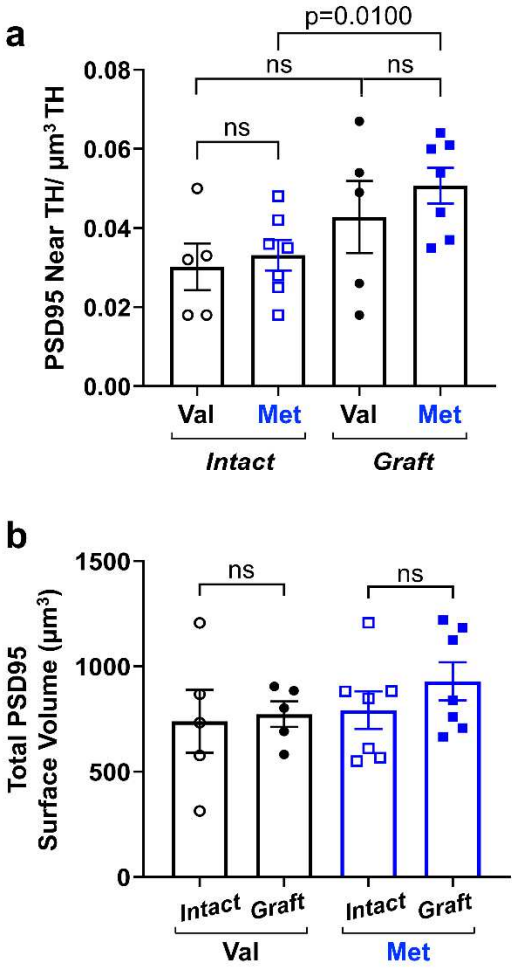


Figure 3.16 PSD95 surface structures and overall PSD95 surface volume
(a) Quantification of PSD95 surface structures in synaptic apposition (≤ 0.6 µm) to THir DA fibers in grafted and intact striatum. **(b)** Total PSD95 surface volume in grafted and intact striatum. Figure reproduced with permission from Mercado et al [1]. Abbreviations: ns, not significant; PSD95, postsynaptic density protein 95; TH, tyrosine hydroxylase

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CHAPTER 4: IMPACT OF THE VAL66MET *BDNF* SNP ON THERAPEUTIC EFFICACY OF DOPAMINE NEURON GRAFTING AND MECHANISMS OF BRAIN REPAIR IN MIDDLE-AGED PARKINSONIAN RATS

Abstract

The Val66Met single nucleotide polymorphism (SNP) in the gene for brain-derived neurotrophic factor (*BDNF*) is a common genetic variant that alters therapeutic outcomes for individuals with Parkinson's disease (PD). We previously investigated the effects of this SNP on the experimental therapy of neural grafting for PD and demonstrated that young rats (grafted at 8m.o.) carrying the variant allele exhibited enhanced graft function and developed aberrant graft-induced dyskinesias (GID), in contrast to wild-type rats. Aging is the primary risk factor for PD and is known to reduce efficacy of neural grafting. In the present experiment we investigated whether aging interacts with this SNP to further alter cell transplantation outcomes. We hypothesized that aging would (1) dampen the enhancement of graft function associated with this genetic variant and (2) exacerbate GID in all grafted subjects. To test this hypothesis, middle-aged wild-type (Val/Val) and Met/Met rats (grafted at 15m.o.) were rendered unilaterally parkinsonian, primed with levodopa, then transplanted with ventral mesencephalic dopamine neurons from wild-type embryonic day 14 donors. Graft function was assessed over 10 weeks following engraftment. Reductions in levodopa-induced dyskinesia severity served as the primary measure of graft function and tests of OFF-time parkinsonian sensorimotor dysfunction served as a secondary measure. In this experiment we demonstrate that: (1) behavioral enhancement associated with rs6265 is maintained with advanced age, and (2) advanced age is permissive to the induction of GID in subjects of both genotypes. Understanding mechanisms underlying the impact of the Val66Met *BDNF* variant in association with aging will aid in the

development of safe and optimized therapeutic approaches for remodeling the parkinsonian striatum.

Introduction

Precision medicine is a novel approach to healthcare based on the concept of identifying individual characteristics among patient populations that might contribute to variability in disease phenotypes and/or treatment efficacy. In Parkinson's disease (PD), the primary goal of precision medicine is to deliver "the right treatment to the right person at the right time… to implement safe, effective, and precise interventions with minimal complications" [1]. In the current era of rapidly advancing scientific concepts and ever-improving state-of-the-art technology, it has become increasingly apparent that transitioning to a precision medicine approach will be key to advancing efforts to unravel the complex heterogeneity in clinical efficacy of PD therapeutics that is reflected in the inherent heterogeneity of the disease itself. Certainly, precision medicine will ensure that the safest and most effective treatment is given to each patient, tailored to the needs of the individual based on characteristics such as (but not limited to) age, sex, lifestyle, comorbidities, and/or genetics. In recent years the PD community has begun to gradually shift toward personalized therapeutics with a central focus on understanding genetic variation and developing treatments specific to genetic forms of PD [2, 3]. However, while the field is beginning to understand the impact of genetics on patient response to therapeutics, the impact of common genetic variants on heterogeneity in clinical outcomes for the experimental approach of neural grafting – which is currently reemerging in the clinic – has yet to be explored.

In the innovative experiments discussed at length in Chapter 3, I provide the first evidence that a common single nucleotide polymorphism (SNP) in the gene for brain-derived neurotrophic factor (*BDNF*) increases the therapeutic efficacy of embryonic dopamine (DA) neuron transplantation while concomitantly increasing side-effect burden in a novel rat model of this SNP. Present in approximately 15-20% of the global human population [4, 5], the rs6265 SNP (commonly referred to as Val66Met) impairs BDNF transport and packaging into secretory vesicles, ultimately reducing activity-dependent BDNF release [6-9]. BDNF is crucially involved in the establishment, maturation, structure, and function of synapses and neuronal connectivity (e.g., [10, 11]). It also mediates survival of DA neurons of the substantia nigra pars compacta (SNc) [12] and maintenance of striatal medium spiny neurons (MSNs) [13], the two principal cell types involved in the nigrostriatal pathway that degenerates in PD. Moreover, BDNF also has well-characterized effects on the survival and function of *grafted* SNc DA neurons [14-16]. Based on this collective evidence, I hypothesized that rs6265-mediated BDNF dysfunction is an unrecognized contributor to the limited clinical benefit observed in a subpopulation of individuals with PD despite robust survival of grafted DA neurons, and further, that this SNP is involved in the development of aberrant graft-induced dyskinesias (GID). While my first study demonstrated that the Val66Met SNP is indeed correlated with GID development, it also revealed, unexpectedly, that homozygous rs6265 SNP status is associated with *improved* graft function as evidenced by more rapid and complete reversal of levodopa-induced dyskinesias (LID) [17]. The results of this initial proof-of-principle study are highly relevant to the field of cell transplantation therapy as the field is reinitiating clinical

neural grafting trials for PD after a decade-long worldwide moratorium [18-20]. However, this potentially impactful experiment considered only one factor – rs6265 genotype – and the effects of its interactions with other individual characteristics such as age of the graft recipient remain to be investigated.

Aging is an inevitable and irreversible process in which a large number of degenerative structural and functional changes occur cumulatively over time, both in the brain and in the periphery. For example, in the aged brain, dendritic spine density is decreased in structures such as the hippocampus, prefrontal cortex, and striatum [21-23], the immune system is dysregulated and neuroinflammatory responses are exaggerated (e.g., [24, 25]), and neurotrophic response to neurotoxic injury is impaired [26, 27]. Given these normal deteriorative changes, it is reasonable to assume that therapeutic approaches for PD might be less effective in elderly individuals. Indeed, transplanting new cells into an aged and neurotrophically impoverished environment has been shown to negatively impact survival and synaptic integration of transplanted cells in aged, parkinsonian rats, leading to inferior recovery of motor impairment when compared to young rats [28, 29]. This pattern of reduced graft efficacy with advanced age is also evident in humans with PD. For instance, in early cell transplantation clinical trials for PD, it was discovered that older patients [30] and those with more advanced disease [31] experienced little, if any, clinical benefit while younger patients and those with milder disease saw clinically significant improvement of parkinsonian motor symptoms at 1-2-year follow-up assessments. These findings prompted experts to conclude that cell transplantation therapy is best suited to patients with milder pathology, and indeed current clinical trials have recruited patients in earlier PD stages

(e.g., [19]). However, performing an invasive surgical procedure early in the disease when pharmacological treatments are still highly effective at ameliorating motor symptoms is generally not ideal. Theoretically, if the mechanisms underlying reduced functional benefit of DA neuron grafts in the aged and/or severely parkinsonian brain are more thoroughly understood, this therapy could then be modified to address these yet unknown factors in order to be clinically effective in this patient population. As such, studying interactions between patient age and other factors (e.g., common genetic variants) known to alter treatment efficacy in PD is a reasonable first step toward the development of more effective therapies for elderly populations of patients.

The Val66Met *BDNF* polymorphism has been studied in association with aging in a variety of contexts. Most prominently, the modulatory effects of this *BDNF* variant on aging-associated changes in cognitive performance have been extensively characterized. Generally, aging-related deterioration of brain plasticity and cognitive function is correlated with alterations in BDNF activity and availability [32-34]. Along the same line, the “resource-modulation hypothesis” proposed by Lindenberger et al. [35] posits that the loss of physiological “resources” in the brain during normal aging allows for the effects of common genetic variants such as rs6265 to become more prominent, thus contributing to variability in cognitive function among older adults. This has been demonstrated in particular for aging carriers of the rs6265 *BDNF* polymorphism. For instance, Li et al. [36] reported that older individuals carrying the rs6265 SNP performed worse in a backward recall task than older Val66Val homozygotes, while this difference was not observed in younger participants. Additional studies expand upon this finding by demonstrating that the Val66Met *BDNF* SNP exacerbates aging-related decline in

other cognitive tasks including multiple measures of memory performance and perceptual speed [37-39], and further, that aging-associated decline in posterior hippocampal activation during a declarative memory task is accelerated in Met allele carriers [40]. Additional evidence has established a compounding effect of the Val66Met *BDNF* SNP in aging-related volume reduction of the dorsolateral prefrontal cortex in that Met allele carriers showed greater volume loss in this structure with aging when compared to Val66Val homozygotes [41]. Similar Met allele-associated volume loss with aging has also been reported in the amygdala [42].

In contrast, Harris et al. [43] demonstrated that homozygosity for the Met allele was associated with *better* performance in a non-verbal reasoning task in older individuals, while another group reported that elderly Met allele carriers performed better on the Stroop task [44], were better able to perform goal-directed tasks in the presence of auditory distractions [45], and showed superior memory-based task-switching abilities [46] compared to Val66Val homozygotes. In line with this contradictory evidence, a related study demonstrated that the Met allele also confers protective effects following brain injury in older individuals. In particular, Failla and colleagues [47] showed that the variant rs6265 genotype was associated with higher survival probability following severe traumatic brain injury (TBI) in persons >45 years old, but not in younger individuals.

In all, the evidence implicating a significant role for the Val66Met *BDNF* variant in modulating normal aging-related decline in cognitive function is abundant, though whether the presence of this SNP is helpful or harmful appears to depend on the cognitive task being performed. As for whether the resource-modulation hypothesis

holds true for the interaction between aging and the Val66Met *BDNF* variant in relation to clinical effectiveness of therapeutics such as neural grafting, the present study is the first (to the best of my knowledge) to investigate this empirical question.

As neural grafting clinical trials are rapidly reemerging, there are several important limitations to this therapeutic approach that have yet to be fully elucidated and remedied. Specifically, it remains unknown why a subpopulation of individuals with PD experiences limited functional graft efficacy despite robust survival of transplanted neurons and extensive synaptic integration into the host brain [48]. In addition, obstacles that interfere with graft efficacy in aged, parkinsonian subjects and the mechanisms underlying GID pathogenesis are yet unknown. In Chapter 3, we showed that the variant rs6265 Met allele mediates the relationship between atypical excitatory neurochemical signatures in grafted DA neurons and aberrant GID development in young rats. However, while the field is beginning to recognize the importance of considering patient age and genetics when choosing the most appropriate therapeutic approach, it remains uncertain how aging impacts GID development or how aging might interact with the rs6265 *BDNF* variant to alter therapeutic responses in persons with PD. Furthermore the majority of persons with PD can be characterized as elderly [49], and aging is widely recognized as the primary risk factor for PD [50, 51]. Therefore, the use of an aged animal model is highly warranted and will be crucial in advancing efforts to more accurately recapitulate the aged, parkinsonian environment into which new DA neurons are to be transplanted in the clinic. Accordingly, in the current study, I employed the rs6265 knock-in rat model developed by our group (detailed in Chapter 3 and [17]) to investigate the effects of the interaction between the rs6265 genotype and

advanced age on therapeutic efficacy and the development of graft-derived side-effects following cell transplantation in a rat model of PD.

Based on evidence indicating that graft-derived motor benefit is inferior in aged parkinsonian rats [28, 29], and that young, homozygous rs6265 rats show enhanced graft-derived benefit (Chapter 3 and [17]), I hypothesized that the effects of aging will supersede those of the resource-modulation hypothesis and that this paradoxical enhancement will persist into old age, but to a lesser extent than that observed in young animals. Furthermore, based on knowledge that aging-related deterioration of brain plasticity is correlated with alterations in BDNF activity and availability [32-34], and that abnormal patterns of graft-host synaptic connectivity have been hypothesized to contribute to GID pathogenesis ([31, 52, 53] but see also Chapter 2: An Unexpected Side-Effect: Addressing GID), I also hypothesized that GID will be more prevalent in this older animal model regardless of genotype, and that the rs6265 SNP will further exacerbate this side-effect according to the resource-modulation hypothesis. The present study expands upon the results of the experiments detailed in Chapter 3 and contributes to the field by providing the first-ever investigation of the potential interaction between advanced age and the rs6265 SNP and its effects on therapeutic efficacy of cell transplantation in an animal model of PD. In addition, to the best of my knowledge, this study is the first to examine GID in a middle-aged, parkinsonian animal model.

Methods

Animals

In the current experiments, adult male Sprague-Dawley rats (12-13 m.o. at time of lesion; *N* = 27 rs6265 rats, *N* = 24 wild-type rats) were aged in-house within our Val68Met colony as described above in Chapter 3. Due to a limited quantity of wild-type rats born in-house, the wild-type group was supplemented with Sprague-Dawley rats derived from the same genetic background from which our colony is derived (CD® International Genetic Standardization Program, Charles River Laboratories, Wilmington, MA, USA). These additional rats were purchased as 6 m.o. retired breeders and aged in-house for 6 months before experiments began.

In these proof-of-principle studies, wild-type (Val68Val) and homozygous SNP (Met68Met) rats were used to maximize the probability of observing an effect associated with the variant allele. Rats were housed on a 12-hour light/dark cycle (lights on at 06:00) and given *ad libitum* access to food and fresh water. Due to long-term health complications associated with excessive weight gain in aging Met68Met rats, all rats in the present study were given rodent chow designed for longevity and normal body weight management (Teklad Global 14% Protein Rodent Maintenance Diet, Teklad Diets, Madison WI, USA). They were switched to this diet at 3 m.o. (except for the purchased wild-type rats, which were switched at 6 m.o. upon receipt from the vendor) and were maintained on this diet until sacrifice. As in Chapter 3, rats were housed two per cage until initiation of levodopa treatment and dyskinesia rating. At this time, the rats were individually housed to allow accurate behavioral assessment in their home cage.

All rats were provided with environmental enrichment once they were individually housed.

Over the course of the experiment, some animals were excluded from further analysis. Specifically, a small number of Met68Met rats (*N* = 3) were humanely euthanized due to illness and rapid weight loss presumed to be a result of spontaneous pituitary adenomas, which are common in aging rats (e.g., [54, 55]) and were observed postmortem in some of these aging animals, curiously occurring exclusively in rs6265 subjects in this study. Several additional rats were removed from the study due to deaths unrelated to the experiment (*N* = 8), while others were excluded because they did not develop LID (*N* = 8; a small percentage of outbred Sprague-Dawley rats are typically LID-resistant (e.g., [56])). At the time of sacrifice, *N* = 17 rs6265 rats and *N* = 15 wild-type rats remained in the study. One additional rs6265 rat was excluded postmortem due to severe deformity/compression of the brain resulting from a large pituitary adenoma.

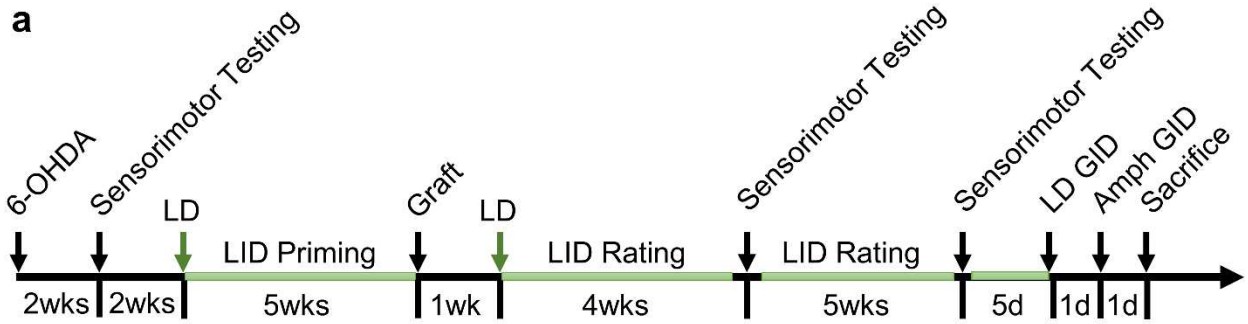
While Sprague-Dawley rats are generally not considered “aged” until 18 months of age [57], the cohort in the present study is composed of slightly younger, middle-aged rats (14-15 m.o. at time of grafting; 17-18 m.o. at sacrifice). This is because, as mentioned above, Met68Met rats in our hands commonly exhibited long-term health complications associated with excessive weight gain (e.g., diabetes), which rendered aging of large numbers of these rats long-term capricious. These health complications only became apparent as the rats reached mature ages (~ 6 m.o. and above), and are likely a result of disruptions to normal metabolic function dependent on BDNF signaling

as has been observed in BDNF$^{+/-}$ mutant rodents and humans carrying the rs6265 *BDNF* polymorphism [58-67].

All experimental procedures were approved by the Michigan State University Institutional Animal Care & Use Committee. “Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) were followed along with specific national and international laws in accordance with the ethical standards established by the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Experimental Design Overview

Similar to the experiments detailed in Chapter 3, rats were rendered unilaterally parkinsonian via intranigral stereotaxic injection of 6-hydroxydopamine (6-OHDA) **(Figure 4.1)**. Two weeks following 6-OHDA surgery, drug-free sensorimotor behavior was evaluated using the drag test and the “sticky paws” test to indirectly assess lesion status and to collect baseline behavioral data. Three weeks after sensorimotor testing, rats began priming with daily levodopa to induce stable levodopa-induced dyskinesias (LID). After 5 weeks of levodopa priming, all rats received an intrastriatal graft of ventral mesencephalic (VM) DA neurons from wild-type embryonic day 14 (E14) rat donors or a cell-free sham graft. Levodopa was withdrawn for one week following graft surgery, after which levodopa treatment was reinitiated. LID were evaluated in parkinsonian rats for 10 weeks following DA neuron grafting and served as the primary behavioral endpoint. Drug-free sensorimotor behaviors were assessed again at 5- and 10-weeks post-grafting as a measure of “OFF-time” graft function. As an indicator of graft dysfunction,



b

Host Genotype	Graft Genotype	Final *N*
WT	WT	8
WT	Sham	7
Met68Met	WT	8
Met68Met	Sham	8

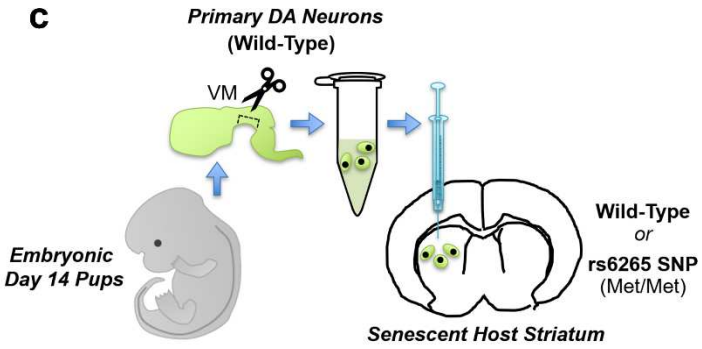


Figure 4.1 Experimental design
(a) Experimental timeline of surgical procedures, drug administration, and behavioral evaluation. **(b)** Experimental groups and final group size upon completion of the study. **(c)** Schematic depicting the experimental design. Ventral mesencephalic tissue was dissected from wild-type embryonic day 14 Sprague-Dawley rat pups, immediately dissociated, then transplanted into middle-aged rats of both wild-type and homozygous Met/Met genotypes. Abbreviations: 6-OHDA, 6-hydroxydopamine; Amph GID, amphetamine-mediated GID assessment; DA, dopamine; GID, graft-induced dyskinesias; LD, levodopa; LD GID, levodopa-mediated GID assessment; LID, levodopa-induced dyskinesias; SNP, single nucleotide polymorphism; VM, ventral mesencephalon; WT, wild-type

graft-induced dyskinesias (GID) were evaluated 24 hours following the final LID assessment. Rats were sacrificed 24 hours following GID evaluation. After the study was concluded, ear clippings collected at sacrifice were genotyped to confirm Val68Met SNP status.

