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ABSTRACT

Mutations in *leucine-rich repeat kinase 2 (LRRK2)* are the most common known cause of inherited Parkinson's disease (PD), and *LRRK2* is a risk factor for idiopathic PD. How LRRK2 function is regulated is not well understood. Recently, the highly-conserved 14-3-3 proteins, which play a key role in many cellular functions including cell death, have been shown to interact with LRRK2. In this study, we investigated whether 14-3-3s can regulate mutant LRRK2-induced neurite shortening and kinase activity. In the presence of 14-3-3θ overexpression, neurite length of primary neurons from BAC transgenic G2019S-LRRK2 mice returned back to wildtype levels. Similarly, 14-3-3θ overexpression reversed neurite shortening in neuronal cultures from BAC transgenic R1441G-LRRK2 mice. Conversely, inhibition of 14-3-3s by the pan-14-3-3 inhibitor difopein or dominant negative 14-3-3θ further reduced neurite length in G2019S-LRRK2 cultures. Since G2019S-LRRK2 toxicity is likely mediated through increased kinase activity, we examined 14-3-3θ's effects on LRRK2 kinase activity. 14-3-3θ overexpression reduced the kinase activity of G2019S-LRRK2, while difopein promoted the kinase activity of G2019S-LRRK2. The ability of 14-3-3θ to reduce LRRK2 kinase activity required direct binding of 14-3-3θ with LRRK2. The potentiation of neurite shortening by difopein in G2019S-LRRK2 neurons was reversed by LRRK2 kinase inhibitors. Taken together, we conclude that 14-3-3θ can regulate LRRK2 and reduce the toxicity of mutant LRRK2 through a reduction of kinase activity.

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Introduction

Parkinson's Disease

Epidemiology of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, behind Alzheimer's disease (AD) [1]. It primarily affects older adults and is predicted to increase in prevalence as the human population ages [2]. One million Americans are affected by PD with around 60,000 new cases being diagnosed each year. Worldwide, an estimated 7 to 10 million people are thought to be affected [3]. The Parkinson's Disease Foundation estimates the total direct and indirect (via lost work days) economic impact to be $25 billion in the US alone [3]. This highlights the importance of uncovering treatments to slow disease progression as none currently exist.

PD was first characterized in 1817 by Dr. James Parkinson in "An Essay On Shaking Palsy", although it wasn't officially termed Parkinson's disease until the late 1880s when Jean-Martin Charcot pushed to honor the late Dr. Parkinson for his work in elucidating and describing the disease [4]. PD is a progressive disorder that affects both motor and non-motor function in patients suffering from disease. Roughly 85-90% of PD patients have what is termed idiopathic or sporadic PD, meaning the cause of disease is unknown, while the remaining cases have a genetic cause [5, 6]. Age is the most potent risk for PD as the average age of onset is ~60 years of age [1]; however a small subset (about 5-10%) are deemed early onset with manifestation of symptoms before the age of 40 [7].

Symptoms

Motor symptoms are the traditional clinical criteria used for diagnosing PD. The four cardinal motor symptoms include rigidity, postural instability, bradykinesia (slowness of movement) and tremor [8]. The most noticeable symptom is the "pill rolling" rest tremor that presents in over 70% of patients [9]. This tremor occurs when the particular limb is at rest. Bradykinesia manifests as slowness of movement and reduced dexterity, while problems such as difficulty rising from a seated position and shuffling gait, fall under the category of postural instability. As the disease progresses, patients will display more noticeable issues maintaining balance with an increased likelihood to fall, leading to higher risk of injury. In addition to these classic "cardinal" symptoms, there are other motor manifestations such as dystonia, speech impairment, hypomimia (masked facial expression), decreased eye blink rate, micrographia (tiny writing), hypophonia (increasingly soft voice) and difficulty turning in bed [10-14].

Almost 90% of patients experience non-motor symptoms as well [15]. These can range from psychiatric problems, such as anxiety and depression, to cognitive impairment to autonomic dysfunction, including constipation, orthostatic hypotension and urinary incontinence. Anxiety is one of the more common non-motor symptoms with up to one third of patients suffering from anxiety [16, 17]. Patients with PD are also at a six-fold increased risk for developing dementia [18]. Dementia typically manifests as changes in concentration and judgment, speech and language difficulty, hallucinations, delusions or paranoia, depression, irritability and potentially even sleep disturbances [19]. Interestingly, the first symptoms that can manifest for PD patients are anosmia (loss of sense of smell) and constipation [20]. However, the most common non-motor symptom

appears to be sleep disturbances as almost 98% of patients deal with some form of sleep disturbance [21]. This includes restless leg syndrome (RLS), periodic limb movements of sleep (PLMS), REM sleep behavior disorder (RBD), insomnia, daytime sleepiness, and vivid dreaming [22]. Some sleep related movement disorders associated with PD, such as RLS and PLMS, have been shown to be responsive to dopamine replacement therapy, yet many other sleep issues like insomnia and vivid dreaming are not. Additionally, dopamine replacement therapy can cause excessive daytime sleepiness [22].

Available Treatments for Motor Symptoms

Standard treatment for PD patients focuses on the use of either dopamine replacement therapies or inhibitors preventing dopamine breakdown at the synaptic junction. The gold standard for dopamine replacement therapy is use of levodopa (L-DOPA) to replace the decrease in dopamine production caused by neuronal loss in the substantia nigra. L-DOPA is a dopamine precursor that crosses the blood-brain barrier and is converted into dopamine by the enzyme, DOPA decarboxylase. This treatment was discovered in the late 1960s [23] and continues to be the best option available to combat the motor symptoms of PD. While beneficial in reducing severity of motor symptoms, over time patients can develop complications such as dyskinesias and motor fluctuations, such as the "wearing-off" phenomenon and "on-off" fluctuations during treatment [24]. Combining the fact that L-DOPA, a drug that has been the standard treatment for PD for over 40 years and has a notable side effect profile, with the fact that no currently available drug slows the progression of disease highlights the need for improved treatments for PD patients.

Surgical therapies do exist for PD patients who are experiencing complications of L-DOPA treatment over time. Deep brain stimulation (DBS) is the most frequently used surgical intervention [25]. This is done by implantation of a device called an implantable pulse generator (IPG) into either the thalamus, the globus pallidus or the subthalamic nucleus. The electrode then provides high frequency electrical stimulation. One hypothesis is that this stimulation blocks abnormal nerve signals that cause PD symptoms. Only patients whose symptoms cannot be controlled adequately with medications are considered for IPG implantation. Additionally, only patients who showed some improvement while taking medications for PD will see improvement with DBS. If effective, however, DBS can help reduce bradykinesia, stiffness and tremors. It can also allow for lower drug treatments on patients which leads to reduced dyskinesias associated with drug treatment [26].

Available Treatments for Non-motor Symptoms

Beyond treatment for the cardinal motor symptoms of PD, there are emerging treatments to deal with autonomic dysfunction associated with PD. Autonomic dysfunction refers to problems in the autonomic nervous system (ANS), including issues such as orthostatic hypotension, sexual and urinary dysfunction and constipation. These are important symptoms to treat as well since ANS dysfunction affects 60-70% of PD patients and causes significant discomfort and increased morbidity [27-29]. In fact, a survey of 163 patients showed that difficulties with balance, sleep disturbance, cognitive impairment and drooling were rated as the most disabling symptoms [30]. This highlights the impact of non-motor symptoms that do not respond to levodopa as being significantly disabling to patients.

There are available treatments to manage many of these issues. In orthostatic hypotension, the first line drugs to treat patients are midodrine, fludrocortisone and droxidopa [31, 32]. Midodrine is an α1-receptor agonist that acts on alpha adrenergic receptors in the blood stream to increase vascular tone and subsequently increase blood pressure. Fludrocortisone causes fluid retention in the body which increases total blood volume and therefore increases blood pressure [31]. Droxidopa works by increasing levels of norepinephrine in the peripheral nervous system (PNS), which enables the body to maintain blood flow upon standing, thus preventing a sudden drop in blood pressure when one stands up from a seated position [32]. Additionally, patients are advised to avoid external stimuli such as large meals, hot baths, alcohol and sudden postural changes to prevent sudden drops in blood pressure that may cause fainting.

Bladder dysfunction occurs in up to 71% of PD patients [33, 34], making it one of the more common autonomic disturbances. Symptoms can range from frequency, urgency, decreased urinary stream, intermittent stream, having to strain to void, or incomplete emptying [27]. These can be caused by dysfunction of the internal urethral sphincter, which needs to relax to allow urine to exit. Muscarinic receptor antagonists such as tolterodine or oxybutynin have shown varying degree of success in treating urinary incontinence [35].

Sexual dysfunction is also a common problem in PD patients although it can be hard to distinguish between PD related sexual dysfunction and dysfunction caused by advanced age or chronic illness perhaps. For example, one study showed that 60% of men with PD have symptoms of sexual dysfunction compared with 37% in the general population [36]. Symptoms of sexual dysfunction include sexual dissatisfaction, erectile

dysfunction, impaired orgasm and in women, diminished vaginal lubrication. The only medicinal intervention is the use of sildenafil to treat erectile dysfunction; however, adjuvant therapies can be used to improve satisfaction between partners.

Constipation is one of the most frequent autonomic disturbances in PD, occurring in 76% of patients [37]. This is thought to be caused by decreased motility in the gastrointestinal (GI) system and in fact does correlate with disease severity and duration [38]. Treatment consists of altering one's diet to include more fiber, stool softeners, and the use of laxatives.

Notably, there are many non-pharmacological approaches including exercise, speech therapy and nutrition to lessen the impact of progressing motor symptoms. For example, exercise regimens have been shown to improve flexibility and strength in patients as well as improve cognition [39-46]. Simple education about Parkinson's disease has helped to decrease fear and anxiety in newly diagnosed patients [47]. Proactive dietary changes and proper nutrition can offset weight loss and help to avoid specific PD related issues such as constipation. However, while these non-pharmacological approaches can help to reduce the severity of disease in the short term, these are also only temporary solutions that target the symptoms instead of the underlying cause of disease.

Prognosis

PD progresses rather aggressively in the first years of disease with multiple placebo-controlled studies showing a 30-40% decrease in unified Parkinson's disease rating scale (UPDRS) over the first 18 months after diagnosis [48]. However, it is

difficult to predict the exact course that the disease will manifest as the severity of individual symptoms varies from person to person. It is important to note that as age progresses, so does disease progression. A 20 year PD study showed that mortality rates for PD patients continued to rise faster than the normal population as age increased and that this was attributed to issues that do not respond to current treatments, such as dementia, dysphagia– leading to increased risk for aspiration pneumonia, falls and fractures resulting from falls [49]. In addition, the same 20 year study showed that dementia occurred in most of the patients in the study, with 75% of patients in the study being diagnosed with dementia [49]. However, many patients who were not diagnosed with dementia died early in the course of disease or did not have a psychological evaluation performed within the last year of their lives, leaving their mental status at the time of death unknown. These two caveats suggest that the rate of dementia in the test population would have been higher if patients had survived further into the study [49].

Different subtypes have been used to attempt to classify patients to help determine progression with mixed results and debate about the specific criteria for each subtype [50]. It is the heterogeneity of symptoms in PD that make determining disease prognosis difficult. For example, one study suggested that older age of onset and the presence of rigidity/hypokinesia or postural instability and gait dysfunction (PIGD) led to a more rapid rate of motor progression and early cognitive decline, whereas the presence of tremor as an initial symptom predicts a slower progression and a longer response to levodopa therapy [51]. In contrast, two studies challenge this theory by proposing that tremor and PIGD dominant subtypes were not distinct subtypes but rather were just different stages of disease as PIGD is less responsive to dopamine therapy than tremors,

in agreement with the data that the cardinal motor features of PD progress at different rates and show variable response to L-DOPA treatment [52, 53].

In summary, because of the variability in disease progression from patient to patient, it remains difficult in giving the prognosis to an individual PD patient in terms of how quickly the disease will progress and which symptoms will become the most severe. Generally speaking, however, mortality rates increase for PD patients, which is attributed to the likelihood of older patients to be more prone to falling, causing fractures and potential serious injury if the fall is from a significant height. Additionally, aspiration pneumonia occurs more frequently in PD populations due to increase in dysphagia, in fact 10 times more often in PD patients than in the general population [54]. In elderly patients, pneumonia can be more challenging to successfully treat, leading to poorer outcomes. Indeed, research has shown that death caused by pneumonia is increased in PD patients over the general population, 20% to 9% respectively [55]. Most patients with PD will develop dementia. Indeed, the Sydney multicenter 20 year study showed that 25 of the 30 remaining PD patients that survived 20 years were diagnosed with dementia, with 2 more developing dementia after the end of the study [49]. In short, available treatments can lessen the severity of disease, yet PD will continue to progress and symptoms will continually worsen over time, further highlighting the need to understand the mechanism of disease to develop better treatments for PD patients.

Cellular Pathology of Parkinson's Disease

From a pathological perspective, PD is marked by the loss of dopaminergic neurons in the substantia nigra pars compacta region of the brain [56]. By the time motor

symptoms of PD begin to occur, 60-70% of the neurons in the substantia nigra have already been lost [57]. Loss of neurons prior to onset of clinically diagnosable symptoms makes treating PD even more complicated. It is important to note that while loss of dopaminergic cells is the hallmark of disease, PD is not localized to just the substantia nigra. Braak and colleagues suggest that Lewy body pathology begins in either the olfactory bulb, or from the enteric nervous system in the gut and that this passes into the CNS through the vagus nerve [58]. Indeed, early symptoms of PD include anosmia (loss of sense of smell) and constipation [59]. The staging system developed by Braak and colleagues (Braak staging or Braak hypothesis) correlates progression of disease with alpha-synuclein pathology in post-mortem brains. For example, the Braak model shows the disease to commence in the lower brainstem and the anterior olfactory structures [60], perhaps explaining why constipation and anosmia are the first symptoms to appear in PD patients. From there, the disease progresses rostrally through the medulla and midbrain to the basal forebrain before eventually making its way to the cerebral cortex [60]. In agreement, as the Lewy pathology increases in severity throughout the brain, there is a correlative increase in clinical severity as well, with the location of Lewy pathology also correlating with the manifestation of symptoms associated with that brain region [61].

The hallmark pathology seen in PD patients is the formation of intracellular inclusions termed Lewy bodies. These Lewy bodies are comprised mainly of the protein alpha-synuclein, which plays a role in synaptic plasticity and learning [62], in supplying synaptic vesicles to presynaptic terminals [63], in regulating vesicle trafficking and refilling [64] and in regulating the phosphorylation, and thus the action, of tyrosine hydroxylase [65, 66]. While the exact biological consequence of Lewy body formation is

unknown, ubiquitin is also found in Lewy bodies with alpha-synuclein, suggesting a failure of the ubiquitin-protease system that breaks down proteins targeted for degradation [67].

Causes and Risk Factors

Genetic

While PD is largely a sporadic disease with ~85% of cases being of unknown etiology, mutations in several genes have been identified as being causal to PD. The most common genes associated with PD include SNCA (alpha-synuclein) [68] and LRRK2 [69, 70], which are both inherited in an autosomal dominant manner, as well as PINK1 [71], DJ-1 [72] and Parkin [73], which are autosomal recessively inherited [74]. Parkin is part of the E3 ubiquitin ligase complex in the ubiquitin-protease system, suggesting it plays a role in helping to ubiquitinate proteins targeted for degradation [75]. Parkin has also been shown recently to play a role in mitophagy, which is the degradation of mitochondria through autophagy. Parkin is recruited to the mitochondrial surface by PINK1 under conditions inducing mitochondrial membrane depolarization [76-78]. Stabilized by PINK1, parkin can ubiquitinate mitochondrial proteins and induce autophagic clearance of mitochondria [79]. DJ-1 is normally localized to the outer mitochondrial membrane where it protects mitochondria from oxidative stress [80-82], implicating a role in maintaining mitochondrial homeostasis as a convergence of function between multiple genes associated with PD. However, despite the protective roles these three proteins play, parkin/DJ-1/PINK1 triple-knockout mice failed to develop nigral

neurodegeneration [83]. This finding is interesting given the protective functions of the endogenous proteins.

Environmental Factors

There have also been many environmental factors suggested to be risk factors for PD. The biggest risk factor for developing the disease is age as the incidence of PD is 17.4 in 100,000 in people 50-59 years of age and this increases to 93.1 in 100,000 in people 70-79 years of age [84]. Additionally, head trauma, as seen with boxers such as Muhammed Ali, is also a risk factor for developing parkinsonism [85]. Exposure to the pesticides paraquat [86] and rotenone [87] has been linked to causing PD. In addition, there is a gender bias with men being more likely to develop PD [84]. Other environmental and occupational risk factors, such as rural living, drinking well water or exposure to heavy metals, such as iron, manganese, copper lead, amalgam, aluminum or zinc, have been shown to increase the incidence of PD [88]. Interestingly, tobacco smokers and coffee drinkers (for caffeine) have been shown to have decreased risk of developing PD [89-93], perhaps through acting on the nicotinic cholinergic system in the brain in the case of smoking [94]. Interestingly, recent research has shown that the ease by which smoker could quit smoking correlated with PD. For example, Ritz et al. showed that fewer patients with PD over controls had ever established a smoking habit and that those who were more easily able to quit smoking were more likely to develop PD [95]. They suggest this is because of a decreased responsiveness to nicotine during the prodromal phase of PD [95]. Despite multiple environmental risks being identified, PD still remains largely idiopathic.

One of the challenges in treating PD is that the onset of motor symptoms, which are used for disease diagnosis, do not manifest until about 70% of the dopaminergic cells of the substantia nigra pars compacta (SNc) have been destroyed [96]. This increases the difficulty in treating PD patients as major damage has already occurred prior to the onset of symptoms. However, certain nonmotor symptoms, such as anosmia, sleep abnormalities, constipation, depression and pain, may manifest early in the course of disease [97, 98], and could serve as premotor features that may predict that a person is more likely to develop motor deficits later on [99]. Determining the underlying cause for why this specific cell type is vulnerable in disease has been difficult as well. It is thought that perhaps reactive species from dopamine metabolism lead to increased oxidative stress and mitochondrial dysfunction [100-102]. Interestingly, dopaminergic neurons show abnormalities in complex 1 of the mitochondria, which also leads to alpha-synuclein aggregation and increased cell death. Complex 1 inhibition is also the mechanism of action of rotenone and 1-methyl-4-phenylpyridinium (MPP+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), that causes dopaminergic specific cell death. Notably, there is some evidence suggesting that dopaminergic neurons have a reduced capacity for calcium buffering [103-105], which alters the amount of intracellular calcium, eventually leading to cell death. However, while no definitive reason for SNpc specific depletion has been identified, there is indirect evidence pointing to increased oxidative stress and mitochondrial dysfunction as being likely culprits in causing dopaminergic cell death in PD patients.

Mechanisms of neurodegeneration

Based on the known causes of PD, different models have been developed in the hopes of elucidating potential mechanisms in disease pathogenesis. For example, the use of neurotoxins such as MPTP and rotenone has shown mitochondrial dysfunction to be a potential pathway in neuronal cell death [106-110]. MPTP and rotenone are mitochondrial complex 1 inhibitors that selectively kill dopaminergic cells in the substantia nigra. Primates administered with MPTP developed many of the clinical and pathological features of PD [106, 107], and mice were shown to have depleted dopamine production in the substantia nigra [108]. In agreement with the idea of mitochondrial dysfunctional, mutations in Parkin and PINK1 cause PD [71, 111-113]. Parkin and PINK1 are proteins that regulate mitophagy, the process by which dysfunctional mitochondria are broken down and recycled by the cell. Mutations in these proteins lead to problems with mitophagy that prevent the proper breakdown of damaged mitochondria. These mutations lead to morphologically aberrant and functionally impaired mitochondria [114-116]. In addition, mutations in alpha-synuclein and LRRK2 have been shown to cause mitochondrial dysfunction as well; implying mitochondrial damage may be a point of convergence in the pathogenesis of disease [117-120].

In addition to mitochondrial dysfunction, the formation of Lewy bodies is considered to be the pathological hallmark of PD. Misfolding of the protein alpha-synuclein occurs in a large percentage of PD patients and the location of Lewy pathology correlates with disease [60, 61]. Indeed, Braak et al. have described a staging system for PD that details the procession of Lewy bodies formation throughout the brain [60]. More recently, alpha-synuclein has shown to exhibit prion-like properties. This was found

during a study where healthy neurons were transplanted into the brains of PD patients and these transplants eventually developed Lewy bodies [121-123]. Further research into the mechanism of alpha-synuclein uptake has shown that alpha-synuclein can enter the cell through endocytosis and/or propagate through adjacent cells [124, 125]. Indeed, aggregated transplanted alpha-synuclein can cause endogenous alpha-synuclein to misfold, creating Lewy bodies in otherwise healthy cells [125]. This is potentially how Lewy pathology progresses throughout the brain of PD patients, with alpha-synuclein aggregation being transmitted from cell to cell, inducing protein misfolding in each newly infected cell.

Alpha-synuclein aggregation has also been shown to compromise autophagy. Inhibiting macroautophagy with bafilomycin has been shown to cause accumulation of mutant alpha-synuclein [126]. Conversely, using rapamycin to stimulate autophagy has shown increased clearance of both wildtype and mutant alpha-synuclein, thus implicating autophagic dysfunction in the pathogenesis of alpha-synuclein derived disease [126]. Notably, PD brains show abnormal expression of lysosomal proteins in the substantia nigra [127]. Specifically, alpha-synuclein binds to LAMP-2A receptors on lysosomes, blocking these receptors and inhibiting chaperone-mediated autophagy (CMA) [128]. Thus, alpha-synuclein can further accumulate and induce oxidative stress within the cells by preventing the clearance of misfolded proteins targeted for degradation, leading to mitochondrial dysfunction and eventual cell death [129].

There is also mounting evidence suggesting neuroinflammation plays a role in PD pathogenesis [130]. Microglial activation occurs in the substantia nigra of patients with PD and is also seen in animal models that have been treated with MPTP [130]. A recent

study showed that people who took ibuprofen had a decreased risk of developing PD [131]. Interestingly, this did not expand to aspirin, acetaminophen or any other non-steroidal anti-inflammatory (NSAID) indicating an ibuprofen specific pathway was conferring protection [131]. However, the mechanism that triggers neuroinflammation is up for debate. One hypothesis is that neuromelanin, a byproduct of dopamine synthesis, is released by dying neurons in the substantia nigra and is taken up by glial cells, leading to their activation [132, 133]. This leads to the upregulation of NF-κB and p38 mitogen-activated protein kinase (MAPK) inflammation signaling pathways [132, 133]. Alternatively, CD8+ and CD4+ T cell infiltrates are found in the brains of PD patients, suggesting the presence of some endogenous pathological antigen [134]. The antigen suggested to activate this adaptive immune response is an epitope of oxidatively-modified alpha-synuclein [134], which is in agreement with data showing an increase in neuroinflammation in the brains of alpha-synuclein overexpressing mice [135]. Despite the occurrence of neuroinflammation in PD patients, it appears that while inflammation certainly contributes to the progression of PD, it may still be secondary to whatever the primary insult causing disease may be.

Despite the proposed pathways leading to neurodegeneration, one still as of now unanswered question is why this specific region of the brain is vulnerable to degeneration. Initially, it was thought that oxidation of dopamine increased cellular stress through increased production of free radicals [136, 137]. However, L-DOPA treatment has been shown to slow the rate of clinical PD progression and this model does not explain why dopaminergic neurons outside of the substantia nigra are largely spared [138-141]. Another hypothesis is that the rapid, spontaneous firing of these neurons

leads to problems buffering the constant influx of calcium, leading to increased mitochondrial damage [142]. Indeed, the use of dihydropyridines, commonly used antihypertensives that antagonize L-type calcium channels seem to exhibit a protective effect against PD [143, 144]. Despite the potential mechanisms that lead to nigral specific degeneration, no specific pathway has been confirmed to this point.

In summary, PD remains a large challenge to the medical field. From the increasing patient numbers as the population continues to age to the lack of effective treatments to slow progression of disease, PD remains one of the largest problems yet to be solved. While PD itself does not directly cause death, it is associated with increased morbidity and mortality. What is needed now is better insight into the cause and progression of disease as well as research into ways to halt disease progression and means to replace the extensive cell loss that has already occurred by the time PD is diagnosed. Through this, we will hopefully be able to find better, disease-modifying treatments to help patients stricken with PD in the near future.

LRRK2 – The Most Common Genetic Cause of PD

Protein Structure and Domains

Leucine rich repeat kinase 2 (LRRK2) was first identified as causal to PD in a study of Japanese families back in 2002. Soon thereafter, the locus, first termed *PARK8*, has been reported in families from North America of European descent [69, 70, 145]. Since then, research into the biology of LRRK2 has amplified as its role in PD has become more important. A 2008 study showed that mutations in LRRK2 account for

around 4% of familial cases and 1-2% of sporadic cases [146]. This is increased greatly in certain populations such as North African Arabs and Ashkenazi Jews that can have anywhere from 20-40% of the population carrying this gene [146, 147]. It is worth noting that the LRRK2 gene has incomplete penetrance as many individuals have survived to old age without any clinical signs of PD [148]. More recent studies have identified single nucleotide polymorphisms (SNPs) that are risk factors for disease, further increasing the importance of elucidating mechanisms of LRRK2 mediated toxicity in PD [149-151].

LRRK2 is a rather large 286 kDa protein comprised of 2527 amino acids [152]. Its name derives from the large leucine rich repeat domain in the central region of the protein. LRRK2 is a member of the ROCO family of proteins which are characterized by a Ras of complex (ROC) and a C-terminal of ROC (COR) domain [152, 153]. This domain is primarily responsible for the binding and hydrolysis of GTP. In addition, on the C-terminal side of the COR domain is an active kinase domain, but the true physiological target of this domain has yet to be elucidated [154].

Endogenous LRRK2 Function

The true function of LRRK2 is not well understood, although work looking into the normal distribution of LRRK2 in the cell has provided some clue as to its normal function. LRRK2 is normally localized to vesicular structures such as autophagic vacuoles, multivesicular bodies and areas of endocytic uptake at the plasma membrane [155-157]. There is also some evidence that LRRK2 interacts with cytoskeletal proteins such as tubulin and actin [158-160], and that this interaction requires intact kinase

function and is enhanced in LRRK2 mutations [158]. This would suggest a role in anchoring cellular components to the cytoskeleton, or perhaps in maintaining/building the microtubules that make up the cytoskeleton or even in influencing neurite outgrowth. Taken together, it is also possible that LRRK2 plays a role in vesicle sorting and transport. Indeed, it has been shown that *lrrk* knockout in *Drosophila* causes alterations in the positioning of lysosomes within the cytosol [161]. In addition, LRRK2 may play a regulatory role in autophagy. Inhibition or knockout *in vitro* has been shown to enhance autophagy while mutations in LRRK2 suppress autophagy [162, 163]. In LRRK2 knockout mice, there is a notably impairment in autophagy [164-166], strengthening the *in vivo* role of LRRK2 in regulating vesicular dynamics.

Additionally, the GTPase activating protein ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1) has been shown to interact with and enhance both LRRK2 GTPase and kinase activity [167], providing evidence of the interplay between the GTPase and kinase domains in LRRK2. It is within the ROC/COR and kinase domains that a majority of the pathogenic mutations that cause PD have been identified. For example, the G2019S missense mutation, which is the most common mutation of LRRK2 found in PD patients, is found in the kinase region of the protein. This mutation can account for anywhere from 2.8-6.6% of familial cases [168].

LRRK2 Kinase and GTPase activity

Once the sequence and catalytic regions of LRRK2 had been identified, much research has delved into measuring the kinase and GTPase activities of the protein. Understandably, it was thought that mutations in LRRK2 may have a consistent effect in

altering the protein's activity. Early studies did indeed show that the G2019S mutation in particular had a twofold to fourfold increase in kinase activity over wildtype LRRK2 [169, 170]. However, these studies also showed that other LRRK2 mutations only had a modest increase or no effect on kinase activity at all [170]. However, the prevailing idea still remains that enhanced LRRK2 kinase activity is required for toxicity [171-173]. This is supported by the data showing that mutant LRRK2 induced neurite shortening can be reversed by LRRK2 kinase inhibition [174, 175].

Work looking into the effects on the ROC-COR GTPase domain of LRRK2 has pointed to a role for GTPase activity in disease. Multiple studies have confirmed that LRRK2 does in fact bind GTP [153] and that pathogenic mutations in LRRK2 do not alter the binding affinity of LRRK2 to GTP [176-178]. However, mutations in the ROC-COR domain have been shown to have slower hydrolysis of GTP to GDP [167, 176, 177, 179, 180]. These data support the idea of enhanced protein activity leading to toxicity, as LRRK2 is in a more active conformation when bound to GTP, thus leaving the protein in an active conformation for a longer period of time before it is able to hydrolyze GTP to GDP and deactivate [181].

It is important to note that some studies implicate the dimerization of LRRK2 to control GTPase activity of the protein [182]. The dimerization motif of LRRK2 has been shown to be in the COR domain, suggesting that mutations in the COR domain of the protein may affect LRRK2 dimerization and therefore would explain the diminishing GTPase activity of mutant LRRK2 [183]. However, it is difficult to measure GTPase activity *in vitro*, as many proteins need to be overexpressed (i.e. not at physiological

levels) to see changes in activity, thus bringing into question how actively LRRK2 can turnover GTP to GDP without the addition of other proteins above endogenous levels.

One other potential idea is the interplay between the GTPase and kinase domains. The thought is that if small GTPases can regulate other kinases (like Ras and Raf), then perhaps GTP binding to LRRK2 can influence the kinase activity of itself [178, 184]. However, multiple studies have shown that while mutations that reduce LRRK2 binding to GTP have a negative effect on kinase activity, using a non-hydrolyzable GTP homologue (to keep LRRK2 in its "active" GTP bound state for longer) did not increase kinase activity as expected [185, 186]. While nothing definitive has been shown connecting the activity of the two domains within LRRK2, it is worth noting that both kinase activity and GTPase activity are required to incur toxicity as inhibiting one region or the other can reduce toxicity [167, 171-173, 187].

In short, there does not seem to be a clear consensus about the exact mechanism of toxicity in LRRK2 mutations. The majority of data out there seems to suggest that mutations in LRRK2 enhance overall function. However, because it is still unknown exactly what LRRK2 does at the cellular level, determining the effects of LRRK2 mutations *in vivo* is challenging. My work here describes the biochemical changes between LRRK2 and a recently identified set of interactors, the 14-3-3s, in hopes of finding ways to modify LRRK2 to ultimately affect the toxicity of LRRK2 mutations.

The 14-3-3 Protein family

14-3-3s were first identified during a characterization study of brain proteins back in 1967 [188]. Their name derives from the elution fraction following DEAE-cellulose chromatography and their migration position after starch gel electrophoresis. At the time, not much was noted about these proteins other than their size at ~30kDa. However, since their discovery, much has been elucidated about this family of proteins that makes them one of the more important brain proteins in mammals.

14-3-3s are highly conserved across species and are ubiquitously expressed in many organs, although they are highly expressed in the brain and make up ~1% of all brain protein. In mammals there are seven identified isoforms – β, γ, ε, σ, ζ, θ and η [189]. Plants can have up to 15 isoforms, while only two isoforms have been identified in *Drosophila* and *C. elegans* [190, 191]. 14-3-3s typically form dimers, either homo- or heterodimers that can interact with a large number of proteins [192, 193]. To this point, over 200 different interacting partners have been identified, including kinases, enzymes, cytoskeletal proteins, proteins involved in cell cycle control, proteins involved in regulating transcription and proteins that control apoptosis [194-196]. Since they can bind so many different proteins and can bind multiple ligands simultaneously, acting as a scaffold to facilitate protein interactions, it is understandable that 14-3-3s play a role in so many cellular processes.

The Many Interactors of 14-3-3s

14-3-3s commonly bind to substrate through phosphoserine/threonine motifs [197, 198]. There are a couple notable exceptions such as exoenzyme S and

p190RhoGEF that do not need a phosphorylated residue to interact with 14-3-3s [199-203]. Regardless, structural analysis of 14-3-3s has shown a conserved amphipathic binding groove that binds ligands [197, 198, 204-206]. Since each 14-3-3 contains a binding site, this allows for dimerized 14-3-3 to bind two separate proteins and bring them together to interact [197, 198, 205]. They may also bind to multiple sites on a single protein to affect its conformation and/or phosphorylation state. Additionally, 14-3-3s can regulate intracellular localization of certain proteins by binding and thus exposing or hiding targeting sequences on the protein [207]. For example, 14-3-3s bind to the pro-apoptotic proteins Bad and Bax and prevent them from shuttling to the mitochondria where they can induce caspase mediated apoptosis [208, 209]. 14-3-3s also bind multiple transcription factors such as Cdc25 and the forkhead transcription factors and keep them out of the nucleus, sequestered in the cytoplasm [210, 211]. There are numerous other proteins that are controlled by 14-3-3s in the same manner including the plasma membrane proteins Raf-1 [212], Rin1 [213], KSR1 [214] and G-protein regulators RGS3 [215] and RGS7 [216].

