

The Role of Dynamin Like Protein 1 in Parkinson's Disease

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Abstract

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in Parkinson's Disease

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Parkinson's disease (PD) is a neurodegenerative disease diagnosed by the presence of various motor symptoms, which result from loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). In addition to these motor defects, numerous non-motor symptoms occur and appear prior to the onset of clinical symptoms. Significant information implicates mitochondrial dysfunction in the pathogenesis of PD, with recent evidence showing changes in mitochondrial dynamics may be involved. Although the role of astrocytes has become increasingly recognized as an important factor in promoting neuronal health, their contributions towards PD have yet to be fully realized. This thesis focuses on various aspects related to detection of PD in patients as well as mechanisms of PD that occur through mitochondrial defects, which could represent targets of therapy. Chapter 2 considers the current state of biomarkers in PD and how the development of preclinical markers could be achieved to potentially allow for therapies aimed at preventing neuronal loss. Chapter 3 focuses on mitochondrial involvement in PD and how therapies may act to alleviate mitochondrial deficits. Chapters 4 and 5 examine how astrocytes and excitotoxicity play a role in PD particularly, due to changes in mitochondrial dynamics related to the fission promoting protein dynamin like protein 1 (Dlp1), and how the downstream effects of this could be a target of therapy.

Using human tissue and following previous mass spectrometry data, Dlp1 expression was demonstrated to be decreased in the SNpc of PD patients. This decrease occurred in both neurons and astrocytes within the SNpc, a finding that was extended to the same cell types in the frontal cortex of patients without observable cortical degeneration.

In pursuing the effects of this decrease in astrocytes, it was observed that knockdown of Dlp1 resulted in extensive interconnection and elongation of mitochondria, combined with impairment in their movement and localization. Further, knockdown of Dlp1 in astrocytes hindered their ability to protect against the excitotoxic effects of glutamate, which was protected against by blocking NMDA receptors. No changes in expression or localization of the major astrocytic glutamate transporters were observed. Instead, these effects can be tied back to differences in intracellular Ca^{2+} that occur in response to glutamate, as the intracellular Ca^{2+} levels were elevated in astrocytes after Dlp1 was knocked down. This was due to impaired mitochondrial buffering of Ca^{2+} that originates from the extracellular space.

These results identify a novel mechanism of mitochondrial dysfunction due to alterations in dynamics, in astrocytes, as a means through which neurodegeneration in PD could develop. Further, the results that Dlp1 is decreased in the cortex prior to the appearance of degeneration indicates that depression of Dlp1 expression is an early event in PD. They also show that targeting excitotoxicity could be an effective means of alleviating PD. Such therapies may prove to be effective in preventing neuron loss, if treatment is administered prior to the onset of clinical symptoms, which is dependent upon the development of preclinical biomarkers.

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Chapter 1:

INTRODUCTION TO PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting between 1-4% of the population over the age of 60[1]. While the causes of PD have yet to be determined, the greatest risk factor for developing PD remains age, as the prevalence of PD increases starting between ages 55 and 60[1,2].

PD is clinically diagnosed by the presence of characteristic motor symptoms, including resting tremor, bradykinesia, rigidity, and postural instability[3]. The underlying cause of these symptoms is loss of dopamine (DA) signaling in the striatum due to death of DAergic neurons that project there from the substantia nigra pars compacta (SNpc)[4,5], which is confirmed by the finding that DA replacement with levodopa (L-DOPA) alleviates these symptoms[6]. Protein aggregates called Lewy bodies, comprised largely of α -synuclein, are a hallmark of the disease[4], and are found throughout the brains of PD patients, first in the remaining DAergic neurons in the SNpc of PD patients and later in cortical regions as the disease progresses[7].

These inclusions correlate with disease progression, even prior to the onset of clinical symptoms[7], and may contribute to a variety of non-motor symptoms that have been associated with PD, including the earliest stages. Such symptoms include autonomic dysfunction, impaired olfaction, alterations in sleep patterns, and changes in cognitive function related to memory, speech, visual perception, and executive function[8-11]. This indicates that brain function in regions beyond the SNpc are affected in PD, which is not surprising as Lewy pathology is observed throughout the central nervous system[12]. Further, it also shows that PD is a systemic disorder that can affect the quality of life for those afflicted by it in many ways, beyond the classical motor dysfunction.

Depression, constipation, anosmia, and rapid eye movement sleep behavior disorder are some symptoms that are particularly interesting, as they have been shown to preclude the onset of motor symptoms, in some instances by several years. These symptoms, however, are fairly non-specific and can stand on their own as conditions, or are similarly present as preclinical symptoms in other neurodegenerative disorders[13]. Understanding different patterns that develop in sub-populations of PD patients will allow for early therapeutic intervention in PD. This is critical as halting or preventing neurodegeneration prior to the onset of motor symptoms will likely be more effective at treating PD than treating after disease onset.

Although the pathway through which neurodegeneration occurs is still unclear, several mechanisms of neuron death have been proposed. As protein aggregates are a hallmark of PD, dysfunction of chaperone proteins or the ubiquitin-proteasome system has been hypothesized to occur, resulting in the accumulation of damaged proteins[14,15]. Protein aggregates may also be indicative of other processes going on, particularly excessive production of reactive oxygen species (ROS). Evidence of elevated oxidative stress has been observed in PD patients, and may result in damage to neurons directly, or could damage proteins resulting in their aggregation[16-18]. The finding of elevated oxidative damage also sheds light on mitochondrial dysfunction as a mechanism of PD, as mitochondria are a primary source of ROS and their dysfunction results in elevated production[19]. Indeed, mitochondrial activity is believed to be impaired in PD and inducing mitochondrial dysfunction results in PD[19,20]. As their dysfunction could not only result in the death of neurons but also in the aggregation of proteins involved in PD, mitochondrial dysfunction represents an important aspect of PD pathogenesis that could be targeted to prevent neuronal loss and PD.

Chapter 2 will describe the current state of biomarker discovery in PD and discusses how these techniques may be employed in strategies to develop markers capable of detecting PD prior to the onset of clinical symptoms (i.e. in the prodromal stages of disease). Chapter 3 discusses how mitochondrial dysfunction has been implicated in PD and how current mechanisms of therapy could target these defects. It also considers how changes in mitochondria could be implicated in neurodegeneration and PD and represent a novel target of therapy. Chapters 4 and 5 consider how alterations in mitochondrial dynamics in astrocytes associated with PD could contribute to excitotoxic neuron death.

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Chapter 2:

BIOMARKERS OF PARKINSON DISEASE: CURRENT STATUS AND FUTURE PERSPECTIVES

This chapter is slightly modified from the following co-authored publication that is available through the publisher listed on the National Center for Biotechnology Information's PubMed Database (<http://www.ncbi.nlm.nih.gov/pubmed/>)

Wang, J. Hoekstra, J.G, Zuo, C., Cook, T.J., Zhang, J. (2013) Biomarkers of Parkinson's disease: current status and future perspectives. *Drug Discover Today*. 18 (3-4): 155-62. PMID: 22982303.

Introduction

Although current methods for the diagnosis of PD are fairly effective, the misdiagnosis rate of PD can range from 10-50% by movement disorder specialists [1] due in part to the fact that there are no sensitive and specific biomarkers validated for clinicians to differentiate PD from other movement disorders with overlapping clinical symptoms. Apart from diagnostic utility, biomarkers for PD are also needed for monitoring disease progression and efficacy of interventions, which are currently assessed by severity of motor symptoms.

An ideal PD biomarker should meet the following qualifications: high sensitivity and specificity validated by neuropathological examination, satisfactory test–retest reproducibility, easy accessibility, inexpensive, and offer the ability to monitor disease progression without being biased by age, compensatory mechanisms, or treatments. While no such biomarker to date fulfills all of these criteria for PD, this review will discuss two areas of research, neuroimaging and biochemical markers, which have demonstrated obvious potential in diagnosing the disease and monitoring its progression. Future biomarker investigations relating to prodromal biomarkers and markers related to nonmotor symptoms are also discussed.

Neuroimaging Biomarkers

To date, the most mature PD biomarkers for nigrostriatal neurodegeneration are those employing neuroimaging methodologies. Currently, controversy surrounds whether these techniques can be effective in differentiating clinically overlapping parkinsonisms and/or objectively assessing PD progression. The major methods used as well as their current clinical and research utility are discussed below and summarized in **Table 1**.

Dopaminergic Imaging

Aromatic Amino Acid Decarboxylase

6-[¹⁸F]-fluoro-L-3,4-dihydroxyphenylalanine (¹⁸F-dopa) positron emission tomography (PET) was the first neuroimaging approach validated for measuring and assessing presynaptic DAergic neuronal integrity. To reach the striatum, ¹⁸F-dopa is transported across the blood-brain barrier, taken up by axonal terminals of DA neurons, decarboxylated by aromatic amino acid decarboxylase (AADC), converted into ¹⁸F-DA, and stored in vesicles. The key determinants for ¹⁸F-dopa uptake are the density of the axonal

terminal plexus and AADC activity, which reflect the number of remaining nigral DAergic cells. Patients with early PD show 50% decreased ^{18}F -dopa uptake in the putamen [2], with the greatest reduction occurring in a gradient pattern in the posterior dorsal putamen contralateral to the side of symptom onset [3,4]. Although such imaging appears to be effective in confirming symptomatic PD, its utility in assessing PD progression [5,6] and detecting prodromal PD are questionable because ^{18}F -dopa PET may underestimate the degree of degeneration due to compensatory up-regulation of AADC in remaining terminals [7].

Dopamine Transporter

The dopamine transporter (DAT), a protein expressed on the membrane of presynaptic DA terminals and involved in the reuptake of DA, can be assessed with PET using several tracers, including ^{11}C -CFT, ^{18}F -FP-CIT, ^{11}C -RTI-32, and ^{11}C -methylphenidate. Single photon emission computed tomography (SPECT), a technology used routinely in clinical practice, can also be used to image DAT via ^{123}I -b-CIT, ^{123}I -FP-CIT, ^{123}I -IPT, ^{123}I -altropane, or $^{99\text{m}}\text{Tc}$ -TRODAT-1 [8]. DAT imaging is used as an *in vivo* marker, reflecting integrity and number of DA neurons. A few studies have reported significantly reduced striatal DAT in more than 95% of parkinsonism cases [9], including those at early stages [10].

With respect to monitoring PD progression and efficacy of putative agents, the value of DAT imaging remains to be established. Although some investigations suggest that striatal uptake of ^{18}F -FP-CIT correlates with Hoehn and Yahr (H&Y) score [11], other clinical trials show that DAT imaging density declined faster in PD patients treated with levodopa, despite improved clinical motor scores [12]. Symptomatic therapy may therefore influence imaging results in their relation to clinical diagnosis.

Vesicular Monoamine Transporter 2

Vesicular monoamine transporter 2 (VMAT2), a membrane protein that transports monoamines from the cytosol into secretory vesicles in monoaminergic neurons, is exclusively expressed in the brain and plays an essential role in DA reuptake. VMAT2 is imaged using ^{11}C - or ^{18}F -dihydrotetrabenazine (DTBZ) PET and is the newest approach for the assessment of nigrostriatal projections [13]. In patients with PD, striatal ^{11}C -DTBZ is significantly reduced with the putamen exhibiting the greatest decrease [13]. VMAT2 imaging appears to be less sensitive to compensation and pharmacologic regulation [14], giving it the potential to provide the most reliable measurement of the density of DAergic terminals in PD.

Nevertheless, the application of ^{11}C -DTBZ is limited because its short half-life requires a cyclotron on-site [15]. A novel ^{18}F -labeled tetrabenazine derivative, ^{18}F -fluoropropyldihydrotetrabenazine, is currently under development and is showing promise as a longer-lived and lower-cost alternative [15].

Caveats and Potential Solutions

Among the three techniques discussed above, DAT imaging is the most commonly used to date. However, several limitations associated with its use should be emphasized. DAT imaging likely overestimates the reduction in terminal density in early PD due to compensatory down-regulation in remaining neurons. Also, current DAT radioligands bind with other monoamine transporters, particularly serotonin (SERT), which is especially problematic as SERT is the dominant monoamine transporter in the midbrain [16]. Lastly, DAT imaging cannot reliably differentiate between PD and other forms of parkinsonism [17], making the production of additional methods in conjunction with DAT imaging necessary to define a PD biomarker. Recently, fluorine-18 labeled 2- β -carbomethoxy-3 β -(4-chlorophenyl)-8-(2-fluoroethyl)-nortropane (^{18}F -FECNT) was developed as a novel PET tracer with high affinity for DAT and a much lower affinity for the norepinephrine transporter and SERT. Compared to current PET DAT tracers, ^{18}F -FECNT has higher test–retest reproducibility and may be able to track striatal and nigral DA denervation [18], making it potentially useful for longitudinal evaluation of PD progression.

As previously mentioned, another shortcoming of DAergic neuroimaging is that the techniques assess nigrostriatal DA function, rather than true pathology, rendering the obtained values subject to compensatory mechanisms, in which there is up-regulation of ^{18}F -dopa uptake and down-regulation of DAT binding [14]. To partially circumvent this problem, postsynaptic DA receptors can be examined by the PET ligand ^{11}C -raclopride for $\text{D}_{2/3}$ receptor [19] or SPECT tracer ^{123}I -iodobenzamide for D_2 receptors. Increased levels of D_2 receptor availability can be observed in early stages of *de novo* PD, which is useful in differentiating PD from atypical parkinsonisms [20]. Nonetheless, whether these new targets are truly unaffected by compensatory modulations or medications remains to be investigated.

Non-Dopaminergic Imaging

An active area of research in neuroimaging markers is assessment of brain functions and structures beyond nigrostriatal DAergic degeneration. These include Parkinson disease-related spatial covariance pattern (PDRP), cholinergic function imaging, and magnetic resonance imaging (MRI). Such strategies

incorporate the fact that PD affects non-nigrostriatal DAergic regions of the brain. Transcranial sonography (TCS), also a non-dopamine imaging method, will be discussed in the context of prodromal diagnosis, where utility of imaging methods will be integrated with biochemical markers.

Metabolism Network Imaging in Brain

Investigation with ^{18}F -FDG PET revealed that PD is associated with a specific metabolic network characterized by increased pallido-thalamic and pontine metabolism associated with metabolic reductions in the lateral premotor and posterior parietal cortical regions [21,22], termed PDRP. In one study, PDRP network analysis classified idiopathic PD with 84% sensitivity and 97% specificity, helping differentiate PD from atypical parkinsonisms such as multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) [23]. Furthermore, PDRP activity precedes the appearance of motor symptoms by approximately 2 years [24], indicating its potential usefulness in diagnosing PD at its prodromal stage. These facts, coupled with its high level of reproducibility [25] and correlation with clinical severity [26], make PDRP a promising biomarker to track PD progression, monitor therapeutic intervention [27], and could potentially fill voids left by nigrostriatal imaging methods mentioned above.

Cholinergic Dysfunction Imaging

In addition to PDRP, imaging brain regions that are not part of the nigrostriatal tract shows some promise in diagnosing and distinguishing PD from other neurological diseases. New imaging techniques for assaying cortical acetylcholinesterase activity with ^{11}C -MP4A PET reveal a deficit of cholinergic function throughout the cortex in parallel with the loss of striatal DAergic function in PD [28]. This observation is significant, especially with the realization of non-motor components in PD (e.g. cognitive impairment) which is intimately associated with the cholinergic system.

MRI

MRI is a form of neuroimaging that is particularly useful in ruling out secondary causes of parkinsonism due to the fact that it is capable of detecting abnormalities in the structure of various brain regions. Recent advances in high-field MRI technology have been increasingly employed in diagnosing PD and are more sensitive towards demonstrating iron deposits in the midbrain of early PD patients [29]. Additionally, diffusion tensor imaging (DTI) for evaluating regional fractional anisotropy has shown changes in the olfactory tract [30], which could be related to anosmia or hyposmia (a symptom that

appears in the prodromal stage – see discussion below), and reduction in the nigra of PD patients [31] which inversely correlates with H&Y score [32].

Biochemical Biomarkers

Biochemical markers, especially those reflecting PD pathogenesis, are sorely needed in samples that are readily accessible clinically [e.g. cerebrospinal fluid (CSF), blood, and saliva]. To date, the most extensively tested candidate biochemical markers are those present in CSF and typically stem from genetic investigations which link the candidate to PD development. A few major biochemical markers and their current clinical and research utility are listed in **Table 2**. However, none of these markers completely fulfill the criteria defined earlier as an ideal biomarker for PD. Most new candidate markers are discovered by ‘-omics’ technology, and are generally in a preliminary stage with the results obtained in small cohorts using cross-sectional study designs. Validation is therefore needed using large cohorts, particularly those with samples collected longitudinally. Finally, similar to the neuroimaging field, major challenges to the biochemical marker field also include defining prodromal biomarkers and those related to nonmotor dysfunctions.

α -Synuclein, DJ-1, and tau

α -Synuclein has been intensely researched as a PD biomarker due to the fact that it is a key protein in Lewy bodies, the pathological hallmark of PD, and mutations or multiplication of its gene are known to cause familial PD [33,34]. α -Synuclein has been reported to be decreased in CSF from patients with PD compared to controls in most well-controlled investigations [35-38], although it does not appear to be able to distinguish between various synucleinopathies independently [38]. One study has reported an inverse relationship between α -synuclein levels and H&Y score [35], indicating measuring α -synuclein levels may not only be useful as a biomarker of PD but also of disease progression. However, the latter observation has not been replicated in most recent studies [37]. Additionally, oligomeric forms of α -synuclein have been found to be elevated in the CSF of PD patients as compared to controls. When only oligomers were measured, the sensitivity and specificity were calculated to be 75% and 87.5%, respectively, which increase to 89.3% and 90.6% when the ratio of oligomers/total α -synuclein is calculated [39]. Similar elevations have also been observed in plasma [40]. A more recent study reports that the CSF level of phosphorylated α -synuclein (PS-129) appears to be more effective than native α -synuclein in

differentiating PD from MSA and PSP and correlates with disease severity as assessed using the Unified Parkinson's Disease Rating Scale (UPDRS) [41], making it a potential candidate to complement the neuroimaging methods discussed above.

DJ-1 is an extensively studied antioxidative protein which, like α -synuclein, is implicated in PD pathogenesis as mutation of its *PARK7* gene results in familial autosomal recessive forms of the disease. Some reports indicate that DJ-1 is increased in CSF from PD patients compared to controls [42], a finding which was replicated in plasma and extended to show that DJ-1 levels correlate with disease stage as approximated by H&Y scores [43]. Other studies, however, contradict this conclusion by showing DJ-1 to be decreased in CSF from PD patients compared to controls, and show an apparent age-dependent increase in DJ-1 levels [37]. The discrepancy might be related to methodological variations or contamination of CSF by blood, which contains a comparatively high level of DJ-1 [44].

In addition to α -synuclein [45,46], genome-wide association studies have found associations between the gene encoding tau (*MAPT*) and PD. However, this observation has largely arisen in cohorts of European descent [45] and was not found in a Japanese cohort, indicating there may be geographical differences in genes associated with PD [46]. Studies with larger cohorts have reported that CSF levels of tau and phospho-181 tau (p-tau) are decreased in symptomatic PD compared with controls [47,48].