Nigrostriatal 6-OHDA Lesion Surgery

Rats were anesthetized with inhalant isoflurane (2-3%; Sigma, St. Louis, MO, USA) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Each rat received two 2-µl injections of 6-OHDA (5mg/ml) using a 5 µl Hamilton syringe with a 26-gauge needle. 6-OHDA was administered at a flow rate of 0.5 µl/min into the SNc (4.8 mm posterior, 1.7 mm lateral, 8.2 mm ventral, relative to bregma) and medial forebrain bundle (MFB; 4.3 mm posterior, 1.6 mm lateral, 8.6 mm ventral, relative to bregma); 2 µl at each site. After surgery, rats received carprofen (Rimadyl®; 5 mg/kg) as analgesic treatment. Nigral lesion status was confirmed postmortem using medial terminal nucleus DA cell enumeration per [68].

Drug-Free "OFF-Time" Sensorimotor Behavioral Evaluation

Drug-free sensorimotor behaviors were assessed at 8 weeks pre-graft (baseline) and weeks 5 and 10 post-graft. These assessments served as a secondary measure of graft function and allowed for evaluation of parkinsonian motor deficits during OFF periods (i.e., time between levodopa doses that in patients is characterized by reduced or absent levodopa efficacy and low plasma levodopa concentrations). Accordingly, on test days, drug-free behavioral assessments were conducted immediately prior to

receiving the daily levodopa dose (i.e., 24 hours following the previous levodopa injection). Given the advanced age of the subjects in the present study, these drug-free tests were used in place of amphetamine rotational behavior to avoid potential mortality and/or undue stress associated with the high dose of amphetamine (5 mg/kg) that is used for this particular task.

Drag Test

The drag test is a modification of the postural adjustment test [69]. In essence, the drag test measures the ability of the rat to maintain balance during a postural stability challenge (i.e., dragging the rat backward while it balances weight on its forepaws). On test days, each rat was removed from the home cage and placed on a hard surface while an investigator grasped the rat by the tail, approximately 2 cm above the base of the tail. The tail was lifted until the hindpaws were suspended in the air and the rat's body was at an approximately 45-degree angle from the surface, allowing forepaws to remain in contact with the surface. The rat was dragged backward over a fixed distance of 3 m at a constant speed (~1 m/s) while a second investigator filmed the rat. In normal rats, this maneuver induces a forepaw tapping response in order to stabilize the off-balance center of gravity while being dragged. In unilaterally 6-OHDA-lesioned rats, the affected forepaw is instead extended rigidly to brace against the forced movement and tapping behavior is significantly reduced, while normal tapping response is maintained in the unaffected forelimb. Each test day consisted of 3-4 consecutive trials per animal. Video recordings were later watched at half-speed and a blinded investigator recorded the number of taps made by both the left and right

forepaws. Data are represented as the average number of taps across three trials for each forepaw.

Sticky Paws Test

The “sticky paws” test is a tactile stimulation task that is sensitive to nigrostriatal damage and is used to quantify somatosensory impairment in 6-OHDA-lesioned rats [70]. In this test, an adhesive sticker is placed on each forepaw. After returning to the home cage, intact rats quickly remove the sticker from both paws using their teeth, while unilaterally lesioned rats are much slower to accomplish the same task with the affected/parkinsonian paw. In the present experiment, food and water were removed from the home cage during behavioral evaluation on test days to prevent interference from these items. The test consisted of two consecutive trials. At the start of each trial, the rat was removed from the home cage and a 1 cm x 2 cm adhesive label (Avery Products Corporation, CCL Industries Inc.) was placed on the distal-radial aspect on the top of each forepaw per [70]. The rat was immediately placed back in the home cage and a timer was started. Each trial ended after the rat successfully removed both stickers or two minutes had elapsed, whichever occurred first. Latency to sticker removal and forepaw preference (i.e., the first forepaw from which the rat attempted to remove the adhesive sticker) were recorded for each trial. Data are represented as the average total latency to successfully remove the stickers (in seconds) across two trials at each time point. Rats were handled prior to testing to acclimate them to investigator manipulation.

Levodopa Administration

Beginning 5 weeks after 6-OHDA surgery, rats were primed for 5 weeks with once daily (M-Fr) injections of levodopa and benserazide (12 mg/kg, 1:1; subcutaneous (s.c.)). Levodopa was withdrawn for one week following graft surgeries to prevent potential toxic interaction of the drug with grafted cells [28, 71], after which it was reinitiated and administered daily (M-Fr) throughout the remainder of the study.

Levodopa-Induced Dyskinesia Rating

In the current study, LID were evaluated on pre-graft days 1, 6, 12, 20, and 26, and at six post-graft timepoints (weeks 3, 4, 5, 7, 9, and 10 post-engraftment). LID were rated according to the LID severity rating scale developed by our lab [72, 73], as described above in Chapter 3. On rating days, food and water were removed from the home cage to prevent interference or distractions from these items during behavioral evaluation. LID were assessed in one-minute intervals at 20, 70, 120, 170, and 220 minutes following levodopa/benserazide (12 mg/kg, 1:1, s.c.). All rats were rated by the same blinded investigator throughout the duration of the experiment.

Preparation of Donor Tissue

VM tissue containing developing A8–A10 DA cell groups was dissected from wild-type E14 Sprague-Dawley rat pups. The VM tissue was collected and pooled from multiple donors in cold calcium–magnesium free (CMF) buffer, then immediately dissociated per [28]. Briefly, dissected embryonic tissue was incubated for 10 min in CMF buffer containing 0.125% trypsin at 37°C. The cells were triturated in 0.005%

DNase using a Pasteur pipette (2.0 mm tip diameter) followed by a sterile 3cc 22-gauge syringe. The resulting suspension was layered into a 15 ml conical tube containing 5 ml sterile fetal bovine serum, then pelleted by centrifugation at 190 × g for 10 min at 4°C. The pellet was then resuspended in 1.0 ml of Neurobasal™ medium (Gibco®; Thermo Fisher Scientific, Waltham, MA, USA). Cell number and viability were estimated using the trypan blue exclusion test. Final suspensions were prepared at a density of 66,666 cells/μl, twice that used in the young cohort of rats based on [28]. Cells were kept on wet ice during transplantation surgery and used within 4 h of preparation. Cell-free Neurobasal™ medium was used for sham surgeries.

Cell Transplantation

After levodopa priming, rats were assigned to DA graft and sham graft groups such that the mean pre-graft LID ratings were similar between groups. Rats designated to the DA graft group received an intrastriatal transplant of 400,000 VM cells from E14 wild-type donors. This number of cells was chosen based on previous work by our group demonstrating that older rats (17-26 m.o. at time of grafting) exhibit reduced survival of grafted cells and delayed and inferior recovery of motor function compared to young rats [28, 29]; this inferior graft performance was observed even when older rats received proportionally larger grafts to increase the number of surviving grafted cells [28]. Accordingly, in the present study we chose to increase the number of grafted cells to maximize the probability of observing meaningful LID amelioration in these aging subjects (15 m.o. at time of grafting). As before, cells were deposited at a single rostral-caudal striatal site (0.2 mm anterior, 3.0 mm lateral, relative to bregma), distributed

along three dorsal-ventral coordinates at this site corresponding to 5.7, 5.0, and 4.3 mm ventral to the bottom edge of the skull [17, 28]. Each dorsal-ventral coordinate was injected with 2 µl of the VM cell suspension for a total volume of 6 µl (0.5 µl/min). Sham grafted rats received 6 µl of cell-free vehicle (2 µl at each site) using the same stereotaxic coordinates and flow rate. The needle was left in place for 4 min following the final injection of cells or cell-free media before being withdrawn. Levodopa was discontinued for 1 week post-grafting to prevent any toxic interaction of the drug with grafted cells [28], after which levodopa was reinitiated on a daily basis (M-Fr).

Graft-Induced Dyskinesia Rating

As in Chapter 3, in the present study we employed two previously established approaches for modeling GID: levodopa- and amphetamine-mediated. To evaluate amphetamine-mediated GID, rats received a single intraperitoneal (i.p.) dose of amphetamine sulfate (2 mg/kg) and were returned to the home cage for behavioral evaluation. The amphetamine-mediated dyskinetic behavior was assessed by a blinded investigator using the same method and rating scale described above for LID rating. Both levodopa- and amphetamine-mediated GID were examined at 10 wks post-engraftment. Amphetamine-mediated GID were evaluated 24 hours after levodopa-mediated GID were rated, and 24 hours prior to sacrifice.

Necropsy

Rats were sacrificed according to the same protocol described above in Chapter 3 and in [17]. Briefly, rats were deeply anesthetized with phenytoin/pentobarbital

euthanasia solution (250 mg/kg pentobarbital, i.p.; VetOne, Boise, ID, USA) then intracardially perfused with 200 ml room temperature heparinized 0.9% saline followed by 200 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were immediately post-fixed in 4% paraformaldehyde for 24 hours at 4°C, then submersed in 30% sucrose solution and stored at 4°C until sectioning. Brains were sectioned coronally with a sliding microtome at 40-µm thickness, then stored at -20°C in cryoprotectant. Ear clippings were collected during necropsy and stored at -80°C to be used for automated genotype confirmation (Transnetyx Inc., Cordova, TN, USA).

Tyrosine Hydroxylase Immunohistochemistry

A 1:6 series of 40-µm coronal tissue sections was used for tyrosine hydroxylase (TH) immunohistochemistry. All steps were performed at room temperature. Sections were thoroughly rinsed in tris-buffered saline containing 0.3% Triton X-100 (TBS-Tx) then incubated in 0.3% hydrogen peroxide for 20 min and blocked in 10% normal goat serum (NGS) for 90 min. After blocking, the sections were incubated for 24 hours with rabbit anti-TH primary antibody **(Table 4.1)** and 1% NGS. The next day, tissue sections were incubated for 90 min with biotinylated goat anti-rabbit secondary antibody and 1% NGS (Vector Laboratories, Inc., Burlingame, CA, USA; Cat No. BA-1000), then developed with avidin/biotin enzyme complex (Vector Laboratories, Inc., Burlingame, CA, USA; Cat No. PK6100) and 3,3'-diaminobenzidine (0.5 mg/ml).

Antigen	Assay	Primary Antibody	Vendor	Catalog Number	Dilution	Secondary Antibody [a]
Synaptopodin	TH-Synaptopodin	rabbit anti-synaptopodin	Synaptic Systems	163002	1:4000	A21207
Tyrosine Hydroxylase	TH-Synaptopodin	mouse anti-TH	Millipore	Mab318	1:1000	SuperBoost™
Tyrosine Hydroxylase	TH-VGLUT2	rabbit anti-TH	Millipore	AB152b	1:1000	SuperBoost™
VGLUT2	TH-VGLUT2	mouse anti-VGLUT2 IgG1	Abcam	Ab79157	1:300	2045303

Table 4.1 Targeted antigens and corresponding antibodies

[a] NOTE: Secondary antibody catalog numbers are Alexa Fluor®-conjugated, purchased from Invitrogen®

Stereological Quantification of Graft Cell Number & Graft Volume

A blinded investigator stereologically quantified TH-immunoreactive (THir) cells in the grafted striatum per [17, 28] using the Stereo Investigator® Optical Fractionator workflow for total enumeration estimation (MBF Bioscience, Williston, VT, USA). Grafted THir cells were counted within a 200 µm × 200 µm counting frame superimposed on a 200 µm × 200 µm grid using a 20x objective (numerical aperture 0.75) on a Nikon Eclipse 80i microscope. This was completed in 4-12 serial (1:6) TH-immunolabeled sections throughout the grafted striatum. The number of grafted tissue sections varied between animals depending on the extent of rostrocaudal spread of the graft. The optical disector height was 20 µm and the guard zones were 2.0 µm.

The same striatal tissue sections used for cell counting were used for graft volume estimation. Graft volumes were estimated with the Stereo Investigator® Cavalieri Estimator probe. A blinded investigator traced contours around the central region of the graft containing THir somas in serial tissue sections. A grid of randomly placed sampling sites (50-µm spacing) was superimposed over the contours. Volume data are expressed as total estimated graft volume corrected for over-projection (mm^3).

Stereological Quantification of Neurite Outgrowth

Graft-derived reinnervation of the host striatum was stereologically quantified using the Stereo Investigator® Spaceballs workflow. The TH-immunolabeled striatal tissue section containing the largest portion of the graft was chosen for analysis. Stereological estimates were taken proximal and distal to the graft, in all directions

relative to the graft (i.e., dorsal, lateral, ventral, medial). As before [17], the “proximal” region was defined as 100-500 μm from the graft edge and the “distal” region was defined as 700-1100 μm from the graft edge. Using systematic random sampling of sites within a grid superimposed over each region of interest, the Spaceballs probe was applied. The probe was spherical with a 5.0 μm radius and 1.0 μm guard zones. For comparison, additional sites were sampled using the same protocol in the intact striatum. Neurite density measurements were collected by a blinded investigator using the 60x oil immersion objective (numerical aperture 1.40) on a Nikon Eclipse 80i microscope. Data are expressed as estimated neurite length per probe volume ($\mu m/mm^3$).

Immunofluorescence

One representative grafted striatal tissue section was used per animal in each immunofluorescent assay. To visualize mRNA **(Table 4.2)**, the manual RNAscope® Multiplex Fluorescent V2 kit (Advanced Cell Diagnostics, Newark, CA, USA) was used with Opal dyes (Akoya Biosciences, Marlborough, MA, USA) according to manufacturer instructions. Following RNAscope®, the tissue sections were stained for TH protein using the Alexa Fluor™ 488 Tyramide SuperBoost™ kit (Invitrogen®; Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer instructions. An exception was the DA receptor mRNA assay, in which TH protein was labeled with an Alexa Fluor™ secondary antibody (goat anti-rabbit 488) in place of a Tyramide SuperBoost™ kit.

RNA Target	Probe	Accession Number	Catalog Number
Vglut2	Rn-Slc17a6	NM_053427.1	317011
Tph2	Rn-Tph2	NM_173839.2	316411
Bdnf	Rn-Bdnf-CDS	NM_012513.4	409031
Drd2	Rn-Drd2-C3	NM_012547.1	315641-C3

Table 4.2 mRNA targets and corresponding RNAscope® probes

For immunofluorescence assays in which only protein (no mRNA) was labeled and quantified, Alexa Fluor™ 488 Tyramide SuperBoost™ kits (goat anti-rabbit IgG and goat anti-mouse IgG) were used first with anti-TH primary antibodies specific to each assay **(Table 4.1)**. Additional target antigens were stained as follows after completion of the TH SuperBoost™ secondary. Briefly, TH-labeled tissue sections were rinsed in TBS-Tx, then blocked in TBS-Tx containing 10% serum for 1 hour at room temperature. The sections were then incubated with primary antibodies relevant to each assay **(Table 4.1)** for 24 hours at room temperature (VGLUT2 primary antibody) or 48 hours at 4°C (synaptopodin (SP) primary antibody) in the dark. Tissue sections were then labeled with Alexa Fluor® secondary antibodies (1:400 dilution; **Table 4.1**) for 90 min at room temperature in the dark, then incubated for 1 min in TrueBlack® (Biotium, Inc., Fremont, CA, USA) to quench endogenous lipofuscin autofluorescence. The sections were then mounted onto 2% subbed slides and coverslipped with Vectashield® anti-fade mounting medium with DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

Fluorescent Image Acquisition

As described in Chapter 3, confocal images (1024 × 1024) were acquired on a Nikon A1 laser scanning confocal system equipped with a Nikon Eclipse Ti microscope and Nikon NIS-Elements AR software (v. 5.02). For detailed synaptic characterization, z-stacks were acquired through the mounted tissue sections using the 60x oil-immersion objective (numerical aperture 1.40) with a digital zoom of 1.67x for a final magnification of 100x. A z-step of 0.3 µm was used per [74] with a scan speed of ⅛ frame/sec. In synaptic characterization analyses, two images (125 µm × 125 µm) were

collected in the grafted striatum – one in the “proximal” zone and one in the “distal” zone relative to the lateral edge of the graft as defined above. For comparison, additional images were acquired in the intact contralateral striatum using identical parameters.

For general mRNA detection and cell counting, z-stacks of the graft were acquired using a 10x or 20x objective (numerical aperture 0.45 or 0.75, respectively), with a z-step of 2 µm and a scan speed of ⅛ frame/sec. Multiple images were acquired at these increased magnifications to capture the entire graft present within each striatal tissue section.

For striatal DA receptor D2 (*Drd2*) mRNA quantification, confocal images (1024 × 1024) were acquired using a 20x objective. Two images were collected in the dorsolateral striatum of both the 6-OHDA-lesioned hemisphere and the intact contralateral hemisphere (total of four images per animal). The tissue was also stained for TH protein to ensure that regions selected for analysis in grafted animals did not include somas of grafted cells but were close enough to the graft that THir fibers were still abundant. Images were collected from both DA grafted and sham grafted groups in mature rats and, for comparison, in the same young rats used in Chapter 3 experiments and in [17].

Imaris® Fluorescent Image Quantification

Fluorescent images were analyzed with Imaris® software (v. 9.3.1 and v.9.6.0, Oxford Instruments, Bitplane Inc., Concord, MA, USA). For all analyses except those described below, three-dimensional (3D) z-stacks were imported into Imaris® and

analyzed per [17]. In each analysis, one representative tissue section was used from each animal.

TH & VGLUT2 Proteins

Z-stacks of tissue immunolabeled for TH and VGLUT2 were imported into Imaris®. The Imaris® surface function was used with semi-automatic thresholding to reconstruct THir fibers in 3D. Then, the spots function was applied to VGLUT2 protein puncta, using the same parameters across all images. A binary mask was applied to the TH surface object and VGLUT2 spots were filtered using the masked channel to select VGLUT2 spots with centers located inside the TH surface object. Data are represented as the number of VGLUT2 puncta with centers located inside THir fibers, normalized to TH surface volume (μm^3).

TH Protein & Vglut2 mRNA

Z-stacks of tissue labeled for *Vglut2* mRNA and TH protein were imported into Imaris® and converted to native Imaris® file format. The analysis was quantitatively similar to that described in Chapter 3, but modified slightly for simplification. Specifically, a TH surface was created semi-automatically followed by manual adjustment using the marching cubes function so that all THir somas were reconstructed with a 3D surface object. The spots function was used to reconstruct *Vglut2* mRNA puncta in 3D, using the same parameters in all images. The MATLAB® "Split into Surface Objects" Imaris® XT plugin was applied to the spots and the resulting data were exported. Data are expressed as the sum of values from all images collected per animal. Care was taken to ensure that the same cells appearing in adjacent images were not counted more than once. Only THir somas containing ≥ 2 *Vglut2* mRNA puncta were included for analysis.

Striatal Drd2 mRNA

Acquired confocal images were imported into Imaris® and the spots function was applied to *Drd2* mRNA puncta. Identical parameters were used across all images. Data are expressed as the total number of labeled mRNA puncta within the field of view, averaged between both images acquired in each region.