The major role for 14-3-3s in neurons is to promote cell survival. As mentioned previously, 14-3-3s play an anti-apoptotic role in the inhibition of Bad and Bax [208, 209]. In addition, 14-3-3s regulate transcription factors that promote cell cycle re-entry and subsequent apoptosis in neurons by preventing transcription factors like Cdc25 [217], SRPK2 [218] and FOXO1 [219] from translocating to the nucleus from the cytosol. Specific to dopaminergic neurons, 14-3-3s interact with phosphorylated tyrosine hydroxylase (TH) to enhance its activity in producing dopamine [220-222]. Interestingly, alpha-synuclein has the opposite effect by binding dephosphorylated TH and reducing

activity [65]. It is thought that 14-3-3 binding to alpha-synuclein can sequester 14-3-3s to prevent 14-3-3s from binding pro-apoptotic factors to promote cell survival and thus lead to an increase in cell death [223]. Indeed, there is an increase in 14-3-3/alpha-synuclein complex formation in the substantia nigra of PD patients, [136].

14-3-3s in Neurodegenerative Disease

From a pathological standpoint, 14-3-3s have been linked to multiple diseases. The presence of 14-3-3s in the cerebral spinal fluid (CSF) of patients with Creutzfeldt-Jakob disease (CJD) was attributed to high levels of 14-3-3s being released from dying neurons as 14-3-3s do not normally appear in CSF [224, 225]. In AD, 14-3-3s have been found to be part of neurofibrillary tangles (NFTs) [226]. Indeed, 14-3-3s do interact with tau protein and it is thought that 14-3-3s might facilitate tau phosphorylation by the kinase GSK3β [227, 228]. Studies suggest that 14-3-3s bind to tau and alter its conformation, making it more accessible to kinases for phosphorylation, and that 14-3-3s can protect the phosphorylated tau from being dephosphorylated, leading to hyperphosphorylated tau and the formation of NFTs [228, 229]. In spinocerebellar ataxia type 1 (SCA1), a polyglutamate disease, mutant ataxin-1 has a higher binding affinity for 14-3-3s, correlating with the number of polyglutamine repeats the mutant protein contains [230]. When combined in *Drosophila*, both 14-3-3 and mutant ataxin-1 lead to higher levels of neurodegeneration as opposed to mutant ataxin-1 alone, suggesting 14-3-3 binding to mutant ataxin-1 potentially protects the protein from dephosphorylation or even degradation [230]. To further cloud the situation, a mouse model of SCA1 exhibits either protection or increased cell death in different regions of the brain with 14-3-3ε overexpression [231]. In Huntington's disease (HD), 14-3-3s have been shown to

colocalize with mutant huntingtin when it aggregates [232], which could lead to an increase in apoptosis the same way 14-3-3s are sequestered by alpha-synuclein in Lewy bodies in PD. Additionally, 14-3-3s interact with Huntington's disease binding protein 2 (HDBP2) and shuttle it from the cytosol to the nucleus where it regulates transcription of huntingtin [233, 234].

14-3-3s in Parkinson's Disease

In PD, 14-3-3s have been shown to associate with multiple pathogenic proteins identified as causing PD. Notably, 14-3-3s have been shown to colocalize in Lewy bodies with alpha-synuclein [235, 236] and there is increased coimmunoprecipitation of 14-3-3s with alpha-synuclein in brains of PD patients [75]. This suggests that 14-3-3s are being sequestered in Lewy bodies and are unable to perform their natural anti-apoptotic role in the cell [223, 237]. Previous research in this lab has shown downregulation of the 14-3-3ε, γ and θ isoforms at the mRNA level of an alpha-synuclein overexpression mouse model [237, 238]. Conversely, when any of these three isoforms were overexpressed, there was reduced alpha-synuclein aggregation. Overexpression of human14-3-3θ or the *C. elegans* 14-3-3 homolog reduces dopaminergic neuronal loss in a *C. elegans* alpha-synuclein overexpression model [237]. 14-3-3ε, γ and θ overexpression were also protective against cell loss from rotenone or (MPP+) treatment [237]. 14-3-3 inhibition promotes dopaminergic cell loss in a MPTP mouse model [239]. Additionally, 14-3-3ζ binds to phosphorylated tyrosine hydroxylase (TH), an important enzyme in dopamine synthesis. Interaction between 14-3-3ζ and TH is thought to lead to prolonged activation of TH and increased dopamine synthesis [220-222].

14-3-3s have been shown to interact with other proteins that have been identified to be pathogenic in PD. Recent research has shown that 14-3-3η binds and negatively regulates parkin, which is an autosomally recessive mutation that causes PD [240]. Mutations to parkin disrupt the interaction between 14-3-3η and parkin, and alpha-synuclein also disrupts this interaction [75]. While at this point it is unclear how disruption of the interaction leads to manifestation of disease, it suggests that 14-3-3s play a potential role in pathogenesis of disease through parkin. Lastly, 14-3-3s have been recently shown to interact with LRRK2, but this will be covered more thoroughly in the next section.

14-3-3s may play a significant role in many neurodegenerative disorders. From AD and PD to amyotrophic lateral sclerosis (ALS – Lou Gehrig's disease), Huntington's disease (HD) and SCA1, 14-3-3s interact with many of the proteins that cause these diseases. While many of the pathways leading to cell death still need to be elucidated to determine the exact role 14-3-3s play in neurodegeneration, it is obvious that they play an important part in maintaining healthy neuronal function and delaying/preventing cell death over time. This study aims to highlight the potential therapeutic value of 14-3-3s in LRRK2-related causes of PD.

The Interaction Between LRRK2 and 14-3-3s

Binding Characterization

14-3-3s have recently been shown to interact with LRRK2 [241]. 14-3-3s are known to bind to phosphoserine/threonine motifs, which are also present in LRRK2

[242]. To date, four serines have been identified that are important in binding 14-3-3s. They are S910, S935, S955 and S973 [242, 243]. Nichols et al. first highlighted the importance of phosphorylation at two of these sites (S910 and S935) in order to maintain 14-3-3 interaction [242]. Their study showed that mutation of either of these sites to an alanine (a non-phosphorylatable amino acid) caused a complete loss of 14-3-3 interaction as measured by coimmunoprecipitation [242]. What was also noticed was that when this interaction was disrupted by the alanine mutations, the cytoplasmic localization was altered. LRRK2's normally diffuse cytoplasmic localization changed into the formation of LRRK2 puncta within the cell [242].

Other studies have shown that pathogenic mutations in LRRK2 cause changes in phosphorylation of these four serine sites and have variable effects on 14-3-3 binding and LRRK2 localization within the cell [242, 243]. Other groups have shown that multiple familial mutations in LRRK2 disrupt 14-3-3 binding including the R1441C/G/H, Y1699C and I2020T, although the G2019S mutation has no observed change in 14-3-3 binding [242-246]. For example, Doggett et al. showed that the R1441C, R1441G, Y1699C and I2020T mutations all had either reduced or absent LRRK2 phosphorylation at all four serine sites while the G2019S still had relatively normal phosphorylation [243], which confirms previous results shown by Nichols et al [242]. Interestingly, the same study showed that mutation of the S955 and S973 to alanines did not lead to the formation of LRRK2 puncta, suggesting that perhaps phosphorylation of these two sites may not be as crucial for 14-3-3 binding as the S910 and S935 sites, but may act to stabilize the interaction between the two [243]. It is important to note that this does not appear to have been studied at this point.

Functional Importance of Interaction

Of note, another recent study has looked at the functional importance of the 14-3-3/LRRK2 interaction [247]. While many studies show that the 14-3-3 to LRRK2 interaction is disrupted by multiple pathogenic mutations and that this can lead to LRRK2 redistribution within the cell, few have looked at the functional significance of the LRRK2 interaction. Fraser at al. recently highlighted an important role for 14-3-3s in regulating LRRK2 release in exosomes [247]. Treatment with the pan-14-3-3 inhibitor difopein disrupted the interaction between 14-3-3s and LRRK2 and caused a reduction of LRRK2 release in exosomes from HEK293T cells, thus clarifying one function of the LRRK2/14-3-3 interaction [247]. Additionally, Muda et al. showed that addition of protein kinase A (PKA) and 14-3-3γ caused a reduction in wildtype-LRRK2 kinase activity as measured by autophosphorylation and phosphorylation of the *in vitro* substrate of LRRK2, moesin [244]. This emphasizes the potential role of 14-3-3s in regulating LRRK2 kinase activity.

Hypothesis About the Role of 14-3-3s Against Toxic LRRK2

The overall goal of this dissertation project is to examine whether 14-3-3 proteins can regulate LRRK2 toxicity. My overall hypothesis is that 14-3-3s can reduce LRRK2 toxicity by regulating kinase activity. Figure 1 provides a brief pathway outlining what we believe is happening with LRRK2 mutations. Mutant LRRK2 has decreased or ablated S910/S935 phosphorylation which disrupts the interaction with 14-3-3s. As a result, LRRK2 distribution is altered from its normal, diffuse cytosolic distribution to

forming LRRK2 puncta within the cytosol. The disruption in the interaction between 14-3-3s and LRRK2 also results in an increase in LRRK2 kinase activity that leads to neurite shortening. Based upon previous work by other groups as well as our own, we believe that 14-3-3s can regulate LRRK2 and will provide protection against LRRK2 mediated toxicity by reducing LRRK2 kinase activity.

In chapter 3, I examine the ability for 14-3-3 to modulate and regulate LRRK2 phosphorylation at the S910 and S935 residues as well as its localization within the cell. In chapter 4, I examine the ability for 14-3-3θ overexpression to reduce LRRK2 mediated toxicity and for 14-3-3 inhibition to potentiate LRRK2 toxicity as measured by neurite outgrowth. In chapter 5, I examine 14-3-3's ability to regulate LRRK2 kinase activity as we believe 14-3-3θ overexpression is protective by reducing aberrant kinase activity seen in mutant LRRK2.

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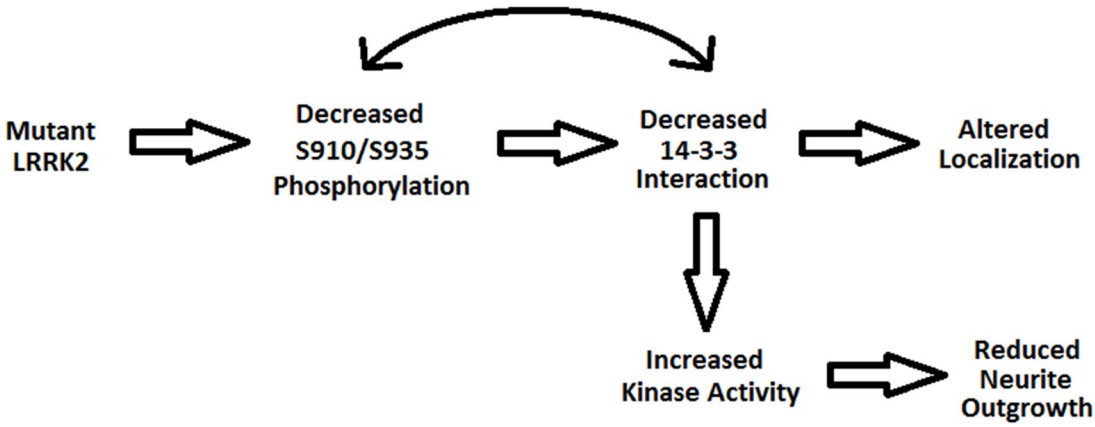

Figure 1. Mutant LRRK2 has decreased phosphorylation at serines 910 and 935 which reduces the ability for 14-3-3s to bind, possibly reciprocally leading to further dephosphorylation of LRRK2. By disrupting the interaction between LRRK2 and 14-3-3s, the result is the formation of LRRK2 puncta and increased kinase activity, the latter of which has been shown to reduce neurite outgrowth.

Models and Methods

In trying to elucidate the mechanisms underlying the cause of Parkinson's disease (PD), many different models have been developed to try to recapitulate the disease. While PD is a largely idiopathic disease, there have been several genes identified that either cause or increase the risk of developing PD, along with multiple environmental risk factors for PD. Much time and effort has gone into developing PD models in the hopes of discovering pathways that lead to disease that are targetable for therapeutics. Here, we will review many of the characterized models of PD and discuss why we chose the LRRK2 model. That will be followed with a review of the assays used to examine LRRK2 function and how we adapted them to test the effects of 14-3-3s in the LRRK2 model.

Mouse Models of Parkinson's Disease

Neurotoxin Models

Many models of Parkinson's disease (PD) are focused on recapitulating the loss of dopaminergic neurons in the substantia nigra in the hopes of recreating the same motor phenotype as seen in PD patients. Indeed, multiple neurotoxins have been used that target mainly dopaminergic neurons; namely rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is the nontoxic precursor to 1-methyl-4-phenylpyridinium (MPP+) that causes neurodegeneration. These neurotoxins are mitochondrial complex 1 inhibitors that cause selective loss of dopaminergic neurons through an increase in oxidative stress on the cells [1-3]. Interestingly, while MPTP has

been shown to cause PD symptoms in humans and primates, although not in rats [4], it does not induce Lewy body pathology, leading to uncertainty about how well the mechanism of MPTP toxicity is related to PD progression [5-7]. Rotenone treatment reproduces the behavioral features of PD and the degenerating neurons form intracellular inclusions that resemble Lewy body pathology seen in human patients [8-10]. These inclusions do contain alpha-synuclein and ubiquitin, similar to normal Lewy bodies [11]. However, the drawback to using rotenone in mice is the lack of reproducibility in the amount of animals that develop lesions and the size and location of the lesions and the associated mortality rate [12, 13], although these issues appear to have been mitigated somewhat by utilizing different routes of rotenone administration [14]. Since both neurotoxins target dopaminergic neurons specifically, they have been utilized as models to test potential therapeutics against preventing neuronal cell death.

Genetic Models of PD (Excluding LRRK2)

Alpha-synuclein

Multiple genetic mutations that cause PD have been made into mouse lines in the hopes of developing a model that will fully recapitulate disease. Namely, point mutations in the *SNCA* gene encoding alpha-synuclein as well as gene duplications increase in the likelihood for alpha-synuclein to aggregate [15, 16]. This alpha-synuclein pathology is seen not only in PD, but also in other "synucleinopathies" such as dementia with Lewy bodies (DLB) or multiple system atrophy (MSA) [17]. In essence, using models that exhibit alpha-synuclein pathology to test potential therapeutics may lead to benefits against multiple disorders and will not be limited to just PD.

There have been multiple missense mutations in alpha-synuclein that have been identified in familial PD thus far. They are the A30P, A53T, E46K, H50Q and G51D mutations [18-21]. In addition, gene duplication and triplications also can lead to PD, which would indicate that alpha-synuclein expression levels may also influence PD progression [16, 22]. These mutations in *SNCA* have spawned multiple transgenic mouse models to try to recapitulate disease with varying degrees of success, although notably no significant nigrostriatal degeneration has been seen in most of them.

The A30P and A53T mutations have been inserted into mouse lines under the expression of various promoters. Under the mouse prion promoter [23, 24] and hamster prion promoter [25], there was significant alterations in behavior, such as age related spatial memory deficits and reduction in anxiety like behavior, suggesting learning and memory deficits [26-28]; however, despite the memory and behavioral phenotypes matching with human PD, they did not show any nigral cell loss [23-25]. Under the TH or Thy-1 promoter, a few studies showed TH+ cell loss, but no alpha-synuclein pathology was present [29-34]. The most positive results came from utilizing a murine Thy-1 promoter where the A53T mice had loss of dopamine levels in the striatum, but only mild dopaminergic cell loss occurred in the substantia nigra, although there was alpha-synuclein pathology present in this model [35-39].

A line of tetracycline inducible transgenic mice have also been recently developed [40]. These mice overexpress A53T alpha-synuclein under the Pitx3 promoter and show robust neurodegeneration in the midbrain as well as significant deficits in motor function. In addition, they also exhibit decreased dopamine release and dopaminergic neurons show Golgi fragmentation and impairment of autophagy [40].

Besides these transgenic mouse lines, several viral vectors have been used to drive alpha-synuclein overexpression. In contrast to the alpha-synuclein transgenic mouse lines, the viral models exhibit robust alpha-synuclein pathology and clear dopaminergic cell loss [41-45]. Injection of AAV human wildtype or A53T alpha-synuclein into the substantia nigra of rats causes progressive, age-dependent loss of dopaminergic neurons, motor impairment and the formation of alpha-synuclein based cytoplasmic inclusions [41-45]. The cell loss was preceded by degeneration of striatal axons and terminals and the formation of alpha-synuclein inclusions in axons and dendrites [41-43, 46]. Notably, these models have a degree of variability, yet they are a valuable tool for testing out new, neuroprotective therapeutics.

Parkin

Mouse lines expressing mutations in other PD related genes have also been created to examine the role these genes may play in PD neurodegeneration. Parkin is an E3-ubiquitin ligase that plays an important role in facilitating mitophagy in damaged mitochondria [47]. Mutations in parkin have been shown to cause early-onset PD in an autosomal recessive manner and are also seen in some young-onset sporadic cases as well [48, 49]. Parkin null mice do not show dopaminergic cell loss; only some alterations in dopamine metabolism were noted [50-53]. Furthermore, when these parkin null mice were treated with MPTP, there was no increase in toxicity in the dopaminergic cells over wildtype, suggesting some sort of compensatory mechanism in reducing mitochondrial dysfunction in these parkin knockout mice [54-56]. Notably, a more recent parkin knockout model has been shown to develop dopaminergic cell neurodegeneration [57]. Lentiviral-Cre injection into the substantia nigra of adult parkin flox/flox mice leads to

progressive nigral cell loss 10 months after parkin deletion. Over time, these mice see an accumulation of PARIS (a substrate of parkin), with downregulation of PGC1-α (a regulator of mitochondrial biogenesis and function) eventually culminating with mitochondrial dysfunction and cell death [57]. This is perhaps indicative of the mechanism by which parkin mutation leads to PD, although this needs to be studied further.

There are also two mutant parkin mouse models that have been developed. The first is an AAV overexpression of wild-type and T240R-parkin that showed progressive and dose-dependent dopaminergic cell death in rats [58]. The second model showed more promise as it exhibited more of a motor phenotype than the T240R model. A Parkin-Q311X-DAT-BAC mouse exhibits late onset and progressive hypokinetic motor deficits, age-dependent dopaminergic degeneration in the substantia nigra and a reduction of striatal dopamine and dopaminergic terminals in the striatum [59]. While both models exhibit some degree of dopaminergic cell loss, the Q311X truncated parkin mouse shows a more clear motor phenotype as well as pathology in the striatum.

PINK1

Mutations in PTEN-induced putative kinase 1 (PINK1) have also been shown to cause early-onset autosomal recessive PD [60, 61]. PINK1 knockout in *Drosophila* showed robust neurodegeneration and mitochondrial defects [62], but PINK1 knockout and knockdown models in mice show muted results [63]. PINK1 knockout in mice showed an age-dependent, modest reduction in striatal dopamine as well as a reduction in locomotor activity [63, 64]. However, they do not show any major abnormalities in the

dopaminergic neurons or in striatal dopamine levels, nor do they show any Lewy body formation or nigrostriatal degeneration up to 18 months of age [63, 64]. A PINK1 null mouse (exon 4-5 deletion) was shown to have a small, progressive loss of dopamine in the striatum, but no degeneration in the substantia nigra [65]. The overall effects of PINK1 knockdown are modest in recapitulating disease in mice.

DJ-1

Lastly, mutations in DJ-1 lead to early-onset, autosomal recessive PD [66]. Like PINK1, DJ-1 is responsible for protecting cells against oxidative stress [67]. For example, studies have shown that cell lines not expressing DJ-1 are more susceptible to apoptosis from oxidizing agents like hydrogen peroxide or paraquat [68, 69]. Similar to PINK1, when DJ-1 knockout models were created, there was decreased locomotor activity [70, 71]. Characterization of the mouse line showed similar problems with dopamine transmission and mitochondrial dysfunction, but no overt neuronal loss in the substantia nigra occurred [70-72]. Analysis of neurons from these mice did show elevated mitochondrial oxidative stress, which may suggest that DJ-1 impairment makes neurons more susceptible to apoptosis under stress from other risk factors for PD [73].

Summary of Non-LRRK2 Mouse Models

Mouse models for genetic mutations in PD have had varying results in recapitulating disease progression seen in humans. Neurotoxins are good at selective dopaminergic cell loss, yet because alpha-synuclein pathology does not occur (in the case of MPTP), questions pertain to whether or not the pathways leading to neuronal loss are the same as in human PD. Genetic mouse models may give insight into disease

mechanisms, but they fail to mimic clinical features of disease or to show dopaminergic neuronal loss. Alpha-synuclein genetic models can provide insight into specific mechanism for disease, suggesting that mitochondrial dysfunction and overactive PARP1 from DNA damage may play a role leading to apoptosis [74]. Alpha-synuclein viral models are better recapitulating the human PD phenotype in mice, yet suffer from a degree of variability. Parkin knockout models show increased mitochondrial dysfunction and the Parkin flox/flox mouse showed progressive nigral cell loss when parkin expression was nullified in adult mice [57]. The PINK1 and DJ-1 knockout mouse lines showed small changes in mitochondrial respiration, but no degeneration or overt motor phenotype. In summary, these models suggest an underlying problem of mitochondrial dysfunction, yet because the disease is not fully recapitulated in these models, whether or not therapeutics that show protection in these models will translate to human patients remains to be seen.

LRRK2 Mouse Models

Kinase Domain Mutations

In addition to the genes mentioned in the previous section, mutations in LRRK2 have been shown to be the most common genetic mutations in PD [75]. Many of the most common mutations in LRRK2 have been made into transgenic and/or knock-in mouse lines, with variable results as to how well each mutation recapitulates PD.

The G2019S mutation is the most common LRRK2 mutation [76]. It is a point mutation in the kinase domain of the protein that has been shown to increase kinase

activity of the protein 2-4 fold [77, 78]. This mutation has been introduced in bacterial artificial chromosome (BAC) transgenic mice as well as knock-in mice. The BAC transgenic mice show a reduction in extracellular striatal dopamine and age-dependent progressive motor deficits [79, 80]. However, the behavioral testing suggested that the changes in behavior are connected to increased fear and anxiety in the G2019S mice as their motor function was not overall impaired [79]. In addition, G2019S BAC transgenic mice show an increased amount of tau accumulation in neurons over wildtype-LRRK2 BAC transgenic mice suggesting that tau pathology is not linked to LRRK2 overexpression levels, but rather to the G2019S mutation specifically [79]. Another BAC transgenic mouse developed by Li et al. showed an age-dependent decrease in striatal dopamine content and decreased striatal dopamine release and uptake. However, there was no measured loss of dopaminergic neurons in the substantia nigra at 12 months [81]. Interestingly, a separate G2019S transgenic mouse line developed by Darren Moore's group overexpressing G2019S under the CMV-enhanced human platelet-derived growth factor β-chain (CMVE-PDGFβ) promoter group showed progressive degeneration of dopaminergic neurons of the substantia nigra [82]. However, these mice did not show any changes in striatal dopamine levels or exhibit any changes in locomotor activity, contrary to what was seen in the other BAC transgenic lines [79, 82]. The phenotypic differences between the BAC transgenic and other transgenic lines can be explained perhaps by different expression patterns in the mouse lines. Notably, there was a point of phenotypic convergence between the G2019S mouse lines, which is reduced neurite outgrowth [79, 80, 82, 83]. This has been seen both *in vitro* and *in vivo*; this will be discussed further in the next section.

In addition to these transgenic lines, there have been multiple G2019S knock-in mouse lines developed [84]. One G2019S knock-in line created by Herzig et al. showed no behavioral or motor deficits, although they noticed pathology in the kidneys related to LRRK2 expression there [84]. This group did not describe dopamine release or dopaminergic cell health in this knock-in mouse model. A second G2019S knock-in line created by Yue et al. had reduced dopamine release at 12 months of age as well as mitochondrial abnormalities in the striatum of these mice, which they describe as consistent with mitochondrial fission [85]. No impairment in mobility was seen, but these mice did exhibit fear and anxiety during behavioral testing [85], similar to what was described in the BAC transgenic mice.

While the G2019S mutation is the most common mutation in the kinase domain of LRRK2, the I2020T mutation also affects the kinase domain, although it does not cause the same increase in overall LRRK2 kinase activity that the G2019S mutation does. An I2020T mouse line expressed from the cytomegalovirus (CMV) promoter in the substantia nigra exhibited impaired locomotion compared to their nontransgenic littermates [86]. Further analysis showed that while there was no apparent loss of dopaminergic neurons in either the substantia nigra or striatum, there was reduced striatal dopamine. Like the G2019S mouse lines, these mice also exhibited reduced neurite outgrowth as compared to nontransgenic controls [86].

GTPase Domain Mutations

The G2019S mutation is important because it is the most common mutation observed in PD. However, there have been many other pathogenic mutations identified

that affect other, non-kinase domain regions of the protein; many of them in the GTPase domain. As such, there have been multiple mouse lines produced with mutations in the GTPase domain.

After the G2019S mutation, the R1441C is the second most common LRRK2 mutation [76]. A mouse line conditionally expressing R1441C in midbrain dopaminergic neurons displayed nuclear abnormalities, but no overt neurodegeneration [87]. A R1441C knock-in mouse also appears normal and has no dopaminergic neurodegeneration or loss of striatal dopamine [88]. They do note that the R1441C mutation impairs dopamine D2 receptor function, which impairs stimulated dopamine neurotransmission by D2 receptor agonists like quinpirole [88].

While not as common as the R1441C mutations, the R1441G mutation targets the same residue and this mutation is also associated with PD. A R1441G BAC transgenic mouse line has been developed that shows age-dependent and L-DOPA-responsive slowness of movement that is associated with diminished dopamine release [89]. Additionally, these mice have been further characterized to show reduced neurite outgrowth, similar to what is seen in the G2019S and I2020T mouse lines [89].

LRRK2 Knockout Models

Multiple LRRK2 knockout (KO) lines have been established to determine the normal function of LRRK2. These mice are viable and have an intact nigrostriatal pathway up to 2 years of age [90-92]. Other studies of this mouse line have established that LRRK2 knockout causes an elongation of neurite outgrowth [83, 93]. Interestingly, the kidneys of LRRK2 KO mice are a darker color than normal, but still function

normally and have been shown to be resistant to rhabdomyolysis-induced acute kidney injury [94]. These mice otherwise display normal neural and behavioral phenotypes.

Neurite Outgrowth in LRRK2 Models of PD

While the mouse models of LRRK2 have variable effects on dopamine synthesis and motor function, one of the commonalities seen across multiple LRRK2 mutations is the reduction in neurite outgrowth [80, 82, 83, 89, 93]. This has been repeatedly seen both *in vitro* and *in vivo*, thus emphasizing the role that LRRK2 plays in neurite outgrowth. LRRK2 has been implicated in multiple, diverse molecular pathways, but the connection to neurite outgrowth has largely been implicated because of LRRK2's noted interaction with actin and tubulin [95, 96]. Indeed, one of the first studies looking at neurite outgrowth with LRRK2 mutations showed that transfection of cDNA to express the G2019S, I2020T and R1441G mutations in primary cortical neurons led to a significant reduction in total neurite outgrowth with these mutations [93]. This effect has also been shown in differentiated SH-SY5Y neuroblastoma cells [97]. Upon creation of various mouse lines expressing these LRRK2 mutations, characterization has shown neurite shortening to also occur [80, 82, 83, 89]. Dachsel et al. first showed that hippocampal neurons from BAC transgenic G2019S mice exhibited neurite shortening in primary cultures from postnatal mice [83]. This was followed up by Winner et al. who showed that adult BAC G2019S mice have reduced neurogenesis and that neurite outgrowth was stunted *in vivo* [80]. Other groups have shown that different G2019S transgenic mouse lines exhibit the same neurite shortening [82]. In addition to G2019S,

the R1441G and I2020T BAC transgenic mouse lines also exhibit reduced neurite outgrowth [86, 89]. Together, this highlights the use of neurite outgrowth as a consistent, reliable method for measuring LRRK2 toxicity across multiple LRRK2 mutations.

It has been shown that LRRK2 mediated neurite shortening can be reversed by introducing LRRK2 kinase inhibitors [83, 93, 98], thus linking LRRK2 kinase activity with neurite outgrowth. Overexpression of the Rho GTPase Rac1, which regulates actin cytoskeleton remodeling and inhibits the GTPase-activating protein ArfGAP1, can rescue neurite shortening as well [99]. This suggests an interplay between the GTPase domain and the kinase domain of LRRK2 in affecting neurite outgrowth. Nevertheless, the link between LRRK2 kinase activity and neurite outgrowth will play an important part of the discussion going forward.

Neurite Shortening vs. Retraction

Of note, there is some discourse on neurite shortening and whether this reflects a developmental phenomenon where neurons expressing LRRK2 mutations develop shorter neurites or whether neurites initially grow out and later retract and degenerate For example, one of the first groups looking at neurite outgrowth with LRRK2 mutations saw that transfecting primary cortical cultures with mutant G2019S-LRRK2 after day *in vitro* seven (DIV 7) caused neurite lengths to shorten over time compared to wildtype LRRK2 [93]. By day 15, neurite lengths had appeared to stabilize at 25% of their pre-transfected length. As such, they termed this effect to be neurite retraction [93]. Further research by other groups has agreed that neurite lengths are shorter in G2019S carrying neurons, but that this occurs because of a reduced rate of neurite outgrowth over time and that given

enough time, neurites from G2019S mice may in fact catch up and match the lengths of normal neurons [100]. This second scenario appears to make more sense in terms of disease progression as other reports show no overt morphological differences in the brains of adult LRRK2 mouse models [81, 90, 91, 101], suggesting a developmental delay with mutant LRRK2. Additionally, it is possible that a developmental delay could impart vulnerability later on as most cases of PD do not manifest until later in life [100].

Potential LRRK2 Neurite Outgrowth Pathways

In the search for the true substrate for LRRK2, many cytoskeletal proteins have been discovered to be phosphorylated by LRRK2. This perhaps provides the pathway(s) by which mutant LRRK2 are affecting neurite outgrowth. For example, LRRK2 has been shown to phosphorylate moesin [95], a protein involved in attaching the actin cytoskeletal network to the plasma membrane. Indeed, overphosphorylation of the ERM (ezrin, radixin and moesin) family of proteins that tether filamentous actin to the cytoplasmic membrane causes retardation in neurite outgrowth [102]. Additionally, LRRK2 has been shown to interact with and phosphorylate β-tubulin [103, 104], which can alter microtubule stability. Importantly, LRRK2 can phosphorylate tubulin associated tau, but not free soluble tau [105]. Protein tau is responsible for stabilizing the microtubule network and when it becomes phosphorylated, leaves the network, causing destabilization and eventual breakdown of microtubules. Indeed, the mutant LRRK2 mouse lines exhibit not only neurite shortening, but also elevated levels of phosphorylated tau suggesting that perhaps mutant LRRK2 is overly phosphorylating tau leading to destabilization of microtubules [82, 89]. To further emphasize the role of tau, increased phosphorylation of tau at the T181 residue has been shown to cause the same

neurite shortening phenotype and that this residue is also the one phosphorylated by LRRK2 [105, 106]. It is plausible that mutant LRRK2 causes neurite shortening through phosphorylation of tau; however there are multiple pathways that mutant LRRK2 may also act on to cause neurite shortening.

14-3-3 Mouse Model

The LRRK2 mouse model described above shows highest transgene expression in the hippocampus. In order to examine the effects of 14-3-3θ overexpression on mutant LRRK2, we decided to utilize our HA-tagged 14-3-3θ overexpressing mouse line that also has high transgene expression in the hippocampus. This mouse line was created by cloning a human 14-3-3θ construct, tagged with a hemagglutinin (HA) epitope tag at the C-terminal end into a Thy1.2 expression cassette [107] to drive neuron specific expression of 14-3-3θ. Following digestion with NdeI and EcoRI, the Thy1.2 construct containing HA-tagged 14-3-3θ was purified and microinjected into C57BL/6 fertilized mouse oocytes. Founder mice were bred with C57BL/6 mice from Jackson labs, and pups were examined for expression of HA-tagged 14-3-3θ in mouse brain by both immunohistochemistry and western blotting. Two of the founder lines showed diffuse neuronal expression of HA-tagged 14-3-3θ (M5 and F1 lines). We chose to utilize the M5 mouse line for the experiments detailed in these experiments because of high levels of expression in the hippocampus, which is where highest expression of the LRRK2 transgene also occurs in the BAC transgenic mice we utilized. Of note, we have also evaluated these 14-3-3θ mice behaviorally and observe no differences from wildtype

littermates in body weight, gross brain morphology, motor function, learning, or anxiety (Fig. 1).

We also wished to look at 14-3-3 inhibition to determine if inhibiting this neuroprotective protein may further increase the insult caused by the mutant LRRK2 transgene. We utilized two separate mouse lines, one that expresses difopein, a pan-14-3-3 inhibitor, as well as a mouse line that expresses a dominant negative (DN) 14-3-3θ isoform [108]. Difopein (dimeric fourteen-three-three peptide inhibitor) is a high-affinity 14-3-3 competitive antagonist peptide that inhibits 14-3-3/ligand interactions by binding within the amphipathic groove of 14-3-3s without selectivity among the 14-3-3 isoforms [109]. The difopein mouse line has been recently characterized as displaying behavioral deficits that correspond to the symptoms of schizophrenia [110]. Mice expressing difopein exhibited impairments in associative learning, working memory and in long-term synaptic plasticity [108, 110]. These mice also were less sociable when placed in a cage with a novel mouse, indicating social withdrawal [110]. Neuronal characterization showed a reduction in dendritic complexity and spine density in forebrain excitatory neurons, which is thought to be caused by the disruption of 14-3-3 dependent regulation of phosphorylated cofilin on actin cytoskeletal dynamics [110]. Interestingly, the mice had higher dopamine levels in the striatum; however, this was not thought to be a direct consequence of 14-3-3 inhibition in the mice because difopein transgene expression was not detected in the dopaminergic neurons of the substantia nigra [110].

