Parkinson's Disease and Mechanisms of Fast Axonal Transport

BY

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THESIS

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This thesis is dedicated to my husband, Arzhang Zereshki and my aunt Nicole Szilagyi, for without them I would have not made it here.

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LIST OF ABREVIATIONS

6-OHDA	6-hydroxydopamine
A30P	alanine 30 to proline
A53T	alanine 53 substituted to threonine
AD	Alzheimer's disease
AFM	Atomic Force Microscopy
araC	cytosine β-D-arabinofuranoside
BDNF	brain derived neurotrophic factor
C	chemiluminescence,
CIP	calf intestinal phosphatase
DAT	dopamine transporter
DHC	dynein heavy chain
DIC	dynein intermediate chain
DIC	dynein intermediate chain
DIC1	dynein intermediate chain 1
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
E46K	glutamate 46 to lysine
EMEM	Eagle's Minimum Essential Medium
F	immunofluorescence
FAT	fast axonal transport
FBS	Fetal Bovine Serum
fSynuclein	mutated forms of α-synuclein

LIST OF ABREVIATIONS (continued)

FUdR	5-fluoro-2'-deoxyuridine
GSK3β	glycogen synthase kinase 3 β
HB	homogenization buffer
His	histidine
IP	immunoprecipitation
IPTG	isopropyl β-D-1-thiogalactopyranoside
KHC	kinesin heavy chain
KLC	kinesin light chain
LB	Lewy bodies
LB	Luria Broth
LN	Lewy neurites
LRRK2	leucine-rich repeat kinase 2
MBO	membrane bound organelles
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropridine
MT	microtubule
MTB	microtubule binding
MW	molecular weight
N27	1RB3AN27
NAC	non-Aβ component
OPI	oxaloacetate, pyruvate, and insulin
PAGE	polyacrylamide gel electrophoresis

LIST OF ABREVIATIONS (continued)

PBS	phosphate buffered saline
PBST	PBS Tween
PD	Parkinson's Disease
PDZ	PSD-95, Discs large, ZO-1
PenStrep	Penicillin Streptomycin
PKC	protein kinase C
PKCδ	protein kinase C δ
PKCμ	protein kinase C
PKD	protein kinase D
PMA	phorbol 12-myristate 13-acetate
RA	retinoic acid
S	serine
SB	sample buffer
SNpc	substantia nigra pars compacta
TB	Terrific Broth
TH	tyrosine hydroxylase
Urd	1-β-D-ribofuranosyluracil
WT	wild type

SUMMARY

Parkinson's Disease (PD) is a devastating neurodegenerative disorder that affects one million people in US alone. Its risk increases with age and as the population gets older, the financial and public health impact is expected to rise significantly. Traditionally, PD has been characterized by severe loss of dopaminergic neurons in the substantia nigra and their connections to the striatum although it is now known that these neurons are neither the first nor the only ones to be affected. When about 80% of the synapses from nigral dopaminergic neurons are no longer functional, the shortage of dopamine in the striatum causes the movement defects that characterize PD. The most common treatment is the administration of the dopamine precursor levodopa, but this only temporarily relieves the symptoms of the disease and it is most effective in the earlier stages. Pathologically, PD is characterized by the presence of Lewy bodies, intracellular protein aggregates whose main component is the protein α-synuclein. Mutations of α-synuclein as well as duplication and triplication of its gene lead to severe cases of early onset familial PD. Taken together this suggests a common pathogenic mechanism for both familial and sporadic PD involving α-synuclein.

Misregulation of axonal transport leads to loss of synaptic function, axonal degeneration and cell death. Previous results from our laboratory have demonstrated that perfusion of squid axoplasm with α-synuclein increase retrograde and decrease anterograde transport, the two components of fast axonal transport. Additional experiments suggest that α-synuclein effects on transport are mediated via non-receptor tyrosine kinase Src and Fyn and an isoform of protein kinase C (PKCμ), results that would be consistent with an effect on the state of phosphorylation of kinesin and

dynein, the molecular motors responsible for transport. Disruption of axonal transport could be a critical pathogenic mechanism in PD therefore this investigation focused on furthering understanding of the role α-synuclein might play in this mechanism.

The first aim was to determine molecular motifs within α-synuclein that affect kinase activity and fast axonal transport. Our laboratory identified the C-terminus of α-synuclein to have a role in modulating effects on axonal transport. Within the C-terminus there are potential non-receptor tyrosine kinase binding sites than could interact with kinases such as Src and Fyn. Generation of constructs of α-synuclein that had the C-terminus deleted abolished the effects pathogenic forms of α-synuclein had on axonal transport in squid axoplasm. Taking together all the observations, a mechanism for α-synuclein and axonal transport was generated. This model proposes that pathogenic forms of α-synuclein such as aggregates, mutated forms or too much protein, through its C-terminus, activate non-receptor tyrosine kinase such as Src and Fyn. Src and Fyn in turn activate PCKμ, which either directly or indirectly, affect the phosphorylation status of the molecular motors kinesin and dynein and therefore affect fast axonal transport.

The second aim was to test the pathogenic mechanism generated using squid axoplasm into a cellular model of PD. Several cell lines were screened and in that we found that the characteristics of a cell line significantly impacts its suitability for use as an expression system. The neuroblastoma cell line SH-SY5Y was selected and clones stably transfected with different forms of α-synuclein were generated. In assessing the molecular motors, we found that there were no changes in their expression in cells

expressing α-synuclein but that dynein interaction with the microtubules was affected. Examination of motor association with membranes may also be affected, indicating disruptions in cargo motor interactions, however a more specific pool of vesicle needs to be isolated in order to determine that with certainty.

Analysis of PKCμ in SH-SY5Y cells yielded surprising results. Treatment of differentiated cells with the phorbol PMA, a PKC activator, showed rapid increase in both activity and quantity of PKCμ within a manner of minutes, indicating that the kinase is tightly regulated. Although very intriguing these results rendered SH-SY5Y cells problematic for studying the effects of α-synuclein on PKCμ and a different system needs to be selected in order to define that. Work on PKCμ led to the serendipitous discovery of an unknown protein that is affected by α-synuclein expression. The identity of this protein has yet to be determined.

In conclusion we have demonstrated that the C-terminus of α-synuclein plays a significant role in activation of pathways that could contribute to PD pathogenesis. The generation of cell lines transfected with α-synuclein led to the discovery of an unknown protein that could hold the key to understanding this protein's mechanism of action and could bring us closer to the development of more effective therapies for PD.

I. INTRODUCTION

A. Parkinson's disease

1. History of Parkinson's disease

Parkinson's disease has been recognized since antiquity. Three thousand year old Indian Ayurvedic medical texts describe a clinical syndrome consisting of tremor and akinesia, the absence of movement, termed *kampavata* [1]. The ancient texts recommended this syndrome be treated with natural products from *Mucuna pruriens,* a plant now known to contain levodopa, the immediate precursor to dopamine although it was not until 1957 that Arvid Carlsson discovered dopamine in the mammalian brain [2]. In the Western world, in 175 A.D., the physician Galen [3], described a "shaking palsy" but the signature description of the disease came in 1817 when James Parkinson, in "Essay on the Shaking Palsy", described the core clinical features of what today is the most common neurodegenerative movement disorder. The essay described observations of six patients and was intended to encourage the study of the disease, which would indeed happen 60 years later, with Jean Martin Charcot who was the first to truly recognize the importance of Parkinson's work and named the disease after him.

According to the Parkinson Disease Foundation [4], there currently are as many as one million Americans and seven to ten million people worldwide that live with PD. It is believed that these numbers are an underestimate, as some cases go undetected. Men are one and a half times more likely to develop Parkinson and the average onset for the disease is 60 years of age. Approximately 3% of people are diagnosed before 50 and the incidence increases with age. For example, while 1% or more of the population over the age of 65 is affected, for the population of the age of 85 and over,

the numbers rise to 3% or more [5]. Such statistics would point not only to a great emotional and physical toll on many patients and their families but also to a great financial burden. The PD foundation also states that “the combined direct and indirect cost of Parkinson’s, including treatment, social security payments and lost income from inability to work, is estimated to be nearly $25 billion per year in the United States alone. Medication costs for an individual person with PD average $2,500 a year, and therapeutic surgery can cost up to $100,000 dollars per patient.” Therefore understanding, providing relief and ultimately curing the disease would not only be in the interest of affected individuals but of society as a whole as well.

2. Clinical Symptoms and Pathology in Parkinson Disease

Much research and effort has gone toward understanding PD and many advances have been made during the last decades. It is emerging that in many of its manifestations this is a very complex and heterogeneous disease. In spite of greater understanding, fundamental questions still challenge us. For example, how do we define the disease and is there more than one type? Are all diagnosed cases actual cases of PD or is Parkinsonism a secondary aspect of multiple diseases? How is PD diagnosed and what criteria should be used: clinical symptoms, neuropathology, genetics? When does the disease start?

Classic, idiopathic PD is seldom seen before the age of 40 and can only be diagnosed with certainty postmortem. Clinically, there are three cardinal features of the disease: rigidity, resting tremor and bradykinesia – slowness of movement [6]. Other symptoms include hypokinesia – reduction in movement amplitude and akinesia – absence of normal unconscious movement, which present themselves as paucity of

normal facial expression, decreased voice volume, drooling, decreased size and speed of handwriting, decreased stride length during walking [7], as well as postural instability and gait dysfunction. The majority of these motor symptoms, in particular bradykinesia, are due to a loss of projections of dopaminergic neurons from the substantia nigra pars compacta (SNpc), specifically those of neurons that project to the putamen in the striatum. The SNpc and the striatum are components of the basal ganglia, which are responsible for a variety of functions, one of which is voluntary movement control. The SNpc modulates motor function in two ways: activation of the direct pathway though dopamine receptors D1, thus promoting voluntary movement, and inactivation of the indirect pathway though dopamine D2 receptors, inhibiting involuntary movements. Degeneration of the dopaminergic pathways affects the balance between the two [8] and current therapies for PD continue to focus on dopamine replacement therapy, in particular administration of levodopa. To date, the most effective therapy, for patients who qualify, is deep brain stimulation in combination with administration of dopamine medication [9]. Such therapies vastly improve the quality of life of patients for a time, and to certain extent longevity, by eliminating many motor impairments but eventually all treatments lose efficacy and none prevent disease progression. Nonetheless, controlling the motor symptoms has revealed a number of non-motor symptoms associated with the disease.

Non-motor manifestations of PD include olfactory as well as autonomic dysfunctions such as constipation, depression, and sleep disorders. Their study is leading to insights as to when the disease might start. Most importantly, they indicate that PD is not a disorder that affects only dopaminergic neurons. For example,

constipation may precede motor symptoms by at least 10 and perhaps more than 20 years, anxiety and neuroticism by 20, rapid eye movement sleep behavior disorder by 12 and anemia by 30 [10]. Work from the laboratory of Heiko Braak has brought attention to early, non-dopaminergic degeneration in PD. Lewy bodies (LB) and Lewy neurites (LN) are intracellular protein aggregates that have been used by neuropathologists to assess PD. They are present postmortem in different nerve cells at predisposed sites in patients with the disease. Using LB and LN distribution, Braak has shown that in the PD brain, the very first neurons affected are not in the substantia nigra but non-catecholaminergic neurons of the dorsal glossopharyngeus-vagus complex, projection neurons of the intermediate reticular zone, specific nerve cell types of the gain setting system (coeruleus-subcoeruleus complex, caudal raphe nuclei, gigantocellular reticular nucleus), olfactory bulb, olfactory tract, and/or anterior olfactory nucleus [11]. The lesions in the olfactory bulb observed before lesions in the SNpc are consistent with hyposmia, reduced ability to smell, or anosmia, inability to perceive odors, early clinical symptoms of disease. Continuing these observations, this work led Braak to propose six stages of pathology in PD. In stage one and two, there are lesions and pathology observed in medulla oblongata and the pontine tegmentum. In stage three there is pathology in the midbrain, the SNpc in particular, continuing with the basal prosencephalon and mesocortex in stage four and ending with the neocortex in stages five and six [12-14]. The motor clinical symptoms start occurring in stages three and four. Recently however, this staging theory has come under question as it fits with some but not all cases of disease. When the severity of cortical LB and other age

related pathologies are enhanced as well as non-dopamine symptoms, this does not correlate well with conventional Braak staging [15].

Given the variety of disease manifestations, studies using cluster analysis of motor and non-motor functions of PD and differences in the severity of non-dopaminergic features can identify four subtypes of the disease [16]. The first is mild in severity of all clinical symptoms and the patients are younger, with earlier age of onset. The second subtype has severe and frequent motor complications, moderately severe sleep and depressive problems, and a larger fraction of the patients are women. The third subtype is characterized by mild and less severe motor complications, the patients being older, with a higher age onset of the disease. Finally, the fourth subtype is characterized by severity in all clinical domains, but tremor is mild and motor complications were less severe than in subtype two. The patients in the study were administered dopaminergic treatment and the results pointed to differences not only in the mechanisms that underlie PD but to complications from treatment as well. The certainty of these categorizations has yet to be determined, especially given secondary effects from medication, but at least two groups of patients emerge, separated by age. In people with younger age of onset, 55 years old on average, tremor is dominant and there is a slow decline in motor function. In people with older age onset, 70 or older, postural imbalance and gait disorder is observed, characterized by akinesia, rigidity, gait and balance impairment, which progress rapidly [15, 17]. All these observations point to the complexity of PD, its heterogeneity on a background of age and genetic diversity, raising the question of whether there are one or multiple causes for the disease.

3. Causes of PD: sporadic versus familial forms. Genetic mutations.

The exact causes of PD have yet to be elucidated. Does aggregation of proteins in the form of LB and LN contribute to neuronal degeneration or are they part of the cell's protective mechanism? What leads to protein aggregation? Previously, the focus was on identifying possible environmental causes and the discovery of a toxin dominated the field. Support for this idea came from cases of postencephalitic PD, as described by Oliver Sacks in the book *Awakenings*, which was a sequela to a viral infection, and the demonstration in the early 1980's that young people who had taken contaminated recreational drugs presented with a rapidly progressive parkinsonian syndrome. The cause of drug related parkinsonism was the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropridine (MPTP), a byproduct of opioid drug making process [18]. The discovery that MPTP leads to severe PD revitalized the idea that environmental toxins could cause it. Subsequent studies showed that paraquat, a herbicide structurally similar to 1-methyl-4-phenylpyridinium (MPP+), the active metabolite of MPTP, and rotenone, an insecticide, produced parkinsonism in animal models and were associated with an increased incidence of PD in workers exposed occupationally [19]. All of these compounds are believed to impair mitochondrial function and increase oxidative stress [20, 21]. Other factors associated with a role in PD pathogenesis or increased susceptibility are the fungicide maneb, chronic manganese exposure, patient sex and estrogen status, head trauma, and ironically, not smoking, not drinking alcohol, and not drinking coffee [22]. However, environmental causes could be associated with only a small fraction of PD cases and did not exhibit LB pathology, which is characteristic of the overwhelming majority of sporadic PD.

In the late 1990's the direction of research took a turn when Polymeropoulos *et al* mapped a gene on chromosome 4q21-q23 that was linked to the PD phenotype in a large kindred with autosomal dominant PD [23]. He identified the mutation in the gene to encode the protein α-synuclein, where the alanine at position 53 was substituted for a threonine (A53T) [24]. Two more mutations within α-synuclein were subsequently discovered to result in autosomal dominant familial PD: the substitution of alanine 30 to proline (A30P) [25] and that of glutamate 46 to lysine (E46K) [26]. The discovery of disease associated genetic mutations redirected and invigorated the field of PD because study of mutations that cause disease might provide insights into the pathogenic mechanism that occurs in sporadic types. Familial cases account for approximately 10% of the cases of PD and to date a total of 18 PD loci have been nominated through linkage analysis (PARK1-15) or identified through genomewide association studies [27]. Work on mutations in genes associated with these disease loci has identified additional proteins associated with PD and led to the development of a variety of animal models that have contributed many insights into PD [28, 29]. These led to suggestions that genetic and environmental factors together can impact predisposition and disease development [30].

The identified mutations involve a variety of proteins and have been extensively reviewed [27, 31-34]. They are of two classes; one autosomal dominant exemplified by mutations in the proteins α-synuclein and leucine-rich repeat kinase 2 (LRRK2). Discovered in 2002, mutations in LRRK2 account for the highest number of familial PD cases and seem to be the highest risk factor of developing the disease. The protein contains multiple domains such as Ras/GTPase, tyrosine kinase, leucine-rich-repeat

domains and there are indications that LRRK2 acts upstream of α-synuclein [35, 36]. The other class of mutations is inherited as autosomal recessive and includes parkin, PINK1 and DJ-1. This latter group generally lacks LB pathology. The diversity of genes that can be associated with susceptibility to PD further emphasizes the heterogeneity of Parkinson's and the difficulty in selecting clear-cut criteria to identify the disease and its causes. The impact of each mutation can vary and can only be associated with a minority of cases. In addition, some might interact functionally with each other [36-38], but it has yet to be determined whether they all relate to a single pathogenic process [39]. The work of this dissertation focuses on the protein α-synuclein and its role in PD pathology.

B. The α- Synuclein Protein

1. Discovery

In 1988 Maroteaux *et al* [40] were studying the synapse, looking for molecules that underlie presynaptic function. Using the elasmobranch *Torpedo califonica,* a cDNA clone was isolated that encoded synuclein, a neuron-specific protein that localized to the nucleus and presynaptic nerve terminal, hence the name synuclein. α-Synuclein's first association with neurodegeneration was through Alzheimer's disease (AD). Analyzing amyloid deposits from AD cortex, Ueda *et al* [41] found that second to the amyloid β peptide, the most abundant component present in the deposits was a 35 amino acid peptide whose full-length cDNA encoded a 140 amino acid protein. The peptide was named the non-Aβ component (NAC) of AD and it corresponded to sequences of two distinct synucleins in the brain, α and β synuclein [42], which were

assigned to chromosomes 4q21 and 5q35, respectively [43]. Subsequent studies identified α-synuclein as the major component of LB in PD and mutations in α-synuclein were the first associated with familial PD. This association was reinforced by the finding that, in addition to the three aforementioned mutations associated with α-synuclein, a genomic duplication [44] and triplication [45] of a region spanning the α-synuclein gene were also found to produce familial PD [45]. Interestingly, duplication of the gene and its disease manifestation mimics very well sporadic cases of PD and there is a direct correlation between how early and aggressive the disease manifests and the amount of protein expressed. These observations indicate that α-synuclein plays a role in PD pathology and that role can be a matter of quality as well as quantity of α-synuclein present.

2. <u>Role and function</u>

The synucleins comprise a family of proteins that include the α, β, and γ forms [46]. The involvement, in particular of α-synuclein, with disease is so extensive that these diseases have been grouped into a category named synucleopathies. The physiological role of α-synuclein remains a mystery. Knockout of various isoforms of synuclein, alone or in combinations of two, appears to have just slight effects on synaptic function and no degeneration in mice [6, 47]. The isoform present in the absence of the other two is up regulated, for example β when α and γ are knocked out [48, 49], indicating that there is compensation during development and a degree of functional redundancy within the synuclein gene family. Only when all three genes were knocked out did mice show an age dependent neuronal dysfunction with changes in synaptic protein composition and axonal structure [50]. A study across species reveals

that synucleins are only present among vertebrates. Curiously all species examined, with the exception of humans, have T at position 53 instead of A. Moreover, mouse A53T α-synuclein is even more prone to aggregation than the human mutant version and raises the question whether A53 in humans is a specialization. It also indicates that other species might have a better mechanism of dealing with synuclein aggregation or that aggregation itself is not what leads to toxicity. Consistent with this idea, overexpression of α-synuclein in animal models of synucleinopathies often does not lead to significant inclusions [51].

The normal functions of α-synuclein remain a matter of conjecture. It has been predicted that α-synuclein may function as a molecular chaperone given its physical and functional homology with 14-3-3 proteins [52] or that it may be a novel class of substrates for G protein coupled receptor kinases as the latter phosphorylate α-synuclein at a single serine, Ser129 [53, 54]. There is evidence that synuclein regulates dopamine release [55, 56], and early studies of the protein in songbirds such as the zebra finch suggest that it may play a role in synaptic plasticity [57]. Most studies have suggested that α-synuclein's role appears to be indeed at the synapse, in vesicle trafficking and in the trafficking of cargoes within the endoplasmic reticulum/Golgi network. A member of the Rab proteins, Ypt/Rab1, restored defects in vesicle trafficking induced by α-synuclein, observations confirmed in yeast, *C.elegans*, *Drosophila* and mammalian cultured neurons [58-60]. Recently, Nemani *et al* have demonstrated that α-synuclein inhibits neurotransmitter release by inhibiting vesicle clustering after endocytosis [61]. Further supporting a role for α-synuclein at the synapse, observations in cultured neurons from brains of transgenic mice

overexpressing fluorescent human α-synuclein indicated there was a loss of critical presynaptic proteins involved in exocytosis and endocytosis such as SNARE, vesicle-associated proteins (VAMP2 and synapsin-1), active-zone proteins (piccolo) and proteins critical in endocytosis (amphiphysin) [62].

To further complicate matters in understanding the role of α-synuclein, more than 300 different posttranslational protein modifications of α-synuclein have been identified, all potentially changing the protein's function. Many of these modifications are presumed to have pathological consequences leading to a focus on the role of α-synuclein within a disease framework rather than under normal, healthy circumstances. Modifications such as proteolysis, phosphorylation, lipidation, oxidation, acylation, and ubiquitination result in changes of the protein's size, charge, structure, and conformation, leading to alterations that may have severe negative effects on cellular function and have been proposed to play roles in various pathogenic mechanisms [63].

3. Structure

α-Synuclein is a small, conserved protein that is predominantly expressed in the brain, in particular the neocortex, hippocampus, striatum, thalamus, and cerebellum [64] but is expressed in other tissues as well including heart, skeletal muscle, pancreas and placenta as well as hematopoietic cells [65]. Structurally, human α-synuclein has an N-terminal amphipathic region containing six imperfect repeats, KTKEGV, a hydrophobic core region that contains the NAC domain, and an acidic C-terminal region. It appears unstructured in aqueous solution but has significant conformational plasticity depending on the composition of its environment, such as pH, chemical concentration of solutions, interacting partners, whether *in vitro* or *in vivo*: i.e. it can remain unstructured, exhibit

folding in either α-helix or β-sheet conformations, form monomeric and oligomeric species, or generate amyloidogenic filaments [32, 66].

α-Synuclein is predominantly localized to presynaptic terminals in the central nervous system, where it is loosely associated with presynaptic vesicles [67]. A few examples presented below will illustrate α-synuclein's variability and flexibility in structure. These are thought to lead to different roles and consequences within the cell depending on its location and conformation [68]. For example, many studies place the protein at presynaptic nerve terminals and nuclear association has been less investigated. It appears that while α-synuclein can translocate across the cell membrane, it does not cross the nuclear membrane readily [69] although imaging techniques can show α-synuclein localization in the nucleus. Studies of amino acid sequence motifs required for membrane translocation show that all three isoforms of synuclein could penetrate the cell membrane. This indicates that the conserved N terminus plays a role in translocation, in particular the imperfect repeats [69]. The more of these sequence motifs that were deleted, the lower the translocation efficiency and the imperfect repeats alone could transverse the membrane. In addition this study suggests that internalization of synuclein is not temperature dependent and does not occur via normal endocytosis. However, other studies show that α-synuclein is transmitted to neighboring neurons via endocytosis [70].

Using single molecule fluorescence resonance energy transfer, Ferreon *et al* [71], have shown that when binding to amphiphilic small molecules of membrane-like partners, α-synuclein transitions between natively unfolded and multiple α-helical structures. This process is dependent on the concentration of binding partners or the

variation in curvature of binding surfaces of micelles or bilayers of lipid-mimetic SDS. *In vitro*, monomeric α-synuclein binds phospholipid membranes and micelles that are negatively charged. It forms N-terminal amphiphatic α-helical structure by turning three full turns every 11 residues [72], its structure stabilizing in the process [73]. Mutations can also have numerous effects on the protein's structure. A53T and E46K exhibit increased membrane-binding affinity while A30P has a decreased affinity presumably due to differences in the way the protein folds [60]. A53T disrupts the formation of the α-helix between residues 51 and 66 which allows the protein to form a β-structure, A30P disrupts the first α-helical domain [60], while E46K, a more recent and therefore less well studied mutation, appears to produce more subtle changes in the α-helical confirmation [74]. Given the tendency to stabilize in association with itself or in association with other proteins or cellular components, α-synuclein's aggregation and its role in pathology have been focused on extensively.

4. Lewy Bodies

The year after Polymeropolus published the discovery of an α-synuclein mutation associated with PD, Spillantini *et al* identified α-synuclein as the main component of LB [75]. Mutations of α-synuclein in PD are rare and their clinical manifestations can differ. For example, patients with A53T mutations distinguish clinically from idiopathic PD while A30P resemble it. Nevertheless PD characterized by α-synuclein containing LB accounts for >90% of sporadic Parkinsonian disorders [67]. These facts effectively place α-synuclein at the center of most cases of PD pathogenesis. Lewy bodies were identified in 1912 by Friedrich Heinrich Lewy, who described inclusion bodies while working on paralysis agitans and in 1919, Tretiakoff would name these inclusions in his

honor. Lewy was the first person to describe the anatomical pathology of Parkinson's disease [76]. If located in the cell body the aggregates are called Lewy bodies and in the neural processes, where they look spindle like, they are called Lewy neurites [8]. It is important to point out that α-synuclein aggregates, LB inclusions, are not unique to PD. They are observed in other diseases as well, such as dementia with LB, the LB variant of Alzheimer's disease and multiple system atrophy. Not unlike amyloid plaques in Alzheimer's disease [77], LB also appear to be a normal process of aging as they were observed in the brains of older individuals that did not exhibit dementia or PD in their life time [78]. Although the main component of LB appears to be α-synuclein, their exact composition can vary and a wide range of proteins have been identified in the inclusions. For example, in addition to α-synuclein, ubiquitin is present and was used to detect LB before α-synuclein was discovered as a component. Recent, more sophisticated staining techniques identified approximately 80 proteins within LB [8]. However, α-synuclein is by far the major constituent of LB.

There are two categories of LB associated with PD. Classical LB, located extensively in the brainstem, are described as spherical eosinophlic cytoplasmic protein aggregates, more than 15 μm in diameter, with an organized structure containing a dense hyaline core surrounded by a clear halo, which under electron microscope shows a dense granulovesicular core surrounded by a ring of 8-10 nm fibrils [7]. The other type of LB, cortical LB, are localized primarily in deep neocortical layers and are less eosinophilic, more homogeneous, lacking the distinctive core and halo [8, 79]. Considering the variability of LB in composition and presence in more than just PD, controversy surrounds these inclusions. Are they a cellular protective mechanism [78],

for example, do the aggregates form as a way for the cell to sequester a bad protein? Are the effects of α-synuclein of such nature that the cell cannot clear them fast enough and therefore packages them into an aggregate? Or are they part of the pathological mechanism [80] and more importantly, what role does the aggregation of α-synuclein play in LB and therefore in PD pathogenesis?

C. Pathogenic Mechanisms of Parkinson's Disease and α-Synuclein

1. Role of aggregation

In terms of cell toxicity and α-synuclein, important questions have emerged: is the protein alone pathological or is LB formation required? If aggregates of the protein are bad, are all aggregates created equally or is there a difference between the different types of aggregates observed? Interestingly, of the three isoforms of synuclein, α-synuclein is the only one that aggregates *in vivo*, although under certain conditions γ-synuclein can also be induced to aggregate [60]. In diseased states, α-synuclein is thought to self-associate, which seems to stabilize the protein, leading to formation of oligomers and subsequent fibrillar forms, as well as aggregates like the LB and LN. Current research favors oligomers as the toxic species and LB as the cell's mechanism of protecting itself against toxicity [32, 63, 67, 81, 82]. One theory is that α-synuclein, in a similar manner to other proteins, follows a hierarchal assembly model of aggregation. In this model, proteins aggregate into oligomers or protofilaments, which elongate via the addition of monomeric, partially folded intermediates to its ends. The rod-like protofilaments then interact with each other to form protofibrils and two or three protofibrils will then intertwine to form mature fibrils [83]. Luk *et al* have shown that

introduction of exogenous α-synuclein fibrils, but not soluble α-synuclein, into various cells that overexpress the protein, led to aggregation [84]. These aggregates had features of human LB and indicate that a seeding mechanism will induce aggregation. In addition, this study also showed that the central portion of synuclein is sufficient for aggregation. This type of aggregating which requires seeding, complemented with the observation that α-synuclein can cross between neurons and from inclusions [70], has led some to compare α-synuclein to prions [85].

Posttranslational modifications appear to play a significant role in α-synuclein aggregation, two of which are notable. Up to 90% of α-synuclein found in LB is phosphorylated while only 4% of normal α-synuclein is [63, 86]. Phosphorylation appears to play a key role in pathogenesis by stabilizing the protein and increasing its ability to form inclusions and interact with other proteins [86, 87]. The first phosphorylated sites identified were Ser129, which is conserved across species, and to a lesser extent Ser87, found only in humans and monkeys [87]. Subsequent studies explored Ser129's role in aggregation and its correlation to the formation of LB [88-91]. Other than serine, α-synuclein also has tyrosines that could be phosphorylated. Members of non-receptor family kinases Src and Fyn primarily phosphorylate tyrosine 125 [92, 93] and it appears that α-synuclein and Fyn distribution and colocalization in the brain is similar which would suggest a common pathway for the two proteins [92]. In addition, Syk physphorylates tyrosines 125, 133, and 136 and this multiphosphorylation event can prevent oligomerization [94]. Indeed it appears that phosphorylation of Tyr125 and Ser129 have opposing effects in aggregation [95]. These observations further support α-synuclein's versatility of interaction upon posttranslational

modifications such as phosphorylation events and the consequences these interactions can have on its structure and potential for aggregation.

In addition to phosphorylation, C-terminus truncations of α-synuclein have generated a great deal of interest. There is evidence that truncation of the protein may be a normal cellular process in the cell and that it may be enhanced in familial PD [96]. It was also found that C-terminus truncations accelerate aggregation of full-length and mutated α-synuclein at substoichiometric levels [96, 97]. This is not necessarily surprising since, as mentioned above, the C-terminus is acidic in nature and tends to remain unfolded. It has four aspartate and eight glutamate residues in amino acids 109-140, whereas basic amino acids are absent. Under physiological conditions, the C-terminus retards aggregation, however this can be overridden by lowering the pH or by the addition of compounds such as spermine, heparin, and $MgCl_2$ [98-101]. There are also five proline residues within the terminus, which may also play roles in synuclein structure and aggregation, as replacement or deletions of these amino acids leads to aggregation. In addition, substitution of alanine for proline results in a more structured, α-helical, conformation of α-synuclein [102].

In vitro aggregation studies of α-synuclein are extensive [68]. Recently, Winner *et all*, using α-synuclein variants that form oligomers but not fibrils, have demonstrated that the formation of α-synuclein into oligomers is accompanied by increased *in vivo* toxicity [81]. Using fluorescence lifetime imaging microscopy, Klucken *et al* observed that in intact cells, it appears that the N and C terminal domains of α-synuclein fold in such a manner that brings them relatively close to one another, strongly within the protein and weaker between two different synuclein proteins [103]. These observations

required an intact cell and indicate that α-synuclein oligomers form in an antiparallel fashion. Moreover they offer a cautionary tale to assumptions made as we draw conclusions about α-synuclein, providing an important reminder that the protein's folding and behavior dependents greatly on its environment. Conclusions we reach from *in vitro* experiments can vary widely from what may happen *in vivo*. Considering how protean α-synuclein is and how specialized neuronal cells are, this in turn would provide an explanation as to why some cells are more vulnerable than others.

2. <u>Cell Specific Vulnerability.</u>

It is obvious that whether sporadic or familial, PD takes a long time to develop and generally it is considered a disease of old age. Why does it take so long to develop and why is it that some cells are affected and others are not? Considering that α-synuclein is quite abundant and highly expressed throughout the brain, what would make one particular neuron more vulnerable than another? Overexpression of human α-synuclein in primary cultures of human embryonic mesencephalon leads to cell death of dopaminergic neurons while other type of cells are not affected [104]. This could be explained by evidence that α-synuclein can interact with dopamine receptors, specifically D2, via its C-terminus [105]. The C-terminus, in particular its ^{125}YEMP^{129} sequence, appears to also mediate interaction with oxidized catechols and leads to oligomerization of α-synuclein [106]. Considering the roles of α-synuclein in vesicle trafficking, it is plausible that α-synuclein may also affect dopamine release at the synapse [6]. Release of DA within the cell makes it vulnerable to oxidation, which can lead to oxidative damage and mitochondrial dysfunction.

Neurons are extremely specialized cells, their overall composition unique to their function. It is most likely a *combination* of the diversity of α-synuclein structures and the specific composition of particular dopaminergic neurons that makes them vulnerable. Dopaminergic neurons of the SNpc have properties unique to themselves unlike the dopaminergic neurons from the ventral tegmental area, which are not affected in PD. For example, nigrostriatal neurons have L-type CaV1.3 calcium channels [107]. Calcium buffering affects dopamine release as well as cellular pH, and pH, as mentioned earlier, can affect α-synuclein folding. In addition, Nath *et al* have shown that *in vitro,* α-synuclein aggregates in the presence of calcium in a dose dependent manner and while *in vivo*, increase of intracellular free calcium yielded α-synuclein aggregates [108]. Moreover, the axons of dopaminergic neurons can be extremely long. In the rat brain, due to branching, single nigrostriatal dopamine neurons have a total cumulative axonal length of 70 cm and can form extensive arborizations that lead one dopaminergic neuron to exert strong influence over a large number of striatal neurons [109]. Such axonal lengths and extensive interactions make these neurons highly dependent on vital cellular and neuronal processes such as axonal transport.

3. <u>Disruption of Axonal Transport</u>

In 1999, using antibodies to α-synuclein, Galvin *et al* showed that there was evidence of axonal pathology in postmortem brains of PD patients suggesting that PD has the characteristics of a dying back neuropathy, where the neuron, starting with the synapse, degenerates slowly toward the cell body and it eventually dies, a process common with other adult-onset neurodegenerative diseases like AD, Huntington disease, and amyotrophic lateral sclerosis. This implies a role for axonal transport

pathogenesis, but the precise role, if any, played by axonal transport in PD, and in neurodegeneration in general, has not been extensively explored until somewhat recently [110-112]. Neurons are the most polarized cells in the body, highly compartmentalized, and as a result they rely heavily on highly regulated axonal transport for proper function. In a rat model of synucleinopathy expressing human mutant A53T α-synuclein, Chung *et al* have observed changes in proteins relevant to synaptic transmission, such as rabphilin 3A and syntaxin, and proteins relevant to axonal transport, such as kinesin, dynein, dynamitin, and dynactin1, *before* neuronal loss [113]. Kinases and their regulation of axonal transport are discussed in larger detail in the next chapter, but given α-synuclein's diversity of interaction, it is difficult to ignore the possibility that the protein may have direct effects on axonal transport. Misregulation of transport though alteration of signaling pathways and therefore phosphorylation events can have dire consequences in a neuron as it relies on transporting components between the cell body and the synapse. Over time alterations in axonal transport can slowly lead to neuronal death, consistent with the slow progression of disease [111, 114-121].

Despite the existence of many unanswered questions – is PD a proteinopathy, does it have one or multiple triggers, what is the extent of its heterogeneity, and how does aging contribute to pathology – Braak has summed up the disease best, stating that sporadic PD "is a progressive degenerative illness of the nervous system that manifests itself clinically after the pathology has reached an advanced stage" [12]. Thus far, it is evident that environmental toxins, age, and genetics factor in and the role each plays varies with circumstance. Two facts emerge: PD is not simply a movement

disorder and α-synuclein has a significant role in the great preponderance of both genetic and familial cases of PD. The purpose of this dissertation is to further understanding of α-synuclein in PD in general, and axonal transport in particular.

D. Statement of hypothesis

PD is a neurodegenerative disease marked by severe loss of dopaminergic neurons in the substantia nigra and their connections to the striatum. Pathologically, it is characterized by the presence of abundant Lewy bodies and Lewy neurites, inclusions that have as their main component the protein α-synuclein. Mutations of α-synuclein have been identified that lead to severe cases of early onset familial PD. Taken together this suggests a common pathogenic mechanism for both familial and sporadic PD involving α-synuclein, so analysis of its effects on neuronal function could offer insights into possible pathogenic mechanisms in PD.

Misregulation of axonal transport can lead to loss of synaptic function, axonal degeneration and cell death. Work from our laboratory demonstrates that perfusion of squid axoplasm with α-synuclein increases retrograde and decreases anterograde transport, the two components of fast axonal transport (FAT). Additional experiments suggest that α-synuclein effects on transport are mediated via an isoform of protein kinase C (PKCμ), results that would be consistent with an effect on the state of phosphorylation of kinesin and dynein, the molecular motors responsible for transport. We hypothesized that in PD, specific domains within α-synuclein alter regulatory pathways for fast axonal transport in neurons through a mechanism involving PKCμ. Two aims tested this hypothesis:

1. Determine molecular motifs within α-synuclein that affect kinase activity and fast axonal transport.

Posttranslational modifications, such as C-terminus truncations of α-synuclein appear to play a role in the protein's aggregation and therefore pathogenesis. Motifs identified in our laboratory as potentially important and not extensively studied are SH2 domain recognition consensus sites within the C-terminus of α-synuclein. *We propose that a SH2 domain recognition site plays an important role it the pathogenesis of α-synuclein within the cell.* In order to establish this role for the C-terminus, truncated forms of α-synuclein in soluble and fibril form were generated and tested in squid axoplasm.

2. Determine the effects of pathological α-synuclein on kinesin and dynein motor proteins and its ability to activate PKCμ.

Previous work form the laboratory has demonstrated that phosphorylation events are key to regulating axonal transport. In addition, it has been shown that pathogenic α-synuclein increases retrograde FAT and decreases anterograde FAT via activation of kinase pathways involving specific PKC isoforms and members of Src family tyrosine kinases. *We propose that pathogenic forms of α-synuclein alter signaling pathways that regulate axonal transport.* A cell model of synucleinpathy was generated and used to assess the effects of wild type and mutated forms of α-synuclein on the molecular motors kinesin and dynein and to determine the activity of PKCμ in the presence of pathological α-synuclein.

II. PATHOGENIC EFFECTS OF α-SYNUCLEIN DOMAINS ON AXONAL TRANSPORT

A. Summary

Squid axoplasm is a unique tool that allows for the study and regulation of axonal transport and its role in the context of neurodegeneration. Perfusion of kinases such as non-receptor tyrosine kinases Src and Fyn or different isoforms of protein kinase C, such as PKCδ and PKCμ, have a dramatic effect on fast axonal transport leading to a rapid increase in retrograde and a slow decrease of anterograde transport. The same effects were observed with perfusions of α-synuclein, fibrils of wild type or soluble A30P and A53T mutants. Coperfusion of α-synuclein with inhibitors of the above mentioned kinases abolish the effects noticed on transport. This suggests a common path of axonal transport regulation that involves α-synuclein, non-receptor tyrosine kinases and PKCμ. Examination of motifs within α-synuclein identifies SH2 domain recognition consensus sites within its C-terminus that could interact with non-receptor tyrosine kinases. C-terminus truncated forms of α-synuclein were made using two vectors, one of which expressed the protein in large quantities. Soluble and fibrilar forms of truncated wild type, A30P and A53T α-synuclein were made and perfused in squid axoplasm with no effect on transport. Taken together these results confirmed that the C-terminus of α-synuclein mediates effects on axonal transport and suggest a mechanism of pathogenesis for α-synuclein where, through an activity requiring the α-synuclein C-terminus, the protein activates non-receptor tyrosine kinases such as Src and Fyn. They in turn increase PKCμ activity, which can regulate transport by affecting the phosphorylation status of the molecular motors.