Statistical Analysis

All LID and GID behavioral data were analyzed using non-parametric Kruskal-Wallis with Dunn's post-hoc multiple comparisons test, with the exception of two-way ANOVAs which were used in the absence of a non-parametric alternative with Šídák's multiple comparisons test to compare peak GID scores between groups. Sensorimotor behavioral tasks were analyzed with two-way ANOVA, or mixed-effects model where appropriate, followed by Šídák's multiple comparisons test.

Unpaired two-tailed *t*-tests were used to analyze graft cell counts and graft volumes within each age group. Neurite outgrowth data were analyzed with two-way repeated measures ANOVA (genotype × distance from graft) and standard two-way ANOVA (age × genotype) followed by Šídák's multiple comparisons test. Two-way ANOVAs (age × genotype) were also used to assess TH-SP data at each striatal location and correlations with behavior were analyzed using non-parametric Spearman correlation.

Vglut2 mRNA data were analyzed using two-way ANOVA (age × genotype; treatment × genotype) with Šídák's multiple comparisons test. Val68Val vs Met68Met comparisons in naïve midbrains were assessed with unpaired two-tailed *t*-tests.

VGLUT2-TH protein colocalization was assessed within age groups using two-way ANOVA (treatment × genotype) with Šídák's multiple comparisons test. Differences between age groups were also analyzed with two-way ANOVA (age × genotype). VGLUT2-TH correlations with behavior were analyzed with Spearman correlation.

Unpaired two-tail *t*-tests were used to compare 5-HT/DA ratios and percentage of DA neurons containing *Bdnf* mRNA between middle-aged Val68Val and Met68Met subjects, while two-way ANOVAs (age × genotype) were used to compare these measures between age groups. Finally, three-way ANOVA (age × genotype × treatment) and a three-way mixed-effects model were used to compare *Drd2* mRNA data between striatal hemispheres within sham and grafted groups separately. Two-way ANOVAs (age × genotype) were used to make comparisons between groups within lesioned and intact striatum separately.

Statistical outliers were identified using ROUT and Grubbs' outlier tests. Parametric statistical tests were used only when the data met assumptions for normality and homogeneity of variances. All statistics were completed using GraphPad Prism® software for Windows (v. 9.0.0).

Results

Enhanced LID amelioration in grafted Met68Met rats is maintained with aging

We previously demonstrated that young, parkinsonian Met68Met rats (8 m.o. at time of grafting) showed greater and more rapid improvement of LID following DA neuron engraftment compared to young wild-type rats (Chapter 3, but see also [17]). In

the present experiment middle-aged Met68Met rats (14-15 m.o. at time of grafting) maintained this behavioral phenotype, showing significant reversal of LID compared to sham grafted rats at the first time point examined (**Figure 4.2a,d**; Week 3: $p = 0.0013$ Met68Met-DA vs Sham; $p = 0.0599$ Met68Met-DA vs Val68Val-DA). The wild-type DA grafted group required an additional week to show significant improvement vs sham (i.e., until week 4 post-graft), and both DA grafted groups maintained a robust level of behavioral recovery for the remainder of the experiment (**Figure 4.2a,d**; Week 4: $p = 0.0369$ Val68Val-DA vs Sham; $p = 0.0176$ Met68Met-DA vs Sham; Week 5: $p = 0.1035$ Val68Val-DA vs Sham; $p = 0.0540$ Met68Met-DA vs Sham; Week 7: $p = 0.0628$ Val68Val-DA vs Sham; $p = 0.0069$ Met68Met-DA vs Sham; Week 9: $p = 0.0151$ Val68Val-DA vs Sham; $p = 0.0079$ Met68Met-DA vs Sham; Week 10: $p = 0.0076$ Val68Val-DA vs Sham; $p = 0.0017$ Met68Met-DA vs Sham). Pre-graft LID during levodopa "priming" was similar between genotypes **(Figure 4.2c)**, in contrast to clinical evidence suggesting that Met allele-carrying individuals with PD develop LID earlier than wild-type Val/Val patients [75].

At the final post-graft time point, middle-aged DA grafted Met68Met rats showed a 70.31 ± 9.89% (mean ± SEM) reduction and middle-aged DA grafted Val68Val rats showed a 66.99 ± 12.71% reduction in LID severity from pre-graft baseline. Similarly, young rats exhibited LID improvement of 55.21 ± 2.22% (Val68Val) - 73.92 ± 12.51% (Met68Met) with *half* the number of cells transplanted into older subjects (200,000 vs 400,000 cells). When represented as percent improvement per grafted cell, the young cohort showed significantly greater improvement per grafted cell compared to the middle-aged cohort at the final post-grafting time point (two-way ANOVA $F(1,24) =$

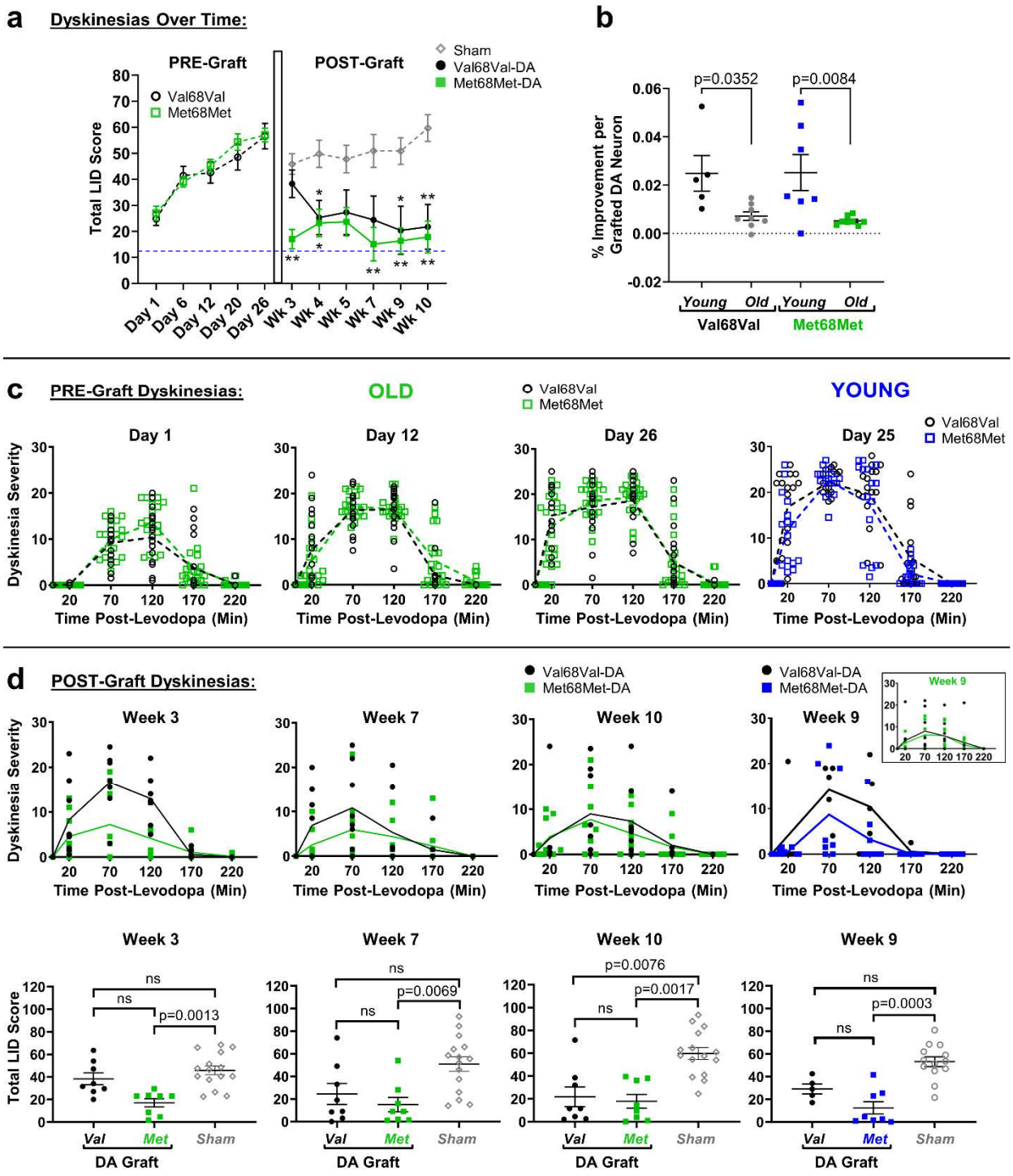


Figure 4.2 Primary behavioral assessment of graft function: Impact of host genotype on LID amelioration in middle-aged, parkinsonian rats

(a) Time course of total LID severity throughout the duration of the experiment including pre-graft LID priming and post-graft behavioral recovery. In sham grafted rats, dyskinesia severity scores did not differ between genotypes. As such, sham grafted animals were combined into one group for all post-graft time points. Horizontal blue dashed line

Figure 4.2 (cont'd)
indicates average LID score at final rating day for young DA grafted Met68Met rats with half the number of transplanted cells compared to middle-aged DA grafted rats. Statistics: Non-parametric Kruskal-Wallis test with Dunn's multiple comparisons test at each time point. Week 3: ***p*=0.0013 Met68Met-DA vs Sham; Week 4: **p*=0.0176 Met68Met-DA vs Sham, **p*=0.0369 Val68Val-DA vs Sham; Week 7: ***p*=0.0069 Met68Met-DA vs Sham; Week 9: ***p*=0.0079 Met68Met-DA vs Sham, **p*=0.0151 Val68Val-DA vs Sham; Week 10: ***p*=0.0017 Met68Met-DA vs Sham, ***p*=0.0076 Val68Val-DA vs Sham. **(b)** LID improvement per grafted DA neuron compared between age groups vs the young cohort of rats described in Chapter 3 and [17], at the final LID rating post-graft. Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test. **(c)** Pre-graft dyskinesia severity time course at days 1, 12, and 26 during LID priming. Final pre-graft LID rating (day 25) for young animals is provided for comparison with the final pre-graft LID rating for middle-aged animals (day 26). **(d)** Dyskinesia severity time course and total LID scores at weeks 3, 7, and 10 post-graft. Final post-graft rating (week 9) for young animals is provided for comparison with final rating (week 10) for middle-aged animals. For direct comparison with young animals at the same time point post-graft, week 9 dyskinesia severity time course is also provided for the middle-aged cohort (inset graph). Kruskal-Wallis test with Dunn's multiple comparisons test at each time point. Abbreviations: DA, dopamine graft; LID, levodopa-induced dyskinesias; ns, not significant

16.07, $p = 0.0005$; Šídák's multiple comparisons test: Val68Val: $t(24) = 2.545$, $p = 0.0352$; Met68Met: $t(24) = 3.161$, $p = 0.0084$; **Figure 4.2b**). There was no genotype effect (two-way ANOVA $F(1,24) = 0.0297$, $p = 0.8647$) or age × genotype interaction (two-way ANOVA $F(1,24) = 0.0579$, $p = 0.8118$).

Both Val68Val and Met68Met rats show partial recovery of "OFF-time" parkinsonian sensorimotor dysfunction with DA grafts

In clinical grafting trials for PD, improvement in OFF-time Unified Parkinson's Disease Rating Scale (UPDRS) scores is often used as a measure of transplant efficacy [30, 31]. However, to the best of my knowledge, "OFF-time" parkinsonian behaviors have not been routinely measured as a behavioral endpoint for DA neuron transplants in aged rat models of PD. In the current experiment, two tests of sensorimotor function that are sensitive to nigrostriatal DA depletion were chosen as secondary behavioral endpoints of graft function: the "sticky paws" test **(Figure 4.3a-d)** and the drag test **(Figure 4.3e-g)**.

In the "sticky paws" task, 6-OHDA-lesioned rats of both genotypes showed significant impairment of the forepaw contralateral to the lesioned brain hemisphere as evidenced by an increase in latency to successfully remove the adhesive label from the forepaw (two-way repeated measures ANOVA $F(1,46) = 66.09$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(46) = 6.648$, $p < 0.0001$, Met68Met: $t(46) = 4.814$, $p < 0.0001$; **Figure 4.3b**). At 5 weeks post-grafting, DA grafted Met68Met rats, but not Val68Val rats, showed a trend toward improvement in latency to remove the adhesive label from the affected forepaw vs baseline (two-way repeated measures

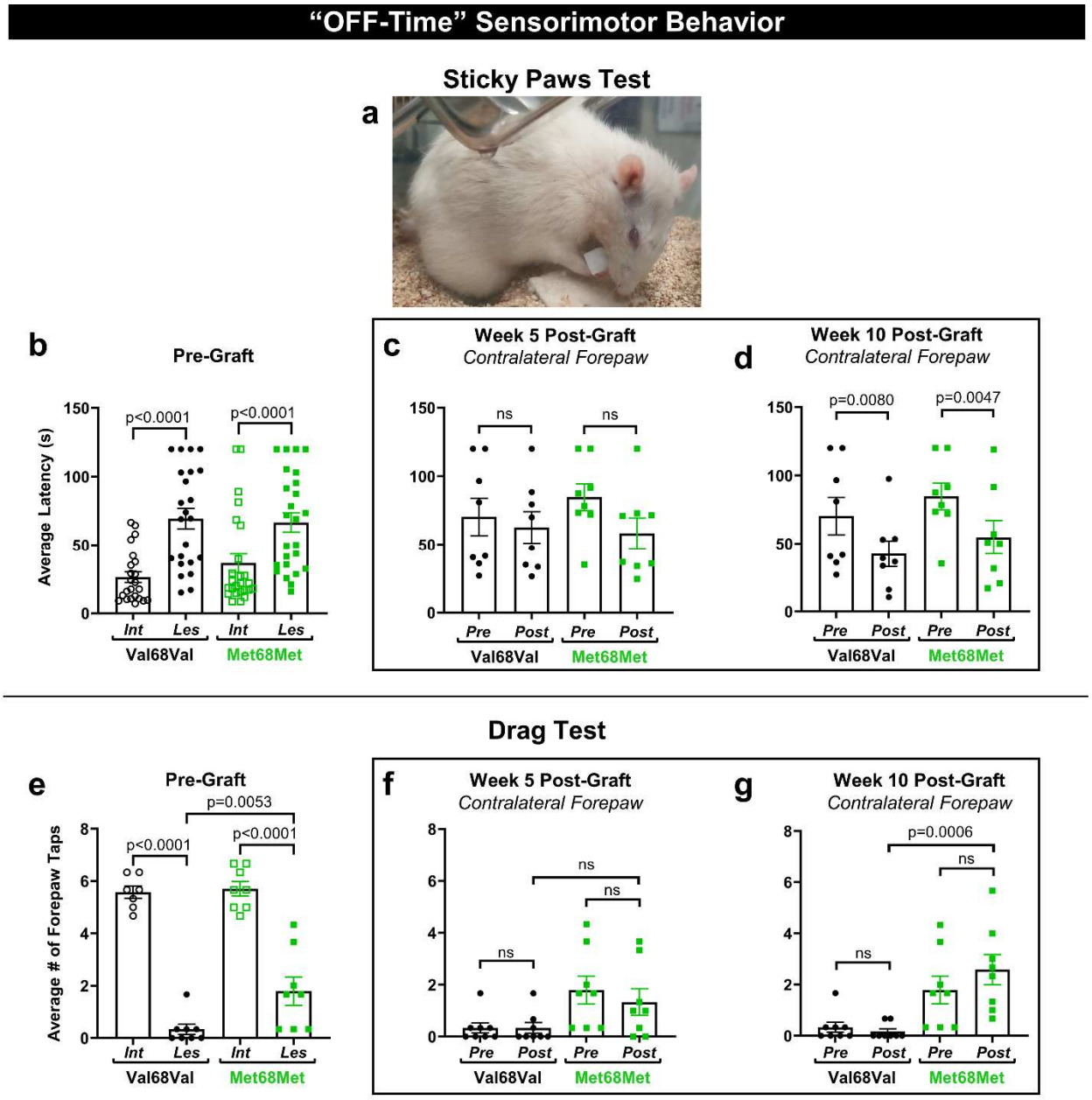


Figure 4.3 Secondary behavioral assessment of graft function: Impact of host genotype on "OFF-time" graft efficacy in middle-aged graft recipients

Behavioral measures of "OFF-time" parkinsonian sensorimotor function and subsequent improvement following grafting. **(a)** Image depicting a middle-aged, parkinsonian rat attempting to remove an adhesive label from the forepaw during the "sticky paws" test. **(b)** Average latency in seconds to remove the adhesive label from both the intact and affected forepaws at baseline, 8 weeks pre-graft. Two-way ANOVA with Šídák's post-hoc test. **(c)** Average latency to remove the adhesive label from the affected contralateral forepaw at 5 weeks post-graft (*post*) and comparison with baseline latency (*pre*). Two-way ANOVA with Šídák's post-hoc test. **(d)** Average latency to remove the adhesive label from the affected contralateral forepaw at 10 weeks post-graft (*post*) and comparison with

Figure 4.3 (cont'd)
baseline latency (*pre*). Two-way ANOVA with Šídák's post-hoc test. **(e)** Average number of forepaw taps by both the intact and affected forepaws at baseline evaluation for the drag test. Two-way ANOVA with Šídák's post-hoc test. Note that one statistical outlier was removed from the Val68Val group for the intact forepaw. **(f)** Average number of taps by the affected paw at 5 weeks post-graft (*post*) and comparison with baseline (*pre*). Mixed-effects analysis with Šídák's post-hoc test. **(g)** Average number of taps by the affected paw at 10 weeks post-graft (*post*) and comparison with baseline (*pre*). Two-way ANOVA with Šídák's post-hoc test. All data are expressed as mean ± SEM. Abbreviations: Int, intact; Les, lesioned; ns, not significant

ANOVA $F(1,14) = 4.605$, $p = 0.0499$; Šídák's multiple comparisons test: Val68Val: $t(14) = 0.6892$, $p = 0.7519$, Met68Met: $t(14) = 2.346$, $p = 0.0673$; **Figure 4.3c**). Both genotypes showed significant improvement from baseline at 10 weeks post-grafting (two-way repeated measures ANOVA $F(1,14) = 25.51$, $p = 0.0002$; Šídák's multiple comparisons test: Val68Val: $t(14) = 3.436$, $p = 0.0080$, Met68Met: $t(14) = 3.706$, $p = 0.0047$; **Figure 4.3d**).

In the drag test, similar to the "sticky paws" task, 6-OHDA-lesioned rats showed a significant impairment of the contralateral forepaw as evidenced by a reduced postural adjusting tapping response (mixed-effects model with repeated measures $F(1,12) = 230.9$, $p < 0.0001$; Šídák's multiple comparisons test for left vs right forepaw: Val68Val $t(12) = 11.97$, $p < 0.0001$, Met68Met $t(12) = 9.438$, $p < 0.0001$; **Figure 4.3e**). There was also a significant genotype effect in that Met68Met rats showed significantly more tapping of the affected forepaw than Val68Val rats ($F(1,14) = 5.380$, $p = 0.0360$; Šídák's multiple comparisons test for affected forepaw in Val68Val vs Met68Met rats: $t(26) = 3.322$, $p = 0.0053$; **Figure 4.3e**). This was not due to reduced 6-OHDA lesion success, as rats of both genotypes showed similar levels of nigral DA neuron loss (mean percent SNc DA neuron loss compared to intact hemisphere ± SEM: Val68Val: 97.03 ± 0.869%; Met68Met: 98.54 ± 0.396%; unpaired t-test $t(9) = 1.479$, $p = 0.1733$). At 5 weeks post-grafting, no improvement was observed in the affected forepaw for either genotype (mixed-effects model $F(1,12) = 0.9194$, $p = 0.3566$; **Figure 4.3f**). Similarly, at 10 weeks post-grafting, there was also no significant improvement in the affected forepaw for

either genotype (two-way repeated measures ANOVA $F(1,14) = 0.5381$, $p = 0.4753$; **Figure 4.3g**).