The DN 14-3-3θ mouse line was beneficial in highlighting the importance of the theta isoform. While difopein inhibits all seven isoforms, 14-3-3θ has only been shown to dimerize with 14-3-3ζ, ε, γ and β, as well as forming homodimers [111-114]. The DN

14-3-3θ would disrupt only the interaction between the theta isoform and its binding partners and would not disrupt the homo- and heterodimerization of the other six isoforms of 14-3-3s as difopein would. Thus, the impact of the DN 14-3-3θ is less than that of difopein, and using DN 14-3-3θ in our studies would investigate the potential importance of the theta isoform in LRRK2 mediated toxicity.

Establishing the Neurite Outgrowth Assay

Given the reproducibility of neurite shortening in several *in vitro* and *in vivo* LRRK2 models, we decided to use neurite outgrowth as a measure of LRRK2 function/dysfunction in order to test how 14-3-3s may regulate LRRK2 toxicity. We cultured hippocampal neurons from postnatal day 0 (P0) pups in a protocol that was adapted from Dachsel et al. [83]. Hippocampal neurons were chosen because transgene expression of both G2019S in the BAC transgenic line as well 14-3-3θ overexpression in our mouse line was highest in the hippocampus, leading us to believe that biochemical changes from overexpression would most likely be observed in these neurons. Briefly, hippocampi were dissected from individual mice and incubated in papain for 20 minutes at 37°C. Cells were thoroughly washed using Neurobasal-A media containing B-27 supplement and 5% FBS before tituration using fire polished glass pipettes. After centrifugation at 1500 rpm for five minutes, pelleted cells were layered on top of a 4% BSA in HBSS and centrifuged at 700 rpm for five minutes. Cells were resuspended and plated at 350,000 cells/well on 18mm glass coverslips coated with poly-D-lysine. After 16 hours, media was removed and replaced by Neurobasal-A media containing B-27

supplement and Arabinose C at 6μM. After 8 days in culture, cells were immunostained for microtubule associated protein 2 (MAP2) and the coverslips mounted on slides. Neurons were imaged under fluorescent microscope and neurites were traced and measured using Neuralucida software.

We validated the assay by assessing the hippocampal cultures from the BAC transgenic G2019S mice to verify that in fact we were seeing neurite shortening in our culture model. Analysis of primary hippocampal cultures from G2019S transgenic mice showed a significant reduction in total neurite lengths as compared to nontransgenic littermates (Fig. 2), in agreement with what had previously reported by multiple groups [80, 83, 93]. In addition, we also examined primary neurite outgrowth (neurite length from the cell body to the first branching point) and the number of branches per neurite. Primary neurite outgrowth was reduced in the G2019S cultures; however, no significant difference was seen in total number of branch points when compared to nontransgenic littermates (Fig. 3A, B). Thus, we decided to use total neurite length as the measure for neurite outgrowth, in agreement with what other groups have done as total neurite outgrowth appears to be the most consistently used measure for LRRK2 toxicity in neurons. We also examined cortical neurons in addition to hippocampal neurons and saw that neurite lengths were reduced in G2019S versus non-transgenic littermates (Fig. 4); however this effect was not statistically significant and was somewhat blunted compared to hippocampal neurons. This was likely due to lower transgene overexpression in the cortex as compared to the hippocampus. Since 14-3-3θ overexpression is also highest in the hippocampus, and we will be crossing this mouse with the LRRK2 mouse lines that

also have highest overexpression in the hippocampus, we decided to exclusively analyze hippocampal cultures from postnatal mice.

Measuring Kinase Activity

Autophosphorylation

To measure how 14-3-3s may regulate G2019S kinase activity, we turned to substrate phosphorylation assays. While there has been some difficulty in the LRRK2 field at identifying the true target of LRRK2's kinase activity, there are multiple *in vitro* substrates that are phosphorylated by LRRK2 that can be measured, including multiple autophosphorylation sites on LRRK2 itself. Notably, phospho-specific antibodies have been developed toward phosphorylated threonine1503 (pT1503) [115] and phosphorylated threonine1967 (pT1967) [116]. Phosphorylation at these sites occurs after an *in vitro* autophosphorylation reaction with LRRK2 and is considered to correlate with kinase activity [115, 116]. We used the T1503 residue as our readout of LRRK2 kinase activity. We performed the kinase assay by transfecting human embryonic kidney (HEK) cells with our LRRK2 plasmids with and without 14-3-3θ. HEK 293T cells were transfected with HA-tagged LRRK2 with or without V5-tagged 14-3-3θ or difopein-YFP. 48 hours after transfection, cell lysates were incubated with HA-antibody conjugated Dynabeads for 30 minutes. Beads were washed with PBS with two high salt washes of PBS with 500mM NaCl before being resuspended in kinase buffer (10mM Tris pH 7.4, 0.1mM EGTA, 20mM of $MgCl_2$, 0.1mM ATP) at 30°C for 30 minutes. The kinase reaction was terminated by incubating samples on ice and beads were then incubated in

Laemmli buffer at 75°C for ten minutes. Samples were then western blotted for pT1503 LRRK2 and for total LRRK2 using an antibody against the HA tag.

While pT1503 is a valid measure, there is another recently discovered autophosphorylation site that corresponds well with LRRK2 kinase activity and can be measured *in vivo* [98]. Sheng et al. discovered and characterized autophosphorylation at the serine1292 (S1292) residue on LRRK2 [98]. Autophosphorylation at this site is elevated in multiple LRRK2 mutations, including the R1441G/C, G2019S and I2020T. Interestingly, when this site was mutated to an alanine (S1292A), neurite shortening was reversed in R1441G/G2019S double transgenic cultures [98]. In addition, when the LRRK2 inhibitor sunitinib was added, S1292 phosphorylation was lost [98]. Taken together, this shows that S1292 phosphorylation correlates to LRRK2 kinase activity and that kinase activity is necessary for neurite shortening to occur, implicating the role of S1292 phosphorylation in pathogenesis. To test for S1292 phosphorylation, we performed the same transfections as described for the pT1503 experiments, except protein lysates collected from the cells were directly western blotted for pS1292 and total LRRK2. No *in vitro* kinase assay was required to induce autophosphorylation.

Substrate Phosphorylation

The kinetics of autophosphorylation and substrate phosphorylation can be different from one another, so it is useful to measure LRRK2 kinase activity by other measures besides autophosphorylation. While the true substrate(s) of LRRK2's kinase activity has yet to be resolved, there are general substrates that have been shown to be phosphorylated by LRRK2. Moesin and myelin basic protein (MBP) are two protein

substrates of LRRK2 as well as the peptide substrate LRRKtide that are commonly used [95]. Phosphorylation of these substrates is generally measured by a ^{32}P incorporation assay where the substrate and LRRK2 are incubated together with radioactive ^{32}P in solution and then radiography is done to measure the amount of ^{32}P that is incorporated by the substrate. We instead chose to use a protein recently identified to interact with LRRK2, the GTPase activating protein that stimulates the GTPase activity of LRRK2, ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1). ArfGAP1 has been shown to be phosphorylated by LRRK2 and to have reciprocal phosphorylation back onto LRRK2 [117, 118].

The kinase assays for ArfGAP1 phosphorylation were performed by transfecting human embryonic kidney (HEK) cells with our LRRK2 plasmids with and without 14-3-3θ. HEK 293T cells were transfected with HA-tagged LRRK2 with or without V5-tagged 14-3-3θ or difopein-YFP. 48 hours after transfection, cell lysates were incubated with HA-antibody conjugated Dynabeads for 30 minutes. Beads were washed with PBS with two high salt washes of PBS with 500mM NaCl before being resuspended in kinase buffer (10mM Tris pH 7.4, 0.1mM EGTA, 20mM of $MgCl_2$, 0.1mM ATP) with 1µg recombinant ArfGAP1 at 30°C for 30 minutes. The kinase reaction was terminated by incubating samples on ice and beads were then incubated in Laemmli buffer at 75°C for ten minutes. Sample was then immunoblotted with a phospho-threonine antibody specific to recombinant protein as well as GST to measure total ArfGAP1 in solution. Densitometric analysis of bands was used to quantify changes in ArfGAP1 phosphorylation under the different transfection conditions. Through both

autophosphorylation and ArfGAP1 phosphorylation assays, we hope to determine the mechanism by which 14-3-3θ overexpression may reduce LRRK2 toxicity.

Summary

Multiple models of PD have been developed in the hopes of fully recapitulating disease so that the underlying mechanism(s) that cause disease can be elucidated and targeted for therapeutics. The models discussed in this chapter display various phenotypes, yet none fully recapitulate the progression of PD in humans. Recently, LRRK2 has become a protein of interest as patients with LRRK2 mutations have an identical clinical presentation as idiopathic PD patients, thus hinting at perhaps an overlap in mechanism. Additionally, mutations in LRRK2 are linked to both familial and idiopathic PD and recently single nucleotide polymorphisms (SNPs) in LRRK2 have been shown to be risk factors for idiopathic PD, further highlighting the importance of LRRK2. Because of the importance of LRRK2 in PD, and the fact that 14-3-3s have been shown to readily interact with LRRK2, we decided to work with the LRRK2 mouse model of PD to determine if 14-3-3s could regulate LRRK2 mediated toxicity.

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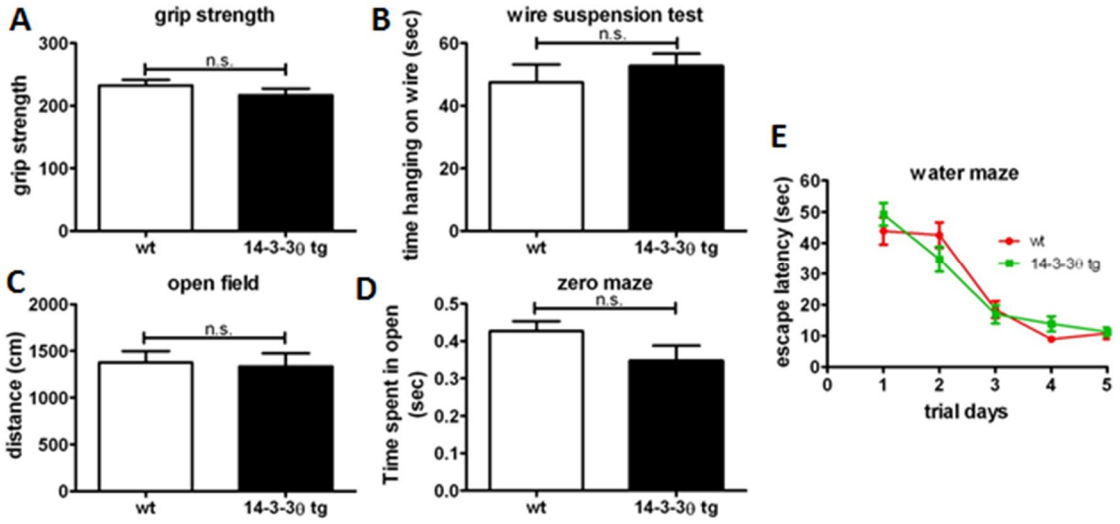

Figure 1. 14-3-3θ overexpressing mice display normal motor and behavioral phenotype

A-E) Mice overexpressing 14-3-3θ display a normal phenotype when subjected to various motor and behavioral tests. n=2 cohorts of 5 mice per cohort. (Data obtained Huiping Ding)

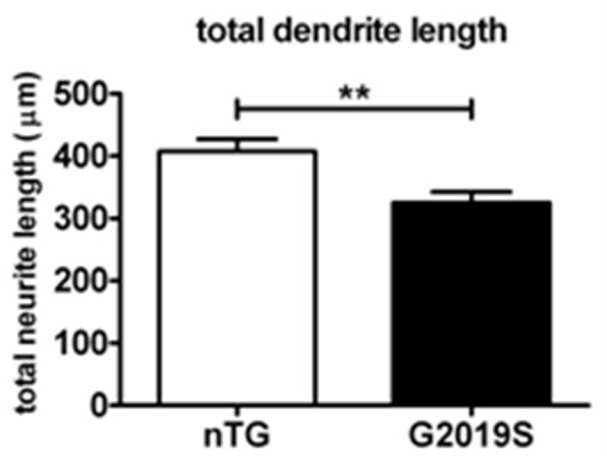

Figure 2. Total neurite length is decreased in G2019S primary cultures

A) Total neurite length analysis of neurons from nontransgenic and G2019S-LRRK2 mouse cultures. n=56 neurons for ntg, n=53 for G2019S-LRRK2 combined from three independent rounds. *$p<0.05$ (unpaired t-test).

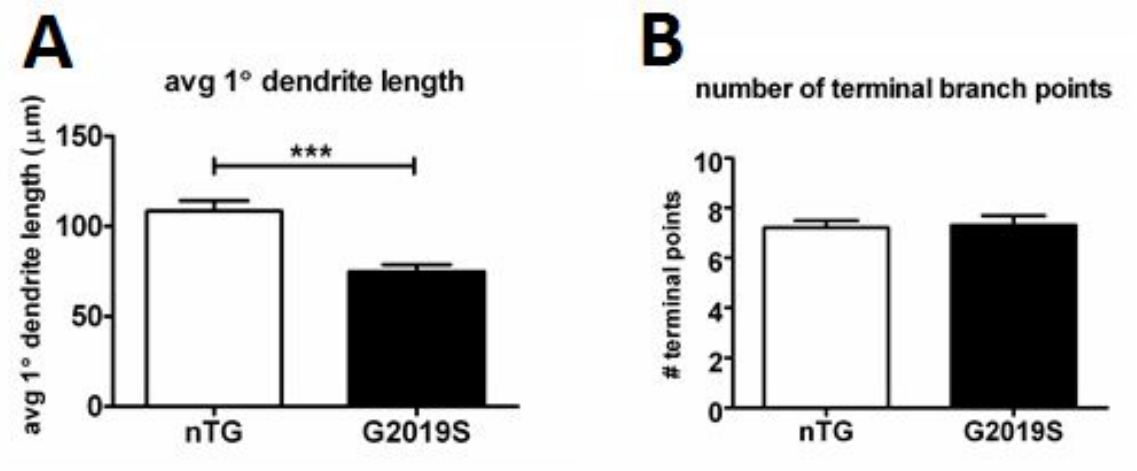

Figure 3. Primary neurite length and number of branches per dendrite

A) Primary neurite length analysis of neurons from nontransgenic and G2019S-LRRK2 mouse cultures. n=56 neurons for ntg, n=53 for G2019S-LRRK2 combined from four independent rounds. *$p<0.05$ (unpaired t-test).

B) Analysis of number of terminal branch points per neuron from nontransgenic and G2019S-LRRK2 mouse cultures, n=56 dendrites for ntg, n=53 for G2019S-LRRK2 combined from three independent rounds. n.s. = not significant

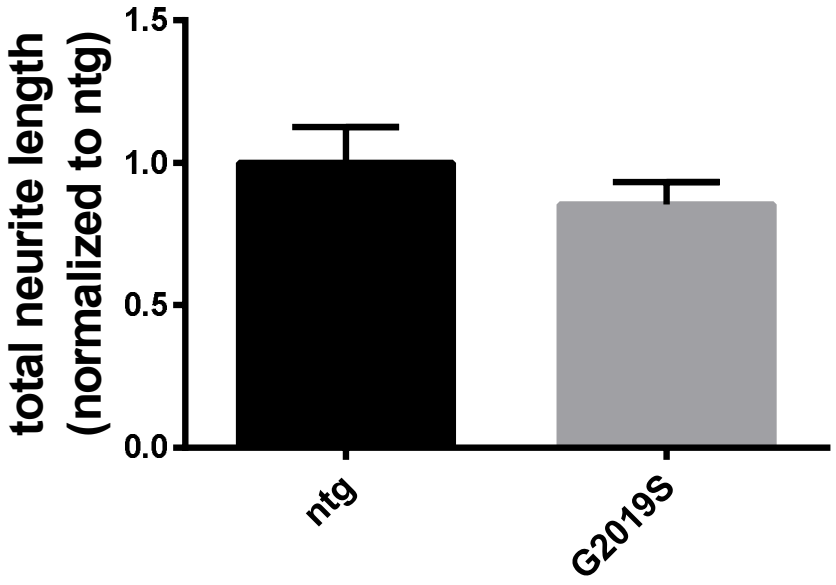

Figure 4. Total neurite length in cortical cultures is not reduced in G2019S-LRRK2

Total neurite length analysis of neurons from nontransgenic and G2019S-LRRK2 mouse cultures. n=73 neurons for ntg, n=62 for G2019S-LRRK2 combined from three independent rounds.

The Regulation of Localization and Phosphorylation of LRRK2 by 14-3-3θ

Introduction

How LRRK2 function is regulated in health and disease is not well understood as the physiological function of LRRK2 is yet to be fully elucidated. However, mutations in LRRK2 are thought to alter the biochemical properties of the protein, including kinase and GTPase activity as well as phosphorylation [1-8]. As discussed in chapter 1, LRRK2 protein interacts with 14-3-3s [6, 7, 9], a family of seven conserved proteins that participate in many cellular functions with an important role in cell survival [10]. 14-3-3s can regulate ligand function by altering subcellular localization of the ligand [11, 12]. For example, 14-3-3s bind to the pro-apoptotic proteins Bad and Bax and prevent them from shuttling to the mitochondria where they can induce caspase mediated apoptosis [13, 14]. 14-3-3s also bind multiple transcription factors such as Cdc25 and the forkhead transcription factors and keep them out of the nucleus, sequestered in the cytoplasm [15, 16].

14-3-3s can also bind to phosphorylated proteins and either enhance phosphorylation or protect against dephosphorylation. 14-3-3s interact with tau protein and it is thought that 14-3-3s might facilitate tau phosphorylation by the kinase GSK3β [17, 18]. Studies suggest that 14-3-3s bind to tau and alter its conformation, making it more accessible to kinases for phosphorylation, and that 14-3-3s can protect the phosphorylated tau from being dephosphorylated [18, 19]. Specific to dopaminergic neurons, 14-3-3s interact with phosphorylated tyrosine hydroxylase (TH) to enhance its activity in producing dopamine [20-22].

14-3-3s interact with LRRK2 at several phosphorylated serine sites: serines 910, 935, and 1444 [7-9]. Several pathogenic LRRK2 mutants have reduced phosphorylation at S910 and S935 and decreased interaction with 14-3-3s [7, 9], suggesting the importance of 14-3-3s in regulating LRRK2 function and toxicity. Mutation of S910/S935 to alanine to disrupt the 14-3-3/LRRK2 interaction causes punctate, perinuclear redistribution of LRRK2 in HEK293 cells [7]. Since 14-3-3s have a noted role in regulating the phosphorylation and localization of various other proteins, we hypothesize that 14-3-3θ overexpression will enhance LRRK2 phosphorylation at S910 and S935. Conversely, if the interaction between LRRK2 and 14-3-3s is disrupted by inhibiting 14-3-3s with difopein, we hypothesize that difopein will decreased LRRK2 phosphorylation and will cause LRRK2 redistribution into perinuclear, punctate foci as seen in the S910A and S935A models.

Results

14-3-3 inhibition blocks interaction of 14-3-3s with LRRK2 and causes alterations in LRRK2 localization

We first investigated the effects of 14-3-3 inhibition on LRRK2 subcellular localization. Dimeric fourteen-three-three peptide inhibitor (difopein) is a high-affinity 14-3-3 competitive antagonist peptide that inhibits 14-3-3/ligand interactions by binding within the amphipathic groove of 14-3-3s without selectivity among the 14-3-3 isoforms [23]. We first confirmed that difopein disrupts the interaction between 14-3-3s and LRRK2. Co-immunoprecipitation of endogenous 14-3-3s with wildtype LRRK2 was

reduced in HEK293T cells co-transfected with LRRK2 and difopein C-terminally tagged with enhanced yellow fluorescent protein (eYFP), as compared to control HEK293T cells co-transfected with LRRK2 and mutant difopein-eYFP that is unable to bind and inhibit 14-3-3s [23] (Fig. 1). Mutant difopein-eYFP contains two mutations of acidic residues (D12 and E14) to lysine residues that block binding to 14-3-3s [23].

We then examined if the subcellular localization of LRRK2 was altered in HEK293T cells in the presence of difopein. HEK293T cells cotransfected with LRRK2 and difopein or mutant difopein were immunostained for LRRK2 expression 48 hours after transfection. Under control conditions, only 8.2% of cells showed LRRK2 localization into small, perinuclear puncta, while LRRK2 was localized into perinuclear puncta in 39.0% of cells cotransfected with difopein (Fig. 2). This is in agreement with the previous results showing that alanine mutations in LRRK2 cause loss of 14-3-3 interaction and aggregate formation [7].

14-3-3s regulate LRRK2 phosphorylation *in vitro*

Binding of 14-3-3s to LRRK2 could protect phosphorylated residues and prevent dephosphorylation of LRRK2 by protein phosphatase 1 at S910 and S935 (PPA1) [24]. We examined whether 14-3-3 inhibition by difopein altered LRRK2 phosphorylation at S910 or S935.

We transfected HEK 293T cells with either wildtype or G2019S-LRRK2 and either difopein or mutant difopein as a negative control. After 48 hours, lysates were collected from the cells and western blotted for phosphorylated S910 or S935. We saw a significant decrease in LRRK2 phosphorylation in the presence of the pan-14-3-3

inhibitor. With wildtype-LRRK2, we saw a 79% decrease in S910 phosphorylation and a 78% decrease in S935 phosphorylation compared to control (Fig. 3). The same result was seen with G2019S-LRRK2, as we observed an 80% and 93% decrease in S910 and S935 phosphorylation, respectively, compared to control (Fig. 3).

Conversely, we tested whether 14-3-3θ overexpression could affect LRRK2 phosphorylation at S910 and S935. HEK cells were transfected with LRRK2 and either 14-3-3θ or green fluorescent protein (GFP). After 48 hours, lysates were collected from the cells and immunoblotted for either phosphorylated S910 or S935. 14-3-3θ overexpression caused a significant increase in phosphorylation at both sites. Compared to GFP control, 14-3-3θ overexpression led to a 60% increase in S910 phosphorylation and a 58% increase in S935 phosphorylation of wildtype LRRK2. Similarly, 14-3-3θ overexpression caused a 64% and 57% increase in S910 and S935 phosphorylation, respectively, of G2019S-LRRK2 (Fig. 4) compared to GFP control

We next tested whether 14-3-3s could regulate S910 and S935 phosphorylation of another pathogenic LRRK2 mutant that shows reduced interaction with 14-3-3s. Notably, the R1441G-LRRK2 mutation has been shown to have either reduced binding [8, 9], or no binding to 14-3-3s [7]. We first confirmed by co-immunoprecipitation whether 14-3-3 binding to R1441G-LRRK2 was altered compared to that with wildtype or G2019S-LRRK2. Lysates from HEK293T cells transfected with a myc-tagged wildtype, G2019S, or R1441G-LRRK2 were immunoprecipitated for myc, and then immunoprecipitates were run on a gel and probed for endogenous 14-3-3s. While R1441G-LRRK2 could still immunoprecipitate endogenous 14-3-3s, the amount of 14-3-3s that was immunoprecipitated was significantly reduced compared to that pulled down

with either wildtype or G2019S-LRRK2 (Fig. 5). We then transfected HEK293T cells with R1441G-LRRK2 with and without 14-3-3θ to determine if 14-3-3θ overexpression could alter S910 and S935 phosphorylation of R1441G-LRRK2. Western blots of these lysates confirmed that S910 and S935 phosphorylation was decreased in the R1441G-LRRK2 background, as previously reported [7-9]. 14-3-3θ overexpression did not increase phosphorylation at either serine site (Fig. 6). This suggests that binding of 14-3-3s directly to LRRK2 is required to promote phosphorylation at S910 and S935.

14-3-3s regulate LRRK2 phosphorylation *in vivo*

We next tested the effects of 14-3-3s on LRRK2 phosphorylation in mice. We first measured S910 and S935 phosphorylation in transgenic mice expressing difopein-eYFP under the Thy1.2 promoter. The difopein mouse demonstrated high levels of difopein-eYFP expression in the hippocampus and other brain regions (Fig. 7). Hippocampal lysates from two month old difopein mice, as well as their nontransgenic littermates, were immunoblotted for phosphorylated S935. In corroboration with the HEK cell data, we observed an 85% decrease in S935 phosphorylation in difopein mice compared to nontransgenic mice (Fig. 8).

We also examined S935 phosphorylation in transgenic mice expressing HA-tagged 14-3-3θ under the Thy-1.2 promoter. Two of the 14-3-3θ founder lines demonstrated high expression of HA-tagged 14-3-3θ in the cortex and hippocampus, with the M5 line showing particularly high levels of exogenous HA-tagged 14-3-3θ in the hippocampus (Fig. 9). While HA-tagged 14-3-3θ expression increased over time in these

mice due to the Thy1.2 promoter, expression was detected early postnatally in the M5 line. Hippocampal lysates from 14-3-3θ mice showed a 2.8-fold increase in S935 phosphorylation compared to lysates from nontransgenic littermates (Fig. 10).

Discussion

In this study, we demonstrate that 14-3-3s can regulate several aspects of LRRK2 biology, including phosphorylation and localization. Disrupting the interaction between LRRK2 and 14-3-3s with the pan-14-3-3 inhibitor difopein leads to LRRK2 aggregation, similar to what other groups have also seen inserting S910A and/or S935A mutations into LRRK2 [7]. 14-3-3θ overexpression increased LRRK2 phosphorylation at S910 and S935 in both wildtype and G2019S-LRRK2 both *in vitro* and *in vivo*, while 14-3-3 inhibition by difopein reduced S910 and S935 phosphorylation. Notably, the effect of 14-3-3θ overexpression on S910 and S935 phosphorylation in R1441G was muted. Since we observed much reduced interaction between 14-3-3s and the R1441G mutant, this suggests that 14-3-3s must bind to LRRK2 to promote or protect LRRK2 phosphorylation at S910 and S935.

Our findings that 14-3-3 inhibition with difopein caused LRRK2 redistribution into perinuclear puncta is consistent with what other previous groups had demonstrated with S910A and S935 LRRK2 mutants [7]. As shown by co-immunoprecipitation, difopein disrupted the interaction between LRRK2 and endogenously expressed 14-3-3s in the HEK cells, confirming that using difopein does indeed prevent the interaction between LRRK2 and 14-3-3s. These findings suggest that 14-3-3s control LRRK2

subcellular distribution. Alternatively, 14-3-3s may help to keep LRRK2 solubilized, and in the presence of 14-3-3 inhibition, LRRK2 can form insoluble aggregates. Notably, the chaperone-like activity of 14-3-3s has been shown to reduce aggregation of proteins [25, 26]. For example, work by Sluchanko et al. has shown that monomeric 14-3-3ζ prevents the aggregation of insulin, alcohol dehydrogenase and phosphorylase kinase under stress conditions that induce aggregation [25]. This chaperone effect was shown to be comparable or even higher than normal heat shock proteins that are normally responsible for preventing aggregation [25]. Specific to PD, 14-3-3η has been shown to reduce alpha-synuclein aggregation intermediates, reducing both aggregation of and toxicity caused by alpha-synuclein [26]. This highlights another role for 14-3-3s in that they can potentially reduce toxicity and either prevent or at least slow the progression of PD.

Next, we decided to examine whether 14-3-3s can regulate LRRK2 phosphorylation. Other groups have identified proteins involved in phosphorylating LRRK2, such as protein kinase A [8] and IκB [27], while another group has shown that inhibition of a protein phosphatase (PP1) is responsible for dephosphorylating LRRK2 and that when PP1 is inhibited, S910 and S935 phosphorylation in LRRK2 increases and binding with 14-3-3s is restored [24]. We surmised that perhaps 14-3-3s could play a role in altering the conformation of LRRK2 and could potentially protect these serine sites from dephosphorylation by PP1 or could enhance phosphorylation by exposing these sites to PKA or IκB. Notably, S910 and S935 phosphorylation of R1441G-LRRK2 was unchanged when 14-3-3θ was co-transfected in HEK cells. This may indicate that because there is reduced interaction between R1441G-LRRK2 and 14-3-3s, more unbound LRRK2 may be subject to dephosphorylation by PP1. Alternatively, because

14-3-3s are not binding to R1441G-LRRK2, LRRK2 conformation is not optimal for being phosphorylated by PKA or IκB.

These initial experiments showed that 14-3-3 levels have a profound impact on LRRK2. When 14-3-3θ was overexpressed both *in vitro* and *in vivo* we saw an increase in phosphorylation of the two key serine sites required for 14-3-3 binding to LRRK2. Conversely, when difopein was used to inhibit all endogenous 14-3-3s, we saw the phosphorylation of S910 and S935 in LRRK2 decrease. This suggests that perhaps 14-3-3s, and specifically the 14-3-3θ isoform, play a role in altering LRRK2 conformation to either prevent dephosphorylation by PP1 or enhance phosphorylation by PKA and/or IκB. Notably, disrupting the interaction with difopein not only causes changes in phosphorylation, but leads to the formation of LRRK2 intracellular aggregates in the cytosol. Since 14-3-3s have a noted role in subcellular localization of other proteins [28, 29], it is likely that 14-3-3s are important in maintaining a diffuse, cytosolic pattern of LRRK2 expression. In summary, this data demonstrates that 14-3-3s play a large role in regulating the phosphorylation and localization of LRRK2.

Materials and Methods

Cell transfection. HEK 293T cells were grown in DMEM containing 10% normal calf serum with 1% penicillin/streptomycin. 24 hours after plating, cells were transfected using Superfect transfection reagent (Qiagen, Germantown, MD) using manufacturer's guidelines. After transfection, cells were incubated in fresh media for 48 hours prior to protein collection.

Immunoblotting. HEK 293T cells or primary cultured neurons were washed in phosphate buffer saline (PBS) and pelleted at 1500x*g* for five minutes. Cell pellets were then sonicated in lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)), followed by centrifugation at 16,000x*g* for 10 minutes. Protein concentrations were assessed by BCA assay (Pierce, Rockford, IL). Samples were boiled for five minutes in DTT sample loading buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 200 mM DTT, 30% glycerol, Bromophenol Blue), resolved on 7.5 or 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking in 5% non-fat dry milk in TBST (25 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20), membranes were incubated overnight in rabbit polyclonal antibody against pan-14-3-3 (1:1000 Abcam, Cambridge, MA), rabbit polyclonal antibody against GFP (1:5000 Abcam), mouse monoclonal antibody against HA (1:1000 Covance, Princeton, NJ), rabbit polyclonal antibody against LRRK2 (1:1000 Abcam), or rabbit polyclonal antibody against alpha-tubulin at 4°C. Membranes were then incubated in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:2000 Jackson ImmunoResearch, West Grove, PA) for two hours and then washed in TBST six times for ten minutes each. Blots were developed with enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ). Images were scanned and analyzed using Un-ScanIT software (Orem, UT) for densitometric analysis of bands.

Hippocampi from mouse brains was homogenized in lysis buffer (Tris/HCl 50mM pH7.4, NaCl 175mM, EDTA 5mM, protease inhibitor and phosphatase inhibitor cocktails) and sonicated for 10 seconds. Cell lysates were then incubated on ice for 30

minutes after the addition of 1% Triton X-100 and then spun at 15000x*g* for one hour at 4°C. The supernatant was saved as the Triton X-100 soluble fraction. Samples were resolved on SDS-polyacrylamide gels, and analyzed by western blotting as described above**.**

Immunoprecipitation. For immunoprecipitation of HA-tagged LRRK2, Protein G Dynabeads (Life Technologies, Grand Island, NY) were incubated with 4µg mouse HA antibody (Sigma, St. Louis, MO) overnight. 500µg of cell lysate was incubated with antibody-conjugated beads for 30 minutes at room temperature. Beads were then washed five times in PBS with 0.02% Tween. After washing, beads were boiled in DTT sample loading buffer and loaded on a SDS-polyacrylamide gel. After transfer to nitrocellulose, the membrane was probed for 14-3-3 proteins using a polyclonal rabbit antibody against 14-3-3s (Abcam).

Generation of 14-3-3θ transgenic line. Mice were used in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC). Human 14-3-3θ tagged with a hemagglutinin (HA) epitope tag at the C-terminal end was cloned into a Thy1.2 expression cassette [30] to drive neuronal expression of 14-3-3θ. Following digestion with NdeI and EcoRI, the Thy1.2 construct containing HA-tagged 14-3-3θ was purified and microinjected into C57BL/6 fertilized mouse oocytes. Founder mice were bred with C57BL/6 mice from Jackson labs, and pups were examined for expression of HA-tagged 14-3-3θ in mouse brain by both immunohistochemistry and Western blotting. Two of the

founder lines showed diffuse neuronal expression of HA-tagged 14-3-3θ (M5 and F1 lines). The M5 line was used for experiments in this paper due to high expression levels in the hippocampus. Hemizygous transgenic mice were identified by genotyping using the following primers: forward primer 5' ATCTCAAGCCCTCAAGGTAAATG, and reverse primer 5' CTCCACTTTCTCCCGATAGTCC.