B. Introduction

Classically, neurons are divided into four morphologically distinct regions: the cell body, dendrites, axon, and synaptic terminals. Within these regions, there are functionally distinct microdomains, each with a unique protein composition. The axon not only conducts action potentials, but also provides the physical conduit for transport of materials between the cell body and synapses. Cargoes such as membrane bounded organelles (MBO), synaptic vesicle precursors, signaling molecules, growth factors, and protein complexes are transported from the cell body, where they are made, through the axoplasm to intracellular target sites in the axon and the synapse. Similarly, trophic signals are transported from the synapse back to the cell body thus reporting on the integrity of target innervation. Axons vary in length, but in humans they can be longer than a meter. These cells depend especially on the efficient and coordinated transport of materials for function and viability [115].

Traditionally, axonal transport has been classified as either fast axonal transport (FAT) or slow axonal transport [122-124]. Slow axonal transport is responsible for the transport of cytoplasmic proteins such as glycolytic enzymes and cytoskeletal proteins [125]. However, aspects of the slow component of axonal transport and its mechanisms are still contentious as what appears to be slow movement represents in fact intricate kinetics of stop and fast go mode of transport [125-127] and further research to understand it is necessary.

Our laboratory focuses primarily on FAT, which is associated with the movement of MBOs and membrane-associated proteins. Axonal transport is dependent on microtubules, which are polar structures that confer directionality. Microtubules are

long, hollow cylinders made up of α and β tubulin subunits, with a fast growing end (plus) oriented toward the synapse and a slow growing end (minus) oriented toward the cell body [128]. The classes of molecular motors involved in axonal transport are kinesins and dyneins [117, 118].

Kinesins are known to be largely plus-end directed motors, and are generally responsible for anterograde transport toward the synapse. Although a few kinesin family members have been associated with some minus-ended transport, thus far none have been proven to be involved in retrograde FAT. Their cargo includes synaptic vesicle precursors and MBOs. The kinesin superfamily includes 45 genes for microtubule-dependent motors in mice and humans and it is grouped into at least 14 subfamilies [118]. The most abundant kinesin motor is kinesin 1, the first discovered and enriched in the nervous system [129, 130]. It is a heterotetramer of two heavy and two light chains. In mammals there are three genes for the heavy chains (kinesin 1A, B, and C) that form homodimers and there are two or more light chain genes that may undergo alternative splicing. Other species, such as *Drosophila melanogaster* and the squid, *Loligo pealei,* have only one gene present for kinesin 1 [131]. The light chains are involved in the binding of specific cargo, while the heavy chains contain a microtubule-binding domain and have ATPase activity that provides the motor with the energy needed to move along the microtubules on their N-terminal, as well as domains in the C-terminal that may interact with cargo [132, 133]. Kinesins have a wide variety of interactions with proteins throughout the cell which regulate and confer to them specific roles and localizations throughout the neuron [134].

Compared to kinesins, the dynein family is small and has a separate evolutionary lineage. It can be divided into three groups: axonemal, involved in powering the beating of cilia and flagella, the evolutionarily conserved intraflagellar dyneins, responsible for the transport of proteins in the axoneme, and cytoplasmic dyneins, responsible for most minus-end transport of cargo in eukaryotes [135, 136]. In neurons, cytoplasmic dyneins are minus-end directed motors also responsible for retrograde transport to the cell body. Their cargo includes neurotrophic signals, endosomes, as well as other organelles and vesicles. The dynein motor is much bigger than kinesin, cytoplasmic dynein being a large multisubunit complex of approximately 1.5 megadaltons. It has a motor domain and variable intermediate, light and heavy chains and given its size and complexity, it has been more difficult to study [137, 138]. Dynein may also interact with proteins that do not belong to the complex itself but that are crucial for adapting the motor to its cellular function, of which the best decribed is dynactin, a large protein complex characterized as an essential dynein activator. [139].

Misregulation, as well as a variety of mutations in the molecular motors or proteins associated with them, leads to neuronal degeneration. For example, genetic defects that lead to abnormal axonal transport result in congenital cranial nerve dysfunction. Specifically, mutations in the stalk domain of kinesin KIF21A are the primary causes of congenital fibrosis of the extraocular muscles [140, 141] and loss of function of the kinesin 1 family member KIF5A is linked to the neurodegenerative disease hereditary spastic paraplegia [142, 143]. Several examples also provide evidence for defects in components of the retrograde transport pathway that lead to neurodegenerative diseases [112]. For example, a mutation in the DCTN1 gene results

in an amino-acid substitution of serine for glycine at position 59 in the highly conserved CAP-Gly motif of the p150Glued subunit of dynactin. Individuals with this mutation develop a slowly progressive, autosomal dominant form of lower motor neuron disease without sensory symptoms [144]. A variety of mice models, such as legs at odd angles, cramping 1, and sprawling mice, point to neurodegeneration due to mutant forms of the cytoplasmic dynein heavy chain [112]. In addition, heterozygous mice with point mutations in the cytoplasmic dynein heavy chain develop progressive motor neuron degeneration, and in homozygotes, this is accompanied by the formation of Lewy-like inclusion bodies [145].

Research from our laboratory provides evidence for the vulnerability of neurons to disruptions in axonal transport and demonstrates the extensive regulation of FAT by kinases and phosphatases [120, 146-149]. The findings imply that disruption of regulatory pathways for FAT may also lead to neurodegeneration. For example, experiments using squid axoplasm have shown that perfusion of active glycogen synthase kinase 3 β (GSK3β) resulted in dramatic inhibition of anterograde, kinesin-1 dependent FAT, with no effects on retrograde FAT [147]. Subsequently, it was demonstrated that GSK3β phosphorylates kinesin light chains, resulting in release of the cargo from the light chains. Also, in a presenilin 1 knock-in mouse model for Alzheimer's disease, mutant presenilin 1 increased GSK3β activity, leading to increased kinesin light chain phosphorylation and a decrease of kinesin bound vesicles [148]. Such observations support the idea that disruption of axonal transport is a contributing factor to neurodegeneration, especially in Alzheimer's disease [120, 150, 151].

Our laboratory has examined the effect of kinases that have potential regulatory roles on axonal transport using the squid, *Loligo pealei*. This organism offers unique advantages to the study of function and regulation of axonal transport. Squid have a giant axon that is 1,000 times larger in diameter than the average human axon thus rendering it a great model for the study of neurons and transport in particular. The method employed for the experiments has been described previously [152-156], however to facilitate understanding of the experimental model and the data generated by the model, a brief overview is given. Dissected squid axoplasm can be extruded from the axon by gently squeezing it out as one might squeeze toothpaste out of a tube. The axoplasm maintains its form and remains organized, allowing continued transport of intracellular organelles in both anterograde and retrograde directions. This motion can be monitored under a microscope, which is attached to a digital video camera. With the aid of cursors matched to the motion on a video screen, one can estimate the group velocity of particles transported in either direction. The activity of a single preparation can be monitored for hours, during which the speed in anterograde and retrograde directions is estimated and used to generate plots such as the one presented in Figure 1. Right-facing arrowheads denote individual anterograde measurements, left-facing arrowheads, individual retrograde measurements and corresponding linear regression lines are determined for each direction. The average anterograde speed is 1.6 - 1.8 μm/sec and retrograde 1.2 - 1.4 μm/sec. This preparation allows for the perfusion of axoplasm with effectors of interest and facilitates an evaluation of their effect on FAT.

Previous screening by the laboratory of a series of kinases for effects on transport by perfusion in squid axoplasm identified several as potential regulators of

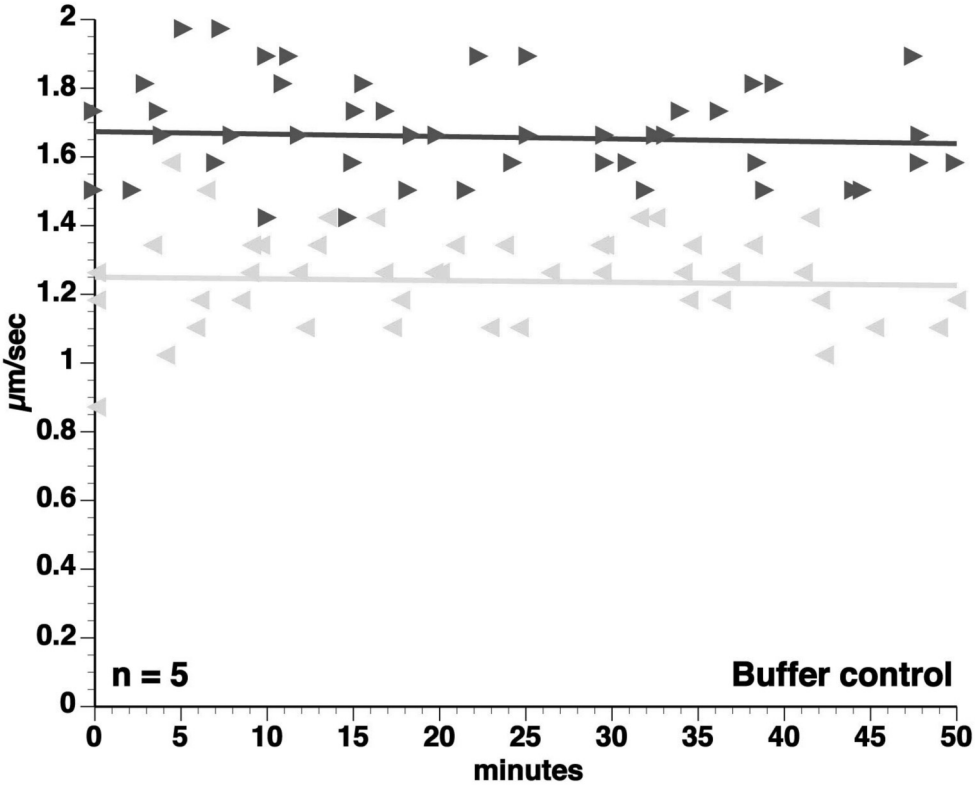


Figure 1: Motility assay of fast axonal transport in isolated squid axoplams

Each black, right-facing arrowhead represents an individual anterograde measurement, and each gray, left-facing arrowhead, an individual retrograde measurement. The measurements for n=5 experiments are represented. A corresponding linear regression line for each direction is indicated by the black and gray lines, and is derived from all of the individual measurements. The average anterograde speed is between 1.6 and 1.8 μm/sec and retrograde is between 1.2 and 1.4 μm/sec. Measurements are taken for 50 min. (ST Brady, personal communication).

FAT. Of these, protein kinase C (PKC) comprise of a family of serine/threonine protein kinases that play roles in many cellular activities [157]. A constitutively active catalytic domain of PKC generated by tryptic digest of a mix of PKCα,β,γ had a distinctive effect on FAT in squid axoplasm: rapid increase in the amount and rate of retrograde FAT accompanied by a slow decline in anterograde FAT (Figure 2A, see also Morfini [158]). This suggested that PKC isoforms might play a role in the turnaround of MBOs in transport and provide an indication of coordinate regulation of anterograde and retrograde FAT. Similar effects were observed by using other isoforms of PKC, such as PKCδ [158, 159] and PKCμ, an atypical PKC (Figure 2B, Brady and Morfini, *unpublished*).

Studies on signaling by the reelin receptor suggested that nonreceptor tyrosine kinases could also affect FAT [160, 161]. The human genome contains 32 non-receptor tyrosine kinases, separated into 10 subfamilies according to kinase domain sequence [162]. Of these, the largest subfamily is the Src family with nine members, including Src and Fyn. Their activation is highly regulated and critical for generating appropriate cellular responses to external stimuli. Src family kinases are implicated in many diseases including cancer, diabetes and congenital syndromes such as X-linked lymphoproliferative syndrome [163-165]. Non-receptor tyrosine kinases possess multiple domains that mediate protein-protein interaction, including both SH2 and SH3 domains. The role of these domains has been found to be important not only in interaction with other proteins but also in self-regulation of activation. In screening of these kinases, Brady and Morfini (*unpublished*) have shown that of the the Src- and Abl-family members, Abl had no effects but that Src and Fyn have effects similar to those

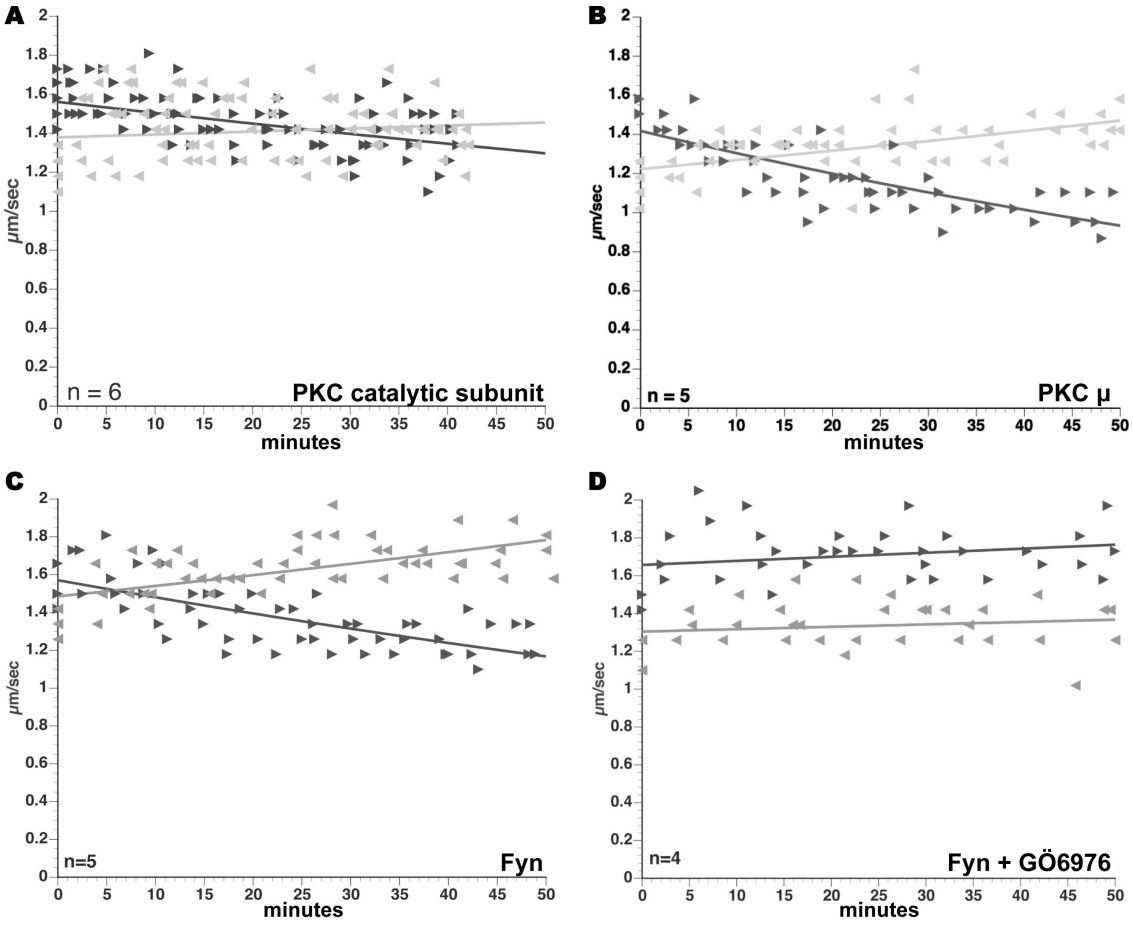


Figure 2: Effects of kinases on axonal transport

Increase retrograde FAT and reduced anterograde FAT is due to effects of conventional PKCs (A), novel PKCμ (B) and non receptor tyrosine kinases such as Fyn (C). The PKC inhibitor Gö6976 blocks the effects of the kinases on transport – only Fyn is shown (D). (ST Brady and G Morfini, *unpublished*)

observed with the PKCs (Figure 2C). Neither kinesin nor dynein are known to have tyrosine phosphorylation sites, so the ability of a PKC inhibitor Gö6976 was tested on the ability of Fyn to affect FAT. The inhibitor abolished the effects of Fyn on FAT (Figure 2D) supporting that these kinases regulate transport through the intermediate of a PKC isoform.

Due to increasing evidence for the complexity of pathways regulating FAT and the observation that many neurodegenerative diseases display altered enzymatic activities that regulate this process, it is conceivable that there could be misregulation of these pathways in Parkinson's disease as well. For example, in the case of MPP+ induced PD, our laboratory has demonstrated that protein kinase C delta (PKCδ) mediates the effect of this toxin on axonal transport [158, 159]. Specifically, MPP+ increased retrograde and reduced anterograde FAT through activation of caspase-3 and PKCδ [158] in a manner similar to PKC isoforms and non-receptor tyrosine kinases. In addition, injection of MPP+ into the presynaptic terminal of the squid giant synapse of MPP+ reduced the availability of presynaptic vesicles, but not their release. MPP+ blocked synaptic transmission and led to a reduction in neurotransmitter vesicles at the presynaptic active zone. The effects were mimicked by the injection of an active caspase-3 and prevented by inhibitors of caspase-3 and PKCδ [159]. From these precedents, it was reasonable to predict that pathogenic forms of α-synuclein could also have effects that result in the alteration of normal FAT mediated by a PKC isoform.

In a similar manner to MPP+, α-synuclein experiments in the laboratory tested what, if any, effect various forms of the protein have on transport. Brady and Morfini (*unpublished*) have next showed that the rate of transport in either direction remained

the same for the duration of the experiment when perfusing with soluble wild type (WT) α-synuclein (Figure 3A). However, perfusion of WT synuclein that has been assembled *in vitro* into filaments like those found in LB *in vivo* [166], decreases anterograde, and increases retrograde transport (figure 3B). These effects are even more dramatic with mutated forms of α-synuclein, where perfusion with either A30P or A53T has effects similar to that of fibrillar WT synuclein: a rapid increase in retrograde and a slow decrease in anterograde transport (Figure 3C,D). These results show that α-synuclein has effects on transport similar to MPP+, PKC isoforms or non-receptor tyrosine kinases and point to important questions: just like MPP+, are α-synuclein effects mediated by caspase-3 and PKCδ or other mechanisms and is there a modification or domain within α-synuclein that plays a particularly significant role?

Coperfusion of α-synuclein with inhibitors of PKC and non-receptor tyrosine kinases had a dramatic effect on transport. For example, coperfusion with Gö6976 shows that it reduces or abolishes the effect synuclein WT type fibrils or mutants have on FAT (Figure 4A,C for mutants only A30P shown). Coperfusion with Gö6983 did not abolish the effects of mutant α-synuclein on FAT as illustrated for A30P (Figure 4D). The difference between these two inhibitors is that Gö6976 inhibits PKCμ, whereas Gö6983 inhibits a wide range of PKCs but does not inhibit PKCμ at the concentrations used. Therefore, Gö6983 can be used to distinguish PKCμ from other PKC isoforms [167]. The different results obtained with these two PKC inhibitors supports the idea that, in the case of α- synuclein, effects on transport are most likely mediated by PKCμ rather than PKCδ. PKCμ was discovered as a novel, atypical member of the protein kinase C family [168]. It is also known as the human homolog of PKD1, the most

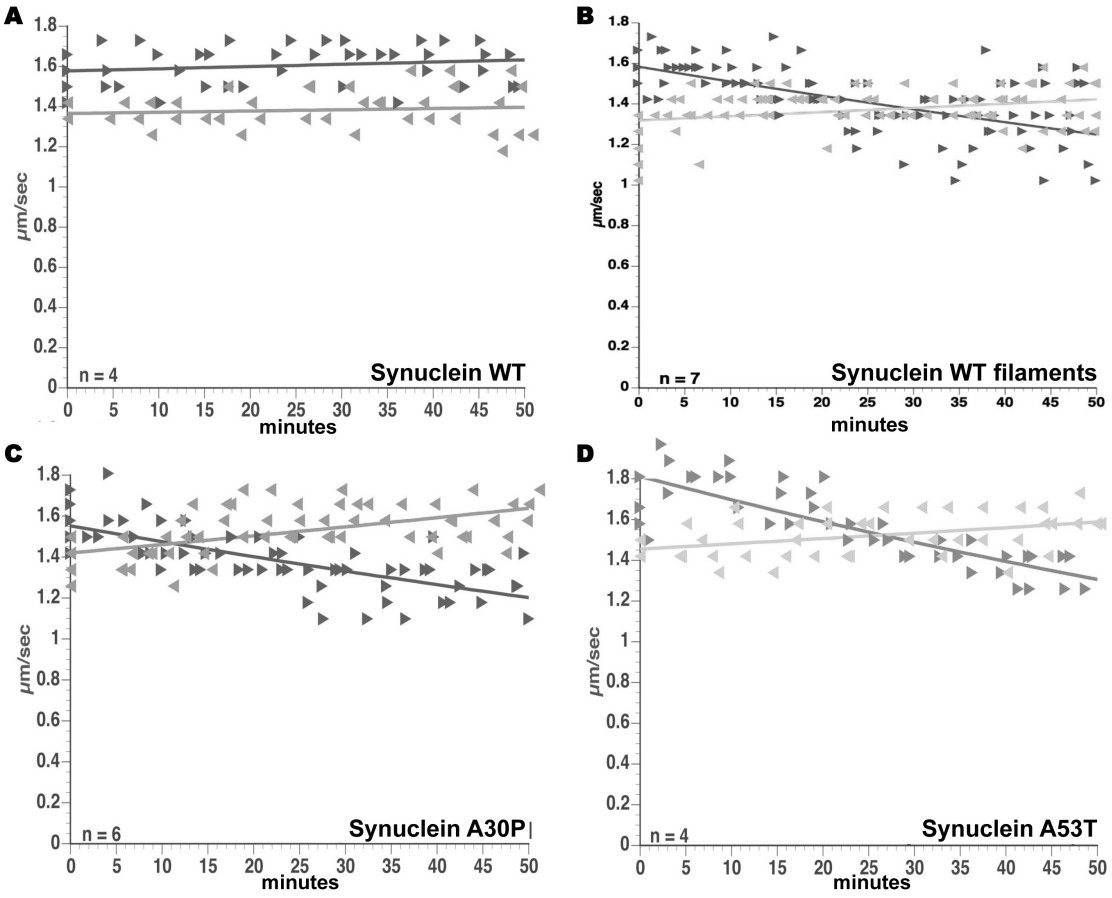


Figure 3: **Effect of various forms of α-synuclein associated with PD on FAT**

Soluble WT α-synuclein has no effect on transport (A) while WT filaments decrease transport in the anterograde direction and increase it in the retrograde one (B). Similarly, both A30P and A53T increase retrograde FAT and decrease anterograde FAT (C, D). (ST Brady and G Morfini, *unpublished*)

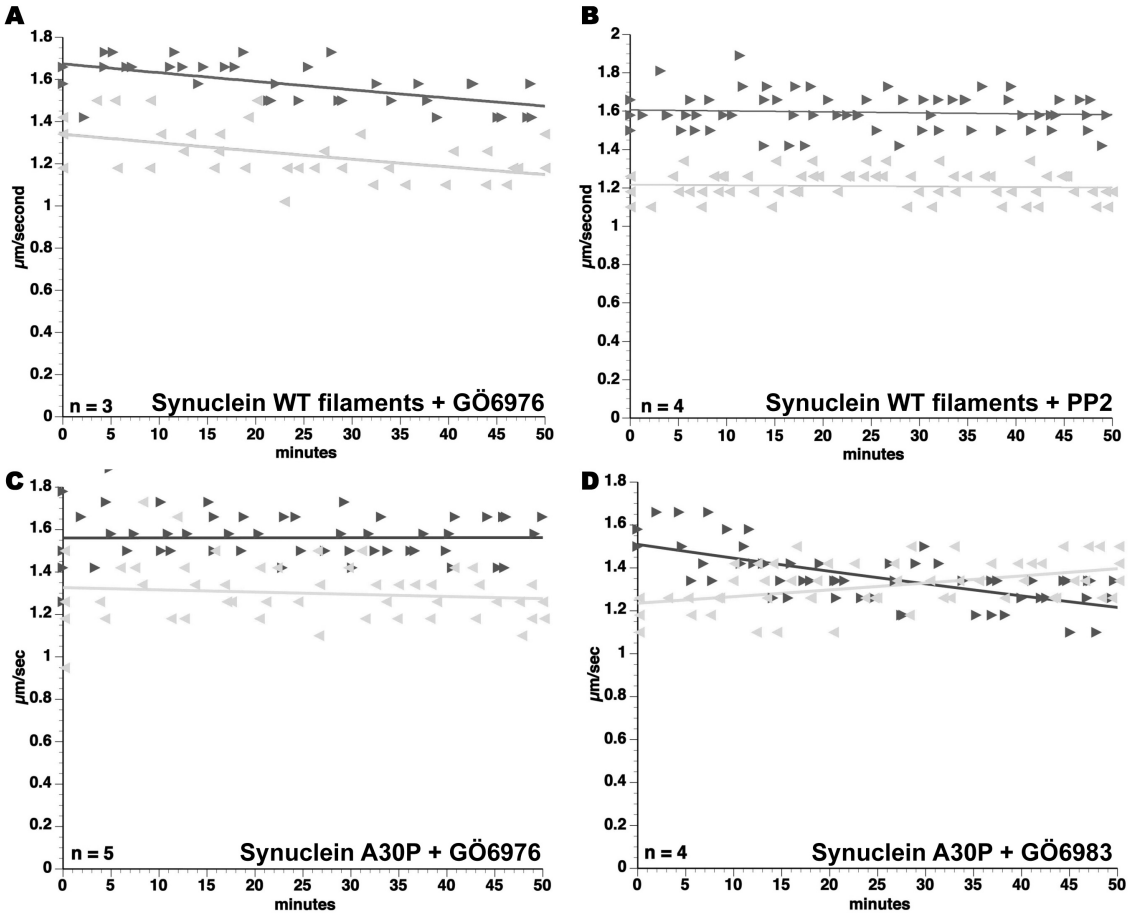


Figure 4: **Coperfusion of pathogenic α-synuclein and kinase inhibitors in squid axoplasm**

Coperfusion of pathogenic forms of α-synuclein such as WT filaments and A30P (A, C) with inhibitors of PKC abolish the effects that the proteins alone have on FAT. Similar effects are observed with the non-receptor tyrosine inhibitor PP2 (B). Coperfusion with GÖ6983 does not abolish the effect on transport, indicating that it is PKCμ that plays a role rather than other PKC isoforms. (ST Brady and G Morfini, *unpublished*)

characterized member of the protein kinase D (PKD) family, which are still serine/threonine protein kinases, but are now classified as a subfamily of the Ca^{2+}/calmodulin-dependent kinase superfamily. The protein undergoes a multistep activation which allows it to regulate membrane, cytoplasmic, and nuclear events [169]. One of its best-characterized roles is in regulation of Golgi membranes and transport carriers to the plasma membrane [170, 171]. PKCμ has been implicated in crosstalk of signaling pathways and a variety of functions such as apoptosis; immune responses; cell proliferation; motility; protein transport and membrane trafficking [172], observations consistent with results from our research group and supportive of a role in axonal transport. In addition, coperfusion with the Src family kinase inhibitor PP2 also abolished the negative effects α-synuclein has on FAT as shown for WT α-synuclein fibrils (Figure 4B). Taken together, these results point to a mechanism of α-synuclein action mediated by both PKCμ and non-receptor tyrosine kinases.

In order to tease out specific motifs or modifications of α-synuclein relevant to pathology, our laboratory next tested the effects of phosphorylated S129 of α-synuclein on transport. Published reports of posttranslational modifications suggested that phosphorylated α-synuclein, in particular synuclein phosphorylated at serine 129 (S129), plays a significant role in aggregation of the protein by making it more prone to this process [86, 90]. Recent studies demonstrated that in PD patients, soluble non-phosphorylated α-synuclein decreases over the course of the disease, becoming increasingly phosphorylated at S129 and insoluble [173]. The amino acids alanine and aspartate can be generally used to eliminate or mimic, respectively, phosphorylation in proteins [174-176]. Constructs of α-synuclein protein where S129 was mutated to an

alanine, S129A, mimics the nonphosphorylated form of α-synuclein while aspartate, S129D, mimics the constitutive phosphorylated form. Both were perfused into isolated squid axoplasm and neither construct had any effect on either retrograde or anterograde transport (data not shown). These results indicate that, at least in the case of transport, phosphorylation of α-synuclein is not necessary or sufficient to produce an effect. One could speculate that phosphorylation of α-synuclein may be the cell's mechanism of removing harmful forms of the protein, i.e. too much soluble synuclein, by targeting it to aggregate into LB. This idea is supported by evidence that up to 90% of α-synuclein present in LB is phosphorylated [63] and that oligomers of α-synuclein, rather than LB, are the more harmful aggregates in the cell [32, 81, 82].

Studies concurrent to squid axoplasm experiments showed that some receptor tyrosine kinases could also mediate regulation of retrograde FAT in a way that is blocked by a broad spectrum PKC inhibitor. Specifically, NGF or BDNF binding to Trk neurotrophin receptor cause a shift in dynein phosphorylation. To identify the kinase pathway involved in this change, our laboratory screened more than 30 different kinase and phosphatase inhibitors. Once more, only the inhibitor Gö6976 was able to block this effect while Gö6983 failed to block phosphorylation of dynein intermediate chain (DIC) (ST Brady and GM Morfini, personal communication). These results indicate that for dynein, phosphorylation after BDNF treatment is again most likely mediated by PKCμ rather than PKCδ. These results indicate that there is a direct correlation between the pathogenic role of α-synuclein and activation of specific kinases such as non-receptor tyrosine kinases and PKCμ. This in turn, has a direct effect on the proper regulation of FAT and axoplasm experiments indicated that this process is not mediated

through the phosphorylation of α-synuclein at S129. The question then is: what is the relationship between α-synuclein and these kinases?

Screening of the sequence of α-synuclein indicates that within its C-terminus there are motifs that could potentially interact with non-receptor tyrosine kinases. If so, deletions of the C-terminus might abolish the effects that pathogenic forms of synuclein have on FAT and some preliminary experiments indicated that may indeed be the case, at least in the case of A30P form of α-synuclein. The following presents my work in generating C-terminus truncated forms of wild type, A30P and A53T α-synuclein and experiments to confirm what effect, if any these forms may have on FAT.

C. Materials and Methods

1. Materials

To generate α-synuclein constructs the plasmid pQE30 was used according to Qiagen recommended protocols. Cloning reactions were performed using T4 DNA ligase (Invitrogen) according to manufacturer's instructions. Plasmids were amplified in DH5α bacterial cells (Invitrogen). All generated plasmids were sequenced at the UIC DNA Services Facility. Restriction enzymes used for cloning purposes were acquired from New England Biolabs and Invitrogen. The pcDNA 3.1+ plasmids containing WT α-synuclein, A30P and A53T mutated α-synuclein were a generous gift from Dr. Virginia Lee. Antibodies used for Western blots: anti α-synuclein (BD Biosciences) 1:2500, anti His (Qiagen) 1:1000 and Jackson anti mouse Ig-HRP 1:15000. Phosphate buffer: 11.55 g KH_2PO_4, 62.7 g K2HPO_4, and 500 mL H_2O. Lysis buffer: 10 mM Tris pH 7.5, 750 mM NaCl, and 1 mM EDTA to which the following was freshly added: 10 mg/ml lysozyme, 5

mg/ml DNase, 1 mg/ml RNase, protease inhibitor cocktail III (Calbiochem), PMSF, antipain dihydrochloride, chymostatin (Sigma). Buffers for affinity chromatography/gel filtration, 0.22 μm filtered: A – 10 mM Tris pH 7.6 and B – 10 mM Tris pH 7.6, 1 M NaCl.

2. Generation of α-synuclein constructs.

Primers for α-synuclein pQE30 cloning – the pQE30 plasmid contains a 6 histidine (6 His) N terminus tag

- full-length: Forward 5' AAA AAA GCA TGC GAT GTA TTC ATG AAA GG 3', Reverse 5' AAA AAA AAG CTT TAG GCT TCA GGT TCG TAG 3'
- C- terminus truncated - the last 21 amino acids were eliminated and a stop codon was introduced: Forward 5' AAA AAA GCA TGC GAT GTA TTC ATG AAA GG 3', Reverse 5' AAA AAA AAG CTT ATC CAC AGG CAT ATC TTC CAG 3'

The PCR reaction was carried out using the pcDNA3.1+ plasmids as templates and Platinum Taq DNA polymerase (Invitrogen) under the following conditions: 94 °C 1 min, 94 °C 30 sec, 54 °C 30 sec, 72 °C 35 sec for 30 cycles, 72 °C 5 min.

Primers for α-synuclein pRK172 cloning – plasmid contains no tags

- full-length: Forward 5' AAA AAA CAT ATG GAT GTA TTC ATG AAA GG 3'
- C- terminus truncated - the last 21 amino acids were deleted by introducing a stop codon: Forward 5' AAA AAA GCA TGC GAT GTA TTC ATG AAA GG 3', Reverse 5' AAA AAA AAG CTT ATC CAC AGG CAT ATC TTC CAG 3'

The PCR reaction was carried out using the pcDNA3.1+ plasmids as templates and Platinum Taq DNA polymerase (Invitrogen) under the following conditions: 94 °C 5 min, 94 °C 15 sec, 54 °C 30 sec, 68 °C 35 sec for 30 cycles, 68 °C 5 min.

The 423 – pQE30, 420 – pRK172 base pairs for full-length and 357 – pQE30, 360 – pRK172 base pairs for C-terminus truncated fragments of α-synuclein were separated by agarose gel electrophoresis and extracted (Qiagen Gel Extraction Kit). Linear, empty vectors were treated with alkaline phosphatase, calf intestinal (CIP) (New England Biolabs), and the fragments were ligated into either pQE30 or pRK172 vector. The prepared DNA was transformed into DH5α cells. Insertion of the fragments was confirmed by Hind III digestion of the vectors. Plasmids containing the inserts were sent for sequencing at the UIC DNA Services facilities to confirm correct orientation and sequence.

3. Expression and purification of α-synuclein protein.

E. coli host strains M15[pREP4] and SG130009[pREP4] were made competent and pQE30 plasmids were transformed into either strain. Several colonies were picked for each and α-synuclein expression was tested. Cells were grown at 37 °C, shaking at 225 rpm. Expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) when cell density reached a concentration of 1 mM at OD_{600}. Tests to optimize expression ware done using several growth media: Luria Broth (LB), 2 x YT media, and LB autoinducible media (Novagen), as well as time courses of 0 – 5 h growth from induction, where samples were collected every hour.

pRLK172 plasmids were transfected into *BL21(DE3) E. coli* strains (Stratagene). Protein expression was tested using Terrific Broth (TB) with phosphate buffer, LB autoinducible media (Novagen) as well as *E. coli* S30 T7 High-Yield Protein Expression System (Promega). Coomassie blue stained gels and western blots were used to assess protein expression. Large-scale protein production was done in 1 L TB media.

The bacteria were pelleted and kept at -80 °C until use. Pellets were resuspended in lysis buffer containing lysozyme and inhibitors. The cells were lysed using a micro-disrupter high pressure cell (Cell Scientific) and cell debris was pelleted by centrifugation at 30,000 rpm for 20 min at 4°C in a Beckman ultracentrifuge Ti45 rotor. The supernatant was boiled for 20 min in glass tubes. The sample was allowed to cool to room temperature and spun again in a Beckman ultracentrifuge Ti45 rotor at 30,000 rpm for 30 min at 4°C. The supernatant was applied to an affinity column monoQ 5/50 GL (GE healthcare) using a BioCad Perfusion Chromatography Workstation and eluted with a linear salt gradient from buffer A (0 M NaCl) to B (1M NaCl). The purity of the fractions collected was assessed by gel electrophoresis stained with Coomassie blue. The purest fractions were pooled and concentrated using 3k MW concentrators (Millipore). Protein concentration was assessed using BCA assay (Pierce) and the molecular weight was confirmed by mass spectrometry.

4. **Generation of α-synuclein fibrils.**

Filaments of α-synuclein were generated based on a modified protocol published by Necula *et al* [166]. Briefly, 8 μM purified protein and 75 μM arachidonic acid, peroxide free (Cayman Chemical) were incubated in assembly buffer (10 mM Hepes pH 7.4, 100 mM KCl, 5 mM DTT) at 37 °C for 4 h. Filaments are stable at room temperature for several days and were stored for longer periods and transport by freezing in liquid nitrogen and kept at -80 °C.

5. **Assessment of fibrillization.**

Filament assembly was assessed using light scattering employing a Beckman DU640 spectrophotometer. Samples of α-synuclein in soluble and fibrillar forms of 1

mL volume each were tested using a glass cuvette. To ensure that differences in readings were not due to the arachidonic acid, blank scans were used appropriately, for soluble samples – assembly buffer and for fibril samples – assembly buffer with 75 μM arachidonic acid. The instrument was used in Wavelength Scan mode, where samples were scanned from 250 to 500 nm.

6. Squid axoplasm perfusions.

Axoplasm perfusion experiments were performed in Woods Hole, MA where the squid were obtained. Squid axoplasm preparation and vesicle motility assays were performed as previously described [156]. Briefly, 2 μM of α-synuclein, either soluble or in fibril form was added to buffer X (175 mM potassium aspartate, 65 mM $MgCl_2$, 5 mM EGTA, 1.5 mM $CaCl_2$, 0.5 mM glucose, pH 7.2) that was diluted in half and supplemented with 2 mM ATP. Of this solution, 20 μL was added to the perfusion chambers and perfused axoplasm was analyzed for 50 min using an inverted Zeiss Axiomat microscope with high-numerical-aperture differential interference optics, 100x planachromatic objective.

