Effects of Mutant Alpha-Synuclein

In the Activation of Retrograde Fast Axonal Transport

BY

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THESIS

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
aFAT	Anterograde Fast Axonal Transport
α-Syn	Alpha-Synuclein
β-Syn	Beta-Synuclein
BDNF	Brain-Derived Neurotrophic Factor
CDyn	Cytoplasmic Dynein
DA	Dopamine
DHC	Dynein Heavy Chain
DIC	Dynein Intermediate Chain
DLIC	Dynein Light Intermediate Chain
fPD	Familial Parkinson's Disease
FAT	Fast Axonal Transport
IRES	Internal Ribosomal Entry Site
KD	Kinase Domain
LBs	Lewy Bodies
LC	Light Chain
MBOs	Membrane Bound Organelles
MT	Microtubule
NGF	Nerve Growth Factor
NT	Neurotrophin
PKCμ	Protein Kinase C μ
PD	Parkinson's Disease
rFAT	Retrograde Fast Axonal Transport
sPD	Sporadic Parkinson's Disease
SFKs	Src Family Kinases
SH2	Src Homology 2
SH3	Src Homology 3
SNpc	Substantia Nigra *pars compacta*

SUMMARY

Parkinson's disease (PD) is a progressive movement disorder affecting motor functions regulated by nigrostriatal pathway. Pathologically, PD is characterized by selective loss of dopaminergic neurons in substantia nigra and by the presence of intraneuronal protein aggregates called Lewy Bodies (LBs). α-synuclein(α-Syn), the major component of LBs is a 140 aa presynaptic protein implicated in synaptic function of neurons, but its role in PD pathogenesis is not completely understood. Autosomal missense mutations or duplication/triplication in α-Syn gene have been shown to cause familial forms of PD (fPD). Genetic evidence suggests that mutations in α-Syn induce a *gain of function* toxic to neurons, but the underlying mechanisms remain unknown. Interestingly, we found the fPD-related α-Syn mutant A30P abnormally activated retrograde fast axonal transport (rFAT) mediated by a Src Family kinase (SFKs)- Protein kinase C*μ* (PKCμ) regulatory pathway, suggesting that the rFAT alteration might represent a critical component of PD pathogenesis. In this research, we proposed two specific aims to evaluate effects of pathogenic forms of α-Syn in the abnormal activation of rFAT. As a result, we observed that A30P α-Syn dramatically increases SFKs autophosphorylation during the activation of rFAT in squid axoplasm. In rat primary neurons, overexpression of pathogenic α-Syn shows slight increase in SFKs autophosphorylation only at early time course. Furthermore, both WT and A30P exhibited activating effect on SFKs autophosphorylation and activity *in vitro*, suggesting that there might be further unknown cell biological factors for A30P specific activation of SFKs. When rat primary neurons were stimulated by neurotrophin, SFKs and PKCμ mediated DIC phosphorylation, indicating SFKs and PKCμ are critical regulators for the molecular event associated with rFAT activation in neurons. However, overexpression of pathogenic forms of α-Syn did not represent significant result linked to rFAT activation. Here, we showed our observations suggesting the role of

pathogenic α-Syn in the molecular events underlying the activation of rFAT, which is expected to provide better understanding of early events during PD pathogenesis.

I. INTRODUCTION

1. PARKINSON'S DISEASE and α-SYNUCLEIN

1. Pathological hallmarks and familial mutations in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease showing selective loss of projection neurons underlying nigrostriatal pathway, representing dying-back pattern of neurodegeneration (Anderson JK et al, 2001; Dauer W and Przedborski S, 2003). Dying-back neurodegeneration undergoes loss of synaptic connectivity and subsequent axonal degeneration prior to neuronal death, but the pathogenic mechanism is still unclear.

PD is pathologically characterized by presence of intraneuronal Lewy bodies (LBs) and loss of dopaminergic neurons in substantia nigra (SNpc) (Fig.1). LBs are intraneuronal protein aggregates composed of mainly α-synuclein (α-Syn) and other cytoskeletal proteins. α-Syn is a 140 aa presynaptic protein which is physiologically implicated in the regulations of exocytosis (Larson KE et al, 2006; Mosharov EV et al, 2006), dopamine biosynthesis (Perez RG et al, 2002) and synaptic transmission (Liu S et al, 2004), but its role during PD pathogenesis is not understood well. However, discovery of three autosomal missense mutations such as A30P, A53T, and E46K and duplication/triplication of α-Syn revealed pathogenicity of α-Syn in familial forms of PD (Kruger R, et al, 1998; Polymeropoulos MH et al. 1997; Zarranz JJ et al, 2004; Chartier-Harlin MC et al, 2004; Singleton AB et al, 2003; Ibanez P et al, 2004), indicating those mutations induce toxic *gain of function* of α-Syn thus resulting in neuronal damage during disease progression. To understand the role of α-Syn in terms of PD pathogenesis, investigators generated fPD animal models using overexpression of adeno-associated viral (AAV) or lentiviral (LV) α-Syn and found α-Syn transgenic animals showed significant neuronal loss in the

injected brain areas and dystrophic neurons (Bianco CLo et al, 2002; Kirik D et al, 2002; Kirik D et al, 2003; Lauwer E et al, 2003), supporting the idea that genetic alterations in α-Syn exhibits neuronal toxicity. However, the molecular mechanisms underlying how α-Syn affects neuronal death still remains unclear. Our laboratory has been studying pathogenic role of α-Syn in the context of PD pathogenesis, and found mutant α-Syn showed toxic *gain-of-function* to affected neurons during early stage of PD progression. Briefly, fPD-linked mutant α-Syn significantly led to FAT dysregulation which is thought to be a critical component associated with pathogenic feature shown in other genetically-linked neurodegenerative diseases (reviewd in Morfini G et al, 2009). This observation initiated our research by relating its pathogenic role with FAT deficits at which mechanisms underlying other genetically linked dying-back neurodegenerative diseases converge during disease progression.

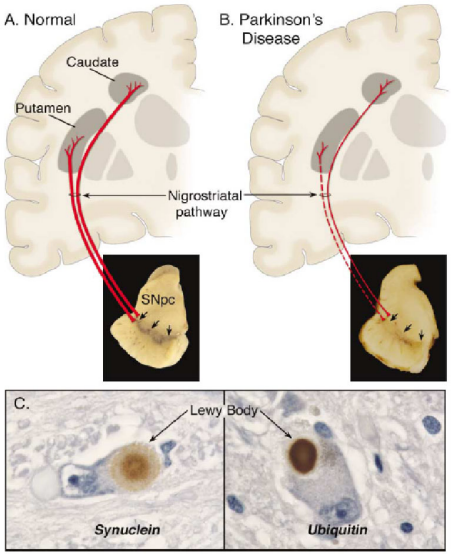

Fig.1. Pathological hallmarks of PD brain.
(A) Normal nigrostriatal pathway(in red). Dopamindergic neurons located in the substantia nigra pars compacta (SNpc, arrows) projects their axons(thin solid red lines) to the striatum (caudate, putamen). The photograph shows normal pigmentation of the SNpc. (B) Diseased nigrostriatal pathway (in red). In Parkinson's disease, the nigrostriatal pathway degenerates (dashed and thin line). The photograph demonstrates depigmentation, representing loss of dopaminergic neurons. (C) Immunohistochemical labeling of intraneuronal Lowy bodies (LBs) in SNpc. Immunostaining with an antibody against α-Syn reveals a LBs (black arrow, left). Immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the LBs(right). *Figure adapted from* ***Dauer W and Przedborski S (2003)***

Locus	Chromosomal Localization	Gene product	Inheritance	Lewy body pathology	Specific clinical symptoms
PARK 1	4q21	α-Synuclein	AD	Yes	Dementia
PARK 2	6q25.2-27	Parkin	AR	No	Early onset, L-Dopa-induced dyskinesias, improvement during sleep, foot dystonia
PARK 3	2p13	?	AD	Yes	Dementia
PARK 4	4q21	α-Synuclein	AD	Yes	Dementia, postural tremor
PARK 5	4p14	UCH-L1	AD	No report	Not described
PARK 6	1p35-36	PINK-1	AR	No report	Early onset, tremor dominant
PARK 7	1p36	DJ-1	AR	No report	Early onset, dystonia, psychiatric alterations
PARK 8	12cen	LRRK2/Dardarin	AD	Yes/but also Tau pathology	Tremor, late onset
PARK 9	1p36	ATP13A2	AR		Kufor-Rakeb syndrome, very early onset
PARK 10	1p32	?	AD (?)	No report	Late onset
PARK 11	2q34	?	AD (?)	No report	Late onset
	5q23	Synphilin-1	Susceptibility	No report	Late onset
PARK 13	2p13	HtrA2/Omi	Susceptibility	No report	Late onset

Table.1. Genetics of Parkinson's Disease.
(AD:autosomal dominant; AR:autosomal recessive)
Adapted from ***Schulz JB (2008).***

2. Structure and role of alpha-synuclein in Parkinson's disease

α-Synuclein (α-Syn) is a 140 aa presynaptically enriched protein which was previously cloned through small peptides found in the brains of Alzheimer's patients (Iwai A et al, 1995). It has other family members including β-, γ-synucleins and all of them have a series of imperfect KTKEGV repeats but vary in their C-terminals (Fig.2). α-Syn also has serine and tyrosine residues that were detected basally phosphorylated in neurons. In terms of structure, α-Syn does not have defined structure in solution and is often called natively unfolded. However, α-Syn has high propensity to form aggregates due to the hydrophobicity of central domains close to the imperfect repeats region, which is thought to be critical property leading to Lewy bodies deposition in affected neurons of PD brains. On the other hand, α-Syn contains a C-terminal 20 aa region which is acidic that exhibits negative effect on aggregation, as shown

by the fact that truncation of the acidic C-terminal domain increases aggregate formation (reviewed in Cookson MR, 2005). As well as aggregation of WT α-Syn proteins, familial mutations such as A53T and A30P are more prone to form fibrillar (A53T) or oligomeric (A30P) forms of α-Syn, which are predicted to eventually contribute as a building block to form insoluble inclusion bodies within neurons (Cookson MR, 2005). High amounts of cytosolic α-Syn aggregates might be a physical burden to neurons, leading to neurotoxicity during the pathogenesis of PD. However, it is not understood how familial mutants such as A53T or A30P, which are usually expressed at physiological concentrations, can pathologically affect neurons even before protein aggregate are detectable. This research aimed to evaluate the role of pathogenic forms of α-Syn in the early stage of Parkinson's disease pathogenesis, specifically focusing on effects of FAT impairments that are implicated in genetically-linked dying-back neurodegenerative diseases (reviewed in Morfini G et al, 2009).