Aging rats develop GID with widespread intrastriatal DA neuron grafts regardless of host genotype

Previously, we showed that young Met68Met rats developed GID in the presence of a widespread intrastriatal graft of E14 primary VM DA neurons, whereas wild-type rats did not (see Chapter 3 or [17]). Here, we show that middle-aged parkinsonian rats developed GID following intrastriatal transplantation of E14 VM DA neurons *regardless of host genotype* **(Figure 4.4)**. Two paradigms for the assessment of GID were employed: amphetamine-mediated and levodopa-mediated. GID were observed in grafted animals of both genotypes using each behavioral assessment paradigm **(Figure 4.4a-d)**. Amphetamine-mediated GID assessment demonstrated that DA grafted rats of both genotypes developed robust GID when compared to sham grafted rats as evidenced by absolute peak GID score (two-way ANOVA $F(1,27) = 32.16$, $p < 0.0001$; Šídák's multiple comparisons test for DA grafted vs sham grafted rats: Val68Val: $t(27) = 2.686$, $p = 0.0243$, Met68Met: $t(27) = 5.381$, $p < 0.0001$; **Figure 4.4a**). Further, peak GID scores were higher in DA grafted Met68Met rats than in DA grafted Val68Val rats (two-way ANOVA $F(1,27) = 5.510$, $p = 0.0265$; Šídák's multiple comparisons test: $t(27) = 2.990$, $p = 0.0118$; **Figure 4.4a**). While levodopa-mediated GID were observed, no statistically meaningful differences were found (two-way ANOVA $F(1,27) = 2.728$,

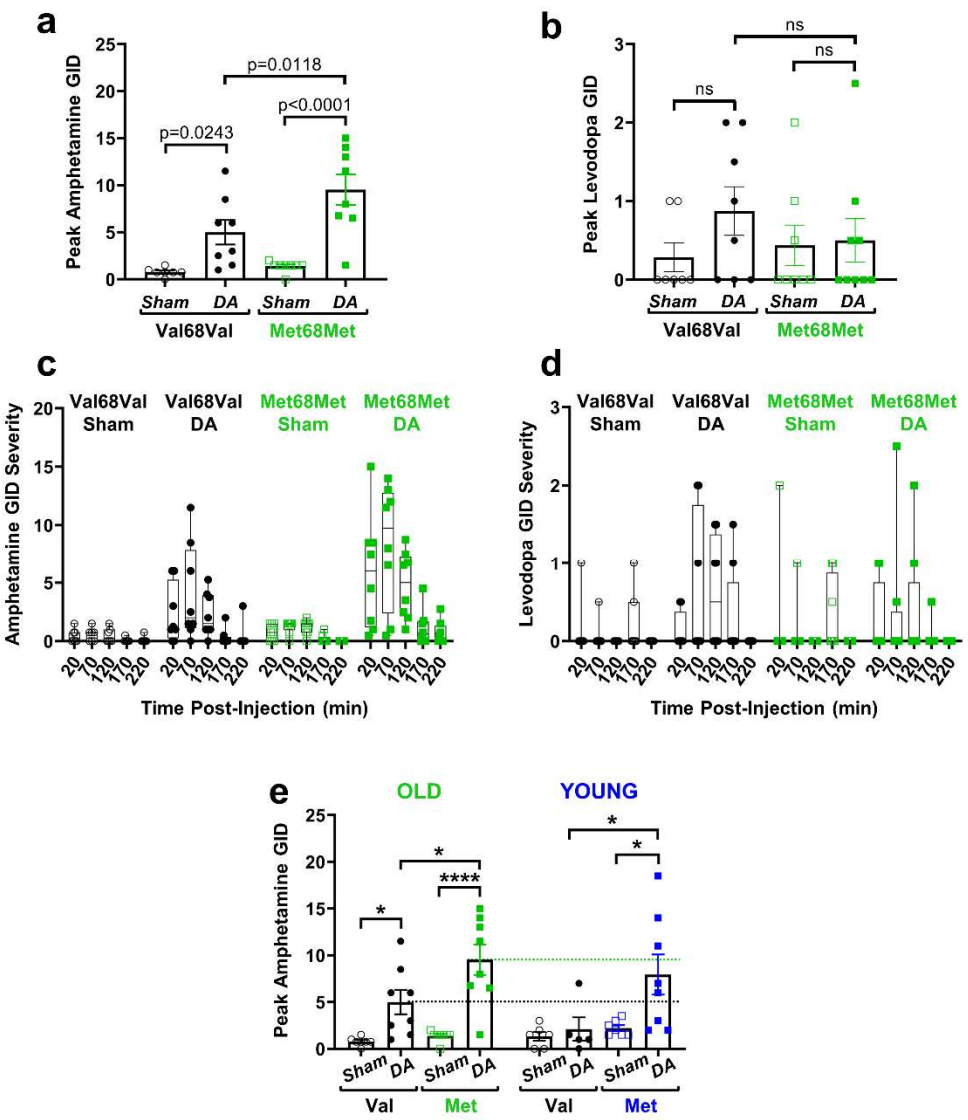


Figure 4.4 Behavioral assessment of graft dysfunction: Impact of host age and genotype on GID

(a,b) Absolute peak (i.e., highest score regardless of time post-injection) amphetamine-mediated (a) and levodopa-mediated (b) GID scores at 10 weeks post-engraftment. Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test. **(c,d)** Time course of amphetamine-mediated (c) and levodopa-mediated (d) GID at 10 weeks post-engraftment. All animals were rated in 1-min intervals at 20, 70, 120, 170, and 220 mins post-injection. **(e)** Absolute peak amphetamine-mediated GID score at 10 weeks post-graft for middle-aged and young grafted rats (young rats are the same animals used for Chapter 3 experiments and in [17]). Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test within each age cohort. Middle-aged: *p=0.0243 Val68Val-DA vs Val68Val Sham; *p=0.0118 Val68Val-DA vs Met68Met-DA; ****p<0.0001 Met68Met-DA vs Met68Met Sham. Young: *p=0.0132 Met68Met-DA vs Met68Met-Sham; *p=0.0219. Horizontal dotted lines indicate the mean score for middle-aged DA grafted groups, extended to show magnitude of increase over GID scores of young DA grafted groups. Abbreviations: DA, dopamine graft; GID,

Figure 4.4 (cont'd)
graft-induced dyskinesias; Met, Met68Met; ns, not significant; Val, Val68Val

p = 0.1102; Šídák's multiple comparisons test for DA grafted vs sham grafted rats: Val68Val: $t(27) = 1.582$, p = 0.2348, Met68Met: $t(27) = 0.7396$, p = 0.7148; **Figure 4.4b**).

Similar to middle-aged subjects, young DA grafted Met68Met rats demonstrated higher absolute peak amphetamine-mediated GID scores than sham grafted Met68Met rats (two-way ANOVA $F(1,22) = 4.881$, p = 0.0379; Šídák's multiple comparisons test: $t(22) = 2.999$, p = 0.0132) and DA grafted Val68Val rats (two-way ANOVA $F(1,22) = 5.231$, p = 0.0322; Šídák's multiple comparisons test: $t(22) = 2.777$, p = 0.0219; **Figure 4.4e**). While GID appeared to be slightly more severe in older subjects **(Figure 4.4e)**, no statistically meaningful differences were detected between age groups for grafted rats of either genotype (two-way ANOVA $F(1,25) = 1.647$, p = 0.2111).

Increased graft-derived neurite outgrowth associated with Met68Met host genotype is lost with aging

In young, parkinsonian rats, we previously demonstrated that E14 VM DA neurons grafted into Met68Met hosts extended denser and more evenly distributed neurites into the surrounding striatum than those grafted into wild-type hosts, despite similar graft survival and graft volumes (see Chapter 3 or [17]). Similarly, I demonstrate here that primary DA neuron transplants in aging rats also possessed similar numbers of surviving DA neurons and similar graft volumes between genotypes (surviving DA neurons: unpaired $t(14) = 0.2324$, p = 0.8196; graft volumes: Welch's $t(8.272) = 1.331$, p = 0.2186; **Figure 4.5a-c**). However, the more extensive neurite outgrowth observed in young Met68Met hosts was no longer apparent in middle-aged Met68Met rats when compared to Val68Val rats, and

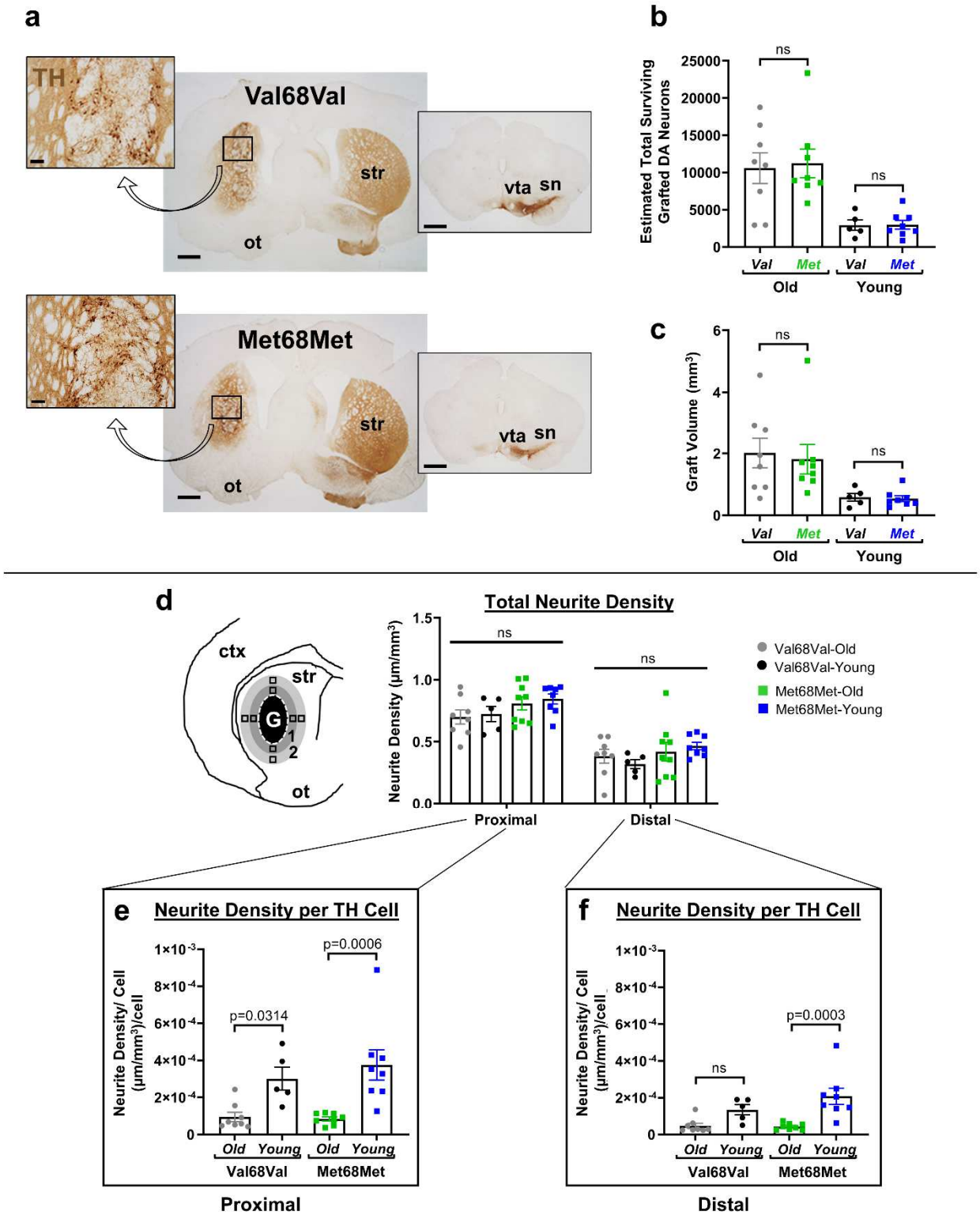


Figure 4.5 Impact of host age and genotype on graft histology and neurite outgrowth

(a) Representative micrographs of tyrosine hydroxylase immunohistochemistry in grafted striatum and corresponding substantia nigra in Val68Val and Met68Met rats. Nigral tissue

Figure 4.5 (cont'd)
sections are included to demonstrate near-complete unilateral nigral cell loss following 6-OHDA lesion. Scale bars = 1000 μm (1x striatum and nigra micrographs), 100 μm (10x micrographs). **(b)** Stereological estimate of total number of surviving grafted DA neurons. Mean ± SEM. Unpaired *t*-test between genotypes. **(c)** Estimated graft volume. Mean ± SEM. Unpaired *t*-test between genotypes. **(d)** Grafted THir neurite densities in proximal and distal regions, in both middle-aged and young rats. Young rats are the same rats used in Chapter 3 experiments and in [17]. Mean ± SEM. Two-way ANOVA (age × genotype) and two-way repeated measures ANOVA (genotype × distance from graft) with Šídák's post-hoc test. Adjacent, schematic illustrating fields of view used for image acquisition and analysis of grafted DA neurite outgrowth. Proximal and distal regions are denoted by "1" and "2," respectively. **(e,f)** Comparison of neurite density per grafted DA neuron between age groups in proximal and distal regions. Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test. Abbreviations: ctx, cortex; DA, dopamine; G, graft; Met, Met68Met; ns, not significant; ot, olfactory tubercle; sn, substantia nigra; str, striatum; TH, tyrosine hydroxylase; Val, Val68Val; vta, ventral tegmental area

this relationship held true for regions both proximal and distal to the graft (two-way repeated measures ANOVA $F(1,14) = 1.600$, $p = 0.2265$; **Figure 4.5d**). In addition, no statistically meaningful differences were observed between age groups in proximal (two-way ANOVA $F(1,26) = 0.3013$, $p = 0.5878$) or distal regions (two-way ANOVA $F(1,26) = 0.0185$, $p = 0.8930$), despite twice as many cells transplanted into older hosts (200,000 and 400,000 cells in young and middle-aged hosts, respectively) **(Figure 4.5d)**. Indeed, neurite density per transplanted DA neuron was greater in young rats than in middle-aged rats, both proximal (two-way ANOVA $F(1,25) = 22.02$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(25) = 2.588$, $p = 0.0314$, Met68Met: $t(25) = 4.165$, $p = 0.0006$; **Figure 4.5e**) and distal (two-way ANOVA $F(1,25) = 20.28$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(25) = 2.082$, $p = 0.0932$, Met68Met: $t(25) = 4.456$, $p = 0.0003$; **Figure 4.5f**) to the graft. No genotype differences were observed for this outcome measure.

Presumed graft-host synaptic connectivity is increased with aging in Met68Met, but not Val68Val, rats

Our group has shown in previous studies that graft-host synaptic connectivity is associated with graft function and that DA neuron transplants in the aged striatum form fewer synaptic connections with host striatal cells ([17, 28] but see also Chapter 3). In the present experiment, I employed the same methodology (i.e., labeling TH and SP proteins as markers of DA neuron fibers and host striatal dendritic spines, respectively) to investigate presumed graft-host synaptic appositions in middle-aged Met68Met and Val68Val rats **(Figure 4.6a,b)**. As before, all SP puncta ≤ 0.6 μm froma THir neurite

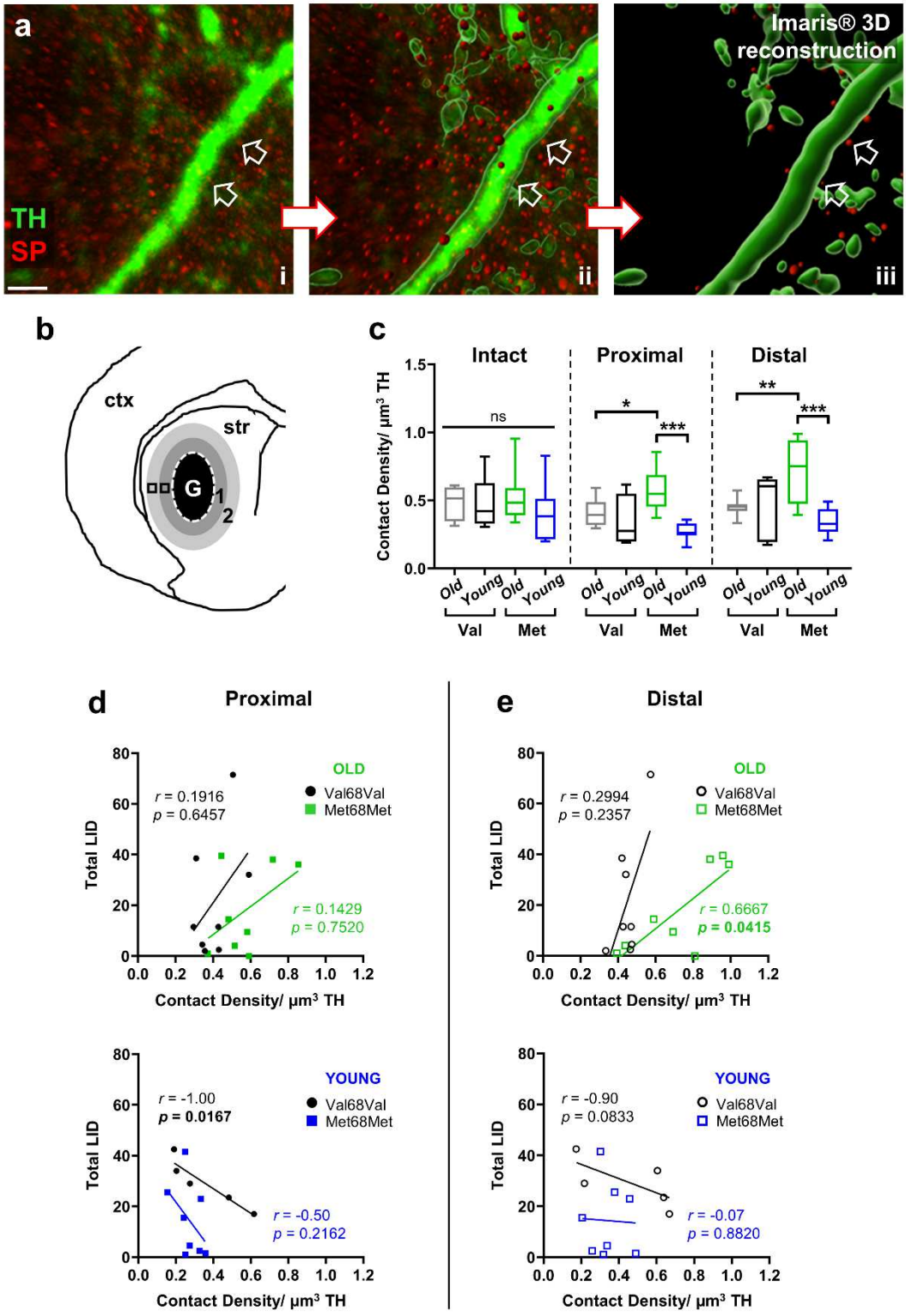


Figure 4.6 Impact of host age and genotype on graft connectivity with host dendritic spines in parkinsonian striatum

(a) Representative micrograph of immunofluorescent staining for tyrosine hydroxylase and synaptopodin to visualize grafted DA neurons and host striatal dendritic spines,

Figure 4.6 (cont'd)
respectively. Original confocal micrograph is depicted in (i), while two different views of the Imaris® 3D reconstruction are presented in (ii) and (iii). Scale bar = 2 μm. **(b)** Schematic illustrating regions in which images for analysis were acquired. Proximal and distal regions are denoted by "1" and "2," respectively. **(c)** TH-SP contact densities in striatum both proximal and distal to the graft, and in the intact contralateral striatum. Data are provided for both young and middle-aged grafted rats. The young cohort consists of the same rats used in Chapter 3 experiments and in [17]. Two-way ANOVA with Šídák's post-hoc test. Proximal: **p*=0.0363 middle-aged Val68Val vs middle-aged Met68Met; ****p*=0.0002 young Met68Met vs middle-aged Met68Met. Distal: ***p*=0.0072 middle-aged Val68Val vs middle-aged Met68Met, ****p*=0.0003 young Met68Met vs middle-aged Met68Met. **(d,e)** Side-by-side comparison of non-parametric Spearman correlations between TH-SP contact density and total LID score at the final post-graft behavioral rating time point in young and middle-aged cohorts. Correlations are shown in both proximal (d) and distal (e) regions. Abbreviations: ctx, cortex; G, graft; LID, levodopa-induced dyskinesias; Met, Met68Met; ns, not significant; SP, synaptopodin; str, striatum; TH, tyrosine hydroxylase; Val, Val68Val

were included for analysis. Grafted Met68Met host striatum contained more TH-SP appositions than Val68Val striatum in regions both proximal (two-way ANOVA $F(1,25) = 0.7091$, $p = 0.4077$; Šídák's multiple comparisons test in middle-aged Val68Val vs Met68Met rats: $t(25) = 2.524$, $p = 0.0363$) and distal (two-way ANOVA $F(1,25) = 1.439$, $p = 0.2416$; Šídák's multiple comparisons test in middle-aged Val68Val vs Met68Met rats: $t(25) = 3.212$, $p = 0.0072$) to the lateral edge of the graft **(Figure 4.6c)**. Interestingly, middle-aged Met68Met rats also possessed more graft-host/TH-SP synaptic appositions than *young* Met68Met rats, both proximal (two-way ANOVA $F(1,25) = 13.02$, $p = 0.0013$; Šídák's multiple comparisons test in middle-aged vs young Met68Met rats: $t(25) = 4.622$, $p = 0.0002$) and distal (two-way ANOVA $F(1,25) = 8.286$, $p = 0.0081$; Šídák's multiple comparisons test in middle-aged vs young Met68Met rats: $t(25) = 4.486$, $p = 0.0003$) to the graft **(Figure 4.6c)**. This is in contrast to previous work by our group demonstrating that graft-host synaptic connectivity was *decreased* with advanced age in parkinsonian, wild-type Fischer 344 rats [28]. In the present study, however, no significant age effect was observed in wild-type rats. In all, a significant age × genotype interaction was observed in which Met68Met, but not Val68Val, rats demonstrated an increase in graft-host TH-SP connectivity with advancing age (proximal two-way ANOVA *Interaction* $F(1,25) = 6.184$, $p = 0.0199$; distal two-way ANOVA *Interaction* $F(1,25) = 9.223$, $p = 0.0055$). This contrasts the intact contralateral striatum in which the endogenous TH-SP contact density was not different between genotypes (two-way ANOVA $F(1,25) = 0.0152$, $p = 0.9027$) or between age groups (two-way ANOVA $F(1,25) = 0.9958$, $p = 0.3279$; **Figure 4.6c)**.