In addition to the cardinal motor indicators of PD, patients also experience significant non-motor symptoms. As such, biomarker investigations have been launched in an attempt to characterize these effects. One such symptom is cognitive impairment, for which a decrease in CSF amyloid- β ($A\beta$) levels has been well established in patients with Alzheimer disease (AD). In CSF samples obtained from PD patients, the $A\beta$ isoforms $A\beta_{40}$ and $A\beta_{42}$ have been found to be decreased as compared to controls. However, in contrast to AD, there is no consistent increase in tau in conjunction with decreased $A\beta$ in patients with PD with cognitive impairment or dementia [47,49,50].

It should be pointed out that due to the heterogeneity of patients with PD, a combination of several markers may be necessary to achieve high sensitivity and specificity. This is evidenced by the fact that the ratio of α -synuclein to the percentage of p-tau (p-tau/tau) is able to distinguish PD from MSA [48]. Furthermore, the ratio of fractalkine, an inflammatory mediator of microglia, to $A\beta_{42}$ positively correlates with PD severity and progression in cross-sectional and longitudinal CSF samples, respectively [48].

Using combinations of protein markers and/or imaging techniques could therefore produce a biomarker that not only diagnoses PD, but also monitors disease progression. Alternatively, markers may also be stratified based on the predominant features of PD, such as tremor or rigidity, or at-risk traits and/or clinical features (discussed below).

Use of 'omics' in Biomarker Development

A variety of profiling techniques have been employed to find novel markers that could facilitate diagnosis and monitoring of PD progression. Indeed, the use of '-omics' technologies have enabled for high throughput studies of metabolites, genes, and proteins in the context of comparing PD patients to controls. This section will discuss the major findings of metabolomic, genomic, gene expression profiling, and proteomic studies aimed at developing PD biomarkers.

Metabolomics

Metabolomics is used to study the profile of small molecules and has been used in a limited capacity to observe differences in metabolites that may aid in PD diagnosis. Previous studies report decreased uric acid and increased glutathione in plasma from PD patients compared to controls [51]. However, the reduced form of glutathione has been reported to be decreased in CSF of patients with Lewy body disease, with PD patients showing a non-statistically significant decrease of this metabolite [52]. By contrast, decreased plasma levels of uric acid have been found in both idiopathic PD and PD caused by mutations in leucine rich repeat kinase 2 (*LRRK2*), indicating decreased uric acid levels may be a universal feature of PD patients [53]. Given both higher serum and CSF urate concentrations at baseline are associated with slower rates of clinical deterioration [54] and serum urate is reported to decrease with disease progression as measured by H&Y score [55], urate may be important in predicting and monitoring PD. Notably, the *LRRK2* study discussed above also calculated projection to latent structures-discriminant analysis (PLS-DA) for related and unrelated controls in addition to patients with idiopathic and *LRRK2* PD. It was found that each form was distinguishable from the control in addition to each other, although common signatures are also noted. Another study using PLS-DA analysis has identified pyruvate as a key metabolite that can distinguish patients with PD from controls [56]. While metabolomic studies may provide a biomarker that can accurately diagnose PD, the limited number of published

studies highlights the need for validation as well as comparisons of PD to other neurodegenerative diseases.

Genomic and Expression Profiles

A majority of the genomic work done relating to PD biomarker research has found the genes *SNCA* and *MAPT*, encoding for α -synuclein and tau, respectively, are associated with PD as described above [45,46]. A variety of studies have also examined differences in mRNA expression between PD and control subjects. Unfortunately, most of these have been performed in brain tissue, making them impractical towards the development of a clinically useful biomarker. There have been a select number of studies that measured transcript differences in blood. One such study looked at the blood mRNA signature and reported a molecular marker of 8 genes that are indicative of a greater risk of PD, in particular decreased expression of *ST13* [57]. Results for the expression of *ST13*, however, were not replicated in a separate study [58] and no differences were observed in a similar study of *GSK3B* in blood [59]. By contrast, several other genes discovered initially, including *HIP2* and *HSPA9* [57], appear to be reproducible in other investigations [60,61].

Proteomic Profiling

Many studies have profiled various proteomes of PD and other neurodegenerative patients with the goal of detecting differences in proteins between these groups. A commonly used biofluid is CSF, as it is in close contact with the location of PD pathogenesis and can be reasonably obtained. One such study utilized proteomic profiling of CSF from patients with PD, AD, and dementia with Lewy bodies and found unique changes for each group compared to healthy controls amongst the roughly 1500 (approximately) proteins identified [62]. Additionally, each group was distinguishable from one another with 95% sensitivity achieved [62]. Later studies validated that levels of brain-derived neurotrophic factor, interleukin 8, vitamin D binding protein, β_2 -microglobulin, haptoglobin, apolipoprotein AII, apoE, tau, and A β 42 could accurately classify 90 of 95 healthy controls, 36 of 48 AD patients, and 38 of 40 PD patients when compared to expert diagnosis [63]. This further supports the concept that utilizing multiple proteins may be a key factor in developing a biomarker for PD. Other profiling studies have been performed and have found different candidate markers [64,65], illustrating that reproducibility is low when employing general profiling methods across diverse cohorts.

Future Directions of Biomarkers in PD

A biomarker that is able to diagnose PD prior to the onset of motor symptoms would provide a better chance to develop interventions capable of arresting or slowing disease progression. To this end, two types of high risk populations, subjects with genetic mutations leading to familial PD and those with clinical symptoms associated with a high conversion rate to PD, are candidates wherein prodromal biomarkers can be potentially identified. Additionally, discovery of biochemical markers is shifting from CSF to other peripheral biofluids that are more easily accessible. Imaging, when used in combination with biochemical biomarkers, may allow for the discovery of a specific profile that can predict PD onset.

Prodromal diagnosis

Tools used for prodromal diagnosis.

The use of neuroimaging has shown the greatest promise in developing prodromal markers for PD. Nigrostriatal DA imaging, the best established marker of motor dysfunction thus far, should be considered the 'gold-standard' in defining subjects at a higher risk for PD prior to the onset of motor symptoms (discussed below). Additionally, TCS, which is readily available in most clinics, may be useful towards prodromal diagnosis of PD. To this end, TCS has been used to observe the lateral midbrain and studies report increased echogenicity ('hyperechogenicity') in the lateral midbrain in about 90% of cases [66]. Compared to the clinical standard, diagnosing PD at baseline by TCS was assessed with a sensitivity of 91% and specificity of 82% [67]. TCS may be useful in developing a prodromal biomarker as one study reports 14 of 39 patients with rapid eye movement behavior disorder (RBD), a group of people at risk of PD (discussed below), showed hyperechogenicity in the nigra [68]. Additionally, for individuals 50 years or older without evidence of PD, the relative risk for incident PD in those with enlarged substantia nigra hyperechogenicity was 17 times higher compared with normoechogenic controls after 37 months of prospective follow-up [69]. It should be noted, however, the size of the TCS signal did not show changes with disease progression when assessed using H&Y or UPDRS scores [70], suggesting it is not an appropriate biomarker for follow-up evaluation of disease severity.

Population at High Risk

To develop a prodromal biomarker for PD, individuals who are at risk of developing PD can be studied prior to disease onset. Two such populations include individuals that have a genetic risk for PD

and people who show symptoms that appear prior to disease onset. Among individuals with a genetic risk for PD, those with mutations in *LRRK2* or glucocerebrosidase (*GBA*) are worth discussing, as their prevalence is relatively high. Studies on asymptomatic *LRRK2* carriers are likely to be especially informative due to the high penetrance rate of *LRRK2* mutations [71]. DAT imaging of two asymptomatic *LRRK2* mutation carriers showed reduced ^{11}C -MP binding but normal ^{18}F -dopa uptake in putamen [72], which is consistent with decreased binding of DAT and increased activity of dopa decarboxylase in prodromal stages of PD. Additionally, *LRRK2* patients were similar to patients with sporadic PD [72]. A separate study reported a greater than expected decline in PET markers (most commonly ^{11}C -DTBZ and ^{11}C -methylphenidate but also ^{18}F -dopa uptake) for some non-symptomatic *LRRK2* carriers [73], indicating PET imaging prior to clinical PD onset may be useful in diagnosing PD. Others have shown that *GBA* mutation carriers have decreased cerebral rates of glucose metabolism in the supplemental motor area [74], further validating the concept that imaging prior to the appearance of motor symptoms may be an important aspect of a prodromal PD biomarker.

Imaging subjects displaying non-motor symptoms that commonly occur prior to the onset of motor symptoms in PD may also be useful in defining a prodromal biomarker. Impaired olfactory function is a common finding in PD patients, occurring early in the course of the disease [75] and may be able to distinguish PD from other movement disorders [76]. Similarly, studies have shown that RBD may precede the onset of parkinsonism [77] in addition to other prodromal symptoms of PD [78], which include depression, constipation, and cardiac dysfunction (the foundation of cardiac scan, see Table 1). DAT imaging has been especially useful towards developing such a biomarker. In a cohort of 361 asymptomatic relatives of PD patients, idiopathic olfactory dysfunction was assessed and nigrostriatal DA neuron function was evaluated via SPECT DAT imaging. A total of 12.5% of the hyposmic first-degree relatives of patients with PD eventually developed PD and all had an abnormal baseline SPECT scan [79]. RBD patients similarly have shown reduced striatal DAT binding using ^{123}I -IPT SPECT [68,80], and to a lesser extent FP-CIT SPECT [81], indicating that neuroimaging, particularly DAT imaging, is capable of detecting prodromal DA dysfunction in subjects who are at risk for PD but have not yet developed motor symptoms, as mentioned above. This was indeed confirmed in our recent investigation (**Figure 1**). Longitudinally monitoring patients that have these conditions and observing the differences between

those that develop PD and control patients may provide valuable insight into prodromal markers of PD as well as markers capable of monitoring disease progression.

Patients with *LRRK2* or *GBA* mutations or with prodromal PD symptoms can also be utilized to develop biochemical biomarkers. Most of the work in this area has focused on *LRRK2* carriers. Plotting PLS-DA scores calculated from asymptomatic *LRRK2* carriers and family members without a mutant copy of *LRRK2* showed that metabolomic profiles of these groups were distinguishable from each other [53]. Additionally, decreased levels of A β and tau species in CSF correlate with decreased DA neuron function in *LRRK2* mutation carriers as detected by imaging via ^{18}F -dopa, ^{11}C -DTBZ and ^{11}C -methylphenidate [82], adding strength to their usefulness as PD biomarkers. Because asymptomatic carriers represent a prodromal state of PD, such findings indicate metabolomics and protein levels may be useful in the development of prodromal biomarkers for PD. Unfortunately, attempts at using α -synuclein or DJ-1 in *LRRK2* patients for the same purpose has not proved successful [83], warranting further investigation into candidate markers in at risk populations. Longitudinal studies on such populations will likely allow for the development of a prodromal biomarker for PD.

Transition from CSF to Peripheral Fluids

Although CSF biomarkers have shown great potential for PD diagnosis, CSF is obtained through lumbar puncture, which is relatively invasive compared to obtaining other biofluids such as blood or saliva. While some studies in plasma have yielded promising results (discussed above) [39,40], further validation is still necessary. One recent study identified α -synuclein and DJ-1 in saliva [84], opening the door for using saliva as a biofluid in which to develop a biomarker for PD. Future studies should aim to integrate neuroimaging techniques as well as markers in biofluids to develop a marker that is not only specific for PD but also allows for preclinical diagnosis and monitoring of disease progression.

Concluding remarks

The current field of PD biomarkers focuses on using neuroimaging in addition to biochemical markers that can be measured in biofluids. Although a perfect biomarker has not been developed, significant progress has been made towards discovering molecular and imaging patterns that can accurately distinguish PD and monitor its progression. Among imaging methods that are currently available, DAT appears to be most widely used towards diagnosing PD, including defining PD at

prodromal stages. However, newer imaging methods are needed to improve differential diagnosis of overlapping parkinsonian disorders and assessing PD progression objectively. Biochemical markers are important not only towards filling the gaps associated with imaging fields, but also in revealing novel molecular targets involved in PD development and progression. Additionally, biochemical markers are typically determined in human fluids, making them advantageous in the aspect that they are easily accessible for application in a routine clinical setting. Future efforts should look to integrate these two fields and extend studies to the prodromal stages of PD in addition to non-motor symptoms. Correlating imaging data with biochemical markers will probably improve the diagnostic accuracy of PD. This is particularly important towards the development of markers capable of detecting PD in the prodromal stages of the disease. Such markers will be critical towards the development of therapies capable of inhibiting neuronal loss, which will likely be the most effective therapy in treating PD.

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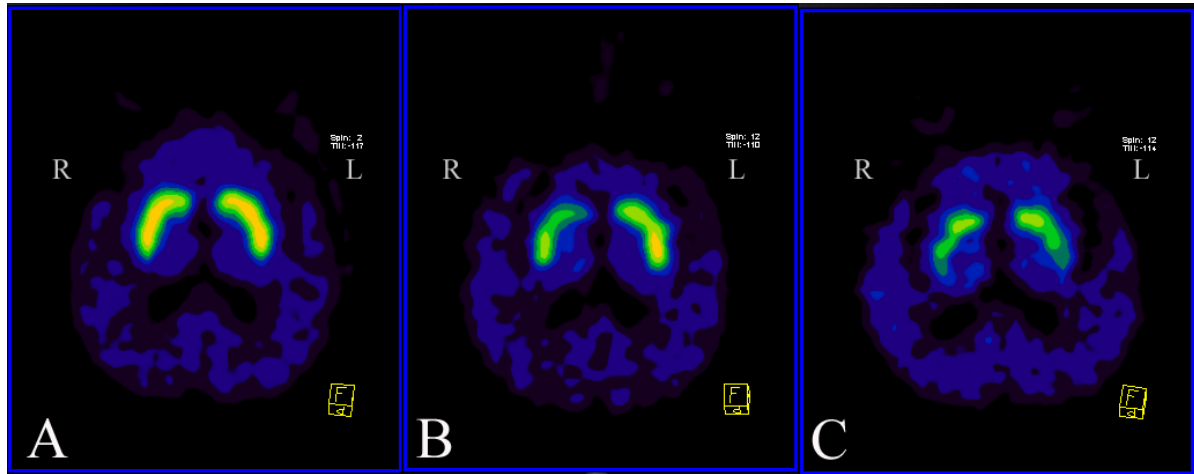


Figure 1: Imaging of dopamine transporter in different types of RBD patients. **A)** Idiopathic RBD with normal DAT imaging and no signs for PD (male, 66 yrs); **B)** Idiopathic RBD with reduction of DAT in imaging but no signs for PD; however, PD was confirmed clinically after follow-up with left limbs affected at onset (male, 70 yrs); **C)** Idiopathic RBD with reduction of DAT in imaging and PD (male, 71 yrs).

Table 1: Neuroimaging Biomarker candidates and their potential utilities in PD

Imaging target	Prodromal diagnosis	Confirming PD at its onset	Differentiation from atypical Parkinsonism	Monitoring progression	Sensitivity and specificity for PD diagnosis
AADC(¹⁸ F-dopa)	Probably useful	Useful	Unlikely useful	Possibly useful	
DAT	Useful	Useful	Unlikely useful	Possibly useful	98% and 83%[85], 92% and 100% [17]
VMAT2	Probably useful	Useful	Investigational	Investigational	
FDG network	Probably useful	Probably useful	Probably useful	Possibly useful	95% and 94% [86]
Dopamine receptors	Non-useful	Non-useful alone	Non-useful alone	Non-useful	
Cholinergic function	Investigational	Investigational	Probably useful (for DLB)	Investigational	
Cardiac autonomic dysfunction	Investigational	Non-useful	Useful (for MSA)	Investigational	
TCS	Probably useful	Useful	Investigational	Unlikely useful	91% and 82% [67]
MRI (DWI, iron deposit)	Investigational	Probably useful	Investigational	Investigational	

Table 2: Biochemical Biomarker candidates and their potential utilities in PD

Biochemical marker(s)	Prodromal diagnosis	Confirming PD at its onset	Differentiation from atypical Parkinsonism	Monitoring progression	Sensitivity and specificity for PD diagnosis
α -Synuclein	Unlikely [82]	Possibly Useful [35-38]	Possibly useful-potentially more useful if PS-129 is measured or it is used with the ratio of p-tau/tau [41],	Possibly Useful [41]	Measuring oligomers 75% and 87.5%; improves to 89.3% and 90.6% when measuring the ratio of oligomers:total [39]
DJ-1	Unlikely [82]	Possibly Useful [42]	Unknown	Possibly Useful [42]	
A β	Possibly Useful [82]	Possibly Useful [47-49]	Unknown	Possibly Useful if used in conjunction with other protein(s), e.g. tau, fractalkine [47-49].	
Tau	Possibly Useful [82]	Possibly Useful [47,48]	Possibly useful if used in a p-tau/tau ratio with α -synuclein [41]	Possibly useful if used in conjunction with A β [47,49,50]	
Uric Acid	Unknown	Possibly Useful [51]	Unknown	Possibly Useful [54]	
Glutathione	Unknown	Possibly Useful [51]	Unknown	Unknown	

Chapter 3:

MITOCHONDRIAL THERAPEUTICS IN PARKINSON'S DISEASE

This chapter is slightly modified from the following co-authored publication which is available through the publisher listed on the National Center for Biotechnology Information's PubMed Database (<http://www.ncbi.nlm.nih.gov/pubmed/>)

Hoekstra, J.G., Montine, K.S., Zhang, J., Montine, T.J. (2011). Mitochondrial therapeutics in Alzheimer's disease and Parkinson's disease. *Alzheimers Res Ther.* 3(3):21. PMID: 21722346.

Overview of mitochondrial function

Mitochondria are organelles serving a wide variety of actions critical to cellular function, several of which are of particular importance to neuronal survival. The primary function of mitochondria is to produce energy in the form of ATP via oxidative phosphorylation, where electrons are transported down the electron transport chain (ETC) while generating a proton gradient. This drives ATP synthase [1]. Mitochondrial function is particularly important to the central nervous system (CNS) since the CNS uses 20% of the body's resting metabolic energy, with 95% of that energy coming in the form of ATP [1]. Neuronal ATP is essential to the function of the Na^+/K^+ and Ca^{2+} ATPases that maintain ion gradients [1,2]. Similarly, mitochondria play a prominent role in Ca^{2+} buffering by sequestering Ca^{2+} using ion transporters [1-3]. These actions of mitochondria are especially important to neurotransmission as well as synapse formation and remodeling [3-5]. However, critical roles for mitochondria go beyond ATP production since mitochondria also control cell signaling pathways and cell survival via apoptosis regulation [6]. Mitochondria are now also understood to be dynamic structures that undergo fission and fusion, and the relationships between mitochondrial dynamics and other 'classical' functions are a matter of intense investigation. For these reasons, mitochondria are commonly implicated in neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD).