Other mouse lines. BAC wildtype and G2019S-LRRK2 hemizygous transgenic mice [31] were backcrossed on a C57BL/6 background and were bred with wildtype C57BL/6 mice from Jackson labs (Bar Harbor, ME). Hemizygous difopein-YFP transgenic mice were obtained from the lab of Yi Zhou [32].

Statistical analysis. GraphPad Prism 6 (La Jolla, CA) was used for statistical analysis of experiments. Kinase assays, western blot experiments, and neurite analyses were analyzed by either student t-test or by 1-way ANOVA, followed by post-hoc pairwise comparisons using Tukey's multiple comparison test.

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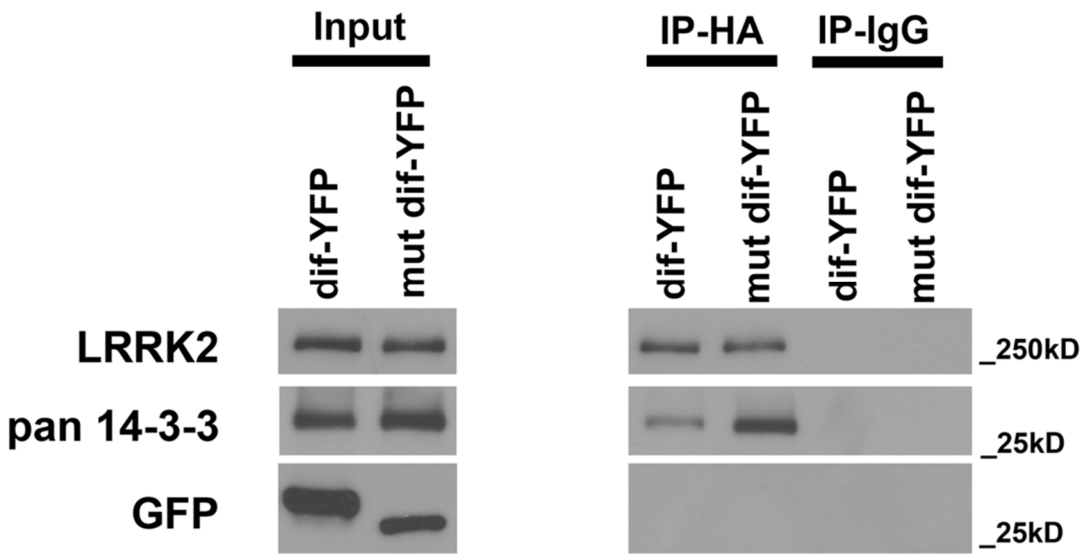

Figure 1. Difopein disrupts the interaction between LRRK2 and 14-3-3s

Western blots from coimmunoprecipitation experiments of endogenous 14-3-3 interaction with HA-tagged LRRK2 protein. Difopein-eYFP migrates slightly higher than mutant difopein-eYFP since difopein has two R18 peptide sequences while the mutant difopein peptide has one copy of the mutated R18 peptide sequence [23].

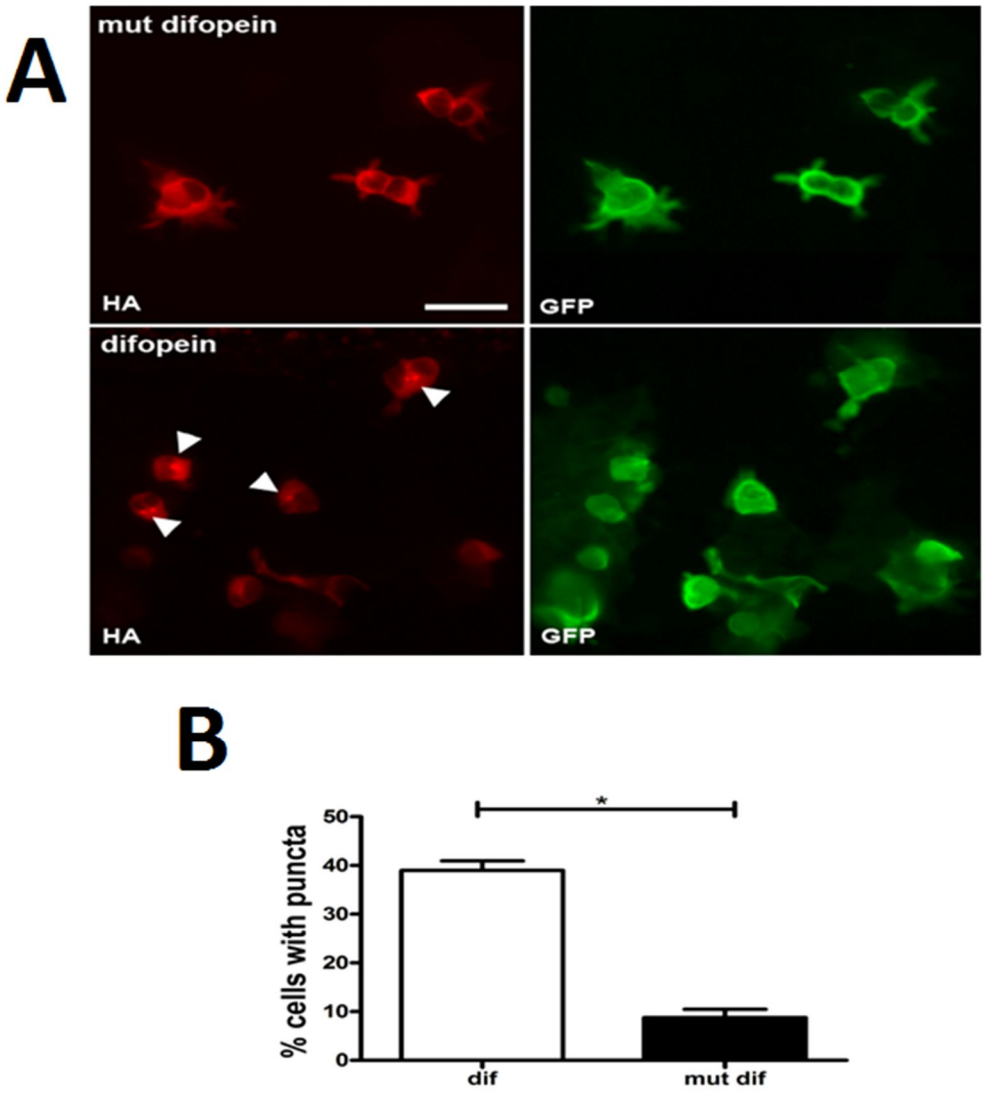

Figure 2. 14-3-3 inhibition with difopein causes LRRK2 aggregation

A) Immunostaining for HA-tagged LRRK2 and eYFP-difopein or eYFP-mutant difopein in transfected HEK293 cells shows robust protein expression and the formation of LRRK2 aggregates with difopein treatment as opposed to mutant difopein treatment. Scale bar = 100µm.

B) Quantification of the number of cells with LRRK2 aggregates shows difopein treatment increases LRRK2 aggregation.

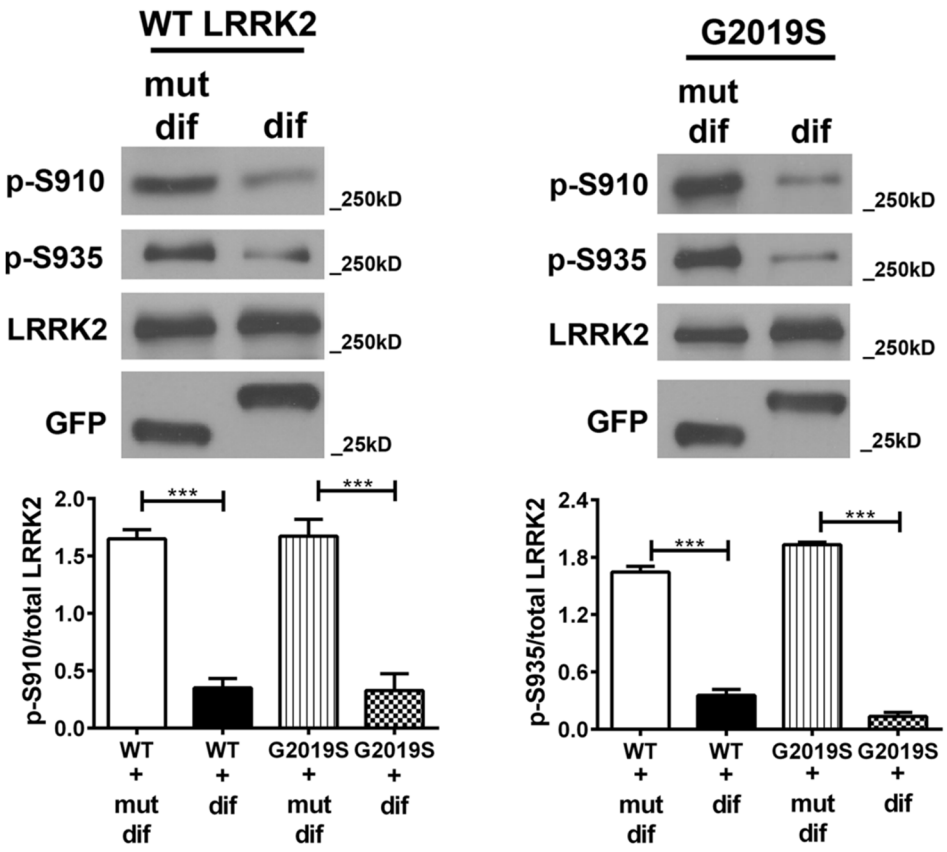

Figure 3. 14-3-3s inhibition decreases LRRK2 phosphorylation at serine 910 and 935

Lysates from HEK 293T cells transfected with difopein and either wildtype or G2019S-LRRK2 were analyzed for LRRK2 phosphorylation at S910 and S935 and total LRRK2 by western blot. Representative western blots are shown. The ratio of phosphorylated LRRK2 to total LRRK2 is quantified from three independent experiments. ***$p<0.001$ (unpaired t-test).

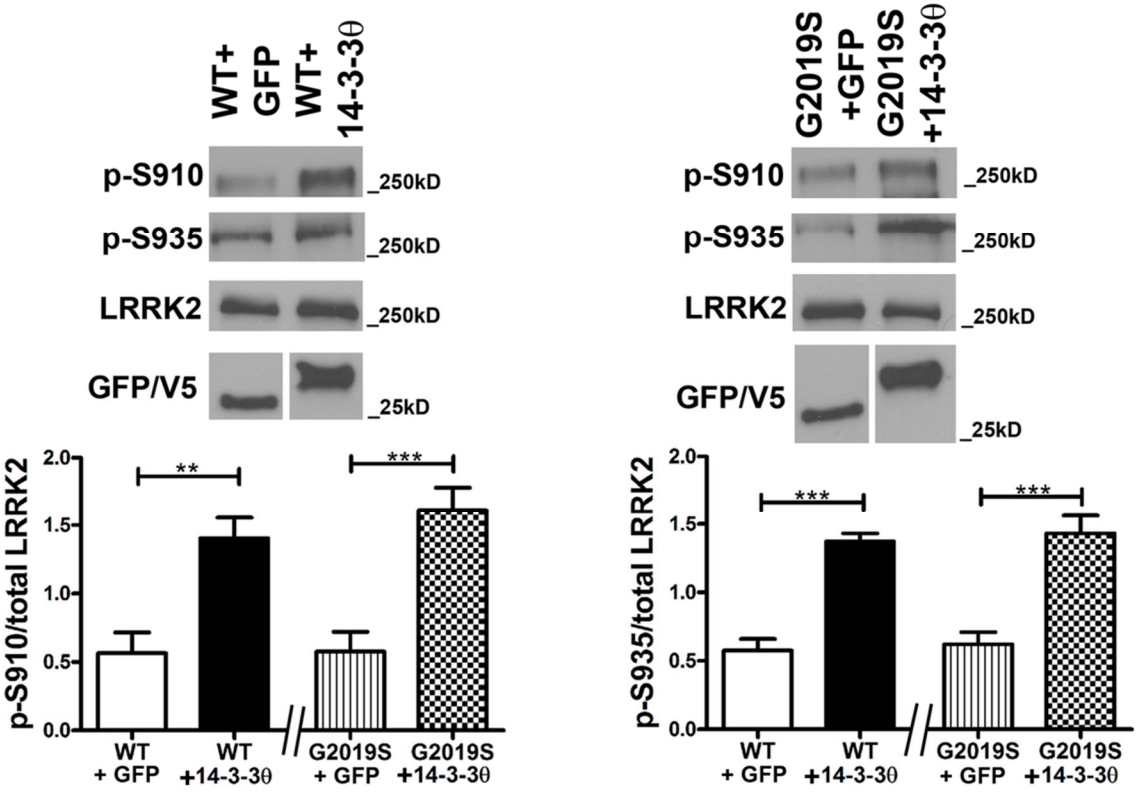

Figure 4. 14-3-3θ overexpression increases LRRK2 S910 and S935 phosphorylation

Lysates from HEK 293T cells transfected with GFP or 14-3-3θ and either wildtype or G2019S-LRRK2 were analyzed for LRRK2 phosphorylation at S910 and S935 and total LRRK2 by western blot. n=3 independent rounds, **p<0.01, ***p<0.001 (unpaired t-test).

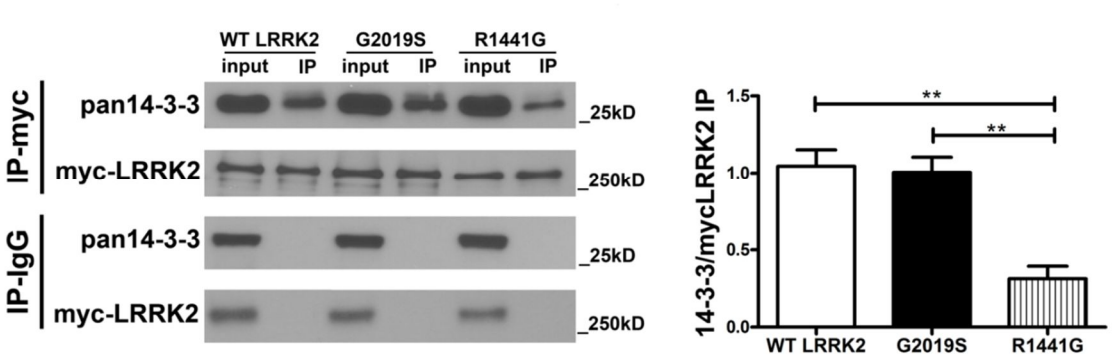

Figure 5. R1441G-LRRK2 has reduced interaction with 14-3-3s

Cell lysates from HEK 293T cells transfected with myc-tagged wildtype, G2019S, or R1441G-LRRK2 were immunoprecipitated with a monoclonal antibody against myc, and resulting immunoprecipitants were analyzed by western blot with a polyclonal rabbit antibody against endogenous 14-3-3s (pan). The ratio of pan 14-3-3 to myc LRRK2 was quantified for three independent rounds. **$p<0.01$ (Tukey's multiple comparison test).

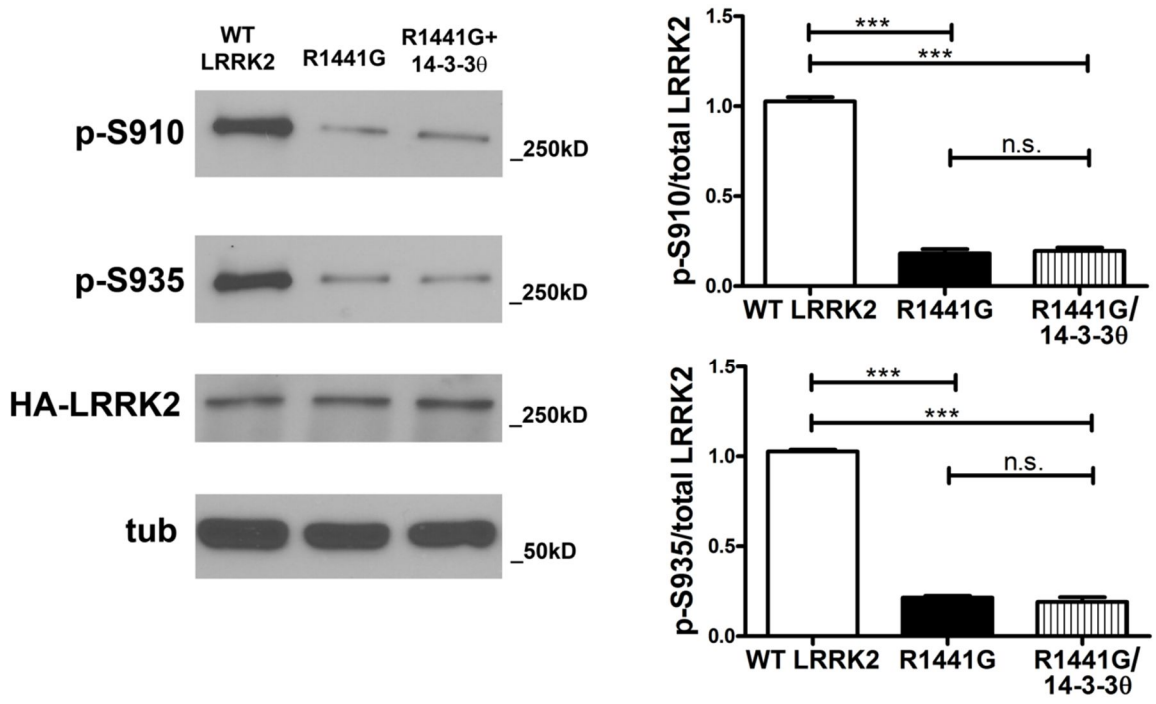

Figure 6. 14-3-3θ overexpression does not alter R1441G-LRRK2 S910 and S935 phosphorylation

Lysates from HEK 293T cells transfected with V5-tagged 14-3-3θ and either wildtype or R1441G-LRRK2 were analyzed for LRRK2 phosphorylation at S910 and S935 and total LRRK2 by western blot. The ratio of phosphorylated LRRK2 to total LRRK2 was quantified for three independent rounds. ***$p<0.001$, ns=not significant (Tukey's multiple comparison test).

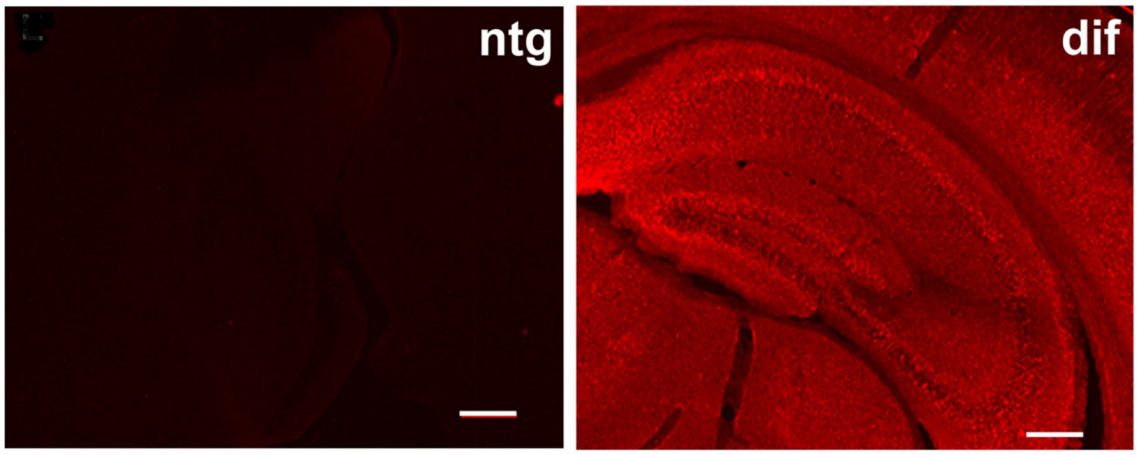

Figure 7. Immunohistochemistry for difopein expression in difopein-e-YFP mice

Immunohistochemistry for difopein-eYFP in coronal sections through the hippocampus of difopein mice and nontransgenic mice. Scale bar = 500µm.

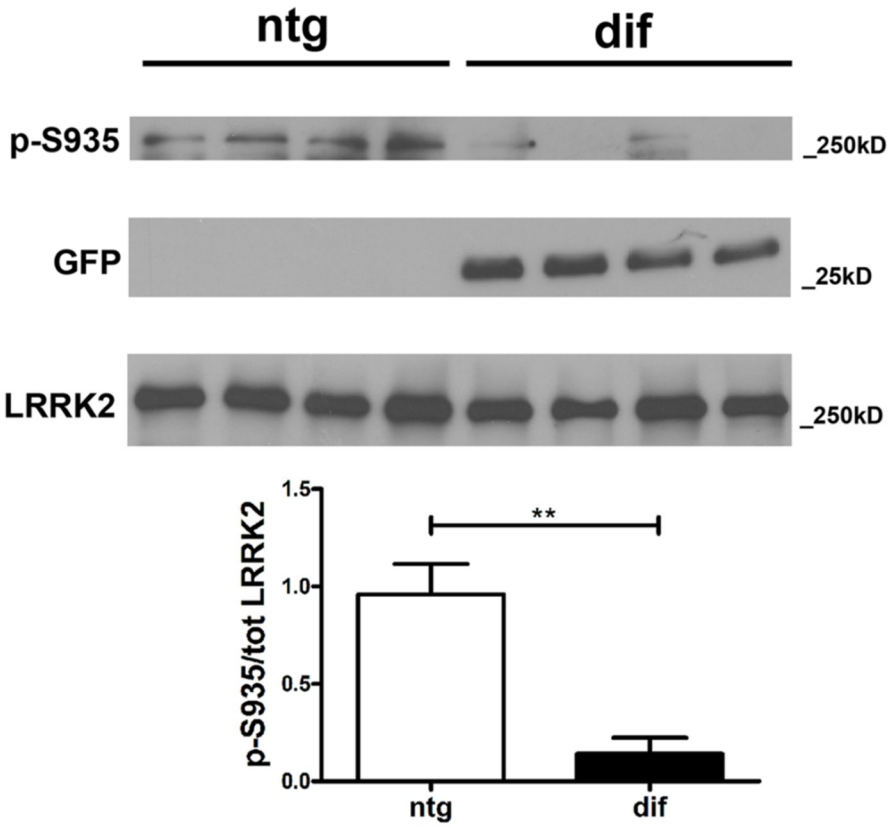

Figure 8. Difopein expression reduces LRRK2 S935 phosphorylation

Quantification of S935 phosphorylation in hippocampal lysates from wildtype and difopein-eYFP transgenic mice. n=4 mice per group, **p<0.01 (unpaired t-test).

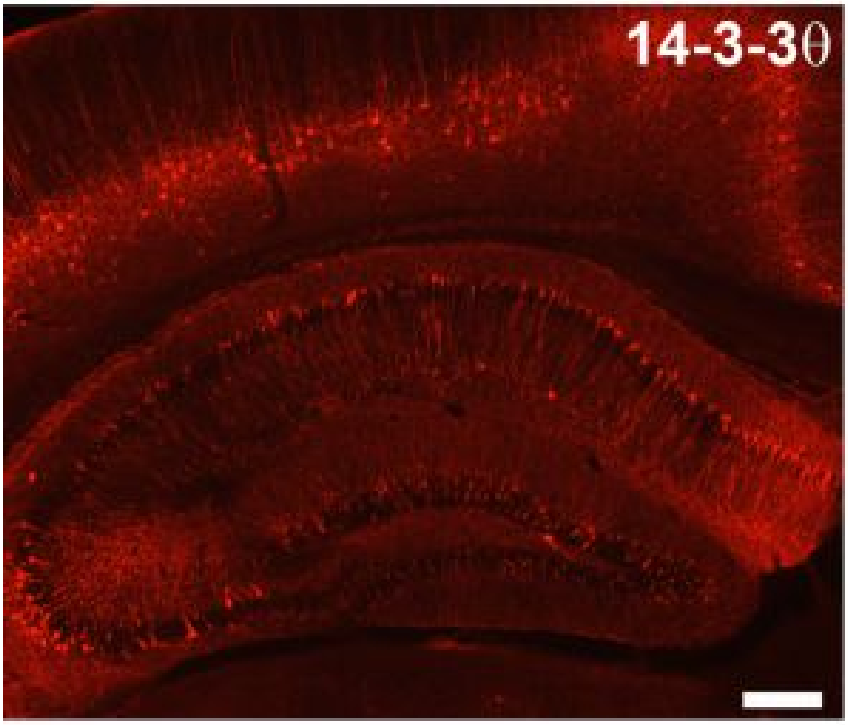

Figure 9. Immunohistochemistry showing robust 14-3-3θ hippocampal expression

Immunohistochemistry for HA-tagged 14-3-3θ in coronal brain section through the hippocampus from a 14-3-3θ mouse. Scale bar = 200μm.

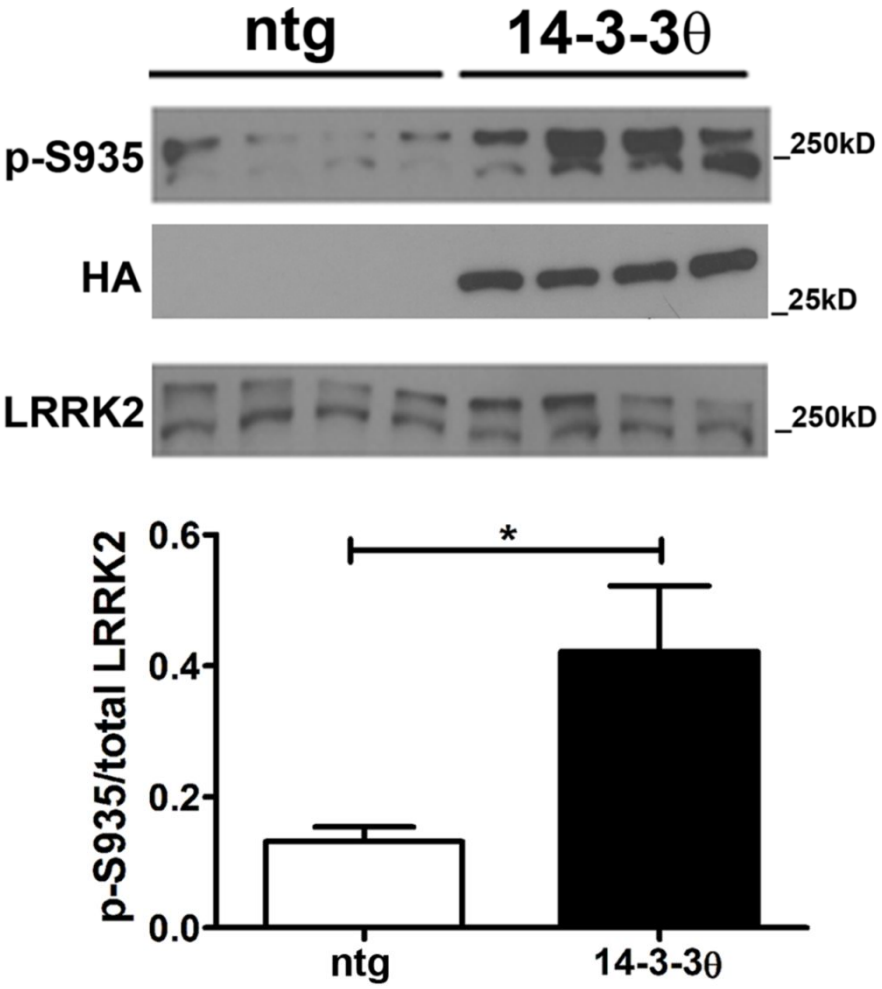

Figure 10. 14-3-3θ overexpression increases LRRK2 S935 phosphorylation *in vivo*

Representative western blots and quantification of S935 phosphorylation in cortical lysates generated from transgenic mice overexpressing 14-3-3θ. n=4 mice per group, *$p<0.05$ (unpaired t-test).

14-3-3s Regulate G2019S-LRRK2-Induced Neurite Shortening

Introduction

Mutations in *leucine-rich repeat kinase 2 (LRRK2*) are the most common known genetic cause of PD with the G2019S mutation as the most common known pathogenic mutation [1-4]. Animal models of this LRRK2 mutation have shown one consistent phenotype: reduced neurite outgrowth [5-8]. G2019S-LRRK2 has been shown to reduce neurite outgrowth both *in vitro* and *in vivo* by multiple groups [5-9]. In fact, several other LRRK2 mutations have also been shown to consistently cause neurite shortening, including the R1441G, Y1699C and I2020T mutations [5, 9, 10]. Thus, neurite outgrowth has become one of the more consistent measures of LRRK2 toxicity. This may be through alterations in tubulin as LRRK2 has been shown to affect neurite outgrowth through regulation of tubulin [11-13].

One set of proteins that have recently been identified as potent interactors of LRRK2 are the 14-3-3 family of proteins. 14-3-3s have been shown to be protective in other models of PD, including reducing the toxic effects of the neurotoxins rotenone and MPP+, as well as reducing dopaminergic cell loss in a *C. elegans* model of PD [14]. This has been supplemented with data demonstrating that 14-3-3θ overexpression provides neuroprotection in a MPTP mouse model, with 14-3-3 inhibition increasing neurotoxicity [15]. 14-3-3s have been shown to also regulate neurite outgrowth through various mechanisms including regulation of the cell adhesion molecules L1 and neural cell adhesion molecule (NCAM), actin depolymerizing factor (ADF), neuron navigator 2 (NAV2), and SLIT and NTRK-like family member 1 (SLITRK1) [16-20].

Taken together, the known neuroprotective effects of 14-3-3s suggest that overexpression of 14-3-3s can reduce mutant LRRK2 mediated toxicity. We hypothesize that 14-3-3θ overexpression will reverse LRRK2 mediated toxicity as measured by neurite outgrowth. We focus on the theta isoform, as previous work has shown it to consistently confer the most protection in multiple models of PD. Conversely, we hypothesize that 14-3-3 inhibition will potentiate neurite shortening seen with LRRK2 mutations.

Results

14-3-3θ overexpression is protective against G2019S-LRRK2-induced neurite shortening in primary neuronal cultures

Several disease-causing mutations, including the G2019S-LRRK2 mutation, cause a decrease in arborization and overall neurite length in primary hippocampal and cortical cultures [5, 8, 9, 21-23]. We confirmed total primary neurite shortening in neuronal cultures from a BAC human G2019S-LRRK2 transgenic mouse line that demonstrates the highest level of G2019S-LRRK2 expression in the hippocampus [5, 6]. To test whether 14-3-3θ overexpression can reverse the effects of the G2019S-LRRK2 mutation on neurite length, we crossed our hemizygous 14-3-3θ overexpressing mice (Fig. 1A, B) with the BAC G2019S-LRRK2 mice and prepared primary hippocampal cultures from pups at postnatal day zero (P0). Primary hippocampal neurons demonstrated cytoplasmic expression of exogenous HA-tagged 14-3-3θ expression, as shown by immunocytochemistry at day-*in-vitro* (DIV) eight (Fig. 2). 14-3-3θ

overexpression in the BAC G2019S-LRRK2 mouse did not alter LRRK2 transgene expression levels (Fig. 3A). Levels of 14-3-3θ overexpression in primary cultures from double transgenic mice were sufficient to cause an increase in S935 phosphorylation in LRRK2 (Fig. 3A, B), suggesting that the amount of 14-3-3θ expression was sufficient to induce biochemical changes in LRRK2. When 14-3-3θ was overexpressed alone, there was no noticeable effect on neurite length compared to nontransgenic cultures (Fig. 4A-E). In cultures from double transgenic mice, 14-3-3θ overexpression reversed the neurite shortening due to G2019S-LRRK2 expression (Fig. 4A-E).

14-3-3 inhibition exacerbates neurite shortening by the G2019S-LRRK2 mutation

We next investigated whether 14-3-3 inhibition would exacerbate neurite shortening induced by G2019S-LRRK2 expression. Neuronal cultures from G2019S-LRRK2 mice were transduced with a tetracycline-inducible lentivirus expressing either difopein-eYFP or mutant difopein-eYFP (control virus) [24], and difopein expression was induced with doxycycline (2 μg/ml) the next day. Mutant difopein-eYFP contains two mutations of acidic residues (D12 and E14) to lysine residues that block binding to 14-3-3s [25]. Difopein expression alone in nontransgenic neurons caused reduced neurite length compared to nontransgenic neurons expressing control mutant difopein (Fig. 5). Difopein expression in G2019S-LRRK2 neurons additionally decreased total neurite length by 25% compared to G2019S-LRRK2 neurons also expressing the control mutant difopein (Fig. 5).

We further confirmed these findings by crossing a transgenic mouse expressing difopein-eYFP [26] with the G2019S-LRRK2 mouse. The difopein mouse demonstrated high levels of difopein-eYFP expression in the hippocampus and other brain regions (Fig. 6A). Primary hippocampal neurons from difopein-eYFP mice showed diffuse, cytoplasmic expression of difopein-eYFP, as demonstrated by immunocytochemistry at DIV8 (Fig. 6B). LRRK2 transgene expression was unchanged when the BAC G2019S-LRRK2 mouse was crossed with the difopein-eYFP mouse. S935 phosphorylation of LRRK2 was reduced in cultures from these double transgenic mice (Fig. 7). Consistent with other experiments (Fig. 5), neurons from G2019S-LRRK2/difopein double transgenic mice showed a 16% decrease in total neurite length compared to neurons from G2019S-LRRK2 mice (Fig. 8A-E).