Presumed graft-host synaptic connectivity is positively associated with LID in aging Met68Met, but not Val68Val, rats

Previously, our group demonstrated that TH-SP synaptic connectivity is associated with graft function [17, 28]. Specifically, an increase in TH-SP connectivity was associated with better graft efficacy in wild-type rats as evidenced by a *decrease* in LID severity. However, a significant correlation was not found between TH-SP connections and LID severity in young Met68Met rats (**Figure 4.6d,e**, but see also Chapter 3 or [17]). Here, I show that increased TH-SP connectivity distal to the lateral edge of the graft is significantly associated with *increased* LID severity in middle-aged Met68Met, but not Val68Val, rats (Val68Val: Spearman $r = 0.2994$, $p = 0.2357$; Met68Met: Spearman $r = 0.6667$, $p = 0.0415$; **Figure 4.6e**). Though positive trends were observed for both genotypes proximal to the graft, these correlations were not significant (Val68Val: Spearman $r = 0.1916$, $p = 0.6457$; Met68Met: Spearman $r = 0.1429$, $p = 0.7520$; **Figure 4.6d**). Finally, no significant correlations were found between TH-SP connectivity and GID.

Grafted DA neurons maintain an immature phenotype in aging hosts as evidenced by VGLUT2 protein and mRNA expression

In Chapter 3, I demonstrated that primary DA neurons transplanted into young, parkinsonian hosts expressed VGLUT2 protein and mRNA, and that this protein was associated with GID in Met68Met subjects. In the present experiment, I investigated whether aging altered these outcomes.

As in young rats, *Vglut2* mRNA was found to be present within some, but not all, grafted DA neurons **(Figure 4.7a)**. On average, 49.78 ± 4.456% and 50.52 ± 5.071% of transplanted DA neurons (presumed both A9 and A10 cell groups) contained *Vglut2* mRNA in Val68Val and Met68Met rats, respectively **(Figure 4.7b)**. This is in contrast to the naïve midbrain, in which significantly fewer endogenous DA neurons contained *Vglut2* mRNA (percent of *naïve midbrain* DA neurons expressing *Vglut2* mRNA: Val68Val: 18.97 ± 2.680%; Met68Met: 16.55 ± 1.712%; two-way ANOVA $F(1,20) = 39.47$, $p < 0.0001$; Šídák's multiple comparisons test: Val68Val: $t(20) = 4.639$, $p = 0.0003$, naïve vs grafted; Met68Met: $t(20) = 4.307$, $p = 0.0007$, naïve vs grafted; **Figure 4.7b**). This pattern of increased *Vglut2* mRNA expression in ectopically transplanted midbrain DA neurons was also demonstrated in young, grafted rats (see Chapter 3 or [17]). *Vglut2* mRNA expression in grafted DA neurons did not differ between young and middle-aged graft recipients (two-way ANOVA $F(1,26) = 2.352$, $p = 0.1372$).

Similar to what was observed previously in young rats, VM grafts placed into middle-aged, parkinsonian striatum contained an abundance of unidentified cells positive for *Vglut2* mRNA that were not immunoreactive for TH **(Figure 4.7a)**. When the total number of *Vglut2* puncta present within the grafted striatal tissue section was compared between genotypes, no differences were observed (two-way ANOVA $F(1,26) = 1.103$, $p = 0.3034$; **Figure 4.7c**). When compared to VM grafts in young rats and corrected for graft size, a significant age effect was observed. Specifically, VM grafts contained significantly more *Vglut2* mRNA relative to the number of grafted DA neurons in young rats compared to middle-aged rats (two-way ANOVA $F(1,26) = 31.96$, $p < 0.0001$; Šídák's multiple comparisons test, young vs middle-aged: Val68Val: $t(26) =$

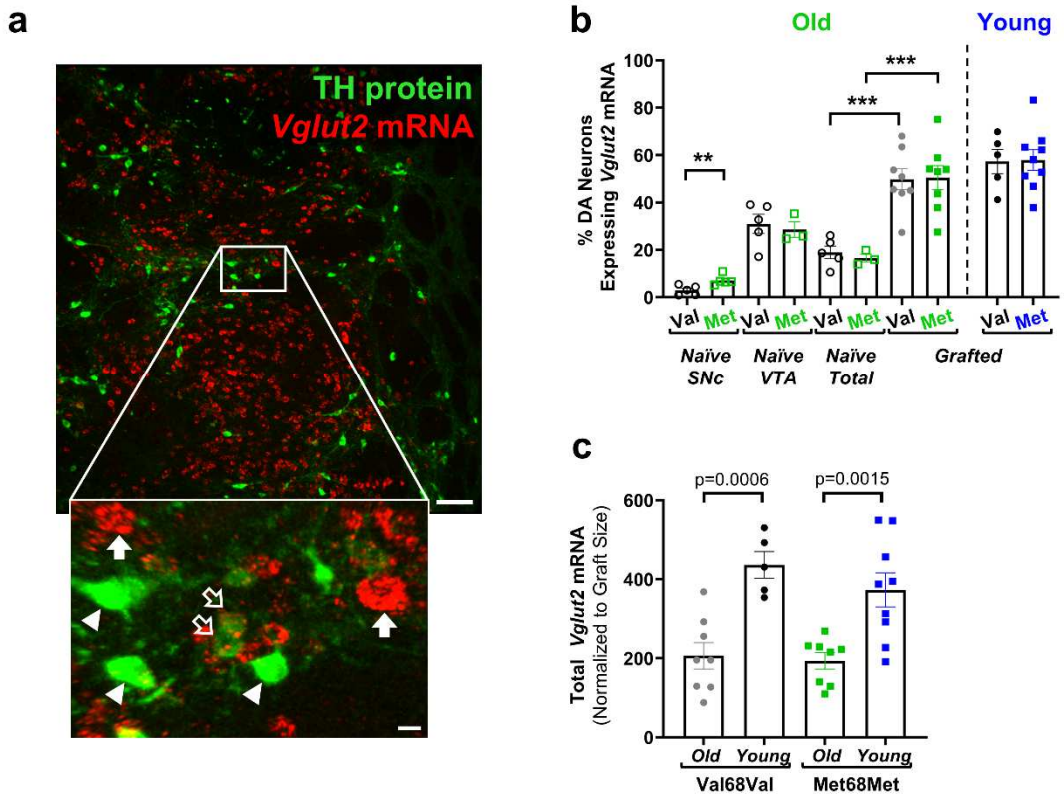


Figure 4.7 Impact of host age and genotype on *Vglut2* mRNA expression in naïve midbrain and grafted DA neurons

(a) Representative confocal micrograph depicting dual-label staining for tyrosine hydroxylase protein and *Vglut2* mRNA. TH+/*Vglut2*+ cells are indicated by open arrows, while TH-/*Vglut2*+ cells are indicated by filled arrows. Arrowheads point to TH+/*Vglut2*- cells. Scale bars = 100 µm and 10 µm (inset). **(b)** Percentage of DA neurons expressing *Vglut2* mRNA in aged, naïve adult rat midbrain and grafted, parkinsonian striatum in young and middle-aged rats. Young rats are the same subjects used in Chapter 3 experiments and [17]. Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test (naïve total midbrain vs middle-aged grafted striatum) and unpaired *t*-tests (naïve Val68Val vs Met68Met). ***p*=0.0067 naïve Val68Val SNc vs naïve Met68Met SNc; ****p* = 0.0003 naïve Val68Val total vs middle-aged grafted Val68Val; ****p* = 0.0007 naïve Met68Met total vs middle-aged grafted Met68Met. Group means in young vs middle-aged *grafted* DA neurons were not significantly different. **(c)** Comparison of total *Vglut2* mRNA present within the graft (combined TH+/*Vglut2*+ and TH-/*Vglut2*+ cells), normalized to graft size (i.e., number of DA neurons present in the tissue section). Mean ± SEM. Two-way ANOVA with Šídák's post-hoc test. Abbreviations: DA, dopamine; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; *Vglut2*, vesicular glutamate transporter 2; VTA, ventral tegmental area

4.168, p = 0.0006; Met68Met: t(26) = 3.825, p = 0.0015; **Figure 4.7c**). Without correction for graft size, grafts in both young and middle-aged graft recipients contained similar levels of *Vglut2* mRNA, despite twice as many total cells transplanted into the middle-aged striatum (two-way ANOVA F(1,26) = 0.6450, p = 0.4292; data not shown).

Using Imaris® 3D reconstruction analysis, VGLUT2 protein was also found within THir neurites in the parkinsonian striatum **(Figure 4.8a)**. While a clear pattern of increased TH-VGLUT2 colocalization in grafted animals (vs naïve) was observed in the young cohort, this pattern of VGLUT2 expression was not maintained with aging **(Figure 4.8b)**. Indeed, no significant differences were observed between (two-way ANOVA F(1,49 = 3.404, p = 0.0711) or within (two-way ANOVA F(3,49) = 1.346, p = 0.2702) genotypes. Because VGLUT2-TH colocalization was similar between proximal and distal regions in middle-aged rats, an average of both regions was used for the remaining analyses.

Strikingly, VGLUT2-TH colocalization was robustly increased across all middle-aged groups compared to young subjects **(Figure 4.8b)**. This aging-associated increase reached statistical significance for the intact contralateral striatum of grafted rats of both genotypes with a greater increase observed in Met68Met rats (two-way ANOVA F(1,26) = 48.40, p < 0.0001; Šídák's multiple comparisons test, young vs middle-aged: Val68Val: t(26) = 3.025, p = 0.0110; Met68Met: t(26) = 7.175, p < 0.0001; significant interaction effect: F(1,26) = 5.533, p = 0.0265), and in the grafted striatum of Met68Met rats only (two-way ANOVA F(1,26) = 10.79, p = 0.0029; Šídák's multiple comparisons test, young vs middle-aged: Val68Val: t(26) = 1.918, p = 0.1279; Met68Met: t(26) =

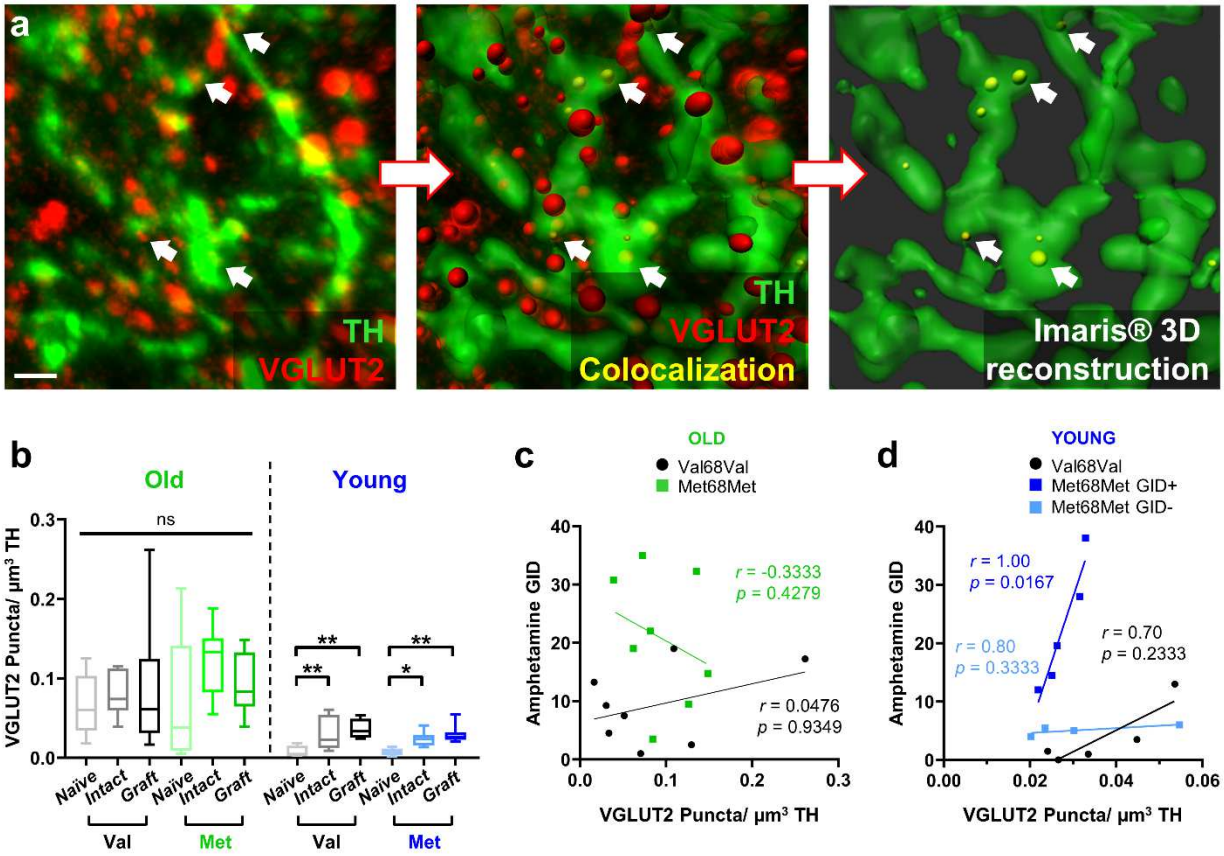


Figure 4.8 Impact of host age on VGLUT2 protein expression in DA neuron grafts and relationship to GID

(a) VGLUT2 colocalization within grafted DA fibers**,** reconstructed in 3D with Imaris®. This reconstruction was created from a representative confocal z-stack of grafted striatal tissue immunolabeled for tyrosine hydroxylase and VGLUT2. Arrows indicate VGLUT2 protein puncta enclosed within grafted DA neurites, shown in yellow. Scale bar = 1 µm. **(b)** Quantification of VGLUT2-TH colocalization, normalized to volume of the TH surface object. Young rats are the same animals used in Chapter 3 experiments and in [17]; data used with permission from the publisher. Two-way ANOVA with Šídák's post-hoc test. Young: ***p*=0.0095 Val68Val-naïve vs Val68Val-Intact, ***p*=0.0014 Val68Val-naïve vs Val68Val-DA, **p*=0.0435 Met68Met-naïve vs Met68Met Intact, ***p*=0.0043 Met68Met-naïve vs Met68Met-DA. Data for young vs middle-aged comparisons are presented in the accompanying text. **(c,d)** Non-parametric Spearman correlations between amphetamine-mediated GID and VGLUT2-TH colocalization in middle-aged (c) and young (d) grafted subjects. Data in (d) was used with permission from [17]. Note different x-axis scales in (c) and (d). Abbreviations: GID, graft-induced dyskinesias; Met, Met68Met; ns, not significant; TH, tyrosine hydroxylase; Val, Val68Val; VGLUT2, vesicular glutamate transporter 2

2.813, p = 0.0184). While VGLUT2-TH colocalization also increased in naïve old (18 m.o.) animals compared to young animals, this increase was not statistically significant.

Finally, while we showed previously that VGLUT2-TH colocalization was positively correlated with amphetamine-mediated GID in young Met68Met rats (see Chapter 3 or [17]), this association was not maintained in middle-aged subjects of either genotype (Val68Val: Spearman r = 0.0476, p = 0.9349; Met68Met: Spearman r = -0.3333, p = 0.4279; **Figure 4.8bc,d**). VGLUT2-TH colocalization was also not correlated with levodopa-mediated GID or LID in middle-aged rats (data not shown).

5-HT neurons present in VM grafts are not associated with GID in aging hosts

In Chapter 3, I showed that 5-HT neurons (identified using an RNAscope® probe for tryptophan hydroxylase 2 (*Tph2*), a marker of 5-HT neurons) were present in VM grafts present in both Val68Val and Met68Met graft recipients, with significantly (but modestly) more 5-HT neurons in Met68Met hosts. Moreover, the number of 5-HT neurons (normalized to the number of grafted DA neurons, termed "5-HT/DA ratio") was not associated with GID in graft recipients of either genotype. In the current experiment, I extended this analysis to middle-aged graft recipients. While 5-HT neurons were observed in all VM grafts, the 5-HT/DA ratio did not differ between genotypes (unpaired $t(14)$ = 0.7394, p = 0.4719) or between age groups (two-way ANOVA $F(1,26)$ = 1.846, p = 0.1859; **Figure 4.9a,b**). Furthermore, the 5-HT/DA ratio was not correlated with amphetamine-mediated GID, levodopa-mediated GID, or LID in these middle-aged subjects **(Figure 4.9c-e)**.