Mitochondrial Involvement in Parkinson's Disease

Mitochondrial dysfunction has been repeatedly associated with PD. Complex I of the ETC is decreased in the SNpc, as well as other tissues[7-9], of PD patients when compared to controls, leading to excessive ROS formation [7]. Oxidative damage to lipids, proteins, and nucleic acids is also elevated in PD brain tissue [10-12]. Toxicants recapitulating most aspects of human PD also implicate mitochondrial dysfunction in PD pathogenesis, particularly through complex I inhibition. These toxicants include 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone [13]. MPTP was discovered when a drug abuser who synthesized and injected himself with MPTP-contaminated meperidine analog presented with parkinsonism; this patient later showed damage to the DA system in the SNpc at autopsy [14]. Other drug abusers subsequently presented with clinical symptoms of PD due to MPTP exposure [13]. MPTP is metabolized to 1-methyl-4-phenylpyridium (MPP^+), which enters DA neurons via the DA transporter. MPP^+ binds to and inhibits complex I of the ETC, leading to DA neuron death and PD

symptoms [13]. Rotenone also acts by inhibiting complex I. Although rotenone is highly lipophilic and can cross the plasma membrane, it causes selective DA neuron degeneration with elevated oxidative stress, indicating that DA neurons may be particularly susceptible to mitochondrial dysfunction [15].

Studies on two genes that are mutated in inherited forms of PD, PINK1 and parkin further implicate mitochondria in PD. In *Drosophila*, ablation of either gene causes flight muscle degeneration, and mitochondria appear enlarged and swollen with fragmented cristae [16-18]. Whereas parkin expression in a PINK1 knockout reverses this phenotype, PINK1 expression in a parkin knockout does not, indicating that parkin acts downstream of PINK1 to affect mitochondria [16-18]. DA neuron degeneration in PINK1 and parkin deficient *Drosophila* also has been observed [18,19] along with sensitivity to compounds that model PD and generate ROS [16]. One hypothesis is that loss of these PD related genes that help regulate mitochondrial function leads to increased sensitivity to neurotoxic insults and DA neuron death.

Mitochondria as Targets of Therapy and Treatment in PD

A majority of current PD treatments (**Table 3**) do not target the mitochondria directly, although mitochondrial protection is more common in PD than AD. The current gold standard to restore DA signaling is the DA precursor levodopa (L-DOPA), which crosses the blood brain barrier, combined with a peripheral decarboxylase inhibitor such as carbidopa, which minimizes the gastrointestinal and cardiovascular side effects of DA [20,21]. Treatment may also include a selective inhibitor of monoamine oxidase B (MAO-B) or catechol-O-methyltransferase (COMT) to decrease DA metabolism [20,21]. DA agonists also have been used but appear less effective than L-DOPA [20,21]. Unfortunately, the effectiveness of L-DOPA therapy often is limited and can be associated with debilitating side effects [21]. L-DOPA has been hypothesized to enhance neurodegeneration [21], since DA metabolism by MAO-B generates ROS [22]. In theory, this oxidative stress would cause mitochondrial dysfunction and further ROS production. MAO-B inhibitors may decrease the amount of oxidative damage potentially caused by DA metabolism [20,21]; however, data from some clinical investigations do not support this hypothesis [23]. Regulation of Ca^{2+} influx shows a slight effect in alleviating dyskinesia in PD patients [20,21]. While regulation of intracellular Ca^{2+} to prevent ROS production is a potential therapeutic target in PD, NMDA receptor antagonists that block Ca^{2+} entry have shown minimal benefits in treating PD [20,21]. A more

promising strategy may be to supplement mitochondria with molecules that improve their function. In this case, not only would ATP production improve, but ROS production by mitochondria might be decreased [20,21,24-26]. Coenzyme Q₁₀ and creatine both may act through such a mechanism, with Coenzyme Q₁₀ improving electron flow and creatine improving high-energy phosphate reservoirs [20,21,24-26]. Any approach that suppresses ROS production might also impact Lewy body formation since ROS can modify α -synuclein, increasing its tendency to aggregate and possibly form Lewy bodies [27,28].

Mitochondrial Dynamics: New direction of mitochondrial research and potential therapeutics

Although mitochondrial dysfunction is extensively associated with PD, direct effective mitochondrial therapy is very limited. A new area of mitochondrial research known as mitochondrial dynamics may provide new opportunities for mitochondrial therapies in PD. Accumulating evidence suggests that mitochondrial morphology and transport can be regulated by fission and fusion, with fusion regulated primarily by mitofusins (Mfn) 1 and 2 and optic atrophy 1 (OPA1), and fission regulated primarily by Fis1 and dynamin-like protein 1 (Dlp1, also known as Drp1) [29]. Several observations support the involvement of mitochondrial dynamics in neurodegeneration. Mutations in Mfn2 lead to Charcot-Marie-Tooth disease and peripheral neuropathy, and mutations in OPA1 to autosomal dominant optic atrophy and loss of optic nerve fibers [3,29,30]. More recently, Dlp1, Mfn1, Mfn2, and OPA1 levels were shown to be decreased in brain tissue from AD patients while Fis1 levels were increased [5]. When these changes were mimicked in primary neurons using RNAi (RNA interference) or gene overexpression, mitochondria were decreased in neurites and dendritic spines, indicating such changes may play a role in AD [5]. This study also showed that Dlp1 overexpression in primary cultures protected neurons against neurotoxic insult, suggesting that increased fusion may be neuroprotective strategy [5]. In a separate study, Dlp1 was genetically ablated in *Drosophila* with a resulting loss of synaptic mitochondria, perhaps due to defects in axonal transport [31], an aspect of neuronal function affected in neurodegenerative diseases [32]. Using the neuromuscular junction (NMJ) to study synaptic actions of Dlp1, the same group observed defects in Ca²⁺ buffering and neurotransmission during prolonged stimulation of the NMJ [31].

Several studies have made strong connections between altered mitochondrial dynamics and PD. LRRK2 has been shown to interact with Dlp1 and can regulate mitochondrial morphology by recruiting it

to the mitochondria[33]. Knockout of Mfn2 in DAergic neurons results in their degeneration as a result of impaired mitochondrial transport[34]. Defects in transport have also been associated with PD, as DJ-1 and PINK1 have been shown to regulate the transport of mitochondria as well as rates of mitochondrial fusion and fission, which is associated with neuronal death[35-37]. Mitochondrial defects associated with PINK1 and Parkin knockout have been described above. Overexpression of Dlp1 or decreased expression of Mfn2 or OPA1 reversed the observed effects on mitochondrial size and shape and on muscle degeneration, showing that increased fission or decreased fusion may be protective [17]. Such effects have been extended to the mammalian system as knockdown of PINK1 in hippocampal neurons results in elongation of the mitochondria, which is rescued by Dlp1[38]. Although a relatively recent area of research, these results suggest that loss of fission or increased fusion could possibly play a role in AD or PD. A proposed connection of mitochondrial fission and fusion to AD and PD is diagrammed in **Figure 2**. Mitochondrial dynamics also plays a role in mitophagy (elimination of dysfunctional mitochondria), making dysfunction of this process critical towards maintaining neuronal health [39]. Of the above-discussed proteins that regulate mitochondrial fission and fusion, Dlp1 is of particular interest in PD pathogenesis as over expression of Dlp1 rescues observed mitochondrial phenotypes and more importantly, has been reported to be decreased in the mitochondrial fraction from the SNpc of PD patients[40]. This indicates that regulating mitochondrial dynamics, particularly through Dlp1, potentially plays a role in PD pathogenesis and represents new target for therapy.

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Table 3. Current treatments for Parkinson's disease

Therapy	Results	Mitochondrial Involvement?
Levodopa and DA-agonists	Improves PD symptoms	May improve mitochondrial function in neurons by restoring nigrostriatal signaling
MAO-B Inhibitors	Blocks oxidative deamination	May improve mitochondrial function in neurons targeted by DA; May decrease ROS produced by mitochondria
COMT Inhibitors	Blocks catechol metabolism	May improve mitochondrial function in neurons by restoring nigrostriatal signaling
Anticholinergic drugs	Most effective in alleviating tremor and rigidity	May improve mitochondrial function in striatal neurons by balancing the DA and acetylcholine
NMDA receptor antagonists	Can suppress dyskinesia	May decrease amount of ROS produced due to excessive intracellular Ca^{2+}
Coenzyme Q ₁₀	Less disability develops in patients given Coenzyme Q ₁₀ compared to placebo in one study [26]	May increase electron flow in ETC and decrease ROS production
Creatine	Not rejected as futile in a Phase II futility clinical trial [25]	May increase high energy phosphate pool and decrease ROS production

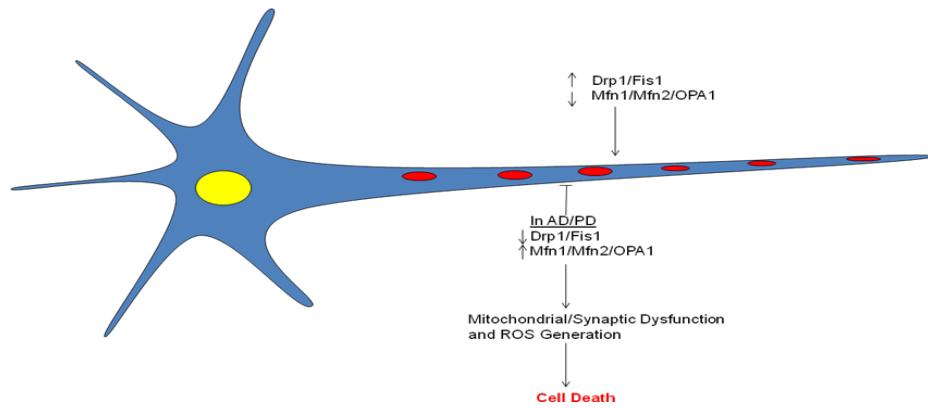


Figure 2. Synaptic function is dependent on proper trafficking of mitochondria to synapses. Mitochondrial trafficking down a neurite to a synapse can be promoted by increased fission (Drp1 or Fis1) or decreased fusion (Mfn1/2 or OPA1). Alterations in the levels of these proteins can lead to synaptic and mitochondrial dysfunction, neurodegeneration, and AD/PD pathogenesis.

Chapter 4:

A ROLE FOR ASTROCYTES IN MITOCHONDRIAL-MEDIATED PD MECHANISMS

Astrocyte Implications in PD

As the primary features of PD are typically attributed to neuronal death, a significant portion of research into mechanisms of PD pathogenesis have focused neurons, largely ignoring the potential contributions of other cell types, such as astrocytes. However, a number of lines of evidence implicate an astrocytic contribution to PD pathology. Immunohistochemical studies on human postmortem samples show that the number of astrocytes, as marked by glial fibrillary acidic protein (GFAP), is increased in the SNpc of PD patients when compared to healthy controls[1,2]. Similarly, aggregates of α -synuclein have been observed in astrocytes in the SNpc of PD patients and the number of astrocytes with these aggregates correlates with disease stage and may parallel the appearance of neuronal inclusions[3,4]. Other studies show that DJ-1, a gene that causes a familial form of PD, is highly expressed in astrocytes, and is increased in astrocytes in PD[5-7].

The above mentioned changes in astrocytes indicate they may be involved in the process of DAergic neuron death in PD. Experimental studies utilizing toxicants, particularly MPTP and 6-hydroxydopamine (6-OHDA), have provided evidence that this may in fact be the case. Astrocytes convert MPTP into MPP^+ [8,9], which leads to specific death of DAergic neurons in the SNpc, and clinical PD through the mechanisms described in detail in Chapter 3. Autopsies performed on patients exposed to MPTP showed an increase in GFAP positive cells in SNpc[10], similar to what is observed in PD. Loss of DAergic neurons and increased astrocytes also occur in MPTP and 6-OHDA PD models, with sustained astrocyte response even after a majority of DAergic neurons have been lost[11-13]. Chemical ablation of astrocytes at the time of MPTP administration reduces the loss of DAergic neurons[14], while blocking MPP^+ uptake by DAergic neurons prevents the observed astrocyte response[13]. This indicates astrocytes respond to damage to DAergic neurons and supports the concept that astrocytes play a contributing role in the loss of DAergic neurons during the pathogenesis of PD.

Additional studies on genes implicated in PD pathogenesis show that astrocytes can be adversely affected and result in neuron death. α -synuclein can be transferred from neurons to astrocytes, which results in the production of cytokines and chemokines[15]. Further, increased expression of α -synuclein in astrocytes impairs their function[16], which may be due to impaired astrocyte mitochondrial Ca^{2+} storage[17]. The observed deficits in astrocyte function commonly result in loss of neurons or impaired

ability of astrocytes to support neuronal growth[16,17]. Astrocytes, therefore, may contribute to DAergic neuron death in PD through production of compounds that are neurotoxic or through impaired neuron protection and support (discussed below).

The Role of Astrocytes in Supporting Neurons

Astrocytes play a critically important role in supporting neuronal function and survival, which occurs through several mechanisms. One such mechanism is through protection of neurons from oxidative damage. Astrocytes express a greater number of antioxidant genes compared to neurons, several of which are up-regulated in the SNpc in PD[18,19]. Indeed, the presence of astrocytes is sufficient to diminish the loss of neurons when stressed with ROS generating compounds[20,21]. Glutathione (GSH), an anti-oxidant molecule, is particularly important. The level of GSH is higher in astrocytes than it is in neurons[22,23] and is decreased in the SNpc in PD[24]. GSH is released by astrocytes to the extracellular space where it can remain or be utilized by neurons to increase their GSH content[25-27]. The importance of this is underscored by the fact that depletion of GSH abolishes the ability of astrocytes to protect neurons from toxic insults[28,29]. Dysfunction of astrocytes could therefore lead to impaired protection against oxidative stress.

Aside from simple protection against oxidative stress, astrocytes can also actively promote the health and survival of neurons through production of trophic factors. Specifically, glial cell line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and mesencephalic astrocyte-derived neurotrophic factor (MANF), are produced by astrocytes and promote survival and differentiation of DAergic neurons *in vitro*[30-32]. Additionally, administration of each protects against loss of DAergic neurons and reverses behavioral deficits in various models of PD[33-35], some of which used astrocytes as a means to deliver these factors to the brain[36,37]. Given the importance of astrocyte release of trophic factors in the survival of DAergic neurons, dysfunction of astrocytes could lead to impaired regulation, and result in loss of DAergic neurons.

Perhaps the most important role astrocytes serve is their ability to regulate neurotransmitters. In particular, astrocytes play a prominent role in taking up excessive amounts of glutamate, an excitatory amino acid, from the extracellular space. When glutamate binds to N-methyl-D-aspartate (NMDA) receptors on the postsynaptic terminal of a synapse, it induces an influx of Ca^{2+} . While this normally

occurs during neurotransmission, excessive stimulation of NMDA receptors results in elevated intracellular Ca^{2+} that can lead to neuronal death and damage in a process termed excitotoxicity (the importance of this is discussed below)[38]. Astrocytes have the ability to take up glutamate from the extracellular space using the glutamate transporter (GLT-1) and glutamate aspartate transporter (GLAST)[39-41]. Glutamate uptake through GLT-1 and GLAST occurs in a Na^+ -dependent manner, which is regulated by the Na^+/K^+ ATPase[39,41]. Dysfunction of astrocyte mediated glutamate management may therefore be a mechanism through which DAergic neurons die in PD. Such dysfunction may be related to changes in mitochondrial dynamics as Dlp1 has previously been identified as a protein that potentially interacts with GLT-1[42]. As astrocytes represent the largest portion of cells in the brain, particularly the SNpc of PD patients, the above-mentioned decrease in Dlp1 in PD (Chapter 2) may be driven by changes in expression in astrocytes and could affect astrocyte glutamate regulation. This could alter astrocyte glutamate regulation and result in excitotoxic neuronal death.

Ca^{2+} Signaling in Astrocytes

The above-mentioned functions help to highlight the primary role of astrocytes, which is to maintain homeostasis and support proper neurotransmission within the brain. Astrocytes rely on Ca^{2+} signaling as a means to regulate their response towards maintaining their environment. These signals proceed primarily through the activation of a variety of metabotropic receptors that have been identified in astrocytes. Such activation results in the generation of IP_3 , which induces the release of Ca^{2+} from the endoplasmic reticulum (ER). This release causes the entry of Ca^{2+} from the extracellular space in a process termed store operated Ca^{2+} entry (SOCE)[43]. These signals can be regulated through refilling the ER, removal of Ca^{2+} to the extracellular space, or buffering by the mitochondria[43].

Astrocytic Ca^{2+} signals are critically important towards neuronal functioning, as they play a large role in the regulation of synaptic transmission. Astrocytes have been shown to release a variety of neurotransmitters, particularly glutamate[44,45], through a process termed gliotransmission. Ca^{2+} regulates this process through promoting vesicle fusion with the plasma membrane[46-48]. Additionally, activation of metabotropic receptors regulates the expression of astrocytic glutamate transporters and promotes the clearance of glutamate [49,50]. Astrocyte metabotropic receptor activation can occur in response to neuronal release of neurotransmitters[44,51], indicating astrocytes act as a hub for regulating

neurotransmission. Utilizing Ca^{2+} , astrocytes sense and interpret neuronal activity, and can then shape the signals sent during neurotransmission by taking up or releasing different molecules that act at the postsynaptic terminal. Such processes may have a wide reaching affect, as astrocytic Ca^{2+} waves have been shown to propagate through gap junctions, over long distances[52]. Mitochondria play an important role in this process as exocytosis of glutamate is dependent upon mitochondrial handling of Ca^{2+} [53]. Disturbances in astrocytic mitochondria, potentially due to decreased Dlp1, could negatively impact the regulation of glutamate, and result in excitotoxicity (discussed below).

Excitotoxicity in PD

Excitotoxicity has been implicated in PD pathogenesis. The depletion of DA in the striatum leads to elevated activity of glutamatergic neurons in the subthalamic nucleus, which innervate the SNpc. This results in excessive transmission of glutamate onto DAergic neurons[54], which could result in excitotoxicity. Several studies utilizing MPTP or 6-OHDA have implicated excitotoxicity in PD pathogenesis by showing that extracellular glutamate is increased in the setting of DAergic neuron death[55] and the administration of glutamate receptor antagonists prevent the loss of neurons[55-57]. These models further implicate astrocyte dysfunction in PD pathogenesis as expression of the astrocytic glutamate transporters are decreased[58,59]. Therefore, death of DAergic neurons could result in part from excitotoxicity due to impaired astrocyte regulation of glutamate.

Summary

The effects of astrocyte dysfunction could be particularly relevant to PD, as the primary mechanism through which glutamate is taken up by astrocytes is heavily dependent upon mitochondrial function[60]. Beyond this, impaired rates of fission and fusion as well as decreased mitochondrial transport in astrocytes impairs the ability of astrocytes to protect neurons[20,61]. If the aforementioned decrease in Dlp1 in PD patients indeed occurs as part of the disease process in PD, mitochondrial dynamics could be adversely affected and impair astrocyte function in protecting against excitotoxicity. This would give tremendous insight into a mechanism of PD pathogenesis that could have impacts into the development of strategies for treatments of PD.

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Chapter 5:

RESULTS AND METHODS

This chapter is slightly modified from a co-authored manuscript currently in preparation for submission.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder clinically characterized by both motor and non-motor symptoms[1-3]. The loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) is the primary cause of the motor deficits[4], whereas non-motor symptoms are the result of dysfunction in multiple brain regions[2,3]. Until recently, most investigations have focused on the neuronal pathogenesis. However, accumulating evidence shows that astrocytes, which have multiple neuroprotective roles[5-9], clearly contribute to neuronal loss in PD[10,11].