Difopein is a pan-14-3-3 inhibitor that binds and inhibits all seven 14-3-3 isoforms. To test whether inhibition of the 14-3-3θ isoform is sufficient to promote G2019S-LRRK2-induced neurite shortening, we tested the effect of a dominant negative (DN) 14-3-3θ mutant (R56A/R60A) [27] on G2019S-LRRK2-induced neurite shortening. 14-3-3θ forms both homodimers and heterodimers with only a subset of 14-3-3 isoforms [28-31], such that the effects of DN 14-3-3θ is limited to only 14-3-3θ and those isoforms with which it can heterodimerize, allowing the other six isoforms to dimerize with one another. We crossed the G2019S-LRRK2 mouse line with a transgenic mouse that overexpresses HA-tagged DN 14-3-3θ under the Thy1.2 promoter. This DN 14-3-3θ mouse showed expression of DN 14-3-3θ in the hippocampus (Fig. 9A), and LRRK2 transgene expression was unchanged in primary cultures from double transgenic mice when the BAC G2019S-LRRK2 mouse was crossed with the DN 14-3-3θ mouse (Fig.

9B). Neurons from G2019S-LRRK2/DN 14-3-3θ double transgenic mice showed a 23% reduction in total neurite length compared to neurons from G2019S-LRRK2 mice (Fig. 9C). Expression of DN 14-3-3θ alone caused a 17.3% reduction in total neurite length compared to that from nontransgenic neurons (Fig. 9C). These data indicated that inhibition of 14-3-3θ is sufficient to cause reduced neurite length, highlighting the importance of this particular isoform.

14-3-3θ overexpression is protective against the R1441G mutation

While G2019S is the most common *LRRK2* mutation found in PD patients, there are several other mutants that cause autosomal dominant PD, including R1441G-LRRK2. R1441G-LRRK2 has been shown by other groups to have reduced interaction [32, 33] or no interaction [34] with 14-3-3s compared to wildtype or G2019S-LRRK2. We observed that endogenous 14-3-3s co-immunoprecipitated with myc-tagged R1441G-LRRK2 expressed in HEK293T cells, but at reduced levels when compared to wildtype LRRK2 (Fig. 5 in previous chapter). Similarly, in hippocampal lysates from age-matched BAC wildtype, G2019S, or R1441G-LRRK2 mice, we observed a similar reduction in 14-3-3 co-immunoprecipitation with R1441G-LRRK2 compared to wildtype or G2019S-LRRK2, although the interaction was not completely abolished (Fig. 10). We also observed a reduction in S910 and S935 phosphorylation in R1441G-LRRK2 lysates, which was not reversed by 14-3-3θ overexpression (Fig. 6 in previous chapter).

To test if 14-3-3θ could reverse neurite shortening by R1441G-LRRK2 despite reduced interaction with R1441G-LRRK2, we crossed our 14-3-3θ transgenic mice with

a BAC R1441G-LRRK2 mouse [10]. As previously reported [10], we observed that hippocampal neurons from BAC R1441G-LRRK2 mice showed reduced neurite length (Fig. 11). 14-3-3θ overexpression reversed this neurite shortening by R1441G-LRRK2 (Fig. 11).

LRRK2-independent effects of 14-3-3 inhibition on neurite growth

Both difopein and DN 14-3-3θ caused neurite shortening not only in cultures from G2019S-LRRK2 mice but also in cultures from nontransgenic mice. As hippocampal neurons express endogenous LRRK2 [35, 36], we hypothesized that inhibition of 14-3-3s could allow unmasking of endogenous wildtype LRRK2 to affect neurite length. To test if difopein's effect in nontransgenic cultures is dependent upon the presence of endogenous LRRK2, we examined the effect of difopein on neurite length in the LRRK2 null background. Difopein transgenic mice were crossed with LRRK2 heterozygous knockout (+/-) mice, and hippocampal cultures from the resulting mice were analyzed for neurite length. LRRK2 knockout (LRRK2 -/-) cultures showed a mild increase in total neurite length compared to wildtype (LRRK2 +/+) cultures as previously reported [5, 9, 22]. Difopein caused a similar reduction in neurite length in both wildtype (LRRK2 +/+) and LRRK2 -/- cultures (Fig. 12A). This finding suggests that difopein can reduce neurite length through a LRRK2-independent mechanism.

To test whether DN 14-3-3θ's effect on neurites was dependent on endogenous LRRK2, we crossed DN 14-3-3θ mice with LRRK2 +/- mice. DN 14-3-3θ expression caused a reduction in neurite length, even in the absence of LRRK2 (Fig. 12B).

Discussion

In this study, we demonstrate that 14-3-3s can regulate neurite shortening induced by mutant LRRK2. 14-3-3θ overexpression reversed neurite shortening induced by G2019S or R1441G-LRRK2 mutations, while inhibition of all 14-3-3 isoforms promoted further neurite shortening. 14-3-3θ inhibition with the dominant negative 14-3-3θ similarly enhanced neurite shortening by G2019S-LRRK2, suggesting that inhibition of the 14-3-3θ isoform is sufficient to affect mutant LRRK2-mediated neurite shortening.

The interaction between 14-3-3s and LRRK2 is well established, but the biological significance of this interaction with regards to LRRK2 kinase activity and function is not clear [32-34]. Our findings demonstrate that 14-3-3s can regulate LRRK2 activity and function so that alterations in 14-3-3 binding to LRRK2 may be important in LRRK2-linked disease. Several LRRK2 mutants show reduced binding to 14-3-3s [32-34] that could lead to increased LRRK2 kinase activity and thereby toxicity. In addition, reduced 14-3-3 expression and function has been demonstrated in PD models and human PD [24, 37-39] and could potentially lead to increased LRRK2 kinase activity that could contribute to the neurodegenerative process.

We have previously evaluated the effects of 14-3-3s in several other PD model systems. Overexpression of 14-3-3θ reduces toxicity of both rotenone and 1-methyl-4-phenylpyridinium (MPP^+) *in vitro,* reduces dopaminergic cell loss in an invertebrate alpha-synuclein model, and promotes earlier striatal dopamine metabolite recovery in MPTP-treated mice [15, 24, 40, 41]. Our data expands the evidence for the

neuroprotective effects of 14-3-3s in PD into pathways relevant to late-onset PD and suggests that 14-3-3s may serve as a therapeutic target for intervention in both idiopathic and genetic forms of disease. Indeed, several commercially available drugs induce 14-3-3 expression at both the mRNA and protein levels – suggesting the potential of small molecule treatments to enhance 14-3-3 expression [42-46].

In our study, we demonstrated that 14-3-3θ overexpression can reduce the neurite shortening effects associated with both G2019S and R1441G-LRRK2 expression. G2019S-LRRK2 interacts with 14-3-3s as we and others [32, 34, 47] have observed. R1441G-LRRK2 either does not bind endogenous 14-3-3s [33, 34] or shows dramatically reduced interaction with 14-3-3s depending on the experimental parameters [32, 48]. Here we did detect endogenous 14-3-3 interaction with R1441G-LRRK2, although at reduced levels compared to that of wildtype or G2019S-LRRK2 (Fig 5A-C). Our data are consistent with a model in which 14-3-3s directly interact with mutant LRRK2 to reduce kinase activity.

An interesting finding in our study was that difopein caused neurite shortening in nontransgenic cultures. 14-3-3 proteins have been shown to regulate both axonal and dendritic growth through several mechanisms, including regulation of the cell adhesion molecules L1 and neural cell adhesion molecule (NCAM), actin depolymerizing factor (ADF), neuron navigator 2 (NAV2), and SLIT and NTRK-like family member 1 (SLITRK1) [16-20]. 14-3-3s have also been implicated in protecting phosphorylation sites on Raf1 and MEK, leading to persistent activation of the MEK-ERK pathway which plays a role in neurite outgrowth as well [49-52]. Interestingly, different isoforms cause differential effects on neurite length, with some promoting extension and others causing

retraction. In our study, we observed that inhibition of all 14-3-3s with difopein caused shortened neurites, suggesting that the sum effect of all endogenous 14-3-3s is to promote elongation.

As difopein's effect on neurite outgrowth occurs in wildtype and LRRK2 knockout cultures in a similar manner, it is reasonable to conclude that 14-3-3s affect neurite outgrowth through LRRK2-independent mechanisms. However, these results do not preclude the possibility of 14-3-3s regulating LRRK2 effects on neurite growth. 14-3-3's effects on neurite growth are complex and involve several mechanisms, and in the presence of mutant LRRK2, 14-3-3s can regulate mutant LRRK2-mediated neurite effects through regulation of kinase activity.

In conclusion, our studies reveal that 14-3-3s can regulate mutant LRRK2 action on neurite outgrowth. 14-3-3θ overexpression reduces mutant LRRK2 neurite effects, while 14-3-3 inhibition enhances LRRK2 neurite shortening. Therefore, increasing the expression of 14-3-3 proteins may provide a new therapeutic avenue to addressing PD caused by LRRK2 mutations.

Material and Methods

Cell transfection. HEK 293T cells were grown in DMEM containing 10% normal calf serum with 1% penicillin/streptomycin. 24 hours after plating, cells were transfected using Superfect transfection reagent (Qiagen, Germantown, MD) using manufacturer's guidelines. After transfection, cells were incubated in fresh media for 48 hours prior to protein collection.

Immunoblotting. HEK 293T cells or primary cultured neurons were washed in phosphate buffered saline (PBS) and pelleted at 1500x*g* for five minutes. Cell pellets were then sonicated in lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)), followed by centrifugation at 16,000x*g* for 10 minutes. Protein concentrations were assessed by BCA assay (Pierce, Rockford, IL). Samples were boiled for five minutes in DTT sample loading buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 200 mM DTT, 30% glycerol, Bromophenol Blue), resolved on 7.5 or 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking in 5% non-fat dry milk in TBST (25 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20), membranes were incubated overnight in rabbit polyclonal antibody against pan-14-3-3 (1:1000 Abcam, Cambridge, MA), rabbit polyclonal antibody against GFP (1:5000 Abcam), mouse monoclonal antibody against HA (1:1000 Covance, Princeton, NJ), rabbit polyclonal antibody against LRRK2 (1:1000 Abcam), and rabbit polyclonal antibody against alpha-tubulin (1:2500 Cell Signaling, Danvers, MA). Membranes were then incubated in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:2000 Jackson ImmunoResearch Laboratories, West Grove, PA) for two hours and then washed in TBST six times for ten minutes each. Blots were developed with enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ). Images were scanned and analyzed using Un-ScanIT software (Orem, UT) for densitometric analysis of bands.

Hippocampi and cortices from mouse brains were homogenized in lysis buffer (Tris/HCl 50mM pH7.4, NaCl 175mM, EDTA 5mM, protease inhibitor and phosphatase inhibitor cocktails) and sonicated for 10 seconds. Cell lysates were then incubated on ice

for 30 minutes after the addition of 1% Triton X-100 and then spun at 15000x*g* for one hour at 4°C. The supernatant was saved as the Triton X-100 soluble fraction. Samples were resolved on SDS-polyacrylamide gels and analyzed by western blotting as described above**.**

Immunoprecipitation. For immunoprecipitation of myc-tagged LRRK2, Protein G Dynabeads (Life Technologies, Grand Island, NY) were incubated with 4µg mouse myc antibody (Thermo Fisher, Carlsbad, CA) overnight. 500µg of cell lysate was incubated with antibody-conjugated beads for 30 minutes at room temperature. Beads were then washed five times in PBS with 0.02% Tween. After washing, beads were boiled in DTT sample loading buffer and loaded on a SDS-polyacrylamide gel. After transfer to nitrocellulose, the membrane was probed for 14-3-3 proteins using a polyclonal rabbit antibody against 14-3-3s (1:1000 Abcam).

Generation of 14-3-3θ transgenic line. Mice were used in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC). Human 14-3-3θ tagged with an HA epitope tag at the C-terminal end was cloned into a Thy1.2 expression cassette [53] to drive neuronal expression of 14-3-3θ. Following digestion with NdeI and EcoRI, the Thy1.2 construct containing HA-tagged 14-3-3θ was purified and microinjected into C57BL/6 fertilized mouse oocytes. Founder mice were bred with C57BL/6 mice from Jackson labs, and pups were examined for expression of HA-tagged 14-3-3θ in mouse brain by both immunohistochemistry and western blotting. Two of the founder lines showed diffuse neuronal expression of HA-tagged 14-3-3θ (M5 and F1 lines). The M5

line was used for experiments in this paper due to high expression levels in the hippocampus. Hemizygous transgenic mice were identified by genotyping using the following primers: forward primer 5' ATCTCAAGCCCTCAAGGTAAATG, and reverse primer 5' CTCCACTTTCTCCCGATAGTCC.

Other mouse lines. BAC wildtype and G2019S-LRRK2 hemizygous transgenic mice [6] were backcrossed on a C57BL/6 background and were bred with wildtype C57BL/6 mice from Jackson labs (Bar Harbor, ME). For experiments evaluating the effect of 14-3-3s on LRRK2, BAC G2019S-LRRK2 hemizygous mice were crossed with hemizygous 14-3-3θ transgenic mice. BAC G2019S-LRRK2 hemizygous mice were also bred with hemizygous difopein-YFP transgenic mice or HA-tagged dominant negative (DN) 14-3-3θ mice on a C57BL/6 background obtained from Yi Zhou[26]. LRRK2 heterozygous (+/-) knockout mice [5] were crossed with hemizygous difopein or DN 14-3-3 θ mice. BAC R1441G-LRRK2 mice [10] were obtained from Jackson Labs, and a breeding colony was maintained by crossing with FVB/NJ mice, also from Jackson Labs. To test the effect of 14-3-3θ on neurite shortening phenotype of the R1441G-LRRK2 mice, hemizygous R1441G-LRRK2 mice were crossed with hemizygous 14-3-3θ transgenic mice. Only mice from the F1 generation were used for neurite analysis to maintain 50% C57BL/6 and 50% FVB/NJ strain contributions, as the 14-3-3θ mice were created in the C57BL/6 strain and the R1441G-LRRK2 mice were created in the FVB strain.

Primary Culture Preparation. Hippocampal neurons were isolated from male and female P0 mice. Hippocampi were dissected from individual mice and incubated in papain (Worthington Biochemical, Lakewood, NJ) for 20 minutes at 37°C. Cells were thoroughly washed using Neurobasal-A media (Thermo Fisher Scientific, Waltham, MA)

containing B-27 supplement (Thermo Fisher) and 5% FBS (Sigma) before tituration using fire polished glass pipettes. After centrifugation at 1500x*g* for five minutes, pelleted cells were layered on top of a 4% BSA (Jackson ImmunoResearch) in HBSS and centrifuged at 700 rpm for five minutes. Cells were resuspended and plated on 18mm glass coverslips coated with poly-D-lysine (Sigma). After 16 hours, media was removed and replaced by Neurobasal-A media containing B-27 supplement and Arabinose C at 6μM. 50% media changes were made every three days.

Neurite analysis. At day in vitro 8 (DIV 8), cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were blocked for a minimum of one hour with 5% normal goat serum (NGS) in tris-buffered saline (TBS) and incubated overnight with a primary rabbit antibody against MAP2 (EMD Millipore, Billerica, MA) at 4°C. Cells were rinsed thoroughly with TBS before being incubated with a Cy3-conjugated goat anti-rabbit secondary antibody for two hours. Cells were again thoroughly washed with TBS before coverslips were mounted on slides using Vectashield mounting solution (Vector Labs, Burlingame, CA). Dendrite lengths were measured using Neurolucida analytical software (MBF Bioscience, Williston, VT).

Immunohistochemistry. Mice were anesthetized with ketamine and xylazine and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Brains were dissected, postfixed for 24 hours in 4% paraformaldehyde at 4°C, and then placed into a 30% sucrose solution in PBS for 48 hours. Brains were sectioned coronally on a Leica microtome with cut thickness of 40 μm. Free floating brain sections were blocked in 10% NGS for 30 minutes and then incubated overnight with primary mouse anti-HA antibody (1:1000, Sigma) or primary rabbit anti-GFP antibody (1:1000, Abcam), followed by

incubation with Cy3-conjugated goat anti-mouse antibody and Alexa-488 conjugated goat anti-rabbit antibody (1:500 Life Technologies)

Statistical analysis. GraphPad Prism 6 (La Jolla, CA) was used for statistical analysis of experiments. Kinase assays, western blot experiments, and neurite analyses were analyzed by either student t-test or by 1-way ANOVA, followed by post-hoc pairwise comparisons using Tukey's multiple comparison test.

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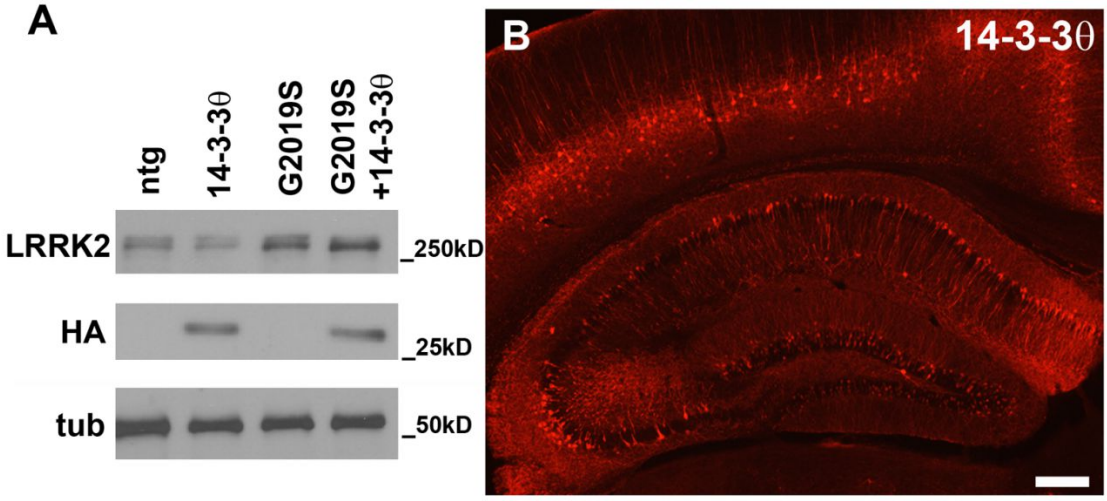

Figure 1. Exogenous 14-3-3θ and LRRK2 hippocampal expression in 14-3-3θ and G2019S-LRRK2 double transgenic mice.

A) Western blots against HA-tagged 14-3-3θ and LRRK2 from lysates of dissected hippocampi from 8 day old nontransgenic, 14-3-3θ, G2019S-LRRK2, and double transgenic littermates.

B) Immunohistochemistry for HA-tagged 14-3-3θ in coronal brain section through the hippocampus from a 14-3-3θ mouse. Scale bar = 200μm.

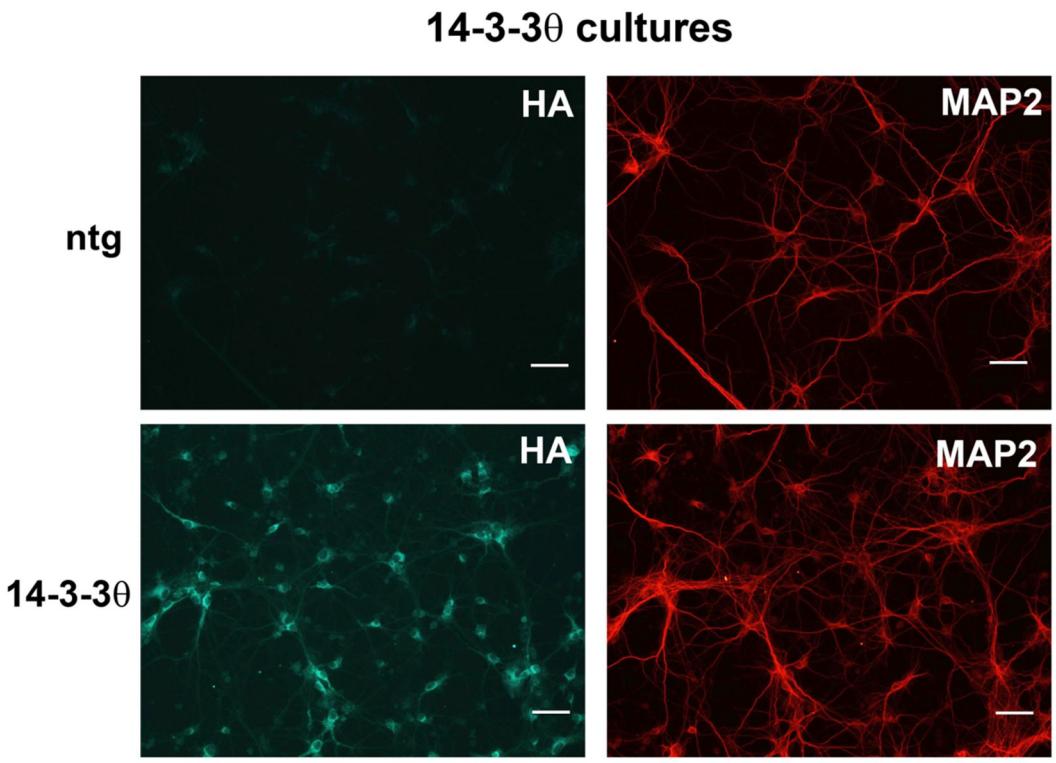

Figure 2. Immunocytochemistry for HA-14-3-3θ in DIV 8 primary hippocampal neuronal cultures

DIV 8 primary cultures from P0 nontransgenic and transgenic 14-3-3θ pups stained for HA (Alexa 488, green) and MAP2 (Cy3, red). Scale bars = 50μm.

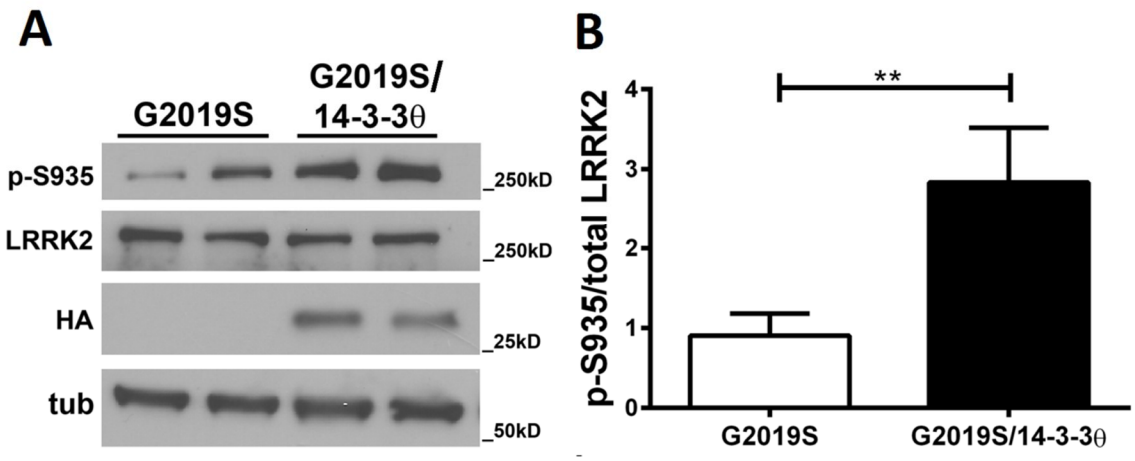

Figure 3. 14-3-3θ overexpression increases S935 phosphorylation on LRRK2 in DIV 8 primary cultures

A) S935 phosphorylation of LRRK2 as determined by Western blot of lysates from DIV 8 primary hippocampal cultures prepared from G2019S-LRRK2, and G2019S-LRRK2/14-3-3θ mice.

B) Quantification of S935 phosphorylation in western blots of lysates from DIV 8 primary hippocampal cultures prepared from G2019S-LRRK2and G2019S-LRRK2/14-3-3θ mice. n=4 mice per group, **p<0.01 (unpaired t-test).

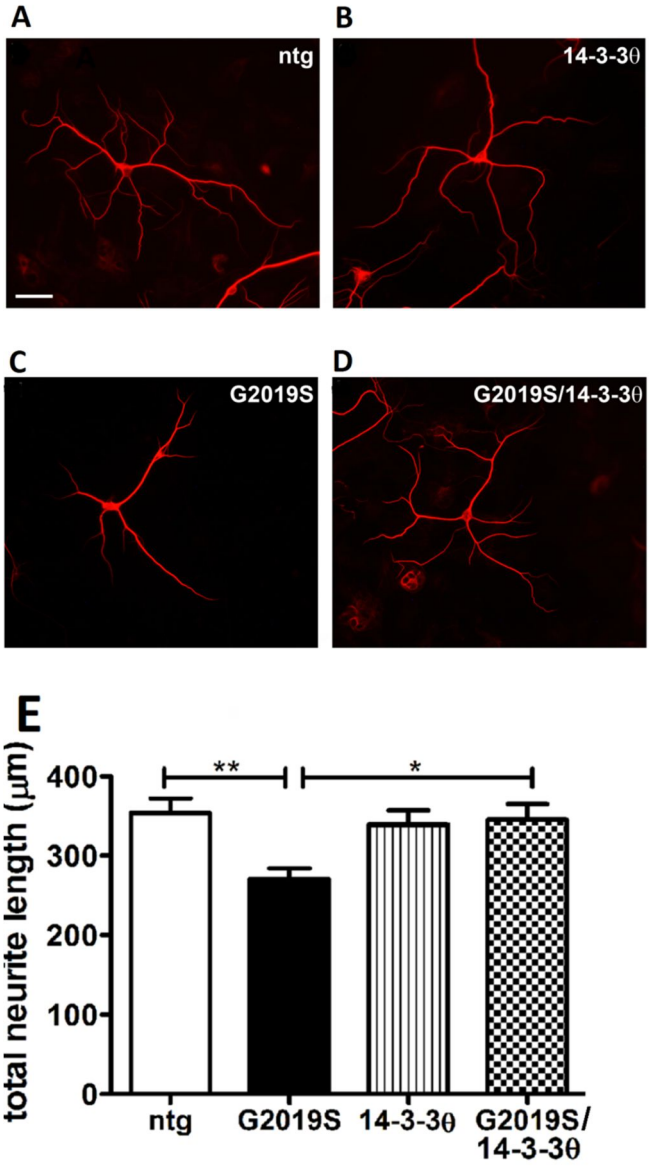

Figure 4. 14-3-3θ overexpression reverses G2019S-LRRK2 mediated neurite shortening in primary hippocampal cultures

A-D) Representative images of neurons stained for MAP2 from each of the four genotypes represented in the cross. Scale bar = 50μm.

E) Total neurite length analysis of neurons from nontransgenic, G2019S-LRRK2, 14-3-3θ, and G2019S-LRRK2/14-3-3θ mouse cultures. n=52 neurons for ntg, n=52 for 14-3-3θ, n=65 for G2019S-LRRK2, and n=44 for G2019S-LRRK2/14-3-3θ combined from three independent rounds. *$p<0.05$, **$p<0.01$ (Tukey's multiple comparison test).

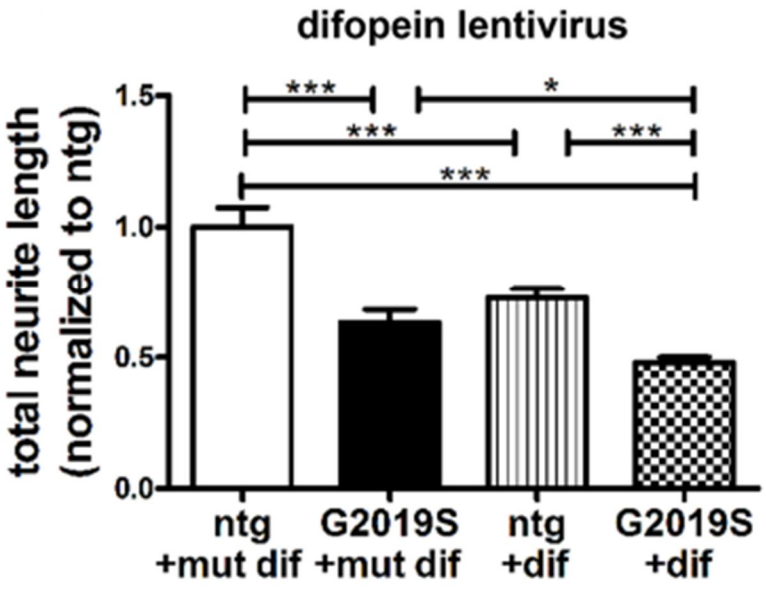

Figure 5. Lentiviral mediated transduction with difopein promotes neurite shortening induced by G2019S-LRRK2

Primary hippocampal cultures from nontransgenic (ntg) and G2019S-LRRK2 littermates were transduced with a doxycycline-inducible lentivirus expressing either difopein-eYFP (dif) or mutant difopein-eYFP (mut dif) and then treated with doxycycline (2 µg/ml). Total neurite length was measured for neurons expressing eYFP. n=22 neurons for ntg/mut dif, n=39 for ntg/dif, n=36 for G2019S-LRRK2/mut dif, and n=66 for G2019S-LRRK2/dif combined from three independent rounds. *$p<0.05$, ***$p<0.001$ (Tukey's multiple comparison test).

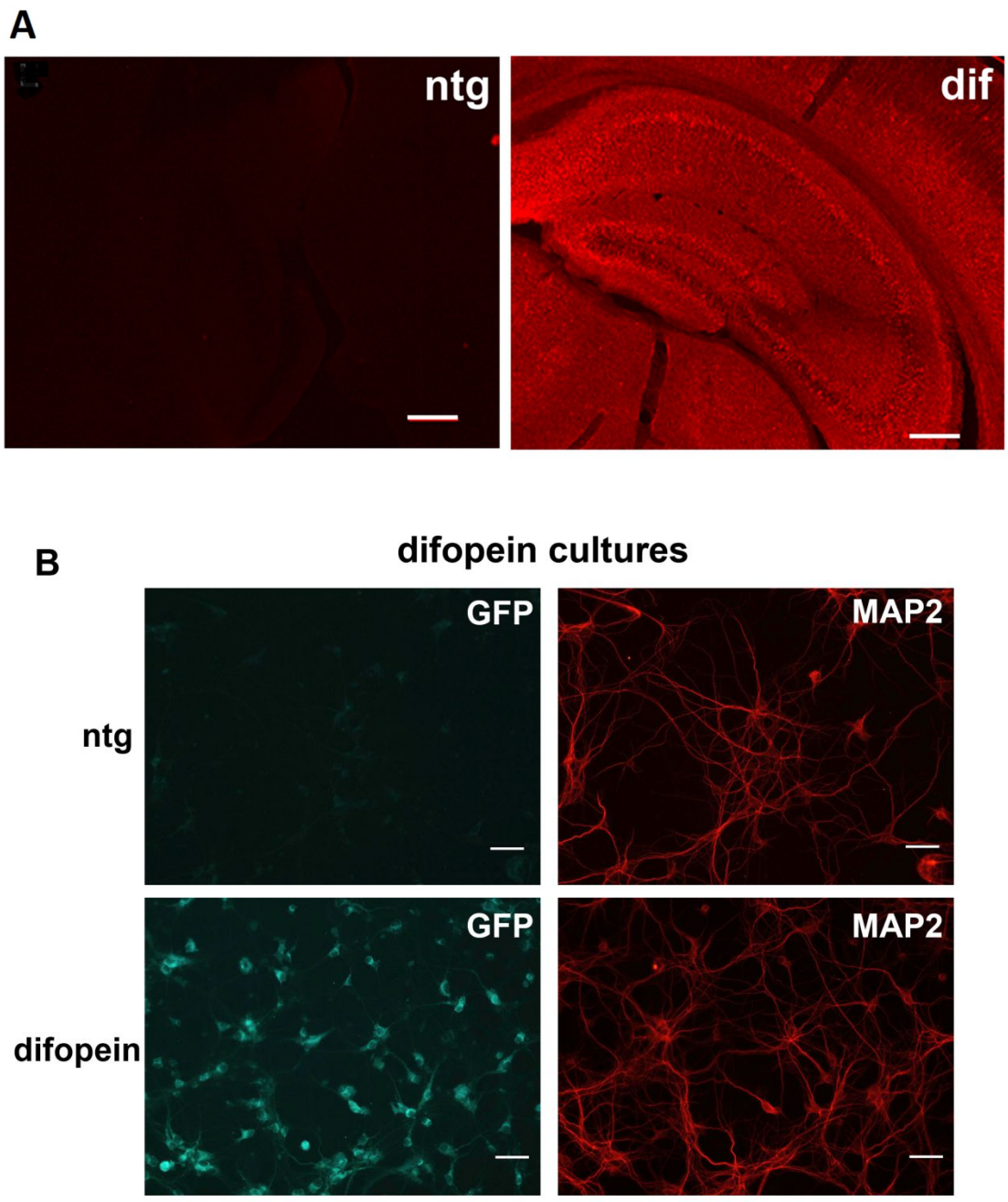

Figure 6. Difopein-eYFP expression in difopein-eYFP transgenic mice

A) Immunohistochemistry for difopein-eYFP in coronal sections through the hippocampus of difopein mice and nontransgenic mice. Scale bar = 500µm.