7. Gel electrophoresis, Coomassie blue staining and western blotting

Samples were prepared with sample buffer (6x Sample Buffer (SB): 0.35 M Tris Base, 10 % sodium dodecyl sulfate, 36% glycerol, 5% β merecaptoethanol, bromophenol blue) and warmed up at 60°C for 5 min or boiled for 1-2 min, depending on the application. 10-20 μg of total protein for cell lysates was loaded onto 4-12% Bis Tris Nupage (Invitrogen) precast gels. Running conditions: running buffer MOPS SDS (Invitrogen NP0001) at 35-45 milliamps. For Coomassie blue staining the gels were placed in Coomassie stain (Coomassie stain: 400 ml methanol, 100 ml acetic acid, 2.5 g

Coomassie Brilliant Blue, 500 ml water, filter with Whatman paper #1) for 30 min and then Coomassie destain (Coomassie destain: 400 ml methanol, 100 ml acetic acid, 500 ml water) overnight. When necessary, gels were dried and preserved using GelAir cellophane support (BioRad 1651779). For western blotting the gels were placed onto Immobilin PVDF membrane (Millipore 1PVH00010, 0.45 μm pore) and transferred into a Hoefner tank with Tobin transfer buffer. Transfer was performed at 100 V for 2h. When blotting for α-synuclein, after transfer, the membranes were boiled in 1x phosphate buffer solution (10x Phosphate buffered saline (PBS): 1.37 M sodium chloride, 0.0268 M potassium chloride, 0.120 M sodium phosphate, pH 7.4) for 3 minutes. Blocking of non-specific binding was done with 5% non-fat dry milk in PBS. Blots were incubated with primary antibody overnight at 4 °C on a rocking platform. After 3 x 10 min washes with PBST (PBST – PBS Tween: 1x PBS, 0.25% Tween), the blots were incubated with secondary antibody linked to horseradish peroxidase prepared in 5% milk in PBST, for 1h at 4°C. After another 3 x 10 min washes in PBST the blots were analyzed for 1) chemiluminescence: using Amersham ECL Plus Western Blotting detection system (GE healthcare) and exposed to autoradiographic film; 2) fluorescence: the membranes were scanned using a Typhoon 9410 (GE).

D. RESULTS

1. Generation of full-length and C-terminus truncated forms of wild type, A30P and A53T mutated forms of α-synuclein.

A well-established role for the SH2 domain of non-receptor tyrosine kinases is in the protein's self-regulation. Phosphorylation of a tyrosine within the C-terminus leads

to the binding of its SH2 domain to the tyrosine. This results in a conformational change within the protein and autoinhibition of the kinase [163, 177]. These SH2 domains bind to specific phosphotyrosines and the specificity of the binding is dependent on the sequence motifs surrounding the tyrosine [178].

Screening of the α-synuclein sequence for functional domains using http://scansite.mit.edu, reveals that within the C-terminus of the protein, surrounding tyrosine 125 or 133, there are two potential SH2 binding domains (Figure 5A). Both of these domains are candidate motifs that could interact with non-receptor tyrosine kinases, such as Src and Fyn, and stabilize them in an active state, thus playing a potentially vital role in α-synuclein pathogenesis. To test this, the last twenty-one amino acids from the C-terminus sequence that include these potential SH2 binding domains of α-synuclein were deleted (Figure 5B). Two vectors, pQE30 and pRLK172, were employed to generate six constructs of α-synuclein: full-length WT, A30P and A53T and C-terminus truncated WT, A30P and A53T.

The first vector, pQE30, was selected because it has a six histidine (His) repeats tag at the N-terminus. Such tags are routinely used in protein purification to simplify the purification process. Generally its size does not interfere with the proper folding of the protein and cutting the tag is also an option if necessary. Western blots using antibodies against the His repeats and α-synuclein indicated that the protein was successfully expressed (Figure 6A). However, Coomassie stained gels showed that large-scale expression of α-synuclein required for protein purification using the pQE30 vector was not achieved for any of the constructs, in spite of selecting multiple clones and manipulating variables such as type of media used or length of time for growth

A

Tyrosine kinase group (Y_kin)				
Src Kinase			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
Y133	0.4164	0.132 %	EMPSEEGYQDYEPEA	2.789
Src homology 2 group (SH2)				
Itk SH2			Gene Card ITK	
Site	Score	Percentile	Sequence	SA
Y125	0.4004	0.074 %	VDPDNEAYEMPSEEG	1.665

B

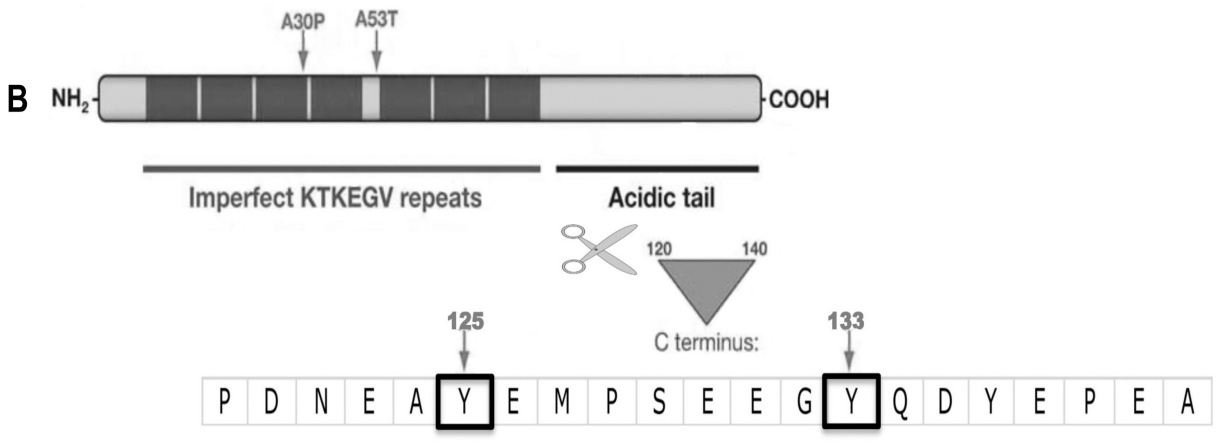


Figure 5: C-terminus truncation of α-synuclein

Scanning of the α-synuclein sequence using http://scansite.mit.edu with high stringency indicated that sequences surrounding tyrosine 125 (Y125) and 133 (Y133) are potential recognition sites for SH2 domains. The sequence scored in the best 0.132% for Y133 and 0.074% for Y125 of sites when compared to all records used in the search (A). The last 20 amino acids that include these sequences in full-length wild type, A30P and A53T α-synuclein were deleted to generate corresponding forms of C-terminus truncated α-synuclein.

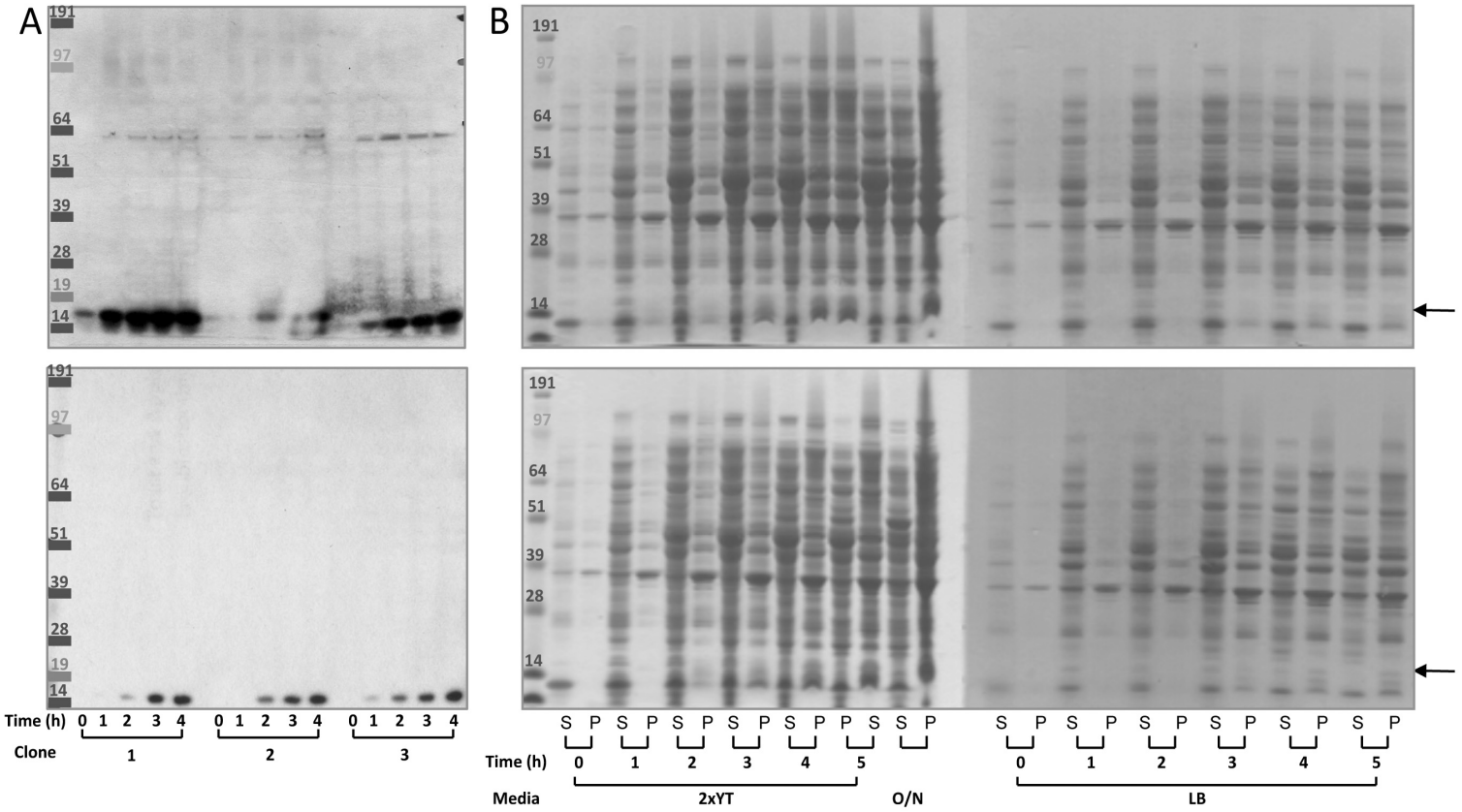


Figure 6: α-synuclein expression in SG13009 *E. coli* strains

Western blots show that tests of different bacterial clones express α-synuclein with a his tag in a time dependent manner A: 3 clones showed; top panel – blot using α-synuclein antibody; bottom panel – blot using his tag antibody. Expression levels however were too low to be useful for large scale purification needed, regardless of time and type of media used for growth B: Coomassie gels, top – full-length wild type α-synuclein; bottom – C-terminus truncated α-synuclein. Arrows indicate where the protein is expected.

(Figure 6B). A possible explanation for the low expression levels of protein is that the pQE30 vector starts with the amino acids RGS. According to the N-end rule in bacteria, proteins starting with the amino acids arginine (R), lysine (K), leucine (L), phenylalanine (F), tyrosine (Y) and tryptophan (W), have been shown to have a very short lifetime in *E. coli* [179, 180]. It may be that although α-synuclein is expressed using the pQE30 vector, its start sequence targets the protein for rapid degradation, thus making it unsuitable for use in large-scale purification. A large-scale purification of α-synuclein was needed in order to obtain sufficient amounts – milligrams – of pure protein to be used for the preparation of fibrils.

The second vector, pRLK172, was especially designed and used for high expression of proteins in *E. coli*. It was modified from the plasmid pAR3038, a derivative pBR322 carrying 23 base pairs of the promoter of gene 10 of the bacteriophage T7. pRLK172 was created so that it contains the T7 gene promoter and can be propagated at very high copy number in *E. coli* due to removal of the pBR322 copy number control region [181]. Two types of media, as well as a new cell free expression system, were used for expression of the protein (Figure 7A). The media used were TB and LB, both autoinducible. The cells were grown overnight and in both cases the protein expression was very robust. The cell free expression system did not express α-synuclein as well and was not used further. Given that the materials for TB preparation were available in house while autoinducible LB media was only available prepared from the company Novagen, TB was used for subsequent conditions. All six constructs were created and each tested for expression. The C-terminus truncated constructs of A30P and A53T α-synuclein ran higher on the gel, when compared to their

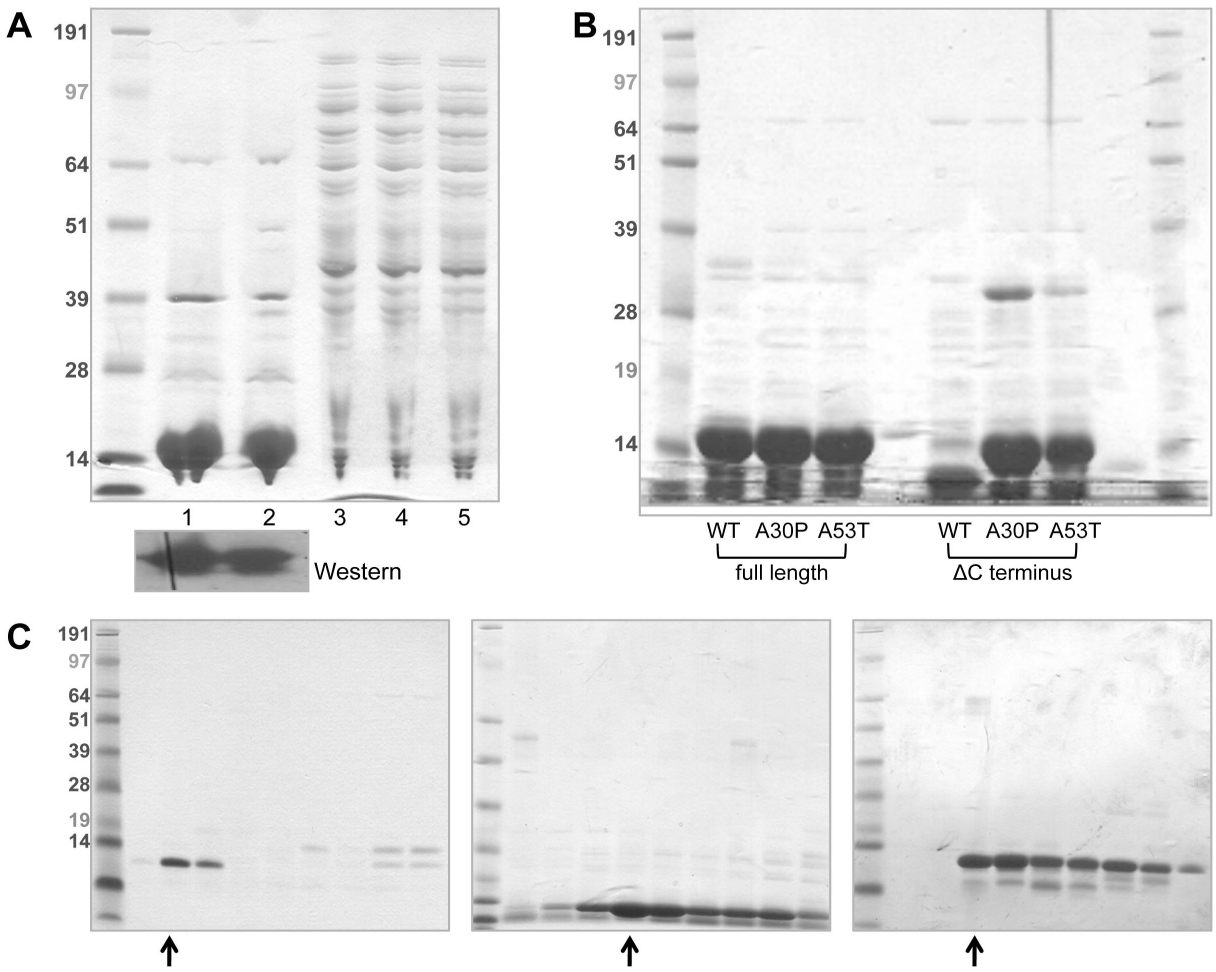


Figure 7: Expression and purification α-synuclein constructs

A. Several conditions were tested for expression 1: TB, 2: LB – both 1 and 2 used autoinducible media and overnight growth. Protein overexpression of α-synuclein was confirmed with Western blotting (below) using antibodies for α-synuclein 3,4,5: cell free expression system, which did not yield very high protein levels. B. All constructs were expressed robustly. The A30P and A53T truncated forms α-synuclein (ΔC terminus) migrated higher on the gel compared to their WT counterpart but the DNA sequence used for each construct was correct. C. The proteins were purified using size exclusion chromatography and only the purest fractions were selected as exemplified here by the arrows for C-terminus truncated WT α-synuclein.

WT counterpart (Figure 7B), in spite of the fact that the molecular weights for all three truncated proteins and their predicted overall charge do not differ – the predicted isoelectric point for all three is 6.11 (the value was obtained using the protein calculator from http://www.scripps.edu/~cdputnam/protcalc.html). However the isoelectric point, in this case, was just an estimation and even though the differences are only of one amino acid, it is possible for that to be sufficient to affect the behavior of the protein, including the manner in which it migrates on a gel. As mentioned in the introduction, the mutations do affect the way α-synuclein folds and therefore its interactions [60]. Given that the DNA sequences were correct, all six constructs were used to continue on to the purification process. Pure protein was isolated using affinity chromatography and only the fractions that had the highest purity of protein were pooled together and used for the experiments as exemplified in Figure 7C for wild type, C-terminus truncated α-synuclein.

2. Generation of full-length and C-terminus truncated forms of wild type, A30P and A53T mutated forms of α-synuclein fibrils.

Based on a protocol adapted from Necula *et al* [166], α-synuclein fibrils were generated using arachidonic acid. Arachidoic acid was shown to induce α-synuclein fibrilization at various concentrations of the protein in short periods of time [166, 182]. The Binder laboratory from Northwestern University had previously modified Necula's method of making α-synuclein fibrils and tested fibril formation using electron microscopy for the experiments described in the introduction. Therefore, for subsequent batches of α-synuclein and the following squid axoplasm experiments, confirmation of aggregation by light scattering methods was sufficient. Substantial

increases in scattered light were observed in samples incubated under fibrilization conditions compared to the same amount of soluble α-synuclein protein as showed in Figure 8.

Although the extent of aggregation by this method was tested using electron microscopy and light scattering, we had the opportunity, in collaboration with the LaDu laboratory at UIC, to test the formation of the fibrils made using Atomic Force Microscopy (AFM). However, attempts to demonstrate fibril formation by AFM under conditions used for squid axoplasm perfusion were unsuccessful (not shown). This is most likely due to the low concentration of protein and aggregates used for axoplasm perfusion (2.5 μM). Previous studies suggest that visualization of α-synuclein filaments by AFM requires a minimum initial protein concentration of 15 μM protein [83], well above the 2.5 μM concentration used in the squid axoplasm.

3. **Effects of C-terminus truncated forms of wild type, A30P and A53T mutated forms of α-synuclein in soluble and fibril form on axonal transport.**

As presented above, fibrillar forms of WT α-synuclein as well as A30P and A53T mutated forms have a significant impact on fast axonal transport. Perfusions with freshly prepared protein confirmed and reproduced those results for all three forms of the protein: increase retrograde and decreased anterograde transport (Figure 9 A and C, only WT and A30P shown). The next step was to test the effects of C-terminus truncated forms of α-synuclein. The truncated forms of the protein, WT, A30P and A53T, were 119 amino acids long and lacked the last 21 amino acids that include the consensus site for an SH2 recognition domain (see Figure 5B). WT α-synuclein fibrils made of C-terminus truncated protein did not have any effect on transport in either

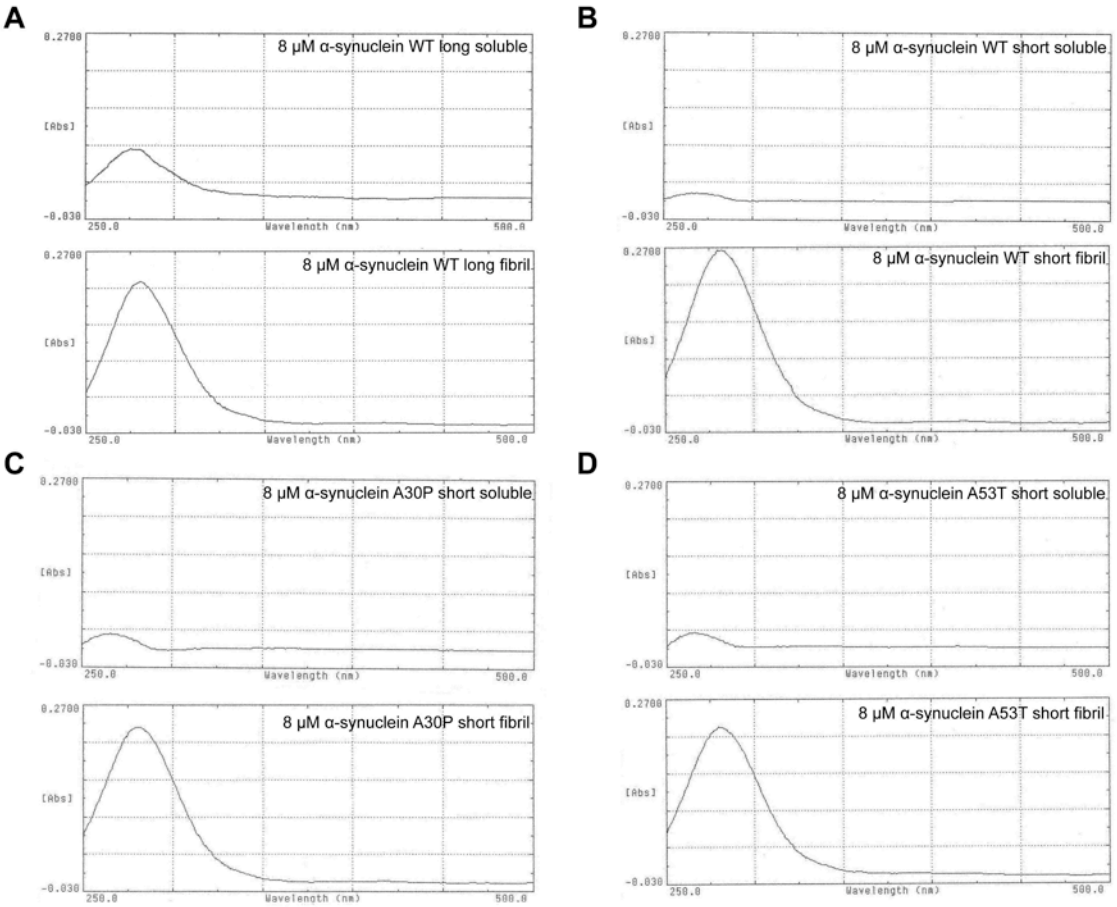


Figure 8: Light scattering of α-synuclein fibrlils

Top panels of A, B, C and D show light scattering at 90° for soluble forms of 8 μM α-synuclein, while the bottom panels show light scattering for each of the same corresponding forms of α-synuclein but which were treated to undergo fibrillization. Notice how for the same volume and amount of starting material, when compared to the soluble conditions, the peaks are taller and wider and the light scatters more, indicative of fibril formation.

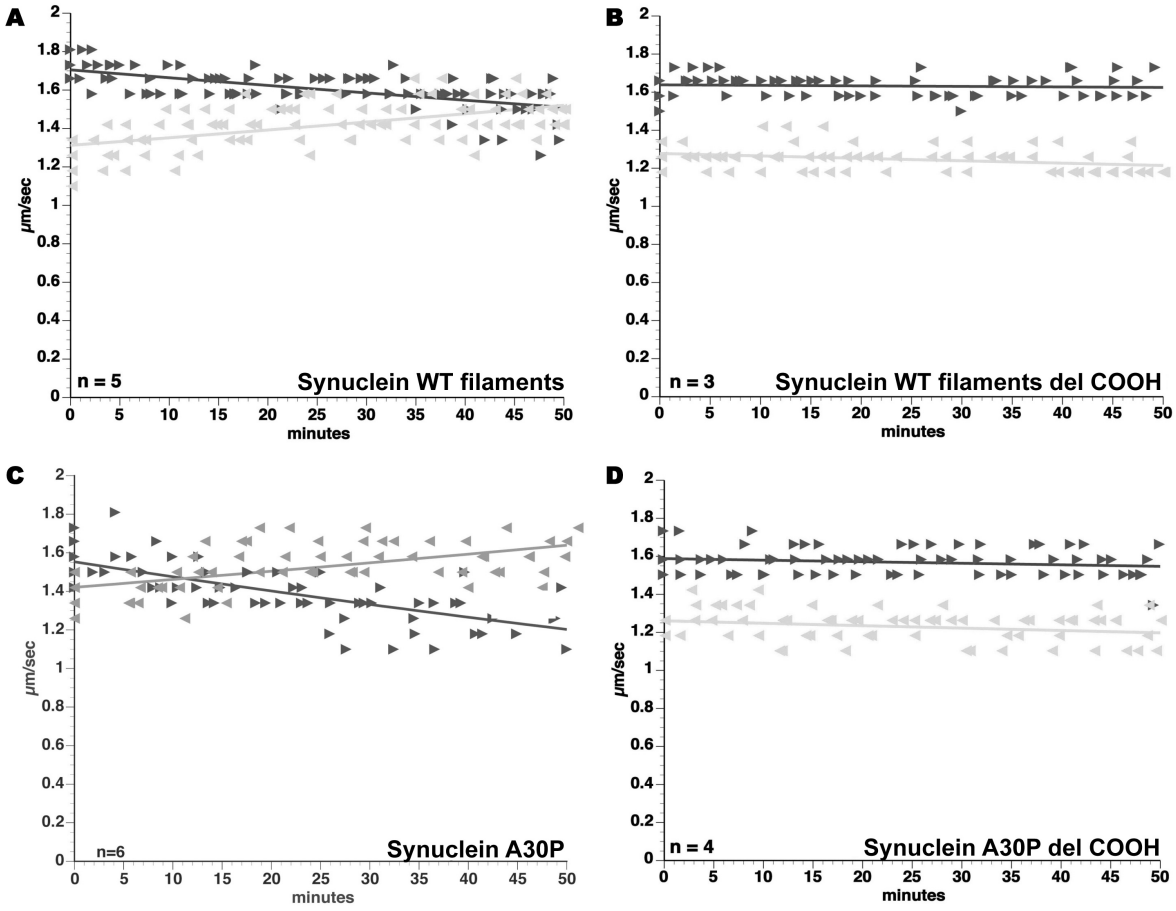


Figure 9: Effects of α-synuclein on axonal transport

Wild type 2.5 μM α-synuclein in fibril form (A) as well as 10 μM mutated forms of the protein, A30P shown (C), affect transport in both anterograde – decrease – and retrograde – increase – transport, as previously demonstrated (see Figure 3). C-terminus truncated forms of the protein however, have no effect on transport (B and D).

anterograde or retrograde direction (Figure 9B) and neither did truncated forms of mutated α-synuclein (Figure 9D, both mutants tested, only A30P represented). Moreover, fibrils made of mutated forms of truncated protein also did not have any effect on transport. Taken together with observations that phosphorylated α-synuclein at S129 does not have an effect on transport, these results clearly indicate a significant role for the C-terminus of α-synuclein in its pathogenic mechanism, independent of S129 phosphorylation.

E. Discussion

Our work demonstrates that pathogenic forms of α-synuclein such as WT fibrils, A30P and A53T mutants have a significant effect on the proper flow of FAT. This effect is similar to that observed with kinases such as non-receptor tyrosine kinases like Src and Fyn or the protein kinase C isoform, PKCμ. Coperfusion of pathogenic forms of α-synuclein with inhibitors to these kinases abolished the effects observed on transport, indicating that α-synuclein effects are mediated through these kinases. Moreover, perfusion of pathogenic forms of C-terminus truncated α-synuclein such as WT fibrils and soluble or fibril forms of A30P and A53T α-synuclein do not have any effect on transport compared to buffer control.

There is evidence that non-receptor tyrosine kinases can directly interact with PKCμ. Stortz *et al* has found that PKCμ can be activated by non-receptor tyrosine kinases such as Src through phosphorylation of a specific tyrosine, Y463 [183, 184]. Coperfusion of PKCμ inhibitors with α-synuclein or non-receptor tyrosine kinases abolishes the effects these have on transport pointing to a similar mechanism of action

where α-synuclein activates Src and/or Fyn and these kinases in turn activate PKCμ possibly by phosphorylating it at Y463.

In its inactive conformation, the SH2 domain of Src is engaged with Tyr530 [185]. Phosphorylation of this tyrosine leads to a conformational change of the protein that opens it up leading to its activation. It is likely that ligand sites for potential recognition by SH2 domains identified within the C-terminus of α-synuclein can act as competitors to Src's own Tyr530, thus converting the protein to an active conformation. The fact that elimination of the C-terminus from α-synuclein abolishes the negative effects the protein has on transport supports this idea. Using solid state NMR, Heise *et al* has shown that the C-terminus in α-synuclein fibrils is rather mobile and lacks a defined secondary structure [186]. In brains from patients suffering from dementia with LB, antibodies against the C-terminal region of α-synuclein immunostained LBs [187], indicating that this part of the protein is exposed. These studies of α-synuclein aggregation and fibril formation indicate that the folding of the protein is such that the C-terminus may always be available for interaction.

These results are exciting and would provide an explanation for a mechanism that encompasses all types of pathogenic forms of α-synuclein. Whether soluble or in aggregated form, the C-terminus is always potentially available to activate non-receptor tyrosine kinases. Phosphorylation of α-synuclein at S129 may be a way for neurons to target the protein for LB formation in order to deal with and prevent the activation of a cascade of pathways that can have a multitude of negative effects. An important conclusion is that it is not the *quality* or the *quantity* of protein that has a negative effect

but rather how either one leads to the increase availability of a domain that under proper circumstances integrates with normal neuronal function.

When taken together a model of pathogenesis for α-synuclein emerges (Figure 10). Various forms of pathogenic α-synuclein such as: mutated forms of the protein, too much protein or protein under various aggregated states such as fibrils, oligomers or LB, activate a non-receptor tyrosine kinase by binding to its SH2 domain, keeping the protein in an active state. This kinase in turn will activate PKCμ. Subsequently, PKCμ will affect the phosphorylation status of the motors, kinesin and dynein, and this will have significant consequences on axonal transport and therefore ultimately on neuronal survival.

These results point toward a novel pathogenic mechanism for loss of neurons in PD and other synucleinopathies. Normal pathways for regulating transport via PKCμ may be altered by local increases in PKCμ activity. The squid experiments provide insights into a potential pathogenic mechanism for α-synuclein, but validation of this model in mammalian neurons is needed. Chapter 3 will focus on the generation of a cellular model of PD and the evaluation of this mechanism in that system.

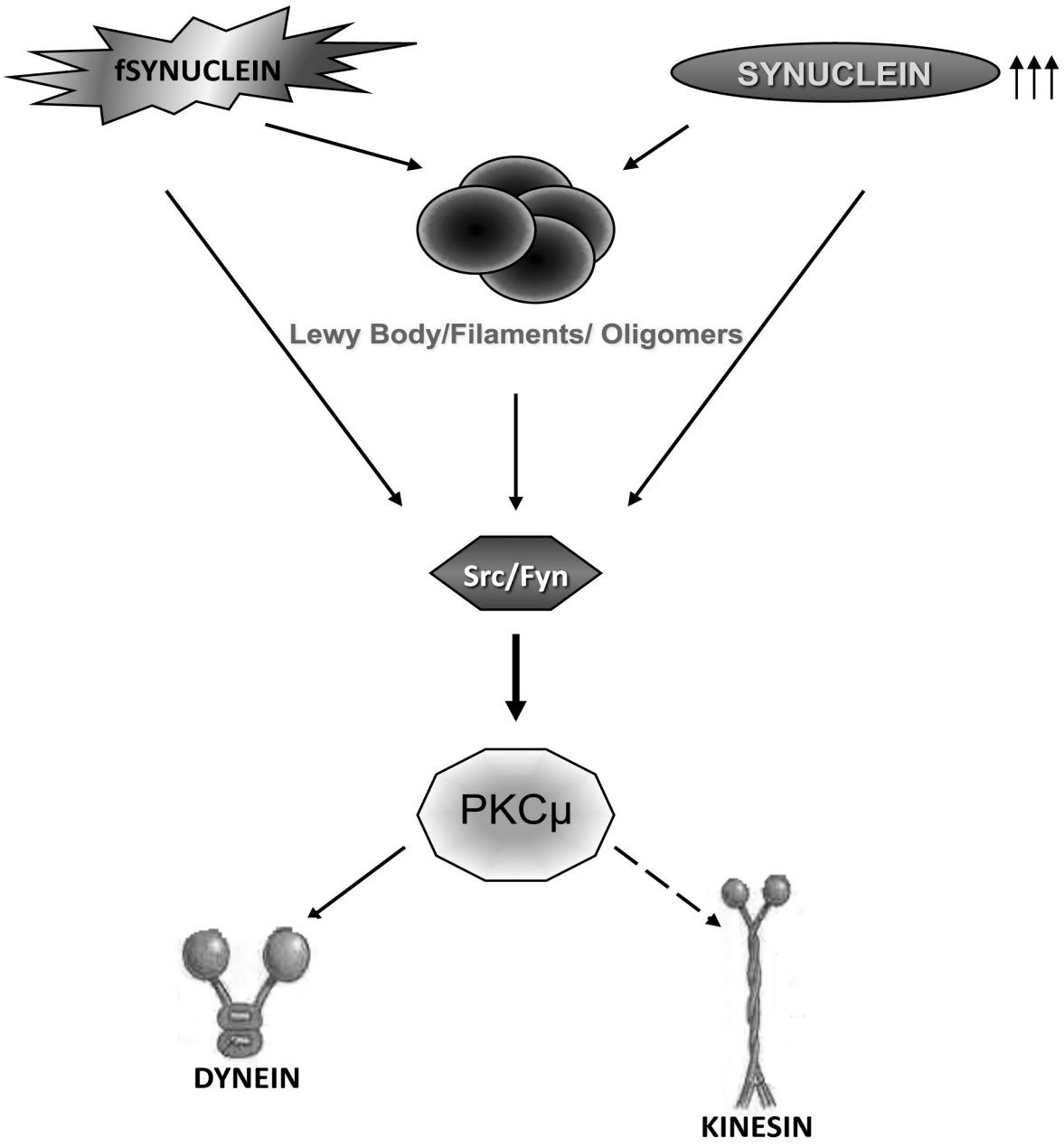


Figure 10: Model of α-synuclein pathology in FAT

α-Synuclein in either mutated forms (fSynuclein), overexpressed (↑↑↑) or in aggregated forms such as LB, filaments or oligomers activates, though its C-terminus, a non-receptor tyrosine kinase such as Src or Fyn, which in turn activate PKCμ. Activated PKCμ will affect the phosphorylation of dynein and kinesin in a direct or indirect manner, which will affect proper flow of axonal transport.

III. EFFECTS OF α-SYNUCLEIN EXPRESSION ON AXONAL TRANSPORT IN CELLS

A. Summary

Using squid axoplasm, a hypothesis for axonal transport and pathology of α-synuclein in PD was developed that underlines a role for several kinases, one of them PKCμ. In order to develop a cellular model of PD and to test this hypothesis, a number of neuroblastoma cell lines were screened. The cell lines SK-N-SH and SH-SY5Y emerged as most suitable to generate cells stably transfected with WT, A30P and A53T α-synuclein. Several clones were developed and analyzed for each cell line. SK-N-SH have a mixed population of epithelial and neuronal like cells and working with this line suggested that α-synuclein is taken in at different rates by the cells – the epithelial cells appeared more permissive to α-synuclein transfections and therefore expressed higher levels of the protein. This led to wildly different clones, some with no detectable expression of α-synuclein whereas in others the expression was very high. Immunocytochemistry demonstrated that, although α-synuclein was expressed, within a clone not all cells expressed the protein. Potential variability of expression within a clone, and a lengthy differentiation protocol led to focus on SH-SY5Y cells.

Transfected clones of SH-SY5Y expressed α-synuclein robustly. As selection criteria, similarity in cell morphology as well as α-synuclein expression, were taken into account for each cell line used. Differentiation was tested, expression was quantified in western blots and imaging confirmed that all cells within a clone expressed α-synuclein. Imaging at higher magnification showed that there was no apparent aggregation of α-synuclein in the time frame examined. It is not uncommon in stable transfections,

especially when working with toxic proteins, for protein expression levels to decrease with each passage, and work with the SH-SY5Y cells was no exception.

Characterization of the molecular motors kinesin and dynein in clones expressing WT or A53T α-synuclein showed that there were no significant changes in the expression of either when compared to untransfected cells. Use of microtubule binding assays demonstrated that kinesin binding to microtubules was unaffected however, there was evidence that dynein associated less with the microtubules in cells expressing α-synuclein. These observations will need further validation as nucleotide binding to dynein is more complex than that of kinesin and the assays employed were designed specifically for the latter. Interaction of the motors with their cargo was assessed by examining their association with membranes and our results indicated that this indeed might be affected in clones expressing either WT or A53T α-synuclein. A high degree of variability in the results indicated however that a method that isolates specific pools of vesicles is required.

Evaluation of PKCμ revealed that in SH-SY5Y cells, this kinase appears to be tightly regulated. Western blots showed that in differentiated cells PKCμ is very low to undetectable. Treatment of differentiated cells with the PKC activator phorbol 12-myristate 13-acetate (PMA) led to an increase in *both* total and phosphorylated PKCμ. These changes occurred fast, within a matter of minutes, which indicates that PMA either inhibits a mechanism that under differentiating conditions targets PKCμ for rapid degradation or activates PKCμ in a manner that makes it more available for recognition by the antibodies used. Use of PKCμ antibodies also led to the discovery of an unknown protein dependent on α-synuclein expression. This protein's apparent

molecular weight was approximately 170 kDa and it shares a sequence within the C-terminus of PKCμ. Searches of this amino acid sequence were unique to PKCμ, which led to the possibility that this unknown protein may potentially be a new isoform of PKCμ. The identity of this protein has yet to be determined and its identification could be key to understanding the mechanism of α-synuclein pathology. The results also suggest that SH-SY5Y cells may not be the most suitable system to test the model of pathogenesis developed in squid axoplasm and alternatives need to be considered for the future.

B. Introduction

The previous chapter illustrates that the squid axoplasm is a useful setting for studying components of neuronal transport and how neurons may be affected in neurodegeneration. The system helped demonstrate that signaling pathways affected in diseased neurons change transport and led to the hypothesis that in PD, motifs within the C-terminus of α-synuclein alter regulatory pathways for fast axonal transport through a mechanism involving PKCμ. It is also important to point out that although altered transport may not be the primary cause of PD, the kinases we identified as potentially significant, play important roles in the cell as a whole. Therefore, up regulation of kinase activities such as Src or PKCμ can have severe consequences on proper cell function, homeostasis and ultimately, survival. An essential next step would be to test our hypothesis in other models of PD and determine whether the kinases identified in squid axoplasm are affected in these models as well. Ideally, the most appropriate venue would be PD patients, but ethical and practical considerations for such

experiments would make that quite difficult. Given that the clinical symptoms of PD are due to loss of neuronal connections between the substantia nigra and the striatum, research has focused on the neurons most affected in this region. For this purpose, one animal model of PD was created by destroying DA neurons in the SNp using the reagent 6-hydroxydopamine (6-OHDA), a neurotoxin selective to noradrenergic and dopaminergic neurons. However this model is not useful to study the role of α-synuclein in neurons, as one would need intact cells to observe the effects of the protein. Animal models of α-synuclein pathology of PD that faithfully reproduce the disease have been difficult to develop [188, 189]. In rodents, as in most vertebrates for that matter, the A53T form of α-synuclein that causes PD in humans is expressed endogenously, and the models, even when they exhibit pathological phenotypes, are often different than those observed humans. Use of primary neuronal cultures from rats and mice can be difficult as they require a lot of animals and are time consuming. These cultures also have a limited life span, and it is nearly impossible to obtain homogeneous cell populations. In addition, primary cultured neurons can be tough to transfect for expression of proteins of interest. By contrast, neuroblastoma cell lines are relatively inexpensive and easy to use. They have been used as *in vitro* models with a wide variety of applications such as pharmacological studies, the study of the effect of viruses, effect of toxins, protein-protein interactions and transplant studies [190]. Neuroblastoma cell lines can potentially provide an unlimited supply of material as they retain their ability to differentiate into neurons when treated with differentiating agents such as retinoic acid (RA). In addition, they are easy to transfect and relatively homogeneous. The following work focused on screening and selecting a

neuroblastoma cell line suitable for developing an α-synuclein cellular model of PD pathology. Stably transfected cell lines were developed and subsequently used to test the hypothesis developed using squid axoplasm experiments.