Decreased respiratory chain activity[12] and increased oxidative damage in the SNpc[13-15] are felt to be complicit in neuronal demise in PD. While several proteins regulate these and other mitochondrial functions[16], the mitochondrial fission promoting protein dynamin like protein 1 (Dlp1, also known as Drp1) is of particular interest because the over-expression of Dlp1 rescues mitochondria from abnormalities associated with PINK1 and Parkin, two proteins implicated in PD[17,18]. In fact, we have shown by mass spectrometry that Dlp1 expression is decreased in mitochondria harvested from PD brain tissue, further suggesting Dlp1's role in PD[19]. However, the cellular origin and consequences of decreased Dlp1 expression remains to be fully elucidated.

The current study focuses on astrocytes because a recent investigation found that Dlp1 potentially interacts with the glutamate transporter GLT-1[20], which is specifically expressed by astrocytes[6]. In theory, disrupting astrocyte regulation of extracellular glutamate (also a function of the glutamate aspartate transporter [GLAST][9]) could result in excessive extracellular glutamate. This excess glutamate could prolong the opening of neuronal N-methyl D-aspartate (NMDA) receptors[21], resulting in excessive Ca^{2+} entry, and, ultimately, neuronal death. Indeed, this phenomenon of excitotoxicity has been implicated in PD and animal models of PD[22-26] as well as in other neurodegenerative diseases.

To test the hypothesis that decreased astrocytic Dlp1 expression contributes to neurodegeneration in PD, we measured astrocytic and neuronal Dlp1 expression (SNpc and cortex) in both PD and healthy control patients and explored the molecular mechanisms related to astrocytic dysfunction resulting from decreased Dlp1 expression.

RESULTS

Dlp1 Expression is Decreased in Astrocytes and Neurons from PD Patients

In our prior study using mass spectrometry and pooled SNpc samples, we found that PD patients had lower Dlp1 expression compared to healthy controls[19]. The initial goal of our current study was to demonstrate our previous findings in individual PD cases with a different technology. Therefore, the SNpc mitochondrial fraction from 4 PD and 4 control patients was examined via western blot with an anti-Dlp1 antibody. In agreement with our previous work[19], Dlp1 protein expression was significantly lower in PD patients (Fig. 3A, B).

The astrocytic Dlp1 expression levels were measured in the SNpc of PD and control patients by immunofluorescence. We found an obvious decrease of Dlp1 expression in PD patients (Fig. 3C, D). Additionally, neuronal Dlp1 expression was measured in DAergic (Fig. 4A, B; Information for human tissue are in Supplemental Table 4) and non-DAergic neurons (Fig. 4C, D) in the SNpc of PD patients and controls. Again, Dlp1 immunofluorescence was significantly decreased in both DAergic and non-DAergic neurons (Fig. 4A-D). Next, we asked whether the observed decrease in Dlp1 expression also occurred in histologically normal cortex from PD patients (i.e. PD patients at a relatively early stage, without apparent cortical neurodegeneration). Remarkably, Dlp1 expression was decreased in both cell types (Fig. 5A-D).

Dlp1 Affects Astrocytic Mitochondrial Morphology and Localization

To understand the biological effects of decreased Dlp1 expression in astrocytes, we performed Dlp1 knockdown by siRNA in primary cortical rat astrocytes (Fig. 6B inset). The astrocytes co-transfected with 488-tagged siRNA and a Mito-DsRed2 plasmid were stained for GFAP. The gross morphological changes were readily observed and primarily characterized by an elongated, fused mitochondrial network (Fig. 6A). The mitochondria located in the astrocyte processes have a clear morphology. Therefore, astrocyte processes were utilized to quantitatively compare morphological and localization differences after Dlp1 knockdown. The mitochondria in astrocytes transfected with Dlp1 siRNA were significantly

longer than the mitochondria in astrocytes transfected with non-specific (control) siRNA (Fig. 6A, B). Additionally, the area and perimeter of mitochondria were significantly larger in astrocytes after Dlp1 knockdown (Fig. 6A, C, D). Furthermore, there were fewer mitochondria within astrocyte processes after Dlp1 knockdown (Fig. 6E), indicating that knockdown of Dlp1 resulted in extensive fusion of mitochondria. Live cell imaging was used to measure net anterograde and retrograde mitochondrial movement to determine if Dlp1 knockdown altered mitochondrial motility (Fig. 7A). In both directions, the net distance traveled by mitochondria was greater in astrocytes transfected with control siRNA compared to astrocytes transfected with Dlp1 siRNA (Fig. 7B, C; Supplemental Movies 1 and 2). This evidence suggests that Dlp1 plays a significant role in astrocytic regulation of the morphology, localization, and motility of their mitochondria.

Astrocytic Dlp1 Protects Neurons from Excessive Glutamate

A co-culture system of rat astrocytes (with or without Dlp1 siRNA) and neurons from the ventral mesencephalon (VM) was utilized to determine if decreased astrocytic Dlp1 impairs the astrocytic protection of neurons from excitotoxic excess glutamate. When DAergic neurons were co-cultured with astrocytes transfected with control siRNA, 10 μ M glutamate did not affect DAergic neurite length or the number of branch points (highlighted by tyrosine hydroxylase [TH+]) (Fig. 8A, C, D). Conversely, when DAergic neurons were co-cultured with astrocytes transfected with Dlp1 siRNA (decreased Dlp1 expression), the addition of 10 μ M glutamate decreased DAergic neurite length and the number of branch points. The administration of the NMDA receptor antagonist MK-801 at the time of glutamate treatment prevented these morphologic changes (Fig. 8A, C, D). This evidence indicates that astrocytic Dlp1 promotes the protection of DAergic neurons from glutamate excitotoxicity.

Similar measurements were performed on non-DAergic MAP2-positive neurons to assess if this effect was specific to TH+ neurons. As was the case with TH+ neurons, treatment with 10 μ M glutamate did not affect neurite length or branch point number of non-DAergic neurons in co-culture with astrocytes transfected with control siRNA (Fig. 8B-D). However, in non-DAergic neurons co-cultured with astrocytes

transfected with Dlp1 siRNA, the addition of 10 μ M of glutamate resulted in decreased neurite length and branch point number. Again, this effect could be prevented by the addition of MK-801 (Fig. 8B-D). These observations suggest that decreased astrocytic Dlp1 expression impairs their ability to protect neighboring neurons from the harmful effects of excess glutamate. Astrocytic neuronal protection should be dependent upon proper function of the glutamate transport system. Therefore, we assessed the uptake of H³-D-aspartate to investigate whether the loss of astrocytic Dlp1 impaired the glutamate transport system. The cultures with astrocytes transfected with Dlp1 siRNA took up less H³-D-aspartate than cultures with astrocytes treated with control siRNA (Fig. 8E). This result supports the hypothesis that astrocytic glutamate transport is impaired by decreased Dlp1 expression.

Because Dlp1 is reported to interact with GLT-1[20], we investigated whether decreased Dlp1 expression alters the expression and/or localization of GLT-1. A western blot analysis of astrocytic GLT-1 showed no change in protein expression when transfected with Dlp1 siRNA (Fig. 9A). We immunofluorescently stained GLT-1 in astrocytes transfected with Dlp1 siRNA to observe astrocytic GLT-1 localization. Again, the localization of GLT-1 did not appear to be affected by Dlp1 expression (Fig. 9B). In similar experiments, we examined the other major astrocytic glutamate transporter, GLAST. Similarly, Dlp1 siRNA did not interfere with GLAST expression or localization (Fig. 9C, D). Therefore, astrocytic Dlp1 knockdown altered the glutamate transport system, but this effect did not occur through changes in the expression or localization of the major astrocytic glutamate transporters.

Dlp1 Affects Astrocytic Regulation of Calcium Entry

Cultured astrocytes were imaged in the presence of the Ca²⁺ sensitive dye Oregon Green BAPTA to determine if decreased Dlp1 alters an astrocyte's intracellular Ca²⁺ response to extracellular glutamate. Astrocyte responses to glutamate could be categorized into four general responses. Under control conditions, a minority of cells responded with a single, large increase in intracellular Ca²⁺, which could be either prolonged (taking longer than 60 seconds to return to baseline) or attenuated (taking 60 seconds or less to return to baseline). We categorized these cell responses as type 1 and 2, respectively (Fig. 10A). The remaining cells responded with intracellular Ca²⁺ oscillations, and we categorized these cell

responses by oscillation frequency. Type 3 responses were characterized by less frequent oscillations (< 0.19 Hz), and type 4 responses were characterized by more frequent oscillations (≥ 0.19 Hz) (Fig. 5A). Control astrocytes demonstrated mostly type 3 and 4 responses, while astrocytes with decreased Dlp1 had fewer type 3 and 4 responses and a significantly greater percentage of type 1 responses (Fig. 10B). These results indicate that astrocytic Dlp1 knockdown results in elevated intracellular Ca^{2+} in response to glutamate.

Astrocyte responses to glutamate are initiated by an intracellular release of Ca^{2+} from the endoplasmic reticulum, which precedes Ca^{2+} -induced Ca^{2+} entry from the extracellular space in a process referred to as store-operated calcium entry (SOCE)[27]. Additionally, the binding of glutamate to ionotropic receptors allows Ca^{2+} influx from the extracellular space[27]. Therefore, the Ca^{2+} response could be driven by intracellular and/or extracellular Ca^{2+} in astrocytes with decreased Dlp1 expression. To determine how extracellular Ca^{2+} contributes to the four aforementioned calcium responses, glutamate-induced Ca^{2+} responses were observed in the presence of the calcium chelator EGTA. When astrocytes were bathed with 2mM EGTA, no type 1 responses were observed (Fig. 10b). This result suggests that extracellular Ca^{2+} is necessary for the extended Ca^{2+} entry observed in type 1 responses. However, the pattern of Ca^{2+} waves was not different between astrocytes transfected with control or Dlp1 siRNA.

Decreased Dlp1 Impairs Mitochondrial Buffering of Extracellular Ca^{2+}

It is well-known that mitochondria buffer intracellular Ca^{2+} [28]. Given that the morphology and distribution of mitochondria are altered in astrocytes after Dlp1 knockdown, the Ca^{2+} buffering capacity of mitochondria could be limited when challenged with increased extracellular glutamate. Rhod-2 AM, a fluorometric dye sensitive to mitochondrial Ca^{2+} , was used to assess the mitochondrial buffering capacity of astrocytes transfected with control or Dlp1 siRNA in response to glutamate. Dlp1 siRNA transfected astrocytes had lower mitochondrial peak Ca^{2+} amplitudes than those of control siRNA transfected astrocytes (Fig. 11A, B). Therefore, knockdown of astrocytic Dlp1 impairs mitochondrial Ca^{2+} buffering in response to glutamate. Additionally, the differences observed in cellular Ca^{2+} responses to glutamate were mediated by the regulation of extracellular Ca^{2+} . Therefore, mitochondrial buffering of glutamate

induced extracellular Ca^{2+} entry probably plays a fundamental role in regulating overall cellular Ca^{2+} responses.

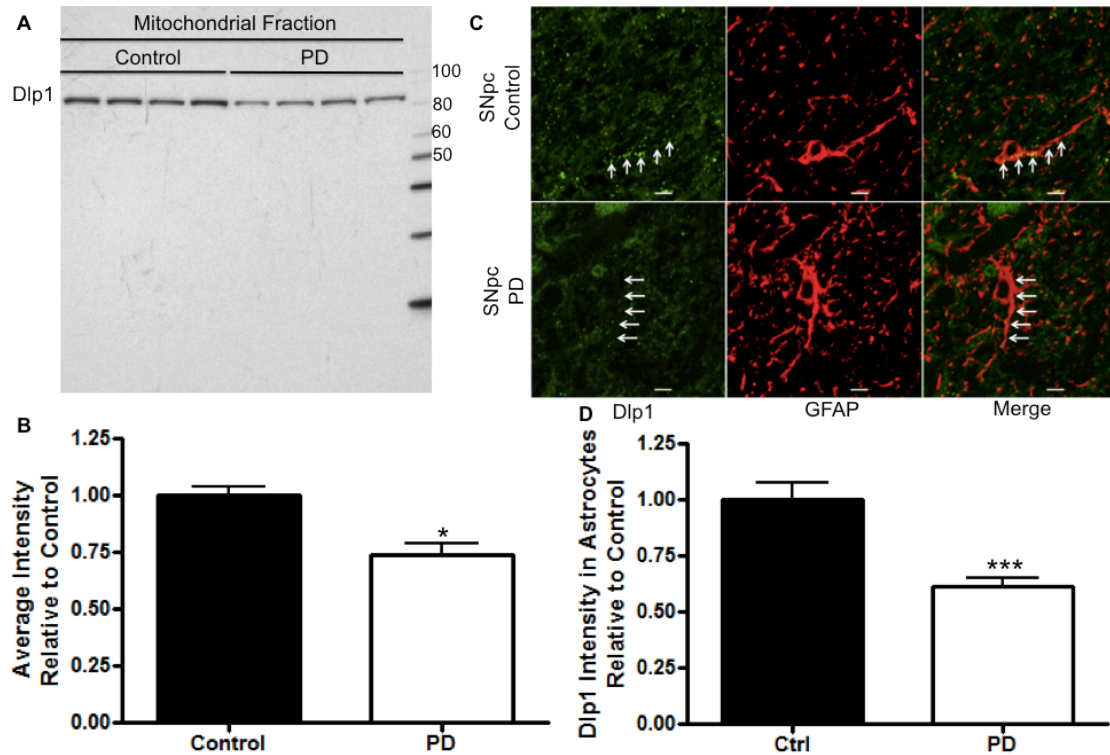


Figure 3. Dlp1 expression is decreased in the SNpc of PD patients. (A) Western blot and (B) densitometry of the mitochondrial enriched fraction of the SNpc from control patients (N=4) and PD patients (N=4), showing decreased Dlp1 in mitochondria-enriched tissue (The units for molecular weight markers on the right of the western blot are kDa; *p<0.05 by two sided unpaired t-test). (C) Fluorescent staining of Dlp1 (Green) and GFAP (Red) in the SNpc of control patients and PD patients. (D) Quantification of Dlp1 intensity in astrocytes from the SNpc of control (N=6 cases, 109 cells) and PD patients (N=6 cases, 118 cells). Scale bar represents 20 μ m (***p<0.001 by two sided unpaired t-test). Data are presented as mean \pm s.e.m.

Patient Details						
Case	Diagnosis	Region	Age	Sex	PMI (Hours:Minutes)	Braak Score
1	Ctrl	SNpc	55	M	9:30	NR
2	Ctrl	SNpc	70	F	6:00	0
3	Ctrl	SNpc	79	M	7:50	0
4	Ctrl	SNpc	78	M	4:00	0
5	Ctrl	SNpc	79	F	2:30	II
6	Ctrl	SNpc	81	M	7:30	I
7	PD	SNpc	57	M	17:45	Mild
8	PD	SNpc	82	F	11:00	I
9	PD	SNpc	81	M	6:30	NR
10	PD	SNpc	80	M	6:30	I
11	PD	SNpc	58	F	>24	0
12	PD	SNpc	73	M	3:50	0
13	Ctrl	FC	78	M	4:00	0
14	Ctrl	FC	78	M	6:00	II
15	Ctrl	FC	81	M	5:30	I
16	Ctrl	FC	81	M	6:00	II
17	Ctrl	FC	82	F	3:30	III
18	Ctrl	FC	91	F	11:00	III
19	PD	FC	73	M	3:50	0
20	PD	FC	78	M	5:00	I
21	PD	FC	81	M	6:30	NR
22	PD	FC	82	F	11:00	I
23	PD	FC	91	F	4:10	V
24	PD	FC	89	F	<12	V

Table 4. Table of clinical data for patients and controls from study in SNpc and cortex. (Ctrl=control patient, PD=Parkinson's disease patient, SNpc=substantia nigra pars compacta, FC=Frontal cortex, PMI=Post mortem interval)

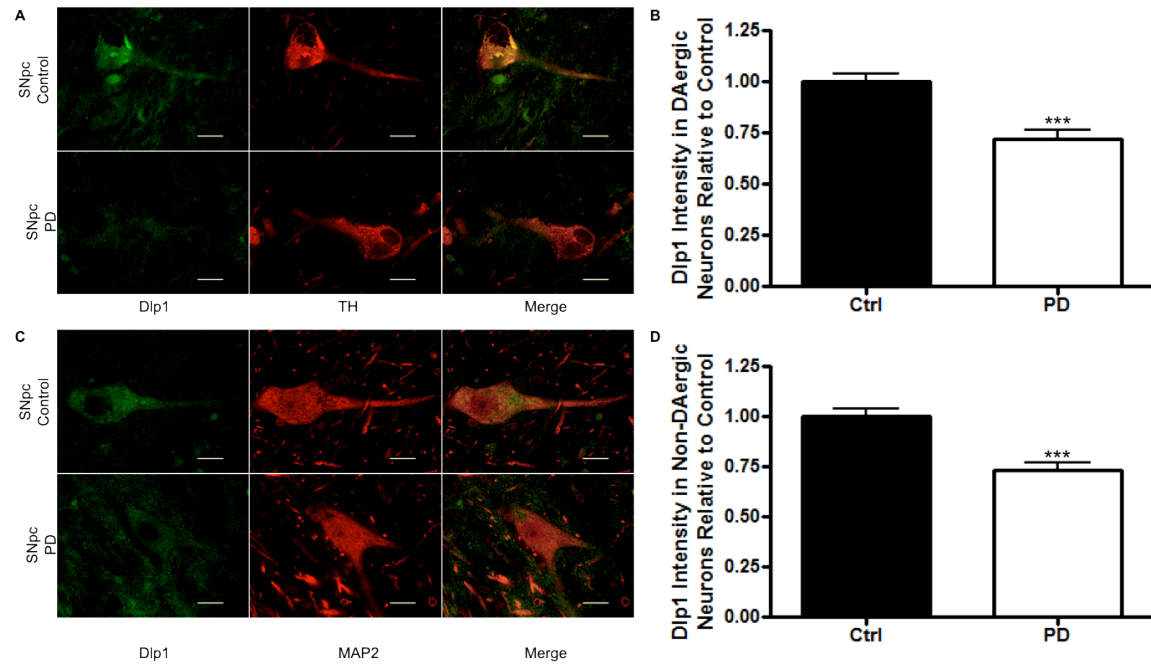


Figure 4. Dlp1 expression is decreased in neurons in the SNpc of PD patients. (A) Fluorescent staining of Dlp1 (Green) and TH (Red) in the SNpc of control and PD patients. (B) Quantification of Dlp1 intensity in DAergic neurons from SNpc of control (N=6 cases, 253 cells) and PD patients (N=6 cases, 143 cells). (C) Fluorescent staining of Dlp1 and MAP2 (Red) without neuromelanin in SNpc of control and PD patients. (D) Quantification of Dlp1 intensity in non-DAergic neurons from control (N=6 cases, 185 cells) and PD patients (N=6 cases, 175 cells). Scale bar represents 20 μ m (**p<0.001 by two sided unpaired t-test). Data are presented as mean \pm s.e.m.