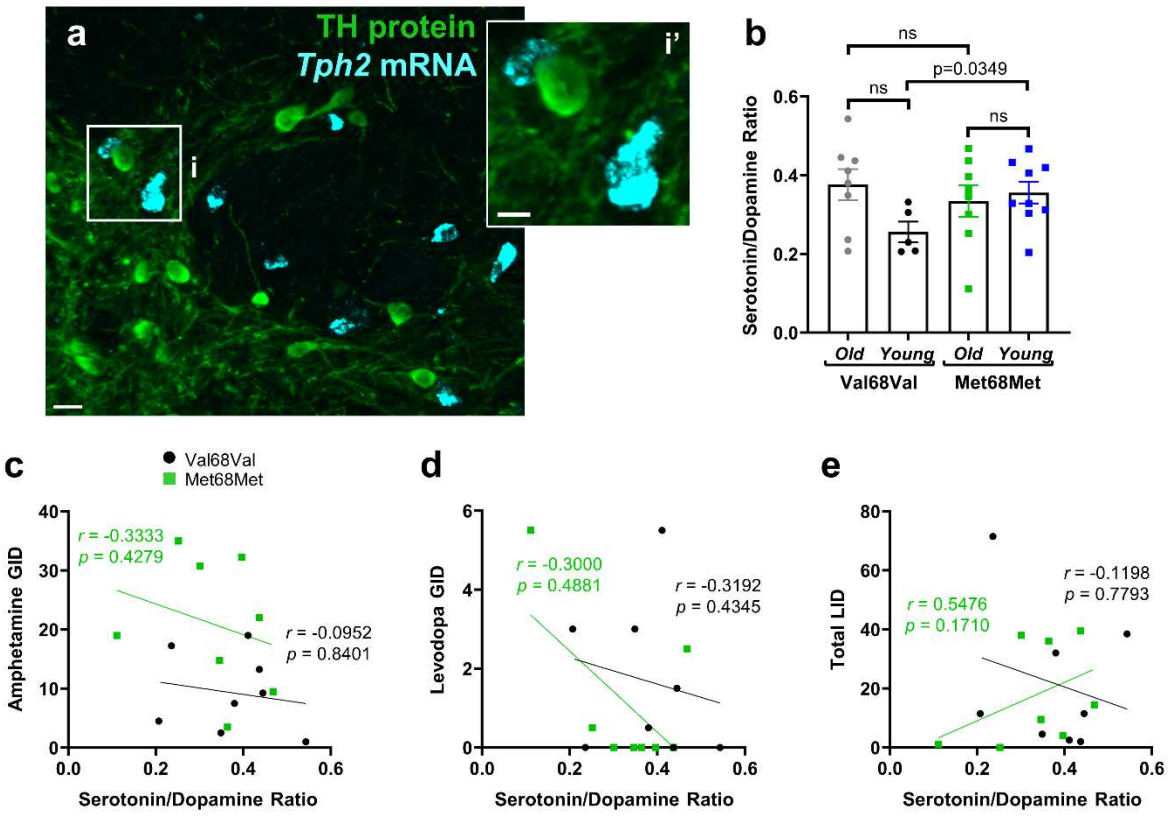


Figure 4.9 Impact of host age and genotype on 5-HT/DA cell composition in VM grafts

(a) Representative confocal micrograph showing 5-HT neurons interspersed among DA neurons in a VM graft placed in middle-aged, parkinsonian striatum. Box indicated by (i) is shown with increased magnification in (i'). Scale bars = 20 μm and 10 μm (inset). **(b)** Quantification of 5-HT neurons relative to the number of DA neurons located within the same grafted striatal tissue section. Mean ± SEM. Unpaired *t*-test (middle-aged Val68Val vs middle-aged Met68Met) and two-way ANOVA with Šídák's post-hoc test (young vs middle-aged). Young rats and statistics are the same as those used in Chapter 3 experiments and [17]; data used with permission from the publisher. **(c-e)** Non-parametric Spearman correlations between 5-HT/DA ratio and amphetamine-mediated GID (c), levodopa-mediated GID (d), and LID (e). Abbreviations: GID, graft-induced dyskinesias; LID, levodopa-induced dyskinesias; ns, not significant; TH, tyrosine hydroxylase; *Tph2*, tryptophan hydroxylase 2

The number of grafted DA neurons expressing *Bdnf* mRNA is reduced in aging hosts

Bdnf mRNA was observed in all grafted subjects **(Figure 4.10a)**. Similar to young graft recipients described in Chapter 3, a variety of cell phenotypes was observed in VM grafts in middle-aged graft recipients. Specifically, the grafts consisted of TH+/*Bdnf*+ DA neurons, unidentified TH-/*Bdnf*+ cells, and TH+/*Bdnf*- DA neurons **(Figure 4.10a: i-iii)**. On average, 69.02 ± 2.791% and 76.34 ± 2.937% of grafted DA neurons contained *Bdnf* mRNA in middle-aged Val68Val and Met68Met hosts, respectively **(Figure 4.10b)**. While grafts in Met68Met hosts contained a slightly higher percentage of *Bdnf*+ DA neurons, this finding was not statistically significant (unpaired $t(14) = 1.806$, $p = 0.0924$). When compared to young graft recipients, the percentage of grafted DA neurons containing *Bdnf* mRNA was significantly reduced in middle-aged rats of both genotypes (two-way ANOVA $F(1,24) = 24.81$, $p < 0.0001$; Šídák's multiple comparisons test, young vs middle-aged: Val68Val: $t(24) = 3.856$, $p = 0.0015$; Met68Met: $t(24) = 3.170$, $p = 0.0082$; **Figure 4.10b**). Lastly, the number of *Bdnf* mRNA puncta per grafted DA neuron did not differ between genotypes (data not shown).

Upregulation of striatal *Drd2* mRNA expression following 6-OHDA lesion is exacerbated in Met68Met subjects

Drd2 mRNA was quantified in the dorsolateral striatum of sham grafted and DA grafted subjects, in both 6-OHDA-lesioned and intact contralateral striatal hemispheres **(Figure 4.11a)**. In grafted rats, data were collected in regions of the lesioned striatum that had been reinnervated with graft-derived THir fibers. In sham grafted subjects,

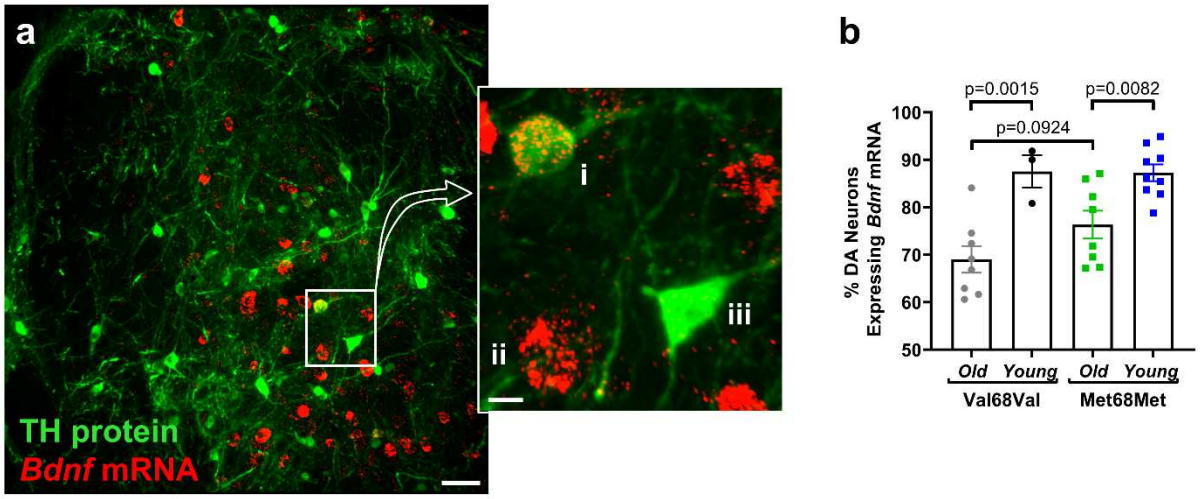


Figure 4.10 Impact of host age and genotype on *Bdnf* mRNA expression in DA neuron grafts

(a) Representative confocal micrograph depicting *Bdnf* mRNA expression in a VM graft placed into middle-aged, parkinsonian striatum. In the inset image, a TH+/*Bdnf*+ cell is indicated by (i), a TH-/*Bdnf*+ cell by (ii), and a TH+/*Bdnf*- cell by (iii). Scale bars = 50 µm and 10 µm (inset). **(b)** Percentage of grafted DA neurons expressing *Bdnf* mRNA in young and middle-aged hosts. Young rats are the same rats used in Chapter 3 experiments and [17]; data used with permission from the publisher. Mean ± SEM. Unpaired *t*-test (middle-aged Val68Val vs middle-aged Met68Met) and two-way ANOVA with Šídák's post-hoc test (young vs middle-aged). Abbreviations: *Bdnf*, brain-derived neurotrophic factor; DA, dopamine; TH, tyrosine hydroxylase

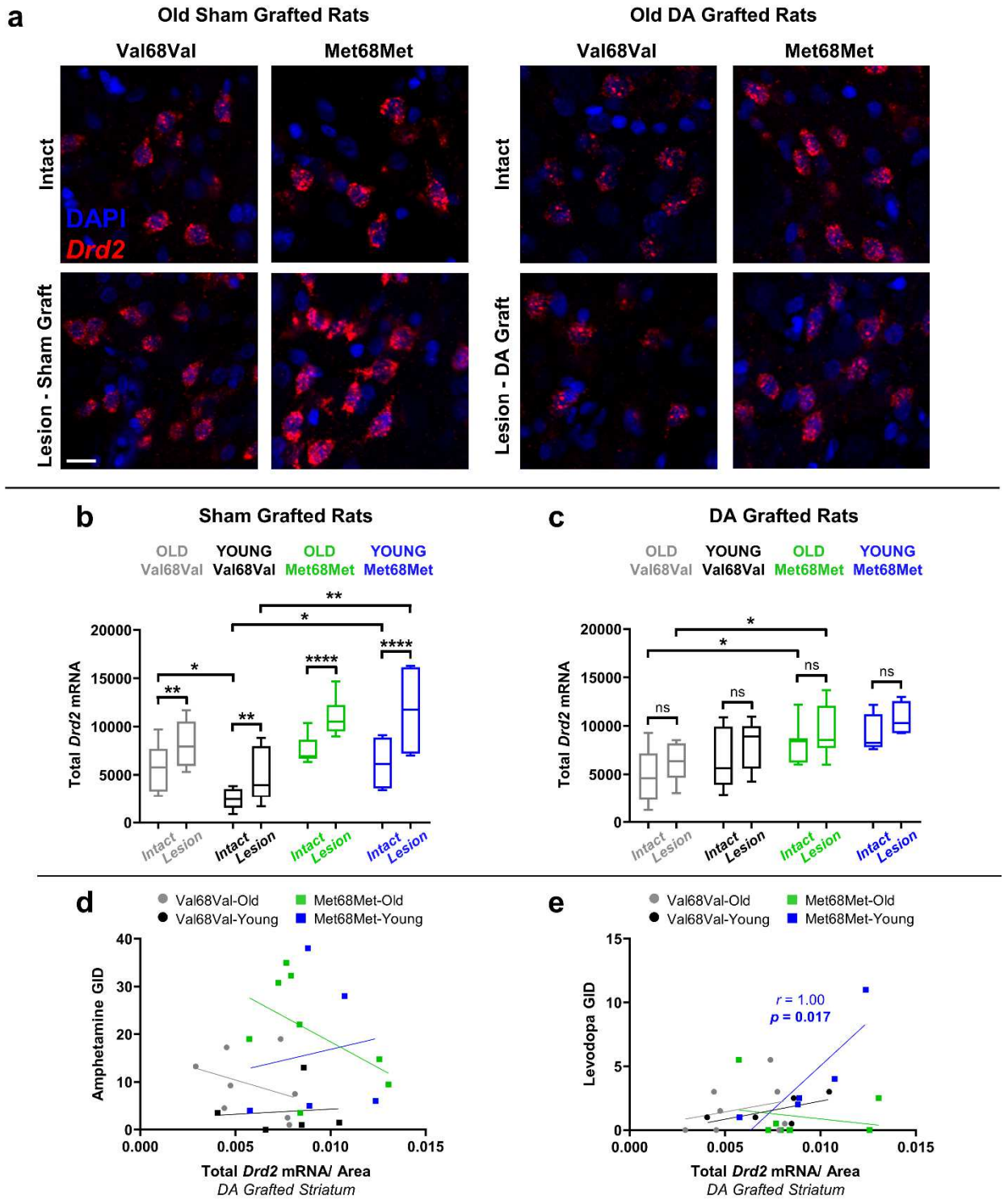


Figure 4.11 Impact of host age and genotype, 6-OHDA lesion, and grafting on striatal *Drd2* mRNA expression in sham grafted and DA grafted subjects
(a) Representative confocal micrographs depicting *Drd2* mRNA in dorsolateral striatum of middle-aged rats and counterstained with DAPI. Images are shown from sham grafted and DA grafted subjects, in both lesioned and intact striatum. Scale bar = 15 μm.

Figure 4.11 (cont'd)
(b) Quantification of total *Drd2* mRNA in sham grafted subjects. Three-way ANOVA with Šídák's post-hoc test (lesion/sham vs intact) and two-way ANOVA with Šídák's post-hoc test (age × genotype) within lesioned and intact hemispheres (young vs middle-aged; Val68Val vs Met68Met). ***p*=0.0057 middle-aged Val68Val intact vs sham graft; ***p*=0.0093 young Val68Val intact vs sham graft; *****p*<0.0001 middle-aged Met68Met intact vs sham graft; *****p*<0.0001 young Met68Met intact vs sham graft; **p*=0.0300 middle-aged Val68Val intact vs young Val68Val intact; **p*=0.0258 young Val68Val intact vs young Met68Met intact; ***p*=0.0060 young Val68Val sham graft vs young Met68Met sham graft. **(c)** Quantification of total *Drd2* mRNA in DA grafted subjects. Three-way mixed-effects analysis (lesion/DA vs intact) and two-way ANOVA with Šídák's post-hoc test (age × genotype) within lesioned and intact hemispheres (young vs middle-aged; Val68Val vs Met68Met). **p*=0.0365 middle-aged Val68Val intact vs middle-aged Met68Met intact; **p*=0.0346 middle-aged Val68Val DA graft vs middle-aged Met68Met DA graft. **(d,e)** Non-parametric Spearman correlations between total striatal *Drd2* mRNA per area and amphetamine-mediated GID (d) and levodopa-mediated GID (e). Correlation statistics are presented with more detail in the accompanying text. Abbreviations: DA, dopamine; *Drd2*, dopamine receptor D2; GID, graft-induced dyskinesias

there was a clear increase in *Drd2* mRNA expression in dorsolateral 6-OHDA-lesioned striatum compared to the intact contralateral striatum (three-way ANOVA $F(1,19) = 129.0$, $p < 0.0001$; Šídák's multiple comparisons test: Middle-aged Val68Val: $t(19) = 4.207$, $p = 0.0057$; Young Val68Val: $t(19) = 3.994$, $p = 0.0093$; Middle-aged Met68Met: $t(19) = 6.753$, $p < 0.0001$; Young Met68Met: $t(19) = 7.778$, $p < 0.0001$; **Figure 4.11b**). In addition, there was a significant treatment × genotype interaction (*Interaction* $F(1,19) = 10.37$, $p = 0.0045$). Specifically, *Drd2* upregulation in response to striatal DA depletion in the lesioned hemisphere is exacerbated in Met68Met rats (both young and middle-aged) compared to wild-type rats. *Drd2* expression was also significantly higher in young Met68Met sham grafted rats compared to young Val68Val sham grafted rats in both intact (two-way ANOVA $F(1,19) = 10.38$, $p = 0.0045$; Šídák's multiple comparisons test: $t(19) = 2.742$, $p = 0.0258$) and lesioned (two-way ANOVA $F(1,19) = 14.21$, $p = 0.0013$; Šídák's multiple comparisons test: $t(19) = 3.399$, $p = 0.0060$) hemispheres.

DA grafts normalize striatal *Drd2* mRNA expression following 6-OHDA-mediated DA depletion

In contrast to sham subjects, *Drd2* mRNA expression in the lesioned striatum of DA grafted subjects was not significantly different from the intact contralateral striatum (mixed-effects model $F(1,21) = 18.41$, $p = 0.0003$; Šídák's multiple comparisons test: Middle-aged Val68Val: $t(21) = 2.402$, $p = 0.2680$; Young Val68Val: $t(21) = 1.968$, $p = 0.5386$; Middle-aged Met68Met: $t(21) = 2.163$, $p = 0.4039$; Young Met68Met: $t(21) = 2.187$, $p = 0.3885$; **Figure 4.11c**). *Drd2* mRNA expression was, however, significantly higher in middle-aged Met68Met striatum compared to middle-aged Val68Val striatum in

both intact (two-way ANOVA $F(1,22) = 8.200$, $p = 0.0090$; Šídák's multiple comparisons test: $t(22) = 2.546$, $p = 0.0365$) and lesioned (two-way ANOVA $F(1,21) = 8.427$, $p = 0.0085$; Šídák's multiple comparisons test: $t(21) = 2.580$, $p = 0.0346$) hemispheres.

Striatal *Drd2* mRNA expression is associated with levodopa-mediated GID in young Met68Met rats

Existing evidence strongly supports the notion that DRD2 is involved in GID pathogenesis [76, 77]. Accordingly, I sought to examine whether *Drd2* mRNA is associated with GID in the present experiments. When the data were expressed as *Drd2* mRNA per area of the field of view (in pixels), *Drd2* expression was not associated with amphetamine-mediated GID in young or middle-aged rats of either genotype (Middle-aged Val68Val: Spearman $r = -0.4286$, $p = 0.2992$; Young Val68Val: Spearman $r = 0.2000$, $p = 0.7833$; Middle-aged Met68Met: Spearman $r = -0.6190$, $p = 0.1150$; Young Met68Met: Spearman $r = 0.3000$, $p = 0.6833$; **Figure 4.11d**). *Drd2* expression was, however, associated with levodopa-mediated GID in young Met68Met rats (i.e., the group of young rats that developed GID), but not in middle-aged rats of either genotype (Middle-aged Val68Val: Spearman $r = 0.0982$, $p = 0.8220$; Young Val68Val: Spearman $r = 0.6669$, $p = 0.2667$; Middle-aged Met68Met: Spearman $r = -0.2182$, $p = 0.6190$; Young Met68Met: Spearman $r = 1.000$, $p = 0.0167$; **Figure 4.11e**).

Discussion

Recent years have seen a rapid resurgence in clinical trials for the experimental therapeutic approach of cell transplantation for PD. Following technological

advancements in stem cell biology, several clinical trials for stem cell transplantation are currently in preparation or have already begun ([20, 78], but see also Chapter 2: Reemergence of Clinical Trials: Are We Ready?). While steps have been taken to optimize patient selection and cell transplantation methods, several noteworthy issues remain to be resolved. For instance, the unexpected development of GID in a considerable number of patients who received VM DA neuron transplants in controlled clinical trials was a primary impetus behind the decision to enact a worldwide moratorium for neural grafting clinical trials nearly 20 years ago. While many theories explaining the pathogenesis of GID have been proposed, a definitive explanation has yet to be realized and is likely to be more complex than can be accounted for by any single contributing factor. In addition, it remains uncertain why some individuals with PD experience little or no clinical benefit despite the presence of large, robust transplants (e.g., [48, 79]), and currently unknown factors within the aged brain continue to limit the applicability of this experimental approach to elderly populations [28, 80].

Aging is the primary risk factor for PD [50, 51] and the majority of persons with PD can be characterized as elderly [49]. Aging is an irreversible process that is characterized by the progressive deterioration of normal physiological states and a concomitant increase in vulnerability to disease and death. As an individual ages, both physical and cognitive abilities decline [81]. Likewise, the resource-modulation hypothesis proposed in 2008 by Lindenberger et al [35] posits that the loss of physiological resources in the brain during aging creates an environment in which the effects of common genetic variants such as rs6265 become more apparent, a phenomenon which is thought to contribute to variability in cognitive function among

elderly populations. In the first experiment detailed in Chapter 3, I demonstrated that the Val66Met *BDNF* SNP (rs6265) paradoxically enhances graft function and contributes to the development of GID in young, parkinsonian rats homozygous for the variant Met allele [17]. Here, I expanded upon these findings by investigating the effects of aging and its interaction with the rs6265 SNP on GID development and graft function in the context of the resource-modulation hypothesis.

A Role for Aging in GID Development

This study is the first to examine GID in middle-aged, parkinsonian rats. In general, it is thought that focal "hot spot" grafts are associated with GID development because they result in discrete zones of hyperinnervation, incompletely reinnervating the DA-denervated striatum (e.g., [30, 73, 82]). However, we recently demonstrated that young rats homozygous for the rs6265 SNP developed GID in the presence of large, widespread intrastriatal grafts, while wild-type rats did not (Chapter 3 and [17]). According to another hypothesis regarding GID pathogenesis, abnormal patterns of graft-host synaptic connectivity underlie this aberrant graft-induced side-effect [31, 52, 53]. Indeed, abnormal graft-host synaptic integration has been observed in rodent and non-human primate models of PD and in postmortem studies of clinical PD cases [83-86], and these abnormal connections were correlated with GID in parkinsonian rats [84]. Furthermore, normal aging is associated with dendritic spine loss [21-23] and impaired synaptic plasticity (e.g., [87, 88]), phenomena which may prime the aged brain for amplified GID development. Given that aging-associated deterioration of synaptic plasticity is correlated with changes in BDNF availability and activity [32-34], and that

levels of available BDNF are further reduced in association with the rs6265 SNP, it is reasonable to hypothesize that the presence of the variant Met allele would exacerbate GID in aged, parkinsonian subjects. In support of this hypothesis, there was a trend toward more severe GID in middle-aged Met68Met rats compared to young Met68Met rats. Evidence from this study also demonstrates for the first time that middle-aged, wild-type rats develop GID in the presence of widespread DA neuron grafts. Notably, middle-aged Met68Met homozygotes presented with significantly more GID than their wild-type Val/Val counterparts, supporting the hypothesis that the Met allele exacerbates GID in aged, parkinsonian subjects.