C. Materials and methods

1. Materials

Antibodies: A list of antibodies for molecular motors and kinases analyzed is provided in table I below. Antibodies were handled as recommended by suppliers and final dilutions from purchased stocks used for western blots are given. Depending on the application, the conditions for some experiments were further optimized.

Cell lines: 1RB3AN27 (N27) were obtained from Dr. Gerardo Morfini (University of Illinois at Chicago, tel: (312) 996-6869, email: gmorfini@uic.edu); CHP 134, IMR5, NLF, NB69 were gifts from Dr. Nao Ikegaki (University of Illinois at Chicago, tel: (312) 996-6048, email: ikegaki@uic.edu); SK-N-MC (Catalog # HTB-10, passage # P40), SK-N-SH (Catalog # HTB-11, passage # P36), SH-SY5Y (Catalog # CRL-2266, passage # P23) were purchased from ATCC.

Cell culture media: Recipes for specific media conditions required for each cell line is provided bellow:

Media for N27, CHP-134, IMR5 and NLF cells

RPMI – 1640 w/Glutamine GIBCO, Catalog No. 11875-093

10% FBS (Fetal Bovine Serum), GIBCO, Catalog No. 26140

1% PenStrep (Penicillin Streptomycin), GIBCO, Catalog No. 15140

TABLE I

ANTIBODIES USED

Name	Type	Dilution	Company	Species
α-synuclein	Primary	1:4,500 (C) 1:1,000 (F)	BD Transduction 610786	Mouse
KHC (H2)	Primary	1:60,000 (C) 1:1,000 (F)	Brady lab	Mouse
KLC (63-90)	Primary	1:5,000 (C)	Brady lab	Mouse
Dynein Heavy Chain (DHC)	Primary	1:500 (C) 1:250 (F)	Santa Cruz sc-9115	Rabbit
DIC	Primary	1:1,000 (F)	Santa Cruz sc-13524	Mouse
PKD/PKCμ	Primary	1:1,000	Cell Signaling 2052	Rabbit
Phospho-PKD/PKCμ (Ser916)	Primary	1:1,000	Cell Signaling 2051	Rabbit
Phospho-PKD/PKCμ (Ser744/7468)	Primary	1:1,000	Cell Signaling 2054	Rabbit
PKCμ(C-20)	Primary	1:1,000	Santa Cruz sc-639	Rabbit
GSK3β	Primary	1:40,000 (C) 1:1,000 (F)	BD Transduction 610201	Mouse
α Tubulin DM1A	Primary	1:12,000 (C) 1:3,000 (F)	Sigma-Aldrich T9026	Mouse
Synaptophysin	Primary	1:3,000	Zymed 18-0130	Rabbit
SNAP-25	Primary	1:10,000 (C) 1:1,000 (F)	Santa Cruz sc-20038	Mouse
Histone H3	Primary	1:1,000 (F)	Cell Signaling 9508	Rabbit
Mouse (HRP conjugated)	Secondary	1:40,000	Jackson 115-035-146	Mouse
Rabbit (HRP conjugated)	Secondary	1:20,000	Jackson 111-005-045	Rabbit
Cy5	Secondary (fluorescent)	1:2,000	Amersham PA45010V	Mouse
Cy5	Secondary (fluorescent)	1:2,000	Amersham PA45012V	Rabbit
Note: C - chemiluminescence, F - immunofluorescence				

Cell culture media *(continued)*:

Media for NB69 cells

RPMI – 1640 w/ L-glutamine and 25 mM HEPES (Mediatech # 10-041-CV)

10% FBS hybridoma screened (Lonza Walkersville 14-901E)

1% PenStrep, GIBCO, Catalog No. 151401% L-glutamine GIBCO

1% OPI (oxaloacetate, pyruvate, and insulin) made according to Sigma recommendations

Media for SK-N-SH and SK-N-MC cells

EMEM (Eagle's Minimum Essential Medium) ATCC, Catalog No. 30-2003

10% FBS (Fetal Bovine Serum), GIBCO, Catalog No. 26140

1% PenStep (Penicillin Streptomycin), GIBCO, Catalog No. 15140

Media for SK-N-SH cells with G418

Media for SK-N-SH cells with 1.4 mg/ml G418, Sigma, Catalog No. A 8601-5G, CAS 108321-42-2

Media for SH-SY5Y cells

DMEM/F12 1:1 (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (HAM) 1 x (+) L-Glutamine, (+) 15mM HEPES, GIBCO 11330

10% FBS (Fetal Bovine Serum), GIBCO, Catalog No. 26140

1% PenStrep (Penicillin Streptomycin), GIBCO, Catalog No. 15140

1x Glutamax, GIBCO, Catalog No. 35050

Media for SH-SY5Y cells with G418

Media for SH-SY5Y cells with 1 mg/ml G418, Sigma, Catalog No. A 8601, CAS 108321-42-2

For better clarity, other materials, such as reagents and solutions, are described with the method for which they are specifically used. Unless otherwise noted the reagents were obtained from Sigma and all the solutions were prepared using Milli-Q or equivalently purified water.

2. General cell handling procedures

Reagents and Solutions: trypsin – 0.05% trypsin-EDTA GIBCO 25300, dimethyl sulphoxide (DMSO) Hybri-Max Sigma D2650, trypan blue – Lonza 17-942E, *ROLB*: 10mM Hepes, 0.5% triton X-100, 0.05 M sodium fluoride, 0.025 sodium pyrophosphate, 0.08 M sodium β glycerophosphate, potassium phosphate, 5 mM EDTA, 0.01% sodium azide, pH 7.4; *Inhibitors added fresh to ROLB*: 0.2 mM sodium orthovanadate (Sigma S6508), 0.2 mM mycrocystin R-R (EMD 475815), 50 nM okadaic acid (Calbiochem), 0.1 mM K252a kinase inhibitor (Calbiochem 420298), 0.1 mM staurosporine (Calbiochem 569397), 1:200 dilution of phosphatase inhibitor cocktail II (Calbiochem 524625), 1:200 dilution of protease inhibitor mammalian cocktail (Sigma P8340), 1:100 dilution of phosphatase inhibitor cocktail 3 (Sigma P0044); *10x phosphate buffered saline (PBS)*: 1.37 M sodium chloride, 0.0268 M potassium chloride, 0.120 M sodium phosphate, pH 7.4.

Cells were housed in a 37°C incubator at 5% CO_2 and 60% relative humidity, and were visualized using an inverted microscope (Aus JENA Telaval 3).

Detachment for passing or long-term storage: cells were rinsed and then incubated with trypsin for 2 - 5 min or PBS with 0.5 mM EDTA solution for 5 minutes. Cells stained with trypan blue were counted using a hemocytometer (Fisher 0267110). For long-term storage, cells were detached and pelleted by spinning at 800 rpm. The

media with trypsin was then removed and the cells were resuspended in 1 ml, 5% DMSO media solution and frozen in liquid nitrogen.

Cell harvest for biochemistry and immunochemistry: ROLB with inhibitors was added to the plate and cells were scraped. The cell lysate was transferred to an Eppendorf tube and sonicated in 10 sec bursts then spun at 14,000 rpm for 10 min, at 4°C. The pellet was discarded and the supernatant was transferred to new tubes and used as needed. Total protein concentration was assessed using Pierce BCA Protein Assay Kit (Thermo Scientific 23227).

3. Poly-lysine coating

Glass coverslips for immunocytochemsitry or dishes used for differentiation of cells were coated with poly-L-lysine hydrobromide (Sigma P1399). Poly-L-lysine was diluted to 0.01 mg/ml in 500 mM Na Bromide (Sigma S9640), pH 8.2 that was filtered at 0.22 μm. Solution was added enough to cover the surface area, e.g. for 10 cm dishes 4.5 - 5 mL solution was used. Glass coverslips were sterilized by exposure to UV radiation for 12 hours. Dishes and coverslips were incubated with poly-lysine for periods ranging from 2 – 12 h and then rinsed once with 500 mM Na Bromide and twice with media before platting.

4. Cell differentiation protocol

SK-N-SH cells

Cells were plated in normal media conditions at 4 – 5 x 10^5 per 10 cm dishes and after 2 days were treated with 10 μM retinoic acid (RA – Calbiochem 554720). Cells were maintained in RA for 1 - 2 weeks until they reached 100% confluence or for a maximum of two weeks. The cells were subsequently replated on polylysine-coated

dishes in media containing 5% FBS and mitotic inhibitors: 10 μM 5-fluoro-2'-deoxyuridine (FUdR), 10 μM 1-β-D-ribofuranosyluracil (Urd), 10 μM cytosine β-D-arabinofuranoside (araC).

SH-SY5Y cells

Cells were plated onto polylysine-coated dishes and grown to a confluence of 50 - 60% or higher, depending on the clone and the application. The media was then changed to one containing only 5% FBS and 10 μM RA. After three days half of the media was removed and replaced with serum-free media and 10 μM RA. After an additional two days the RA-media was replaced with serum-free media to which 50 ng/ml recombinant human brain derived neurotrophic factor (BDNF – Millipore GF029) had been added. The cells were then harvested after 2-3 days of BDNF treatment.

5. <u>Stable cell line generation</u>

Determination of antibiotic resistance (G418) for SK-N-SH and SH-SY5Y cells

Untransfected cells were plated in a 96-well plate – 100 μl of cell suspension per well, decreasing the number of cells in each well by half, starting with 2000 cells in well 1 to 0 cells in well 12. Media containing G418 (G418 Sulfate – Cellgro 61-234-RC) was added decreasing G418 concentration for each well in steps of 0.2 mg/mL starting with 1.4 mg/mL in row A to 0 mg/ml in row H. Cells were incubated and monitored for cell death. After 7 days the appropriate G418 concentration to use for transfected cells was decided by selecting the G418 concentration in the wells above the ones that shows complete cell death by this time. For SK-N-SH: 1.4 mg/mL. For SH-SY5Y: 1 mg/mL.

Transfections

Plasmid pcDNA3.1+ containing WT, A30P and A53T α-synuclein from Dr. V. Lee (University of Pennsylvania School of Medicine, phone: 215-662-6427, email: vmylee@mail.med.upenn.edu) were used to generate transient or stably transfected cell lines. Transfections were performed according to Lipofectamine 2000 protocol (Invitrogen #11668-027). To generate stably transfected cell lines, SK-N-SH and SH-SY5Y cells were transfected and then treated with media containing G418, 1.4 mg/mL for SK-N-SH and 1 mg/mL for SH-SY5Y. Cells were picked from surviving colonies and plated individually, each colony generating an individual cell line.

6. Gel Electrophoresis, Coomassie blue staining and western blotting

Coomassie gels and western blots were performed as previously described in Chapter I.

7. Immunocytochemistry

Reagents and solutions: paraformaldehyde (Electron Microscopy Sciences # 15712; *10x phosphate buffered saline (PBS)*: 1.37 M sodium chloride, 0.0268 M potassium chloride, 0.120 M sodium phosphate, pH 7.4; *PBST*- PBS Tween: 1x PBS, 0.25% Tween®20 – Fisher BP337-500; Triton X-100 – Sigma 9002-93-1; goat serum; Vectashield Mounting Medium – Vector Labs H-1000), ProLong® Gold antifade reagent with DAPI – Invitrogen # P36935.

Coverslips (1.5 Glass 18 mm round, Warner Instruments, CS-18R15, Cat: 64-0714) were set in 12-well plates and treated with polylysine as described above. Cells were counted as described in part 2 of these materials and methods, plated 50,000 in each well and differentiated. Once the differentiation process was done, the samples

were fixed with 4% paraformaldehyde. After 2x washes with PBS the cells were permeabilized by incubation for 10 min with PBS with 0.1% Triton X-100. After 3x washes for 5 min with PBS the samples were blocked with 10% goat serum in 0.4% PBS Triton for 1 h at RT. Following another 3x washes with PBST, 10 min each, α-synuclein primary antibody – at various dilutions – was added and incubated at 4°C overnight. The coverslips were washed again 3x with PBST and working in a dim environment the secondary antibody – goat anti-mouse conjugated to Alexa 594, 1:400 dilution in PBS – was added and incubated for 1 h at RT or 37°C. The samples were then washed 5x with PBST for 10 min each and mounted onto microscope slides (frosted, precleaned, Fisherbrand # 12-550-343) using Vectashield Mounting Medium mixed with ProLong® Gold antifade reagent with DAPI. The slides were kept in the dark for minimum 3 h or overnight, at 4°C before visualizing with an inverted, fluorescence imaging microscope (Zeiss Axiovert 200M) using Openlab imaging software (Cellular Imaging PerkinElmer).

8. <u>Polymerization of tubulin for microtubule binding assay</u>

Reagents and solutions (for both 8 and 9): *2x BRB80 buffer*: 180 mM BRB80, 10 mM MgCl2, 2 mM EGTA, pH 6.8; *Inhibitors for BRB buffer*: 1 μM staurosporine, 1 μM K252a, 50 nM okadaic acid, 1 μM Mycrosystin, 1:100 dilution of protease inhibitor cocktail mammalian (Sigma P8340), 1:100 dilution of phosphatase inhibitor cocktail (Sigma P0044); GTP (Sigma G8877); AMP-PNP (Sigma A2647); ATP (Sigma A8937); tubulin 2.0 mg/ml prepared and purified in house; sucrose; taxol (Alexis Biochemical ALX-351-001);

Purified frozen tubulin (prepared in the laboratory for general use according to published protocols [191]) was quickly thawed and diluted to 2 mg/ml with 1x BRB80 buffer supplemented with 1 mM DTT and 1 mM GTP. Taxol was added stepwise in the following manner:

a. 1/10 volume of 20 μM taxol, incubate at 37°C for 5 – 10 min
b. 1/10 volume of 200 μM taxol, incubate at 37°C for 5 – 10 min
c. 1/10 volume of 2000 μM taxol, incubate at 37°C for 15 min

The solution was set on a 20% sucrose 1x BRB80 37°C warm cushion that was twice the volume of the solution added and spun at 40K at 37°C for 20 min, with 5 min acceleration and 5 min deceleration. The supernatant was aspirated and the resulting pellet was washed with 100 μl 1x BRB80. The pellet was resuspended in 80% of starting volume with warm BRB80 supplemented with 1mM DTT and 20 μM taxol by pipetting up and down carefully. Polymerized microtubules (MTs) prepared in this manner can be stable for couple of weeks at RT.

9. <u>Microtubule binding assay</u>

Reagents and solutions: see section 8 of this material and methods.

Cells were plated and differentiated. At the end of the differentiation protocol, the media was aspirated and the cells were scrapped on ice with 1x 80mM HEPES pH 7 substituted with the following inhibitors: 1 μM staurosporine, 1 μM K252a, 50 nM okadaic acid, 1 μM microcystin, 1:100 dilution of protease inhibitor cocktail mammalian, 1:100 dilution of phosphatase inhibitor cocktail. The cells were lysed by passage three times through a 27-gauge needle. The lysates were then spun at 4°C, at 14,000 rpm for 5 min. The pellet was discarded and the supernatant was transferred to a new tube,

which was then spun at 55,000 rpm for 10 min in TLA 100.3 rotor at 4°C. The resulting supernatant was allowed to warm up to RT and then 0.02 mM taxol with either 5 mM ATP or 5 mM AMP-PNP were added. The solutions were mixed gently and allowed to sit for 5 min at RT. Pre-assembled MT were added to each condition and incubated for 15 min at 37°C. The mixture was then loaded onto a 1x BRB80, 25% sucrose cushion supplemented with 20 μM taxol, protease inhibitor cocktails (both Calbiochem and Sigma), 1 μM microcystin and 2 mM of ATP or AMP-PNP. The samples were then centrifuged at 50,000 rpm for 30 min with 5 min acceleration and 5 min deceleration in a TLA 100.3 rotor at RT (~ 24°C). The supernatant was then carefully transferred to a new set of tubes and underwent methanol precipitation by mixing with methanol in a 1:1 ratio and setting the tubes overnight at -20°C. The precipitate was spun down at 14,000 rpm, 4°C for 15 min and the resulting pellet was resuspended in 2x SB. The MT pellets were washed with 80 mM Hepes buffer, pH 7 and then resuspended in 100 μl 2x SB. The resulting samples were then evaluated using western blotting.

10. <u>Cellular Fractionation</u>

Reagents and solutions: homogenization buffer (HB): 0.25 M Sucrose, 1 mM EGTA, 10 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1 mM DTT; *Protease inhibitors for HB*: 1:100 dilution of protease inhibitor cocktail (Sigma P8340), 1:200 dilution of phosphatase inhibitor cocktail set II, 1:100 dilution of phosphatase inhibitor cocktail 3 (Sigma P0044), 1 μM staurosporine, 1 μM K252a, 1 μM PKI (Upstate 12-151); dithiothreitol (DTT) – Amresco M-109.

Untransfected cells and cells from cell lines expressing WT and A53T α-synuclein were plated in 4 x 10 cm dishes and differentiated according to the protocol

described above. Once the cells were ready, everything was performed at 0 - 4 °C. The cells were collected in HB buffer and passed through a 23-gauge needle twice and then through a 27-gauge needle five times. Total protein concentration was assessed using the BCA assay (Pierce) and the concentration was adjusted to be the same for all samples. Equal volumes of each sample were taken and spun at 600 g for 5 min at 4°C. The resulting pellet, P1 was resuspended in 5 μL DNase (Sigma D5025), 5 μL RNase (Sigma R5125), 40 μL HB, and 50 μL 2x SB. The supernatant was transferred to fresh tubes and spun at 300,000 g for 2 h using a TLA 100.2 or TLA 100.3 rotor and its tubes. The speeds used for each of the rotors were: 83,000 for TLA 100.2 and 84,000 for TLA 100.3 with 5 min acceleration and 5 min deceleration. The resulting pellet, P2, was resuspended in 100 μl of 2x SB. The supernatant was added to an equal volume or more of methanol and set at -20°C overnight. The next day it was spun at 14,000 rpm, 4°C for 10-15 min and the resulting pellets resuspended in 100 μl of 2x SB. All of the resulting samples were then used for western blot analysis.

11. <u>Immunoprecipitation assays</u>

Reagents and solutions: *1x cell lysis buffer for immunoprecipitation (IP)*: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100; *inhibitors for cell lysis IP buffer*: 1:100 dillution of protease inhibitor cocktail (Sigma P8340), 1:200 dilution of phosphatase inhibitor cocktail set II, 1:100 dilution phosphatase inhibitor cocktail III; protein G beads (Pierce 30399); ChromPure Rabbit IgG, whole molecule agarose beads (Jackson ImmunoResearch 011-000-052); blocking peptide for PKCμ antibody (Santa Cruz sc-639 P); blocking peptide (Santa Cruz, sc-639 PL, special order without bovine serum albumin).

Cells expressing A53T α-synuclein were plated and differentiated in 10 cm dishes. Media were removed and the cells were scraped with IP cell lysis buffer. For each experiment, 3 - 4 dishes were pooled together to increase protein concentration. The cell lysate was agitated in a rotator for 20 min at 4°C. The sample was then sonicated on ice, 3 x 5 sec bursts and then centrifuged for 10 min at 14,000 g, 4°C. The supernatant was transferred to a new tube and precleared by incubating with protein G beads equilibrated in IP buffer for 30 - 60 min in a rotator at 4°C. The sample was then spun for 5 min at 4°C at 10,000 x g to pellet the beads. The resulting supernatant was transferred to a fresh tube to which 10 - 15 μL of sc-639 PKCμ primary antibody (0.2 μg/μL) was added and incubated with gentle rocking overnight at 4°C. The next day, 50 - 100 μL of equilibrated protein G beads were added and incubation continued for 3 h longer. The samples were then centrifuged for 30 sec at 10,000 rpm, at 4°C. The supernatant underwent methanol precipitation as described in cellular fractionation. The bead pellet was washed 3x with 300 μL of 1x cell lysis buffer. The protein was eluted adding a blocking peptide 5 - 10 times in excess of the antibody and incubating overnight at 4°C with rotation. A second elution was done using 100 μL of 2x SB. All of the resulting samples were analyzed using western blotting.

12. <u>Mass spectrometry</u>

For identification of proteins, gels containing samples of interest were submitted for mass spectrometry analysis to the UIC facilities – CBC/RRC Proteomic and Informatics Services.

13. Statistical analysis

Statistical analysis of data was examined by two-tailed student's T test, two-way ANOVA, followed by Dunn's multiple comparisons test using GraphPad InStat software (GraphPad Sofware Inc.). Values were considered statistically significant at * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

D. Results

1. Screening and selection of neuroblastoma cell lines

In selecting a cell line to work with, one needs to consider practical issues such as the availability of a cell line, maintenance and time requirements but also, and more importantly, scientifically relevant issues such as how well established the cell line is and whether it would be suitable to the needs and the questions to be addressed. In this particular case, does the cell line considered express the proteins required for transport and those found relevant in our squid model of pathogenesis? Taking into account the heterogeneity of PD, it could be tricky to select a cell line that would be an ideal candidate to model the disease. The effects of α-synuclein on the brain are global, the protein is widely expressed and each cell, depending on its specific functions and cellular content, will deal with it in diverse ways. Different neurons will be affected differently or perhaps not at all and although not the first ones to degenerate, dopaminergic neurons of the SNpc are clearly the most symptomatic. Therefore the goal was to work with a neuronal cell line that expressed proteins of interest and that had dopaminergic properties to assure that dopamine-specific pathways important in pathogenesis would be taken into account.

Working with the MPP+ model of PD, our laboratory has used the immortalized rat mesencephalic cell line 1RB3AN27 (N27). N27 cells have been used extensively as a model for PD because they express activity of tyrosine hydroxylase (TH) and the dopamine transporter (DAT) and both increase upon differentiation with dibutyryl cAMP alone or dibutyryl cAMP 1 dehydroepiandrosterone [192]. These cells have a doubling rate of approximately 26 h and were immortalized by transfecting rat fetal mesencephalon cells with the plasmid vector pSV3 neo, which carries the large T antigen gene of the SV40 virus and proved successful in inducing immortalization [192, 193]. As a candidate for α-synuclein transfections however, this cell line proved to be unsuitable for our experiments since a member of our research group discovered that these cells do not have dynein intermediate chain 1 (DIC1) present, although they do have DIC2 (S. Aspengren, personal communication). In previous data, mentioned in Chapter I, where cultured neurons were treated with BDNF, DIC was phosphorylated and this phosphorylation was inhibited when the PKC inhibitor Gö6976 was used. These observations were specific to DIC1. To address this issue, a series of alternate cell lines were researched and screened. A list of the cell lines examined is listed in table II.

TABLE II

LIST OF CELL LINES CONSIDERED

Cell Line	Type	Origin	Dopaminergic peoperties	Source
1RB3AN27	immortalized	rat, mesencephalic	DA, DA transporter, tyrosine hydroxylase	Morfini lab
CHP134	neuroblastoma	human, adrenal area, neuronal	tyrosine hydroxylase activity	Ikegaki lab
IMR5	neuroblastoma	human	N/A	Ikegaki lab
NLF	neuroblastoma	human	N/A	Ikegaki lab
Nb69	neuroblastoma	human, brain	DA and its metabolites (high levels of norepinephrine), tyrosine hydroxylase	Ikegaki lab
SK-N-MC	neuroblastoma	human, brain, neruoepithelioma from supraorbital area	DA and its metabolites, tyrosine hydoxylase, DA D1 receptor	ATCC
SK-N-SH	neuroblastoma	human	dopamine-beta-hydroxylase activity	ATCC
SH-SY5Y	neuroblastoma	human, clonal derivative of SK-N-SH	dopamine-beta-hydroxylase activity	ATCC

First considered were the neuroblastoma cell lines CHP-134, IMR5, NLF, and Nb69. These were screened for the expression of proteins relevant to this project such as α-synuclein, dynein, kinesin, Fyn, Src and PKCμ. Nb69 emerged as a cell line with appropriate properties. It is a human neuroblastoma cell line that is catecholamine-rich with pharmacological properties similar to dopamine neurons as it expresses high levels of dopamine and its metabolites, in particular, norepinephrine [194]. These cells were transiently transfected for 24 h with WT, A30P and A53T forms of α-synuclein. Preliminary results showed that there is an increase in the activation of PKCμ (Figure 11), however subsequent experiments did not produce the same observations. In addition, evidence from the literature indicates that differentiation of these cells into neurons with reagents such as RA, reduced the levels of tyrosine hydroxylase activity [195] and that RA may not even induce biochemical differentiation of the Nb69 neuroblastoma [196]. Considering the variability of these cells and that ideally we would want to work with a well differentiated neuron-like cell line, NB69 cells were not considered further as a potential candidate to test our model of α-synuclein pathogenesis.

The next cell lines considered were SK-N-MC, SK-N-SH and SH-SY5Y. Studies of human neuroblastoma cell lines led to the identification of three distinct cellular phenotypes: *N* for neuroblastic, *S* for non-neuronal, substrate-adherent, epithelial-like and *I* for intermediate to N and S, which can become either I or S type [197]. Although SK-N-MC cells have functional D1 dopamine receptors [198], they are of the S type and tend to be fibroblast like so the focus was shifted to the SK-N-SH and SH-SY5Y cell lines. SK-N-SH cells have a population doubling time of 44 h and are comprised of both

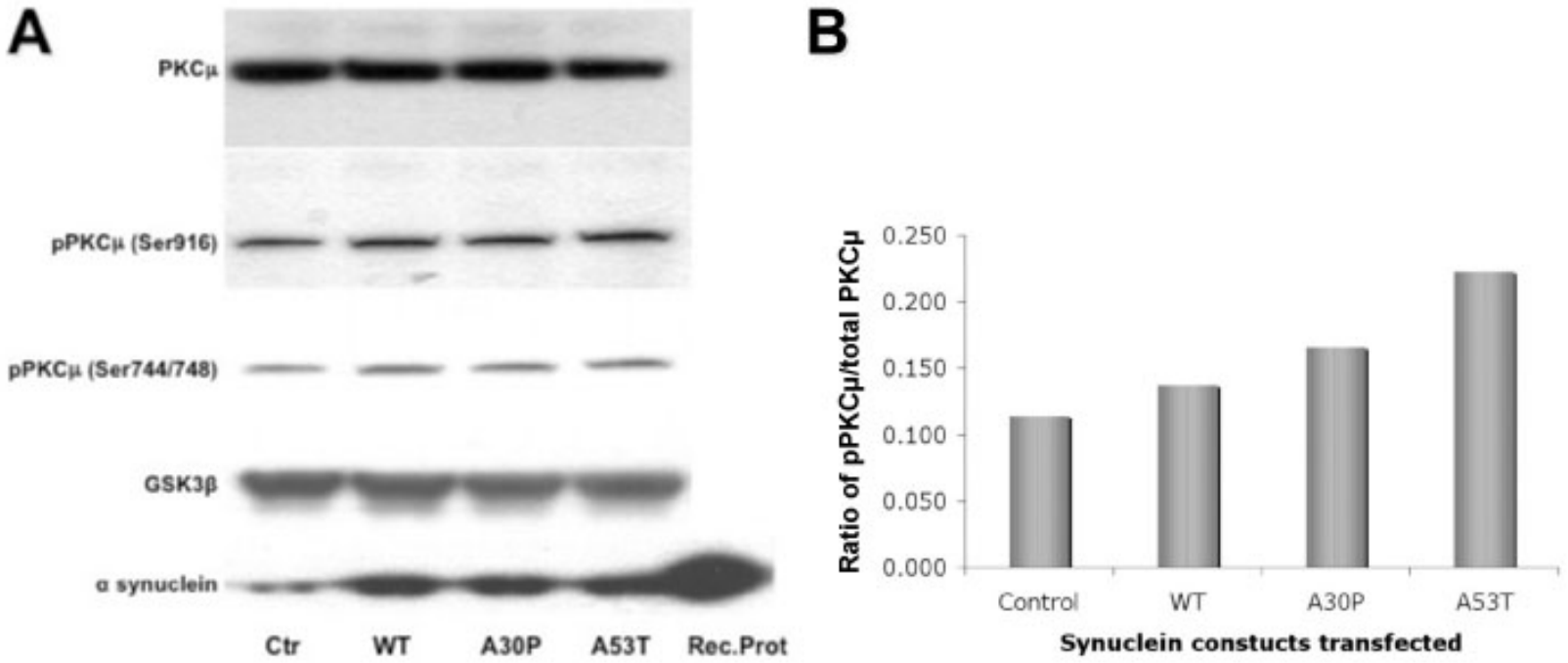


Figure 11: Effects of α-synuclein in NB69 cells

Undifferentiated Nb69 cells were transfected with WT, A30P and A53T α-synuclein for 24 h. Expression of GSK3β, α-synuclein and PKCμ were analyzed by western blot; untr – untransfected cells, rec prot – purified recombinant wt α-synuclein (A). Preliminary results revealed that the ratio of phosphorylated PKCμ (Ser916) to total PKCμ increased in NB69 cells transfected with mutated α-synuclein and to a lesser extent with WT α-synuclein (B). However, subsequent repetitions of the experiments were not able to reproduce these results.

N and S type cells [199]. The presence of epithelial, S type cells was a concern for differentiation into neurons, however Jain *et al* developed a protocol, which used mitotic inhibitors in addition to RA [200]. This method yields a virtually epithelial free, differentiated neuronal population that can survive in culture for long periods of time. The SH-SY5Y cell line was generated by thrice subcloning the N-type cells of the SK-N-SH cell line although it retains a low population of the S-type cells [201]. Both lines have dopamine-beta-hydroxylase activity and have been used as models for dopaminergic neurons therefore both were selected to work with.

2. **Development and characterization of stable cell lines expressing wild type, A30P and A53T forms of α-synuclein**

Cell lines stably transfected with α-synuclein were developed in both SK-N-SH and SH-SY5Y cell lines using the vector pcDNA3.1+. This vector was developed with the antibiotic neomycin resistance gene in it. The gene confers resistance to cells expressing it so when they are treated with media containing the antibiotic neomycin (G418), the cells survive, while cells that do not express it die. Stable transfections are preferred to transient ones since in the latter case temporal variation in α-synuclein expression might lead to variations in production of down–stream proteins that would be difficult to track. In addition, transient transfection experiments are typically performed on average within 24h, which may not be long enough to observe changes associated with late onset PD. Expression levels of α-synuclein in stably transfected clones of the SK-N-SH neuroblastoma cell line are illustrated in Figure 12. Several clones were developed for each WT, A30P and A53T α-synuclein construct. The levels of protein expression varied wildly among different clones. For example, in

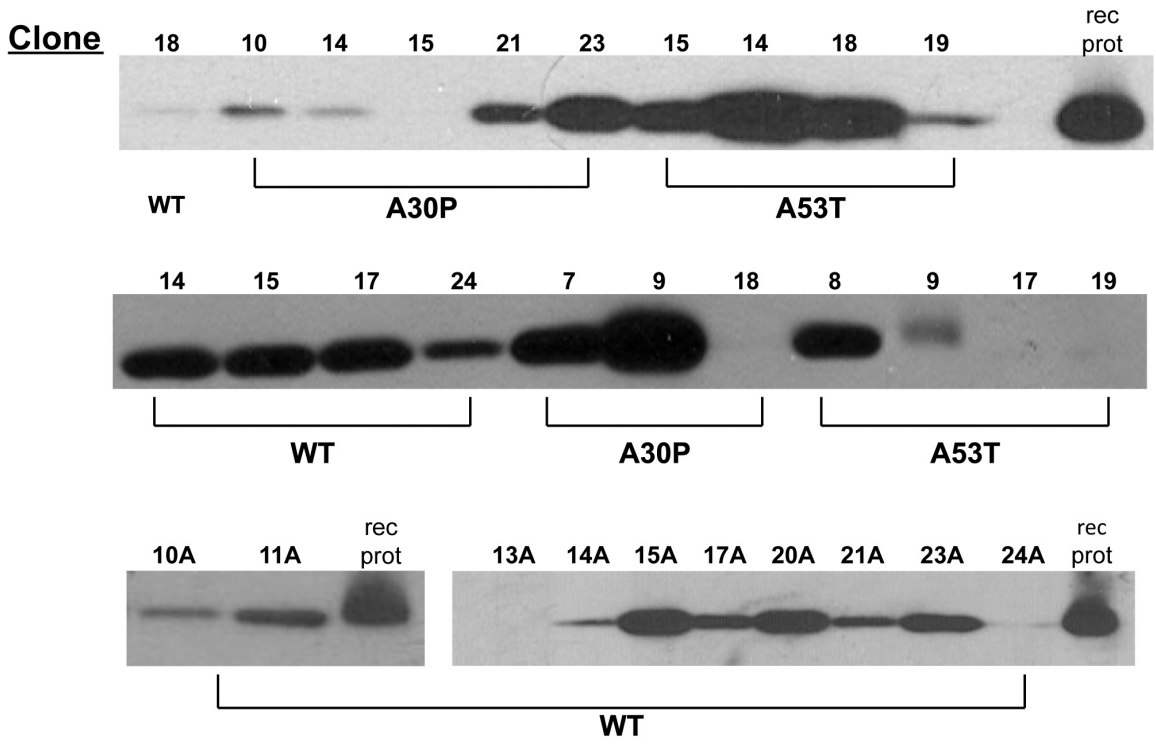


Figure 12: Expression of α-synuclein in clones of SK-N-SH

For each α-synuclein construct several clones were developed. Western blotting shows that expression of α-synuclein varies wildly among clones, from undetectable levels such as in clone 15, expressing A30P, top row, to very high levels like in clone 9 expressing A30P (middle row) or 14 expressing A53T (top row panel); concentrations were the same for each sample and 10 μg of total protein per lane was loaded; rec prot – purified recombinant WT α-synuclein.

clone 15 expressing A30P α-synuclein (Figure 12, top row) there was no detectable expression of the protein, whereas in clone 9 the levels were very high (Figure 12 middle row).

In addition to western blotting, a few clones were selected and the expression of α-synuclein was examined using immunocytochemistry. This proved to be a powerful and necessary technique because imaging demonstrated that, although α-synuclein is expressed within a clonal line, not all cells express the protein (Figure 13). The results suggest that for SK-N-SH cells the success of α-synuclein transfection is cell type specific. Epithelial, S-type cells, were more permissive with higher rates of α-synuclein transfection and therefore these type cells had higher levels of protein expression. These observations were further confirmed when, upon differentiation of selected clones, all cells died during the differentiation process. These results indicated that in some clones only the epithelial cells were expressing α-synuclein because N-type cells without the α-synuclein vector die in the presence of G418 and when the mitotic inhibitors were added during differentiation, the epithelial cells, the S-type, died as well. Given the potential variability of expression within a clone and a lengthy differentiation protocol (3 to 4 weeks) for the SK-N-SH cells, the rest of the efforts were focused on the SH-SY5Y cell line.

The neuroblastoma cell line SH-SY5Y has been well characterized and extensively used in a variety of applications by our laboratory as well as other researchers [201-206]. As opposed to SK-N-SH, an advantage to the SH-SY5Y cells is that the differentiation protocol is brief, lasting on average 7-8 days. Encinas *et al* [202] developed this protocol where, using sequential treatment of RA and then BDNF, nearly

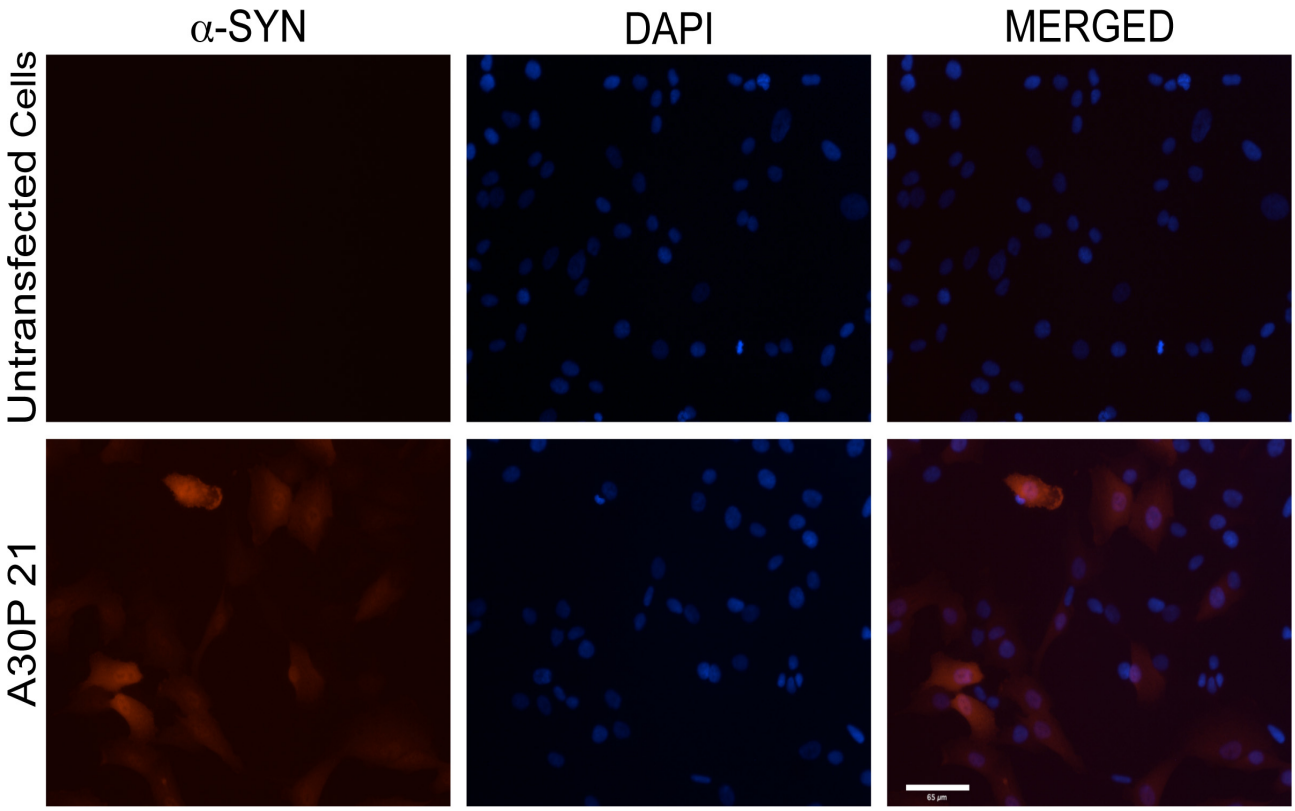


Figure 13: Expression of α-synuclein in stably transfected SK-N-SH cells

Comparing untransfected cells (top row) to transfected cells (bottom row), α-synuclein is expressed in SK-N-SH cells as illustrated in clone 21, featuring the A30P mutant form of α-synuclein. However not all cells within the clone express the protein as evident from the merged image. Size bar: 65 µm. Red – α-synuclein. Blue – DAPI, nucleus.

pure populations of human neuron-like cells are obtained. As with SK-N-SH, several transfected clonal lines were successfully established (Figure 14A). An interesting observation was that for WT α-synuclein a greater number of clones needed to be selected in order to obtain cell lines that exhibited robust expression of the protein. In addition, regardless of construct, many SH-SY5Y clones developed exhibited a higher number of the S-type and/or I-type cells. Given that S-type cells are substrate adherent, they attach more strongly to the dish than the N-type cells. Passage of the cells with a solution of PBS/EDTA is gentler in detaching cells and it was employed to eliminate S-type cells. Therefore, morphologically, the clones were selected to look as similar as possible to untransfected SH-SY5Y cells (Figure 14B). In combination with the differentiation protocol developed by Encinas [202], this ensured that I worked with a homogeneous, neuronal-like population of cells.