B) DIV 8 primary cultures from P0 nontransgenic and difopein transgenic pups stained for GFP (Alexa 488, green) and MAP2 (Cy3, red). Scale bar = 50µm.

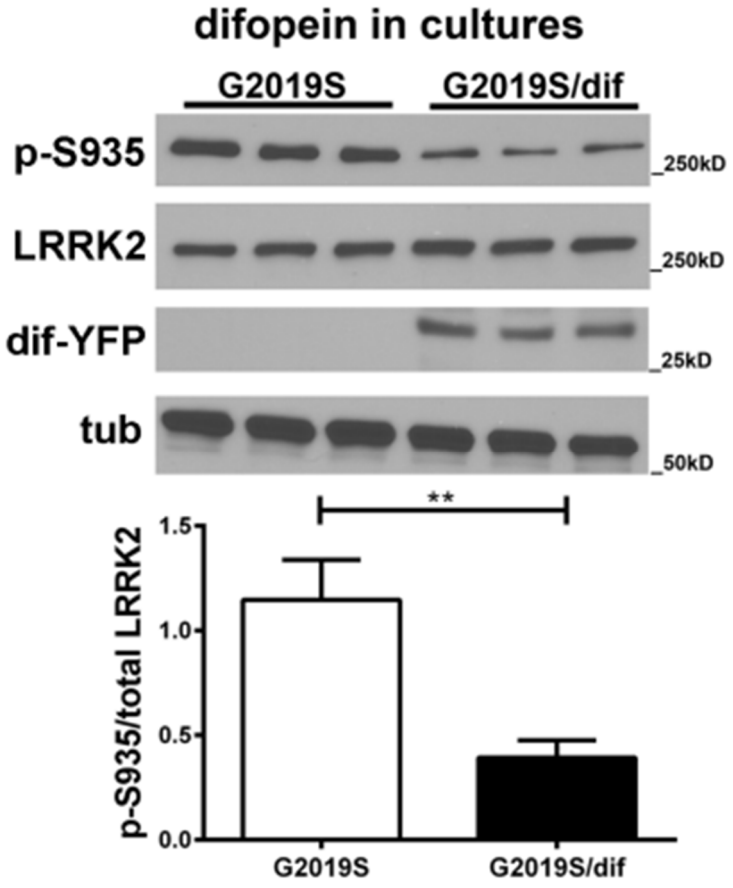

Figure 7. Difopein reduces S935 LRRK2 phosphorylation in cultures from G2019S-LRRK2 mice

Western blot for S935 phosphorylation of lysates from DIV 8 primary hippocampal cultures from G2019S-LRRK2 and G2019S-LRRK2/difopein transgenic mice. n=3 mice per group, **$p<0.01$ (unpaired t-test).

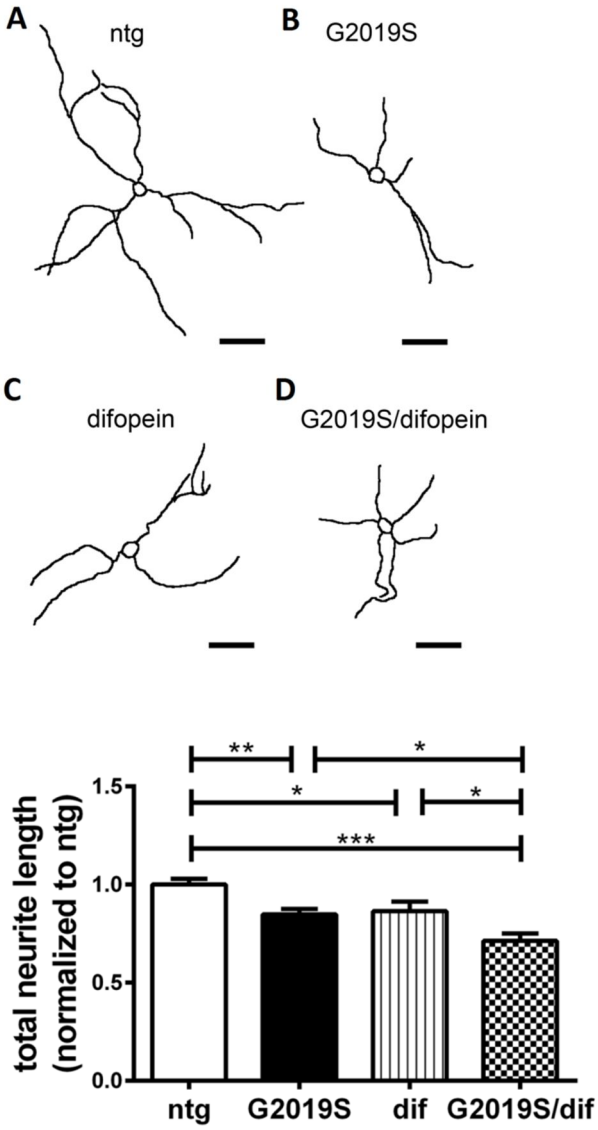

Figure 8. 14-3-3 inhibition with difopein promotes neurite shortening induced by G2019S-LRRK2.

A-D) Representative tracings of neurons analyzed in each resulting genotype in the cross. Scale bar = 50μm.

E) Total neurite length analysis of neurons from nontransgenic, G2019S-LRRK2, difopein, and G2019S-LRRK2/difopein cultures. n=34 neurons for ntg, n=17 for dif, n=34 for G2019S-LRRK2, and n=22 for G2019S-LRRK2/dif combined from three independent rounds. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Tukey's multiple comparison test).

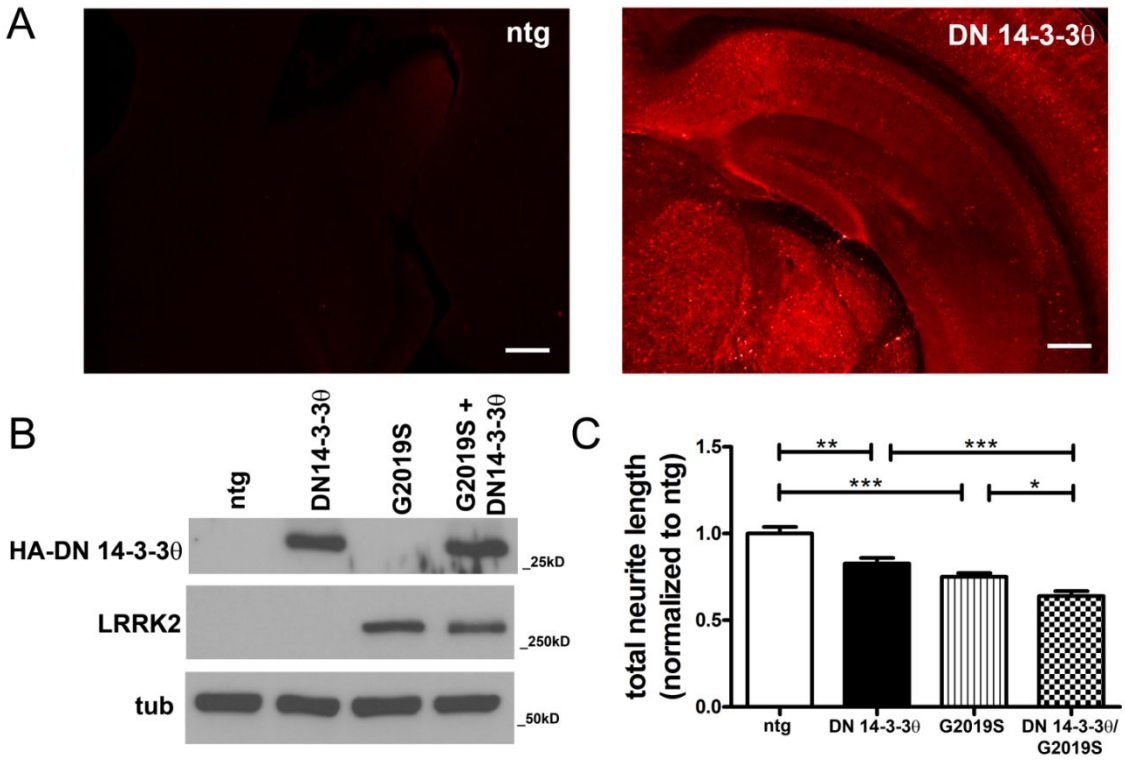

Figure 9. Dominant negative 14-3-3θ expression also promotes G2019S-LRRK2 induced neurite shortening

A) Immunohistochemistry for HA-tagged DN 14-3-3θ in coronal sections through the hippocampus of nontransgenic and DN 14-3-3θ mice. Scale bar = 500µm.

B) Western blot for HA-tagged 14-3-3θ and LRRK2 of lysates from DIV 8 primary hippocampal cultures from nontransgenic, DN 14-3-3θ, G2019S-LRRK2, and double transgenic mice.

C) Total neurite length analysis of neurons from nontransgenic, G2019S-LRRK2, DN 14-3-3θ, and G2019S-LRRK2/DN 14-3-3θ cultures. n=25 neurons for ntg, n=50 for DN 14-3-3θ, n=75 for G2019S-LRRK2, and n=35 for G2019S-LRRK2/DN 14-3-3θ combined from three independent rounds. *p<0.05, **p<0.01, ***p<0.001 (Tukey's multiple comparison test).

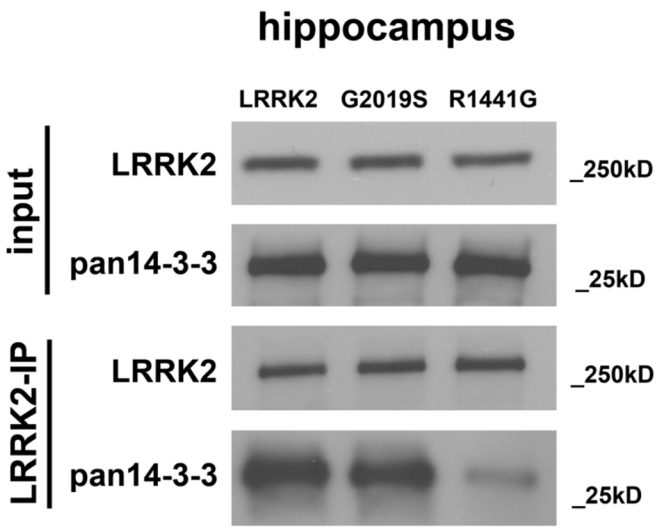

Figure 10. R1441G-LRRK2 has reduced co-immunoprecipitation to 14-3-3s in the hippocampus of transgenic mice

Hippocampal lysates from 8 day old BAC wildtype, G2019S, or R1441G-LRRK2 transgenic mice were immunoprecipitated for LRRK2 and the resulting immunoprecipitates were probed for endogenous 14-3-3s by western blot.

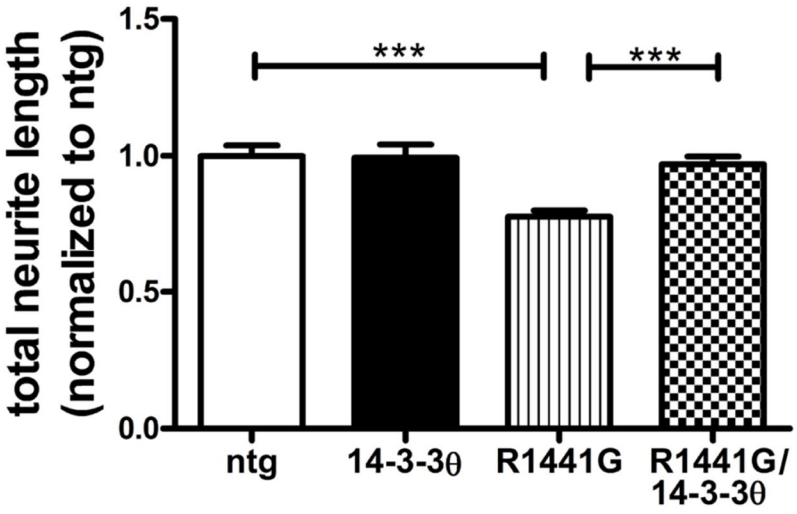

Figure 11. 14-3-3θ overexpression ameliorates R1441G-LRRK2 induced neurite shortening

Total neurite length analysis of neurons from nontransgenic, R1441G-LRRK2, 14-3-3θ, and R1441G-LRRK2/DN 14-3-3θ cultures. n=29 neurons for ntg, n=22 for 14-3-3θ, n=48 for R1441G-LRRK2, and n=55 for R1441G-LRRK2/14-3-3θ combined from three independent rounds. ***$p<0.001$ (Tukey's multiple comparison test).

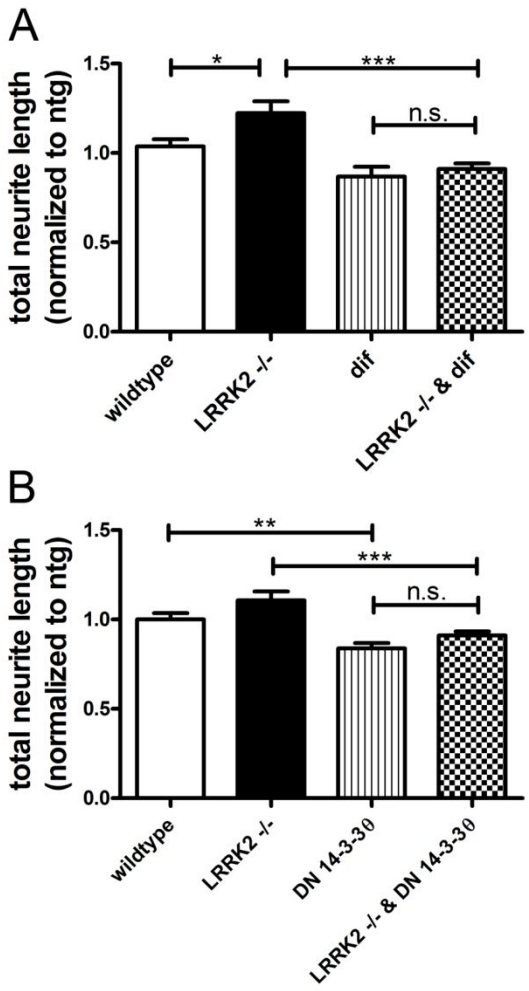

Figure 12. Difopein and DN 14-3-3θ cause neurite shortening in the absence of LRRK2 expression

A) Total neurite length analysis of neurons from difopein-eYFP crossed with LRRK2 -/- cultures. n=22 neurons for wildtype (wt), n=16 for LRRK2 -/-, n=16 for dif, and n=22 for LRRK2 -/- + dif combined from four independent rounds. *p<0.05, ***p<0.001, ns=nonsignificant (Tukey's multiple comparison test).

B) Total neurite length analysis of neurons from DN 14-3-3θ crossed with LRRK2 -/- cultures. n=23 neurons for wt, n=25 for LRRK2 -/-, n=44 for DN 14-3-3θ, and n=39 for LRRK2 -/- + DN 14-3-3θ combined from four independent rounds. **p<0.01, ***p<0.001, ns=nonsignificant (Tukey's multiple comparison test).

14-3-3 Alters the Kinase Activity of LRRK2

Introduction

In the previous chapter, we have shown that 14-3-3s can regulate mutant LRRK2 toxicity, as measured by neurite shortening. Here we wish to examine the potential mechanism by which 14-3-3s are protective against mutant LRRK2 toxicity. Current evidence points to increased kinase activity as a key mechanism for mutant LRRK2 neurotoxicity [1-5]. Several pathogenic *LRRK2* mutations cause a reduction in neurite growth, and inhibiting kinase activity can reverse this effect [6-10]. This suggests that aberrant LRRK2 kinase activity is driving the neurite shortening phenotype seen in these mutations. Indeed, pathogenic mutations in LRRK2 occur mostly in either the kinase or GTPase domains of the protein. Previous work has shown variable effects of these mutations on both the kinase and/or GTPase activity of the protein [1, 3, 4, 7, 11-22], although one idea is that there is interplay between the kinase and GTPase regions and that each of the pathogenic mutations lead to an overactive phenotype, either through increased kinase activity or by maintaining an "on" GTP-bound conformation [23, 24]. Other groups have suggested that mutations in the GTPase domain decrease GTPase activity, but enhance GTP binding, leading to a LRRK2 conformation that stays bound to GTP for longer in what may be considered an active conformation. This then leads to an overall enhanced active state of LRRK2 [23, 24]. Consistently, G2019S-LRRK2 has been shown to have increased kinase activity by multiple groups, as shown by autophosphorylation and by phosphorylation of substrates [1, 14, 25-28].

14-3-3s have a notable role in regulating the kinase activity of target proteins. Work by Muda et al. has shown that 14-3-3γ overexpression can reduce LRRK2 kinase

activity as measured by autophosphorylation and substrate phosphorylation [29], supporting the idea of a regulatory role of 14-3-3s over LRRK2. 14-3-3s have also been shown to regulate the kinase activity of other proteins. For example, 14-3-3s have also been shown to support Raf kinase activity and serve as a catalytic cofactor [30, 31], enhance tryptophan kinase activity [32], increase p53 DNA binding activity [33], inhibit ASK-1 kinase activity [34, 35] and inhibit RGS activity [36]. Specific to dopaminergic neurons, 14-3-3s interact with phosphorylated tyrosine hydroxylase (TH) to enhance its activity in producing dopamine [37-39].

Based on the data above, we hypothesize that that 14-3-3θ's protective effects against G2019S-LRRK2-mediated toxicity occur through reduction of kinase activity. We predict that 14-3-3θ overexpression can reduce the increase in kinase activity seen in mutant LRRK2. Conversely, we predict that inhibition of 14-3-3 will cause increased LRRK2 kinase activity.

Results

14-3-3θ regulates LRRK2 kinase activity

The G2019S-LRRK2 mutation is thought to confer toxicity through an increase in its kinase activity [1-5, 14], and neurite shortening has been shown to be kinase dependent [5-7]. To test if 14-3-3θ protects against G2019S-LRRK2 neurotoxicity by reducing its kinase activity, we examined the effect of 14-3-3θ on both wildtype and G2019S-LRRK2 kinase activity. HEK293T cells were transfected with HA-tagged wildtype or G2019S-LRRK2 with or without 14-3-3θ, and then immunoprecipitated for

HA-tagged LRRK2 using a monoclonal antibody against HA prior to performing the kinase assay. Kinase activity was measured by the level of autophosphorylation at threonine 1503 (T1503) in LRRK2 [11]. We measured a 1.7-fold increase in T1503 phosphorylation with the G2019S-LRRK2 when compared to wildtype LRRK2 (Fig. 1A). When 14-3-3θ was co-transfected with G2019S-LRRK2, T1503 phosphorylation decreased by 50%. The effect of 14-3-3θ on G2019S-LRRK2 was dose-dependent, with a greater reduction of T1503 phosphorylation with increasing amounts of 14-3-3θ expression (Fig. 1A).

We next tested the effects of 14-3-3 inhibition on LRRK2 kinase activity in HEK293T cells. HEK293T cells cotransfected with difopein and wildtype LRRK2 showed a 2.4-fold increase in kinase activity compared to cells transfected with wildtype LRRK2 and mutant difopein (Fig. 1B). G2019S-LRRK2 showed a 2.2-fold increase in kinase activity compared to wildtype LRRK2, and in the presence of difopein, G2019S-LRRK2 kinase activity was increased by 74% (Fig. 1B).

As another measure of LRRK2 kinase activity, we examined LRRK2 phosphorylation at S1292. LRRK2 autophosphorylation at the S1292 site correlates with LRRK2 kinase activity [5]. S1292 phosphorylation was increased by 1.7-fold in HEK293T cells transfected with G2019S-LRRK2 compared to cells transfected with wildtype LRRK2 (Fig. 2A). When 14-3-3θ was cotransfected with G2019S-LRRK2, S1292 phosphorylation was reduced by 46% (Fig. 2A). The effect of 14-3-3θ on LRRK2 was dose-dependent, with a greater reduction of S1292 phosphorylation with increasing amounts of 14-3-3θ expression with G2019S-LRRK2 (Fig. 2A). Conversely, S1292 phosphorylation increased by 62% when G2019S-LRRK2 was transfected together with

constructs expressing difopein (Figures 2B). Difopein also increased S1292 phosphorylation 3.5-fold when co-transfected with wildtype LRRK2 (Fig. 2B).

Next, we measured S1292 phosphorylation in lysates from microdissected hippocampi from six-week-old mice. G2019S-LRRK2/14-3-3θ double transgenic mice showed a 79% decrease in S1292 phosphorylation compared to G2019S-LRRK2 littermates (Fig. 3A). In contrast, G2019S-LRRK2/difopein mice showed a 2.3-fold increase in hippocampal S1292 phosphorylation compared to G2019S-LRRK2 mice (Fig. 3B).

Finally, we measured the effect of 14-3-3θ overexpression on the phosphorylation of a known *in vitro* substrate of LRRK2, ADP-ribosylation factor GTPase-activating protein 1 (ArfGAP1) [21, 40]. Lysates from HEK293T cells transfected with LRRK2 with and without 14-3-3θ were immunoprecipitated for HA-tagged wildtype or G2019S-LRRK2, and then recombinant ArfGAP1 was added to the immunoprecipitate prior to the kinase reaction. As expected, G2019S-LRRK2 caused a 2-fold increase in ArfGAP1 phosphorylation compared to wildtype LRRK2 (Fig. 4). Co-transfection of 14-3-3θ with G2019S-LRRK2 decreased ArfGAP1 phosphorylation to wildtype LRRK2 levels, while 14-3-3θ co-expression with wildtype LRRK2 did not affect ArfGAP1 phosphorylation (Fig. 4).

14-3-3θ binds LRRK2 directly to regulate kinase activity

To determine if a direct interaction between 14-3-3θ and LRRK2 is required in order for 14-3-3θ to regulate LRRK2 kinase activity, we tested the ability of 14-3-3θ to

regulate the kinase activity of LRRK2 mutants that cannot bind 14-3-3s. As previously shown [41-43], the S935A mutation of LRRK2 eliminates the ability of 14-3-3s to bind LRRK2, which we confirmed by immunoprecipitation (Fig. 5). Phosphorylation at T1503 was similar with the S935A mutant compared to wildtype LRRK2. Likewise, the S935A mutation did not alter phosphorylation at T1503 in G2019S-LRRK2 (Fig. 5). While 14-3-3θ overexpression reduced T1503 phosphorylation of wildtype and G2019S-LRRK2, it did not reduce T1503 phosphorylation in the presence of the S935A mutation in either wildtype or G2019S-LRRK2 (Fig. 5). These results suggest that 14-3-3θ must directly interact with LRRK2 in order to regulate LRRK2 kinase activity.

G2019S-LRRK2 has no effect on 14-3-3 phosphorylation

In response to the previous data showing 14-3-3θ overexpression and pan-14-3-3 inhibition alters LRRK2 kinase activity, we determined whether or not mutant LRRK2 had any effect on 14-3-3 phosphorylation. 14-3-3 phosphorylation occurs at three separate sites, S58, S148 and S/T232. Phosphorylation at the S58 site occurs in all 14-3-3 isoforms except σ and θ and has been shown to regulate dimerization of 14-3-3s [44, 45]. Phosphorylation at the S184 site occurs in the 14-3-3β, ε, σ and ζ isoforms and regulates ligand binding [46-48]. Phosphorylation at the S/T232 site is not well understood, although it is thought to regulate ligand binding as the C-terminal loop can fold back into the peptide-binding pocket [49, 50]. Importantly, phosphorylation at both S58 and S184 has been linked to the release of pro-apoptotic factors and cell death [46-

48, 51], leading to the general conclusion that increased 14-3-3 phosphorylation negatively affects its anti-apoptotic role in the cell.

To examine 14-3-3 phosphorylation, HEK293T cells were transfected with either GFP, wildtype or G2019S-LRRK2. Lysates were collected and immunoblotted for phospho-S58 and phospho-S232. Despite the overexpression of either wildtype or G2019S-LRRK2, there was no alteration in either S58 or S232 phosphorylation of 14-3-3 (Fig. 6A). This was also confirmed *in vivo* from hippocampal lysates from 5 week old BAC transgenic G2019S mice and nontransgenic littermates. G2019S transgenic mice had no change in S58 or S232 phosphorylation over their nontransgenic littermates (Fig. 6B), thus confirming mutant LRRK2 does not alter 14-3-3 phosphorylation.

LRRK2 kinase inhibitors reverse enhanced neurite shortening in G2019S-LRRK2/difopein double transgenic cultures

If the effect of 14-3-3s on G2019S-LRRK2 toxicity were mediated by the ability of 14-3-3s to regulate LRRK2 kinase activity, we predicted that the exacerbation of G2910S-LRRK2 induced neurite retraction by difopein would be reversed by LRRK2 kinase inhibitors. Primary cultures from G2019S-LRRK2/difopein double transgenic mice were treated with the LRRK2 kinase inhibitor, HG-10-102-01 [52] at 1.5μM at DIV6, and cultures were then fixed and stained for neurite analysis at DIV8. HG-10-102-01 caused a reversal of neurite shortening in G2019S-LRRK2/difopein primary neuron cultures (Fig. 7A). HG-10-102-01 did not alter neurite length in LRRK2 -/- cultures, demonstrating that HG-10-102-01 effects on neurite length were caused by LRRK2

inhibition (Fig. 8). These findings suggest that the exacerbation of neurite shortening in G2019S-LRRK2/difopein cultures is mediated in part through increased LRRK2 kinase activity.

We next tested the effects of HG-10-102-01 treatment in primary cultures from G2019S-LRRK2/14-3-3θ double transgenic mice. Untreated G2019S-LRRK2/14-3-3θ neurons showed neurite lengths comparable to nontransgenic neurons (Fig. 7B). In cultures from G2019S-LRRK2/14-3-3θ mice, the addition of HG-10-102-01 caused further increase in neurite length compared to control G2019S-LRRK2/14-3-3θ neurons (Fig. 7B). These findings suggest that 14-3-3θ overexpression and LRRK2 inhibitors may act synergistically to promote neurite length.

Discussion

In this study, we demonstrate that 14-3-3s can regulate LRRK2 kinase activity and that the effect of 14-3-3θ overexpression on mutant LRRK2-induced neurite shortening is likely mediated through effects on LRRK2 kinase activity. 14-3-3θ overexpression reduced G2019S-LRRK2 kinase activity both *in vitro* and *in vivo*, as measured by autophosphorylation and phosphorylation of a known *in vitro* LRRK2 substrate, ArfGAP1. Notably, 14-3-3θ's effect on kinase activity was dose dependent. Conversely, difopein expression increased LRRK2 kinase activity *in vitro* and *in vivo*. The ability of 14-3-3θ to reduce LRRK2 kinase activity was dependent on direct binding of 14-3-3θ to LRRK2, as 14-3-3θ had no effect on the kinase activity of the non-14-3-3 binding S935A LRRK2 mutant. Finally, treatment of primary cultures from G2019S-

LRRK2/difopein double transgenic mice with a selective LRRK2 kinase inhibitor HG-10-102-01 reversed the enhanced neurite shortening observed in these double transgenic cultures.

The interaction between 14-3-3s and LRRK2 is well established, but the biological significance of this interaction with regards to LRRK2 kinase activity and function is not clear [29, 42, 43]. Our findings demonstrate that 14-3-3s can regulate LRRK2 kinase activity and function so that alterations in 14-3-3 binding to LRRK2 may be important in LRRK2-linked disease. Several LRRK2 mutants show reduced binding to 14-3-3s [29, 42, 43] that could lead to increased LRRK2 kinase activity and thereby toxicity. In addition, reduced 14-3-3 expression and function has been demonstrated in PD models and human PD [53-56] and could potentially lead to increased LRRK2 kinase activity that could contribute to the neurodegenerative process.

It is possible that there may be intermediary steps required for 14-3-3θ to regulate LRRK2 kinase activity, especially in the case of R1441G-LRRK2 which has reduced binding to 14-3-3s. To determine whether direct interaction between 14-3-3θ and LRRK2 is required for the ability of 14-3-3θ to regulate LRRK2 kinase activity, we evaluated the effect of 14-3-3θ overexpression on the S935A LRRK2 mutant which cannot bind 14-3-3s. 14-3-3θ was unable to reduce kinase activity of wildtype or G2019S-LRRK2 when mutated at S935 to disrupt the interaction with 14-3-3s. Thus, we conclude that 14-3-3θ acts to inhibit kinase activity by direct binding to LRRK2. Notably, we did not see any reciprocal effect on 14-3-3 as there was no change in 14-3-3 phosphorylation when G2019S LRRK2 was present as opposed to wildtype-LRRK2, leading us to believe 14-3-3s play a regulatory role over LRRK2.

There are several possible ways 14-3-3s may regulate LRRK2 kinase activity. 14-3-3s normally act as dimers to bind enzymes and cause conformational changes to alter activity. 14-3-3s have been shown to affect several kinases through these direct interactions [57, 58]. Our data with the S935A-LRRK2 mutant suggest that 14-3-3θ does need to directly bind to LRRK2 to regulate LRRK2 kinase activity. One possibility is that 14-3-3θ binding could stabilize wildtype and G2019S-LRRK2 into a kinase inactive conformation. 14-3-3θ overexpression could allow for more 14-3-3θ to bind LRRK2 to hold it in an inactive state, while disruption of 14-3-3 binding could promote the formation of active-state LRRK2.

Our kinase assay data and our LRRK2 kinase inhibition studies suggest that difopein's exacerbation of effects on G2019S-LRRK2 mediated neurite shortening is likely secondary to difopein upregulating LRRK2 kinase activity. Our kinase assay studies demonstrated that difopein increases G2019S-LRRK2 kinase activity, both with respect to autophosphorylation and *trans* phosphorylation of a LRRK2 kinase substrate ArfGAP1. G2019S-LRRK2's effect on neurite length is kinase dependent, as LRRK2 kinase inhibitors can reverse neurite shortening caused by G2019S-LRRK2 expression [5]. Finally, the specific LRRK2 kinase inhibitor HG-10-102-01 reverses difopein's exacerbation of neurite shortening of G2019S-LRRK2 neurons. 14-3-3's effects on neurite growth are complex and involve several mechanisms, and in the presence of mutant LRRK2, 14-3-3s can regulate mutant LRRK2-mediated neurite effects through regulation of kinase activity.

If 14-3-3θ acts to restore neurite length in G2019S-LRRK2 cultures by inhibiting LRRK2 kinase activity, we would predict that additional LRRK2 kinase inhibition should

not have any additional effect on neurite length in the presence of 14-3-3θ overexpression. However, the addition of LRRK2 kinase inhibitor showed a synergistic effect on neurite length in 14-3-3θ/G2019S-LRRK2 cultures. One possibility is that the amount of 14-3-3θ overexpression in our cultures does not fully inhibit all LRRK2 kinase activity. Indeed, our *in vitro* kinase assays show that 14-3-3θ did not fully eliminate G2019S-LRRK2 kinase activity. The addition of the LRRK2 inhibitor may cause neurite elongation by fully inhibiting any remaining active LRRK2 within the cell.

In conclusion, our studies reveal that 14-3-3s can regulate mutant LRRK2 kinase activity and action on neurite outgrowth. 14-3-3θ overexpression reduces mutant LRRK2 kinase activity, while 14-3-3 inhibition enhances LRRK2 kinase activity. Therefore, increasing the expression of 14-3-3 proteins may provide a new therapeutic avenue to addressing PD caused by LRRK2 mutations.

Materials and Methods

Cell transfection. HEK 293T cells were grown in DMEM containing 10% normal calf serum with 1% penicillin/streptomycin. 24 hours after plating, cells were transfected using Superfect transfection reagent (Qiagen, Germantown, MD) using manufacturer's guidelines. After transfection, cells were incubated in fresh media for 48 hours prior to protein collection.

Immunoblotting. HEK 293T cells or primary cultured neurons were washed in phosphate buffered saline (PBS) and pelleted at 1500x*g* for five minutes. Cell pellets were then sonicated in lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM

EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)), followed by centrifugation at 16,000x*g* for 10 minutes. Protein concentrations were assessed by BCA assay (Pierce, Rockford, IL). Samples were boiled for five minutes in DTT sample loading buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 200 mM DTT, 30% glycerol, Bromophenol Blue), resolved on 7.5 or 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. After blocking in 5% non-fat dry milk in TBST (25 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween 20), membranes were incubated overnight in rabbit polyclonal antibody against pan-14-3-3 (1:1000 Abcam, Cambridge, MA), rabbit polyclonal antibody against GFP (1:5000 Abcam), mouse monoclonal antibody against HA (1:1000 Covance, Princeton, NJ), mouse monoclonal antibody against FLAG (1:1000 Sigma, St. Louis, MO), rabbit polyclonal antibody against LRRK2 (1:1000 Abcam), rabbit polyclonal antibody against alpha-tubulin (1:2500 Cell Signaling, Danvers, MA), rabbit polyclonal antibody against phosphorylated T1503 LRRK2 (1:1000 Abcam), or rabbit polyclonal antibody against phosphorylated S1292 LRRK2 (1:1000 Abcam) at 4°C. Membranes were then incubated in HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:2000 Jackson ImmunoResearch Laboratories, West Grove, PA) for two hours and then washed in TBST six times for ten minutes each. Blots were developed with enhanced chemiluminescence method (GE Healthcare, Piscataway, NJ). Images were scanned and analyzed using Un-ScanIT software (Orem, UT) for densitometric analysis of bands.