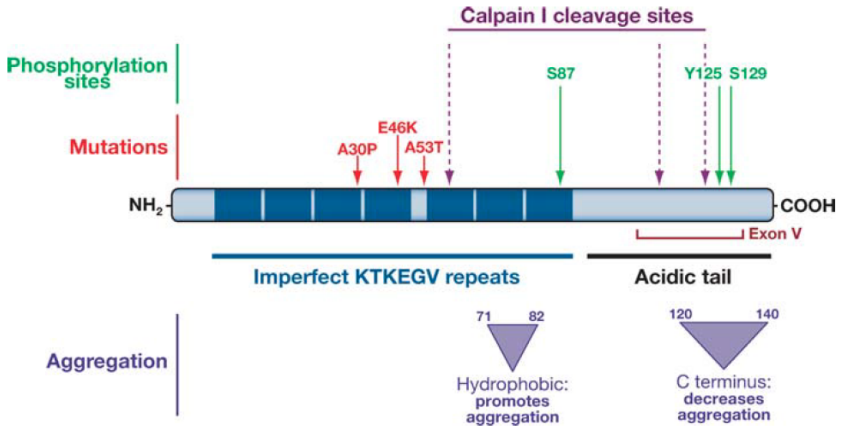

Figure 2. Motifs in the α-synuclein protein.
The natively unfolded **α**-synuclein protein is shown in a linear form. Shaded areas represent the imperfect KTKEGV repeats. Human mutations are shown in red and map to the repeat region. At the C-terminal end of the protein is an acidic tail, containing several sites of phosphorylation (green). The C-terminus also contains the alternatively spliced exon V and a calpain I cleavage site. The acidic tail tends to decrease protein aggregation, whereas a hydrophobic region near the imperfect repeats promotes aggregation. *Adapted from* ***Cookson MR (2005)***.

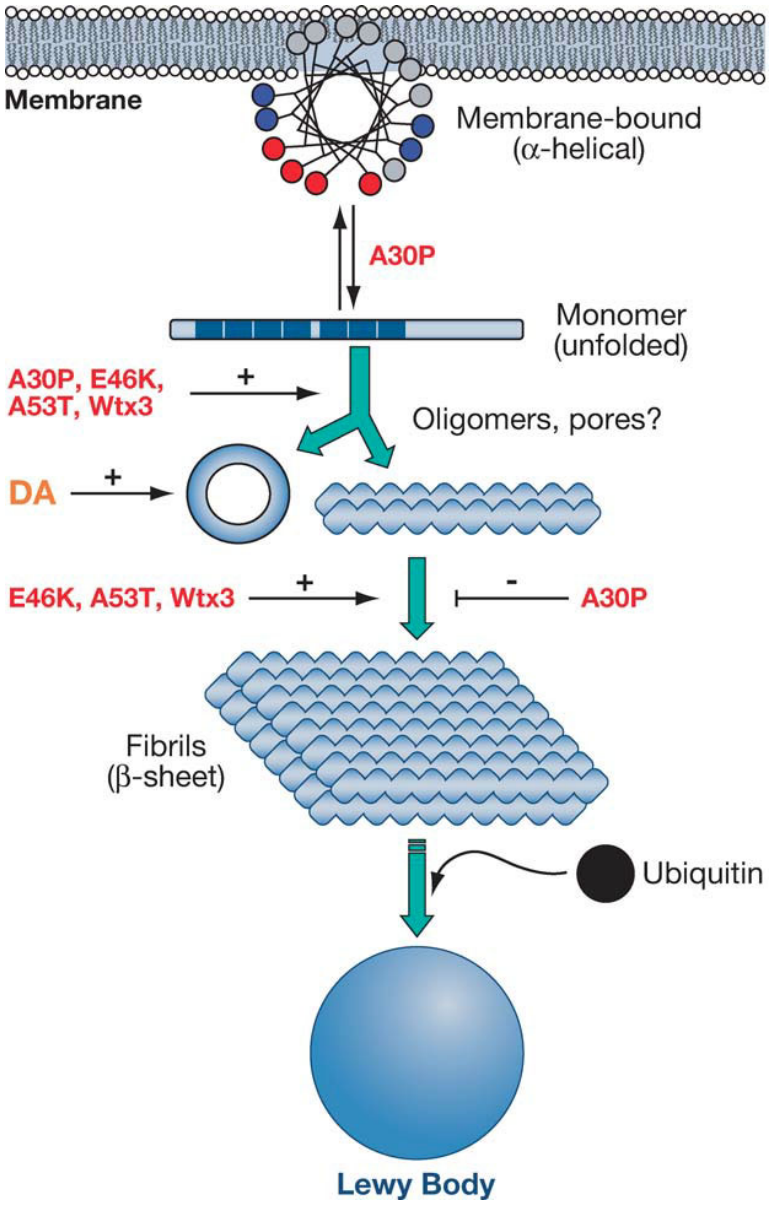

Figure 3. The pathogenic cascade of α-synuclein aggregation.
α-Synuclein exists in solution as an unstructured monomer, shown as a linear structure, similar to Figure 2. Inside the cell, the monomer is in equilibrium with membrane-associated forms with higher helical content, shown schematically as an amphipathic helix. In the helix, blue and red circles indicate charged residues, gray circles are nonpolar and hydrophobic amino acids. The A30P mutation disfavors membrane binding. The green arrows indicate the pathogenic formation of aggregated species. All mutations reported to date increase the rate of formation of oligomers or protofibrils, which may also produce pores. Oligomers and other intermediates are kinetically stabilized by dopamine (DA). However, these are transient species that further aggregate to form mature fibrils, which are stabilized by **β**-sheet-like interactions and are highly insoluble. The formation of Lewy bodies is presumed to be a consequence of fibrillization. Events such as the attachment of ubiquitin (black dot in the figure) are thought to be secondary to the initial aggregation and deposition processes. *Adpated from* ***Cookson MR (2005).***

2. SRC FAMILY KINASES REGULATION

Src family kinases (SFKs) are the largest subfamily of non-receptor tyrosine kinases, which consists of 9 members of Src, Yes, Fyn, Yrk, Fgr, Lyn, Hck, Lck, and Blk. Among these, 5 members Src, Yes, Fyn, Lyn, and Lck are found in CNS (Salter MW et al, 2004), and are implicated in various signaling pathways such as reelin signaling, NMDAR (N-Methyl-D-aspartic acid Receptor)-dependent signaling, and receptor tyrosine kinases-mediated growth factor signaling (Salter MW et al, 2004; Bock HH et al, 2003; Becham D et al, 2007; Baldwin ML et al, 2006; Iwasaki Y et al, 1998; Kalia LV et al. 2004;). SFKs are structurally characterized by the presence of a C-tail regulatory region, a catalytic domain, a SH2 (Src homology 2) kinase linker domain, a SH2 domain, a SH3 (Src homology 3) domain, a unique domain and a N-terminal SH4 (Src homology 4) domain (Fig.4). Functionally, SH2 and SH3 domains are key regulatory regions of which intramolecular interactions with relevant targets determine the activation state of SFKs (reviewd in Roskoski Jr. R, 2005; Superti-Furga G, 1995; Williams JC et al, 1998). SH2 domain interacts with a conserved motif, which contains C-terminal phosphorylated tyrosine, where their interaction induces closed conformation of SFKs (Morgan MF et al, 1990), called "inactivation state". On the other hand, the SH3 domain is able to interact with either the SH2 kinase linker region or a poly proline region "PxxP" (where 'x' is any amino acid) (Ren R et al, 1993). In SFKs regulation, the SH2 kinase linker is the key interacting partner of SH3 domain, in which their interaction keeps the kinase in closed conformation resulting in inactivation state. Therefore, coordination of those intramolecular interactions mediated by SH2 and SH3 domains is critical for the regulation of SFKs activity. When SH2- or SH3-mediated intramolecular interactions are disrupted by external activation signals, SH2 and SH3 domains are released from their interacting targets such as C-terminal phosphor-Tyr527 and SH2-kinase linker, respectively, which will lead to open conformation (Fig.5, Cooper JA 1993;Sicheri F et al, 1997; Salter MW et al, 2004; Roskoski R Jr, 2005). The open conformation allows

the kinases to undergo *trans*-autophosphorylation (Sun G et al, 2002) of Tyr416 at the activation loop of protein kinase domain (Fig.4, PKD). Then, Tyr416-autophosphorylated SFKs become activated entering "activation state" which exhibits catalytic activity in the presence of ATP.