Another innovative facet of the current experiments is the investigation of VGLUT2 protein and mRNA in transplanted DA neurons as a phenotypic indicator of neuronal immaturity and the identification of abnormal glutamatergic synapses established by grafted DA neurons. Interestingly, while the presence of this vesicular glutamate protein within transplanted DA neurites was strongly correlated with GID in *young* Met68Met rats, this finding did not hold true for *middle-aged* subjects of either genotype despite more VGLUT2 protein in neurites extending from DA neurons transplanted into middle-aged hosts. These divergent findings suggest that yet unknown factors inherent to the aged brain contribute to GID expression in parkinsonian subjects, while also highlighting the importance of using aged animal models to study aging-related disorders of the nervous system such as PD.

Moving forward, investigating ways in which aging-associated impairment of synaptic plasticity and dendritic spine loss might be mitigated is warranted. With the right treatment provided at the right time, the restoration of lost dendritic spines and

enhancement of innate plasticity mechanisms could make the aged (and/or Val66Met) striatum more amenable to the establishment of physiologically meaningful synaptic connectivity with newly transplanted DA neurons, thus lessening or even preventing the occurrence of GID. To this end, one approach is to supplement BDNF signaling mechanisms, which are known to deteriorate with aging (e.g., [23, 26, 27, 88, 89]) and are impaired in association with the Val66Met SNP [6]. One approach by which BDNF signaling might be enhanced is by selective activation of the high-affinity BDNF receptor, tyrosine receptor kinase B (TrkB). Interestingly, daily intraperitoneal administration of the small molecule TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF), has been shown to rescue hippocampal synaptic plasticity and spatial memory in aged, cognitively impaired rats [90]. It has also been shown to prevent age-related decline in fear learning and memory in rats by increasing spine density in the amygdala and prefrontal cortex in addition to the hippocampus [91]. Similarly, lifestyle factors such as caloric restriction, physical exercise, cognitive stimulation, and environmental enrichment have the potential to improve synaptic plasticity and cognition and extend the healthspan of the aging brain by acting on a variety of molecular mechanisms including BDNF expression and activity [88]. Previous work in parkinsonian animal models has shown that supplementing VM grafts with neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) or BDNF can increase survival of transplanted cells, enhance graft-derived reinnervation of the host striatum, and improve graft function [14, 92-95]. However, to the best of my knowledge, the effects of this method of treatment on the restoration of striatal spine density and plasticity and/or the

establishment of appropriate graft-host synaptic connectivity in the aged and/or Val66Met striatum have not yet been extensively investigated.

Graft-Derived Recovery of OFF-Time Sensorimotor Function

Traditionally, drug-mediated behaviors have been used to investigate graft efficacy in parkinsonian rodent models, namely rotational behavior mediated by apomorphine or amphetamine [29, 96], and more recently, LID [17, 28, 72]. While amelioration of LID has been documented following clinical DA grafting [52], the primary measures used to assess graft efficacy in clinical studies frequently include changes in OFF-time parkinsonian motor symptoms [19, 30, 31]. Therefore, to more closely emulate clinical scenarios, we used drug-free sensorimotor tasks in the present experiment as a secondary measure of graft function. While graft-mediated recovery of complex sensorimotor behaviors such as skilled forelimb use has been extensively characterized in parkinsonian rodent models (e.g., [95, 97-102]), the ability of VM grafts to recover 6-OHDA-induced deficits in sensorimotor behaviors has not been investigated in *aged* animal models, to the best of my knowledge.

In the present study, grafted animals of both genotypes showed improvement in the “sticky paws” task, indicating some degree of restoration of sensorimotor function. However, this occurred without improvement in postural adjusting behavior in the drag test. As postural instability is generally refractory to DA medication in PD, this is not surprising. Overall, these data suggest that these behaviors are mediated by different underlying mechanisms that respond differentially to cell transplantation therapy. In addition, at baseline and 10 weeks post-transplantation, Met68Met rats showed

significantly more tapping of the contralateral forepaw in the drag test than wild-type rats. Both groups showed similar levels of nigral cell loss, indicating that this increase in forepaw tapping is due to factors other than disparate 6-OHDA lesion success. As anxiety is associated with the rs6265 *BDNF* variant both clinically and in an animal model (e.g., [8, 103]), a possible explanation for the increased forepaw use in Met68Met subjects is a genotype-mediated anxiety response allowing for increased use of the affected forepaw despite near-complete nigrostriatal DA depletion. Indeed, a phenomenon termed “kinesia paradoxica” has been described in persons with PD in which a powerful and urgent stimulus such as emotional or physical stress can elicit movements that a patient could not otherwise perform [104, 105]. This abnormal pattern of motor control is hypothesized to result from motor systems essentially “bypassing” the usual basal ganglia circuits to execute a movement upon receiving input from visual or auditory cues or from life-threatening events [105]. Considering the differing impact of neural grafting on the recovery of these fairly complex sensorimotor tasks, it is suggested here that future animal studies of cell transplantation for PD should include measures of OFF-time motor and sensorimotor behaviors in addition to the traditional measures of graft efficacy. This would allow investigators to better emulate clinical studies and to develop a more complete understanding of the utility of neural transplantation as an effective clinical strategy to alleviate parkinsonian motor symptoms.

The Effects of Aging on Grafted Neurite Outgrowth in rs6265 SNP Carriers

In young, parkinsonian Met68Met rats transplanted with VM DA neurons, we showed that graft-derived neurite outgrowth is enhanced compared to that observed in grafted wild-type rats (see Chapter 3 and [17]). As BDNF is well-known for its ability to promote growth and branching of axon terminals and facilitate the establishment of mature neuronal circuitry [10], the finding that graft-derived neurite outgrowth is *enhanced* in an environment consisting of significantly reduced BDNF availability is peculiar. Moreover, BDNF expression in grafted DA neurons was not different between genotypes, indicating that BDNF generated by this population of grafted cells does not account for the observed increases in neurite outgrowth in young rs6265 hosts.

The mechanism(s) underlying this paradoxical phenomenon, which as reviewed extensively in Chapter 3 is not unique to our model, remains unknown. It also remains uncertain how aging might interact with the rs6265 SNP to alter striatal reinnervation in parkinsonian subjects. Previous work from our group demonstrated that neurite density *per grafted cell* was significantly reduced in both middle-aged and old-aged parkinsonian rats when compared to young rats [28]. In the present experiment, we replicated this finding and showed that aging-related decreases in neurite density per grafted cell were similar in magnitude between genotypes. Indeed, similar neurite densities were observed in young and middle-aged graft recipients despite twice as many transplanted cells in older hosts. Furthermore, we demonstrated that the enhancement of graft-derived neurite outgrowth that was observed in young Met68Met rats was not maintained with aging, even just into middle-age, in this scenario in which an abundance of VM cells were engrafted into the parkinsonian striatum. Indeed, the

increased number of transplanted cells might impose a ceiling effect on the observed neurite densities in middle-aged subjects that could mask a true genotype effect. Future studies employing less abundant graft cell density will be needed to determine definitively whether the Met allele still confers any degree of neurite outgrowth enhancement in the aged brain.

Despite similar neurite densities between genotypes after 10 weeks of maturation in the present study, Met68Met rats exhibited an earlier and more robust reversal of LID compared to Val68Val rats (week 3 post-grafting), replicating our previous finding in young Met68Met rats that LID amelioration was enhanced (see Chapter 3 and [17]). However, in these middle-aged rats at week 4 post-grafting and throughout the remainder of the experiment, grafted wild-type rats maintained a level of behavioral recovery that was similar to that observed in Met68Met subjects. Accordingly, this evidence could be taken to suggest that the variant rs6265 genotype in graft recipients encourages *faster* neurite outgrowth from grafted DA neurons at earlier time points post-engraftment, and as grafts mature, slower neurite outgrowth in wild-type graft recipients eventually reaches the same density as that of transplanted cells in Met68Met hosts, at least in the presence of an abundance of engrafted VM cells. Again, additional studies in which fewer VM cells are grafted may be instrumental in elucidating whether the effect of the Met/Met genotype on neurite outgrowth is indeed lost or diminished in the aged brain. Because these paradoxical findings have direct clinical implications, further investigation into the underlying mechanism(s) is highly warranted.

Interaction Between Host Age and Genotype on Grafted DA Neuron Connectivity with Host Striatal MSNs

Graft-host synaptic connectivity with host striatal dendritic spines was increased in middle-aged Met68Met rats compared to their wild-type counterparts. Counter to my expectations, this measure was also increased in middle-aged Met68Met rats when compared to young Met68Met rats. This evidence contrasts previous work by our group which demonstrated that grafted DA neuron connectivity with host striatal dendritic spines *decreased* with aging in wild-type rats [28]. Notably, we found that increased TH-SP contact density was associated with worsening LID (i.e., reduced graft function) at the final post-graft behavioral rating in middle-aged, grafted Met68Met rats. This association is opposite from that which was observed in young wild-type rats. Specifically, an increase in TH-SP synaptic connectivity was found to be associated with an *improvement* in LID severity in young wild-type rats, which is to be expected if grafted DA neurons are making physiologically normal synaptic contacts/appositions with host striatal MSNs ([17, 28], but see also Chapter 3). The pattern of reduced LID in association with increased TH-SP synaptic contact density was not observed in young or middle-aged Met68Met rats, suggesting that the establishment of physiologically normal synaptic contacts between grafted DA neurons and Met68Met host striatum is not the primary factor underlying enhanced LID amelioration in rs6265 rats. Indeed, in Chapter 3 I speculated that grafted DA neurons may be unable to form appropriate/mature synaptic connections in rs6265 SNP-carrying hosts as a result of aberrant morphological changes to striatal dendritic spines (see Chapter 3:Abnormal Target Plasticity: Evidence for Aberrant Graft-Host Synaptic Connectivity in rs6265 SNP

Carriers). In the present study, this hypothesis is further supported by evidence of a statistically meaningful association of increased LID with increased TH-SP contact density only in Met68Met subjects, which suggests that these synaptic appositions are not physiologically normal. In all, when this evidence is observed through the lens of the resource-modulation hypothesis, it suggests that aging exacerbates the development of non-physiological graft-host synaptic connectivity in Met68Met hosts, which could then impair graft efficacy in elderly subjects carrying the rs6265 *BDNF* SNP or contribute to the pathological induction of GID.

Effects of Graft Recipient Age and Genotype on *Bdnf* mRNA Expression in Grafted DA Neurons

Given the significant reduction of activity-dependent BDNF release associated with the rs6265 SNP [6], we investigated whether transplanting wild-type DA neurons into parkinsonian Met68Met rats would induce an upregulation of *Bdnf* mRNA in grafted DA neurons compared to those transplanted into wild-type hosts, and whether aging also impacted *Bdnf* expression in these cells. Because BDNF signaling is tightly linked with mechanisms of DA release and uptake [106-111], we reasoned that changes in BDNF expression in grafted DA neurons could alter DA signaling in the grafted striatum, ultimately altering graft efficacy. In contrast to this hypothesis, we did not find evidence of *Bdnf* mRNA upregulation in grafted DA neurons in association with the Met allele. We did, however, find that the percentage of grafted DA neurons containing *Bdnf* mRNA was decreased in middle-aged hosts of both genotypes and that there was a trend toward more extensive downregulation in wild-type versus Met68Met graft recipients. In

agreement with the resource-modulation hypothesis, this evidence suggests that, at least in the middle-aged brain, the Met allele in host striatum is associated with a modest increase in *Bdnf* mRNA expression in grafted DA neurons relative to wild-type hosts (or conversely, the Met allele *mitigates* aging-associated reduction of *Bdnf* content in grafted DA neurons). Carrying this reasoning further, one could infer that unknown compensatory mechanisms within the young brain might counteract the need for increased BDNF production in grafted DA neurons. An alternative explanation is that, as the brain ages, endogenous BDNF release falls below a threshold level of BDNF availability that is necessary for normal function of transplanted DA neurons in Met/Met hosts, thus triggering a compensatory increase in *Bdnf* expression in this population of transplanted cells.

Despite evidence of modest *Bdnf* mRNA upregulation in middle-aged Met68Met rats (or conversely, less aging-related downregulation of *Bdnf*) relative to wild-type rats, the total percentage of grafted DA neurons expressing *Bdnf* mRNA fell significantly below that which we observed in young transplant recipients. Similarly, it is known that striatal BDNF upregulation in response to injury is reduced in the aged brain [26, 27], suggesting that advancing age alters BDNF signaling in the striatum. We show here that even young grafted DA neurons are subjected to *Bdnf* mRNA downregulation when transplanted into an aged environment. This finding carries important translational implications as decreased BDNF expression in nigrostriatal DA neurons could impact striatal synaptic plasticity and DA signaling, which in turn could significantly alter graft function. However, it is important to note that mRNA production does not necessarily

correlate 1:1 with protein quantity, and thus additional investigation into BDNF protein expression changes in grafted DA neurons is warranted.

Impact of the Variant Met Allele on Striatal *Drd2* mRNA Expression

The D2 DA receptor, DRD2, has been implicated in GID pathogenesis in part related to the reduction of this behavior following administration of the DRD2 antagonist buspirone [76, 77]. Accordingly, in the present experiment I sought to examine striatal *Drd2* mRNA expression in association with GID development in parkinsonian rats as a first step in evaluating this mechanism in our model. Because BDNF signaling mediates DA release [106-111], it is reasonable to assume that the Val66Met *BDNF* variant alters DA release in the striatum. Consistent with this hypothesis, others have shown that locomotion induced by amphetamine (a drug that stimulates DA release) is altered in $BDNF^{+/-}$ heterozygous mice [112] and the subjective response to acute amphetamine is reduced in humans carrying the Val66Met SNP [113]. Similarly, we showed previously that amphetamine-induced rotational behavior is significantly reduced in young Met68Met rats compared to wild-type rats (Chapter 3 and [17]). Based on this evidence, I hypothesized that *Drd2* mRNA is upregulated in Met68Met striatum, presumably in response to reduced nigrostriatal DA release. Indeed, in the present experiment I observed that *Drd2* mRNA was upregulated in Met68Met striatum compared to wild-type striatum, specifically in young, sham grafted rats and in middle-aged, DA grafted rats. Furthermore, striatal *Drd2* upregulation in response to 6-OHDA-mediated DA depletion was exacerbated in both young and middle-aged Met68Met rats compared to wild-type rats.

While levodopa-mediated GID in young Met68Met rats was statistically correlated with increased *Drd2* expression, this was not the case in all other conditions, which was in general contrary to my predictions. However, it is important to note that the absence of a meaningful relationship between *Drd2* **mRNA** expression and GID does not imply that DRD2 protein and/or DRD2 binding affinity are also not correlated with this aberrant graft-derived side-effect. Therefore, additional research is needed to determine the extent to which striatal DRD2 contributes to GID pathogenesis.

Conclusion

A new and exciting era of reinvigorated clinical trials in the field of cell transplantation for PD has recently begun (e.g., [19, 20]). As the field of PD research progresses toward a future defined by precision medicine, it will be crucial to address the heterogeneity in patient populations that undoubtedly contributes to variable therapeutic outcomes including those observed in past neural grafting clinical trials [30, 31]. One approach to deconstructing the complex heterogeneity of patient response to therapy is to identify common genetic variants that might alter clinical efficacy of therapeutics such as cell transplantation. Among these, the Val66Met/rs6265 *BDNF* SNP was recently shown to reduce therapeutic efficacy of oral levodopa in persons with early-stage PD [114]. Using a novel rat model of the Val66Met SNP, we recently demonstrated that this *BDNF* variant also affects therapeutic outcomes for the experimental therapeutic of neural grafting in young, parkinsonian subjects (see Chapter 3 and [17]).

In the current experiment, we expanded upon these findings by investigating the impact of the Val66Met SNP and its interaction with aging on therapeutic efficacy of DA neuron transplantation in middle-aged, parkinsonian rats homozygous for the variant Met allele. The primary findings from this study demonstrate that: (1) some aspects of enhanced behavioral efficacy in association with the Met allele are maintained with advancing age, and (2) advanced age is permissive to the induction of GID in subjects of both genotypes. While this proof-of-principle study leaves much to be investigated with future work, it carries important translational implications. Primarily, this work underscores the importance of taking global (e.g., aging) and specific (e.g., genetics) individual characteristics into consideration when prescribing therapeutic treatment for persons with PD so that the safest and most effective treatment may be prescribed for each patient. In addition, this work is extremely timely as clinical trials for neural transplantation are rapidly reemerging on an international scale [18, 20]. Notably, several of these new clinical trials are taking place in East Asian countries (China and Japan), where population allelic frequency estimates for the Val66Met SNP are highest (up to 72%, [4, 5]). Therefore, the evidence provided here is in strong agreement with the recommendation that clinical participants be genotyped for the Val66Met/rs6265 SNP so that the impact of this common *BDNF* variant in individuals with PD may be evaluated during interpretation of study results.

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CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTION

In this rapidly evolving era of precision medicine, individualized treatment will be key to advancing PD therapeutics in coming years. While mainstay treatments such as oral levodopa therapy are generally effective and robust, they are not equally effective across all patient populations (e.g., [1, 2]). Therefore, one could argue that a “one size fits all” approach to medicine, while it has had an important place in the development of modern medicine, is not sufficient if the field is to meet its overarching goal of delivering “the right treatment to the right person at the right time…” [3].

Experimental therapeutics for PD have also reached an exciting new era as a result of landmark discoveries and scientific advancements in recent decades. For instance, much of today’s translational PD research is built upon recent findings including the related discoveries of a causal link between α-syn mutations and genetic forms of PD [4] and the presence of misfolded α-syn as the primary component of Lewy pathology [5], as well as the development of effective protocols for differentiating stem cells into authentic DA neurons (e.g., [6-11] but see also Chapter 2: Alternative Cell Sources). One experimental therapeutic approach for PD that is currently experiencing a strong revitalization and generating international interest is the practice of transplanting new DA neurons to replace those that are lost to the disease. The future is at stake for this particular approach as it has been the recipient of considerable skepticism (e.g., see [12, 13] for review) following the disappointing outcomes of early clinical trials. Indeed, the obstacles that must be overcome if neural grafting is to become an effective clinical therapeutic option are substantial. As neural transplantation has yielded mixed results in previous clinical trials, and as the field of PD research has begun the gradual shift to a precision medicine approach [14-17], a prudent first step

toward preparing this experimental therapeutic for success is to elucidate factors (i.e., individual patient characteristics) that could alter the efficacy and clinical applicability of this approach among patient populations. It is in this context that my research takes place. In the following paragraphs I describe ways in which my research has contributed to the field, then end the chapter with a discussion of new empirical questions that have arisen from this work and suggestions for future research.

Implications for the Field of Cell Transplantation for PD

Assessing GID in the Context of Precision Medicine

Following a decade-long worldwide moratorium, clinical neural grafting trials for PD are rapidly reemerging [18-20]. Accordingly, the novel research presented here is especially timely and brings to light new findings that may be useful as scientists and clinicians endeavor to provide personalized treatment and maximize the therapeutic benefit of neural grafting as a clinical approach. For example, this research has demonstrated for the first time that DA neurons grafted into the parkinsonian striatum express VGLUT2 protein and mRNA – a phenotype indicative of immature DA neurons that typically disappears with maturation in the SNc [21, 22]. Though transplanted DA neurons expressed VGLUT2 equally between wild-type and r6265 SNP-carrying hosts within each age cohort, only in young Met/Met rats was this phenotype associated with the development of the deleterious graft-derived side effect, GID. Interestingly, this phenotype was not associated with GID in middle-aged animals of either genotype despite a higher proportion of VGLUT2 protein located within grafted DA neuron fibers,

suggesting that VGLUT2 expression in grafted DA neurons is likely not a major factor contributing to the development of GID in middle-aged subjects. This research also provides evidence of atypical, putative excitatory synapses made by DA neurons in the parkinsonian striatum, a finding that was also strongly associated with GID in young Met/Met rats. In all, this evidence supports the hypothesis that aberrant patterns of graft-host synaptic connectivity underlie GID pathogenesis in rs6265 SNP carriers, at least in younger subjects. Additional research is warranted to better define the role of VGLUT2 expression and its possible interaction with other factors inherent to the aged striatum in the development of GID in older subjects.