Hippocampi and cortices from mouse brains were homogenized in lysis buffer (Tris/HCl 50mM pH7.4, NaCl 175mM, EDTA 5mM, protease inhibitor and phosphatase inhibitor cocktails) and sonicated for 10 seconds. Cell lysates were then incubated on ice

for 30 minutes after the addition of 1% Triton X-100 and then spun at 15000x*g* for one hour at 4°C. The supernatant was saved as the Triton X-100 soluble fraction. Samples were resolved on SDS-polyacrylamide gels and analyzed by western blotting as described above**.**

Immunoprecipitation. For immunoprecipitation of HA-tagged LRRK2, Protein G Dynabeads (Life Technologies, Grand Island, NY) were incubated with 4µg mouse HA antibody (Sigma) overnight. 500µg of cell lysate was incubated with antibody-conjugated beads for 30 minutes at room temperature. Beads were then washed five times in PBS with 0.02% Tween. After washing, beads were boiled in DTT sample loading buffer and loaded on a SDS-polyacrylamide gel. After transfer to nitrocellulose, the membrane was probed for 14-3-3 proteins using a polyclonal rabbit antibody against 14-3-3s (1:1000 Abcam). For immunoprecipitation of FLAG-tagged LRRK2, immunoprecipitation was performed using anti-FLAG affinity gel (Sigma) following manufacturer's protocol.

LRRK2 kinase assay. HEK 293T cells were transfected with HA-tagged LRRK2 with or without V5-tagged 14-3-3θ or difopein-YFP. 48 hours after transfection, cell lysates were incubated with HA-antibody conjugated Dynabeads for 30 minutes. Beads were washed with PBS with two high salt washes of PBS with 500mM NaCl before being resuspended in kinase buffer (10mM Tris pH 7.4, 0.1mM EGTA, 20mM of $MgCl_2$, 0.1mM ATP). The samples were then shaken at 30°C for 30 minutes. The kinase reaction was terminated by incubating samples on ice and beads were then incubated in Laemmli buffer at 75°C for 10 minutes. Sample was then run on a 7.5% SDS-acrylamide gel, transferred to nitrocellulose, and probed for phosphorylated LRRK2 at T1503 using a primary rabbit

antibody against phospho-T1503 (1:1000, Abcam) and for total LRRK2 using an antibody against the HA tag (1:1000, Covance).

For measuring LRRK2 kinase activity via ArfGAP1 phosphorylation, the same kinase assay protocol was followed, but 1µg recombinant ArfGAP1 (Abnova, Taipei City, Taiwan) was added to the kinase buffer prior to the 30 minute incubation at 30°C. Samples were electrophoresed on 7.5% SDS-acrylamide gel, transferred to nitrocellulose, and probed for phospho-threonine (Cell Signaling) or total ArfGAP1 using an antibody against the GST tag on the recombinant protein (GST-HRP, Pierce).

Generation of 14-3-3θ transgenic line. Mice were used in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee (IACUC). Human 14-3-3θ tagged with an HA epitope tag at the C-terminal end was cloned into a Thy1.2 expression cassette [59] to drive neuronal expression of 14-3-3θ. Following digestion with NdeI and EcoRI, the Thy1.2 construct containing HA-tagged 14-3-3θ was purified and microinjected into C57BL/6 fertilized mouse oocytes. Founder mice were bred with C57BL/6 mice from Jackson labs, and pups were examined for expression of HA-tagged 14-3-3θ in mouse brain by both immunohistochemistry and western blotting. Two of the founder lines showed diffuse neuronal expression of HA-tagged 14-3-3θ (M5 and F1 lines). The M5 line was used for experiments in this paper due to high expression levels in the hippocampus. Hemizygous transgenic mice were identified by genotyping using the following primers: forward primer 5' ATCTCAAGCCCTCAAGGTAAATG, and reverse primer 5' CTCCACTTTCTCCCGATAGTCC.

Other mouse lines. BAC wildtype and G2019S-LRRK2 hemizygous transgenic mice [60] were backcrossed on a C57BL/6 background and were bred with wildtype C57BL/6 mice from Jackson labs (Bar Harbor, ME). For experiments evaluating the effect of 14-3-3s on LRRK2, BAC G2019S-LRRK2 hemizygous mice were crossed with hemizygous 14-3-3θ transgenic mice. BAC G2019S-LRRK2 hemizygous mice were also bred with hemizygous difopein-YFP transgenic mice on a C57BL/6 background obtained from Yi Zhou [61]. LRRK2 heterozygous (+/-) knockout mice [6] were crossed with hemizygous difopein or 14-3-3θ mice.

Primary Culture Preparation. Hippocampal neurons were isolated from male and female P0 mice. Hippocampi were dissected from individual mice and incubated in papain (Worthington Biochemical, Lakewood, NJ) for 20 minutes at 37°C. Cells were thoroughly washed using Neurobasal-A media (Thermo Fisher Scientific, Waltham, MA) containing B-27 supplement (Thermo Fisher) and 5% FBS (Sigma) before tituration using fire polished glass pipettes. After centrifugation at 1500x*g* for five minutes, pelleted cells were layered on top of a 4% BSA (Jackson ImmunoResearch) in HBSS and centrifuged at 700 rpm for five minutes. Cells were resuspended and plated on 18mm glass coverslips coated with poly-D-lysine (Sigma). After 16 hours, media was removed and replaced by Neurobasal-A media containing B-27 supplement and Arabinose C at 6μM. 50% media changes were made every three days. For certain experiments, the LRRK2 inhibitor HG-10-102-01, was used to treat cultures at 1.5μM for the final 48 hours in culture prior to immunostaining.

Neurite analysis. At day in vitro 8 (DIV 8), cells were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Cells were blocked for a minimum of one

hour with 5% normal goat serum (NGS) in tris-buffered saline (TBS) and incubated overnight with a primary rabbit antibody against MAP2 (EMD Millipore, Billerica, MA) at 4°C. Cells were rinsed thoroughly with TBS before being incubated with a Cy3-conjugated goat anti-rabbit secondary antibody for two hours. Cells were again thoroughly washed with TBS before coverslips were mounted on slides using Vectashield mounting solution (Vector Labs, Burlingame, CA). Dendrite lengths were measured using Neurolucida analytical software (MBF Bioscience, Williston, VT).

Statistical analysis. GraphPad Prism 6 (La Jolla, CA) was used for statistical analysis of experiments. Kinase assays, western blot experiments, and neurite analyses were analyzed by either student t-test or by 1-way ANOVA, followed by post-hoc pairwise comparisons using Tukey's multiple comparison test.

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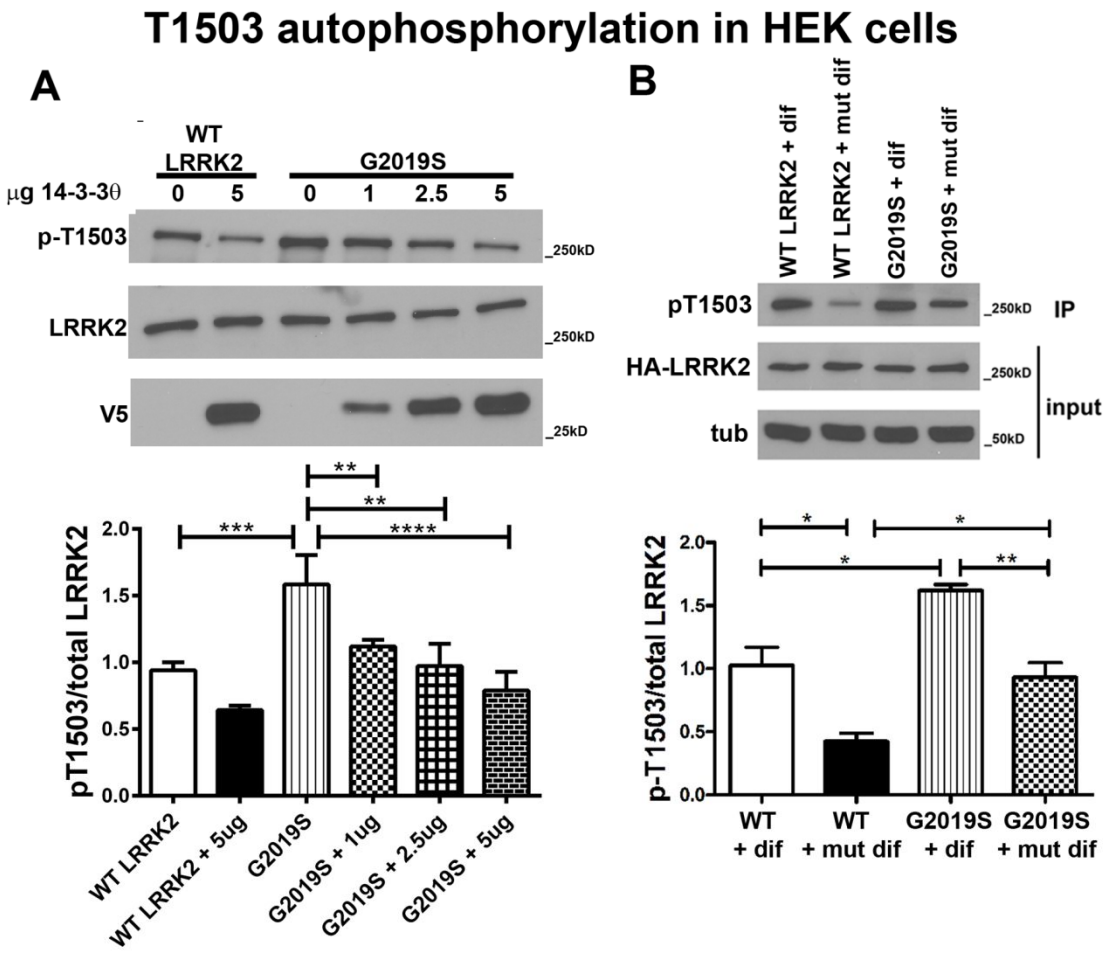

Figure 1. 14-3-3s regulate LRRK2 T1503 autophosphorylation *in vitro*

A) Lysates from HEK293T cells transfected with wildtype or G2019S-LRRK2 together with increasing amounts of V5-tagged 14-3-3θ plasmid were generated. LRRK2 was immunoprecipitated with a HA-specific antibody to isolate LRRK2 for *in vitro* kinase reactions. Following kinase reaction, immunoprecipitates were then analyzed for the LRRK2 autophosphorylation residue phospho-T1503 and total HA-tagged LRRK2 by western blot. n=3 independent rounds. **p<0.01, ***p<0.001, ****p<0.0001 (Tukey's multiple comparison test).

B) Lysates from HEK293T cells transfected with wildtype or G2019S-LRRK2 together with difopein-eYFP or mutant difopein-eYFP were immunoprecipitated with a HA-specific antibody prior to the kinase assay. After the kinase reaction, immunoprecipitates were probed for phospho-T1503 and total HA-tagged LRRK2 by western blot. n=3 independent rounds. *p<0.05, **p<0.01 (Tukey's multiple comparison test).

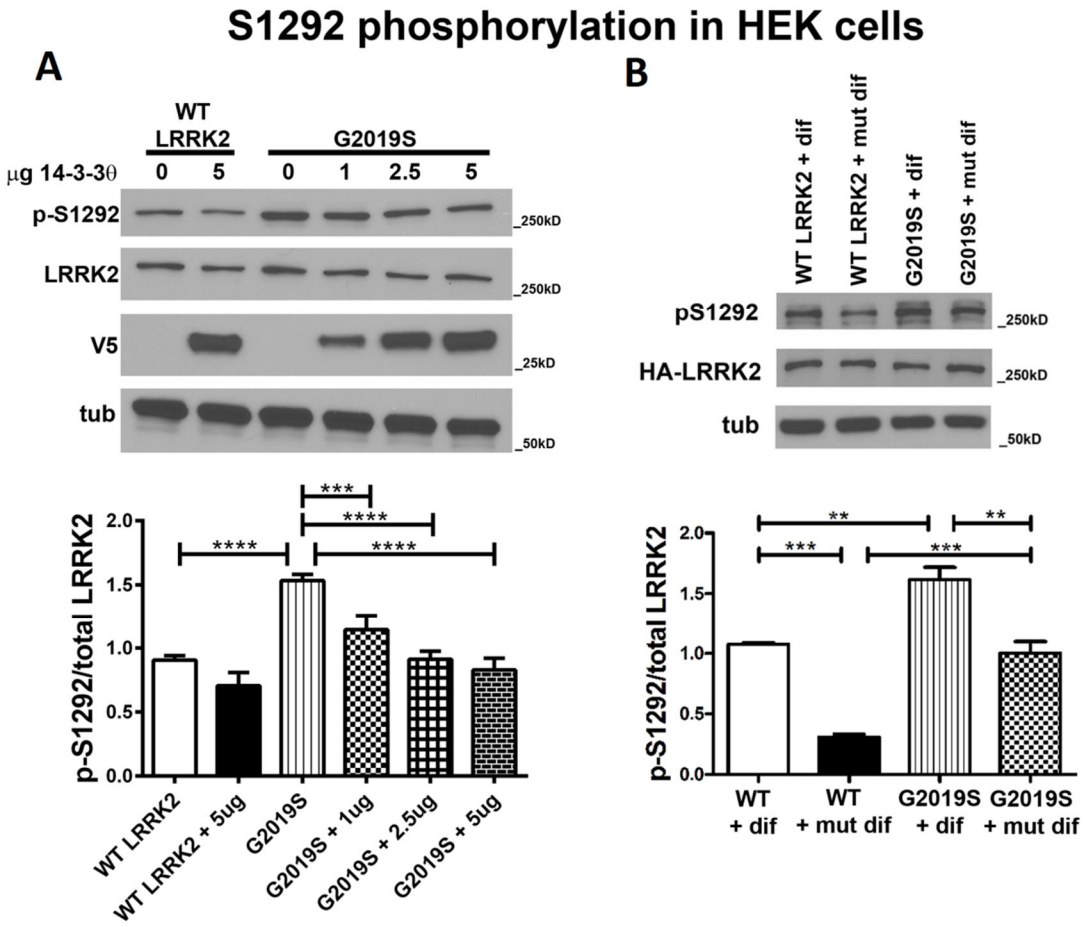

Figure 2. 14-3-3s regulate LRRK2 S1292 autophosphorylation *in vitro*

A) Lysates from HEK 293T cells transfected with wildtype or G2019S-LRRK2 along with increasing amounts of V5-tagged 14-3-3θ plasmid were analyzed for LRRK2 autophosphorylation at S1292 and total LRRK2 by western blot. n=4 independent rounds. ***p<0.001, ****p<0.0001 (Tukey's multiple comparison test).

B) Lysates from HEK 293T cells transfected with wildtype or G2019S-LRRK2 along with difopein-eYFP or mutant difopein-eYFP were analyzed for LRRK2 autophosphorylation at S1292 and total LRRK2 by western blot. n=3 independent rounds. **p<0.01, ***p<0.001 (Tukey's multiple comparison test).

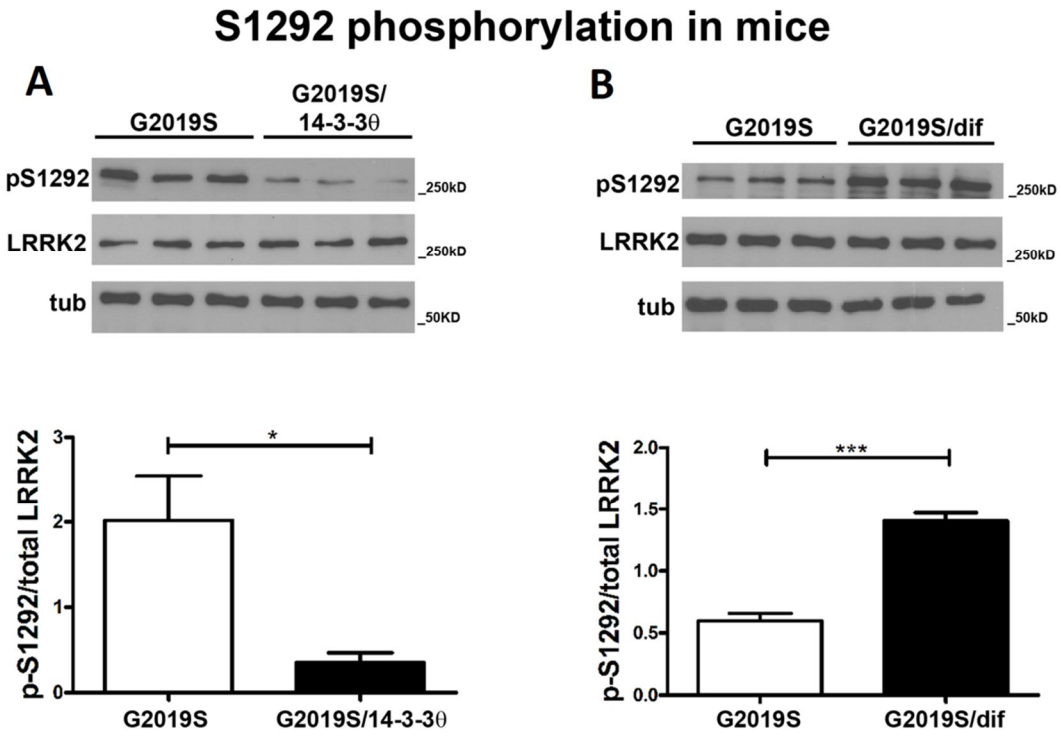

Figure 3. 14-3-3s regulate LRRK2 S1292 autophosphorylation *in vivo*

A) Hippocampal lysates from 6-week-old G2019S-LRRK2 or G2019S-LRRK2/14-3-3θ littermates were analyzed for LRRK2 autophosphorylation at S1292 and total LRRK2 by western blot. n=3 mice per group, *$p<0.05$ (unpaired t-test).

B) Hippocampal lysates from 6-week-old G2019S-LRRK2 or G2019S-LRRK2/difopein-eYFP littermates were analyzed for LRRK2 autophosphorylation at S1292 and total LRRK2 by western blot. n=3 mice per group, ***$p<0.001$ (unpaired t-test).

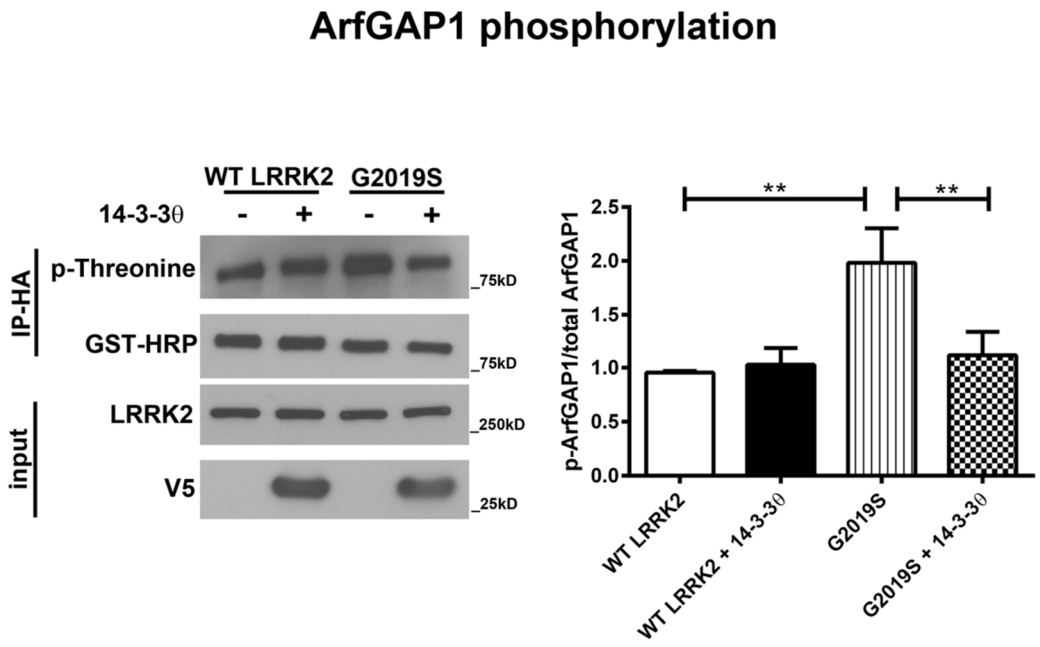

Figure 4. 14-3-3s regulate ArfGAP1 phosphorylation by LRRK2

Lysates from HEK293T cells transfected with wildtype or G2019S-LRRK2 with and without V5-tagged 14-3-3θ were immunoprecipitated with an HA-specific antibody prior to performing the kinase assay. Kinase assays were performed with immunoprecipitates together with recombinant GST-tagged ArfGAP1. Following incubation in kinase buffer, immunoprecipitates were then probed for phospho-threonine and GST by western blot. n=3 independent rounds. **$p<0.01$ (Tukey's multiple comparison test).

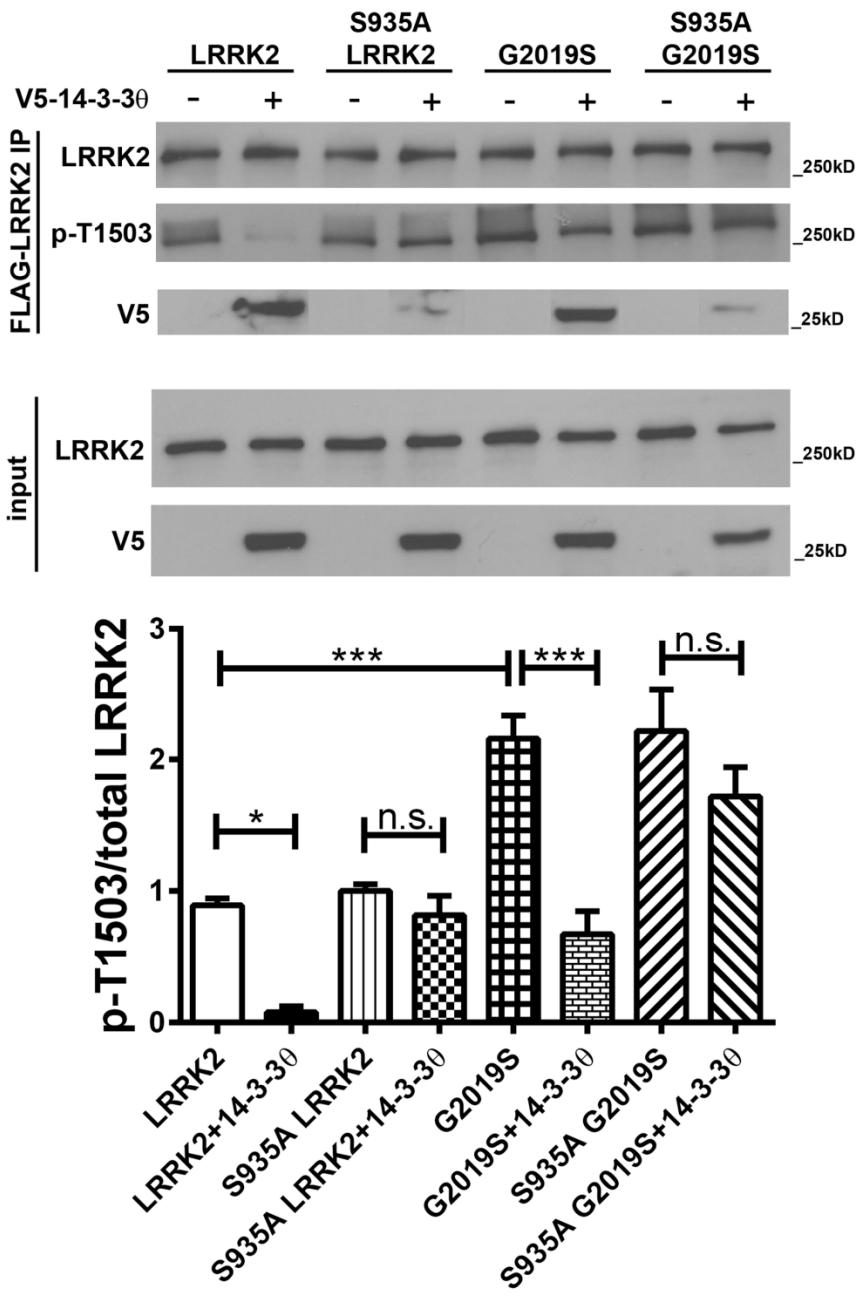

Figure 5. Binding to LRRK2 is required for 14-3-3θ regulation kinase activity

HEK 293T cells were cotransfected with either FLAG tagged wildtype or G2019S-LRRK2 with and without the S935A mutation and V5-tagged 14-3-3θ. Lysates were immunoprecipitated with FLAG antibodies. Kinase assays were performed with immunoprecipitates. Resultant immunoprecipitants were analyzed by western blot for phospho-T1503, and for LRRK2 and V5 to verify that 14-3-3 co-immunoprecipitation was disrupted by the S935A mutation. n=4 independent rounds. *$p<0.05$, ***$p<0.001$, n.s.=not significant (Tukey's multiple comparison test).

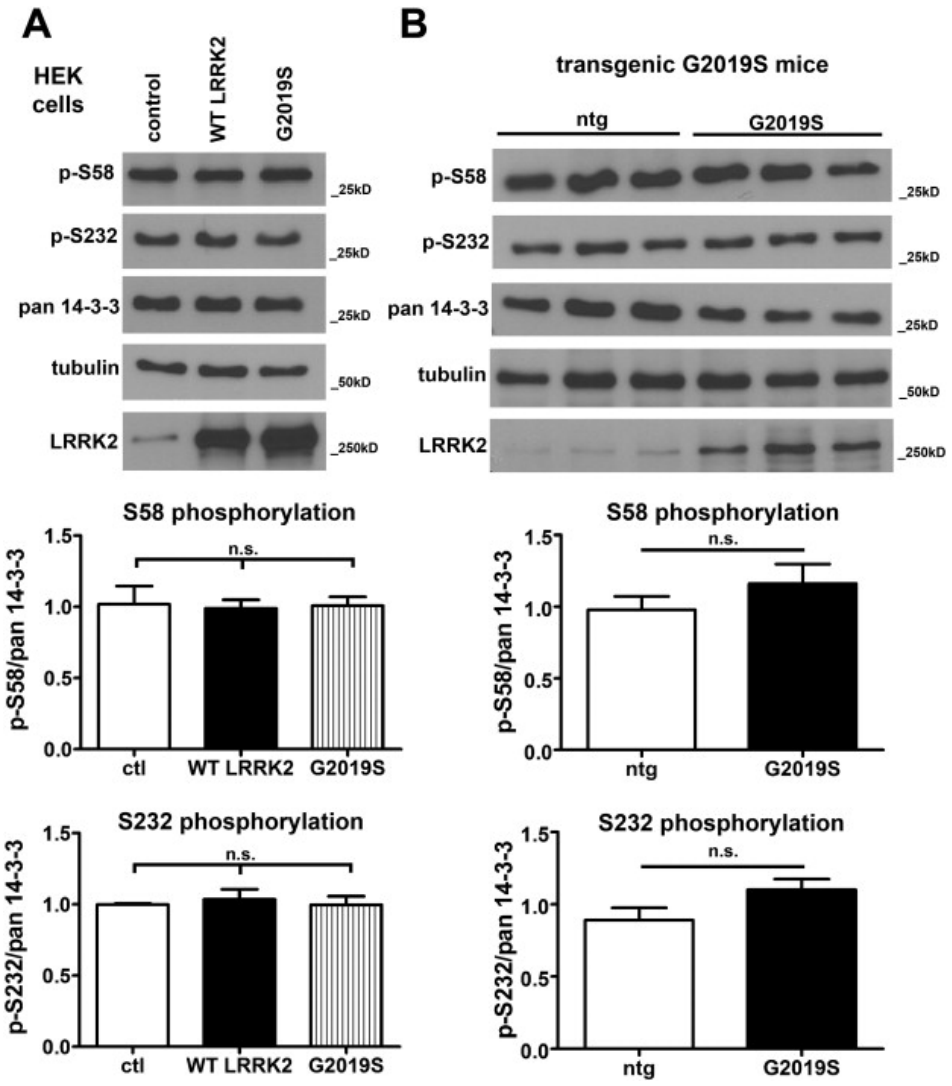

Figure 6. Wildtype and mutant LRRK2 expression does not affect 14-3-3 phosphorylation at S58 or S232.

A) HEK 293T cells were transfected with wildtype or G2019S LRRK2, and cell lysates were analyzed for phospho-S58 or phospho-S232 by Western blot. Untransfected HEK 293T cells were used as control. Representative Western blot is shown. Tubulin was used as loading control. The ratio of phospho-S58 to pan 14-3-3 and the ratio of phospho-S232 to pan 14-3-3 are quantified for five independent runs. n.s. (1 way ANOVA).

B) Hippocampal lysates from non-transgenic or G2019S littermates were analyzed for phospho-S58 or phospho-S232 by Western blot. Representative Western blot is shown. Tubulin was used as loading control. n = three mice per genotype. n.s. (Student's unpaired t-test).

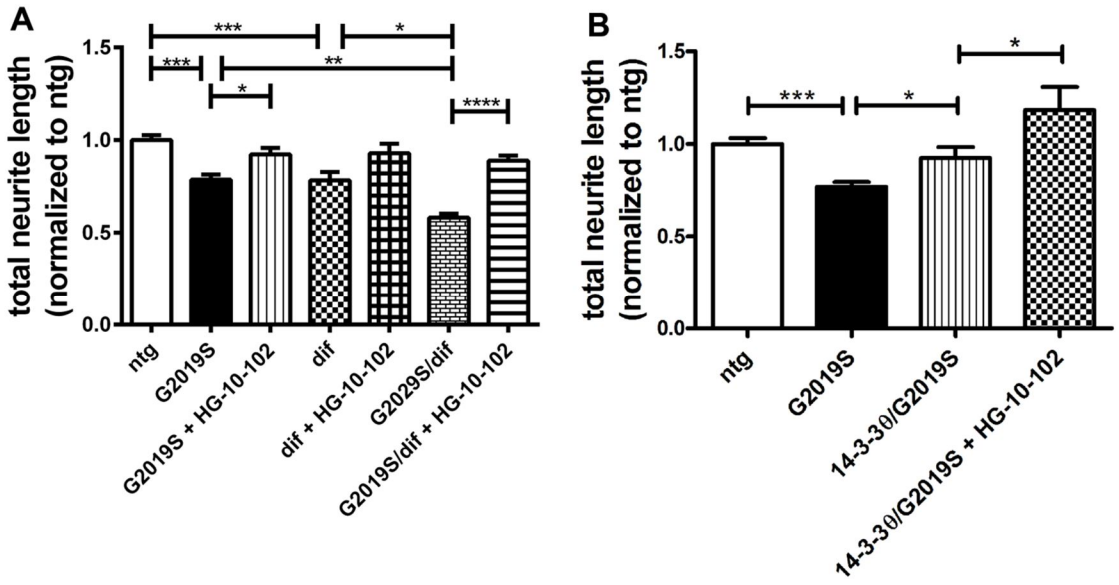

Figure 7. The LRRK2 kinase inhibitor HG-10-102-01 reverses the enhanced neurite shortening observed in the G2019S-LRRK2/difopein double transgenic mice.

A) Total neurite length analysis of primary hippocampal neurons from nontransgenic, G2019S-LRRK2, difopein-eYFP, and G2019S-LRRK2/difopein-eYFP mouse cultures treated with HG-10-102-01 (1μM) for 48 hours prior to fixing and staining. n=45 neurons for ntg, n=21 for G2019S-LRRK2, n=25 for G2019S-LRRK2 + HG-10-102, n=24 for dif, and n=21 for dif + HG-10-102, n=23 for G2019S-LRRK2/dif, n=24 for G2019S-LRRK2/dif + HG-10-102 combined from four independent rounds. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (Tukey's multiple comparison test).

B) Total neurite length analysis of neurons from nontransgenic, G2019S-LRRK2, 14-3-3θ, and G2019S-LRRK2/14-3-3θ mouse cultures treated with HG-10-102-01 (1μM) for 48 hours prior to fixing and staining. n=27 neurons for ntg, n=44 for G2019S-LRRK2, and n=20 for G2019S-LRRK2/14-3-3θ, n=11 for G2019S-LRRK2/14-3-3θ + HG-10-102 combined from three independent rounds. *$p<0.05$, ***$p<0.001$ (Tukey's multiple comparison test).