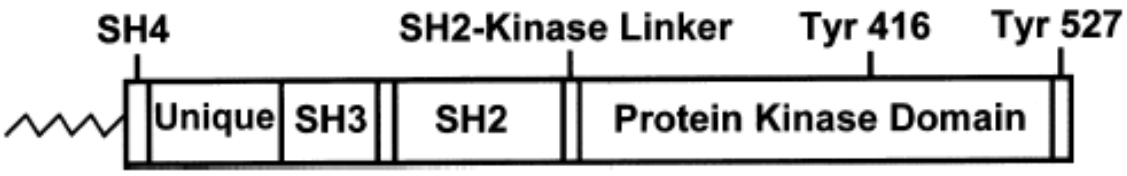

Fig.4. Organization of Src Family Kinases.
Except for the aliphatic myristoyl group attechated to the SH4 domain, the relative length of the domains is to scale. The chicken numbering system is displayed. *Adapted from* ***Roskoski R Jr. (2004).***

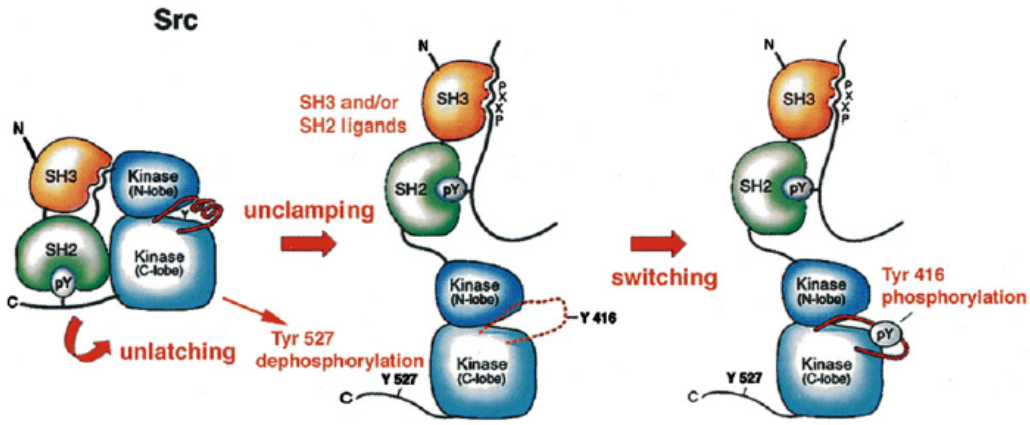

Fig.5. Src Family Kinase activation by unlatching, unclamping, and switching.
The regulation of SFKs activation is schematically drawn. Two intramolecular interactions and tyrosine phosphorylations are critically regulating the activation state of SFKs (SH2:Src homology 2 domain; SH3:Src homology 3 domain). *Adapted from* ***Roskoski R Jr. (2004)***

3. FAST AXONAL TRANSPORT DEFICITS IN NEURODEGENERATIVE DISEASES

1. Fast axonal transport dysregulation and dying back neurodegeneration

Neurons are structurally distinct from other types of cells in that they form extremely polarized structures, thus totally depending on FAT to provide proper communication between cell bodies and far distant synaptic terminals. Healthy communication includes anterograde transport of essential materials produced in cell bodies to synaptic terminals and retrograde transport returning molecules that need to be degraded or that contain neurotrophic signals from synapses. Thus, it is predicted that FAT alterations will lead to imbalance in transport of materials, which will be followed by synaptic dysfunction and neurodegeneration. Interestingly, in early stage of most adult onset neurodegenerative diseases, loss of synaptic connectivity and axonal degeneration is commonly observed, indicating that different mechanisms underlying each disease converge at dysfunctional FAT to produce dying-back neurodegeneration. For example, *loss of function* mutations in molecular motor genes were sufficient to cause dying-back neurodegeneration, through the process of FAT impairment (Reid E et al., 2002; Hafezparast M et al., 2003; Puls I et al., 2003, 2005; Farrer MJ et al., 2009; reviewed in Morfini G et al., 2009). However, a variety of dying-back neurodegenerative diseases do not possess genetic mutations in molecular motor genes, which suggests that mutations in other genes might have different mechanisms underlying deregulation of FAT during disease progression. As schematically summarized in Fig.6, not only mutations in the subunits of molecular motors but also mutations in cytoskeletal or signaling molecules even including toxic reagents were shown to result in dysregulation of FAT through abnormal activity of each disease-specific kinases, eventually contributing to synaptic dysfunction and axonal degeneration. Investigating mechanisms explaining which specific kinases are playing a role for FAT deregulation in the context of each disease will give us an insight to identify proper targets for development of diagnosis as well as therapeutics.

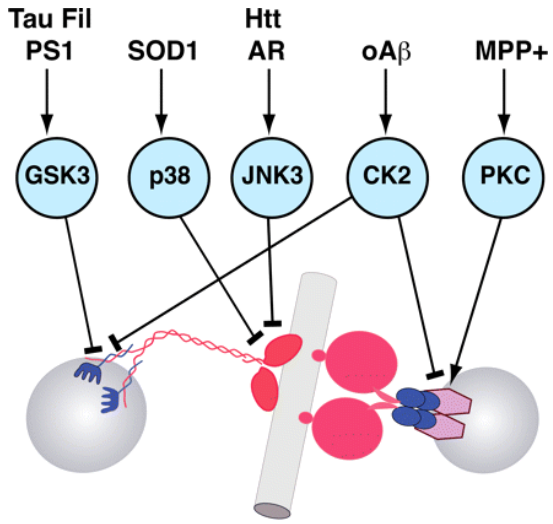

Fig.6. Kinase-dependent regulation of molecular motors in neurodegenerative diseases.
In genetically linked-neurodegenerative diseases, it has been shown altered functionality of molecular motors (Left: Kinesin; Right: Cytoplasmic dynein) by various kinases (GSK3, P38, JNK3, CK2, and PKC) associates with deficits in fast axonal transport, which will eventually result in neuronal dysfunction followed by dying-back degeneration. (Tau, microtubule-associated protein tau; PS1, Presenilin 1; Htt, Huntingtin; A.R, Androgen receptor; α-Syn, α-synuclein; GSK-3, glycogen synthase kinase-3; JNK3, cJun N-terminal kinase 3; CK2, Casein kinase 2; PKC, Protein kinase C). *Adapted from* ***Morfini G et al (2009).***

2. Retrograde transport of neurotrophin in neurodegenerative diseases

Neurotrophins (or neurotrophic factors) are target-derived signaling molecules implicated in axonal guidance, outgrowth, and survival during development and adulthood. The NGF (Nerve growth factor) family of neurotrophins includes NGF, BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3), NT-4 (neurotrophin-4), NT-6 (neurotrophin-6), and NT-7 (neurotrophin-7) (reviewed in Reynolds AJ et al, 2000). To transduce neurotrophin signals, neurotrophins bind to its receptor called Trk family (Tropomyosin receptor kinase) such as TrkA, TrkB, TrkC (reviewed in Reynolds AJ et al, 2000) leading to autophosphorylation and dimerization. The dimerized neurotrophin/Trks complex then become internalized by endocytosis, forming a "signaling endosome" (Howe CL et al, 2004). The signaling endosome is transported retrogradely along the axonal tracks by rFAT mechanism. When signaling endosomes arrive at the cell body, retrogradely transported signaling endosomes are released into the cell bodies so as to affect downstream molecular events such as gene transcription and translation. It is widely accepted that neurotrophins and their Trk receptors convey external trophic signals to neuronal cell bodies by rFAT, suggesting that abnormality of rFAT will be detrimental to neuronal survival.

Various studies reported alterations in neurotrophin level in PD brains, suggesting a possibility that changes in neurotrophin stability or neurotrophin expression (Howells DW et al., 2009; Parain K et al., 1999; Kruttgen A et al., 2003). Specifically, PD brains showed reduced level of BDNF, proNGF, NGF, and TrkA in substantia nigra (Kruttgen A et al. 2003, Siegel GJ et al., 2000; Murer MG et al., 2001; Fahnestock M et al., 2001; Aguado F et al., 1998). The decrease in the level of neurotrophins can be explained by the fact that affected brain region undergoes a neurotoxic event which down regulates proteins level of various neurotrophins. This suggests a possibility that the protein level of neurotrophins is reduced by abnormal returning to cell bodies to be degraded or by decreased expression within the cell bodies. Interestingly, our preliminary study using isolated squid axoplasm revealed that fPD mutant

α-Syn significantly increased the velocity of rFAT, suggesting abnormal activation of rFAT may be associated with the reduction in neurotrophin levels during PD progression. This observation led us to hypothesize that pathogenic α-Syn may contribute to synaptic deficits and subsequent neurodegeneration during PD pathogenesis by upregulating rFAT, which may lead to abnormal return of neurotrophins or neurotrophin receptors subsequently reducing neurotrophin levels in neurons.