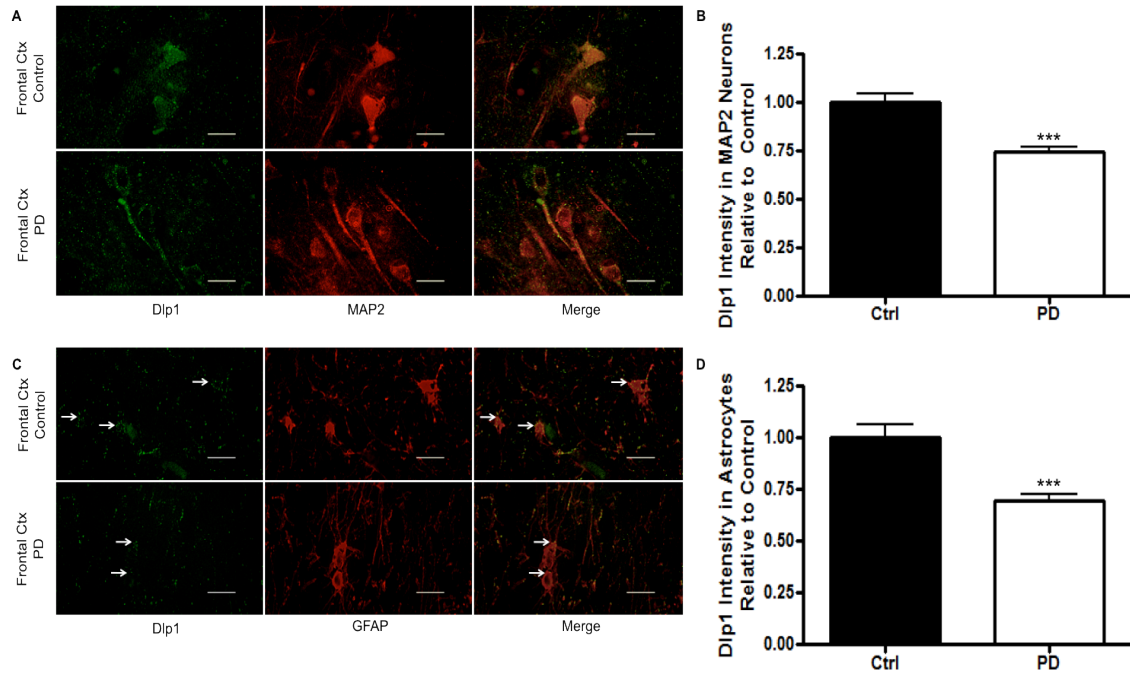


Figure 5. Dlp1 expression is decreased in neurons and astrocytes in the frontal cortex of PD patients. (A) Fluorescent staining of Dlp1 (Green) and MAP2 (Red) in frontal cortex of control and PD patients. (B) Quantification of Dlp1 intensity in neurons from control (N=6 cases, 357 cells) and PD patients (N=6 cases, 385 cells). (C) Fluorescent staining of Dlp1 and GFAP (Red) in frontal cortex from control and PD patients. (D) Quantification of Dlp1 intensity in astrocytes from control (N=6 cases, 230 cells) and PD patients (N=6 cases, 229 cells). Scale bar represents 20 μ m (***) p <0.001 by two sided unpaired t-test, Ctx=Cortex). Data are presented as mean \pm s.e.m.

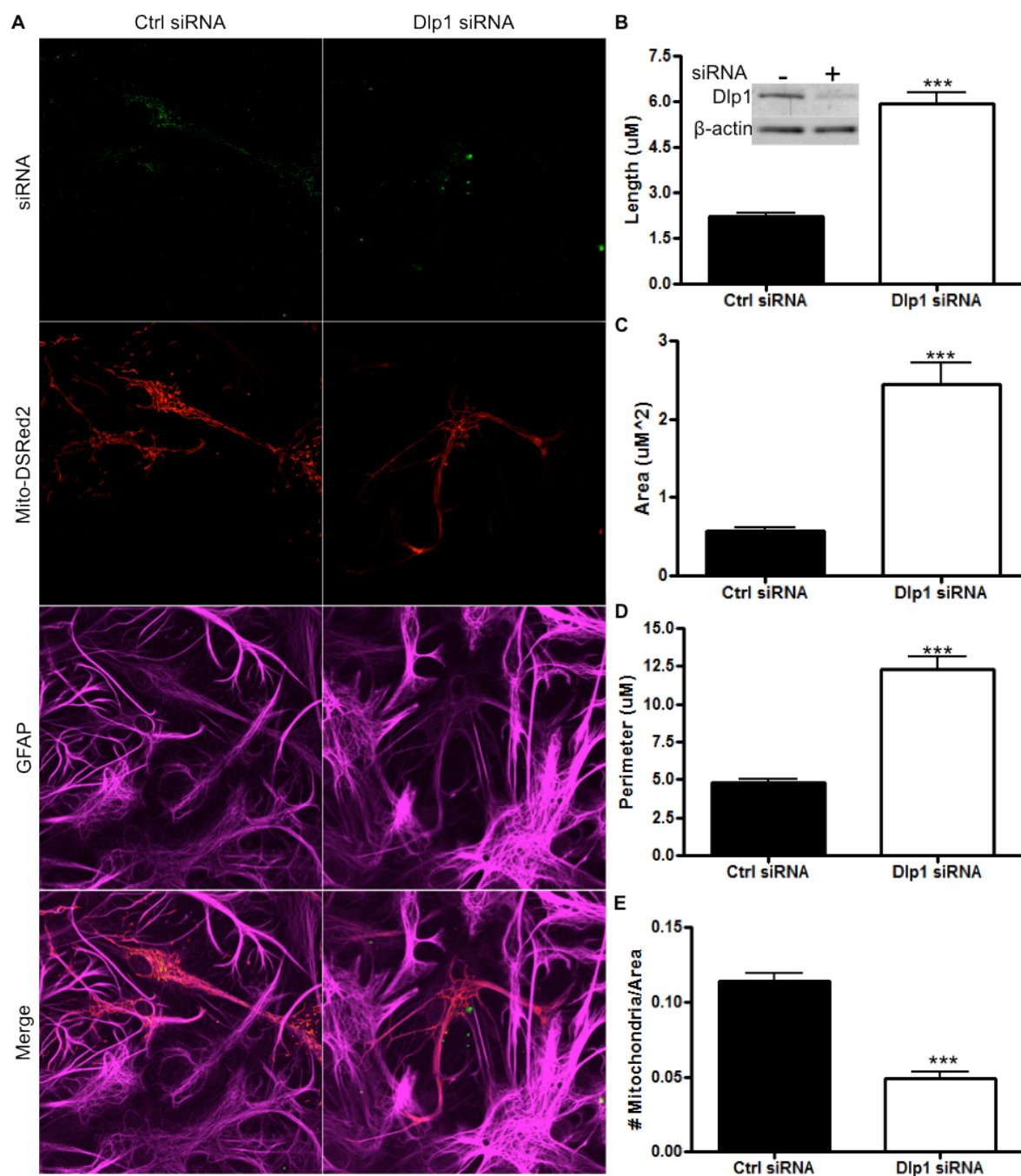


Figure 6. Knockdown of Dlp1 in astrocytes results in elongation and interconnection of mitochondria. (A) Representative images of rat astrocytes transfected with non-specific (Ctrl) siRNA or siRNA targeting Dlp1, each labeled with a 488 tag, and a Mito-DsRed2 plasmid to observe mitochondria. Cells that were positively transfected with both siRNA and Mito-DsRed2 were identified by the presence of green and red fluorescence. Western blot (b inset) confirming siRNA decreased Dlp1 expression. Length (B), area (C), perimeter (D), and density (E) of mitochondria from processes of transfected astrocytes (N=5 replicate; 50-56 cells per group, ***p<0.001 by two sided unpaired t-test). Data are presented as mean \pm s.e.m.

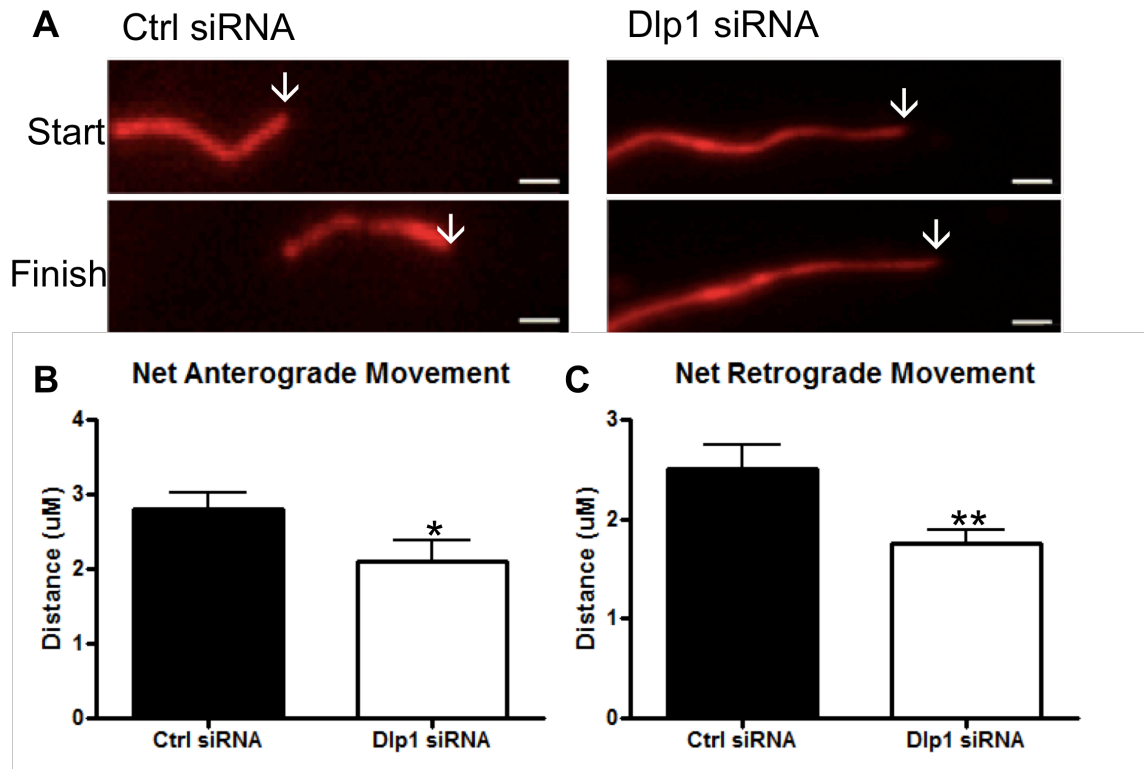


Figure 7. Knockdown of Dlp1 in astrocytes decreases mitochondrial movement. (A) Representative image for measurements of distance for astrocytes transfected with ctrl or Dlp1 siRNA. Arrows on the top panels mark beginning position and those on the bottom panels mark the ending position. (B) Net movement for mitochondria away from the cell body (* $p<0.05$ by two sided unpaired t-test). (C) Net movement for mitochondria towards the cell body (N=6 replicates; 28 cells per group ** $p<0.01$ by two sided unpaired t-test). Scale bar represents 1 μm . Data are presented as mean \pm s.e.m.

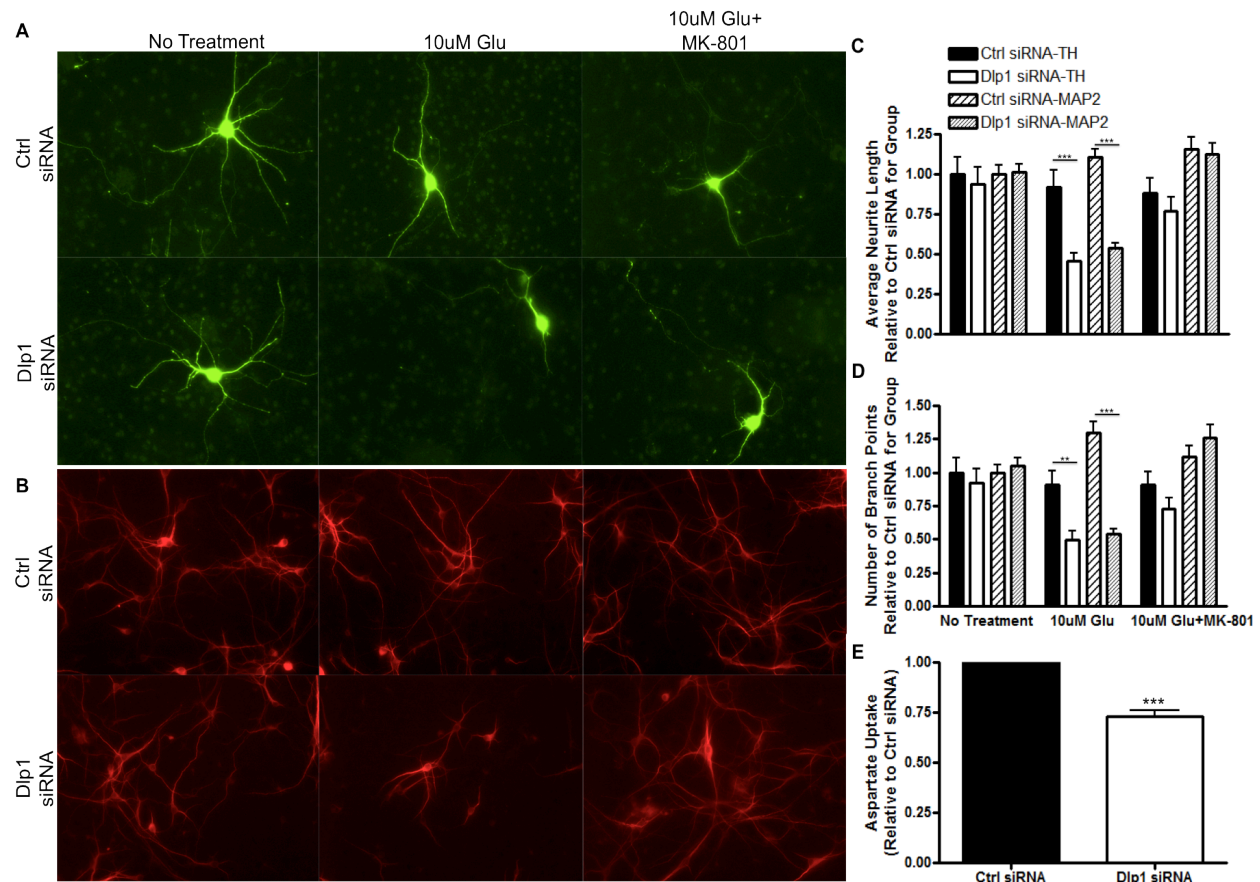


Figure 8. Astrocytic Dlp1 decreases the ability of astrocytes to protect neurons against the effects of excess glutamate. (A) Representative images of DAergic neurons, indicated by TH staining, co-cultured with astrocytes transfected with ctrl (top row) or Dlp1 (bottom row) siRNA and treated as indicated. Neurons cultured with Dlp1 transfected astrocytes show shorter processes and fewer branch points after glutamate treatment. (B) Representative images of non-DAergic neurons, indicated by MAP2 staining cultured with astrocytes transfected and treated similar to (A). (C) Average neurite length for images in (A) (black and white bars) and (B) (striped bars; 3 replicates, 72-101 cells/group for TH+, ~650-800cells/group for MAP2. *** $p < 0.001$ by two way ANOVA with Bonferroni correction). (D) Average number of branch points for images in (A) (black and white bars) and (B) (striped bars; 3 replicates, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni correction). Neurite lengths and number of branch points were normalized to the average value for the No Treatment Ctrl siRNA group for each replicate and each value was used for analysis. (E) Aspartate uptake was decreased in cultures similar to (A) and (B) when astrocyte Dlp1 was decreased (*** $p < 0.001$ by two sided unpaired t-test). Data are presented as mean \pm s.e.m.

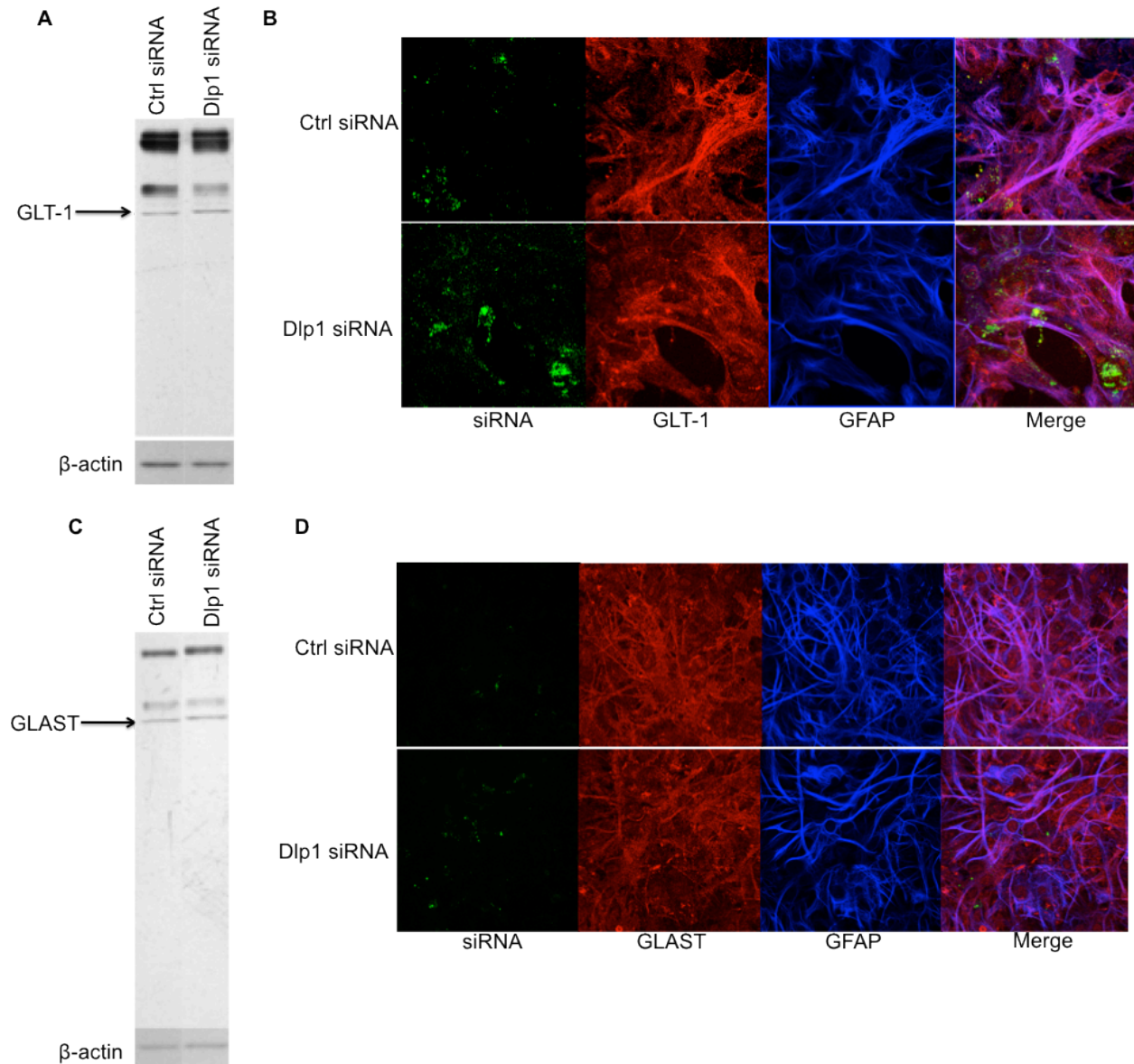


Figure 9. Astrocytic Dlp1 does not affect GLT-1 or GLAST expression or localization. (A) Expression (arrow) and (B) localization of GLT-1 in astrocytes transfected with ctrl or Dlp1 siRNA (identified with 488 tagged siRNA for localization). (C) Expression (arrow) and (D) localization of GLAST transfected with ctrl or Dlp1 siRNA (identified with 488 tagged siRNA for localization).