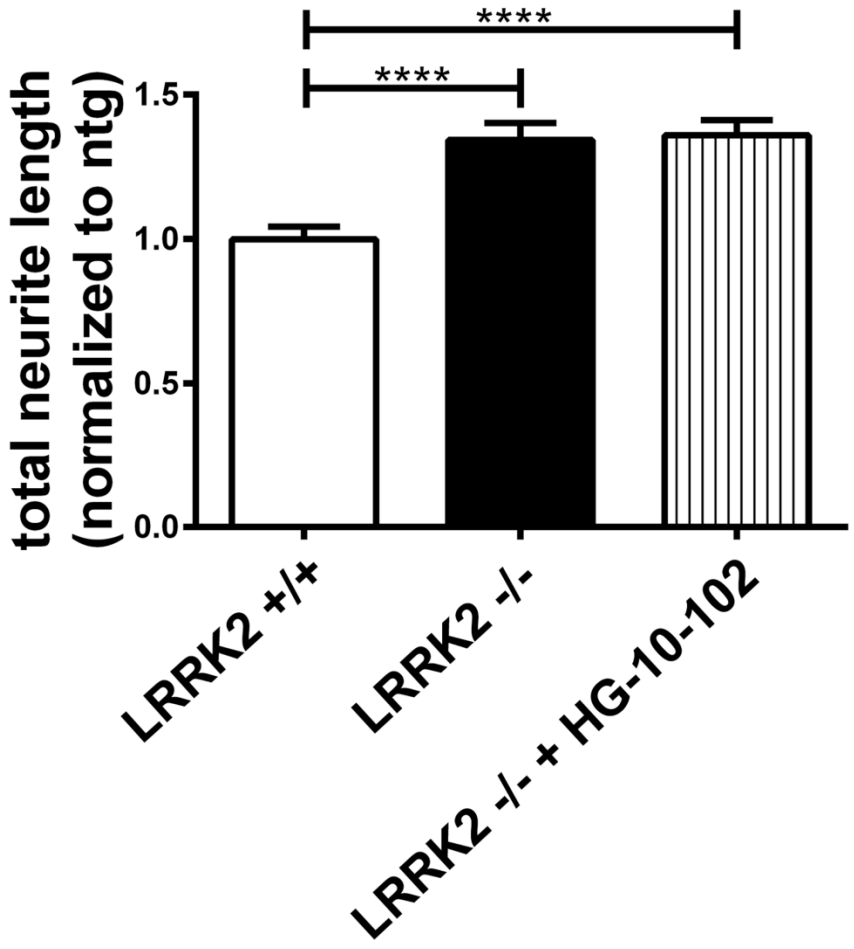

Figure 8. HG-10-102-01 does not alter neurite length in LRRK2 knockout neurons.

Total neurite length analysis of neurons from LRRK2 +/+ and LRRK2 -/- mouse cultures treated with HG-10-102-01 (1μM) for 48 hours prior to fixing and staining. n=30 neurons for LRRK2 +/+, n=22 neurons for LRRK2 -/-, n=23 neurons for LRRK2 -/- + HG-10-102 combined from three independent rounds. ****$p<0.0001$ (Tukey's multiple comparison test).

Conclusions

Here we investigated the role of 14-3-3 as a neuroprotective agent in LRRK2 models of Parkinson's disease (PD). Previous work has demonstrated that 14-3-3s provide a protective effect against neurotoxic models of PD, as well as in a *C. elegans* genetic model of PD [1-3]. Here, our work also demonstrates that 14-3-3s protect against LRRK2 models of PD. Given that mutations in LRRK2 present a similar clinical phenotype as idiopathic PD [4], there is reason to believe that treatments for LRRK2 mediated PD may also extend to idiopathic PD as well.

14-3-3s in LRRK2 Mediated Neurodegeneration

The data provided in this dissertation aimed to examine the role of 14-3-3s in regulating LRRK2 activity and dysfunction. In summary of the data shown in this dissertation, we established that 14-3-3s play a regulatory role over LRRK2 in altering phosphorylation and localization. In addition, 14-3-3θ overexpression reversed mutant LRRK2 toxicity in both the G2019S and R1441G background. Lastly, 14-3-3 expression levels altered LRRK2 kinase activity with overexpression reducing LRRK2 kinase activity and inhibition causing an increase in kinase activity. Conversely, we observed that 14-3-3 inhibition reduces LRRK2 phosphorylation at S910/935, potentiated mutant LRRK2-mediated neurite toxicity, and increased LRRK2 kinase activity. Figure 1A provides an updated look at the model introduced in chapter 1 and shows how we believe 14-3-3θ overexpression affects LRRK2 toxicity. Conversely, Figure 1B shows how 14-3-3 inhibition negatively affects the same pathway and potentiates LRRK2 mediated toxicity. Based on our data, we propose the following model for mutant LRRK2

toxicity: LRRK2 mutations, such as R1441G, result in reduced LRRK2 binding as noted by reduced S910/935 phosphorylation, which then leads to altered LRRK2 subcellular localization and increased kinase activity, which ultimately results in reduced neurite outgrowth and toxicity (Figure 1).

With regard to G2019S, we propose a modification to the above model. Notably, the G2019S mutation is unique from many other LRRK2 mutations in that the S910 and S935 phosphorylation is not reduced and 14-3-3 binding is not disrupted normally. Instead we propose that 14-3-3s regulate G2019S toxicity by direct interaction with G2019S to regulate kinase activity and thus toxicity. Figure 2A and 2B shows how G2019S affects kinase activity and how 14-3-3θ overexpression is protective in reducing G2019S-LRRK2 mediated toxicity and kinase activity. This is potentially through direct interaction between 14-3-3θ and G2019S-LRRK2, preventing excess autophosphorylation and kinase activity.

In chapter 3, we first demonstrated that manipulation of 14-3-3s can cause biochemical changes of LRRK2. Indeed, the data showed that 14-3-3 inhibition blocked the interaction between 14-3-3s and LRRK2 and that this led to redistribution of LRRK2 into perinuclear punctae, in agreement with reports from other groups. In addition, we saw that 14-3-3θ overexpression caused an increase in LRRK2 phosphorylation at both the S910 and S935 sites, two key residues that need to be phosphorylated in order for 14-3-3s to bind LRRK2. Conversely, inhibition with the pan-14-3-3 inhibitor difopein caused a reduction in phosphorylation. This suggests that 14-3-3 binding to LRRK2 protects LRRK2 from interacting with protein phosphatase 1 (PP1), a phosphatase that has been shown to dephosphorylate LRRK2 [5]. By protecting LRRK2 from being

dephosphorylated by PP1, we see increased phosphorylation at S910 and S935 when 14-3-3s are overexpressed.

In chapter 4 we examined whether 14-3-3s could protect against mutant LRRK2 mediated toxicity. We saw that overexpression of 14-3-3θ can reverse mutant LRRK2 mediated neurite shortening in both the G2019S and R1441G mutations. In addition, inhibition with either difopein or dominant negative (DN) 14-3-3θ further potentiated neurite shortening by G2019S, indicating that disrupting the interaction between 14-3-3s and LRRK2 may allow mutant LRRK2 to further reduce neurite outgrowth. LRRK2 has been suggested to influence neurite outgrowth through regulation of tubulin by multiple groups [6-8]. In addition, mutant LRRK2 has been shown to phosphorylate tau [9], which may cause a destabilization of microtubules and eventually lead to breakdown of the microtubule network. These are two potential mechanisms by which LRRK2 may be influencing neurite outgrowth that are reversed by 14-3-3θ overexpression.

In chapter 5, we identified the mechanism by which we believe 14-3-3θ overexpression conferred protection. Indeed, it is believed that aberrant kinase activity is driving toxicity from mutant LRRK2 [10]. We saw that 14-3-3θ overexpression reduced kinase activity as measured by both autophosphorylation and substrate phosphorylation with ArfGAP1, while 14-3-3 inhibition promoted kinase activity. An interesting idea is that LRRK2 is dependent on dimerization for its kinase activity [11], and combining this with the fact that disrupting the LRRK2/14-3-3 interaction causes LRRK2 to relocalize in perinuclear puncta opens the possibility that these puncta are LRRK2 oligomers with increased kinase activity. More simply, 14-3-3θ overexpression could increase binding to mutant LRRK2 and reduce LRRK2 kinase activity by altering the conformation of

LRRK2. Notably, 14-3-3s need to bind LRRK2 to reduce kinase activity as inserting the S935A mutation into LRRK2 to disrupt 14-3-3 binding prevented the reduction of LRRK2 kinase activity, suggesting direct interaction is required. Regardless of the exact mechanism, the data supports the conclusion that 14-3-3θ overexpression reduces LRRK2 kinase activity.

14-3-3s in neurodegenerative disease

The role of 14-3-3s in neurological disease is a bit diverse, as 14-3-3s play a prominent role in multiple neurodegenerative disorders. In most cases, 14-3-3s are conferring protection. Work previously done by this lab has shown that 14-3-3ε, γ and θ are downregulated at the mRNA level in an alpha-synuclein overexpression mouse model and that overexpression of these three isoforms can confer protection in the MPP+ and rotenone neurotoxin models of PD. Additionally, 14-3-3θ overexpression can prevent dopaminergic cell loss in a *C. elegans* model of PD. In Alzheimer's disease (AD), 14-3-3s interact with amyloid beta (Aβ) and this interaction is thought to be important as it may reduce toxicity caused by Aβ [12]. In Huntington's disease (HD), 14-3-3s have been shown to colocalize with mutant huntingtin when it aggregates [13], which could lead to an increase in apoptosis the same way 14-3-3s are sequestered by alpha-synuclein in Lewy bodies in PD. Additionally, 14-3-3s interact with Huntington's disease binding protein 2 (HDBP2) and shuttle it from the cytosol to the nucleus where it regulates transcription of huntingtin [14, 15]. 14-3-3ζ overexpression has also been shown to increase mutant huntingtin aggregation [16, 17], which is thought to be protecting neurons against toxicity when the abnormal processing of misfolded proteins overwhelms

the quality control systems in the cell [17]. In acute neurodegeneration, such as strokes and seizures, 14-3-3 levels have been shown to be altered as well in response to insult [18-20], mostly providing neuroprotection. In fact, overexpression of 14-3-3ε protected against neuronal loss in a model of cerebral ischemia and reperfusion injury in rats [21]. In many models of neurodegeneration, 14-3-3s show a neuroprotective effect in preventing neuronal death and reducing mutant protein aggregation.

However, the role of 14-3-3s in neurodegenerative disease is a bit confounded by data from multiple disease models. For example, in Spinocerebellar ataxia (SCA), 14-3-3s bind to mutant ataxin-1, which stabilizes the protein and slows its normal degradation, allowing mutant ataxin-1 to form aggregates [22]. In a SCA1 mouse model, overexpression of 14-3-3ε has been shown to either be protective or cause neuronal death in different regions of the brain [23], making its roll in SCA1 a bit confusing. Additionally, in AD, 14-3-3s colocalize with tau protein and protect tau from being dephosphorylated [24-27]. This may contribute to hyperphosphorylation of tau and the formation of neurofibrillary tangles, as many groups have shown [24, 25, 27].

From an overall standpoint, the role of 14-3-3s in neurodegeneration appears to be protective in terms of preventing programmed cell death, yet a couple models suggest that certain 14-3-3 isoforms may contribute to disease pathology by inadvertently protecting mutant proteins from being degraded.

Determining Isoform Specificity in LRRK2 Models

Now that we have established that 14-3-3θ can be protective against mutant LRRK2 mediated neurite shortening, an interesting follow-up would be to test the effects of the other isoforms in this model. We have seen that 14-3-3θ overexpression causes neurite lengths to normalize and a concurrent reduction in LRRK2 kinase activity, but whether or not the other isoforms can achieve the same level of protection is unknown. For example, in the alpha-synuclein transgenic mouse model, only three isoforms show a decrease in expression at the mRNA level [3]. This is significant because it shows that certain isoforms may be more important in providing neuroprotection over others. Indeed, the reason this work focused on the theta isoform was because previous work from this group had shown not only that 14-3-3θ expression was decreased in the alpha-synuclein model [28, 29], but that overexpression of the theta isoform consistently conferred the most protection against toxic models of PD [1-3]. It is worth noting that 14-3-3ε, and γ also conferred protection in the MPP+ and rotenone backgrounds *in vitro*; however, 14-3-3ε and γ did not prevent dopaminergic cell death in a *C. elegans* model [3]. Importantly, a functional link between LRRK2 and 14-3-3s have been established as Fraser et al. showed that disrupting the interaction between 14-3-3s and LRRK2 with difopein or LRRK2 inhibitors prevents LRRK2 secretion through exosomes, indicating 14-3-3s may traffic LRRK2 to forming exosomes to be secreted [30]. Conversely, overexpression of 14-3-3ε promotes LRRK2 release in exosomes. In addition, they also saw that 14-3-3ε overexpression rescued LRRK2 aggregation that occurs when HEK-293T cells were treated with a LRRK2 inhibitor [30], indicating a regulatory role for 14-3-3s in maintaining a normal, cytosolic distribution of LRRK2. This in agreement with

our data showing that disruption of the 14-3-3/LRRK2 interaction with difopein causes LRRK2 aggregation. This shows that other isoforms than just the 14-3-3θ isoform can regulate LRRK2.

Expression Levels in LRRK2 Models

One potential follow-up experiment to determine which isoforms might be altered in mutant LRRK2 mouse lines would be to look at isoform specific expression at both the protein and mRNA levels in the hippocampi of mutant LRRK2 mice. This may give insight as to whether or not mutant LRRK2 affects 14-3-3 expression, although we have previously seen that mutant LRRK2 does not have any effect on 14-3-3 phosphorylation (chapter 5, figure 6). We did notice that total 14-3-3 expression was consistent between mice overexpressing wildtype, G2019S and R1441G LRRK2, yet it would be interesting to see if certain isoforms are downregulated and there is a compensatory upregulation of other isoforms to achieve the same total level of 14-3-3 expression. If certain isoforms are downregulated, this may help to identify other pathways that are being altered in mutant LRRK2 expressing lines and provide a new, potential therapeutic targets.

LRRK2 Mediated Neurite Shortening

In addition to the data showing that 14-3-3θ influences mutant LRRK2 mediated neurite shortening, multiple other 14-3-3 isoforms have been implicated in altering neurite outgrowth through a variety of pathways [31-35]. Our data suggests that 14-3-3θ overexpression normalized neurite length by directly interacting with and reversing mutant LRRK2 action. To examine the effects of other 14-3-3 isoforms in reversing mutant LRRK2 mediated neurite shortening, the most feasible model would be to transfect primary hippocampal cultures from the BAC transgenic G2019S mouse line that

we utilized for our experiments. Other groups have shown that neuronal cultures can be successfully transfected using reagents such as Lipofectamine [36] or by using Amaxa electroporation [37-39]. Additionally, the other 14-3-3 isoforms could be made into lentiviral constructs that could be used to transduce primary cultures to achieve specific 14-3-3 isoform overexpression as well.

Mechanisms of LRRK2 Mediated Neurite Outgrowth

From a mechanistic standpoint, there is some evidence that LRRK2 interacts with tubulin and actin and that through alteration of these proteins, LRRK2 may be directly interfering with microtubules and the actin cytoskeleton [6-8]. Specifically, LRRK2 mutations have been shown to interact with microtubules and cause increased phosphorylation of tau [40-43]. Tau protein is involved in stabilizing microtubules and when phosphorylated, separates from the microtubules, leading to destabilization and eventual breakdown. It would therefore be interesting to see if 14-3-3 overexpression can affect increased tau phosphorylation observed with LRRK2 mutations. By *in vitro* transfection of differentiated SH-SY5Y neuroblastoma cells with mutant LRRK2 and the different 14-3-3 isoforms, we could examine changes in tau phosphorylation. Additionally, we could measure tau phosphorylation in hippocampal lysates of G2019S/14-3-3θ double transgenic mice versus nontransgenic and G2019S controls to demonstrate whether this effect occurs *in vivo* as well. Since the aberrant kinase activity of mutant LRRK2 is potentially causing the increase in tau phosphorylation, we would predict that 14-3-3 overexpression would reduce tau phosphorylation by reducing LRRK2 kinase activity. The caveat in looking at tau phosphorylation is that 14-3-3s have been shown to protect tau from being dephosphorylated [26, 27, 44, 45], possibly

contributing to the hyperphosphorylated state of tau which form neurofibrillary tangles that are common in Alzheimer's disease (AD) pathology. Despite this, observing how tau phosphorylation changes with 14-3-3 overexpression in the mutant LRRK2 background would be interesting in further examining potential mechanisms by which 14-3-3s regulate mutant LRRK2 induced neurite shortening.

LRRK2 Kinase Activity

Beyond neurite outgrowth, looking at changes in LRRK2 kinase activity with overexpression of other 14-3-3 isoforms would help to determine which isoforms can confer protection against LRRK2 mutations. Our data has shown *in vitro* and *in vivo* reduction of LRRK2 kinase activity in the G2019S mutation with 14-3-3θ overexpression and that this effect requires direct interaction as introduction of the S935A mutation prevented a reduction in kinase activity. Notably, Sheng et al. showed that phosphorylation of the S1292 residue in LRRK2 corresponded to LRRK2 kinase activity and that phosphorylation at this site was increased in many of the most common pathogenic LRRK2 mutations [46]. Indeed, we saw that S1292 phosphorylation was increased both *in vitro* and *in vivo* with the G2019S mutation and that 14-3-3θ overexpression reduced phosphorylation levels back down to nontransgenic levels. By co-transfecting cells with the different LRRK2 mutants and the other isoforms of 14-3-3, we can show which isoforms can alter LRRK2 kinase activity and how many different LRRK2 mutations they can confer protection against by measuring S1292 phosphorylation. This can also be observed *in vivo* by injecting the hippocampi of BAC transgenic G2019S mice with viruses that overexpress the different 14-3-3 isoforms and look at S1292 phosphorylation in the hippocampi of these mice.

It would also be prudent to look at substrate phosphorylation as well since the kinetics of autophosphorylation and substrate phosphorylation are not the same. There have been multiple *in vitro* substrates identified for LRRK2, including moesin, myelin basic protein (MBP), ArfGAP1 and LRRKtide [47, 48]. Phosphorylation of these substrates is generally measured by a ^{32}P incorporation assay although variable results have been shown in regards to the different LRRK2 mutations. Many of the mutations do not shown any change in substrate phosphorylation over wildtype; however, the G2019S mutation has consistently shown increased substrate phosphorylation similar to autophosphorylation. By co-transfecting G2019S LRRK2 with the different 14-3-3 isoforms, we can determine if any other isoforms can reduce LRRK2 kinase activity in addition to the theta isoform.

Potential 14-3-3 Effects on the GTPase Domain of LRRK2

Notably, inhibition of the GTPase domain of LRRK2 has also been shown to reverse neurite shortening seen in LRRK2 mutations [49]. It is thought that there is interplay between the GTPase and kinase domains of LRRK2 [50] and that inhibition of either domain can reduce the toxicity of the mutant protein [49, 51, 52]. Like changes in kinase activity, mutations in LRRK2 have been shown to have variable effects on GTPase activity with some mutations having little to no effect while others may enhance GTP binding but slow GTP hydrolysis [49, 50, 53]. Since 14-3-3s also have a noted role in altering protein conformation, it is plausible that increased 14-3-3 binding to LRRK2 may either enhance or inhibit GTP binding and hydrolysis by LRRK2. Also, 14-3-3s have be known to alter the activity of other GTPases such as Ras [54], Rac1 [55] and

Cdc42 [56]. As no one has currently measured the effects of 14-3-3 overexpression on LRRK2 GTPase activity, it would be useful to determine if 14-3-3 overexpression can first alter GTP binding by measuring the amount of LRRK2 that binds to a GTP immobilized γ-aminohexyl-GTP-sepharose resin. In addition, GTPase activity can be measured by utilizing a colormetric assay that measures the release of free phosphate when GTP is hydrolyzed to GDP. Through these steps, it can be shown whether 14-3-3 overexpression has any effect on LRRK2 GTP binding and hydrolysis.

14-3-3s in LRRK2-Independent Neurite Outgrowth

Notably, difopein and DN 14-3-3θ expression in our culture system caused a LRRK2-independent neurite shortening phenotype. In the case of difopein, it is likely that we are influencing neurite outgrowth through other pathways besides LRRK2 mediated outgrowth as multiple isoforms have been shown to influence neurite outgrowth through alternate pathways. For example, neuron navigator 2 (NAV2) has been shown to regulate neurite outgrowth and axonal elongation [35]. Work by Marzinke et al. has shown 14-3-3ε to interact with NAV2 and that when 14-3-3ε expression is knocked down, neurite outgrowth is diminished in a similar manner to when NAV2 itself is knocked-out [35]. Additional work by Ramser et al. has shown that 14-3-3ζ binds to and promotes phosphorylation of the cell adhesion molecule L1, which influences L1's ability to control neurite outgrowth [33]. Other groups have identified the link between 14-3-3s and neurite outgrowth regulators such as neural cell adhesion molecule (NCAM) [32], actin depolymerizing factor (ADF) [34] and SLIT and NTRK-like family member 1 (SLITRK1) [31]. In summary, by expressing difopein we are likely affecting multiple

outgrowth pathways simultaneously and the overall effect of 14-3-3 inhibition is neurite shortening. In the case of DN 14-3-3θ, it is likely that because 14-3-3s dimerize with each other to bind other proteins, by inhibiting the interaction of the theta isoform with the other isoforms it interacts with we are disrupting the interaction between 14-3-3s and these neurite outgrowth regulating proteins.

The work looking at neurite outgrowth is important in disease pathogenesis because recent work has shown axonal and dendritic degeneration to occur in many genetic mutations associated with PD [57-61]. For example, Orimo et al. has shown that alpha-synuclein pathology begins in the distal axon and proceeds retrograde to the cell body in patients with PD [61]. This is in agreement with work by other groups showing that alpha-synuclein aggregates are most abundant in the neuropil at the presynaptic cleft in patients with dementia with Lewy Body (DLB) [60], another synucleinopathy related to PD. Furthermore, characterization of the R1441G BAC transgenic mouse showed fragmented axons and multiple other LRRK2 mutations show overall neurite outgrowth to be stunted [41]. Taken together, this suggests that axonal dysfunction may be an early feature of PD and that measurement of neurite outgrowth can be a viable method to test the effectiveness of potential therapeutics for PD.

Potential Drawbacks to Extended Therapeutic Use

The use of 14-3-3θ as a therapeutic for PD has good potential thus far. However, because of the diverse cellular and metabolic pathways that 14-3-3s are involved in, there is the potential for many side effects from treatment that increases 14-3-3 expression levels. Our mouse lines that overexpress 14-3-3θ under the Thy1.2 promotor in the brain

seem to tolerate the overexpression well. The mice have normal life spans and motor testing has shown no changes in motor function or behavior. However, looking forward to patients, the role of 14-3-3s in the pathogenesis of other diseases, such as cancer should be considered. For example, 14-3-3σ has tumor suppressing activity [62, 63] through its interaction with Akt [64], yet the ζ, β and θ isoforms have been shown to be oncogenic, as they have been shown to be overexpressed in various cancers [65-79], although this does not necessarily mean causality as 14-3-3 upregulation could reflect some compensatory mechanism in response to insult. Conversely, high expression of 14-3-3ζ is associated with poor prognosis in breast cancer [65] and elevated 14-3-3ζ expression is seen in patients with non-small cell lung cancers and head and neck cancers like squamous carcinoma [77], opening up the possibility that the anti-apoptotic effects of 14-3-3s might be leading to preservation of tumor cells.

In addition to cancer, 14-3-3s have been shown to be pathogenically involved in other non-neurodegenerative diseases, such as joint inflammation. 14-3-3η and γ have been shown to be elevated in the synovial fluid of patients with joint inflammation and that they may play a causal role in recruiting and/or activating matrix metalloproteinases that cause joint damage in arthritis [80]. This indicates the variable role that 14-3-3s play in disease and highlight the potential downfalls of altering 14-3-3 expression patterns. These are potential issues that can arise from long-term overexpression of 14-3-3s in patients, especially those with a family history of these diseases.

Summary

Accumulated data presented in this dissertation, combined with previous data from this group show that 14-3-3θ may have strong therapeutic potential in PD. Previous work has shown alterations in 14-3-3 expression levels in alpha-synuclein models of PD with multiple isoforms being down-regulated [28, 29] and that overexpression of certain 14-3-3 isoforms can be protective against multiple toxic model of PD [1-3]. The work presented in this dissertation shows that 14-3-3θ overexpression is beneficial against two different mutations in LRRK2. Additionally, mutations in LRRK2 are the most common genetic mutation that causes PD and accounts for a percentage of sporadic cases as well [81-85]. Notably, LRRK2 PD patients have a clinical phenotype that is indistinguishable from sporadic PD [83, 85]. This highlights not only that LRRK2 is a prime target for treatment in PD, but that because 14-3-3θ confers protection against LRRK2, that it has strong therapeutic potential.

To conclude, we believe 14-3-3θ overexpression has viable therapeutic potential. We have seen a reversal in neurite shortening from PD causing LRRK2 mutations. How well this translates to human patients remains to be seen, however, since mouse models do not perfectly replicate human disease. This is characteristic of many potential therapeutics, however, since initial research must begin in cell and mouse lines before being deemed safe and efficacious enough to move to patient trials. Nevertheless, 14-3-3θ overexpression has a growing amount of positive data to support the therapeutic potential to one day move into patient trials. The work provided in this dissertation shows the reversal of toxicity in the LRRK2 mouse model of PD and describes the mechanism by which 14-3-3θ may be protective. Previous work by our group has shown

14-3-3θ to be protective against other accepted models of PD as well, both *in vitro* and *in vivo*. In short, the therapeutic potential of 14-3-3θ is present, but more research would be useful in determining the possibility of potential side effects from prolonged protein overexpression as persistent overexpression of 14-3-3θ would be required to slow degeneration. Current treatments for PD are effective in reducing motor symptoms and improving patient's quality of life, however, there remains no treatment that can stop, or even slow disease progression. We feel that 14-3-3θ has the potential to slow down neurodegeneration in PD and increase lifespan and quality of life for PD patients.

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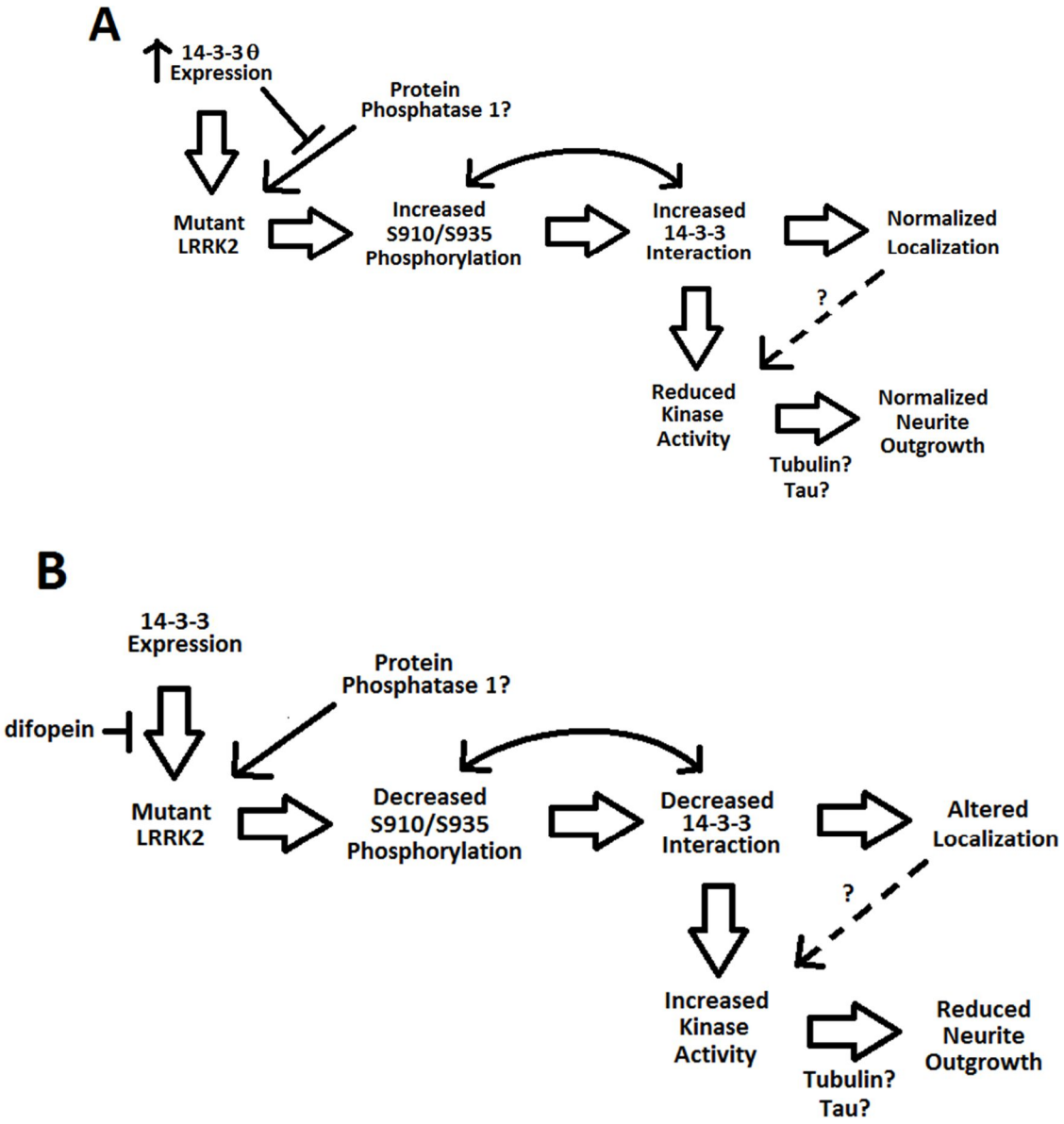

Figure 1. A) 14-3-3θ overexpression prevents LRRK2 dephosphorylation, potentially mediated through protein phosphatase 1 (PP1). This increases S910 and S935 phosphorylation and allows for normal cytosolic distribution. Kinase activity is reduced to normal levels which results in normal neurite outgrowth, potentially through tubulin and/or tau regulation. B) Difopein disrupts the interaction between 14-3-3s and LRRK2, potentially allowing PP1 to further dephosphorylate LRRK2 at S910 and S935. This leads to increased LRRK2 kinase activity and the formation of LRRK2 puncta, which may influence LRRK2 activity by allowing LRRK2 monomers to come together and dimerize. The end result is reduced neurite outgrowth caused by increased LRRK2 kinase activity.

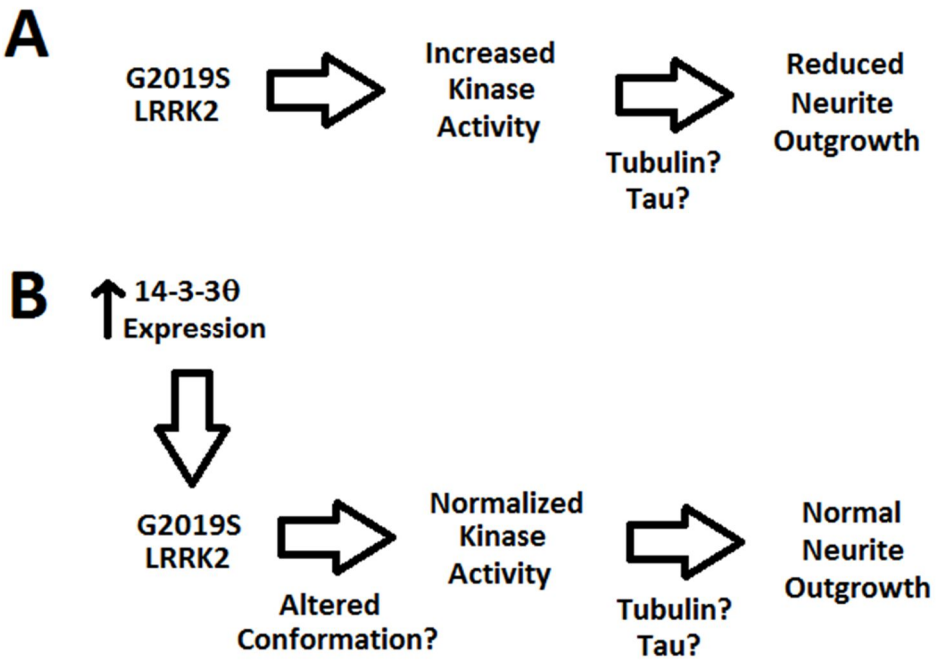

Figure 2. A) G2019S-LRRK2 does not have altered S910 or S935 phosphorylation compared to other LRRK2 mutations and also has normal cytosolic distribution. G2019S does have increased kinase activity that reduces neurite outgrowth. B) Increased 14-3-3θ expression binds to G2019S-LRRK2 and reduces kinase activity to normal levels, possibly through altering the conformation of LRRK2 to prevent excess autophosphorylation and kinase activity. This then allows for normal neurite outgrowth.

APPENDIX A

IACUC APPROVAL FORMS

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 12, 2014

TO: TALENE ALENE YACOUBIAN, PhD
CIRC-560D
(205) 996-7543

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Disruption of 14-3 3s in Models of Parkinson's Disease
Sponsor: Internal
Animal Project_Number: 150109820

As of January 14, 2015 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	264
Mice	B	54

Animal use must be renewed by January 13, 2016. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 150109820 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
CH19 Suite 403 | CH19 Suite 403
933 19th Street South | 1530 3rd Ave S
(205) 934-7692 | Birmingham, AL 35294-0019
FAX (205) 934-1188

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: December 12, 2014

TO: TALENE ALENE YACOUBIAN, PhD
CIRC-560D
(205) 996-7543

FROM: Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL – Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on December 12, 2014.

Title: Disruption of 14-3-3s in Models of Parkinson's Disease
Sponsor: Internal

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC) | Mailing Address:
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