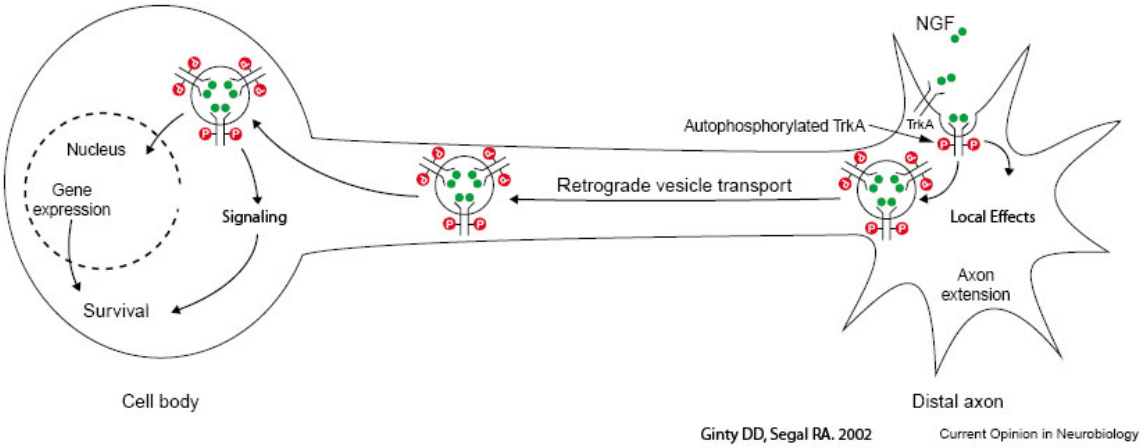

Fig.7. Schematic diagram showing retrograde transport of neurotrophin signaling endosomes. Trk receptors on distal axons are phosphorylated and internalized upon neurotrophin binding. The ligand–receptor complex internalizes through clathrin-mediated endocytosis. Some of the vesicles become signaling endosomes and are transported retrograde to the cell body using a dynein-based retrograde FAT. The vesicle-associated Trk receptor remains autophosphorylated and capable of promoting a unique set of signals upon arrival at the cell bodies(NGF:Nerve growth factor; TrkA:Tropomyosin receotor kinase A). *Adapated from* ***Ginty DD and Segal RA (2002).***

II. BACKGROUND STUDIES

1. Fast axonal transport alteration induced by fPD-linked mutant alpha-synuclein

It has been shown that genetic mutations in molecular motor genes are associated with FAT impairment (Hirokawa and Takemura, 2003; Reid et al, 2002; Hafezparast et al, 2003; Puls et al, 2003, 2005; Farrer et al, 2009; Roy et al, 2005), eventually resulting in dying-back neurodegeneration (reviewed in Morfini G et al., 2009). In PD brains, there was obvious dying-back pattern of neurodegeneration, which is represented by synaptic loss and subsequently degenerating axons of projection neurons in the nigrostriatal pathway. This dying-back feature shown in PD brains led us to evaluate whether particular mutations given in familial forms of PD also share the pathogenic pathway associated with FAT impairment and further dying-back neurodegeneration as shown in other genetically-linked neurodegenerative diseases. First of all, we examined the effect of fPD mutant α-Syn on both anterograde and retrograde FAT through vesicle motility assay using isolated squid axoplasm. This method is established in our laboratory, which has been used for determining effects of recombinant kinases or pathogenic polypeptides in studies of neurodegenerative diseases pathogenesis (Morfini G et al, 2002, 2006, 2007a, 2007b, and 2009; Bosco DA et al, 2010). In this experiment, we found fPD mutant A30P α-Syn significantly activated the rate of rFAT (Fig.8, B) while WT α-Syn did not show any changes (Fig.8, A). Perfusion of recombinant A30P and A53T α-Syn into the axoplasm activated rFAT while slightly decreased aFAT, suggesting that there may be abnormal returns of synaptic components, which will result in dysfunctional synapses. As a downstream effector of the rFAT activation, SFKs was identified to mediate the effect of mutant α-Syn on rFAT. Perfusion of recombinant Fyn (Fig.8, D) or Src (Data not shown) recapitulated similar rFAT activation which was

shown in A30P α-Syn perfused axoplasm. Coperfusion studies using SU6656 a pharmacological inhibitor for SFKs represented that SFKs inhibition no longer showed A30P induced rFAT activation (Data not shown), confirming that SFKs are critical mediators for the rFAT activation by A30P. *All data from these experiments indicated that mutant α-Syn alters FAT mediated by the activity of SFKs, which may be closely associated with FAT impairment shown in other genetically-linked neurodegenerative diseases.*

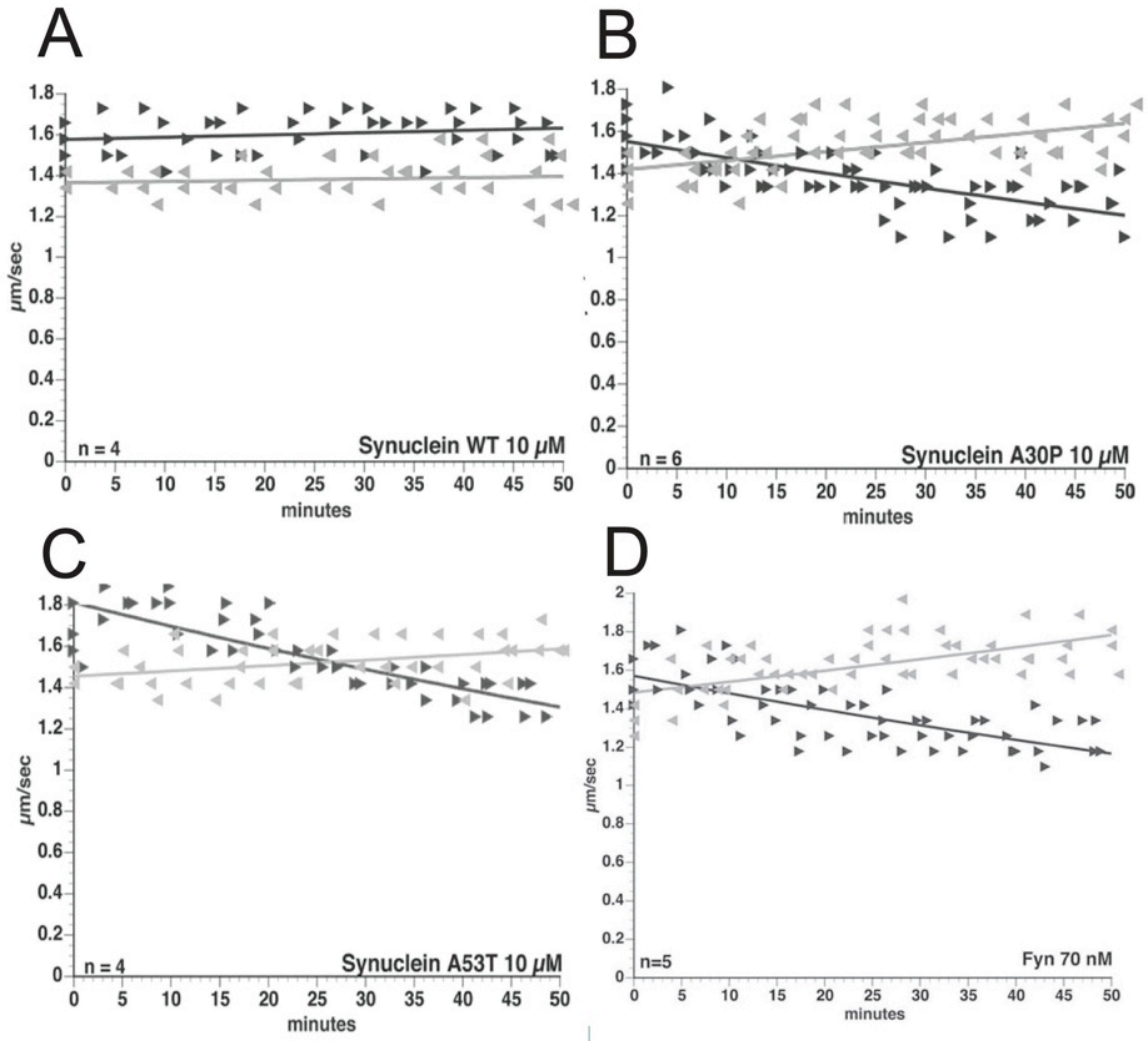

Fig.8. Mutant α-Syn activates rFAT mediated by src family kinases.

(A) Perfusion of recombinant WT α-Syn into isolated squid axoplasm did not affect FAT. (B,C) Perfusion of fPD mutant α-Syn altered the rate FAT bidirectionally. It increased RT but reduced AT, compromising the balance between bidirectional FAT. (D) Perfusion of Fyn revealed similar pattern of FAT alterations shown by fPD mutant α-Syn. ***Courtesy of Drs.Brady ST and Morfini G***.

2. Src family kinases and protein kinase C mu as mediator for fast axonal transport alteration

We also found mutant α-Syn altered FAT mediated by protein kinase Cμ (PKCμ). When PKCμ specific inhibitor Gö6976 was coperfused with A30P, the FAT alterations were prevented. Also, coperfusion of PKC substrates (MARCKS peptide) which blocks PKC activity showed similar protection against A30P effects as Gö6976 did prevent, indicating PKCμ mediates A30P-induced FAT alterations. Furthermore, Gö6976 coperfusion with recombinant Fyn significantly blocked Fyn induced FAT alterations, suggesting SFKs-PKCμ regulatory pathway underlies the A30P altering effects on FAT. The molecular events explaining activation of SFKs and PKCμ by A30P was further discussed in Chapter V Results section.