This novel research also supports the contention that DRN 5-HT neurons present in VM grafts are not associated with GID. Specifically, I observed that the presence of 5-HT neurons in VM grafts was not associated with GID in young or middle-aged subjects of either genotype. However, the limited evidence provided here does not rule out a modulatory role for this cell population in GID pathogenesis – secondary to the DA system – which has been indicated in work by other groups [23-26].

I also examined the association between GID development and additional factors that have been previously hypothesized to contribute to GID (see Chapter 2: An Unexpected Side-Effect: Addressing GID). First, based on the hypothesis by Freed and colleagues [27] that dopaminergic fiber overgrowth from grafted neurons is linked to GID, I investigated whether there was a relationship between grafted dopaminergic fiber outgrowth and GID in our animal model and did not find a meaningful correlation (data not shown). This is in line with findings put forth by other groups who also did not find a relationship between these two variables [28, 29]. Next, I investigated whether the

development of GID was linked to pre-grafting LID severity, as has been previously suggested [25, 30, 31], and again did not find a statistically meaningful correlation between these factors in young or middle-aged rats (data not shown). This finding is in agreement with both preclinical and clinical evidence [28, 32]. Finally, based on the hypothesis that buspirone (a 5-HT_{1A} partial agonist that also displays DRD2 antagonistic properties) reduces GID through its actions as a DA receptor antagonist [33, 34], I examined whether *Drd2* mRNA expression was upregulated in Met/Met striatum compared to wild-type striatum, and whether striatal *Drd2* was linked to the severity of GID. I found that *Drd2* mRNA was indeed upregulated in the dorsolateral striatum of Met/Met rats, and that striatal *Drd2* mRNA expression was linked with GID in young, but not middle-aged, parkinsonian Met/Met rats. These divergent results between young and middle-aged subjects underscore the importance of using aged animal models to study disorders of the aging nervous system.

The research presented in this thesis importantly emphasizes that mechanisms underlying GID pathogenesis are far from being fully understood, and that GID may be more dependent on individual characteristics such as patient age and genotype than was previously thought. Accordingly, it is of the utmost importance that cell transplantation research continues to investigate this graft-derived side-effect and the potential mechanisms underlying its development. Indeed, the findings presented in my research come at a critical time in which clinical trials are being reinitiated, with an especially large trial taking place in China (NCT03119636). Considering that the Val66Met SNP is extremely prevalent in East Asian countries with allelic frequency estimates as high as 72% ([35, 36]), and that this genotype is linked with more severe

GID in both young and middle-aged subjects in this preclinical work, it would be prudent for patients in this clinical study to be genotyped for this common *BDNF* mutation to assist in the interpretation of the study results. This work also suggests that understanding the relationship between the Val66Met SNP and GID in human PD graft recipients from past clinical trials is warranted. In all, once a more complete understanding of GID pathogenesis is attained, the field can then begin to assess which patients are most likely to develop GID, and thus, would be better candidates for other advanced therapies. This would ultimately aid in the overall goal of minimizing treatment side-effects by providing the right treatment to the right patient.

Considering the Role of Patient Genotype When Assessing Graft Function

The present work contributes additional novel information regarding the impact of graft recipient genotype on graft efficacy. Specifically, while more pronounced in young but also evident in middle-aged Met/Met rats, the Met/Met genotype was associated with earlier and more robust LID amelioration (our primary measure of graft function) compared to wild-type rats. In young rats, the variant genotype was also associated with enhanced neurite outgrowth from grafted DA neurons despite similar numbers of surviving DA neurons, which likely underlies the observed enhancement of graft function secondary to better reinnervation of the DA-depleted striatum. However, while the rs6265 SNP was associated with increased fiber outgrowth of grafted DA neurons in young subjects, this effect was not observed in middle-aged rats 10 weeks post-grafting, suggesting that at least in middle-aged subjects, a separate mechanism(s) is responsible for the enhanced LID amelioration that was observed in Met/Met rats. That

said, in Chapter 4 I speculated that extensive grafted DA fiber outgrowth might have occurred more rapidly in middle-aged Met/Met rats compared to that which occurred in middle-aged wild-type rats, which could explain why Met/Met subjects exhibited robust LID amelioration at the first time point post-graft, while wild-type subjects required additional time to reach this level of behavioral recovery. In addition, because the middle-aged subjects presented in Chapter 4 were transplanted with a generous number of VM cells, additional studies in which fewer cells are grafted may be instrumental in elucidating whether the effect of the Met/Met genotype is indeed diminished in the aged brain. Regardless, the mechanism(s) underlying the seemingly paradoxical phenomena of robust neurite outgrowth and enhanced graft function in association with the variant rs6265 Met allele remain to be investigated. In all, our findings hold potentially important implications for clinical studies as they may help guide clinicians and cell transplantation experts in identifying factors that contribute to heterogeneity in clinical outcomes after other previously identified factors such as variable cell transplantation methodologies are addressed.

This work also demonstrated novel age- and genotype-specific effects on graft host-synaptic integration and associated graft function. Specifically, increased grafted DA neurite synaptic connectivity with host striatal dendritic spines (i.e., TH-SP contact density) was associated with a greater reduction in LID in young wild-type, but not Met/Met, rats. This finding of increased graft function in association with increased graft-host connectivity in wild-type rats is supported by previous work by our group [37], which also demonstrated that TH-SP connectivity decreased with aging. Interestingly, when the same analysis was conducted in middle-aged rats in the present studies, TH-

SP connectivity unexpectedly *increased* with aging in Met/Met rats, but not wild-type rats. Further, increased TH-SP connectivity was associated with worsening LID in middle-aged Met/Met rats, suggesting that aging interacts with this SNP to alter graft function in Met allele carriers perhaps by promoting the establishment of abnormal synaptic connections. Translationally, these findings could help to explain, at least in part, why some patients experience minimal graft efficacy despite robust survival and synaptic integration of grafted DA neurons. However, more research is needed before this hypothesis can be substantiated.

Applicability to Related Fields

This work carries additional implications for related research in areas such as TBI, stroke, and spinal cord injury. Perhaps most importantly, my research lends support to the argument that the Val66Met *BDNF* SNP confers protection and genotype-specific advantages in certain situations which might normally be associated with detrimental effects. Indeed, work in these fields has demonstrated a role for the rs6265 SNP in enhancing recovery following TBI, peripheral nerve injury, and stroke, contrary to initial hypotheses that this SNP would be disadvantageous in these situations [38-41]. In addition, the rs6265 Met allele has been associated with better performance in a variety of cognitive tasks in aging individuals compared to Val/Val homozygotes [42-45]. These findings parallel our observations of enhanced graft efficacy and increased neurite outgrowth from DA neurons grafted into Met/Met hosts. Collectively, while these findings are logically inconsistent with what one would expect to occur given a brain environment consisting of significantly reduced BDNF release, they may help to explain

why the rs6265 SNP is so common in human populations. Indeed, it is reasonable to assume that such a common genetic variant must be advantageous at least to some extent in order to have persisted so prevalently in the human population.

Limitations and Alternative Approaches

While the present work adds novel and valuable information to the field of cell transplantation for PD, it is not without limitations and has opened doors to an extensive array of new research questions. The foremost limitation is the lack of Val/Met heterozygotes in this research. This was done intentionally for logistical reasons (e.g., so that a feasible number of experimental subjects could be used) and to maximize our chances of observing a potential effect of this common genetic variant, if any, in these initial proof-of-principle studies. However, because the majority of rs6265 SNP-carrying individuals in the human population are of the heterozygous Val/Met genotype [35], it is imperative that future experiments include heterozygous Val/Met rats to improve translation of these studies to human populations. Similarly, only male rats were used in these proof-of-principle experiments, both to simplify interpretation of results and to maintain feasibility of the experiments while using group sizes large enough to produce statistically meaningful data. Importantly, BDNF is known to interact with sex hormones, contributing to sex differences in BDNF expression, signaling, and function (for review, [46, 47]). Therefore, it is crucial that future research experiments incorporate female subjects.

The next notable limitation is our use of embryonic cells for transplantation. As addressed in detail earlier (see Chapter 2: Alternative Cell Sources), there are several

important limitations associated with the use of embryonic tissues, primarily ethical considerations and difficulties with obtaining a sufficient supply of cells on demand. These shortcomings have prompted grafting experts to shift focus to transplantable cells sourced from pluripotent stem cell populations, which form the basis of the most recently initiated clinical trials [19]. Therefore, to increase the translational impact of this work, it is suggested that future research investigate the effects of the rs6265 SNP on measures of graft function and dysfunction in models of stem cell transplantation.

Another drawback of the research presented here is the fact that our older cohort of rats, while of an advanced age, was not truly “aged”. Rather, at the start of the experiment these animals were middle-aged by typical standards, which dictate that Sprague-Dawley rats reach old age at around 18 m.o. [48]. As described in Chapter 4, this experiment was initiated when these animals were 12-13 m.o. due to concerns regarding the long-term health of rs6265 rats. Specifically, in our hands these animals exhibited excessive weight gain beginning at around 6 m.o., which contributed to the development of health complications such as diabetes and may be related to the development of pituitary adenomas exclusively in rs6265 rats in these experiments. Indeed, BDNF dysfunction (including that observed in association with the Val66Met *BDNF* mutation) is associated with metabolic dysfunction and eating disorders in preclinical animal models and in humans [49-58], in which the pituitary gland in conjunction with the neighboring hypothalamus plays a major role (e.g., [59]). Though at the conclusion of the experiment, our middle-aged cohort of rats had reached the age of 17-18 months, the novel evidence presented here may not be directly comparable, for example, to oldest-old human populations. However, the rats used in this experiment

were still of an advanced age, and accordingly, it is reasonable to assume that the data that we have collected is representative of early patterns of aging-associated change that may go on to become increasingly more apparent with advancing age.

In addition, it is important to note that rats are not humans – a factor that inherently limits the translational aspect of any work in animal models. Therefore, it is possible that our findings do not directly reflect outcomes that might be observed in human populations. For example, while the rate of LID development did not differ between genotypes in our rodent model, there is evidence to suggest that human carriers of the Val66Met mutation develop LID faster than those who do not carry this SNP (though this finding is not consistent among studies, see [60-62]). Therefore, until supporting evidence is obtained from clinical studies, prudence is advised when extrapolating the results of the current experiments to human PD populations.

A discussion of the limitations of the research projects presented in this thesis is not complete without considering alternative approaches to address the same empirical questions from a different perspective. Firstly, it would be prudent to take the experimental timeline out further to allow the grafted cells to continue to mature. This is based on evidence presented by Soderstrom et al [63] in which patterns of abnormal graft-host synaptic connectivity and GID occurred more frequently in grafts aged to 21 weeks compared to those which were allowed to mature for 10 weeks, as was done in the present experiments. Moreover, the middle-aged, grafted Met/Met rats used in these studies curiously appeared to experience unstable LID improvement over time (data not shown). Specifically, while the group as a whole showed robust behavioral improvement, especially at the first behavioral rating time point at 3 weeks post-grafting,

some of the subjects in this group experienced progressively worsening LID throughout the remainder of the experiment despite showing an excellent response at the first post-graft behavioral rating, while others showed an initial worsening followed by a “second wave” of behavioral improvement (data not shown). Therefore, as patients must live with transplanted cells for the rest of their lives – often for decades – it would be clinically relevant to determine whether the enhanced graft function that we observed in both young and middle-aged Met/Met rats is maintained with further graft maturation, and whether the variant genotype has a compounding effect on the tendency for GID to become more prominent with graft maturation.

Next, a primary strength of the work presented here is the inclusion of detailed and sophisticated studies using immunofluorescence combined with modern 3D image analysis techniques to examine neurochemical evidence of abnormal glutamatergic/excitatory synapses made by grafted DA neurons. However, the story that we have presented would be considerably stronger with electron microscopy evidence to allow us to visualize these synapses with greater clarity and confirm that they do indeed exist.

Lastly, it would be justifiable and clinically relevant to perform a more extensive battery of behavioral assessments in both rs6265 and wild-type grafted rats. For example, because we could not rule out an anxiety response as a potential mediator of forepaw postural adjusting behavior in Met/Met rats during the drag test, it would be useful to characterize, and thus control for, anxiety behaviors in this novel rat model. Furthermore, because OFF-time, drug-free motor function is often used clinically as a measure of graft efficacy [18, 27, 29], developing a similar testing protocol for rodent

models would help to improve the translational aspect of this work and would allow for a more refined assessment of the effects of this *BDNF* SNP on graft function and efficacy.

Future Research Directions

In all, while my research addresses important knowledge gaps in the field and provides significant contributions that have the potential to change the way cell transplantation therapy is approached clinically (i.e., consideration given to both global and specific factors during patient selection), it also poses a vast number of intriguing new research questions. As mentioned above, it will be imperative to repeat these experiments using heterozygous Val/Met animals as the heterozygous genotype is far more common in human populations. In addition, because we showed that Met/Met host striatum is capable of inducing enhanced neurite outgrowth in wild-type transplanted cells, it would also be important to understand the consequences of transplanting primary DA neurons from Met allele-carrying donors into wild-type hosts to determine how neurite outgrowth and behavioral recovery might be affected under these conditions. For example, one might expect the presence of the Val66Met *BDNF* SNP in transplanted cells to enhance neurite outgrowth from these cells *per se*, similar to that which has been observed in *in vitro* models with iPSC-derived motor and cortical neurons carrying this SNP (personal communication, Dr. Colin K. Franz, Rehabilitation Medicine, Northwestern University; see also Chapter 3 above) and in a mouse model of this SNP [41]. One could hypothesize that extensive neurite networks derived from these mutant cells might also establish more physiological synaptic connections if grafted into wild-type host striatum, based on the expectation of more “normal” dendritic

spines in wild-type hosts compared to Met allele-carrying hosts (see [64]), which could in turn potentially promote graft efficacy. Alternatively, extensive neurite networks derived from rs6265 cells if transplanted into rs6265 hosts might be expected to establish more extensive *aberrant* synaptic connections with the host striatum, based on our research findings that graft-host connectivity is altered in parkinsonian Met/Met hosts, which would in turn negatively impact graft efficacy. Indeed, cases of reduced or even no therapeutic benefit despite the presence of large, robust grafts that densely reinnervate the DA-depleted striatum have been observed clinically [65, 66].

In due course, it will be important to corroborate our preclinical findings in clinical studies. To this end, Dr. Jeffrey Kordower has generously provided us with postmortem brain tissue samples from the patient described in [66], which we have thus far used to examine our ability to genotype an individual from fixed postmortem tissue. Ultimately, the goal will be to determine the genotype(s) of both the patient and the transplanted cells. The patient whose brain tissue was provided to us experienced no graft-derived clinical benefit and also developed GID [66]. Thus, genotyping this particular patient could provide invaluable corroborative insight into the role of this SNP in determining clinical grafting outcomes. Interestingly, Kordower and colleagues [66] speculated that the grafted cells in this patient produced a low level of DA that was sufficient to cause diphasic dyskinesias independent of levodopa without providing antiparkinsonian benefit (see **Figure 1.6** for reference). Because BDNF mediates striatal DA release and uptake [67-72], it is possible that reduced BDNF release associated with the Val66Met variant genotype could be at least partially responsible for the hypothesized reductions in DA release from the cells that were transplanted into this patient. If confirmation of the

Val66Met SNP as a mediator of graft function/dysfunction in individuals with PD is attained, the next goal would be that future clinical grafting studies would incorporate genotyping of both patients and transplanted cells for this SNP in the interest of providing the safest and most effective therapy for each individual patient.

Another potential future research direction is to transplant stem cell-derived DA neurons into rs6265 rats. There has been concern from experts in the field that stem cell-derived neurite outgrowth is not as extensive in the parkinsonian striatum as that derived from primary DA neurons ([73, 74] but see also Chapter 2: Alternative Cell Sources). However, it is conceivable that if stem cells were transplanted in hosts carrying the rs6265 SNP, or if stem cells themselves carrying this SNP were transplanted into wild-type hosts, this genotype would promote enhanced neurite outgrowth similar to that which we observed with wild-type primary DA neurons in young Met/Met rats. Similarly, based on evidence proposing a role for the Met-prodomain as an independent ligand with modulatory effects on synaptic structure and plasticity (see Chapter 3 above for details), future research efforts aimed at investigating the effects of this peptide *per se* on neurite outgrowth and graft-host synaptic integration would be worthwhile.

In addition, while there was a correlation with levodopa-mediated GID in young Met/Met rats, there were no correlations between striatal *Drd2* mRNA expression and GID in my other experimental groups, despite evidence supporting the involvement of DRD2 in the clinical expression of GID (e.g., [33, 34]). Importantly, mRNA dynamics do not always translate seamlessly to protein function, and thus future studies aimed at examining striatal DA receptor *protein* expression, binding affinities, and activation will

be necessary to establish a clearer picture of the role of striatal DRD2 in GID pathogenesis. Understanding the dynamics of DRD2 protein and mRNA expression in grafted DA neurons themselves may also be valuable. Finally, and importantly, examining DRD2 expression in relation to other DA receptors such as DRD1 would be highly useful since the abnormal co-activation of DRD1 has been hypothesized to be necessary for DRD2-mediated GID induction [33].

Mechanisms underlying aging-related striatal dendritic spine loss and whether reversing this spine loss might improve graft function is another research area that could provide clinically useful insight to improving efficacy of DA terminal replacement therapy. Given that BDNF signaling supplementation with the TrkB agonist, 7,8-DHF, has been shown to rescue synaptic plasticity in the hippocampus, amygdala, and prefrontal cortex of aged rats [75, 76], a judicious first step in this direction would be to test the impact of administering a TrkB agonist to aged rats as a means to prepare the striatum for engraftment by increasing the density of dendritic spines available for synaptic integration. Indeed, Soderstrom and colleagues demonstrated that preserving striatal dendritic spines with the calcium channel antagonist, nimodipine, improves grafting outcomes in young parkinsonian rats [77] (though calcium channel antagonism does not appear to account for aging-related spine loss [78]). Similarly, based on the hypothesis that the variant Val/Met genotype promotes the formation of immature dendritic spines in the striatum, one might argue that BDNF signaling supplementation, for example with 7,8-DHF, could promote the maturation of dendritic spines and the formation of physiologically meaningful synapses between the Val/Met host striatum

and grafted cells. Whether this action might attenuate GID induction in rs6265 SNP carriers would be valuable and warrants further investigation.

Finally, as the field of PD research advances toward a promising future of personalized approaches to PD treatment, my research supports the importance of this methodology and suggests research directions that could advance these ambitious efforts. While stated above, I would like to reiterate that it will be critical for future research to examine the effects of other individual characteristics such as lifestyle, sex, environment, and other common genetic variants, and how these factors interact with each other to alter clinical outcomes of PD therapeutics.

Looking to the Future: An Important Role for Precision Medicine

The evidence presented in this thesis supports the idea that individual differences characteristic of heterogenous patient populations (i.e., patient genotype and age) play an important role in stimulating GID pathogenesis, bringing to light new potential contributing factors. It also emphasizes the current lack of understanding regarding factors inherent to the aged brain that contribute to reduced graft efficacy, and shows for the first time that GID develop in subjects of advanced age despite the presence of widespread intrastriatal grafts.

Based on the collective evidence presented here, it is strongly suggested that these underappreciated factors be considered as the experimental therapeutic approach of cell transplantation for PD is being taken to the clinic once again. It is clear from a plethora of clinical and preclinical data that, moving forward, the key to elucidating the solutions to these outstanding issues and untangling the complex

heterogeneity of patient response to therapeutics lies in the modern approach of precision medicine. While the PD community is beginning the transition into this new era [14-17], it is an ambitious goal that will likely take decades of extensive, collaborative efforts to reach its full potential. In the meantime, there is much work to be completed regarding identifying global and specific factors that alter therapeutic outcomes not only for neural grafting, but also for other experimental and established therapeutic alternatives for PD. Importantly, these factors *must* be addressed if DA terminal replacement strategies are to be optimized as viable therapies for persons with PD. At last, the reader is left once again with the following quote from neural transplantation pioneer, Dr. John Sladek:

> *The issues encompassed by fetal grafting research and its application to humans deserve our dispassionate and timely attention. As a society we have not yet had sufficient time to fully explore and understand the many issues attendant to embryonic cell grafting for neurodegenerative and other disorders… the scientific rationale continues to build for neural grafting as a therapy for neurological disease. Now, however, we could benefit from more* ***patience*** *rather than more* ***patients****.* [79]

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