Considering that in PD there are duplications and triplications of the synuclein gene that lead to the disease, it would be reasonable to expect that overexpression of WT α-synuclein in SH-SY5Y cells would have some effect on the cells. Therefore untransfected cells were included in the experiments and used as controls. Some notable differences between transfected and untransfected cells were observed. By and large, cells expressing any of the α-synuclein constructs were less robust upon differentiation than their untransfected counterparts. In general, in order to differentiate SH-SY5Y cells, it is sufficient to start with 50-60% cell confluence, however for transfected clones, 80% or more was preferred otherwise cells were generally more fragile looking, detached easily, requiring very gentle handling, and on occasion the differentiation protocol would have to be aborted. This indicates that increased α-

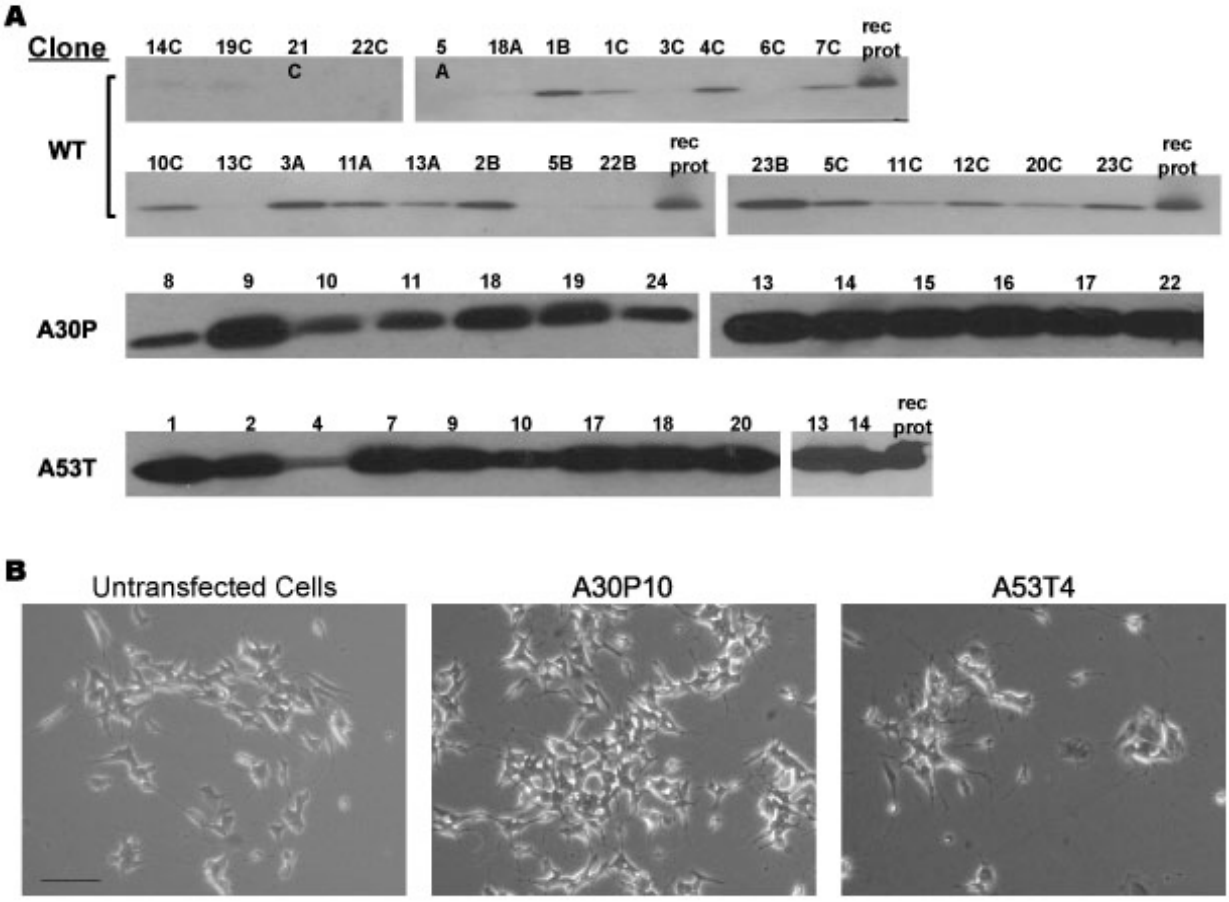


Figure 14: Expression of α-synuclein in clones of SH-SY5Y

Western blotting shows robust α-synuclein expression for A30P and A53T SH-SY5Y clones, however a larger number of WT clones had to be selected and screened for (A). Both expression (A) and cell morphology (B) was taken into account as a selection criteria. Each clone was selected so morphology resembled untransfected cells as closely as possible. Concentrations were the same for each sample and 10 μg of total protein per lane was loaded. Images were taken at 10x magnification, scale bar 100μm.

synuclein expression makes the cells appear sickly and that less confluent and single cells deteriorate faster while at a greater confluence, close proximity to each other allows them to form networks and better survive. Such observations may explain why in some patients there seems to be a critical threshold of disease, they are symptomatic but after a certain amount of time the symptoms are more acute and the disease progresses faster. It is conceivable that as more and more cells die, when there are just a few left these die at a faster rate, as the network around them is gone.

Imaging of differentiated SH-SY5Y clones confirmed that, unlike SK-N-SH, all cells in the selected clones expressed α-synuclein. The protein was distributed throughout the cell and immunofluorescence was brighter at the nucleus, but this might reflect a longer path-length through the nucleus than through surrounding cytoplasm (Figure 15A). No significant aggregates where observed in the time frame examined, even at higher magnification, consistent with other reports [207] (Figure 15B).

It is not an uncommon phenomenon in stable transfections, especially when working with toxic proteins, for the levels of protein expression to go down with increasing passage numbers [203]. My work with the SH-SY5Y cells led to similar observations. For example, clone A30P 10 lost expression of α-synuclein after six passages from initial transfection. Additional cell lines tested, such as clone A30P 16 and WT 12C, also lost their α-synuclein expression. Given these observations, only SH-SY5Y cells expressing WT and A53T α-synuclein were examined further and Western bots showed that for these clones the protein expression levels were significantly higher than in untransfected SH-SY5Y cells (Figure 15 C and D).

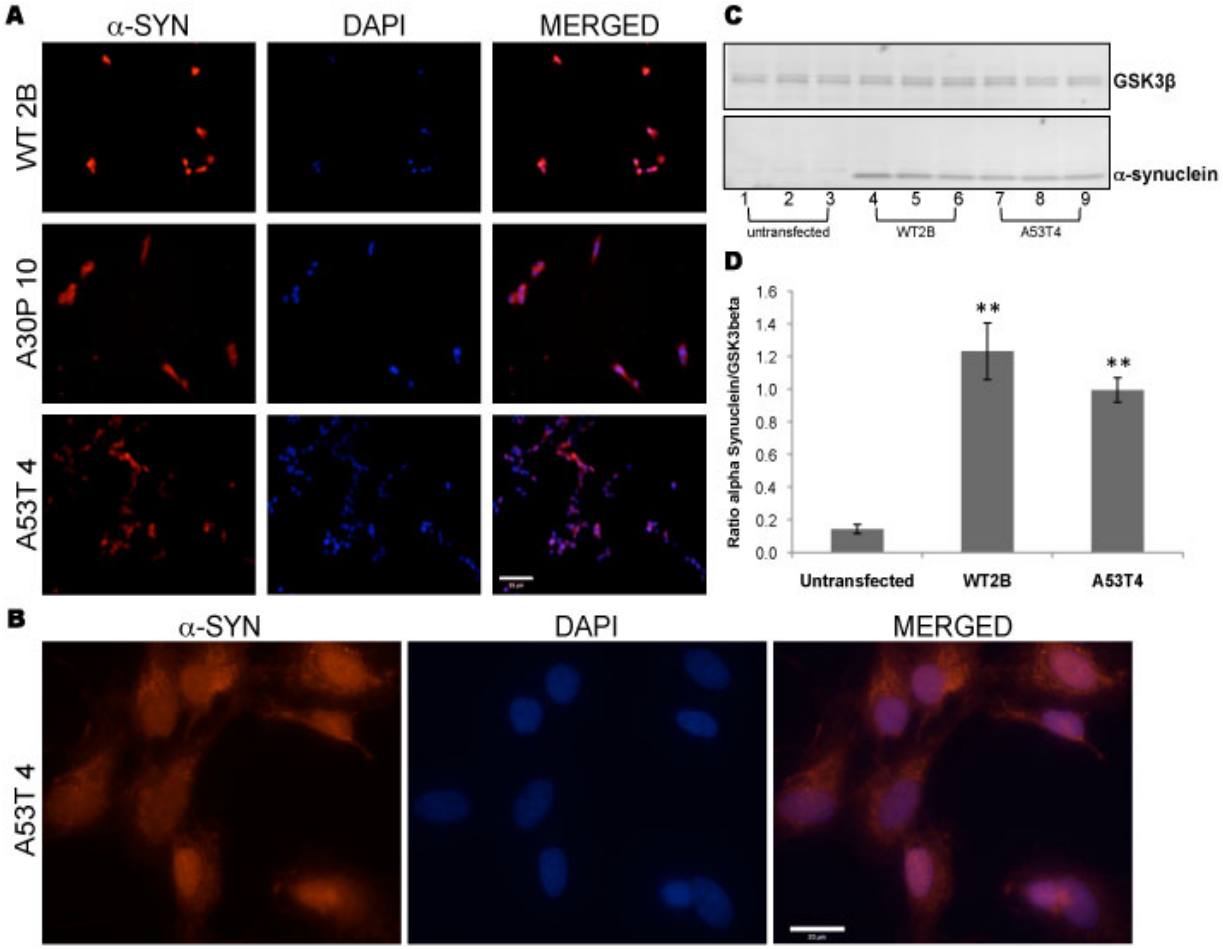


Figure 15: Expression of α-synuclein in stably transfected SH-SY5Y cells

Several clones were selected and imaged for α-synuclein expression. Illustrated are representative clones for each construct WT, A30P, A53T - 20x magnification, size bar 65 μm (A). Higher magnification shows that the protein localizes in the nucleus although it is distributed throughout the cell as well - 63x magnification, size bar 20 μm (B – only A53Tclone shown). Red – α-synuclein. Blue – DAPI, nucleus. Western blots show α-synuclein expression levels (C), significantly higher in transfected clones compared to untransfected cells; n = 3 two-tailed T test: Untr-WT2B P 0.0034, Untr-A53T P 0.004; One way ANOVA P 0.0010 (D). Quantification is shown only for clones selected to work with further (see text for details).

3. Characterization of molecular motors: expression of kinesin and dynein

Representative clones expressing WT and A53T α-synuclein were selected to analyze the molecular motors in SH-SY5Y cells. Western blotting using antibodies for kinesin heavy and kinesin light chain as well as dynein intermediate and dynein heavy chain assessed the expression of the motors. Although the expression of α-synuclein is not the same between WT and A53T clones, in both cases the levels of expression are significantly higher when comparing each clone to untransfected cells (see Figure 14D) and therefore the focus was on whether there were differences between transfected and untransfected cells. Comparisons between untransfected cells and cells expressing WT and A53T α-synuclein are presented (Figure 16). Some variation from sample to sample was observed. For example, in clones expressing WT α-synuclein there appears to be a decrease in the expression of the kinesin heavy chain when compared to untransfected cells (Figure 16A2). However, repeating experiments with increasing numbers of samples tested showed that overall there were no statistically significant differences at the 0.05 level in either kinesin or dynein protein expression. These observations may not be surprising; by the time changes in motor expression are necessary the neurons could be too compromised for this to occur. In addition, squid axoplasm experiments support the idea that α-synuclein effects on axonal transport are primarily of motor regulation rather than of changes in their expression and therefore this was assessed next.

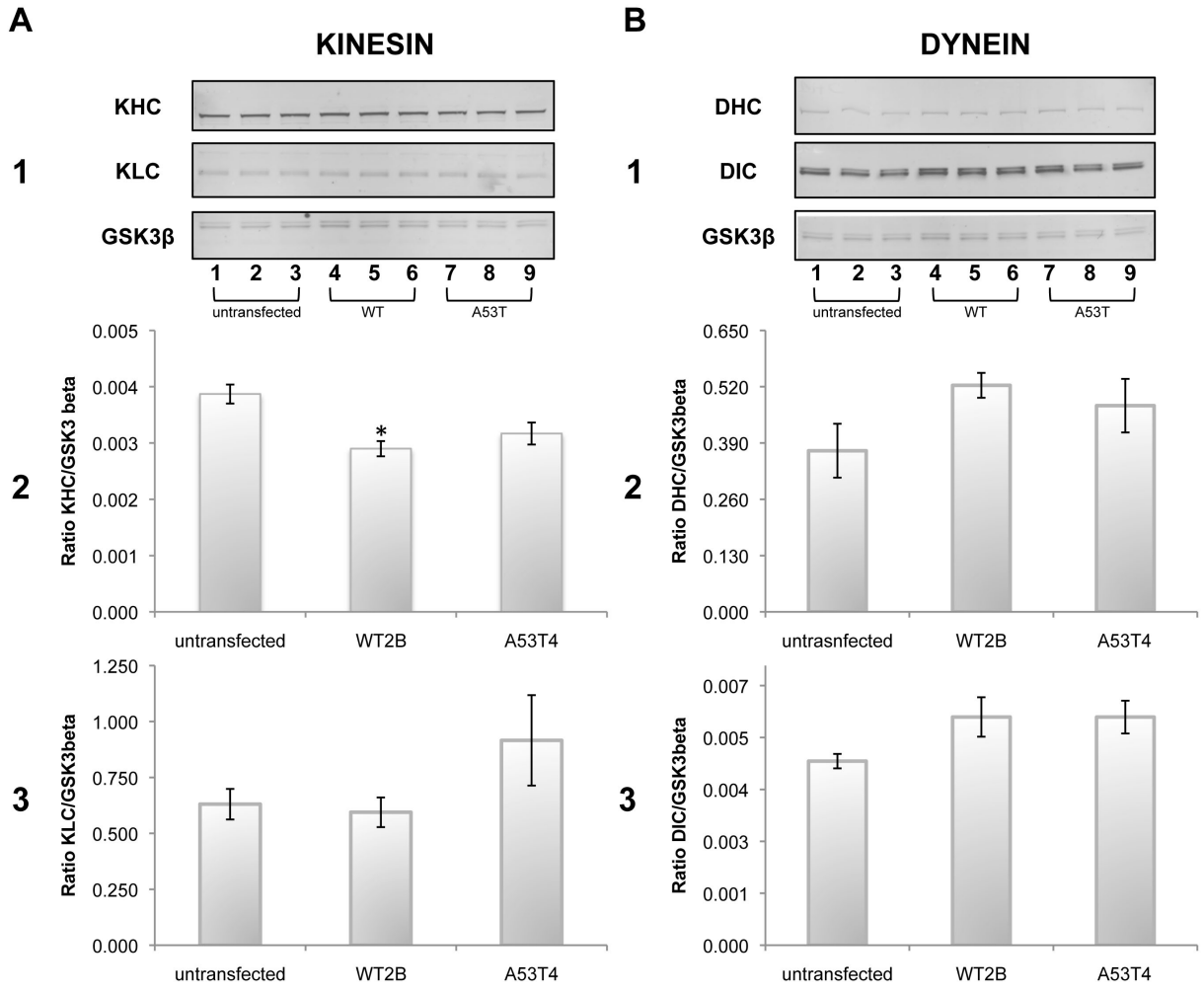


Figure 16: Expression of molecular motors in stable SH-SY5Y clones

Western blotting assessed the expression of kinesin (A1) and dynein (B1). Cells expressing either WT or A53T α-synuclein do not show significant changes in levels of either motor. Although kinesin heavy chain expression in clones of WT α-synuclein appears significantly lower compared to untransfected cells (A2), subsequent repeats of the experiment do not reflect this change. Levels of motors normalized to GSK3β. KHC (H2) - kinesin heavy chain. KLC (63-90) - kinesin light chain. DIC - dynein intermediate chain. DHC – dynein heavy chain. Error bars in graphs indicate standard error of the mean. Statistical analysis: One way ANOVA, P value 0.0159 for KHC untransfected vs. WT.

4. Characterization of molecular motors: microtubule binding assays

Misregulation of FAT can occur in a variety of ways. One is direct disruptions of the motors themselves. This can take place by affecting the motor's ability to bind to its cargo or to carry it by walking along microtubules. Microtubule binding (MTB) assays are experiments that evaluate interaction between the motors, in particular kinesin, with the microtubules. This process is regulated by phosphorylation of kinesin [117, 208]. Kinesin binding to the microtubules is ATP dependent, where hydrolysis of ATP leads to release of kinesin from the microtubules. In the presence of AMP-PNP, a non-hydrolyzable analogue of ATP, kinesin remains bound to the microtubule [209, 210]. However if kinesin is phosphorylated on microtubule binding domains, the motor cannot bind to the microtubule. Briefly, in these experiments, cells are lysed, spun, and the supernatants, which contain soluble kinesin protein, are collected and incubated with previously assembled pig derived microtubules in the presence of either ATP or AMP-PMP (Figure 17). The samples are then spun on a sucrose cushion and the pellets are collected. The supernatants are precipitated in methanol and then all samples are run on a western blot. The presence of the motor in either fraction was quantified. Under normal conditions, in the presence of ATP, kinesin is expected to be mostly in the supernatant and in the AMP-PNP condition kinesin is expected to be mostly or in larger amounts in the pellet, associated with or bound to the microtubules. If motor phosphorylation is affected in any way, then changes in the interaction of the motor with the microtubules are expected, specifically there should be less kinesin bound to the microtubules in the AMP-PNP condition. For SH-SY5Y cells expressing either WT (Figure 18A) or A53T (Figure 18B) α-synuclein, no significant changes in the binding of

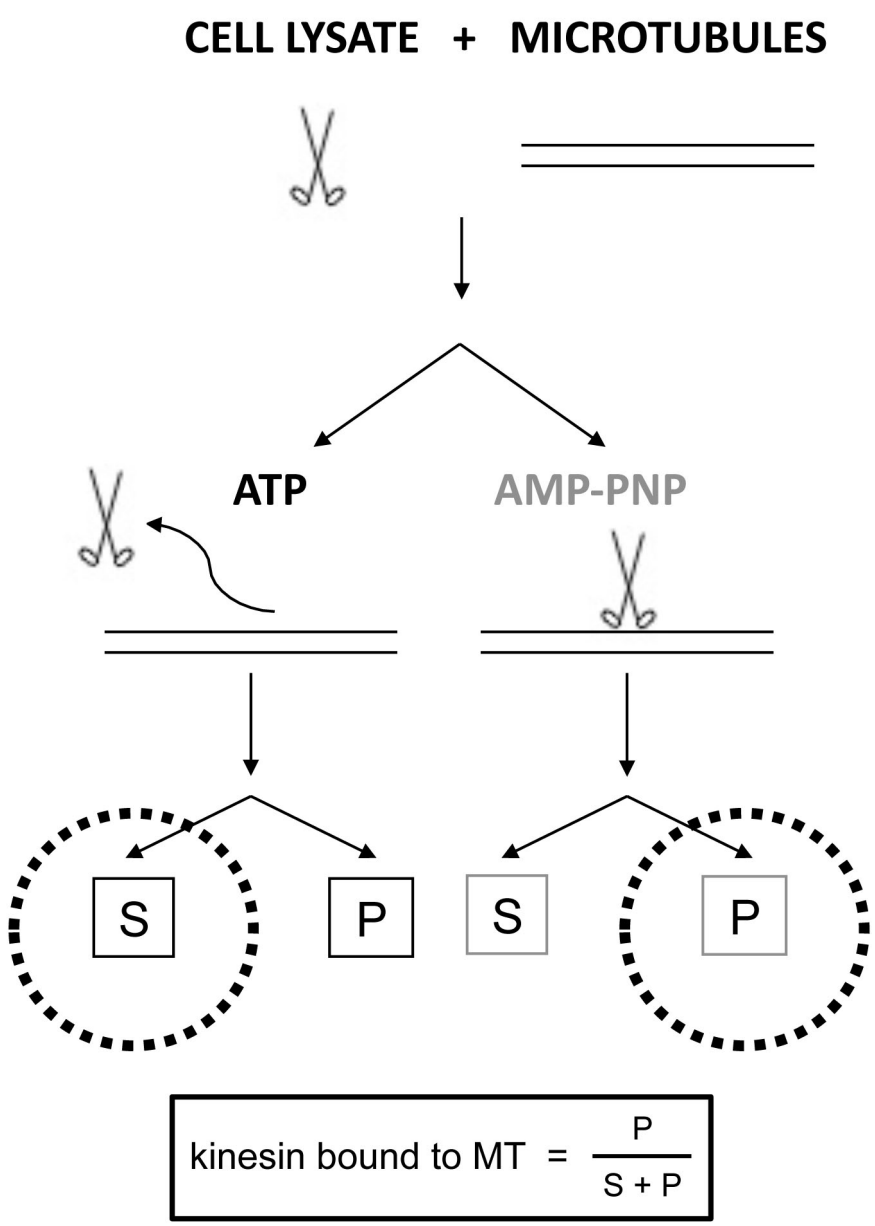


Figure 17: Overview of microtubule binding assay

Cell lysates from SH-SY5Y cells containing kinesin and purified, *in vitro* assembled microtubules were incubated with either ATP or AMP-PNP. In the presence of ATP, kinesin is expected in the supernatant (S) and in the presence of AMP-PNP, kinesin is expected to associate more with the microtubules and therefore be present in larger amounts in the pellet (P). The S and P fractions for each condition were analyzed using western blots and the amount of kinesin bound to the microtubule was calculated using the above formula.

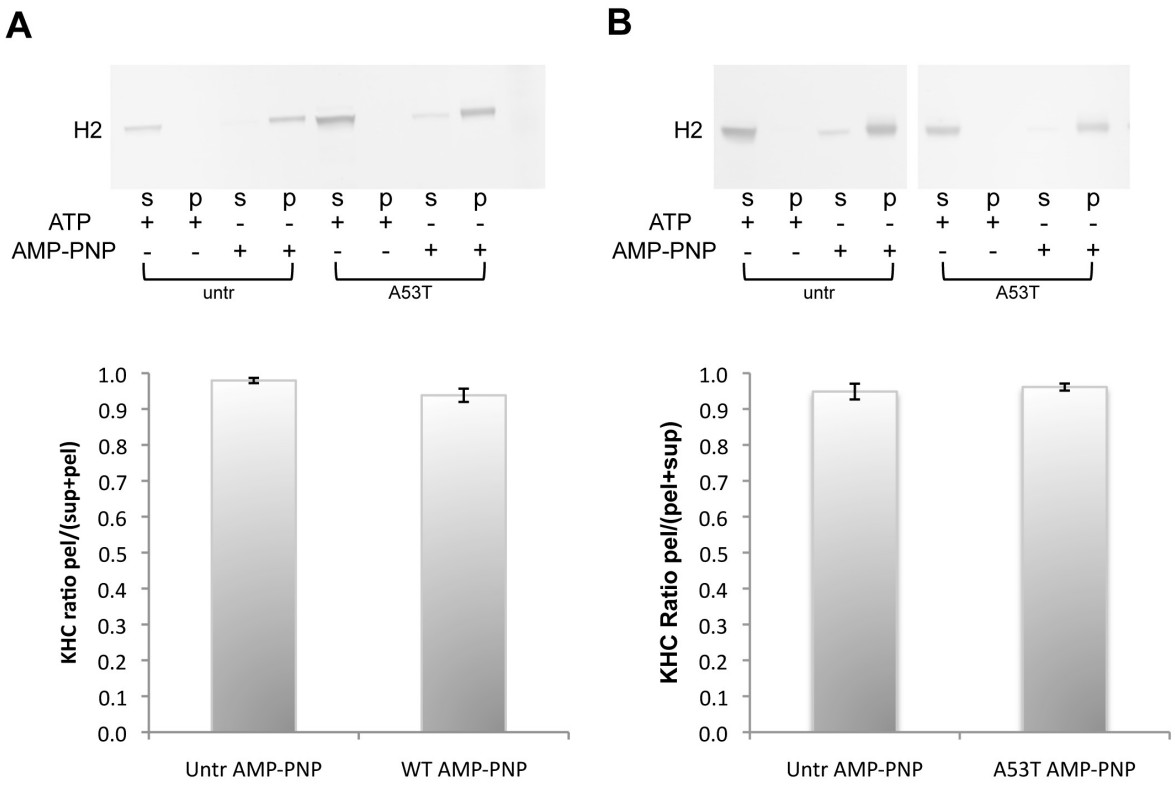


Figure 18: α-Synuclein expression does not affect kinesin binding to microtubules

No significant changes are observed in kinesin (H2) binding to the microtubules in cells transfected with either wild type (A) or A53T (B) α-synuclein. Error bars in graphs indicate standard error of the mean. Experiment repeated 3 times; n=4 per experiment, Statistical analysis: two-tailed T test.

kinesin to the microtubules were observed. These results are consistent with observations of transport in squid axoplasm, where perfusion of α-synuclein has the effect of a slight decrease on kinesin based, anterograde transport but a dramatic increase in dynein based, retrograde transport. MTB assay is an experiment that most accurately evaluates kinesin binding because kinesin has only one site to hydrolyze ATP. Dynein binding to microtubules is more complex as they have evolved from AAA ATPases and have six distinct AAA domains, four of which contain functional nucleotide binding sites. The first one, AAA1 is essential for motility while the role of ATP binding and hydrolysis for AAA2-4 is less understood and AAA5and 6 lost their conserved binding sites [138]. Thus, dynein has both nucleotide sensitive and nucleotide insensitive binding sites. In addition to walking on microtubules, dynein is also known to carry microtubules as cargo. This would make results of microtubule binding assays with dynein difficult to interpret. However, considering that retrograde transport is dramatically affected, the AMP-PNP fractions used for AMP-PNP stabilized binding of kinesin were probed with the DIC antibody (Figure 19). Antibodies for dynein intermediate chain were used to determine whether microtubule binding to this motor is affected. Although the heavy chain is the part of the protein that interacts with the microtubules, changes in microtubule binding should also be detected using antibodies against the intermediate chain since dynein heavy chains and intermediate chains interact with each other to form a large protein complex *in vivo* [139]. Additionally, the antibodies available for the intermediate chain are much more effective for fluorescence Western blotting and therefore it would allow for better quantification than the dynein heavy chain antibody.

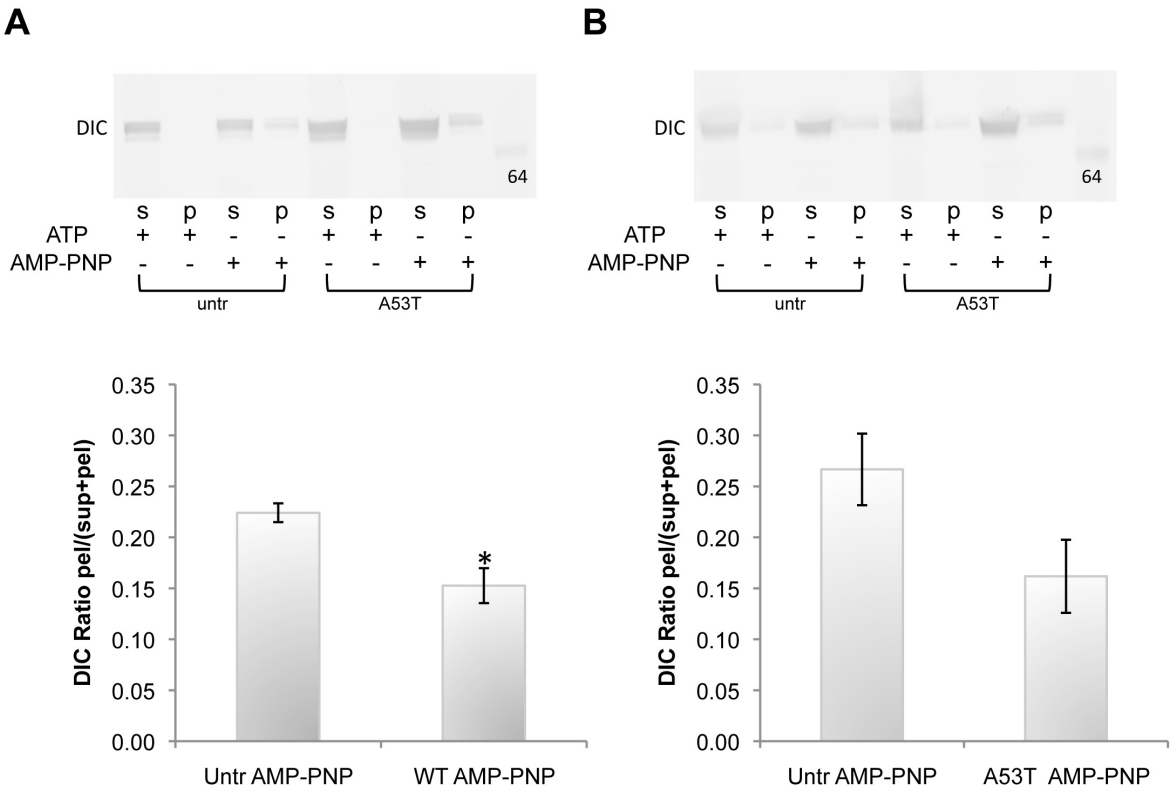


Figure 19: Microtubule binding assay, evaluation of dynein

A significant change in dynein binding to the microtubules is observed in cells transfected with WT (A) α-synuclein for the AMP-PNP condition but not in cells expressing A53T (B) α-synuclein, although it follows a similar trend. 64 - MW marker. The antibody binds to DIC – 74 kDa. Error bars in graphs indicate standard error of the mean. n = 4; Statistical analysis: two-tailed T test P value 0.0103.

The results indicate that there is a decrease in dynein binding to the microtubules in the AMP-PNP condition in samples expressing WT or A53Tα-synuclein. In addition, the AMP-PNP pellet fraction of transfected clones shows a slight shift in dynein molecular weight, which could be indicative of increased phosphorylation. In an *in vitro* study of nucleotide dependent behavior of single molecules of cytoplasmic dynein on microtubules, Miura *et al* has demonstrated that in the presence of AMP-PNP, a relatively large amount of dynein molecules was associated with microtubules, and they remained on the microtubules for tens of seconds, whereas in the presence of ATP the association was not as strong [211]. This might not be as surprising since in order to move along the microtubules the interactions with the motors has to be relatively loose rather than bind tightly to it. Since dynein associates less with the microtubules in the presence of AMP-PNP in clones transfected with α-synuclein, it is feasible that an increase in its phosphorylation levels correlates with increased retrograde transport as observed in squid axoplasm experiments. However additional experiments would be needed to determine with certainty the phosphorylation levels of dynein in these clones and whether this affects the nucleotide sensitivity of dynein binding to microtubules.

5. Characterization of molecular motors: membrane association assay

A variety of methods have been developed in order to partition different components of a cell such as organelles and other subcellular compartments [212]. Vesicle binding assays evaluate axonal transport by assessing the binding of the motors to their cargo. There are several methods through which this can be approached such as subcellular fractionation, which employs various centrifugation speeds or the use of different percentages of sucrose cushions to separate vesicles through flotation or

sedimentation. In order to see whether there are any changes in SH-SY5Y cell lines expressing WT or the A53T mutated form of α-synuclein, I devised a simple experiment to check the overall distribution of the motors in three main subcellular fractions. Briefly, cells were homogenized using a 1 mL syringe and two different gauge (23 and 27) needles (Figure 20). This method lyses cells while it allows for a degree of integrity of cellular components, in particular membranes, and would give a general indication of how the motors partition i.e., do they associate more with the nucleus, do they attach more to membranes and therefore cargo or are they unbound and therefore more soluble and present in the cytoplasm? The homogenate was separated into three different fractions using different centrifugation speeds: nuclear (P1), membrane (P2) and cytosolic (sup). These fractions represent two classes of membrane-bounded organelles and one soluble fraction and once obtained, fluorescence (Figure 21A) and chemiluminescence (Figure 21B) western blots were used to evaluate the partitioning of various markers, including kinesin (KHC – H2), dynein (DIC), tubulin (DM1A), SNAP25 and, histone (H3). Within one experiment there were four replicates of each cell line: untransfected, WT and A53T α-synuclein and the experiments were repeated 4-6 times. Analysis of transfected and untransfected SH-SY5Y cells using chemiluminescence indicated that there was a decrease in the motors in the P2 (membrane fraction) in cells expressing A53T α-synuclein and an increase in both kinesin and dynein released into the cytoplasm (Figure 21B). In addition, there was an increase in motors in the low speed pellet (nuclear fraction), which may reflect a shift to larger organelles. Florescent western blots, which are more quantitative than chemiluminescence blots, indicated that for each set of experiments there were representative samples that showed changes for

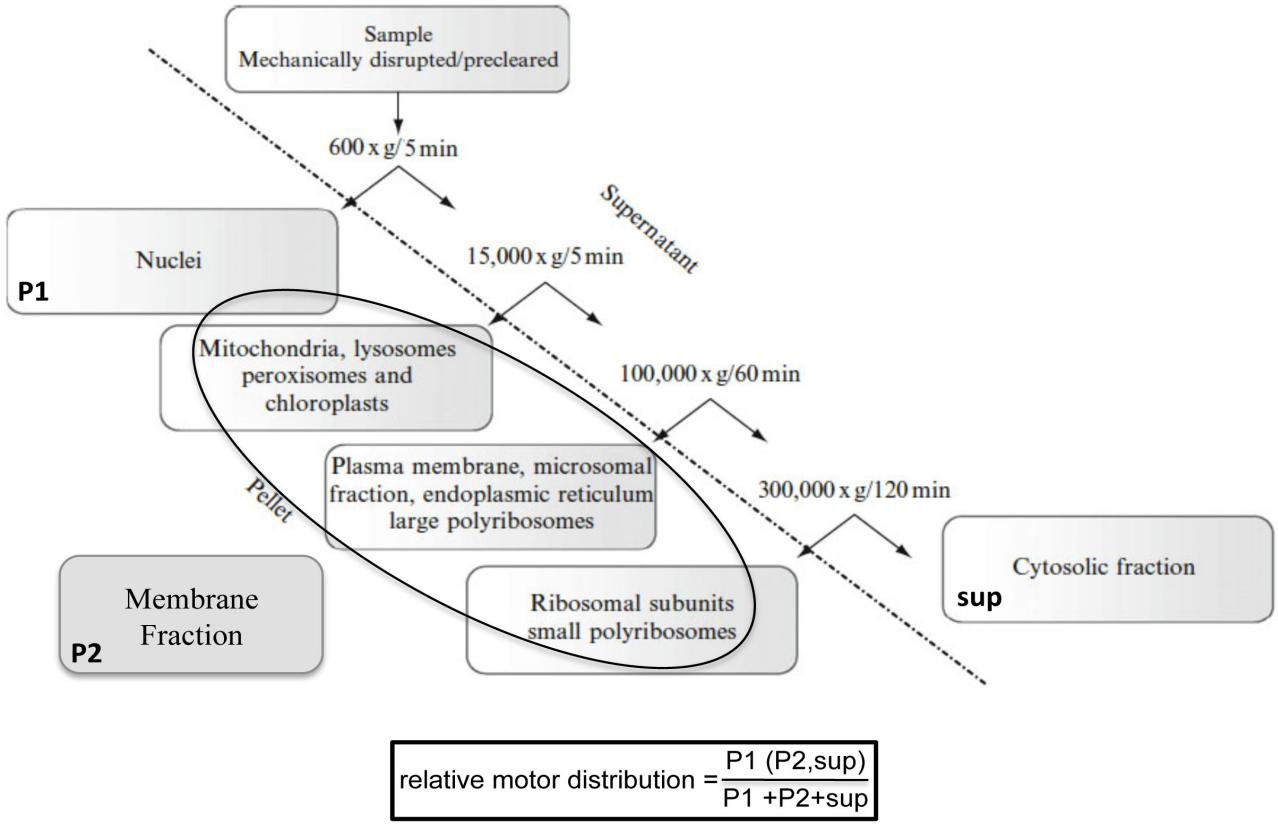


Figure adapted from Michelsen et al, Methods Enzymol, 463, 2009

Figure 20: Overview of membrane association assay protocol

Three subcellular fractions of cell lysates were obtained as a result of a low spin, 600g for 5 min P1 – nuclear fraction, and a high spin, 300,000g for 120 min P2- membrane fraction and sup – supernatant. Dynein and kinesin distribution was assessed in each of the fractions using the enclosed formula. Changes in P2, the membrane fraction, would be indicative of changes of the association of the motors with their cargo.