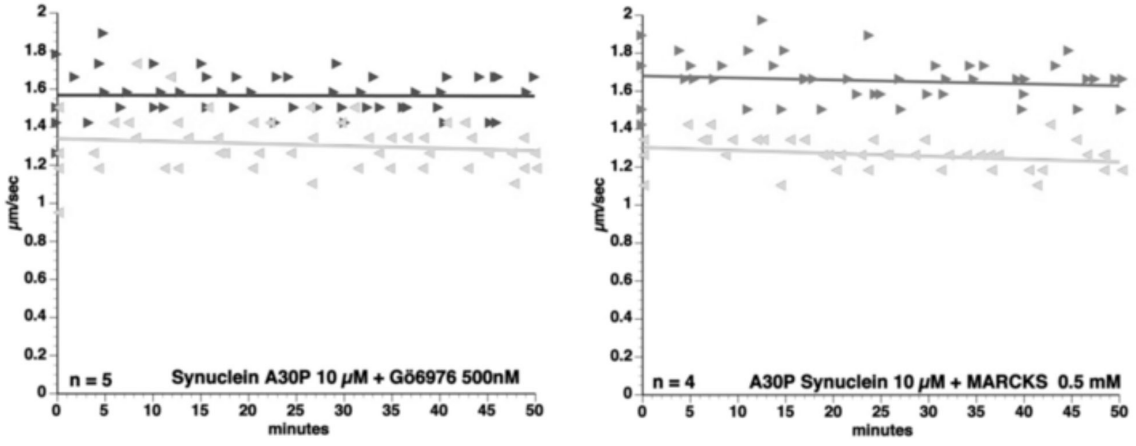

Fig.9. Mutant α-Syn activates rFAT mediated by protein kinase Cμ.
Coperfusion of either Go6976 (Left) or MARCKS (Right) with A30P into isolated squid axoplasm prevented the FAT alterations shown in Fig.8B. ***Courtesy of Drs.Brady ST and Morfini G.***

3. Phosphorylation of dynein intermediate chain by src family kinases and protein kinase C mu

Cytoplasmic dynein (CDyn) is a MT-based primary molecular motor for rFAT and present as a protein complex of two heavy chains (DHCs, dynein heavy chain), two intermediate chains (DICs, dynein intermediate chain), four light intermediate chains (DLICs, dynein light intermediate chains) and light chains (LCs, light chains) (Brill LB and Pfister KK, 2000). These subunits were found as phosphoprotein *in vivo* (Brill LB and Pfister KK, 2000; Dillman JFr and Pfister KK, 1995), suggesting their functionality may be linked to the degree of phosphorylation and regulated by kinases. Our preliminary studies using neurotrophin stimulated rat cortical neurons showed there was significant changes in the migrating pattern (Fig.10, A and B), later characterized as molecular shift due to increased phosphorylation (Fig.10, C and D). This indicates that rFAT activation by neurotrophin is associated with increase in the phosphorylation level of DIC, which is represented by molecular shift in immunoblot analysis. Based on this, *we aimed to evaluate whether fPD-linked mutant α-Syn induced significant molecular shift of DIC as a process for rFAT activation and further determine whether SFKs-PKCμ regulatory pathway mediate the DIC molecular modification during rFAT.*

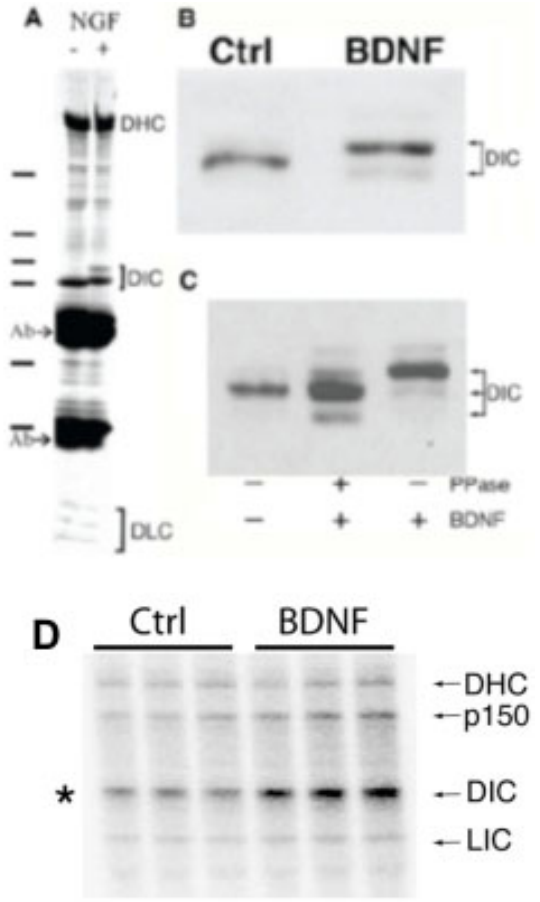

Fig.10. Neurotrophin-induced CDyn phosphorylation in rat primary neurons.
(A) Immunoprecipitations after NGF treatment or (B) BDNF treatment. (C) Significant molecular shift were shown to be caused by phosphorylation as the treatment of alkaline phosphatase (PPase) reversed the molecular shift. (D) Autoradiography results from metaolibally labeled rat cortical neurons after BDNF stimulation. Significant increase in the phosphorylation level of DIC was detected in BDNF stimulated neurons (BDNF, DIC). (DHC:Dynein heavy chain; DIC:Dynein intermediate chain; DLC:Dynein light chain; Ctrl:vehicle treated group; BDNF:Brain-derived neurotrophic factor treated group). *Panel D adapted from* ***Salata MW et al (2001) and Panels A-C Courtesy of Drs. Brady ST and Morfini G.***

III. SPECIFIC AIMS

Parkinson's disease (PD) is the second most common neurodegenerative disease and is characterized by the presence of Lewy bodies (LBs) within neurons along with the loss of dopaminergic neurons in the substantia nigra (SN) (Anderson JK et al, 2001; Dauer W and Prezedorski, 2003). LBs are intraneuronal protein aggregates mainly composed of α-synuclein (α-Syn), a 140 aa presynaptically enriched small protein. In addition to being a major component of LBs, autosomal dominant mutations in α-Syn, such as A30P, A53T, or E46K, or duplications/triplication of WT α-Syn have been implicated in familial forms of PD (Kruger R et al, 1998; Polymeropoulos MH et al, 1997; Zarranz JJ et al, 2004; Chartier-Harlin MC et al., 2004; Singleton AB et al, 2003; Ibanez P et al, 2004). Pathogenic roles of those mutations have been investigated using transgenic animals, in which physiological anomalies such as α-Syn aggregate formation, motor dysfunctions, and neuronal death were detected, indicating genetic alterations in α-Syn manifest neurotoxicity during PD progression (Bianco CLo et al, 2002; Kirik D et al, 2002; Kirik D et al, 2003; Lauwer E et al, 2003). As the other hallmark, selective loss of dopaminergic projection neurons in nigrostriatal pathway has been detected within PD brains, showing a dying-back pattern of neurodegeneration, however, the mechanisms underlying LBs formation and selective neuronal loss is not understood well.

In some genetically-linked dying-back neurodegenerative diseases, it has been shown that mutated proteins induced dysregulation of fast axonal transport (FAT) (reviewed in Morfini G et al. 2009), indicating that FAT impairment as a pathological component induced by disease related mutations during pathogeneses. FAT is microtubule-based transport primarily depending on the mobility of molecular motor proteins called "Conventional kinesin" for anterograde and "Cytoplasmic dynein (CDyn)" for retrograde transport. These molecular motors are required for neurons to communicate

properly between cell bodies and synaptic terminals thus maintaining neuronal functionality and survival. However, the neurons will undergo neurodegeneration shown as synaptic and axonal loss where the normal transport of essential materials such as membrane-bound organelles (MBOs) containing structural or signaling components is disrupted by FAT deregulation. Although FAT impairment has been shown as a critical component for many other dying-back neuropathies (reviewed in Morfini G et al, 2009), it is not understood well whether fPD mutations exhibit pathogenic effect on the regulation of FAT. Interestingly, a recent report showed that moderate expression of mutant α-Syn in transgenic mouse resulted in defective "vacant synapses" exhibiting the absence of critical synaptic proteins (Scott D et al, 2010). This founding strongly suggests a possibility that, in PD, mutant α-Syn may cause FAT deregulation during the pathogenesis, thus leading to mislocalization or abnormal transport of synaptic proteins which will result in dying-back neurodegeneration.

Relating neurotoxicity of mutant α-Syn with dysregulation of FAT, we started evaluating effects of fPD mutant α-Syn on FAT using isolated squid axoplam and found mutant α-Syn significantly activated retrograde FAT (rFAT) via src family kinases (SFKs) and subsequently protein kinase Cμ (PKCμ). Speculating the mechanism underlying SFKs activation by mutant α-Syn, we found C-terminus of α-Syn contains protein regions homologous to SFKs regulatory domains, which suggests α-Syn as an activating factor for SFKs. As shown in isolated squid axoplasm, SFKs and PKCμ were shown to mediate rFAT activation-associated molecular event in neurotrophin stimulated neurons, confirming SFKs and PKCμ are critical regulatory kinases for rFAT activation. These findings led us to hypothesize mutant α-Syn *activates SFKs and PKCμ leading to abnormal rFAT activation which is associated with altered phosphorylation of rFAT motor protein, consequently resulting in synaptic dysfunction during dying-back neurodegeneration of PD.* The objective of this thesis research is to evaluate potential role of mutant α-Syn in rFAT activation, which may be a pathological event critically inducing dying-back

neurodegeneration in PD. We also anticipated that achieving these specific aims would help us understand the early events in the progression of PD more clearly.

Specific Aim 1: To determine the role of pathogenic α-Syn underlying the activation of src family kinases and protein kinase Cμ.