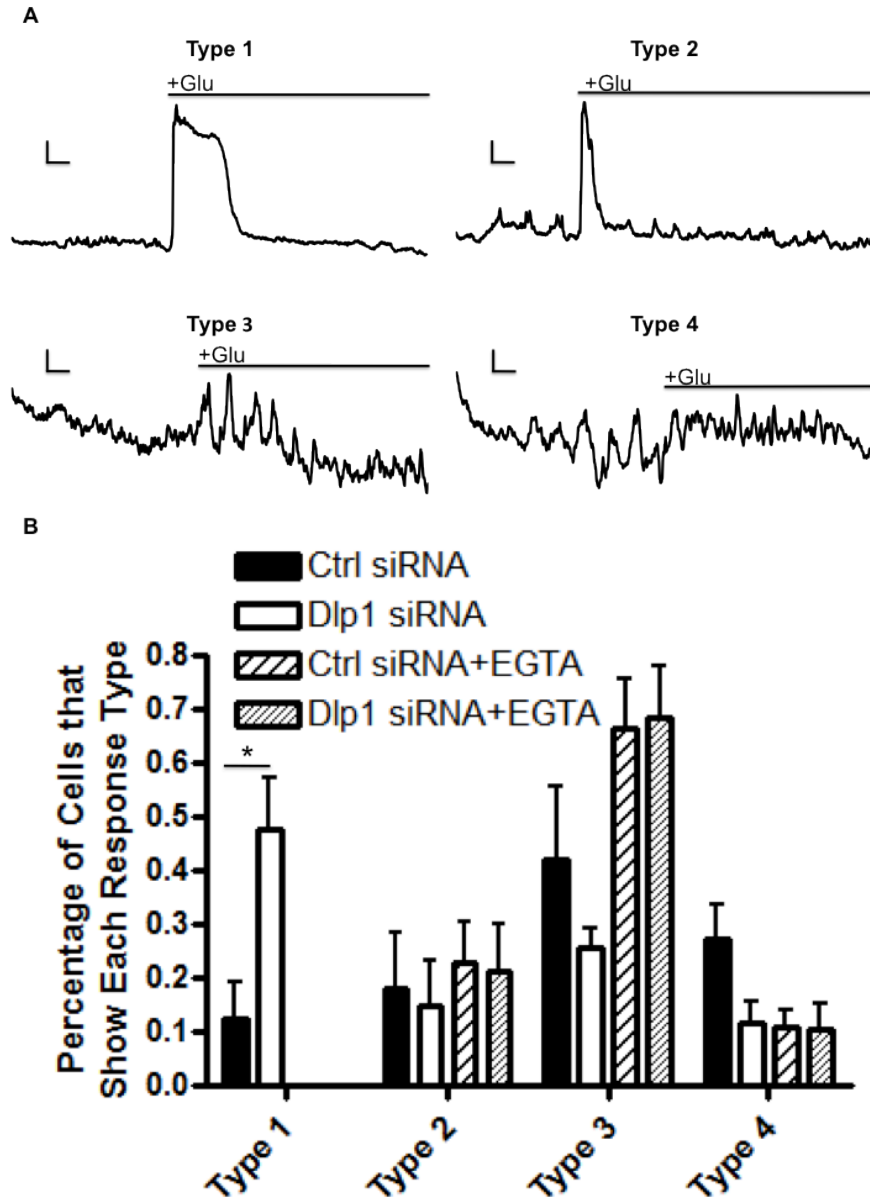


Figure 10. Intracellular Ca^{2+} is increased in response to glutamate when astrocytic Dlp1 is knocked down. (A) Representative images of the 4 primary astrocyte Ca^{2+} responses to glutamate. Traces represent intracellular Ca^{2+} signals from astrocytes treated with glutamate (black bar). For types 1 and 2 the Y scale bar represents 500 fluorescent units (FU) and the X scale bar represents 25 seconds. For type 3 the Y scale bar represents 50 FU and the X scale bar represents 25 seconds. For type 4 for the Y scale bar represents 30 FU and the X scale bar represents 20 seconds. (B) Quantification of percentages of cells showing each type of response to glutamate (N=5, *p<0.05 by two way ANOVA with Bonferroni correction) in normal conditions (black and white bars) or in the presence of extracellular EGTA (striped bars). The percentage of responses in each replicate was calculated and these values were used for analysis. Astrocytes transfected with ctrl siRNA tend to show response types 3 and 4 while those transfected with Dlp1 siRNA show a greater percentage of type 1 responses. No differences are observed between astrocytes with ctrl siRNA and Dlp1 siRNA when EGTA is present and type 1 responses are not observed. Data are presented as mean \pm s.e.m.

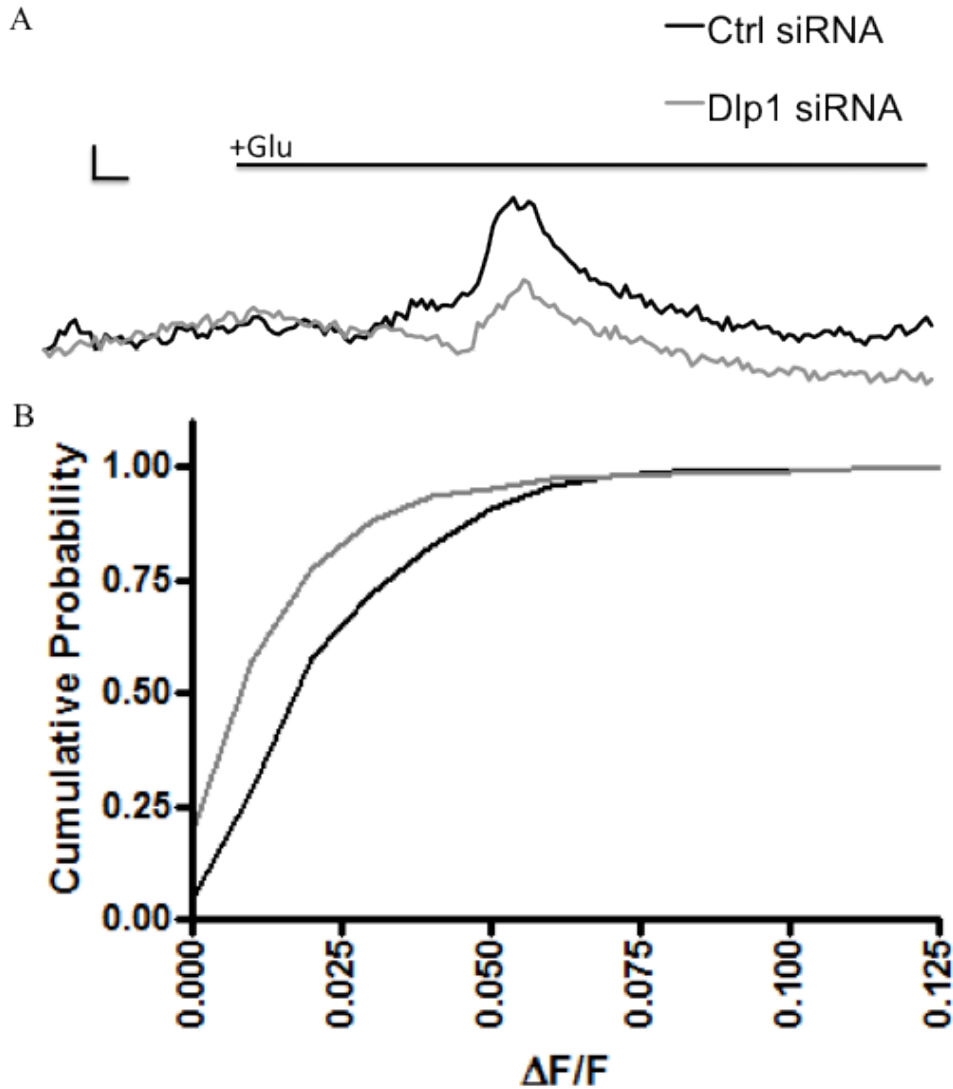


Figure 11. Mitochondrial Ca^{2+} buffering is impaired during glutamate stimulation when astrocyte Dlp1 is knocked down. (A) Representative images of the mitochondrial Ca^{2+} responses to glutamate in astrocytes transfected with ctrl (black line) or Dlp1 (grey line) siRNA. Traces represent mitochondrial Ca^{2+} signals relative to baseline from astrocytes treated with glutamate (black bar). Y scale bar represents 0.01 $\Delta F/F$ and the X scale bar represents 4 seconds. (B) Cumulative probability plot of the peak amplitude ($\Delta F/F$) of mitochondrial Ca^{2+} responses for astrocytes transfected with ctrl (N=7 replicates/473 cells) and Dlp1 siRNA (N=7 replicates/469 cells; $p < 0.001$ by Kolmogorov-Smirnov test).

Methods

Isolation of Mitochondrial Fraction from Human Tissue

Tissue from the SNpc of healthy control and PD patients was suspended in sucrose buffer (20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM PMSF, protease inhibitor cocktail (Sigma), 0.2 mM Na_3VO_3 , 1 mM NaF), homogenized with 10 strokes with a glass homogenizer, and centrifuged at 800 X g for 10 minutes at 4°C. The resulting supernatant was further centrifuged at 10,000 X g for 15 minutes at 4°C to obtain a mitochondrial-enriched fraction. This was resuspended in a buffer composed of 6 M urea, 0.05% SDS, 5 mM EDTA, and 50 mM Tris-HCl (pH. 8.5). Protein concentrations were determined by BCA assay (Pierce).

Western Blot Human Tissue

For Western blot of Dlp1, after quantification of protein using BCA, 10 μ g of protein from the mitochondrial enriched fractions from human tissue were run on 10-20% SDS-PAGE at 100 volts for 10 minutes followed by 160 volts for 80 minutes. Proteins were transferred onto polyvinylidene difluoride membrane at 0.36 amps overnight at 4°C. After transfer, membrane was blocked in 5% milk for 1 hour at room temperature. The membrane was rocked overnight at 4°C with mouse anti Dlp1 (BD Biosciences) diluted 1:2000 in blocking buffer. The membrane was washed twice for 5 minutes and twice for 10 minutes in TBS-T and then incubated in rabbit anti mouse HRP secondary antibody in 3% bovine serum albumin for 1 hour at room temperature. The membranes were washed twice for 5 minutes and twice for 10 minutes in TBS-T and enhanced chemiluminescence (GE Lifesciences) was used to visualize protein bands. Intensity was measured using Quantity One (Biorad). The values for each sample were normalized to the average intensity of Dlp1 from control patients.

Tissue Staining

Fixed and paraffin embedded tissue sections from the midbrain of 6 healthy control patients and 6 PD patients, as well as sections from the frontal cortex of 6 healthy control and 6 PD patients were used to stain for Dlp1 in astrocytes and neurons. For each region, cases were matched for age, gender, and post mortem interval. Sections were deparaffinized by washing 4 times in xylene for 10 minutes each wash, followed by 2 washes in 50% xylene, 50% ethanol for 5 minutes each wash. Tissue was then rehydrated

by washing twice for 5 minutes in 100% ethanol, and once each for 3 minutes in 95%, 70%, and 50% ethanol. Tissue was then quickly rinsed in di-water, and washed twice for 5 minutes in PBS. Antigen retrieval was done in 10mM citric acid pH 6.0 by heating the tissue to boil and then maintaining it at high temperature for 15 minutes. Tissue was allowed to cool for 30 minutes at room temperature and was then washed with 50mM Tris Buffered Saline with 0.05% Tween-20 (TBS-T) 3 times for 10 minutes. Tissue was blocked in 5% normal goat serum, 2% bovine serum albumin, 0.1% Triton 100-X made in TBS-T overnight. The next day, the tissue was incubated with primary antibodies in blocking solution overnight (rabbit anti-Tyrosine Hydroxylase Pel-Freez Biologicals 1:500, mouse anti-Dlp1 BD Biosciences 1:100, rabbit anti-MAP2 Millipore 1:200, rabbit anti-GFAP DAKO 1:500). After incubation with primary antibodies, tissue was washed with 5% normal goat serum, 2% bovine serum albumin in TBS-T 3 times for 10 minutes and were then incubated with secondary antibodies conjugated with alexa fluor 488 or 568 (Life Technologies), each diluted 1:500 in 5% normal goat serum, 2% bovine serum albumin in TBS-T overnight. After incubation with secondary antibodies, tissue was washed 3 times for 10 minutes in TBS-T followed by rocking for 30 minutes in 0.3% Sudan Black dissolved in 70% ethanol. Tissue was rinsed twice in 70% ethanol and washed 3 times for ten minutes in TBS-T. After the third wash, tissue was mounted with Vecatshield with DAPI dye (Vector Laboratories).

Quantification of Dlp1 in Tissue

Images of GFAP positive cells in the SNpc were taken at 100X using a Bio-Rad LS2000 laser scanning confocal microscope. Z-series images were captured for 15 randomly selected fields. For quantification, the middle sections, which had the largest area for the cell marker, were used to quantify Dlp1 intensity. ImageJ was used to quantify the intensity of Dlp1 intensity in each cell. For all other cells types and regions, images were taken at 60X using a Nikon Eclipse Ti microscope. Z-series images were captured for 15 randomly selected fields for GFAP cells in the frontal cortex and 20 fields for neurons in each region. Images were deconvolved and Dlp1 intensity in each cell was measured using NIS-Elements Software (Nikon). Settings on the microscopes being used were kept the same for each cell type from each region. For quantification, each value was normalized to the average intensity of the control patients for the cell type and region being analyzed.

Astrocyte Cultures

Cortical astrocytes from Sprague Dawley rats (postnatal days 0-1) were utilized and cultured into 75cm² vented flasks coated with Poly-D-lysine (5mg/200ml). Flasks were washed with PBS prior to plating astrocytes. Rats were decapitated, brains were removed, and placed into cold dissecting media (DMEM F12, Life Technologies). Cortices were isolated and meninges and blood vessels were removed. Tissue was digested in DMEM F12 with 0.5mM EDTA, 0.2 mg/ml L-cysteine, 15 units/mL papain (Worthington Biochemical), and 10µg/mL DNase (Worthington Biochemical) (10mLs digestion media/3 brains) for 30 minutes at 37°C. Papain was dissolved by incubating solution at 37°C for 20 minutes followed by the addition of DNase and filtration through 0.22µm filter. After digestion, cortical tissue was washed 3 times with culture media (DMEM F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin). After washing, cortices were triturated with a fire polished Pasteur pipette in 10mLs of culture media by passing the tissue through the pipette 10 times. Tissue was allowed to settle and the supernatant was passed through a strainer. The remaining tissue was triturated again in 5mLs of culture media, passed through a cell strainer, and combined with the previous 10mLs of triturated cells. The resulting cells were seeded 2 brains/flask in culture media. The culture media was changed 24 hours later and astrocytes were maintained in culture media until they reach confluency (9-10 days), at which point they were plated for use. Cells were maintained at 37°C 5% CO₂.

Plating Astrocytes for Co-culture with Neurons

Astrocytes to be used for co-culture with neurons were plated onto glass cover slips in a 24-well plate, coated with Poly-D-lysine and washed with PBS as described above. Flasks with astrocytes were shaken to remove microglia, washed once with PBS pH 7.4, and incubated with 0.25% trypsin EDTA at 37°C until cells fell off the flask, at which point an equal volume of culture media was added to each flask to halt the activity of the trypsin. Flasks were washed with the resulting mix of culture media and trypsin and collected into a 50mL conical tube. Cells were centrifuged at 3500 RPM for 10 minutes at room temperature. The resulting pellet was re-suspended 40 times in 2mL of penicillin/streptomycin free media. 8mLs of the same media was added (final volume ~10mLs per flask used) and cells were counted using a

hemocytometer. 25×10^4 cells were plated onto the cover slips at a final volume of 500 μ l in penicillin/streptomycin free culture media (antibiotic free media was used as the plating media for the purposes of transfection as the reagents used are toxic in their presence, according to the manufacturer). Cultures were at least 95% positive for the astrocyte marker glial fibrillary acidic protein, meaning they were highly enriched for astrocytes.

Plating Astrocytes for Western Blot Analysis

Astrocytes to be used for any western blot analysis were plated into 6 well plates coated with Poly-D-Lysine as described above. Astrocytes were harvested from flasks as described above. 10×10^5 cells were plated into each well at a final volume of 2mL in penicillin/streptomycin free culture media.

Plating Astrocytes for Ca^{2+} and Mitochondrial Imaging

Astrocytes to be used for intracellular or mitochondrial imaging were plated onto 35mm dish with a 14mm glass cover slip in the center (MatTek), coated with Poly-D-lysine and washed with PBS as described above. Astrocytes were harvested from flasks and counted as described above. 25×10^4 cells were plated onto the cover slips in the plates at a final volume of 500 μ l in penicillin/streptomycin free culture media, returned to 37°C 5% CO₂ for at least 2 hours to allow cells to adhere to the glass. After 2 hours, 1.5 mLs of antibiotic free media was added so plates had a final volume of 2mLs (see plating astrocytes for co-cultures for why antibiotic free media was used).

Transfections

Transfections were performed with Lipofectamine 2000 according to manufacturer's instructions with slight modifications. Non-specific (control) or Dlp1 siRNA were purchased from Qiagen. For astrocytes to be used for co-cultures, astrocytes were transfected with 2pmol siRNA per well. Specifically, for each well, 50 μ l of Opti-MEM was pipetted into 2 separate eppendorf tubes. 1 μ l lipofectamine was pipetted into one tube while the siRNA was pipetted into the other. Tubes were incubated at room temperature for 5 minutes, at which point the contents of the tube with the siRNA were transferred to the other tube and gently mixed. The mixture was allowed to sit at room temperature for 20 minutes, at which point it was

added to the cultures in antibiotic free media and returned to the incubator. Media was changed to normal culture media after 6 hours. For transfection of siRNA in astrocytes for western blot as well as cytoplasmic or mitochondrial Ca^{2+} imaging, instructions for a 6 well format were followed and 200pmol per plate were used. Similar steps were followed as described for the 24-well plate format except 250µl Opti-MEM and 5µl Lipofectamine was used. For transfections of Mito-DsRed2 plasmid (ClonTech) and 488-tagged siRNA into astrocytes, for the purposes of mitochondrial movement, localization, and morphological measurements, instructions for 6 well plasmid transfection were followed and 4µg plasmid and 100pmol siRNA per plate were used. The procedure is that same as for transfection for western blot or Ca^{2+} imaging. For all transfections, the volumes can be scaled up based on the number of wells or plates being used, and an excess of 2 or 0.5 reactions was made for transfecting 24-well plates or the 6-well plates/imaging dishes, respectively, so there is enough reagent for each plate.