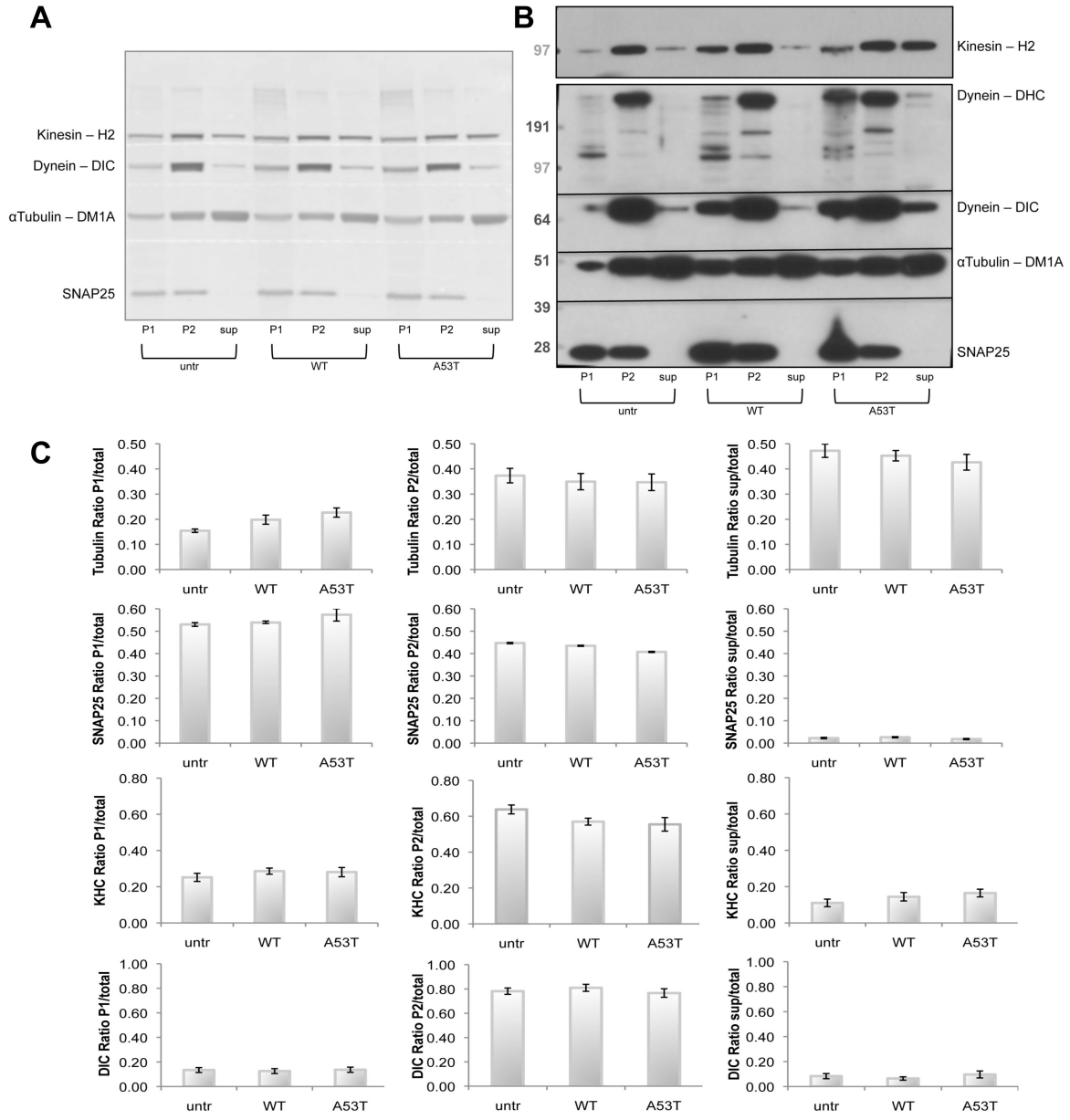


Figure 21: Molecular motor partitioning within cellular fractions

Three cellular fractions – nuclear - P1, membrane - P2, and cytosolic - sup, obtained using two centrifugation speeds (see Figure 20) were analyzed with fluorescent (A) and chemiluminescent (B) western blot techniques. Fluorescent quantification (C) indicates that there are no significant changes in the partitioning of the proteins tested: KHC (H2), DHC, DIC, Tubulin (DM1A), Snap25, histone (H3 – not shown) however in some samples, especially when using chemiluminescence (B) there is visible an increase of molecular motors in the cytosolic and nuclear fractions of transfected cells, in particular A53T α-synuclein. Graph error bars indicate standard error of the mean. Experiments repeated 4 times; n=4 per experiment. Statistical analysis: One-way ANOVA.

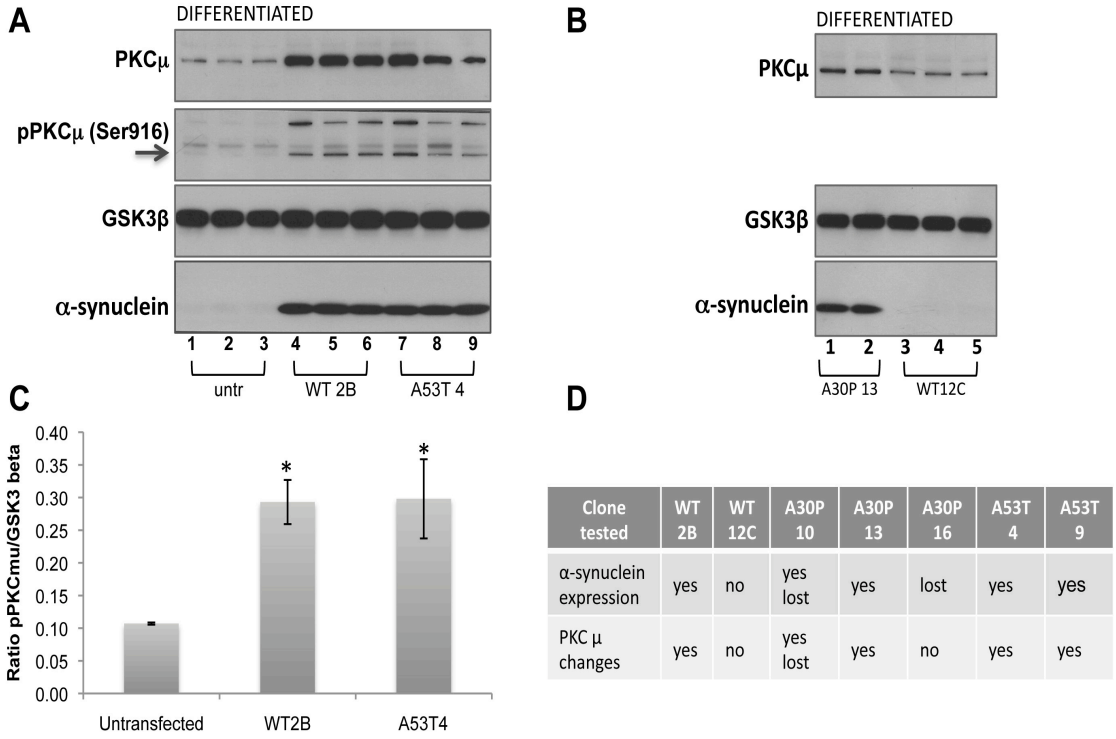
the molecular motors, either kinesin or dynein, while others did. Quantifying all the samples did not indicate any statistically significant differences at the 0.05 levels. Both fluorescence and chemiluminescence methods showed a high degree of variability in the distribution of the motors among the three different fractions even with increasing number of experiments while tubulin, SNAP25 and histone, proteins that provided a measure of the purity of the fractions, consistently showed no significant changes (Figure 21C). This indicated that this method of partitioning of cellular compartments was not suitable to detect changes in the interaction of the motors with membranes, which is reserved to specific pools of vesicles. By looking at the overall profile of cellular membranes, those pools may be masked in this procedure and therefore one where an increase in the number of cellular fractions that would contain these vesicles is necessary.

6. Evaluation of PKCμ and identification of a protein dependent on α-synuclein expression

Key kinases that emerged as important from the squid axoplasm experiments were non-receptor tyrosine kinases such as Src and Fyn as well as PKCμ. The characterization of non-receptor tyrosine kinases and their role in PD is the focus of another project. In addition to characterizing the α-synuclein clones developed and evaluating the molecular motors, the cells were next tested for changes in PKCμ. This was performed using western blotting and polyclonal rabbit antibodies from Cell Signaling. The antibody 2052 identifies total PKCμ and 2051 identifies phosphorylated PKCμ when phosphorylated at Ser 916. This serine has been identified as an autophosphorylation site for PKCμ and correlates with PKCμ catalytic activity [213].

The results indicated that there are changes in the expression and activity of a protein in clones expressing either WT or A53T α-synuclein (Figure 22A) and these changes were significant (Figure 22B). Moreover, when clones, such as A30P 10 and WT 12C, lose the expression of α-synuclein with increased passage numbers, the changes observed in the protein are abolished (Figure 22C). An assessment of all the clones analyzed led to the observation that regardless of α-synuclein construct present, its expression leads to changes in the protein (Figure 22D). Each of the antibodies used identified changes in a band of similar molecular weight. Discussion of experiments involving PKCμ with one of my colleagues, Sun Kyong Lee from the Morfini laboratory, led to the discovery that in western blots the major bands that were immunoreactive with PKCμ antibodies in my samples and the ones detected in Sun Kyong's, were at different positions, indicating significantly different molecular weights for the proteins we were both identifying as PKCμ. A comparison of our samples along with controls provided by Cell Signaling identified two major high molecular weight bands (Figure 23). The protein identified in my samples appears under the molecular weight (MW) marker 191 kDa, and the one identified by my colleague using rat cortical neurons right bellow the MW marker 97 kDa. Interestingly, there was strong immunoreactivity for both bands in the rat cortical neurons (Figure 23, total PKCμ antibody).

For electrophoresis, in gradient gels such as the 4-12% acrylamide ones that we are using, the migration of proteins can vary depending on the running conditions used. It is not uncommon for some proteins of MW higher than 70 - 80 kDa to run differently, usually slower and therefore appear higher on a gel comparative to standard proteins used in MW markers. This discrepancy can be addressed by changing electrophoresis



Clone tested	WT 2B	WT 12C	A30P 10	A30P 13	A30P 16	A53T 4	A53T 9
α-synuclein expression	yes	no	yes lost	yes	lost	yes	yes
PKC μ changes	yes	no	yes lost	yes	no	yes	yes

Figure 22: α-synuclein dependent changes in protein recognized by PKCμ antibodies

SH-SY5Y cells expressing either WT or A53T α-synuclein show a significant change in the increase of expression and activity of a protein recognized by PKCμ antibodies (A). GSK3β was used as loading control and ratios of total and phosphorylated Ser916 PKCμ (pPKCμ) to GSK3β were determined; only pPKCμ shown (C). The changes are not observed when the cells no longer express any of the forms of α-synuclein (B and D). Error bars in graphs indicate standard error of the mean. Statistical analysis: two-tailed T test, P values: Untr-WT2B 0.00534, Untr-A53T4 0.03487, WT2B-A53T4 0.946801

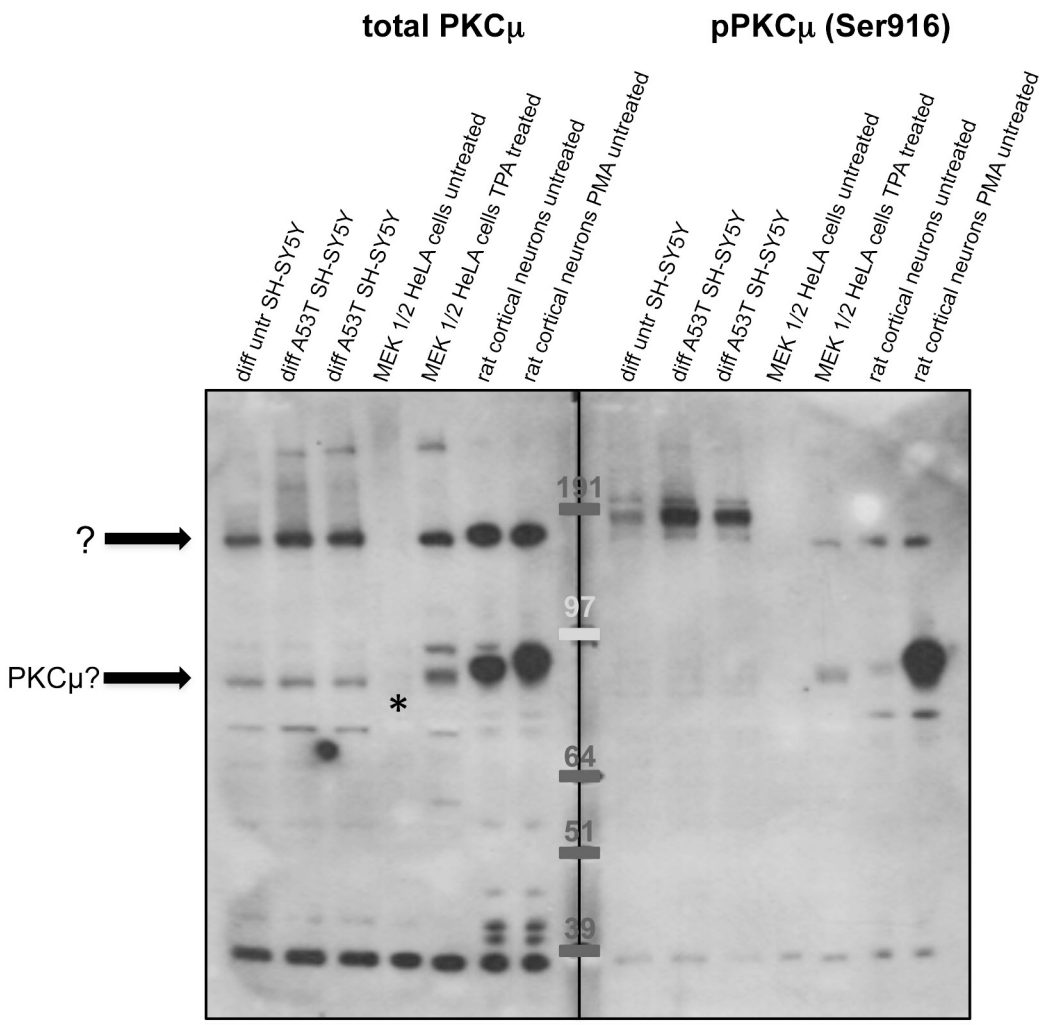


Figure 23: Sample comparison for PCKμ identification

Cell lysates from SH-SY5Y, MEK 1/2 HeLa cells and rat cortical neurons were probed with total and phosphorylated PKCμ antibodies. This identifies two major bands, below MW 191 and 97 kDa respectively, pointing arrows. PKCμ is most likely the lower band, bellow 97 kDa MW marker, as pPKCμ antibody recognizes this band in samples treated with TPA/PMA but not in untreated samples. * Sample of MEC 1/2 HeLa cell lysate untreated from Cell Signaling appears degraded and was later replaced by the company with NIH/3T3 cells. Rat cortical neuron samples provided by Sun Kyong Lee.

conditions such as running the gels for a longer period of time. In combination with the use of primary antibodies this can give one the confidence that proteins of interest are being analyzed. PKCμ has a molecular weight of 115 kDa and therefore it was possible that the bands identified in my samples could be representing PKCμ. However running the gels for a longer period of time did not alter the position of the protein within the gel, it did not run lower, closer to PKCμ's expected molecular weight. In addition, control samples provided by Cell Signaling, Mek 1/2 HeLa cells, and the ones used by my colleague, rat cortical neurons, were treated with phorbol 12-myristate 13-acetate (PMA, also known as TPA), a well-known activator of PKC isoforms. Total PKCμ antibodies recognized the lower band in both treated and untreated samples but phosphorylated PKCμ antibody only recognized the band in PMA treated samples (Figure 23, pPKCμ (Ser916) antibody). This indicated that PKCμ is most likely the band right below MW marker 97 kDa.

A reevaluation of all the data collected led to the conclusion that the immunoreactive protein changing with α-synuclein expression in SH-SY5Y cells may indeed not be the traditional PKCμ. Observations of SH-SY5Y samples indicate there was little or no immunoreactivity for actual PKCμ, the band below MW 97 kDa, while always present for the protein below MW 191 kDa (Figure 23, arrows). Using the given sizes of molecular weight markers used, the apparent MW of the major immunoreactive band the antibodies were recognizing was estimated at approximately 170 kDa, significantly higher than the normal 115 kDa of PKCμ (Figure 24).

In order to differentiate between the two proteins, PKCμ and the unknown protein, and to tell with certainty which protein is actually PKCμ in SH-SY5Y cells, the

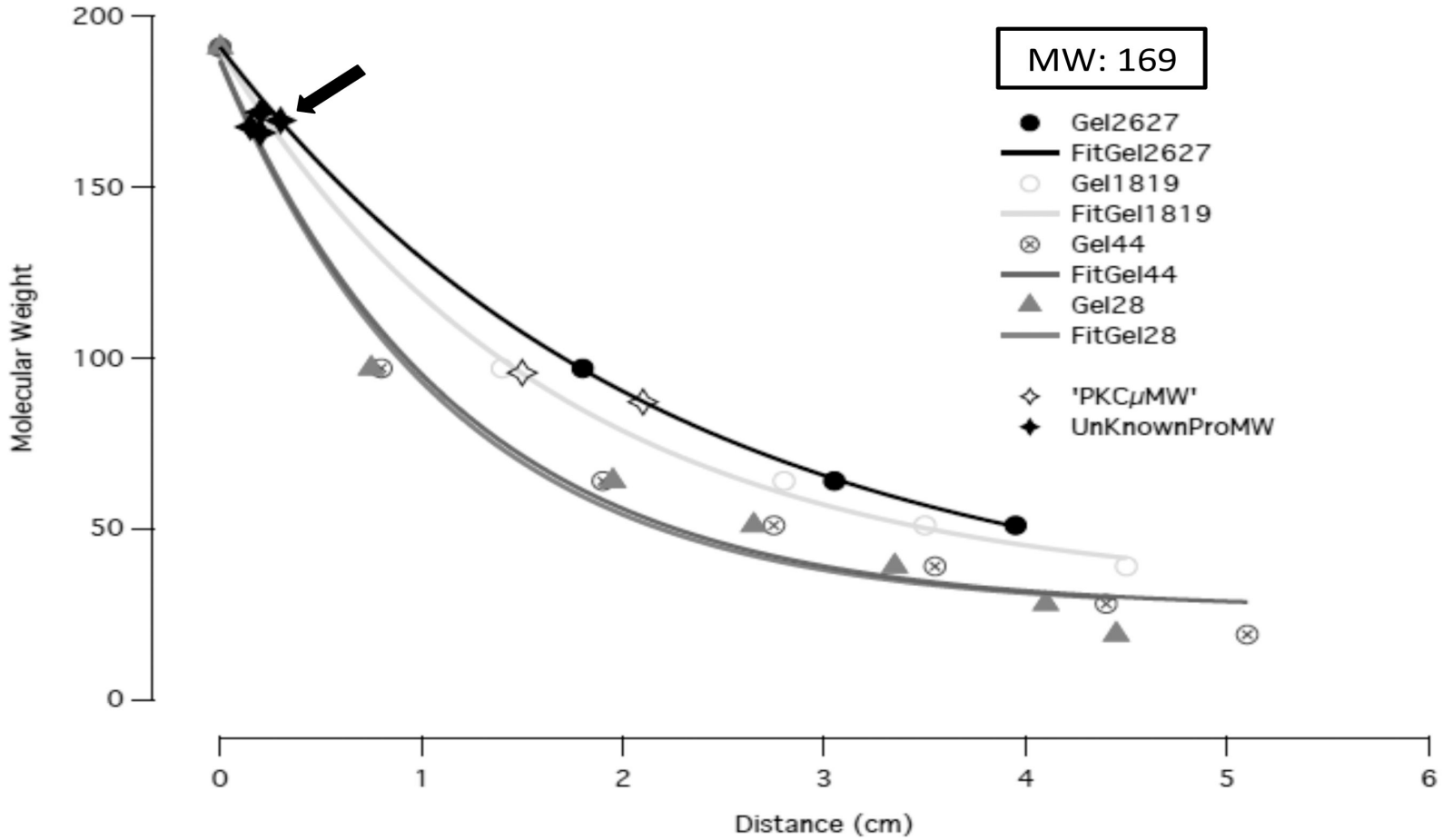


Figure 24: Molecular weight estimation of the unknown protein recognized by PKCμ antibodies

The distance between the two proteins recognized by PKCμ antibodies and molecular markers used for western blotting was measured with a ruler on four gels and plotted as illustrated above. The relative molecular weights of the proteins were determined based on the known sizes of proteins in the molecular weight marker. The size of the unknown protein was estimated to be approximately 170 kDA. The arrow indicates the interpolated MW of the novel species.

cells were treated with PMA. PMA changed phosphorylation levels in PKCμ as expected but not in the protein estimated to be 170 kDa, indicating that this is not traditional PKCμ. In addition, some interesting results were observed with PKCμ itself. When treated with PMA/TPA, these samples should have a higher immunoreactivity for phosphorylated PKCμ and similar reactivity for total PKCμ than untreated cells as observed in samples of NIH/3T3 cells, also used as a positive control for the proper identification of PKCμ. The same is the case for SH-SY5Y cells when blotting with phosphorylated PKCμ antibody. Using two different antibodies for phosphorylated PKCμ, samples treated with PMA indeed show an increase in phosphorylation of PKCμ (Figure 25 A and B right panels). However, when blotting for *total* PKCμ, differentiated SH-SY5Y cells treated with PMA, and regardless of α-synuclein expression status, show considerably higher levels of PKCμ than samples *not* treated with PMA (Figure 25 A and B, left panel). The results are dramatic, the changes occur within ten minutes.

Research in the field has shown that PMA can rapidly activate PKCμ within ten minutes of stimulation [214] and that PKCμ can potentiate DNA synthesis and cell proliferation [215]. However, it is unlikely that so much synthesis of new proteins can occur within a matter of minutes. Renneke *et al* has demonstrated that PKCμ/PKD1 expression correlates closely with keratinocyte proliferation, its activity being very high in basal dividing cells and very low in differentiating cells [216]. In our case, *differentiated* SH-SY5Y cells were treated with PMA. In general, these cells showed low levels of PKCμ expression in cell lysates and often there was little to no immunoreactivity for the protein (Figure 25 A, 0 min PMA). A likely explanation is that PKCμ is tightly regulated and that it is being degraded at a high rate in differentiated

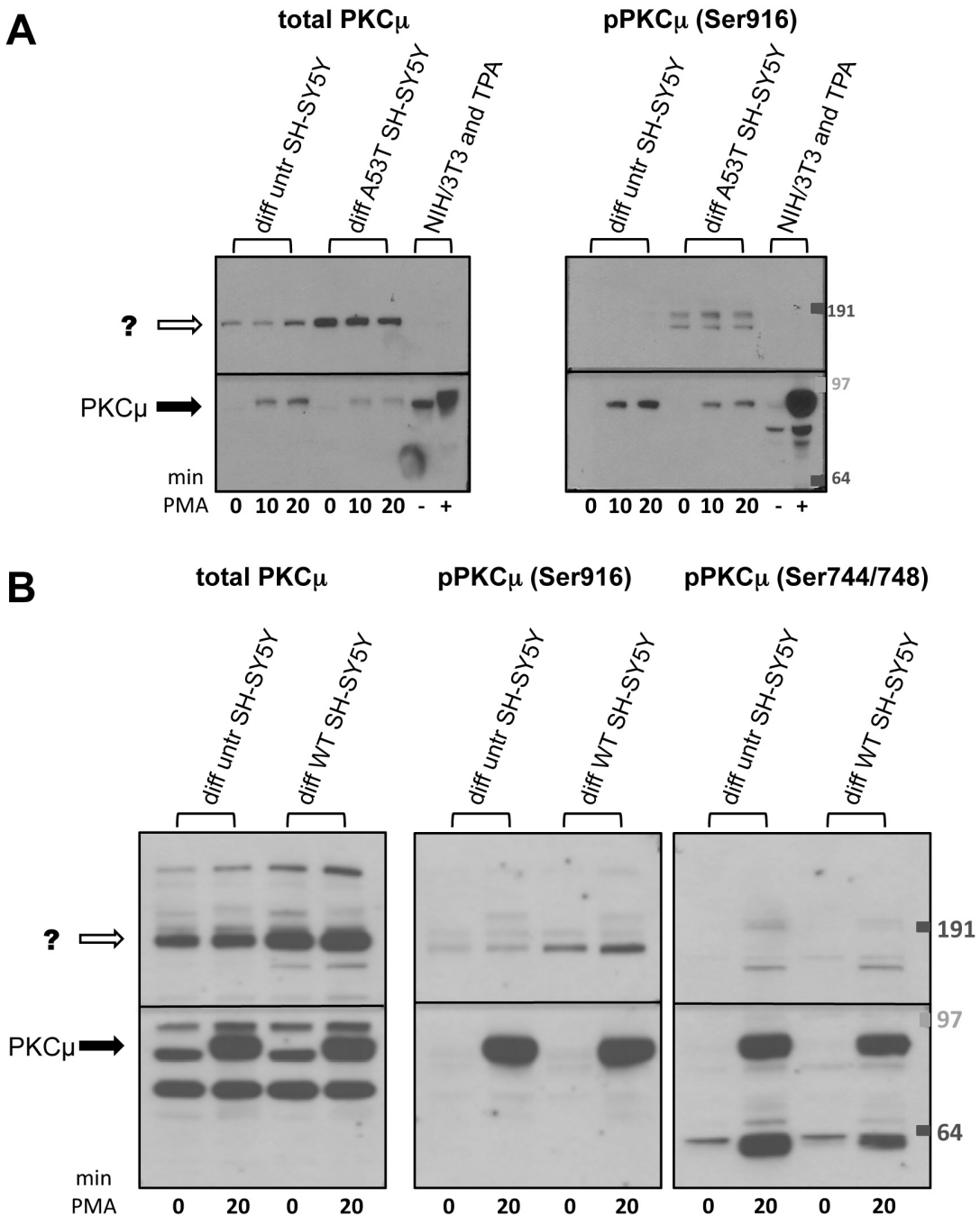


Figure 25: Effects of PMA treatment on SH-SY5Y cells

Treatment of SH-SY5Y cells, either untransfected or expressing A53T (A) or WT (B) forms of α-synuclein, with PMA for 10 or 20 minute show an increase in both *total* (left panels, A and B) and phosphorylated PKCμ. Note that the antibody that recognizes PKCμ when phosphorylated at Ser 744/748 does not recognize the unknown protein (B, third, right panel).

cells. Upon the addition of PMA, this process is rapidly inhibited and it leads to increased levels of both total and phosphorylated PKCμ. Another possible explanation is that upon PMA activation, the conformational changes that occur within PKCμ render sites within the protein more available and therefore more recognizable to the antibody.

It is important to point out that a third PKCμ antibody, Cell Signaling 2054, which recognizes PKCμ only when dually phosphorylated at serines 744 and 748, did not recognize the 170 kDa protein (Figure 25B, third panel). Both 2051 and 2052 PKCμ antibodies are designed to recognize the C-terminus of PKC μ. This led to the hypothesis that the unknown protein must have in common a sequence also found in the C-terminus peptide of PKCμ. A BLAST search of the sequence ERVSIL, the last amino acids of the C-terminus of PKCμ which include the regulatory serine 916, found that this motif was a signature of PKCμ/PKD1, raising the possibility that the higher band identified may be an alternative spliced PKCμ isoform or a closely related protein.

Subsequently, immunoprecipitation techniques were employed to enrich and purify samples containing the unknown protein. Two antibodies available in our laboratory were reported as suitable for immunoprecitpitation techniques for PKCμ, Cell Signaling 2051 and Santa Cruz sc-639. The antibody sc-639 is also a polyclonal antibody and also designed to recognize the C-terminus of PKCμ. In western blotting of SH-SY5Y cell lysates this antibody was even less specific for PKCμ than 2052; it recognized multiple bands and did not bind as well to the protein identified by 2052 and 2051 (not shown).

Given that antibodies can be more suitable for one application than another, such as western blotting versus immunoprecipitation, both 2051 and sc-639 were tested to

determine which would be more appropriate to help detect the unknown protein. In addition, I also hypothesized that if the antibodies preferentially recognized PKCμ, they would then bind more to PKCμ than the unknown protein. A sequential purification step was used where first the cell lysate was depleted of PKCμ and then fresh antibody was added to the leftover lysate so that the unknown protein could bind more. This procedure was tested in two ways: sequential steps of 1) 2051 followed again by 2051 and 2) sc-639 followed by 2051. The most successful immunoprecipitation was with sc-639 and both PKCμ and the unknown protein eluted in the first step (Figure 26A). The sequential step thus proved unnecessary and indicated that both proteins bind to the antibody with similar affinities. The sample containing the unknown protein was loaded in large quantities and run onto an SDS gel for further separation, the gel then stained with Coommassie blue. The region of the gel containing the unknown immunoreactive protein was cut out and submitted to the CBC/RRC Proteomic and Informatics Services at UIC for mass spectrometry analysis (Figure 26B). The analysis yielded 26 possible results, which are summarized in table III. Including homologues and isoforms, this resulted in 128 possible candidates. Upon first inspection, none of the sequences of the proteins identified contained the ERVSIL peptide. However, when an antibody is made, the peptide used for designing the antibody can vary from 8-10 to 18-20 amino acids long. Taking this into account and that all three antibodies used are designed to the C-terminus of PKCμ, it would possible that the sequence recognized in the unknown protein may be higher up than the ERVSIL sequence, which is at the very end of PKCμ.

In another study of PKCμ, using two PKCμ antibodies (sc-639 and P26720), Rennecke *et al* [217], screened 13 murine tissues and 12 cell lines for expression of the

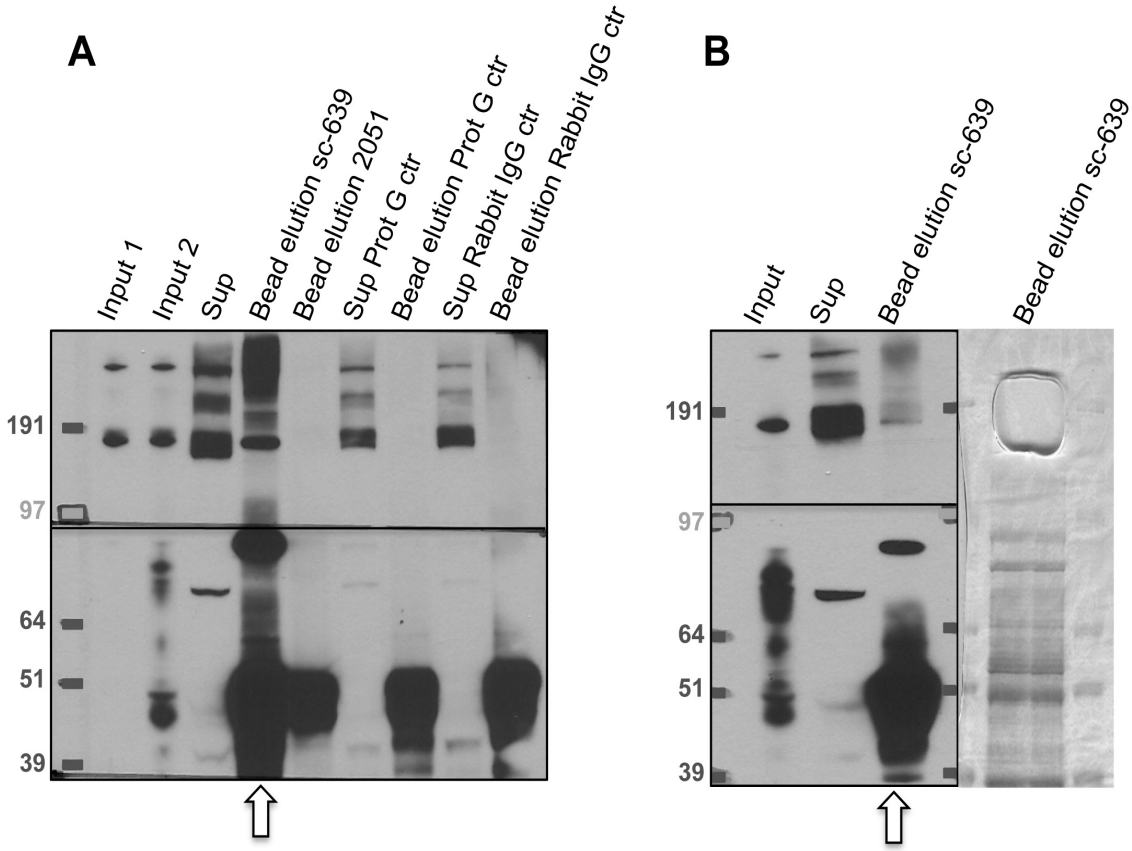


Figure 26: Immunoprecipitation assays

A: The unknown band immunologically related to PKCμ, which runs slightly bellow the marker for MW 191 kDa, elutes from beads with PKCμ - arrows point to representative lanes in both A and B. Input 1 represents cell lysate, input 2, cell lysate that has been cleared with protein G and rabbit IgG beads, sup – supernatant from second bead elution with 2051 antibody; last four lanes: separate aliquots of input 2 were incubated with either protein G or rabbit IgG beads and used as controls to ensure that the protein of interest would not bind directly to the beads. B: The sample was then loaded onto a gel and stained with Coomassie blue. The part containing the protein of interest was cut out and submitted for mass spectroscopy. Left side represents a western blot of Comassie blue stained gel samples on the right.

TABLE III

SUMMARY OF PROTEINS DETECTED

	Protein name	Protein MW (Da)	Protein ID probability	# of unique peptides	Peptide sequence	Previous amino acid	Next amino acid	Best Peptide ID probability
1	trinucleotide repeat containing 15, isoform CRA_b [Homo sapiens]	153,475.60	100.00%	3	ALSSGGSITSPPLSPALPK	R	Y	95.00%
2	keratin 1 [Homo sapiens]	66,050.30	100.00%	8	FLEQQNQVLQTK	R	W	95.00%
3	AT rich interactive domain 1B (SWI1-like), isoform CRA_d [Homo sapiens]	229,867.40	100.00%	9	EITFPPGSVEASQPVLK	R	Q	95.00%
4	plectin [Homo sapiens], gi|1477651|gb|AAB05428.1| plectin [Homo sapiens]	518,465.20	100.00%	3	APVPASELLASGVLSR	R	A	95.00%
5	YLP motif containing 1 [Homo sapiens]	219,969.50	100.00%	13	AIGFVVGQTDWEK	R	I	95.00%
6	complement component 3 precursor [Homo sapiens], AltName: Full=C3 and PZP-like alpha-2-macroglobulin domain-containing protein 1	187,147.20	100.00%	12	DMALTAFVLISLQEAK	K	D	95.00%
7	DEAH (Asp-Glu-Ala-His) box polypeptide 9 [Homo sapiens], Full=ATP-dependent RNA helicase A; AltName: Full=Nuclear DNA helicase II	140,943.50	100.00%	7	DFVNYLVR	R	I	94.50%
8	hCG1782167, isoform CRA_a [Homo sapiens]	241,000.50	100.00%	5	EIDDEANSYFQR	K	I	95.00%
9	heterogeneous nuclear ribonucleoprotein U isoform b [Homo sapiens],	88,928.30	100.00%	3	EKPYFPIPEEYTFIQNVPLEDR	K	V	95.00%
10	HBxAg transactivated protein 2 [Homo sapiens]	295,810.20	100.00%	3	LPGQDESTAGTSEQNDILK	K	V	95.00%
11	carbamoylphosphate synthetase 2/aspartate transcarbamylase/dihydroorotase [Homo sapiens]	242,964.50	100.00%	6	AAFALGGLGSGFASNR	R	E	95.00%
12	keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris), isoform CRA_b [Homo sapiens]	58,810.80	100.00%	5	AETECQNTEYQQLLDIK	R	I	95.00%
13	RecName: Full=Dedicator of cytokinesis protein 7	226,601.40	99.80%	2	FGEDVVEVIK	R	D	95.00%

TABLE III (continued)

SUMMARY OF PROTEINS DETECTED

	Protein name	Protein MW (Da)	Protein ID probability	# of unique peptides	Peptide sequence	Previous amino acid	Next amino acid	Best Peptide ID probability
14	BAT2 protein [Homo sapiens]	228,845.10	100.00%	7	AEPAAPPAAPSTPAPPPAVPK	K	E	95.00%
15	SEC16 homolog A [Homo sapiens], KIAA0310 protein [synthetic construct]	255,183.00	100.00%	7	AGSALPGFANSPAGSTSVVLVPPAHGTLVPDGNK	K	A	95.00%
16	hCG2001591 [Homo sapiens]	193,429.70	100.00%	4	ASAGLLGAHAAAITAYALTLTK	K	A	95.00%
17	PREDICTED: clathrin heavy chain 1 isoform 6 [Macaca mulatta], clathrin, heavy polypeptide (Hc), isoform CRA_a [Homo sapiens]	191,600.90	100.00%	27	ADDPSSYMEVVQAANTSGNWEELVK	K	Y	95.00%
18	CREB binding protein (Rubinstein-Taybi syndrome), isoform CRA_a [Homo sapiens]	265,313.20	99.80%	2	LVQAIFPTPDPAALK	K	D	95.00%
19	DNA-directed RNA polymerase II A [Homo sapiens], AltName: Full=DNA-directed RNA polymerase III largest subunit; AltName: Full=RNA-directed RNA polymerase II subunit RPB1	217,193.10	100.00%	13	AEIQELAMVPR	R	M	95.00%
20	AT rich interactive domain 1A isoform a [Homo sapiens], AltName: Full=ARID domain-containing protein 1A; AltName: Full=SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily F member 1;	242,025.80	100.00%	12	AGPPVPASHIAPAPVQPPMIR	K	R	95.00%
21	eukaryotic translation initiation factor 4 gamma, 1, isoform CRA_c [Homo sapiens]	175,520.10	100.00%	2	GLPLVDDGGWNTVPISK	R	G	95.00%
22	nucleoporin 214kDa, isoform CRA_b [Homo sapiens]	215,383.10	100.00%	8	AAPGPGPSTFSFVPPSK	K	A	95.00%
23	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4 isoform C [Homo sapiens]	181,334.00	100.00%	17	AFLQAILEHEEQDEEEDEVPDDETVNQMIAR	R	H	95.00%
24	KRT9 protein [Homo sapiens]	62,113.00	100.00%	7	DIENQYETQITQIEHEVSSSGQEVQSSAK	K	E	95.00%
25	keratin 2 [Homo sapiens],	65,848.40	99.80%	2	FLEQQNQVLQTK	R	W	95.00%
26	hCG30195, isoform CRA_f [Homo sapiens]	242,820.70	99.80%	2	AVTAFSSTETGSAEGFK	K	S	95.00%

kinase and found that depending on the antibody used, the results were different. Moreover, treatment of cells *in vivo* with TPA resulted in an impaired recognition of PKCμ by the antibody sc-639 but not by P26720. The results led to the conclusion that sc-639, does not recognize activated PKCμ, likely due to its phosphorylation at serine 916. These observations support the idea that the unknown protein has in common a sequence with the C-teminus of PKCμ, but that it may not necessarily include a phosphorylated serine. Widening the screening of the 128 hits against a longer sequence of the PKCμ C- terminus, the last 50 amino acids, still did not yield any individual matches for the protein.

A blocking peptide was available for competition studies for the antibody sc-639. This peptide was tested as an additional elution step in the immunoprecipitation protocol in order to improve the purity of the final product. Contrary to expectations this resulted in the appearance of multiple bands, making it unclear which is the protein of interest (not shown). These observations raise the possibility that the unknown protein could be a form of PKCμ that has posttranslational modifications. The introduction of the peptide may affect these modifications resulting in multiple products. This idea is also supported by observations that the unknown protein appears to be sensitive to freeze thaw cycles or longer term storage. Over time it becomes undetectable using western blots compared to other proteins from the same cell lysate samples. Further revisions to the protocols employed thus far as well as other techniques, such as fractionation methods that would separate PKCμ and the unknown protein from cell lysates will be necessary in order to enrich and improve the purity of the immunoprecipitation product

and thus narrow the possible species of proteins that can be detected by mass spectroscopy and lead to the identification of the unknown protein.