Preliminary results in isolated axoplasm indicated mutant α-Syn activates rFAT via SFKs and PKCμ, but the role of mutant α-Syn underlying the activation of the kinases remains unclear. The activation of SFKs and PKCμ is induced by phosphorylation at specific regulatory residues such as Tyr416 and Tyr463, respectively. Phosphorylation of those regulatory sites allows active conformation of SFKs and PKCμ, leading to functional activation (Cooper JA et al, 1993; Storz P et al, 2003). Our evidence representing that SFKs activity is required for mutant α-Syn induced rFAT activation and the report showing that SFKs activation is associated with PKCμ phosphorylation at Tyr463 (Waldron RT et al, 2004) led us to evaluate effects of α-Syn on the phosphorylation and kinase activities of SFKs and PKCμ. *We hypothesized α-Syn may induce SFKs phosphorylation and PKCμ phosphorylation, leading to kinase activation.* Biochemical experiments using squid and lentivirus-based cell models were used to determine the role of pathogenic α-Syn on the activation of SFKs and PKCμ in Aim1.

Specific Aim 2: To evaluate effects of pathogenic α-Syn on the molecular events of cytoplasmic dynein.

Cytoplasmic dynein (CDyn) is the primary molecular motor for rFAT of which functionality is regulated by phosphorylation. Our pharmacological and biochemical experiments using rat primary neurons indicated that the phosphorylation level of dynein intermediate chain (DIC) increased upon rFAT activation triggered by brain-derived neurotrophic factor (BDNF), which was mediated by SFKs and

PKCμ. Based on this, *we hypothesized that fPD-linked mutant α-Syn may induce phosphorylation of DIC via SFKs-PKCμ regulatory pathway*. Lentivirus-based cell model was used to evaluate the effects of mutant α-Syn on phosphorylation of DIC.

IV. MATERIALS AND METHODS

1. Vesicle motility assay using isolated squid axoplasms

Our previous studies using vesicle motility assays in isolated squid axoplasm have been successful in evaluating axonal effects of recombinant kinases or mutated polypeptides associated with pathogeneses of neurodegenerative diseases (Morfini G et al, 2001; Morfini G et al, 2002; Szebenyi G et al, 2003; Morfini G et al, 2004; Morfini G et al, 2006; Morfini G et al, 2007b; Serulle Y et al, 2007; Lapointe NE et al, 2009). Vesicle motility assay is based on real-time microscopic tracing of membrane bound organelles (MBOs) delivered by FAT. The movement rate of MBOs is recorded by real time imaging and the average rates in anterograde and retrograde were calculated. This established method in our lab was used to determine the effect of mutant α-Syn on rFAT-dependent MBOs delivery (Fig.11). Here, either recombinant SFKs or α-Syn was independently perfused into the axoplasm to see their effects on FAT-dependent MBO movements. Briefly, squid axon segments 2cm in length and 500um in diameter are dissected and extruded onto coverslips. 15-20μl volume of small chamber, which is only 3-5 times volume of the axoplasm facilitates extraction of axoplasm without diffusing axoplasmic components. This helps maintain the integrity of structural organization and machineries related to FAT almost intact. The extruded axoplasm is used to observe bidirectional rate of MBOs movement using video enhanced contrast differential interference contrast (VEC-DIC) microscopy. Also, Zeiss Axiomat with high-resolution DIC optics, a Hamamatsu Photonics high-resolution CCD camera and Argus 20 video image processing system are used for image enhancement and quantification of vesicle velocities. All observations are carried out in real time imaging.

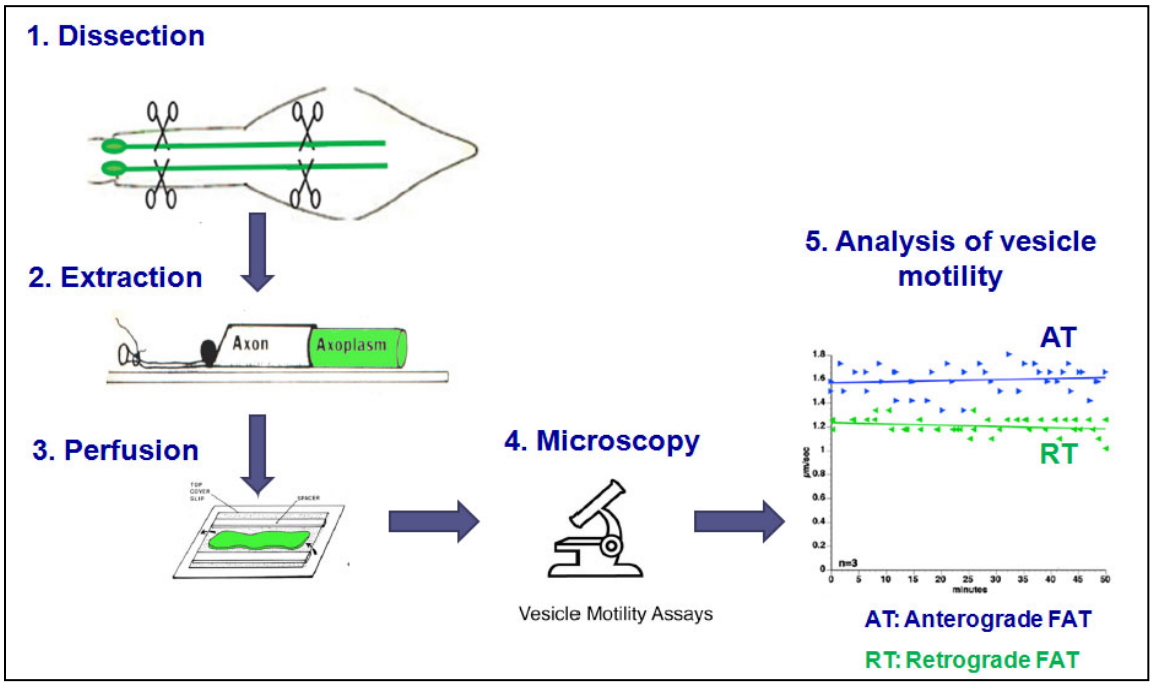

Fig.11. Measurement of fast axonal transport using isolated squid axoplasm.
Courtesy of Dr.Morfini G.

2. Primary culture of rat cortical neurons and lentivirus infection

Primary cultures of E18 rat cortical neurons was carried out following the protocol described in Ivins KJ et al, (1998) and Kaech S and Banker G (2007). Briefly, cortices of E18 rat embryos were dissected out, trypsinized in 0.25% (w/v) trypsin (Invitrogen) for 5 min at 37 °C and dissociated by trituration using pasteurized glass pipettes. Dissociated neurons (9.0 X 10^5 cells/well) were plated on poly-L-Lysine coated 24-well culture dishes containing MEM (Invitrogen) supplemented with 0.6% (w/v) D-glucose and 5% (v/v) fetal bovine serum (Invitrogen). After the cells were attached well to the plate (usually 3~4 hr), we changed the medium with neurobasal medium (Invitrogen) supplemented with N2 (Invitrogen), Glutamax-I (Invitrogen) and B27 (Invitrogen) supplements (1ml of Neurobasal medium with supplements/well). At DIV 1, 0.5ml of medium of each well was removed and added 1.0 X 10^{12} T.U. (Titratable unit=number of viruses/ml) of lentivirus and allowed lentiviral infection for 24hr. Using this concentration of lentiviruses did not show any significant neurotoxicity until postinfection day 14. After 24hr lentivirus infection, 0.5ml of fresh medium was added to each well and the infected cells were allowed to grow until DIV 15 (postinfection day 14). Lentiviral transgene expression was confirmed by fluorescence microscopy detecting DsRed red fluorescence reporter protein and immunoblot detecting α-Syn and DsRed as well.

3. Alpha-synuclein lentiviruses

WT α-Syn and A30P α-Syn lentiviral constructs were generated on the background of LIDsRed-IRES lentiviral vector. LIDsRed-IRES is our home-made lentiviral vector, a derivative from LIDsRed plasmid, in which IRES (Internal Ribosomal Entry Site) sequence allows the vector to produce DsRed red fluorescence reporter protein independently from α-Syn expression. LIDsRed-IRES has been well established for its successful expression of transgenes in rat primary neurons in our lab (Fig.13, B) as well as SHSY5Y cells (Fig.13, A). It contains a β-actin promoter and an upstream CMV enhancer that

allows high expression of transgenes. For lentivirus production, LIDsRed-IRES-WT α-Syn (or A30P α-Syn) plasmid was cotransfected with helper plasmids pCMVΔR8.9 and VSVg in 2:1:1 molar ratio into HEK293T cells cultured in DMEM (Invitrogen) supplemented with 10% (v/v) FBS using calcium phosphate transfection method. After 16hr, the medium was changed with F12 medium (Invitrogen) supplemented with 3% (v/v) FBS and time allowed for growing. At 48hr posttransfection, the supernatant containing lentiviruses produced from HEK293T cells was harvested, filtered through 0.45um low protein binding vacuum filter, and undergone ultracentrifugation for viral isolation. The ultracentrifugation was done using 20% sucrose cushion buffer under the viral supernatant in a swinging rotor at 20,000 g for 4hr. The viral pellets were resuspended in sterilized Mg^{2+}- and Ca^{2+}-free PBS, pH 7.4. Resuspened viral pellets were aliquoted, rapidly frozen in liquid nitrogen and then stored at -80° until use. Once a virus resuspension is thawed, it was allowed to be at 4°C for a week and then discarded if not used by that time.

4. Antibodies

To detect phosphorylation of specific Tyr416 or Tyr527 in SFKs, we used phospho-SFKs Tyr416 (Rabbit, Cell Siganl) or phospho-SFK Tyr527 (Rabbit, Cell Signal), respectively. To detect phosphorylation of specific Tyr463 in PKCμ, we used phospho-PKCμ Tyr463 (Rabbit, AbCam). For detection of phosphorylated tyrosines in general, PY99 (Mouse, Santa Cruz) was used. Mouse monoclonal α-Syn antibody from Transduction laboratory was used to detect transgene expression of α-Syn by immunoblot analysis. Mouse monoclonal H2 antibody recognizing kinesin heavy chain (KHC) was raised in our lab and has been used to successfully represent the protein level of KHC in a variety of cell lysates. DIC 74.1 (Mouse, Santa Cruz), which recognizes both DIC isoform I and II was used to detect DIC in rat cortical neuronal lysates.