Dissecting and Plating Ventral Mesencephalon Neurons for Co-Culture

Neurons from the ventral mesencephalon of embryonic rats days 16-18 were plated on astrocytes plated and transfected on glass cover slips. Sprague Dawley rats (Harlan Animal Research Labs) were anesthetized with CO_2 and pups were removed and placed into neuronal dissection media (Neurobasal A supplemented with B27 and 0.5mM L-glutamine (Life Technologies)) on ice. Meninges were removed and the ventral mesencephalon was dissected out and placed into a 50mL conical with neuronal dissection media on ice. After all brains were dissected, cold neuronal dissection media was replaced with room temperature media and incubated at 30°C for 8 minutes. Tissue was then incubated in digestion media (12 mLs Hibernate A without CaCl_2 (BrainBits), 0.5mM L-glutamine, 15 units/mL papain, 17µg/ml DNase; incubated and filtered as described above) at 30°C for 15 minutes. Tissue was washed 3 times with neuronal dissection media and then triturated in 2 mLs of neuronal dissection media with a fire polished Pasteur pipette. Tissue was allowed to settle and the supernatant was passed through a strainer. Trituration and cell straining was repeated in 2mL of neuronal dissections media 2 more times. The supernatants were combined and spun at 1100 RPM for 5 minutes at room temperature. The resulting pellet was resuspended in 2 mLs of neuronal media (Neurobasal A supplemented with B27, 2mM L-glutamine, and 1% penicillin/streptomycin) and counted using a hemocytometer. Media on astrocytes was

changed from culture media to neuronal media and 12.5×10^4 cells were plated onto astrocytes transfected with control or Dlp1 siRNA. Neurons were allowed to grow for 8 days before treatment.

Glutamate Treatment of Astrocyte-Neuron Co-Cultures

After neurons from the ventral mesencephalon were allowed to grow in Co-Cultures (8 days), neurons were treated with glutamate to assess astrocyte protection against excitotoxic environments. Astrocytes were gently shaken to remove residual microglia. Co-Cultures with astrocytes transfected with either siRNA were treated with normal neuronal media (No Treatment), neuronal media supplemented with $10 \mu\text{M}$ Glutamate ($10 \mu\text{M}$ Glu), a concentration that does not affect neurons in Co-Culture unless potential pathological conditions are simulated, or neuronal media supplemented with $10 \mu\text{M}$ each of Glutamate and MK-801 (Sigma; $10 \mu\text{M} + \text{MK-801}$), which inhibits NMDA receptors, for 10 minutes at 37°C . Treatments were then replaced with normal neuronal media and allowed to recover for 1 hour, at which point Co-Cultures were then fixed with 4% paraformaldehyde for 30 minutes at room temperature and stained as described below.

Staining of Co-cultures and Astrocytes

This is the cell staining protocol for glutamate treated co-cultures as well as astrocytes transfected with 488 tagged siRNA to observe neurons or glutamate transporters, respectively. Co-cultured cells were plated as described above. Astrocytes used for transporter imaging were plated into Poly-D-lysine coated glass coverslips and were transfected and treated similar to astrocytes used for co-culturing. After fixation, cells were washed twice for 5 minutes in PBS, blocked in 4% normal goat serum, 1% bovine serum albumin, and 0.4% triton 100-X to permeabilize cells, for 1 hour at room temperature. Co-cultured cells were then incubated with rabbit anti-tyrosine hydroxylase (Millipore, 1:200) and mouse anti-MAP2 (Abcam, 1:200) while astrocytes without neurons were incubated with either monoclonal mouse anti-GLT-1 (a kind gift from Dr. J. Rothstein, 1:200) or rabbit anti-excitatory amino acid transporter 1 (GLAST, Abcam, 1:200) each with chicken-anti GFAP (Millipore, 1:500) in blocking buffer overnight. Cells were then washed 3 times for 5 minutes each with 0.1% Tween in PBS and were then incubated with secondary antibodies conjugated with alexa fluor 488, 568, or 633 (Life Technologies), diluted 1:500 (488

and 568) or 1:100 (633) in 0.3% triton 100-X. Cells were wash 3 time for 5 minutes again with 0.1% Tween in PBS. Cover slips with stained cells were then mounted on glass slides with Vectashield mounting media with DAPI dye (Vector Laboratories).

Measurement of Neurons After Treatment

To assess the viability of DAergic neurons in response to treatments, images from 10 random fields of TH positive neurons and their processes were captured with a Nikon Eclipse 80i microscope. Processes were traced using Neurolucida to assess average process (neurite) length and number of branch points on processes. To assess the viability of non-DAergic neurons in response to treatments, images from 5 random fields of MAP2 positive, TH negative nerurons were captured with Nikon Elipse 80i microscope and similarly traced.

Western Blot Astrocytes

Astrocytes were cultured and maintained in a manner similar to as described for co-cultures. After plating and transfection in 6 well plates, cells were maintained in DMEM F12, 10% FBS, 1% P/S for 4-5 days, at which point media was changed to neuronal media (Neurobasal A supplemented with B27 (Life Technologies), 2mM L-glutamine, and 1% penicillin/streptomycin). Astrocytes were maintained for 8 days at which point cells were harvested by washing once with ice-cold PBS pH 7.4 and scraped into RIPA buffer, were sonicated on ice, and centrifuged at 15,000 x g for 10 minutes. BCA was performed on the resulting supernatant. For Dlp1 western blot, 25 µg protein was added to Laemmli Sample buffer (Bio-Rad) in the presence of 2-Mercapto ethanol and boiled for 5 minutes. For GLT-1 or GLAST western blot, 25 µg protein was added to Laemmli Sample buffer without 2-Mercapto ethanol and not boiled. Samples were run on 10-20% SDS-PAGE and transferred to PVDF membrane overnight. Membranes were blocked in 5% milk for 1 hour at room temperature followed by incubation with primary antibodies (mouse anti-Dlp1, BD Biosciences 1:2000, mouse anti-excitatory amino acid transporter 2 (GLT-1) Millipore 1:1000, rabbit anti-excitatory amino acid transporter 1 (GLAST) Cell Signaling Technology 1:1000). Membranes were washed twice for 5 minutes and twice for 10 minutes with TBS-T followed by incubation with either rabbit anti-mouse HRP or goat anti-rabbit HRP (Sigma) secondary antibodies at room

temperature for 1 hour in 3% BSA. Membranes were washed twice for 5 minutes and twice for 10 minutes with TBS-T and enhanced chemiluminescence was used to visualize protein bands. Blots were stripped and reblotted with β -actin (mouse anti- β -actin, abcam 1:4000) as a loading control.

Mitochondrial Movement

For mitochondrial movement, astrocytes transfected with 488-tagged siRNA and Mito-DsRed2 plasmid were maintained in a similar manner to how co-cultures were treated. That is, media was changed from culture media to neuronal media at a time similar to when neurons would have been plated and were imaged at a time comparable to when co-cultures would have been treated with glutamate. 3 random fields of astrocytes were selected for each replicate and imaged at 37°C using a Nikon Eclipse Ti microscope and NIS-Elements software. Images were captured every 5 seconds for 5 minutes and analyzed with NIS-Elements software. Movement was assessed by measuring the net distance of mitochondria within the processes towards (retrograde) or away (anterograde) from the astrocyte cell body. Select cells that are positive for green and red indicating transfection of both siRNA and plasmid. In the Nikon software, select Red as the channel to measure with the program as described above for time. After selecting the field, push the FOCUS button on the microscope and bring into focus with the offset controller. To measure the movement, select the endpoint of a mitochondria within the process of an astrocyte and trace a path from that point, through the process, towards the cell body. Run the movie and at the end measure the distance along the same path for the same mitochondria. Repeat this for all mitochondria within the process of the astrocyte. Subtract the final distance from the start distance for each mitochondria. Those with a positive number represent anterograde movement while those with a negative distance represent retrograde movement. Separate them into each group and then make sure all numbers are positive for the purposes of analysis. Average the measurements in separate groups and this is the average distance traveled for each direction for that cell.

Mitochondrial Length and Morphology

After imaging was complete, astrocytes were fixed, washed, blocked, and stained with rabbit-anti GFAP (DAKO, 1:500) as described in above in treating co-cultures. After incubation with primary antibody, cells

were washed and stained with secondary antibodies conjugated with alexa fluor 688 (Life Technologies). Vectashield mounting media with DAPI dye was added to cells and they were covered with a glass coverslip for imaging. 10 randomly selected fields were selected and Z-series images were captured using a Nikon Eclipse Ti microscope. Image processing and mitochondrial measurements were performed using NIS-Elements Software. Files were deconvolved and max intensity projections were generated. Mitochondria within the process were analyzed due to the fact that individual mitochondria were more easily resolved than those in the cell body were. For astrocytes with Dlp1 knocked down, it was not uncommon for mitochondria to continue into the cell body. In this event, mitochondria were cutoff where they reach the cell body. That is, the process was cropped and mitochondria were analyzed after that. The perimeters of mitochondria were selected using the software and from this length and area were automatically calculated. To assess density, the number of mitochondria in the process were counted and divided by the area of the process, which was measured by tracing the process in the program yielding the area. For each measurement of morphology or density, the values for each cell were averaged.

Intracellular Ca^{2+} Imaging

Oregon Green 488 BAPTA AM (Life Technologies) was used to measure astrocyte intracellular Ca^{2+} responses to glutamate. Astrocytes transfected with control or Dlp1 siRNA were maintained in a similar manner to how co-cultures were treated (described in mitochondrial movement section). Any steps involving the use of this dye was protected from light. 50 μ g of the dye was shaken in 10 μ l of 15% Pluronic F-127 in DMSO (Life Technologies) at 4° C for 30 minutes. The dye was diluted to a final concentration of 4 μ M in neuronal media and incubated at 37°C for 30 minutes. To accomplish this, add 90 μ l of warmed media to dye after shaking, vortex for 30 seconds, and dilute 10 μ l into the plates of the cells with 1mL of media. Cells were then washed 3 times for 10 minutes each wash. For the first wash, add 2 mLs of warmed media to the plate, swirl several times, remove and discard 1 mL of media, and return to the incubator. For the remaining 2 washes, add 1 mL of media, swirl, remove and discard 1 mL, and return to the incubator. Cells were imaged after the third wash. Imaging was performed using 60X magnification at 37°C using a Nikon Eclipse Ti microscope and NIS-Elements Software. Fields of cells were randomly selected and intracellular Ca^{2+} data, indicated by fluorescence intensity, was collected every 200ms. Cells

were imaged for approximately 2 minutes, at which point glutamate was added to a final concentration of 100 μ M. Responses were recorded and categorized into 4 response types. Type 1 was a large increase in Ca^{2+} that persisted for longer than 60 seconds. Type 2 responses were a single large increase in Ca^{2+} that was 60 seconds or shorter. Type 3 responses showed large Ca^{2+} oscillations with a lower frequency (defined in this study as less than 0.19 peaks/second). Type 4 responses showed small Ca^{2+} oscillations that had a relatively higher frequency than type 3 responses (defined in this study as 0.19 peaks/second or greater; see figure 5 for examples). 3-4 plates were used per replicate and the percentage of each response type for each group (siRNA used) was calculated. To run the program, select GFP as the channel to use and make the intensity 33%. The program should collect every 200ms. Set the exposure time to 200ms. Use the ND6 (0.6) neutral density filter to minimize quenching the dye. After selecting the field, focus as done for mitochondrial movement. Select a small area with the lowest intensity with a rectangle/box region of interest, right click on the box, and set as background. Select the cells in the field using polygon regions of interest.

Contribution of Extracellular Ca^{2+} to Observed Intracellular Ca^{2+} Wave Pattern

To assess the contribution of extracellular Ca^{2+} to the observed intracellular wave patterns, astrocytes transfected with control or Dlp1 siRNA were cultured and loaded with Oregon green 488 BAPTA as described above. After completion of the wash steps, the media was exchanged with neuronal media supplemented with 2mM EGTA to chelate Ca^{2+} . Cells were treated with glutamate, imaged, and categorized as described above.

Mitochondrial Ca^{2+} Imaging

Rhod-2 AM (Life technologies) was used to measure mitochondrial Ca^{2+} responses in astrocytes transfected with control or Dlp1 siRNA. Astrocytes transfected with control or Dlp1 siRNA were maintained in a similar manner to how co-cultures were treated (described in mitochondrial movement). This dye was used protected from light as was done for intracellular Ca^{2+} responses. 50 μ g was shaken in 10 μ l of 15% Pluronic F-127 in DMSO at 4°C for 30 minutes. The dye was diluted to a final concentration of 4 μ M in neuronal media and incubated at room temperature for 30 minutes. The same technique for

loading cells for intracellular Ca^{2+} can be used here. Cells were washed 3 times with neuronal media and incubated at 37°C for 1 hour, at which point cells were imaged. Imaging was performed using 60X magnification at 37°C using a Nikon Eclipse Ti microscope and NIS-Elements Software. Fields of cells were randomly selected and mitochondrial Ca^{2+} levels, indicated by the fluorescence of the dye, was collected every 1 second. Cells were imaged for approximately 2 minutes, at which point glutamate was added to a final concentration of $100\mu\text{M}$. Mitochondrial Ca^{2+} responses were observed to be a peak and were therefore measured by dividing the change in fluorescence from the baseline (ΔF) by the baseline fluorescence (F). 3-4 plates were used per replicate and the $\Delta F/F$ values for each cell per group (control or Dlp1 siRNA) were plotted as a cumulative probability and differences were assessed using the Kolmogorov-Smirnov test. To run the program, select Red as the channel to use and make the intensity 50%. The program should collect every 1s. Set the exposure time to 200ms. Use the ND4 (0.3) neutral density filter to minimize quenching the dye. After selecting the field focus as was done in mitochondrial movement. Select a small area with the lowest intensity with a rectangle/box region of interest, right click on the box, and set as background. Select the cells in the field using polygon regions of interest.

H^3 -D-Aspartate Uptake

Uptake assays were performed on co-cultures using a Krebs-Ringer solution (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgSO_4 , 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4) in duplicate, similarly to as described elsewhere[29]. Briefly, cells were washed twice with warm Krebs-Ringer solution. After the second wash, cells were incubated with Krebs-Ringer with H^3 -D-Aspartate (($40\text{Ci}/\text{mmol}$, SA $1\text{mCi}/\text{mL}$) $1\mu\text{l}/30\text{mls}$) at 37°C for 10 minutes. Co-cultures were washed 3 times with ice cold Krebs-Ringer. After the third wash, cells were rocked in 1N NaOH for 30 minutes at room temperature. The resulting solutions were collected in scintillation fluid and measured using a scintillation counter. For each replicate, H^3 measurements averaged for each group and normalized to the value for co-cultures where control siRNA was transfected into astrocytes.

Statistical Analysis

Statistical analysis was performed using Graphpad Prism. Dlp1 expression in human tissue in figures 3B and D, 4B and D, and 5B and D both for western blot in the mitochondrial fraction from human SNpc as well as fluorescent staining for Dlp1 in human tissue for each region and cell type, was normalized to the average for the control group for each respective group, are presented as mean \pm s.e.m. and was analyzed with a two sided t-test. Mitochondrial morphology, localization, and movement data in figures 6B-D, and 7A and B are presented as mean \pm s.e.m. and were analyzed using a two sided t-test. Neurite length and branch point measurements seen in figures 8C and D are expressed as mean \pm s.e.m. and were analyzed using two way analysis of variance with the Bonferroni correction. H^3 -D-aspartate uptake assays were run in duplicate. Each group (control and Dlp1 siRNA) were averaged and normalized to the control value for that replicate. Data in figure 8E are presented as mean \pm s.e.m. and were analyzed using column statistics to test if the average is different from the value 1, due to the fact that the control value is 1 for each measurement. Percentages of Ca^{2+} responses after glutamate stimulation for several replicates in figure 10B are presented as average \pm s.e.m. and were analyzed using two-way analysis of variance with Bonferroni correction. The $\Delta F/F$ for mitochondrial Ca^{2+} responses after glutamate stimulation for all replicates are represented as a continuous probability plot for the two groups (control and Dlp1 siRNA) of cells in figure 11B and were analyzed using a two sample Kolmogorov-Smirnov test to assess if the two groups of responses were equal.

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Chapter 6:

DISCUSSION

It is understood that the cause of the motor symptoms observed in PD is loss of DAergic neurons from the SNpc and that the appearance of these motor symptoms occurs at a stage in disease where irreversible neuronal loss has already occurred. While current therapies are effective at alleviating motor symptoms in the short term, the most effective treatment will likely come from preventing neuronal loss prior clinical onset. This makes early detection and understanding the mechanism of neuronal death important towards effective therapeutic intervention. As discussed in Chapter 2, development of imaging and biochemical markers in groups at a high risk of PD will allow for early detection.

The current mechanism(s) through which neurodegeneration in PD occurs is poorly understood. Mitochondrial dysfunction, in the form of impaired ETC function and elevated ROS production, is strongly implied in neuronal death (see Chapter 3). Recent data has shown that changes in mitochondrial dynamics represent another aspect of mitochondrial dysfunction in the development of PD. A large portion of this research has focused on neuronal biology and mechanisms of dysfunction. This largely ignores the potential contributions of other cell types. Astrocytes represent one such cell type as they are the most abundant cell found in the brain, particularly the SNpc, and play a significant role in protecting neurons. If deficiencies in mitochondrial dynamics occur in astrocytes, their ability to maintain adequate neuronal health and function could be compromised. My thesis considers alterations in mitochondrial dynamics due to a decrease in the fission promoting protein Dlp1, which is observed in human tissue, as a mechanism of neurodegeneration. This work focuses on astrocytic contribution to neurodegeneration through impaired glutamate regulation, which results in excitotoxicity. Further, it implies impaired mitochondrial dynamics as a pathogenic event that occurs early in the course of PD, representing a target of therapy to prevent neuronal death.

Dlp1 is Decreased in Human Tissue in PD

Several studies have implicated alterations in mitochondrial dynamics as a mechanism of DAergic neuron loss in PD[1-3]. Western blot was used to show that Dlp1 is decreased in the mitochondrial fraction of the SNpc in PD (Chapter 5), which is in accordance with previous mass spectrometry findings[4]. Immunofluorescent studies showed that this occurs in astrocytes as well as neurons in the SNpc. Decreased neuronal Dlp1 is not a finding unique to PD, as it has also been observed in hippocampal neurons in AD patients[5], indicating a common mechanism of

neurodegeneration. That Dlp1 expression is also depressed in astrocytes shows that PD associated changes in proteins occur in cell types not directly responsible for the clinical presentation. It is therefore likely that astrocytes play an important role in the mechanism of neurodegeneration in PD.

This decrease was also observed in neurons and astrocytes in the frontal cortex of PD patients. Such changes are significant due to the fact that dysfunction in regions of the brain beyond the SNpc is tied to non-motor symptoms observed in PD (discussed below). Additionally, no apparent cortical degeneration or Lewy body formation was observed in the selected cases, suggesting decreased Dlp1 represents an early event in PD pathogenesis, at least in cortex, that precedes, and may therefore contribute to, neuronal death. This makes Dlp1, as well as consequences related to decreased expression, a potential target of therapy to prevent neurodegeneration, particularly if pre-motor detection of PD is achieved (discussed below).