E. DISCUSSION

The goal of the work described in this chapter was to develop a cellular model for the study of PD pathogenesis. A number of cell lines were screened and stably transfected cell lines expressing WT, A30P and, A53T forms of α-synuclein were successfully developed. The cells expressed the protein robustly and although its expression did not lead directly to cell death, it did make them more prone to it, especially upon differentiation, which is consistent with increased toxicity of the expressed gene products. This project did not explore cell death but it would be interesting to see whether, and at what point in these cells' life, pathways that lead to cell death are activated and what these particular pathways may be. Recently, attention has turned to autophagy and how α-synuclein interferes with these processes [218, 219]. It appears that both the ubiquitin-proteasome and the autophagy systems can be overwhelmed in the presence of α-synuclein, which in turn leads to cell death [220]. Even though aggregation, a consequence of these systems being affected, was not observed during the period of time examined it is not unlikely for these systems to be affected in α-synuclein SH-SY5Y transfected clones and perhaps particular insights could be gained by comparing untransfected clones with transfected clones and transfected clones that no longer express the protein.

Upon α-synuclein expression, many of the clones tended to have a high number of S or I-type morphology, which is most likely a state that can better support the

expression of the protein, perhaps not unlike non–neuronal cells *in vivo*. Other clones, such as the clone A30P 10, which maintained largely N, neuronal type morphology, stopped expressing α-synuclein suggesting that cell survival may be dependent on down regulation of expression of the mutant protein. Similar loss of protein expression in SH-SY5Y cells has been noted with other pathogenic proteins. For example, cells lose expression of the Huntington protein when it has 46 polyglutamine repeats, (S. Pollema, personal communication) or, as in the case of the androgen receptor, a gradual disappearance of the expanded repeats of androgen receptor cell population occurs with cell propagation [203].

Squid axoplasm experiments indicate that in the presence of α-synuclein retrograde transport is increased and therefore one might expect increases in dynein, however, in the clones developed, no changes were observed in the expression of the molecular motors, either kinesin or dynein. This was also reflected in the binding of kinesin to MT. There were no significant differences between untransfected, WT and A53T α-synuclein expressing cells. Still, there were indications that there were differences in dynein interaction with the MT. The results pointed to an apparent decrease in their association in the AMP-PNP condition, but the ATP condition has to be examined more closely as well. Dynein is a very complex molecule and the MT binding assay employed was designed for kinesin. A more comprehensive assay needs to be developed that analyzes better the differences in both the ATP and AMP-PNP conditions and takes into account the fact that dynein has nucleotide binding sites that are important not only in motility but regulation as well. Moreover, separate

experiments such as gel shift assay of cell lysates and radioactive techniques that use ^{32}P would help determine the phosphorylation status of the motors, in particular dynein.

In addition, my experiments showed that there were no changes in the distribution of proteins such as histone H3, SNAP25 and tubulin, among three representative subcellular fractions: nuclear, membrane and cytoplasmic but there was variability in the distribution of the molecular motors. This provides evidence that the association of the motors to the membranes may also be affected, but a more extensive isolation and analysis of different vesicle populations is needed, such as the use of sucrose gradients or vesicle immunoisolation. These approaches would allow for the isolation of defined concentrated vesicle populations, some of which would be enriched with membranes that interact specifically with the motors. Such experiments though would require large amounts of cell lysates to ensure that enough motor protein is available for detection.

The experiments presented also suggested that the SH-SY5Y cells might not be the ideal system in which to study the model of pathogenesis developed using squid axoplasm. It has been demonstrated that PKCμ is a protein significant in cancer, its role in tumor and cell proliferation highly controlled [172, 216, 221]. As a neuroblastoma cell line, SH-SY5Y is fundamentally a cancer cell line and the PMA experiments illustrated that PKCμ is tightly regulated in these cells, especially when differentiated. Therefore any effects of α-synuclein expression may be masked by this regulation. Nevertheless, α-synuclein expression clearly has effects in SH-SY5Y cells after differentiation as indicated by the up regulation of an unknown protein recognized by PKCμ antibodies. A summary of these antibodies is listed in table IV.

TABLE IV

PKCμ ANTIBODIES USED

Antibody	Specificity	Epitope
sc-639	total PKCμ *	C-terminus of mouse PKCμ
2051 C.S.	pSer916 PKCμ	residues surrounding Ser916 of mouse PKD
2052 C.S.	total PKCμ	residues surrounding Ser916 of mouse PKD
2054 C.S.	pSer 744/748 PKCμ	residues surrounding Ser744/748 of mouse PKD
Note: ** appears not to recognize pSer916 PCKmu. C.S.=Cell Signaling*		

The specifications of the PKCμ antibodies used and the manner in which they interacted with the unknown protein provide helpful insights into the identification of this protein. Based on the information provided about the epitopes for these antibodies, we can conclude that the protein shares a sequence in common with the C-terminus of PKCμ and it is possible for this sequence to be slightly upstream of Ser916 of PKCμ. Protein database searches of the C-terminus sequence were specific to PKCμ so the results point to the idea that the unknown protein could be a new isoform of PKCμ/PKD1 although it is also possible that the unknown protein is just immunologically related to PKCμ and may in fact be an entirely different protein. Possible candidates could, for example, be proteins that contain a PDZ domain. PDZ (PSD-95, Discs large, ZO-1) domains occur in one or multiple copies within a protein and mediate protein-protein interactions. These interactions can occur via binding to other PDZ domains or, more commonly, by recognition of short amino acid motifs in the carboxyl termini of target

[222, 223]. Kunkel *et al* has shown that the Na^+/H^+ exchanger regulatory factor 1 (NHERF-1), which is a PDZ domain-containing protein, interacts with a PDZ-binding motif found at the C-terminus of PKCμ/PKD1, the last four amino acids VSIL [224]. Since the antibodies that recognized the unknown protein have epitopes designed to the C-terminus of PKCμ, which include this sequence, it is possible that the antibodies identify a protein that interacts with a PDZ domain and/or which has a PDZ domain itself. For example, post synaptic density protein 95, known as PSD-95 or SAP-90, has a PDZ domain which contains within itself the SIL sequence [225]. Many PDZ domain proteins, such as PSD-95, are scaffolding proteins that form complexes or multimers [226-228]. There are several compelling reasons that might make PSD-95 a good candidate for the unknown protein. PSD-95 is part of a family of membrane associated guanylate kinases that serve as synaptic organizers. For example, using distinct protein-protein interactions PSD-95 has been shown to mediate homeostatic accumulation and loss of synaptic AMPA receptors [229] and to regulate synaptic stability at neuronal cholinergic synapses [230]. As a synaptic organizer, PSD-95 would be in close proximity to where α-synuclein is reported to have a role, at the synapse. Moreover, phosphorylation of serines or threonines of the binding sites of PDZ domains, such as the serine in the SIL sequence, can play an important role in regulating PDZ mediated interactions [227]. This would account for recognition of the unknown protein by both total and Ser 916 phosphorylated PKCμ antibodies. Although the molecular weight of PSD-95 is 95 kDA, Kim *et al* has shown that PSD-95 can form heteromers with another PDZ domain protein, PSD-93 [225] and DeGiorgis *et a*l that these two proteins have the same distribution in the postsynaptic density [231]. The molecular

weight of PSD-93 is 110 kDa so together these two proteins would have a molecular weight of 205 kDa, which is not too far off the predicted 170 kDA of the unknown protein. The ability of PSD-95 to form heteromultimers may also explain the observation that the unknown protein is not detected in prepared cell lysates after relatively short periods of time when compared to other proteins, which made it important to work with fresh material. It is possible that PSD-95's association with PSD-93, or other scaffold proteins it may tightly interact with such as NHERF1, degrades over time.

PSD-95 may also be of interest because it has been shown to regulate non-receptor tyrosine kinases such as Src and Fyn [232-234], kinases found by our laboratory to be relevant in regulation of FAT in squid axoplasm. PSD-95 has also been found to play a role in regulating dopamine D1 receptors, which would be a contributing explanation to the vulnerability of particular cell types like dopaminergic neurons of the SNpc [235, 236].

The SH-SY5Y clones analyzed for PKCμ changes were also selected based on morphology. All cells appeared to be mostly of a population of N, neuronal like cells. These cells, although sickly, did not die and maintained the expression of α-synuclein. Therefore it is also possible that the unknown protein could play a role in adaptation and cell survival to toxic effects. Whether a new PKCμ isoform, a scaffold protein with a PDZ binding domain and/or PDZ binding domain recognition site, or any other protein, the identification of this unknown protein could provide key insights into a mechanism that is activated in order to deal with the presence of pathogenic forms of α-synuclein.

This work shows the importance of cell lines in studying disease. They can be a useful tool and have advantages such as ease of use and the possibility of genetic manipulation, but there are limitations, and one must be mindful that some results may be specific to the system employed. Still, this can have the advantage of leading to new ideas into how a problem and its solution can be approached as may be the case with the identification of the unknown protein detected in SH-SY5Y cells. Ultimately, the identification of the unknown protein could play a very important role in elucidating α-synuclein pathogenesis and needs to be further explored.

IV. FUTURE DIRECTIONS

Parkinson's disease is a complex and challenging disease and we have yet to understand its mechanisms of action. Whether it has single or multiple triggers, it is multifactorial and neurologically all encompassing in its progression, with α-synuclein standing at the center of most cases of PD. Understanding this protein still eludes us and there may not be any easy answers given that synuclein's behavior is so dependent on its environment. It appears to be cell type specific and this can make interpretation of observations difficult to determine or the generation of general conclusions complicate.

The efforts of the work presented have focused on enlarging our view of how PD pathogenesis might manifest itself but also continues to raise questions. First, and of significant importance, would be what is the best criteria in defining, selecting and working with a system that models PD? Observations made using squid axoplasm have yet to be validated in mammalian systems and considering the results presented, regarding PKCμ, neuroblastoma cell lines may not be well suited for obtaining these answers. Alternative methods need to be employed to further explore these observations. For example, the use of cultured neurons should be revisited in order to evaluate the effects of α-synuclein on non-receptor tyrosine kinases, PKCμ and the molecular motors. Transfection of these cells was a concern in the past but recently a new method of DNA delivery into cultured neurons, which uses magnetic beads, has been developed with results of high transfection efficiency, low toxicity and long-lasting expression [237, 238]. Given that C-terminus truncations were noted to occur naturally in cells and taking into account our observations, it is possible that truncating α-

synuclein may be a way for the cell to deal with something that is harmful. Parallel transfections of neurons with full-length and C-terminus truncated forms of α-synuclein would allow for comparing the differences in neuronal response between the two forms of the protein. It has been reported that the C-terminus truncated α-synuclein aggregates faster than its full-length counterpart but what about the activation of kinase pathways? Would there be any differences in the activity or expression of non-receptor tyrosine kinases such as Src or Fyn, PKCμ or the molecular motors dynein and kinesin? Such studies could help better elucidate in an *in vivo* setting to what extent deleting the C-terminus of α-synuclein has negative or positive effects on the cell.

The *in vitro* experiments started in this project using atomic force microscopy should continue the study of the C-terminus and α-synuclein aggregation. AFM is a technique that has proven powerful in the identification and preparation of various forms of Aβ aggregates [239, 240]. This technique may be used in a similar manner for α-synuclein and it may be effective in confirming that aggregates of C-terminus truncated α-synuclein are different than its full-length counterpart just as in squid axoplasm truncated fibrils of either WT, A30P or A53T α-synuclein did not have any effect on FAT while fibrils made of the full length protein did.

Although not always statistically significant, the results presented indicate that in cells, α-synuclein might have effects on the molecular motors. In particular, dynein association to the microtubules is affected but to understand this better a microtubule binding assay more specific to dynein needs to be devised. Some studies that analyze general dynein and microtubule interaction present microtubule binding assays that could be modified for these purposes [241-243]. Fractionation of the cellular

components into nuclear, membrane and cytosolic fractions was not sufficient to determine to what extent the interaction of the molecular motors with their cargo and therefore association with membreanes, was affected by α-synuclein. A more refined protocol will be required where specific pools of vesicles are selected. This could be done by scaling up the amount of material used and introduction of sucrose gradients.

The use of the neruobastoma cell line SH-SY5Y produced mixed results in terms of elucidating PD pathogenesis but the observations regarding PKCμ in the neruobastoma cell line SH-SY5Y were intriguing and should be further explored. Are the dramatic changes observed upon PMA activation due to changes in protein conformation and therefore lead to better recognition by the antibody or is there tight regulation of this protein? The latter may be the case as treatment of SH-SY5Y cells with phorbol esters has potent consequences and can induce these cells to differentiate into cells of sympathetic lineage [190, 244] rather than the sympathetic chromaffin lineage observed upon the use of RA. Other studies of PKCμ indicate that PMA stimulation requires the activation of the isoforms PKCε or PKCη for PKCμ to become active [245] and analysis of other PKC isoforms would also be useful. Insights into the role of PKCμ in SH-SY5Y cells would further our understanding of the protein and of neuroblastoma cells in general.

Perhaps the most intriguing part of this work was the identification of an unknown protein recognized by PKCμ antibodies. Increases in this protein's expression levels may be a way for the cell to cope with α-synuclein toxicity and could lead to identification of new pathways that would help us better understand and elucidate the disease. The possibility that the protein could recognize or have a PDZ binding domain

such as PSD-95 is particularly attractive considering that PSD-95 is a scaffold protein that can interact with various proteins and can regulate Src, Fyn and dopamine D1 receptors. PSD-95 could scaffold with another PDZ domain protein, NHERF1. There is evidence that NHERF proteins are present in the brain [246] and that NHERF1 can regulate PKD1/PKCμ activity [224]. In addition, Mok *et al* has demonstrated that the kinesin superfamily motor protein KIF1Bα associates with PSD-95 [247]. Thus PSD-95 could have regulatory effects on the pathway developed using squid axoplasm and might circumvent the role of PKCμ in SH-SY5Y cells transfected with α-synuclein.

Although not presented, probing with PKCμ antibodies the subcellular fractions used for membrane association assays indicated that the distribution of PKCμ and the unknown protein might differ among different fractions. This distribution may differ yet in differentiated versus undifferentiated cells as the expression of some proteins is up or down regulated upon differentiation. Future experiments could take advantage of these differences in order to separate the two proteins, PKCμ and the unknown. In combination with immunoprecipitation protocols this would result in better separation and enrichment of the unknown protein and therefore ease of detection. The identification of the unknown protein could hold the key in getting us closer to understanding PD and α-synuclein pathogenesis.

CITED LITERATURE

1. Manyam, B.V., *Paralysis agitans and levodopa in "Ayurveda": ancient Indian medical treatise.* Mov Disord, 1990. **5**(1): p. 47-8.

2. Carlsson, A., M. Lindqvist, and T. Magnusson, *3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists.* Nature, 1957. **180**(4596): p. 1200.

3. http://www.parkinsons.org/parkinsons-history.html.

4. Foundation, P.s.D. *Statistics on Parkinson's* Available from: http://www.pdf.org/en/parkinson_statistics.

5. de Rijk, M.C., L.J. Launer, K. Berger, M.M. Breteler, J.F. Dartigues, M. Baldereschi, L. Fratiglioni, A. Lobo, J. Martinez-Lage, C. Trenkwalder, and A. Hofman, *Prevalence of Parkinson's disease in Europe: A collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group.* Neurology, 2000. **54**(11 Suppl 5): p. S21-3.

6. Venda, L.L., S.J. Cragg, V.L. Buchman, and R. Wade-Martins, *alpha-Synuclein and dopamine at the crossroads of Parkinson's disease.* Trends Neurosci, 2010. **33**(12): p. 559-68.

7. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.

8. Licker, V., E. Kovari, D.F. Hochstrasser, and P.R. Burkhard, *Proteomics in human Parkinson's disease research.* J Proteomics, 2009. **73**(1): p. 10-29.

9. Vidailhet, M., *Movement disorders in 2010: Parkinson disease-symptoms and treatments.* Nat Rev Neurol, 2011. **7**(2): p. 70-2.

10. Savica, R., W.A. Rocca, and J.E. Ahlskog, *When does Parkinson disease start?* Arch Neurol, 2010. **67**(7): p. 798-801.

11. Del Tredici, K., U. Rub, R.A. De Vos, J.R. Bohl, and H. Braak, *Where does parkinson disease pathology begin in the brain?* J Neuropathol Exp Neurol, 2002. **61**(5): p. 413-26.

12. Braak, H., K. Del Tredici, U. Rub, R.A. de Vos, E.N. Jansen Steur, and E. Braak, *Staging of brain pathology related to sporadic Parkinson's disease.* Neurobiol Aging, 2003. **24**(2): p. 197-211.

13. Hawkes, C.H., K. Del Tredici, and H. Braak, *A timeline for Parkinson's disease.* Parkinsonism Relat Disord, 2010. **16**(2): p. 79-84.

14. Braak, H., E. Ghebremedhin, U. Rub, H. Bratzke, and K. Del Tredici, *Stages in the development of Parkinson's disease-related pathology.* Cell Tissue Res, 2004. **318**(1): p. 121-34.

15. Obeso, J.A., M.C. Rodriguez-Oroz, C.G. Goetz, C. Marin, J.H. Kordower, M. Rodriguez, E.C. Hirsch, M. Farrer, A.H. Schapira, and G. Halliday, *Missing pieces in the Parkinson's disease puzzle.* Nat Med, 2010. **16**(6): p. 653-61.

16. van Rooden, S.M., F. Colas, P. Martinez-Martin, M. Visser, D. Verbaan, J. Marinus, R.K. Chaudhuri, J.N. Kok, and J.J. van Hilten, *Clinical subtypes of Parkinson's disease.* Mov Disord, 2011. **26**(1): p. 51-8.

17. Selikhova, M., D.R. Williams, P.A. Kempster, J.L. Holton, T. Revesz, and A.J. Lees, *A clinico-pathological study of subtypes in Parkinson's disease.* Brain, 2009. **132**(Pt 11): p. 2947-57.

18. Langston, J.W., P. Ballard, J.W. Tetrud, and I. Irwin, *Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis.* Science, 1983. **219**(4587): p. 979-80.

19. Tanner, C.M., F. Kamel, G.W. Ross, J.A. Hoppin, S.M. Goldman, M. Korell, C. Marras, G.S. Bhudhikanok, M. Kasten, A.R. Chade, K. Comyns, M.B. Richards, C. Meng, B. Priestley, H.H. Fernandez, F. Cambi, D.M. Umbach, A. Blair, D.P. Sandler, and J.W. Langston, *Rotenone, Paraquat and Parkinson's Disease.* Environ Health Perspect, 2011.

20. Abdulwahid Arif, I. and H. Ahmad Khan, *Environmental toxins and Parkinson's disease: putative roles of impaired electron transport chain and oxidative stress.* Toxicol Ind Health, 2010. **26**(2): p. 121-8.

21. Cicchetti, F., J. Drouin-Ouellet, and R.E. Gross, *Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models?* Trends Pharmacol Sci, 2009. **30**(9): p. 475-83.

22. Litvan, I., G. Halliday, M. Hallett, C.G. Goetz, W. Rocca, C. Duyckaerts, Y. Ben-Shlomo, D.W. Dickson, A.E. Lang, M.F. Chesselet, W.J. Langston, D.A. Di Monte, T. Gasser, T. Hagg, J. Hardy, P. Jenner, E. Melamed, R.H. Myers, D. Parker, Jr., and D.L. Price, *The etiopathogenesis of Parkinson disease and suggestions for future research. Part I.* J Neuropathol Exp Neurol, 2007. **66**(4): p. 251-7.

23. Polymeropoulos, M.H., J.J. Higgins, L.I. Golbe, W.G. Johnson, S.E. Ide, G. Di Iorio, G. Sanges, E.S. Stenroos, L.T. Pho, A.A. Schaffer, A.M. Lazzarini, R.L. Nussbaum, and R.C. Duvoisin, *Mapping of a gene for Parkinson's disease to chromosome 4q21-q23.* Science, 1996. **274**(5290): p. 1197-9.

24. Polymeropoulos, M.H., C. Lavedan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe, and R.L. Nussbaum, *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease.* Science, 1997. **276**(5321): p. 2045-7.

25. Kruger, R., W. Kuhn, T. Muller, D. Woitalla, M. Graeber, S. Kosel, H. Przuntek, J.T. Epplen, L. Schols, and O. Riess, *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease.* Nat Genet, 1998. **18**(2): p. 106-8.

26. Zarranz, J.J., J. Alegre, J.C. Gomez-Esteban, E. Lezcano, R. Ros, I. Ampuero, L. Vidal, J. Hoenicka, O. Rodriguez, B. Atares, V. Llorens, E. Gomez Tortosa, T. del Ser, D.G. Munoz, and J.G. de Yebenes, *The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia.* Ann Neurol, 2004. **55**(2): p. 164-73.

27. Bekris, L.M., I.F. Mata, and C.P. Zabetian, *The genetics of Parkinson disease.* J Geriatr Psychiatry Neurol, 2010. **23**(4): p. 228-42.

28. Magen, I. and M.F. Chesselet, *Genetic mouse models of Parkinson's disease The state of the art.* Prog Brain Res, 2010. **184**: p. 53-87.

29. Whitworth, A.J., *Drosophila models of Parkinson's disease.* Adv Genet, 2011. **73**: p. 1-50.

30. Horowitz, M.P. and J.T. Greenamyre, *Gene-environment interactions in Parkinson's disease: the importance of animal modeling.* Clin Pharmacol Ther, 2010. **88**(4): p. 467-74.

31. Feany, M.B., *New genetic insights into Parkinson's disease.* N Engl J Med, 2004. **351**(19): p. 1937-40.

32. Moore, D.J., A.B. West, V.L. Dawson, and T.M. Dawson, *Molecular pathophysiology of Parkinson's disease.* Annu Rev Neurosci, 2005. **28**: p. 57-87.

33. Tan, E.K. and L.M. Skipper, *Pathogenic mutations in Parkinson disease.* Hum Mutat, 2007. **28**(7): p. 641-53.

34. Forman, M.S., V.M. Lee, and J.Q. Trojanowski, *Nosology of Parkinson's disease: looking for the way out of a quagmire.* Neuron, 2005. **47**(4): p. 479-82.

35. Cookson, M.R., *The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease.* Nat Rev Neurosci, 2010. **11**(12): p. 791-7.

36. Greggio, E., M. Bisaglia, L. Civiero, and L. Bubacco, *Leucine-rich repeat kinase 2 and alpha-synuclein: intersecting pathways in the pathogenesis of Parkinson's disease?* Mol Neurodegener, 2011. **6**(1): p. 6.

37. Gitler, A.D., A. Chesi, M.L. Geddie, K.E. Strathearn, S. Hamamichi, K.J. Hill, K.A. Caldwell, G.A. Caldwell, A.A. Cooper, J.C. Rochet, and S. Lindquist, *Alpha-synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity.* Nat Genet, 2009. **41**(3): p. 308-15.

38. Giasson, B.I. and V.M. Lee, *Are ubiquitination pathways central to Parkinson's disease?* Cell, 2003. **114**(1): p. 1-8.

39. Hardy, J., *Genetic analysis of pathways to Parkinson disease.* Neuron, 2010. **68**(2): p. 201-6.

40. Maroteaux, L., J.T. Campanelli, and R.H. Scheller, *Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal.* J Neurosci, 1988. **8**(8): p. 2804-15.

41. Ueda, K., H. Fukushima, E. Masliah, Y. Xia, A. Iwai, M. Yoshimoto, D.A. Otero, J. Kondo, Y. Ihara, and T. Saitoh, *Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11282-6.

42. Jakes, R., M.G. Spillantini, and M. Goedert, *Identification of two distinct synucleins from human brain.* FEBS Lett, 1994. **345**(1): p. 27-32.

43. Spillantini, M.G., A. Divane, and M. Goedert, *Assignment of human alpha-synuclein (SNCA) and beta-synuclein (SNCB) genes to chromosomes 4q21 and 5q35.* Genomics, 1995. **27**(2): p. 379-81.

44. Chartier-Harlin, M.C., J. Kachergus, C. Roumier, V. Mouroux, X. Douay, S. Lincoln, C. Levecque, L. Larvor, J. Andrieux, M. Hulihan, N. Waucquier, L. Defebvre, P. Amouyel, M. Farrer, and A. Destee, *Alpha-synuclein locus duplication as a cause of familial Parkinson's disease.* Lancet, 2004. **364**(9440): p. 1167-9.

45. Singleton, A.B., M. Farrer, J. Johnson, A. Singleton, S. Hague, J. Kachergus, M. Hulihan, T. Peuralinna, A. Dutra, R. Nussbaum, S. Lincoln, A. Crawley, M. Hanson, D. Maraganore, C. Adler, M.R. Cookson, M. Muenter, M. Baptista, D. Miller, J. Blancato, J. Hardy, and K. Gwinn-Hardy, *alpha-Synuclein locus triplication causes Parkinson's disease.* Science, 2003. **302**(5646): p. 841.

46. Clayton, D.F. and J.M. George, *The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease.* Trends Neurosci, 1998. **21**(6): p. 249-54.

47. Senior, S.L., N. Ninkina, R. Deacon, D. Bannerman, V.L. Buchman, S.J. Cragg, and R. Wade-Martins, *Increased striatal dopamine release and hyperdopaminergic-like behaviour in mice lacking both alpha-synuclein and gamma-synuclein.* Eur J Neurosci, 2008. **27**(4): p. 947-57.

48. Robertson, D.C., O. Schmidt, N. Ninkina, P.A. Jones, J. Sharkey, and V.L. Buchman, *Developmental loss and resistance to MPTP toxicity of dopaminergic neurones in substantia nigra pars compacta of gamma-synuclein, alpha-synuclein and double alpha/gamma-synuclein null mutant mice.* J Neurochem, 2004. **89**(5): p. 1126-36.

49. Chandra, S., F. Fornai, H.B. Kwon, U. Yazdani, D. Atasoy, X. Liu, R.E. Hammer, G. Battaglia, D.C. German, P.E. Castillo, and T.C. Sudhof, *Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions.* Proc Natl Acad Sci U S A, 2004. **101**(41): p. 14966-71.

50. Greten-Harrison, B., M. Polydoro, M. Morimoto-Tomita, L. Diao, A.M. Williams, E.H. Nie, S. Makani, N. Tian, P.E. Castillo, V.L. Buchman, and S.S. Chandra,

alphabetagamma-Synuclein triple knockout mice reveal age-dependent neuronal dysfunction. Proc Natl Acad Sci U S A, 2010. **107**(45): p. 19573-8.

51. Hamilton, B.A., *alpha-Synuclein A53T substitution associated with Parkinson disease also marks the divergence of Old World and New World primates.* Genomics, 2004. **83**(4): p. 739-42.

52. Ostrerova, N., L. Petrucelli, M. Farrer, N. Mehta, P. Choi, J. Hardy, and B. Wolozin, *alpha-Synuclein shares physical and functional homology with 14-3-3 proteins.* J Neurosci, 1999. **19**(14): p. 5782-91.

53. Arawaka, S., M. Wada, S. Goto, H. Karube, M. Sakamoto, C.H. Ren, S. Koyama, H. Nagasawa, H. Kimura, T. Kawanami, K. Kurita, K. Tajima, M. Daimon, M. Baba, T. Kido, S. Saino, K. Goto, H. Asao, C. Kitanaka, E. Takashita, S. Hongo, T. Nakamura, T. Kayama, Y. Suzuki, K. Kobayashi, T. Katagiri, K. Kurokawa, M. Kurimura, I. Toyoshima, K. Niizato, K. Tsuchiya, T. Iwatsubo, M. Muramatsu, H. Matsumine, and T. Kato, *The role of G-protein-coupled receptor kinase 5 in pathogenesis of sporadic Parkinson's disease.* J Neurosci, 2006. **26**(36): p. 9227-38.

54. Pronin, A.N., A.J. Morris, A. Surguchov, and J.L. Benovic, *Synucleins are a novel class of substrates for G protein-coupled receptor kinases.* J Biol Chem, 2000. **275**(34): p. 26515-22.

55. Larsen, K.E., Y. Schmitz, M.D. Troyer, E. Mosharov, P. Dietrich, A.Z. Quazi, M. Savalle, V. Nemani, F.A. Chaudhry, R.H. Edwards, L. Stefanis, and D. Sulzer, *Alpha-synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis.* J Neurosci, 2006. **26**(46): p. 11915-22.

56. Mosharov, E.V., R.G. Staal, J. Bove, D. Prou, A. Hananiya, D. Markov, N. Poulsen, K.E. Larsen, C.M. Moore, M.D. Troyer, R.H. Edwards, S. Przedborski, and D. Sulzer, *Alpha-synuclein overexpression increases cytosolic catecholamine concentration.* J Neurosci, 2006. **26**(36): p. 9304-11.

57. George, J.M., H. Jin, W.S. Woods, and D.F. Clayton, *Characterization of a novel protein regulated during the critical period for song learning in the zebra finch.* Neuron, 1995. **15**(2): p. 361-72.

58. Cooper, A.A., A.D. Gitler, A. Cashikar, C.M. Haynes, K.J. Hill, B. Bhullar, K. Liu, K. Xu, K.E. Strathearn, F. Liu, S. Cao, K.A. Caldwell, G.A. Caldwell, G.

Marsischky, R.D. Kolodner, J. Labaer, J.C. Rochet, N.M. Bonini, and S. Lindquist, *Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models.* Science, 2006. **313**(5785): p. 324-8.

59. Lashuel, H.A. and H. Hirling, *Rescuing defective vesicular trafficking protects against alpha-synuclein toxicity in cellular and animal models of Parkinson's disease.* ACS Chem Biol, 2006. **1**(7): p. 420-4.

60. Auluck, P.K., G. Caraveo, and S. Lindquist, *alpha-Synuclein: membrane interactions and toxicity in Parkinson's disease.* Annu Rev Cell Dev Biol, 2010. **26**: p. 211-33.

61. Nemani, V.M., W. Lu, V. Berge, K. Nakamura, B. Onoa, M.K. Lee, F.A. Chaudhry, R.A. Nicoll, and R.H. Edwards, *Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis.* Neuron, 2010. **65**(1): p. 66-79.

62. Scott, D.A., I. Tabarean, Y. Tang, A. Cartier, E. Masliah, and S. Roy, *A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration.* J Neurosci, 2010. **30**(24): p. 8083-95.

63. Beyer, K., *Alpha-synuclein structure, posttranslational modification and alternative splicing as aggregation enhancers.* Acta Neuropathol, 2006. **112**(3): p. 237-51.

64. George, J.M., *The synucleins.* Genome Biol, 2002. **3**(1): p. REVIEWS3002.

65. Roy, S., *The paradoxical cell biology of alpha-Synucle.* Results Probl Cell Differ, 2009. **48**: p. 159-72.

66. Uversky, V.N., *A protein-chameleon: conformational plasticity of alpha-synuclein, a disordered protein involved in neurodegenerative disorders.* J Biomol Struct Dyn, 2003. **21**(2): p. 211-34.

67. Lee, V.M. and J.Q. Trojanowski, *Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery.* Neuron, 2006. **52**(1): p. 33-8.

68. Uversky, V.N., *Neuropathology, biochemistry, and biophysics of alpha-synuclein aggregation.* J Neurochem, 2007. **103**(1): p. 17-37.

69. Ahn, K.J., S.R. Paik, K.C. Chung, and J. Kim, *Amino acid sequence motifs and mechanistic features of the membrane translocation of alpha-synuclein.* J Neurochem, 2006. **97**(1): p. 265-79.

70. Desplats, P., H.J. Lee, E.J. Bae, C. Patrick, E. Rockenstein, L. Crews, B. Spencer, E. Masliah, and S.J. Lee, *Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 13010-5.

71. Ferreon, A.C., Y. Gambin, E.A. Lemke, and A.A. Deniz, *Interplay of alpha-synuclein binding and conformational switching probed by single-molecule fluorescence.* Proc Natl Acad Sci U S A, 2009. **106**(14): p. 5645-50.

72. Bussell, R., Jr., T.F. Ramlall, and D. Eliezer, *Helix periodicity, topology, and dynamics of membrane-associated alpha-synuclein.* Protein Sci, 2005. **14**(4): p. 862-72.

73. Davidson, W.S., A. Jonas, D.F. Clayton, and J.M. George, *Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes.* J Biol Chem, 1998. **273**(16): p. 9443-9.

74. Fredenburg, R.A., C. Rospigliosi, R.K. Meray, J.C. Kessler, H.A. Lashuel, D. Eliezer, and P.T. Lansbury, Jr., *The impact of the E46K mutation on the properties of alpha-synuclein in its monomeric and oligomeric states.* Biochemistry, 2007. **46**(24): p. 7107-18.

75. Spillantini, M.G., M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, and M. Goedert, *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.

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

77. Aizenstein, H.J., R.D. Nebes, J.A. Saxton, J.C. Price, C.A. Mathis, N.D. Tsopelas, S.K. Ziolko, J.A. James, B.E. Snitz, P.R. Houck, W. Bi, A.D. Cohen, B.J. Lopresti, S.T. DeKosky, E.M. Halligan, and W.E. Klunk, *Frequent amyloid deposition without significant cognitive impairment among the elderly.* Arch Neurol, 2008. **65**(11): p. 1509-17.

78. Hindle, J.V., *Ageing, neurodegeneration and Parkinson's disease.* Age Ageing, 2010. **39**(2): p. 156-61.

79. Bennett, M.C., *The role of alpha-synuclein in neurodegenerative diseases.* Pharmacol Ther, 2005. **105**(3): p. 311-31.

80. de la Fuente-Fernandez, R., M. Schulzer, E. Mak, A. Kishore, and D.B. Calne, *The role of the Lewy body in idiopathic Parkinsonism.* Parkinsonism Relat Disord, 1998. **4**(2): p. 73-7.

81. Winner, B., R. Jappelli, S.K. Maji, P.A. Desplats, L. Boyer, S. Aigner, C. Hetzer, T. Loher, M. Vilar, S. Campioni, C. Tzitzilonis, A. Soragni, S. Jessberger, H. Mira, A. Consiglio, E. Pham, E. Masliah, F.H. Gage, and R. Riek, *In vivo demonstration that {alpha}-synuclein oligomers are toxic.* Proc Natl Acad Sci U S A, 2011. **108**(10): p. 4194-9.

82. Cookson, M.R., *The biochemistry of Parkinson's disease.* Annu Rev Biochem, 2005. **74**: p. 29-52.

83. Khurana, R., C. Ionescu-Zanetti, M. Pope, J. Li, L. Nielson, M. Ramirez-Alvarado, L. Regan, A.L. Fink, and S.A. Carter, *A general model for amyloid fibril assembly based on morphological studies using atomic force microscopy.* Biophys J, 2003. **85**(2): p. 1135-44.

84. Luk, K.C., C. Song, P. O'Brien, A. Stieber, J.R. Branch, K.R. Brunden, J.Q. Trojanowski, and V.M. Lee, *Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells.* Proc Natl Acad Sci U S A, 2009. **106**(47): p. 20051-6.

85. Angot, E., J.A. Steiner, C. Hansen, J.Y. Li, and P. Brundin, *Are synucleinopathies prion-like disorders?* Lancet Neurol, 2010. **9**(11): p. 1128-38.

86. Smith, W.W., R.L. Margolis, X. Li, J.C. Troncoso, M.K. Lee, V.L. Dawson, T.M. Dawson, T. Iwatsubo, and C.A. Ross, *Alpha-synuclein phosphorylation enhances eosinophilic cytoplasmic inclusion formation in SH-SY5Y cells.* J Neurosci, 2005. **25**(23): p. 5544-52.

87. Okochi, M., J. Walter, A. Koyama, S. Nakajo, M. Baba, T. Iwatsubo, L. Meijer, P.J. Kahle, and C. Haass, *Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein.* J Biol Chem, 2000. **275**(1): p. 390-7.

88. Hasegawa, M., H. Fujiwara, T. Nonaka, K. Wakabayashi, H. Takahashi, V.M. Lee, J.Q. Trojanowski, D. Mann, and T. Iwatsubo, *Phosphorylated alpha-synuclein is ubiquitinated in alpha-synucleinopathy lesions.* J Biol Chem, 2002. **277**(50): p. 49071-6.

89. Fujiwara, H., M. Hasegawa, N. Dohmae, A. Kawashima, E. Masliah, M.S. Goldberg, J. Shen, K. Takio, and T. Iwatsubo, *alpha-Synuclein is phosphorylated in synucleinopathy lesions.* Nat Cell Biol, 2002. **4**(2): p. 160-4.

90. Chen, L. and M.B. Feany, *Alpha-synuclein phosphorylation controls neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease.* Nat Neurosci, 2005. **8**(5): p. 657-63.

91. Anderson, J.P., D.E. Walker, J.M. Goldstein, R. de Laat, K. Banducci, R.J. Caccavello, R. Barbour, J. Huang, K. Kling, M. Lee, L. Diep, P.S. Keim, X. Shen, T. Chataway, M.G. Schlossmacher, P. Seubert, D. Schenk, S. Sinha, W.P. Gai, and T.J. Chilcote, *Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease.* J Biol Chem, 2006. **281**(40): p. 29739-52.

92. Nakamura, T., H. Yamashita, T. Takahashi, and S. Nakamura, *Activated Fyn phosphorylates alpha-synuclein at tyrosine residue 125.* Biochem Biophys Res Commun, 2001. **280**(4): p. 1085-92.

93. Ellis, C.E., P.L. Schwartzberg, T.L. Grider, D.W. Fink, and R.L. Nussbaum, *alpha-synuclein is phosphorylated by members of the Src family of protein-tyrosine kinases.* J Biol Chem, 2001. **276**(6): p. 3879-84.

94. Negro, A., A.M. Brunati, A. Donella-Deana, M.L. Massimino, and L.A. Pinna, *Multiple phosphorylation of alpha-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation.* FASEB J, 2002. **16**(2): p. 210-2.

95. Chen, L., M. Periquet, X. Wang, A. Negro, P.J. McLean, B.T. Hyman, and M.B. Feany, *Tyrosine and serine phosphorylation of alpha-synuclein have opposing effects on neurotoxicity and soluble oligomer formation.* J Clin Invest, 2009. **119**(11): p. 3257-65.

96. Li, W., N. West, E. Colla, O. Pletnikova, J.C. Troncoso, L. Marsh, T.M. Dawson, P. Jakala, T. Hartmann, D.L. Price, and M.K. Lee, *Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is*

enhanced by the familial Parkinson's disease-linked mutations. Proc Natl Acad Sci U S A, 2005. **102**(6): p. 2162-7.

97. Liu, C.W., B.I. Giasson, K.A. Lewis, V.M. Lee, G.N. Demartino, and P.J. Thomas, *A precipitating role for truncated alpha-synuclein and the proteasome in alpha-synuclein aggregation: implications for pathogenesis of Parkinson disease.* J Biol Chem, 2005. **280**(24): p. 22670-8.

98. Hoyer, W., D. Cherny, V. Subramaniam, and T.M. Jovin, *Impact of the acidic C-terminal region comprising amino acids 109-140 on alpha-synuclein aggregation in vitro.* Biochemistry, 2004. **43**(51): p. 16233-42.

99. McClendon, S., C.C. Rospigliosi, and D. Eliezer, *Charge neutralization and collapse of the C-terminal tail of alpha-synuclein at low pH.* Protein Sci, 2009. **18**(7): p. 1531-40.

100. Wu, K.P., D.S. Weinstock, C. Narayanan, R.M. Levy, and J. Baum, *Structural reorganization of alpha-synuclein at low pH observed by NMR and REMD simulations.* J Mol Biol, 2009. **391**(4): p. 784-96.