5. Immunoblotting

All cell lysates were collected in 1% (w/v) SDS dissolved in 20mM Hepes, pH 7.3. Their protein concentration was measured by BCA assay (Pierce), which is compatible with upto 5% SDS in the lysis buffer. Cell lysates were added 6X sample buffer and allowed running in 4-12% bis/tris gradient gel (Invitrogen) at 45mAmp for 2 hr. The separated proteins in the gel was transferred to polyvinyl difluoride (PVDF) membrane in Towbin buffer at 0.4Amp at 4°C for 2hr. After the transfer, the proteins in the membrane was stained by Ponceau S dissolved in 0.1% (v/v) acetic acid and confirmed equivalent amount of proteins were loaded in each well. The membrane was clearly washed in 1X TBS (Tirs-buffered saline) at room temperature (RT) until there was no Ponceau S shown and blocked in 1% (w/v) BSA (bovine serum albumin) dissolved in 1x TBS at RT for 1 hr. Primary antibodies were diluted in 1% (w/v) BSA/1X TBS and then added to membrane for overnight (ON) incubation at 4°C. ON incubated membrane was washed 10 min in TBS containing 0.1% (v/v) Tween 20 (TBST) for 3 times. And then secondary antibody conjugated to horseradish peroxidase (HRP) in 1% (w/v) BSA dissolved in TBST was added and incubated at RT for 1hr. The membrane was washed 10 min in TBST three times and visualized into films using enhanced chemiluminescence (ECL).

6. Recombinant proteins

Recombinant src family kinases (SFKs) are commercially available (Upstate/Millipore). Fyn, a SFKs subfamily member, which showed rFAT activation was tested. The recombinant kinase is N-terminally 6xHis tagged and expressed in Sf21 cells. C-terminally truncated (either exon 5 or exon 6) WT α-Syn constructs have been kindly provided by Dr.Julia M.George. All full-length or truncated constructs were subcloned into pET28a (Novagen) encoding N-terminal 6xHis tag. Recombinated constructs were bacterially expressed and purified using HPLC. Purified proteins were resuspended in 20mM HEPES, pH 7.3.

7. Pharmacological inhibitors

Rat primary cortical neurons at DIV 1 was stimulated by BDNF (20ng/ml) for 20min to trigger rFAT-dependent neurotrophin transport. For pharmacological inhibitor pretreatment, 500nM Gö6976 (PKCμ specific inhibitor), 500nM Gö6983 (Gö6976 analog, which does not inhibit PKCμ activity), 200nM K252a (Trk inhibitor, which blocks neurotrophin receptors), or 50uM SU6656 (SFKs specific inhibitor) were preincubated for 20min before BDNF stimulation. All inhibitors were dissolved in DMSO and negative control group was pretreated with vehicle (DMSO) alone. BDNF stimulation was stopped by addition of 1% SDS lysis buffer dissolved in 20mM Hepes pH 7.3 and cells were harvested for protein concentration measurement and immunoblot analysis.

8. *In vitro* kinase assay

To evaluate the effects of synuclein on Fyn kinase activity *in vitro*, commercially available recombinant kinase Fyn (10~25nM/sample) was coincubated with synucleins and substrates for 5~15min at 30°C in phosphorylation buffer as described (20mM Hepes, pH7.4, 10mM $MnCl_2$, 2mM EGTA, 0.4mM Na_3VO_4, 10mM Magnesium acetate, 1mM dithiothreitol, 0.1mM phenylmethylsulfonyl fluoride, 0.5uM ATP, 10uCi ^{32}P-ATP/sample, Onofri et al, 2007). In the presence of 0.5uM ^{32}P-ATP (10μCi/sample), the recombinant started phosphorylation of substrate and the reaction samples was directly spotted onto P81 phosphocellulose paper to stop the reaction. Spotted resultant was washed in 75mM phosphoric acid and quantified by scintillation counting. To determine effects of synuclein on autophosphorylation of Fyn, recombinant Fyn was coincubated with WT or A30P recombinant synucleins in phosphorylation buffer for 5~15min at 30°C in the presence or absence of substrate. The reaction was stopped by the addition of 6X sample buffer and the resultant samples were analyzed using phospho-SFKs (Tyr416)-specific antibody or undergone autoradiogram to visualize Fyn autophosphorylation.

9. Molecular shift assay for dynein intermediate chain

7.5%-16% Tris-Glycine gradient gel was used to analyze molecular migration of cytoplasmic dynein (CDyn). Rat cortical neuronal lysates in sample buffer were run through the gradient gel and went through immunoblot procedures using DIC 74.1 (Santa Cruz Biotechnology) antibody, which specifically recognizes dynein intermediate chain. DIC migration was visualized by ECL.

V. RESULTS

1. Results for specific aim 1

Rationale and hypothesis

Our observation using isolated squid axoplasm-based vesicle motility assay indicated fPD-linked mutant α-Syn activates rFAT via SFKs and PKCμ, but the molecular events underlying the kinase activation is not understood yet. Tyrosine phosphorylation at specific residues such as Tyr416 of SFKs, or Tyr463 of PKCμ is associated to their functional activation (Cooper JA et al, 1993; Storz P et al, 2003). Phosphorylation of those regulatory sites induces open conformation of SFKs and PKCμ, leading to functional activation. Interestingly, we observed SFKs inhibited by SU6656 (a SFKs inhibitor) did not reproduce A30P induced rFAT activation (data not shown) and perfusion of recombinant Src or Fyn mimicked the A30P altering effects on rFAT (Fig.8, D), indicating A30P leads to SFKs activation for the change in the rate of rFAT. Besides SFKs activation, we also observed PKCμ inhibition using Gö6976 prevented A30P- or recombinant Fyn-induced rFAT activation, indicating that PKCμ is a downstream effector of SFKs in the A30P actions. In the literature, it was shown that non-receptor tyrosine kinases (NRTKs) induced PKCμ activation by increasing Tyr463 phosphorylation under oxidative stress (Storz P et al, 2003). This led us to hypothesize that mutant α-Syn may induce Tyr416 phosphorylation of SFKs, leading to SFKs functional activation, which will further increase Tyr463 phosphorylation of PKCμ. To address the hypothesis, *we examined effects of mutant α-Syn on the phosphorylation and kinase activities of SFKs and PKCμ through biochemical experiments using isolated squid axoplasms and lentivirus-based rat primary neurons*.

Analysis of phosphorylation level of src family kinases and protein kinase C mu in alpha-synuclein perfused squid axoplasm

To evaluate effects of mutant α-Syn on SFKs and PKCμ phosphorylation, either 10uM WT or A30P Syn resuspended in 20mM Hepes, pH 7.3 was perfused into each sister axoplasm for 50 min. Perfused axoplasms were collected, triturated by pipette tips and allowed for immunoblot analysis using phospho-SFKs Tyr416 or phospho-PKCμ Tyr463 antibodies to detect activation of SFKs or PKCμ, respectively. Interestingly, our immunoblot result showed that A30P perfusion significantly increased phospho-SFKs Tyr416 immunoreactivity (Fig.12, 1B), indicating mutant α-Syn activates SFKs by increasing their autophosphorylation. In addition, A30P was perfused to both sister axoplasms in combination with various pharmacological inhibitors, and it revealed SU6656 (SFKs inhibitor) relatively reduced A30P-induced phospho-SFK Tyr416 (Fig.12, 2A and 2B), correlating with our observation from vesicle motility assay which showed SU6656 prevented A30P-induced rFAT activation (Data not shown). All these results suggest A30P activates SFKs by upregulating Tyr416 phosphorylation. Also, we aimed to analyze phospho-SFK at Tyr527 which corresponds to inactive SFKs. Since phospho-SFKs Tyr527 represents inactive state of SFKs, we assumed A30P perfusion might decrease the phsphorylation level of Tyr527. Squid axoplasms perfused with either WT or A30P were analyzed through immunoblot but barely represented positive signals corresponding to phospho-SFK Tyr527. Due to the limitation in the amount of samples, we could not see clear results relating A30P effects on phospho-SFK Tyr527. As with phospho-SFK Tyr527, phospho-PKCμ Tyr463 in squid axoplasms could not be identified due to the multiplicity of immunoreactivity (Data not shown).

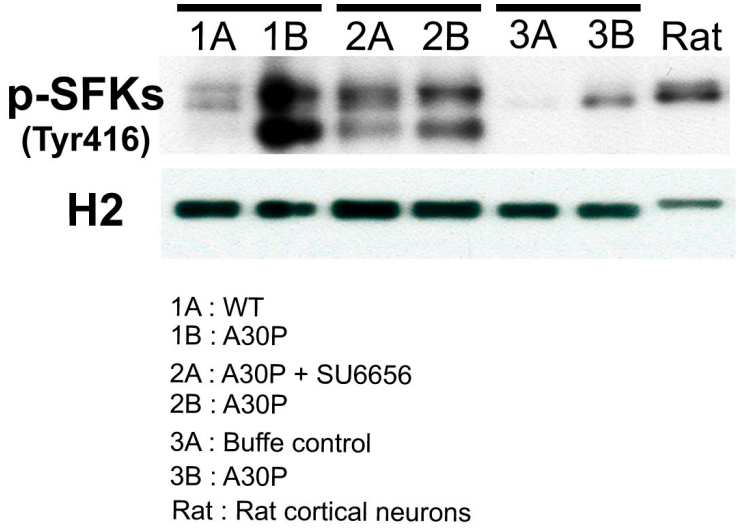

Fig.12. SFKs phosphorylation was increased by pathogenic A30P.
α-Syn in squid axoplasm. Tyr416 phosphorylation of SFKs was significantly increased by the perfusion of mutant (α-Syn into isolated squid axoplasm (1B,2B,&3B). WT or SU6656-perfused axoplasm did not show significant increase in the SFKs phosphorylation. H2 (Kinesin heavy chain) was probed for protein loading control. Phosphorylated SFKs of squid axoplasms were migrating similar with phospho-SFKs from rat cortical neuronal lysate.