Dlp1 Affects Mitochondrial Morphology in Astrocytes

The cellular studies that followed the findings in tissue were performed in astrocytes to observe the effects that Dlp1 had in astrocyte contribution to PD. Astrocytes are increased in the SNpc of PD patients[6,7], and their dysfunction could result in neuronal death (discussed in Chapter 4). Recapitulation of the observed Dlp1 decrease in primary astrocytes results in an increase in length, area, and perimeter of astrocytic mitochondria. This is not surprising given Dlp1 promotes fission and that knockdown or inhibition of Dlp1 has generated similar effects in different cell types[5,8,9]. Aside from morphological changes, knockdown of Dlp1 also dramatically affected the localization of mitochondria. The density as well as the net anterograde and retrograde movement of mitochondria within the processes of astrocytes were all decreased after Dlp1 was knocked down. These results are in keeping with the involvement of mitochondrial dynamics in PD (discussed in Chapter 3) [2,3,10,11], which also indicate that the mechanism through which PD develops occurs through decreased Dlp1-mediated fission. Such changes are detrimental towards astrocyte function as altered mitochondrial dynamics impairs astrocyte neuroprotection (discussed below) [1].

Dlp1 Affects Astrocyte Mediated Neuroprotection

Astrocytes play an important role in promoting neuronal survival as well as proper neuronal function. This occurs through several mechanisms. While protection against ROS and promotion of

neuronal function through trophic factor production are important aspects of astrocyte biology (discussed in Chapter 4), the work in this thesis focused on the role that excitotoxicity plays in neurodegeneration, due to altered mitochondrial dynamics in astrocytes. Astrocytes play an important role in protecting neurons against excitotoxicity[12] through the function of GLT-1[13,14] and GLAST[15], which is heavily dependent upon proper mitochondrial function and localization[16]. The identification of Dlp1 as a potential interacting protein of GLT-1[17] implies that Dlp1 plays an important role in the ability of astrocytes to properly regulate glutamate. Results from Chapter 5 show that loss of Dlp1 in astrocytes decreases glutamate transport in a manner that results in excitotoxic neuron death. This occurs through signaling changes in astrocytes (discussed below) as expression or localization of the astrocyte glutamate transporters was not affected by Dlp1 knockdown. The neuronal effects can be rescued by blocking ionotropic receptors using the NMDA receptor antagonist MK-801. Not only does this provide supporting evidence for the involvement of astrocyte dysfunction as a means of neurodegeneration, but it also indicates that excitotoxicity is a potential mechanism of neuronal death in PD.

The involvement of excitotoxicity in PD (discussed in Chapter 4), however, has not been definitively established. While the findings presented in Chapter 5 support excitotoxicity as a mechanism of neurodegeneration, preferential loss of DAergic neurons, as is observed in PD, did not occur. This can be explained by the fact that glutamate may act as a contributing toxic factor in the presence of a separate DAergic neurotoxic insult, which is supported by various studies. Several agents that kill DAergic neurons require excitotoxic input to function[18]. Additionally, *in vivo* toxicant based PD models cause DAergic neuron loss through generation of ROS and/or inhibition of mitochondrial functions, not through elevation of glutamate[19-24]. The finding that glutamate receptor antagonists rescue DAergic neuron loss[21,23-25] and that loss of Dlp1 in astrocytes results in death of neurons generally, rather than DAergic neurons specifically, implies that excitotoxicity plays a contributing role in PD instead of a causative role. If this is the case, elevated extracellular glutamate due to impaired astrocyte function acts to enhance a neurotoxic insult that is specific to DAergic neurons. The regions from which the astrocytes being studied originate could also explain the lack of specific loss of DAergic neurons. The studies performed in this thesis considered Dlp1 expression in primary cortical astrocytes due to the fact that they are the easiest and largest in quantity to obtain, while the decrease in human tissue being related to is the

SNpc. Astrocytes from this region might have different properties that affect protection of different neuron populations, and decreased Dlp1 from midbrain astrocytes may have specific effects on DAergic neurons. Unpublished data obtained during the course of this thesis shows that cortical astrocytes are less responsive to MnCl_2 treatment than midbrain astrocytes, when using ROS production as a metric, which supports this concept. Further, midbrain astrocytes more effectively promote the growth of DAergic neurons than astrocytes from other regions of the brain[26], meaning dysfunction of astrocytes from this region could have a greater effect on DAergic neurons compared to those from the cortex. Nevertheless, the results presented in Chapter 5 support astrocyte dysfunction and excitotoxicity as contributing factors to PD pathogenesis.

Dlp1 Affects Astrocyte Ca^{2+} Response to Glutamate due to Impaired Mitochondrial Buffering

As discussed in Chapter 3, mitochondrial dysfunction is tightly associated with PD pathogenesis. These defects have been described in several cell types, including those of the SNpc. As the SNpc in PD patients is largely comprised of astrocytes, it is likely that mitochondrial defects in astrocytes contribute to PD pathogenesis. Our results support this through Ca^{2+} imaging experiments. Astrocytes function and communicate via Ca^{2+} signaling in response to various stimuli, including glutamate[27-29]. Alterations in Ca^{2+} signals are therefore a good indication that astrocyte function is similarly altered. When Dlp1 is knocked down, astrocytes tend to have large increases in intracellular Ca^{2+} that last for extended periods of time in response to glutamate. This is dramatically different compared to astrocytes with control siRNA, which tend to have oscillating waves. The overall intracellular Ca^{2+} levels are therefore higher in astrocytes during glutamate stimulation, when Dlp1 is knocked down. Chelation of extracellular Ca^{2+} ablated any wave patterns that showed extended elevations, and produced Ca^{2+} responses that were not dependent on Dlp1 expression. This indicates that Dlp1 is important in regulation of intracellular Ca^{2+} that enters from the extracellular space during glutamate stimulation. This provides mechanistic insight into how elevated astrocytic Ca^{2+} , which has been associated with excitotoxic neuron death elsewhere[30,31], occurs and ties this process to PD pathogenesis. It also implies impaired mitochondrial function, as they regulate intracellular Ca^{2+} (see below).

Mitochondria are localized to regions within cells where Ca^{2+} enters the cytoplasm (i.e. the ER and plasma membrane)[32,33], and maintaining this distribution is dependent upon proper transport.

Glutamate induced Ca^{2+} entry traps mitochondria at the plasma membrane[32]. Further, mitochondrial motility can be regulated by Ca^{2+} signals[34]. These aspects of Ca^{2+} regulation of mitochondrial dynamics localize mitochondria to sites of Ca^{2+} influx to allow for proper buffering of Ca^{2+} , which is critical in regulating Ca^{2+} transients[33,35,36]. As previously discussed, knockdown of Dlp1 in astrocytes results in highly elongated mitochondria that cannot be adequately transported to their destination. It is not surprising, then that mitochondrial buffering is dampened, given how important mitochondrial localization is in Ca^{2+} responses. The diminished mitochondrial buffering results in the elevated cytoplasmic Ca^{2+} , (discussed above, Chapter 5), which yields the deficits in glutamate uptake and neuronal death (Fig. 12A, B). Beyond the effects of glutamate uptake, Ca^{2+} promotes exocytosis of glutamate from astrocytes[37], which is regulated by mitochondria[38]. Impaired mitochondrial buffering could further contribute to excitotoxicity through excessive release of glutamate (Fig. 12A, B).

Therapeutic Implications

The studies discussed above provide insight into a mechanism of neurodegeneration. As Dlp1 was also decreased in the cortex, a region not associated with clinical symptoms of PD, decreased Dlp1 potentially represents an early event in neurodegeneration and may be involved in non-clinical symptoms that occur in PD. Treatment methods that target decreased astrocytic Dlp1 and the effects related to it may, therefore, be effective in treating motor and non-motor symptoms. Chapter 3 discusses how a variety of therapies have been utilized to alleviate the clinical symptoms of PD, and how their mechanisms of action potentially reverse the detrimental effects associated with mitochondrial dysfunction. A majority of these treatments do not directly target mitochondrial dysfunction. Results presented here show that regulation of mitochondrial dynamics represents another potential target of therapy in PD. Specifically, promoting fission by increasing the expression or action of Dlp1, or decreasing fusion, are the most obvious mechanisms to ameliorate dysfunction of mitochondrial dynamics, and have been shown to rescue effects in models of PD[2,3]. This work also shows that mitochondrial deficits in PD occur in a capacity beyond impaired ETC function that results in ATP depletion and ROS generation, as mitochondrial Ca^{2+} buffering is impaired and could similarly be targeted (discussed below).

Ca^{2+} is an important ion in cell signaling and plays an even larger role in neuron and astrocyte function[27-29,39]. The results presented here show that alterations in Ca^{2+} signaling are also involved in PD in several aspects that result in excitotoxicity. Correcting deficits in astrocytes and astrocytic Ca^{2+} during glutamate stimulation represent one such target. This may be achieved by inhibiting astrocyte metabotropic receptors, as they mediate Ca^{2+} signaling [40,41] and astrocyte glutamate transporters[42-44]. Dampening metabotropic receptor action in astrocytes could restore intracellular astrocyte Ca^{2+} signaling thereby promoting glutamate uptake and decreasing excitotoxic neuronal death. Enhancing astrocytic mitochondrial Ca^{2+} buffering via the mitochondrial Ca^{2+} uniporter could similarly alleviate deficits associated with Ca^{2+} , as promoting this function of mitochondria reduces intracellular levels of Ca^{2+} .

Aside from astrocytic Ca^{2+} , neuronal Ca^{2+} and excitotoxicity represent potential targets of therapy. The results from Chapter 5 implicate excitotoxicity in PD pathogenesis. Excitotoxicity occurs when glutamate stimulation through NMDA receptors results in elevated neuronal Ca^{2+} [45]. Administration of NMDA receptor antagonists counters this effect by diminishing Ca^{2+} entry to slow or prevent the loss of neurons. Indeed, results from Chapter 5 support blocking NMDA receptors as a means to protect neurons from astrocyte dysfunction. It should be noted that NMDA receptor antagonists have been included in L-DOPA treatments and have shown promise in treating dyskinesia but still fail to halt disease progression[46,47]. Utilization of NMDA receptor antagonists, therefore, shows great potential at preventing neurodegeneration in PD, provided that treatment is commenced prior to the appearance of clinical symptoms (discussed below).

Necessity for Preclinical Biomarkers in PD

Treatment of PD generally does not occur until a patient presents with the characteristic motor symptoms observed in PD. At this stage of the disease, a majority of DAergic neurons from the SNpc have been irreversibly lost[48] and treatment can only alleviate the symptoms in the short term[49]. The ideal treatment for PD is one that prevents neuronal loss prior to clinical onset of the disease. As discussed above, this could be achieved by rectifying problems associated with mitochondrial dynamics in astrocytes. Metabotropic receptors and the uniporter are widely expressed by both astrocytes and neurons[50,51], making specifically targeting astrocyte Ca^{2+} difficult. Inhibition of excitotoxicity by blocking NMDA receptors represent a more efficient means of preventing excitotoxic neuron death in PD due to

the fact that astrocytic NMDA receptors are less permeable to Ca^{2+} than those found in neurons[52]. This may confer increased specificity of NMDA receptor antagonists to neurons and could prove effective in preventing neuronal death. Animal studies utilizing NMDA receptor antagonists in experimental preclinical stages of PD have been successful in protecting against loss of DAergic neurons as well as behavioral effects[53,54]. This underscores the potential that targeting excitotoxicity has in protecting against the development of PD, and shows that the most effective therapy will result from treatment prior to neuron loss. Treatment of PD therefore hinges on the development of a preclinical biomarker, as discussed in Chapter 2.

Potential Treatment of Non-motor Symptoms in PD

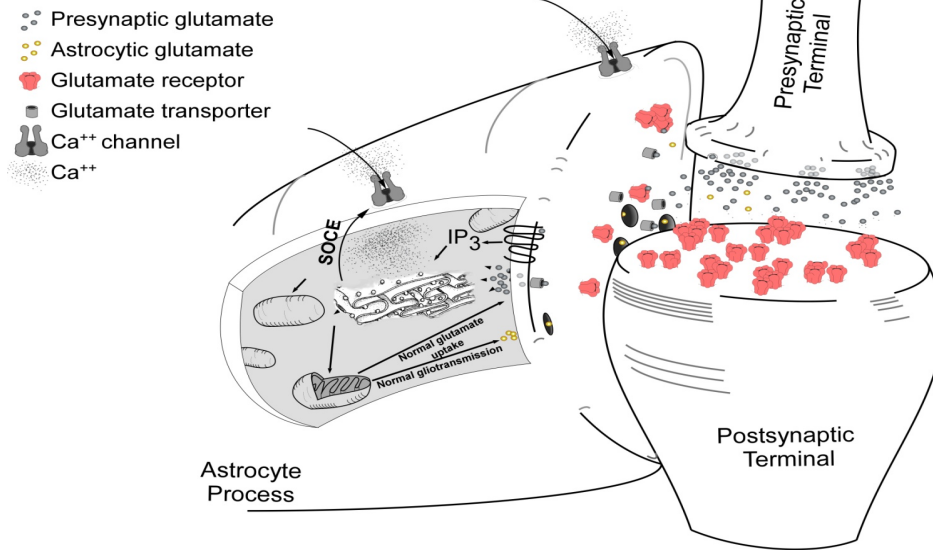
Identification and treatment of patients in the pre-motor stages of PD may not only prevent the onset of clinical symptoms of PD, but could also have the added benefit of alleviating non-motor symptoms. Some symptoms are associated with neurological dysfunction and include depression, impaired olfaction, RBD, as well as changes in cognition related to memory, speech, visual perception, and executive function[48,55-61]. This is not surprising, as α -synuclein lesions have been observed in numerous regions throughout the brain and CNS of PD patients[48,62]. The finding in Chapter 5 that Dlp1 expression is decreased in astrocytes and neurons in the frontal cortex of PD patients indicates that decreased Dlp1, and possibly excitotoxicity, is associated with these symptoms. In addition, the PD cases selected for cortical assessment had no apparent signs of neurodegeneration, while overt signs of cortical degeneration are not apparent until late stages of PD, when Lewy pathology has progressed into this region[48]. Therapeutic intervention to block excitotoxicity prior to the onset of clinical symptoms could similarly alleviate non-motor symptoms. These symptoms are not confined to the CNS as other physiological problems, including constipation and cardiac dysfunction, occur in PD[63,64]. Reports of impaired mitochondrial function in peripheral cells and tissues[65-67] not only supports PD as a systemic disease, but also implicates mitochondrial dysfunction as a potential mechanism responsible for these deficits. Treatment aimed at alleviating mitochondrial affects in the pre-motor phase of PD may also beneficially affect the systemic symptoms associated with PD.

Conclusions and Future Directions

Alterations in mitochondrial dynamics are implicated in PD. This is evidenced by the findings in this thesis that show the Dlp1, a fission promoting protein, is decreased in several cell types and regions in PD patients. In focusing on the effects of this decrease, astrocytes were selected as cells to study as they are implicated in the pathogenesis of PD but their potential contribution towards disease development is poorly understood. Knockdown in astrocytes dramatically affects the morphology, localization, and movement of mitochondria within astrocytes. This impaired astrocyte-mediated protection against glutamate-induced excitotoxicity, which was rescued by the administration of an NMDA receptor antagonist. The mechanism through which this occurs is due to impaired mitochondrial buffering of extracellular Ca^{2+} that enters after glutamate stimulation, resulting in elevated intracellular Ca^{2+} . Mitochondrial dynamics, as well as excitotoxicity, therefore represent targets of therapy to treat PD by mitigating mitochondrial dysfunction. Unfortunately, most treatments that aim to prevent neuronal loss are likely administered at stages in the disease when substantial neuron loss has occurred and interventions may not be effective. The development of biomarkers capable of predicting PD prior to the onset of clinical symptoms will allow for effective intervention to prevent neurodegeneration and halt disease progression. This may also be capable of alleviating non-motor symptoms in PD as they may be caused by similar mechanisms due to altered mitochondrial dynamics or function.

Future studies should aim to elucidate the contribution of excitotoxicity to PD, particularly if it is causative of neurodegeneration or simply contributes to ongoing damage to DAergic neurons. Additionally, other aspects of astrocyte biology could be studied in the setting of decreased Dlp1. Specifically, if changes in Dlp1 in astrocytes affect ROS production, regulation of growth factors, or gliotransmission that could contribute to DAergic neuron loss. Studies looking at how astrocytes from different regions could contribute to DAergic neuron loss would be particularly helpful in this aspect. More importantly, research into mechanisms that target Dlp1 and/or mitochondrial dynamics may represent a means to treat PD. Such strategies include trying to prevent reduction in Dlp1 expression, promoting the action of Dlp1, or identifying the cause of Dlp1 decrease so as to remove humans from exposure to potentially toxic agents. Accomplishing this prior to the onset of clinical symptoms may allow for the prevention of neurodegeneration observed in PD pathogenesis.

Normal synaptic physiology



Decreased astrocytic Dlp1

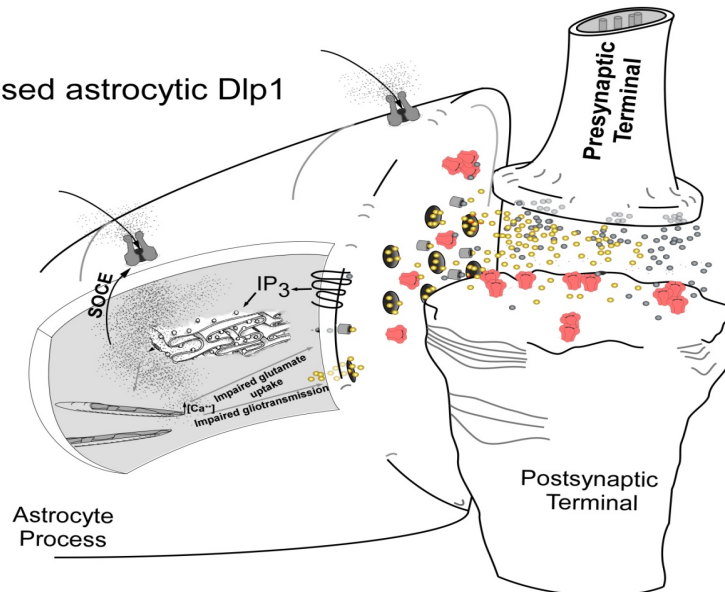


Figure 12. Model of proposed mechanism. (a) During neuronal glutamate release under normal conditions, glutamate binds to its receptor on astrocytes to induce release of Ca²⁺ from the ER, which causes entry of extracellular Ca²⁺. Mitochondria buffer cytoplasmic Ca²⁺ from each source, astrocytic glutamate uptake occurs, and normal neurotransmission proceeds. (b) When Dlp1 is decreased in astrocytes, mitochondria are elongated and mislocalized within astrocytic processes. The binding of glutamate to its receptor on astrocytes during neuronal glutamate release results in Ca²⁺ release from the ER followed by induced entry of extracellular Ca²⁺. However, mitochondria are not adequately positioned to buffer the influx of extracellular Ca²⁺, resulting in elevated levels of intracellular Ca²⁺ that impairs astrocyte-mediated glutamate uptake. Synaptic levels of glutamate are increased as a result, which causes neuronal damage and retraction of the postsynaptic terminal due to excitotoxicity. Astrocytes also release glutamate in a Ca²⁺ dependent manner in a process called gliotransmission. Elevated intracellular Ca²⁺ that results from decreased Dlp1 could promote excessive glutamate release and contribute to increased synaptic glutamate and excitotoxicity.

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