101. Trexler, A.J. and E. Rhoades, *Single molecule characterization of alpha-synuclein in aggregation-prone states.* Biophys J, 2010. **99**(9): p. 3048-55.

102. Meuvis, J., M. Gerard, L. Desender, V. Baekelandt, and Y. Engelborghs, *The conformation and the aggregation kinetics of alpha-synuclein depend on the proline residues in its C-terminal region.* Biochemistry, 2010. **49**(43): p. 9345-52.

103. Klucken, J., T.F. Outeiro, P. Nguyen, P.J. McLean, and B.T. Hyman, *Detection of novel intracellular alpha-synuclein oligomeric species by fluorescence lifetime imaging.* FASEB J, 2006. **20**(12): p. 2050-7.

104. Zhou, W., J. Schaack, W.M. Zawada, and C.R. Freed, *Overexpression of human alpha-synuclein causes dopamine neuron death in primary human mesencephalic culture.* Brain Res, 2002. **926**(1-2): p. 42-50.

105. Kim, S.J., S.Y. Kim, Y.S. Na, H.J. Lee, K.C. Chung, and J.H. Baik, *Alpha-synuclein enhances dopamine D2 receptor signaling.* Brain Res, 2006. **1124**(1): p. 5-9.

106. Mazzulli, J.R., M. Armakola, M. Dumoulin, I. Parastatidis, and H. Ischiropoulos, *Cellular oligomerization of alpha-synuclein is determined by the interaction of oxidized catechols with a C-terminal sequence.* J Biol Chem, 2007. **282**(43): p. 31621-30.

107. Chan, C.S., J.N. Guzman, E. Ilijic, J.N. Mercer, C. Rick, T. Tkatch, G.E. Meredith, and D.J. Surmeier, *'Rejuvenation' protects neurons in mouse models of Parkinson's disease.* Nature, 2007. **447**(7148): p. 1081-6.

108. Nath, S., J. Goodwin, Y. Engelborghs, and D.L. Pountney, *Raised calcium promotes alpha-synuclein aggregate formation.* Mol Cell Neurosci, 2011. **46**(2): p. 516-26.

109. Matsuda, W., T. Furuta, K.C. Nakamura, H. Hioki, F. Fujiyama, R. Arai, and T. Kaneko, *Single nigrostriatal dopaminergic neurons form widely spread and highly dense axonal arborizations in the neostriatum.* J Neurosci, 2009. **29**(2): p. 444-53.

110. Roy, S., B. Zhang, V.M. Lee, and J.Q. Trojanowski, *Axonal transport defects: a common theme in neurodegenerative diseases.* Acta Neuropathol, 2005. **109**(1): p. 5-13.

111. De Vos, K.J., A.J. Grierson, S. Ackerley, and C.C. Miller, *Role of axonal transport in neurodegenerative diseases.* Annu Rev Neurosci, 2008. **31**: p. 151-73.

112. Perlson, E., S. Maday, M.M. Fu, A.J. Moughamian, and E.L. Holzbaur, *Retrograde axonal transport: pathways to cell death?* Trends Neurosci, 2010. **33**(7): p. 335-44.

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

114. Brady, S.T., *Molecular motors in the nervous system.* Neuron, 1991. **7**(4): p. 521-33.

115. Duncan, J.E. and L.S. Goldstein, *The genetics of axonal transport and axonal transport disorders.* PLoS Genet, 2006. **2**(9): p. e124.

116. Gindhart, J.G., *Towards an understanding of kinesin-1 dependent transport pathways through the study of protein-protein interactions.* Brief Funct Genomic Proteomic, 2006. **5**(1): p. 74-86.

117. Hirokawa, N., S. Niwa, and Y. Tanaka, *Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease.* Neuron, 2010. **68**(4): p. 610-38.

118. Hirokawa, N. and R. Takemura, *Molecular motors and mechanisms of directional transport in neurons.* Nat Rev Neurosci, 2005. **6**(3): p. 201-14.

119. LaMonte, B.H., K.E. Wallace, B.A. Holloway, S.S. Shelly, J. Ascano, M. Tokito, T. Van Winkle, D.S. Howland, and E.L. Holzbaur, *Disruption of dynein/dynactin inhibits axonal transport in motor neurons causing late-onset progressive degeneration.* Neuron, 2002. **34**(5): p. 715-27.

120. Morfini, G., G. Pigino, U. Beffert, J. Busciglio, and S.T. Brady, *Fast axonal transport misregulation and Alzheimer's disease.* Neuromolecular Med, 2002. **2**(2): p. 89-99.

121. Morfini, G., G. Pigino, and S.T. Brady, *Polyglutamine expansion diseases: failing to deliver.* Trends Mol Med, 2005. **11**(2): p. 64-70.

122. McEwen, B.S. and B. Grafstein, *Fast and slow components in axonal transport of protein.* J Cell Biol, 1968. **38**(3): p. 494-508.

123. Dahlstrom, A., *Axoplasmic transport (with particular respect to adrenergic neurons).* Philos Trans R Soc Lond B Biol Sci, 1971. **261**(839): p. 325-58.

124. Ochs, S., *Effect of maturation and aging on the rate of fast axoplasmic transport in mammalian nerve.* Prog Brain Res, 1973. **40**(0): p. 349-62.

125. Terada, S., *Where does slow axonal transport go?* Neurosci Res, 2003. **47**(4): p. 367-72.

126. Brown, A., L. Wang, and P. Jung, *Stochastic simulation of neurofilament transport in axons: the "stop-and-go" hypothesis.* Mol Biol Cell, 2005. **16**(9): p. 4243-55.

127. Brown, A., *Axonal transport of membranous and nonmembranous cargoes: a unified perspective.* J Cell Biol, 2003. **160**(6): p. 817-21.

128. Heidemann, S.R., J.M. Landers, and M.A. Hamborg, *Polarity orientation of axonal microtubules.* J Cell Biol, 1981. **91**(3 Pt 1): p. 661-5.

129. Brady, S.T., *A novel brain ATPase with properties expected for the fast axonal transport motor.* Nature, 1985. **317**(6032): p. 73-5.

130. Vale, R.D., T.S. Reese, and M.P. Sheetz, *Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility.* Cell, 1985. **42**(1): p. 39-50.

131. Miki, H., Y. Okada, and N. Hirokawa, *Analysis of the kinesin superfamily: insights into structure and function.* Trends Cell Biol, 2005. **15**(9): p. 467-76.

132. Diefenbach, R.J., J.P. Mackay, P.J. Armati, and A.L. Cunningham, *The C-terminal region of the stalk domain of ubiquitous human kinesin heavy chain contains the binding site for kinesin light chain.* Biochemistry, 1998. **37**(47): p. 16663-70.

133. Hirokawa, N., K.K. Pfister, H. Yorifuji, M.C. Wagner, S.T. Brady, and G.S. Bloom, *Submolecular domains of bovine brain kinesin identified by electron microscopy and monoclonal antibody decoration.* Cell, 1989. **56**(5): p. 867-78.

134. Hirokawa, N., Y. Noda, Y. Tanaka, and S. Niwa, *Kinesin superfamily motor proteins and intracellular transport.* Nat Rev Mol Cell Biol, 2009. **10**(10): p. 682-96.

135. Hook, P. and R.B. Vallee, *The dynein family at a glance.* J Cell Sci, 2006. **119**(Pt 21): p. 4369-71.

136. Pfister, K.K., P.R. Shah, H. Hummerich, A. Russ, J. Cotton, A.A. Annuar, S.M. King, and E.M. Fisher, *Genetic analysis of the cytoplasmic dynein subunit families.* PLoS Genet, 2006. **2**(1): p. e1.

137. Hook, P., *The mechanical components of the dynein motor.* ScientificWorldJournal, 2010. **10**: p. 857-64.

138. Carter, A.P., C. Cho, L. Jin, and R.D. Vale, *Crystal structure of the dynein motor domain.* Science, 2011. **331**(6021): p. 1159-65.

139. Kardon, J.R. and R.D. Vale, *Regulators of the cytoplasmic dynein motor.* Nat Rev Mol Cell Biol, 2009. **10**(12): p. 854-65.

140. Yamada, K., C. Andrews, W.M. Chan, C.A. McKeown, A. Magli, T. de Berardinis, A. Loewenstein, M. Lazar, M. O'Keefe, R. Letson, A. London, M. Ruttum, N. Matsumoto, N. Saito, L. Morris, M. Del Monte, R.H. Johnson, E. Uyama, W.A. Houtman, B. de Vries, T.J. Carlow, B.L. Hart, N. Krawiecki, J. Shoffner, M.C. Vogel, J. Katowitz, S.M. Goldstein, A.V. Levin, E.C. Sener, B.T. Ozturk, A.N. Akarsu, M.C. Brodsky, F. Hanisch, R.P. Cruse, A.A. Zubcov, R.M. Robb, P. Roggenkaemper, I. Gottlob, L. Kowal, R. Battu, E.I. Traboulsi, P. Franceschini, A. Newlin, J.L. Demer, and E.C. Engle, *Heterozygous mutations of the kinesin KIF21A in congenital fibrosis of the extraocular muscles type 1 (CFEOM1).* Nat Genet, 2003. **35**(4): p. 318-21.

141. Traboulsi, E.I. and E.C. Engle, *Mutations in KIF21A are responsible for CFEOM1 worldwide.* Ophthalmic Genet, 2004. **25**(4): p. 237-9.

142. Fichera, M., M. Lo Giudice, M. Falco, M. Sturnio, S. Amata, O. Calabrese, S. Bigoni, E. Calzolari, and M. Neri, *Evidence of kinesin heavy chain (KIF5A) involvement in pure hereditary spastic paraplegia.* Neurology, 2004. **63**(6): p. 1108-10.

143. Reid, E., M. Kloos, A. Ashley-Koch, L. Hughes, S. Bevan, I.K. Svenson, F.L. Graham, P.C. Gaskell, A. Dearlove, M.A. Pericak-Vance, D.C. Rubinsztein, and D.A. Marchuk, *A kinesin heavy chain (KIF5A) mutation in hereditary spastic paraplegia (SPG10).* Am J Hum Genet, 2002. **71**(5): p. 1189-94.

144. Puls, I., C. Jonnakuty, B.H. LaMonte, E.L. Holzbaur, M. Tokito, E. Mann, M.K. Floeter, K. Bidus, D. Drayna, S.J. Oh, R.H. Brown, Jr., C.L. Ludlow, and K.H. Fischbeck, *Mutant dynactin in motor neuron disease.* Nat Genet, 2003. **33**(4): p. 455-6.

145. Hafezparast, M., R. Klocke, C. Ruhrberg, A. Marquardt, A. Ahmad-Annuar, S. Bowen, G. Lalli, A.S. Witherden, H. Hummerich, S. Nicholson, P.J. Morgan, R. Oozageer, J.V. Priestley, S. Averill, V.R. King, S. Ball, J. Peters, T. Toda, A. Yamamoto, Y. Hiraoka, M. Augustin, D. Korthaus, S. Wattler, P. Wabnitz, C. Dickneite, S. Lampel, F. Boehme, G. Peraus, A. Popp, M. Rudelius, J. Schlegel, H. Fuchs, M. Hrabe de Angelis, G. Schiavo, D.T. Shima, A.P. Russ, G. Stumm,

J.E. Martin, and E.M. Fisher, *Mutations in dynein link motor neuron degeneration to defects in retrograde transport.* Science, 2003. **300**(5620): p. 808-12.

146. Morfini, G., G. Szebenyi, B. Richards, and S.T. Brady, *Regulation of kinesin: implications for neuronal development.* Dev Neurosci, 2001. **23**(4-5): p. 364-76.

147. Morfini, G., G. Szebenyi, R. Elluru, N. Ratner, and S.T. Brady, *Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility.* EMBO J, 2002. **21**(3): p. 281-93.

148. Pigino, G., G. Morfini, A. Pelsman, M.P. Mattson, S.T. Brady, and J. Busciglio, *Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport.* J Neurosci, 2003. **23**(11): p. 4499-508.

149. Morfini, G., G. Szebenyi, H. Brown, H.C. Pant, G. Pigino, S. DeBoer, U. Beffert, and S.T. Brady, *A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons.* EMBO J, 2004. **23**(11): p. 2235-45.

150. Mandelkow, E.M., K. Stamer, R. Vogel, E. Thies, and E. Mandelkow, *Clogging of axons by tau, inhibition of axonal traffic and starvation of synapses.* Neurobiol Aging, 2003. **24**(8): p. 1079-85.

151. Stokin, G.B., C. Lillo, T.L. Falzone, R.G. Brusch, E. Rockenstein, S.L. Mount, R. Raman, P. Davies, E. Masliah, D.S. Williams, and L.S. Goldstein, *Axonopathy and transport deficits early in the pathogenesis of Alzheimer's disease.* Science, 2005. **307**(5713): p. 1282-8.

152. Allen, R.D., J. Metuzals, I. Tasaki, S.T. Brady, and S.P. Gilbert, *Fast axonal transport in squid giant axon.* Science, 1982. **218**(4577): p. 1127-9.

153. Brady, S.T., R.J. Lasek, and R.D. Allen, *Fast axonal transport in extruded axoplasm from squid giant axon.* Science, 1982. **218**(4577): p. 1129-31.

154. Brady, S.T., R.J. Lasek, and R.D. Allen, *Video microscopy of fast axonal transport in extruded axoplasm: a new model for study of molecular mechanisms.* Cell Motil, 1985. **5**(2): p. 81-101.

155. Schroer, T.A., S.T. Brady, and R.B. Kelly, *Fast axonal transport of foreign synaptic vesicles in squid axoplasm.* J Cell Biol, 1985. **101**(2): p. 568-72.

156. Brady, S.T., B.W. Richards, and P.L. Leopold, *Assay of vesicle motility in squid axoplasm.* Methods Cell Biol, 1993. **39**: p. 191-202.

157. Parker, P.J. and J. Murray-Rust, *PKC at a glance.* J Cell Sci, 2004. **117**(Pt 2): p. 131-2.

158. Morfini, G., G. Pigino, K. Opalach, Y. Serulle, J.E. Moreira, M. Sugimori, R.R. Llinas, and S.T. Brady, *1-Methyl-4-phenylpyridinium affects fast axonal transport by activation of caspase and protein kinase C.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2442-7.

159. Serulle, Y., G. Morfini, G. Pigino, J.E. Moreira, M. Sugimori, S.T. Brady, and R.R. Llinas, *1-Methyl-4-phenylpyridinium induces synaptic dysfunction through a pathway involving caspase and PKCdelta enzymatic activities.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2437-41.

160. Beffert, U., G. Morfini, H.H. Bock, H. Reyna, S.T. Brady, and J. Herz, *Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta.* J Biol Chem, 2002. **277**(51): p. 49958-64.

161. Beffert, U., E.J. Weeber, G. Morfini, J. Ko, S.T. Brady, L.H. Tsai, J.D. Sweatt, and J. Herz, *Reelin and cyclin-dependent kinase 5-dependent signals cooperate in regulating neuronal migration and synaptic transmission.* J Neurosci, 2004. **24**(8): p. 1897-906.

162. Robinson, D.R., Y.M. Wu, and S.F. Lin, *The protein tyrosine kinase family of the human genome.* Oncogene, 2000. **19**(49): p. 5548-57.

163. Boggon, T.J. and M.J. Eck, *Structure and regulation of Src family kinases.* Oncogene, 2004. **23**(48): p. 7918-27.

164. Hubbard, S.R. and J.H. Till, *Protein tyrosine kinase structure and function.* Annu Rev Biochem, 2000. **69**: p. 373-98.

165. Aleshin, A. and R.S. Finn, *SRC: a century of science brought to the clinic.* Neoplasia, 2010. **12**(8): p. 599-607.

166. Necula, M., C.N. Chirita, and J. Kuret, *Rapid anionic micelle-mediated alpha-synuclein fibrillization in vitro.* J Biol Chem, 2003. **278**(47): p. 46674-80.

167. Gschwendt, M., S. Dieterich, J. Rennecke, W. Kittstein, H.J. Mueller, and F.J. Johannes, *Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes.* FEBS Lett, 1996. **392**(2): p. 77-80.

168. Johannes, F.J., J. Prestle, S. Eis, P. Oberhagemann, and K. Pfizenmaier, *PKCu is a novel, atypical member of the protein kinase C family.* J Biol Chem, 1994. **269**(8): p. 6140-8.

169. Rozengurt, E., O. Rey, and R.T. Waldron, *Protein kinase D signaling.* J Biol Chem, 2005. **280**(14): p. 13205-8.

170. Jamora, C., N. Yamanouye, J. Van Lint, J. Laudenslager, J.R. Vandenheede, D.J. Faulkner, and V. Malhotra, *Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D.* Cell, 1999. **98**(1): p. 59-68.

171. Liljedahl, M., Y. Maeda, A. Colanzi, I. Ayala, J. Van Lint, and V. Malhotra, *Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network.* Cell, 2001. **104**(3): p. 409-20.

172. Wang, Q.J., *PKD at the crossroads of DAG and PKC signaling.* Trends Pharmacol Sci, 2006. **27**(6): p. 317-23.

173. Zhou, J., M. Broe, Y. Huang, J.P. Anderson, W.P. Gai, E.A. Milward, M. Porritt, D. Howells, A.J. Hughes, X. Wang, and G.M. Halliday, *Changes in the solubility and phosphorylation of alpha-synuclein over the course of Parkinson's disease.* Acta Neuropathol, 2011.

174. Huang, W. and R.L. Erikson, *Constitutive activation of Mek1 by mutation of serine phosphorylation sites.* Proc Natl Acad Sci U S A, 1994. **91**(19): p. 8960-3.

175. Leger, J., M. Kempf, G. Lee, and R. Brandt, *Conversion of serine to aspartate imitates phosphorylation-induced changes in the structure and function of microtubule-associated protein tau.* J Biol Chem, 1997. **272**(13): p. 8441-6.

176. Egelhoff, T.T., R.J. Lee, and J.A. Spudich, *Dictyostelium myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization in vivo.* Cell, 1993. **75**(2): p. 363-71.

177. Filippakopoulos, P., S. Muller, and S. Knapp, *SH2 domains: modulators of nonreceptor tyrosine kinase activity.* Curr Opin Struct Biol, 2009. **19**(6): p. 643-9.

178. Machida, K. and B.J. Mayer, *The SH2 domain: versatile signaling module and pharmaceutical target.* Biochim Biophys Acta, 2005. **1747**(1): p. 1-25.

179. Tobias, J.W., T.E. Shrader, G. Rocap, and A. Varshavsky, *The N-end rule in bacteria.* Science, 1991. **254**(5036): p. 1374-7.

180. Shrader, T.E., J.W. Tobias, and A. Varshavsky, *The N-end rule in Escherichia coli: cloning and analysis of the leucyl, phenylalanyl-tRNA-protein transferase gene aat.* J Bacteriol, 1993. **175**(14): p. 4364-74.

181. McLeod, M., M. Stein, and D. Beach, *The product of the mei3+ gene, expressed under control of the mating-type locus, induces meiosis and sporulation in fission yeast.* EMBO J, 1987. **6**(3): p. 729-36.

182. Perrin, R.J., W.S. Woods, D.F. Clayton, and J.M. George, *Exposure to long chain polyunsaturated fatty acids triggers rapid multimerization of synucleins.* J Biol Chem, 2001. **276**(45): p. 41958-62.

183. Storz, P., H. Doppler, F.J. Johannes, and A. Toker, *Tyrosine phosphorylation of protein kinase D in the pleckstrin homology domain leads to activation.* J Biol Chem, 2003. **278**(20): p. 17969-76.

184. Storz, P. and A. Toker, *Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway.* EMBO J, 2003. **22**(1): p. 109-20.

185. Bjorge, J.D., A. Jakymiw, and D.J. Fujita, *Selected glimpses into the activation and function of Src kinase.* Oncogene, 2000. **19**(49): p. 5620-35.

186. Heise, H., W. Hoyer, S. Becker, O.C. Andronesi, D. Riedel, and M. Baldus, *Molecular-level secondary structure, polymorphism, and dynamics of full-length alpha-synuclein fibrils studied by solid-state NMR.* Proc Natl Acad Sci U S A, 2005. **102**(44): p. 15871-6.

187. Takeda, A., M. Hashimoto, M. Mallory, M. Sundsumo, L. Hansen, and E. Masliah, *C-terminal alpha-synuclein immunoreactivity in structures other than*

Lewy bodies in neurodegenerative disorders. Acta Neuropathol, 2000. **99**(3): p. 296-304.

188. Taylor, T.N., J.G. Greene, and G.W. Miller, *Behavioral phenotyping of mouse models of Parkinson's disease.* Behav Brain Res, 2010. **211**(1): p. 1-10.

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

190. Shastry, P., A. Basu, and M.S. Rajadhyaksha, *Neuroblastoma cell lines--a versatile in vitro model in neurobiology.* Int J Neurosci, 2001. **108**(1-2): p. 109-26.

191. Castoldi, M. and A.V. Popov, *Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer.* Protein Expr Purif, 2003. **32**(1): p. 83-8.

192. Prasad, K.N., E.D. Clarkson, F.G. La Rosa, J. Edwards-Prasad, and C.R. Freed, *Efficacy of grafted immortalized dopamine neurons in an animal model of parkinsonism: a review.* Mol Genet Metab, 1998. **65**(1): p. 1-9.

193. Adams, F.S., F.G. La Rosa, S. Kumar, J. Edwards-Prasad, S. Kentroti, A. Vernadakis, C.R. Freed, and K.N. Prasad, *Characterization and transplantation of two neuronal cell lines with dopaminergic properties.* Neurochem Res, 1996. **21**(5): p. 619-27.

194. Mena, M.A., J. Garcia de Yebenes, A. Dwork, S. Fahn, N. Latov, J. Herbert, E. Flaster, and D. Slonim, *Biochemical properties of monoamine-rich human neuroblastoma cells.* Brain Res, 1989. **486**(2): p. 286-96.

195. Handler, A., M.D. Lobo, F.J. Alonso, C.L. Paino, and M.A. Mena, *Functional implications of the noradrenergic-cholinergic switch induced by retinoic acid in NB69 neuroblastoma cells.* J Neurosci Res, 2000. **60**(3): p. 311-20.

196. Ikeda, H., A. Pastuszko, N. Ikegaki, R.H. Kennett, and D.F. Wilson, *3,4-dihydroxyphenylalanine (dopa) metabolism and retinoic acid induced differentiation in human neuroblastoma.* Neurochem Res, 1994. **19**(12): p. 1487-94.

197. Ross, R.A., J.L. Biedler, and B.A. Spengler, *A role for distinct cell types in determining malignancy in human neuroblastoma cell lines and tumors.* Cancer Lett, 2003. **197**(1-2): p. 35-9.

198. Sidhu, A. and P.H. Fishman, *Identification and characterization of functional D1 dopamine receptors in a human neuroblastoma cell line.* Biochem Biophys Res Commun, 1990. **166**(2): p. 574-9.

199. Biedler, J.L., L. Helson, and B.A. Spengler, *Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture.* Cancer Res, 1973. **33**(11): p. 2643-52.

200. Jain, P., M.A. Cerone, A.C. Leblanc, and C. Autexier, *Telomerase and neuronal marker status of differentiated NT2 and SK-N-SH human neuronal cells and primary human neurons.* J Neurosci Res, 2007. **85**(1): p. 83-9.

201. Biedler, J.L., S. Roffler-Tarlov, M. Schachner, and L.S. Freedman, *Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones.* Cancer Res, 1978. **38**(11 Pt 1): p. 3751-7.

202. Encinas, M., M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Cena, C. Gallego, and J.X. Comella, *Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells.* J Neurochem, 2000. **75**(3): p. 991-1003.

203. Avila, D.M., D.R. Allman, J.M. Gallo, and M.J. McPhaul, *Androgen receptors containing expanded polyglutamine tracts exhibit progressive toxicity when stably expressed in the neuroblastoma cell line, SH-SY 5Y.* Exp Biol Med (Maywood), 2003. **228**(8): p. 982-90.

204. Szebenyi, G., G.A. Morfini, A. Babcock, M. Gould, K. Selkoe, D.L. Stenoien, M. Young, P.W. Faber, M.E. MacDonald, M.J. McPhaul, and S.T. Brady, *Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport.* Neuron, 2003. **40**(1): p. 41-52.

205. Morfini, G., G. Pigino, G. Szebenyi, Y. You, S. Pollema, and S.T. Brady, *JNK mediates pathogenic effects of polyglutamine-expanded androgen receptor on fast axonal transport.* Nat Neurosci, 2006. **9**(7): p. 907-16.

206. Voigt, A. and F. Zintl, *Effects of retinoic acid on proliferation, apoptosis, cytotoxicity, migration, and invasion of neuroblastoma cells.* Med Pediatr Oncol, 2003. **40**(4): p. 205-13.

207. Hasegawa, T., M. Matsuzaki, A. Takeda, A. Kikuchi, H. Akita, G. Perry, M.A. Smith, and Y. Itoyama, *Accelerated alpha-synuclein aggregation after differentiation of SH-SY5Y neuroblastoma cells.* Brain Res, 2004. **1013**(1): p. 51-9.

208. Hirokawa, N. and Y. Noda, *Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics.* Physiol Rev, 2008. **88**(3): p. 1089-118.

209. Hirokawa, N., *Kinesin and dynein superfamily proteins and the mechanism of organelle transport.* Science, 1998. **279**(5350): p. 519-26.

210. Parke, C.L., E.J. Wojcik, S. Kim, and D.K. Worthylake, *ATP hydrolysis in Eg5 kinesin involves a catalytic two-water mechanism.* J Biol Chem, 2010. **285**(8): p. 5859-67.

211. Miura, M., A. Matsubara, T. Kobayashi, M. Edamatsu, and Y.Y. Toyoshima, *Nucleotide-dependent behavior of single molecules of cytoplasmic dynein on microtubules in vitro.* FEBS Lett, 2010. **584**(11): p. 2351-5.

212. Michelsen, U. and J. von Hagen, *Isolation of subcellular organelles and structures.* Methods Enzymol, 2009. **463**: p. 305-28.

213. Matthews, S.A., E. Rozengurt, and D. Cantrell, *Characterization of serine 916 as an in vivo autophosphorylation site for protein kinase D/Protein kinase Cmu.* J Biol Chem, 1999. **274**(37): p. 26543-9.

214. Johannes, F.J., J. Prestle, S. Dieterich, P. Oberhagemann, G. Link, and K. Pfizenmaier, *Characterization of activators and inhibitors of protein kinase C mu.* Eur J Biochem, 1995. **227**(1-2): p. 303-7.

215. Zhukova, E., J. Sinnett-Smith, and E. Rozengurt, *Protein kinase D potentiates DNA synthesis and cell proliferation induced by bombesin, vasopressin, or phorbol esters in Swiss 3T3 cells.* J Biol Chem, 2001. **276**(43): p. 40298-305.

216. Rennecke, J., P.A. Rehberger, G. Furstenberger, F.J. Johannes, M. Stohr, F. Marks, and K.H. Richter, *Protein-kinase-Cmu expression correlates with enhanced keratinocyte proliferation in normal and neoplastic mouse epidermis and in cell culture.* Int J Cancer, 1999. **80**(1): p. 98-103.

217. Rennecke, J., F.J. Johannes, K.H. Richter, W. Kittstein, F. Marks, and M. Gschwendt, *Immunological demonstration of protein kinase C mu in murine tissues and various cell lines. Differential recognition of phosphorylated forms and lack of down-regulation upon 12-O-tetradecanoylphorphol-13-acetate treatment of cells.* Eur J Biochem, 1996. **242**(2): p. 428-32.

218. Xilouri, M. and L. Stefanis, *Autophagic pathways in Parkinson disease and related disorders.* Expert Rev Mol Med, 2011. **13**: p. e8.

219. Cuervo, A.M., E.S. Wong, and M. Martinez-Vicente, *Protein degradation, aggregation, and misfolding.* Mov Disord, 2010. **25 Suppl 1**: p. S49-54.

220. Ferrucci, M., L. Pasquali, S. Ruggieri, A. Paparelli, and F. Fornai, *Alpha-synuclein and autophagy as common steps in neurodegeneration.* Parkinsonism Relat Disord, 2008. **14 Suppl 2**: p. S180-4.

221. Guha, S., S. Tanasanvimon, J. Sinnett-Smith, and E. Rozengurt, *Role of protein kinase D signaling in pancreatic cancer.* Biochem Pharmacol, 2010. **80**(12): p. 1946-54.

222. Hung, A.Y. and M. Sheng, *PDZ domains: structural modules for protein complex assembly.* J Biol Chem, 2002. **277**(8): p. 5699-702.

223. Stiffler, M.A., J.R. Chen, V.P. Grantcharova, Y. Lei, D. Fuchs, J.E. Allen, L.A. Zaslavskaia, and G. MacBeath, *PDZ domain binding selectivity is optimized across the mouse proteome.* Science, 2007. **317**(5836): p. 364-9.

224. Kunkel, M.T., E.L. Garcia, T. Kajimoto, R.A. Hall, and A.C. Newton, *The protein scaffold NHERF-1 controls the amplitude and duration of localized protein kinase D activity.* J Biol Chem, 2009. **284**(36): p. 24653-61.

225. Kim, E., K.O. Cho, A. Rothschild, and M. Sheng, *Heteromultimerization and NMDA receptor-clustering activity of Chapsyn-110, a member of the PSD-95 family of proteins.* Neuron, 1996. **17**(1): p. 103-13.

226. Kruger, W.A., G.R. Monteith, and P. Poronnik, *The plasma membrane Ca(2+)-ATPase: regulation by PSD-95/Dlg/Zo-1 scaffolds.* Int J Biochem Cell Biol, 2010. **42**(6): p. 805-8.

227. Lee, H.J. and J.J. Zheng, *PDZ domains and their binding partners: structure, specificity, and modification.* Cell Commun Signal, 2010. **8**: p. 8.

228. Im, Y.J., J.H. Lee, S.H. Park, S.J. Park, S.H. Rho, G.B. Kang, E. Kim, and S.H. Eom, *Crystal structure of the Shank PDZ-ligand complex reveals a class I PDZ interaction and a novel PDZ-PDZ dimerization.* J Biol Chem, 2003. **278**(48): p. 48099-104.

229. Sun, Q. and G.G. Turrigiano, *PSD-95 and PSD-93 play critical but distinct roles in synaptic scaling up and down.* J Neurosci, 2011. **31**(18): p. 6800-8.

230. Parker, M.J., S. Zhao, D.S. Bredt, J.R. Sanes, and G. Feng, *PSD93 regulates synaptic stability at neuronal cholinergic synapses.* J Neurosci, 2004. **24**(2): p. 378-88.

231. DeGiorgis, J.A., J.A. Galbraith, A. Dosemeci, X. Chen, and T.S. Reese, *Distribution of the scaffolding proteins PSD-95, PSD-93, and SAP97 in isolated PSDs.* Brain Cell Biol, 2006. **35**(4-6): p. 239-50.

232. Kalia, L.V., G.M. Pitcher, K.A. Pelkey, and M.W. Salter, *PSD-95 is a negative regulator of the tyrosine kinase Src in the NMDA receptor complex.* EMBO J, 2006. **25**(20): p. 4971-82.

233. Hou, X.Y., G.Y. Zhang, J.Z. Yan, M. Chen, and Y. Liu, *Activation of NMDA receptors and L-type voltage-gated calcium channels mediates enhanced formation of Fyn-PSD95-NR2A complex after transient brain ischemia.* Brain Res, 2002. **955**(1-2): p. 123-32.

234. Tezuka, T., H. Umemori, T. Akiyama, S. Nakanishi, and T. Yamamoto, *PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A.* Proc Natl Acad Sci U S A, 1999. **96**(2): p. 435-40.

235. Gu, W.H., S. Yang, W.X. Shi, G.Z. Jin, and X.C. Zhen, *Requirement of PSD-95 for dopamine D1 receptor modulating glutamate NR1a/NR2B receptor function.* Acta Pharmacol Sin, 2007. **28**(6): p. 756-62.

236. Sun, P., J. Wang, W. Gu, W. Cheng, G.Z. Jin, E. Friedman, J. Zheng, and X. Zhen, *PSD-95 regulates D1 dopamine receptor resensitization, but not receptor-mediated Gs-protein activation.* Cell Res, 2009. **19**(5): p. 612-24.

237. Buerli, T., C. Pellegrino, K. Baer, B. Lardi-Studler, I. Chudotvorova, J.M. Fritschy, I. Medina, and C. Fuhrer, *Efficient transfection of DNA or shRNA vectors into neurons using magnetofection.* Nat Protoc, 2007. **2**(12): p. 3090-101.

238. Fallini, C., G.J. Bassell, and W. Rossoll, *High-efficiency transfection of cultured primary motor neurons to study protein localization, trafficking, and function.* Mol Neurodegener, 2010. **5**: p. 17.

239. Stine, W.B., Jr., K.N. Dahlgren, G.A. Krafft, and M.J. LaDu, *In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis.* J Biol Chem, 2003. **278**(13): p. 11612-22.

240. Stine, W.B., L. Jungbauer, C. Yu, and M.J. LaDu, *Preparing synthetic Abeta in different aggregation states.* Methods Mol Biol, 2011. **670**: p. 13-32.

241. Mitchell, D.R. and F.D. Warner, *Binding of dynein 21 S ATPase to microtubules. Effects of ionic conditions and substrate analogs.* J Biol Chem, 1981. **256**(23): p. 12535-44.

242. Silvanovich, A., M.G. Li, M. Serr, S. Mische, and T.S. Hays, *The third P-loop domain in cytoplasmic dynein heavy chain is essential for dynein motor function and ATP-sensitive microtubule binding.* Mol Biol Cell, 2003. **14**(4): p. 1355-65.

243. Kon, T., K. Imamula, A.J. Roberts, R. Ohkura, P.J. Knight, I.R. Gibbons, S.A. Burgess, and K. Sutoh, *Helix sliding in the stalk coiled coil of dynein couples ATPase and microtubule binding.* Nat Struct Mol Biol, 2009. **16**(3): p. 325-33.

244. Heikkila, J.E., G. Akerlind, and K.E. Akerman, *Protein kinase C activation and down-regulation in relation to phorbol ester-induced differentiation of SH-SY5Y human neuroblastoma cells.* J Cell Physiol, 1989. **140**(3): p. 593-600.

245. Lint, J.V., A. Rykx, T. Vantus, and J.R. Vandenheede, *Getting to know protein kinase D.* Int J Biochem Cell Biol, 2002. **34**(6): p. 577-81.

246. Lee, A., A. Rayfield, D.H. Hryciw, T.A. Ma, D. Wang, D. Pow, S. Broer, C. Yun, and P. Poronnik, *Na+-H+ exchanger regulatory factor 1 is a PDZ scaffold for the astroglial glutamate transporter GLAST.* Glia, 2007. **55**(2): p. 119-29.

247. Mok, H., H. Shin, S. Kim, J.R. Lee, J. Yoon, and E. Kim, *Association of the kinesin superfamily motor protein KIF1Balpha with postsynaptic density-95 (PSD-95), synapse-associated protein-97, and synaptic scaffolding molecule PSD-95/discs large/zona occludens-1 proteins.* J Neurosci, 2002. **22**(13): p. 5253-8.

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Chicago Brain Bee Coordinator, University of Illinois at Chicago, 2008 and 2011

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Chemistry and Mathematics tutor, Diablo Valley College 1998-2000

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Publications

Jeong-Sun Kim, J.S, Dong Hae Shin, **Ramona Pufan**, Candice Huang, Hisao Yokota, Rosalind Kim, Sung-Hou Kim, *Crystal Structure of ScpB from Chlorobium tepidum, a Protein Involved in Chromosome Partinioning*, 2006 Proteins 62 (2): 322-8

Sung-Hou Kim, Dong Hae Shin, Jinyu Liu, Vaheh Oganesyan, Shengfeng Chen, Qian Steven Xu, Jeong-Sun S Kim, Debanu Das, Ursual Shulze-Gahmen, Steve R Holbrook, Bruno A Martinez, Natalia Oganesyan, Andy DeGiovanni, Yun Lou, Marlene Henriquez, Candice Huang, Jarmila Jancarik, **Ramona Pufan,** In-Geol Choi, Jean-Marc Chandonia, Jingtong Hou, Barbara Gold, Hisao Yakota, Steve E Brenner, Paul D Adams, Rosalind Kim, *Structural Genomics of minimal organism and protein fold space*, 2005 Journal of Structural and Functional Genomics 6: 67-30

Debanu Das, Natalia Oganesyan, Hisao Yokota, **Ramona Pufan**, Rosalind Kim, Sung-Hou Kim, *Crystal Structure of Conserved Hypothetical Protein MPN330 (GI: 1674200) from Mycoplasma Pneumoniae*, 2005 Proteins 58 (2): 504-8

Jarmila Jancarik, **Ramona Pufan**, Connie Hong, Sung-Hou Kim, Rosalind Kim, *Optimum Solubility (OS) screening: an efficient method to optimize buffer conditions for homogeneity and crystallization of proteins*, 2004 Acta Crystallographica Section D Biological Crystallography 60 (Pt 9): 1670-3

Shengfeng Chen, Dong Hae Shin, **Ramona Pufan**, Rosalind Kim, Sung-Hou Kim, *Crystal Structure of methenyltetrahydrofolate synthetase from Mycoplasma pneumoniae* (GI: 1 at 2.2 resolution), 2004 Proteins 56 (4): 839-43

Vaheh Oganesyan, **Ramona Pufan**, Andy DeGiovanni, Hisao Yokota, Rosalind Kim, Sung-Hou Kim, *Structure of the Putative DNA-binding protein SP_1288 from Sreptococcus pyrogenes*, 2004 Acta Crystallographica Section D Biological Crystallography 60 (Pt 7): 1266-71

Shengfeng Chen, Alexander F. Yakunin, Ekaterina Kuznetsova, Michael Proudfoot, Didier Busso, **Ramona Pufan**, Rosalind Kim and Sung-Hou Kim, *Structural and Functional Characterization of the hypothetical protein MJ0936 from Methanococcus jannaschii: a Novel Phosphodiesterase*, 2004 Journal of Biological Chemistry 279 (30): 31854-62

Qian Steven Xu, Dong-Hae Shin, **Ramona Pufan**, Hisao Yokota, Rosalind Kim and Sung-Hou Kim, *Crystal Structure of a Phosphotransacetylase from Streptococcus pyogenes*, 2004 Proteins 55 (2): 479-81

Abstracts

R. Pufan, N. Marangoni, S. Pollema, R. Chavez, T. Chilcote, G. Morfini, S. T. Brady, An isoform of protein kinase C is critical in Parkinson's disease pathogenesis. Presented at Society for Neuroscience 2007, Parkinson's disease poster

R. Pufan, G. Morfini, S. T. Brady, α-Synuclein affects PKC activity in a cell model of Parkinson disease. Presented at Society for Neuroscience 2009, Parkinson's disease: Alpha Synuclein nanosymposium

Ramona Pufan, Natalia Marangoni, Sarah Pollema, Ray Chavez, Tamie Chilcote, Gerardo Morfini, Scott T. Brady, α-Synuclein Affects PKC Activity in a Cell Model of Parkinson Disease. Presented at the University of Illinois Chicago, Research Forum