Analysis of phosphorylation level of src family kinases and protein kinase C mu in lentiviral alpha-synuclein infected rat cortical neurons

-Transgene expression:

To evaluate effects of mutant α-Syn on SFKs and PKCμ phosphorylation in cell model, rat primary cortical neurons (E18) were infected with either α-Syn (WT or A30P) or control vector (empty vector, no α-Syn) at DIV 1. At 72hr postinfection, we observed about 80~90% neurons were red fluorescence positive and started showing transgene expression detected through immunoblot analysis using DsRed and α-Syn antibodies (Fig.13). The transgene expression was continued until postinfection day 14. However, α-Syn immunoblot result showed α-Syn protein expression level in A30P infected group was much stronger than in WT infected group. We predicted the reason might be due to differential propensity to form aggregates between WT and A30P, giving a rise to more stability in A30P proteins. This will be further discussed in Chapter VI.

For time course analysis, infected neurons were harvested at different time points from postinfection day 1 (DIV2) to day 14 (DIV15) and gone through immunoblot to detect phosphorylation level of endogenous SFKs and PKCμ using phospho-SFKs or phospho-PKCμ specific antibodies. This analysis allowed us to compare the phosphorylation level of endogenous SFKs (or PKCμ) between different experimental groups.

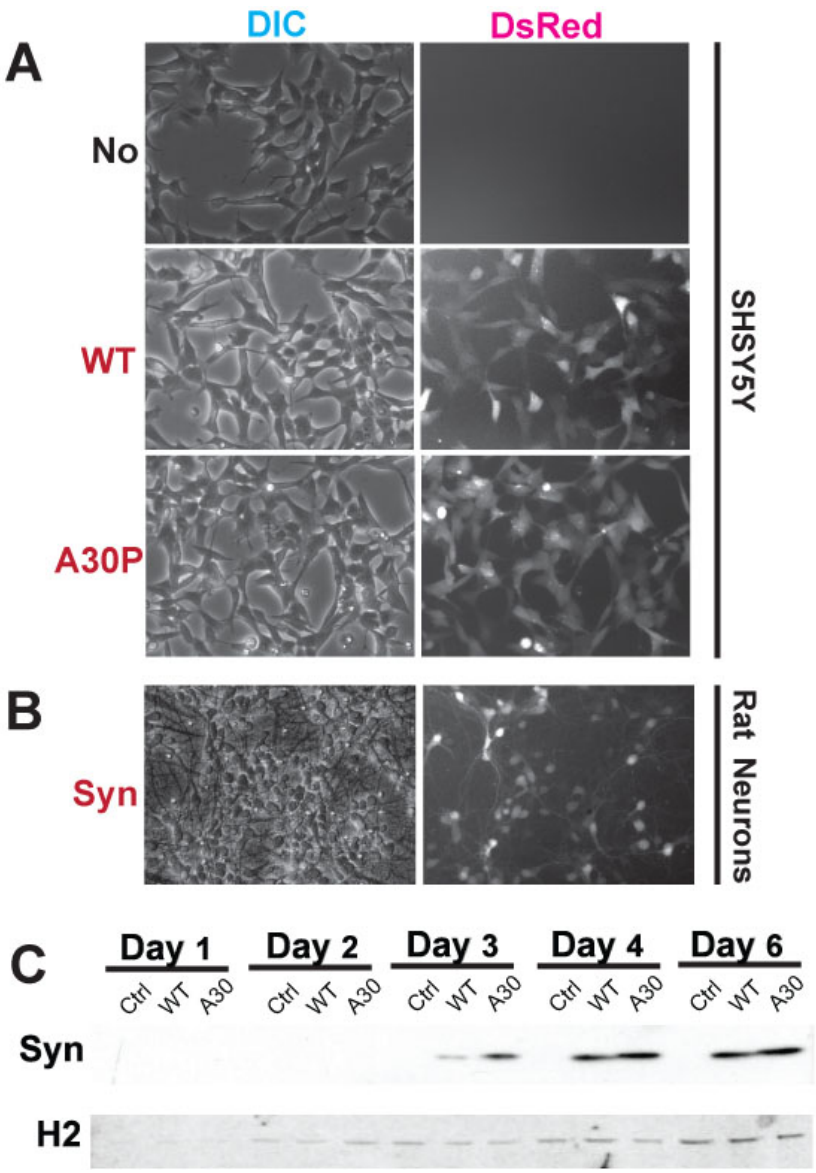

Fig.13. Lentiviral α-Syn expression in SHSY5Y cells and rat primary cortical neurons.

WT or A30P lentiviral α-Syn was infected to SHSY5Y cells (A) or to rat primary cortical neurons (B, C). SHSY5Y cells infected with α-Syn lentiviruses (A, WT and A30P) revealed > 90% lentiviral infectivity shown by red fluorescence (A, DsRed of WT and A30P). At 96hr postinfection, both WT and A30P infected SHSY5Y cells showed diffused expression of DsRed fluorescence proteins throughout the whole cells (A, DsRed in WT and in A30P). Rat primary cortical neurons were also infected with WT or A30P α-Syn lentiviruses and DsRed started detected by fluorescence microscopy at postinfection 72hr (B, DsRed; WT infected neurons were represented in Fig.B). Correlating the result shown in B, immunoblot result also revealed overexpression of α-Syn started appearing at postinfection day 3 (72hr) in both WT and A30P infected rat cortical neurons (C). H2 shows equivalent amount of proteins were loaded between wells. ((A) No, non infected SHSY5Y cells; WT, α-Syn WT infected SHSY5Y cells; A30P, A30P infected; (B) Syn, WT infected rat cortical neurons; DsRed, DsRed fluorescence; (C) Ctrl, empty lentiviral vector infected rat cortical neurons; WT, WT lentivirus infected; A30P, A30P lentivirus infected; Syn, Mouse monoclonal synuclein antibody (Transduction Laboratory); H2, Mouse monoclonal Kinesin heavy chain antibody generated in Brady lab)

-Phosphorylation of endogenous SFKs and PKCμ:

As described above, we aimed to analyze the time course of changes in the phosphorylation of endogenous SFKs and PKCμ in α-Syn lentivirus infected rat cortical neurons. Briefly, either α-Syn (WT or A30P) or control empty vector infected rat cortical neurons were harvested at different time points from DIV2 (postinfection day 1) to DIV15 (postinfection day 14). Collected cell lysates were in 6X sample buffer and run immunoblot for analysis of SFKs phosphorylation (Tyr416 or Tyr527) and PKCμ phosphorylation (Tyr463).

First of all, phospho-SFK Tyr416 increases as DIV progresses (Fig.14A, Ctrl, from DIV2~ DIV7). Phospho-SFK Tyr416 represented doublet signals, which may be due to the cross-reactivity with other SFKs subfamily members. As well as Tyr416 phosphorylation of SFKs, H2 signal also went up as DIV progresses, indicating there was upregulated expression of kinesin heavy chain, which is the primary anterograde transport molecular motor. However, H2 showed that protein loading amount between Ctrl, WT and A30P was pretty even.

At DIV2 and DIV3, which correspond to postinfection day 1 and day 2, respectively, both WT and A30P represented a little stronger immunoreactivity in phospho-SFK Tyr416, but it became all even between control and synuclein groups from DIV 4 until DIV 15 (Fig.14A, DIV4~DIV15). This observation suggests an idea that pathogenic synuclein including overexpressed A30P and WT may activate SFKs by facilitating SFKs autophosphorylation. In addition to phospho-SFK Tyr416, we also probed phospho-SFK Tyr527 to analyze synuclein effects on inactivating phosphorylation of SFKs. Although phospho-SFK Tyr527 is usually thought to be inversely correlated with phospho-SFK Tyr416, we could not see add significant inverse correlation with the result shown in Fig.14A (p-SFK(Tyr416)). Only phospho-SFK Tyr527 of DIV 3 showed the expected inverse correlation (Fig.14B, DIV3) with phospho-SFK Tyr416 of DIV3 (Fig.14A, DIV3), but later immunoreactivities did not represent significant changes between control and synuclein groups (Fig.14B, DIV5~DIV15).

The immunoblot result was not clear enough to identify specific SFKs members affected by α-Syn since all subfamily members migrated at similar MWs. Another challenge in understanding the results was that the changes represented in groups at early time course (DIV2~DIV3) was not consistently shown in the groups of later time course (DIV15). We did not exclude the possibility that early responses by lentivirally expressed synuclein proteins might be sufficient to induce pathogenic effects on endogenous SFKs in neurons even before the transgene expression reaches at maximal rate. Further discussions will suggest a possible explanation about time course responses of lentivirus infected rat primary neurons in Chapter VI.

Unfortunately, phosphorylated PKCμ (Tyr463) was barely detected at the expected molecular weight (~105kDa, Data not shown), indicating there was no dramatic increase in the phosphorylation of PKCμ induced by overexpressed synucleins.

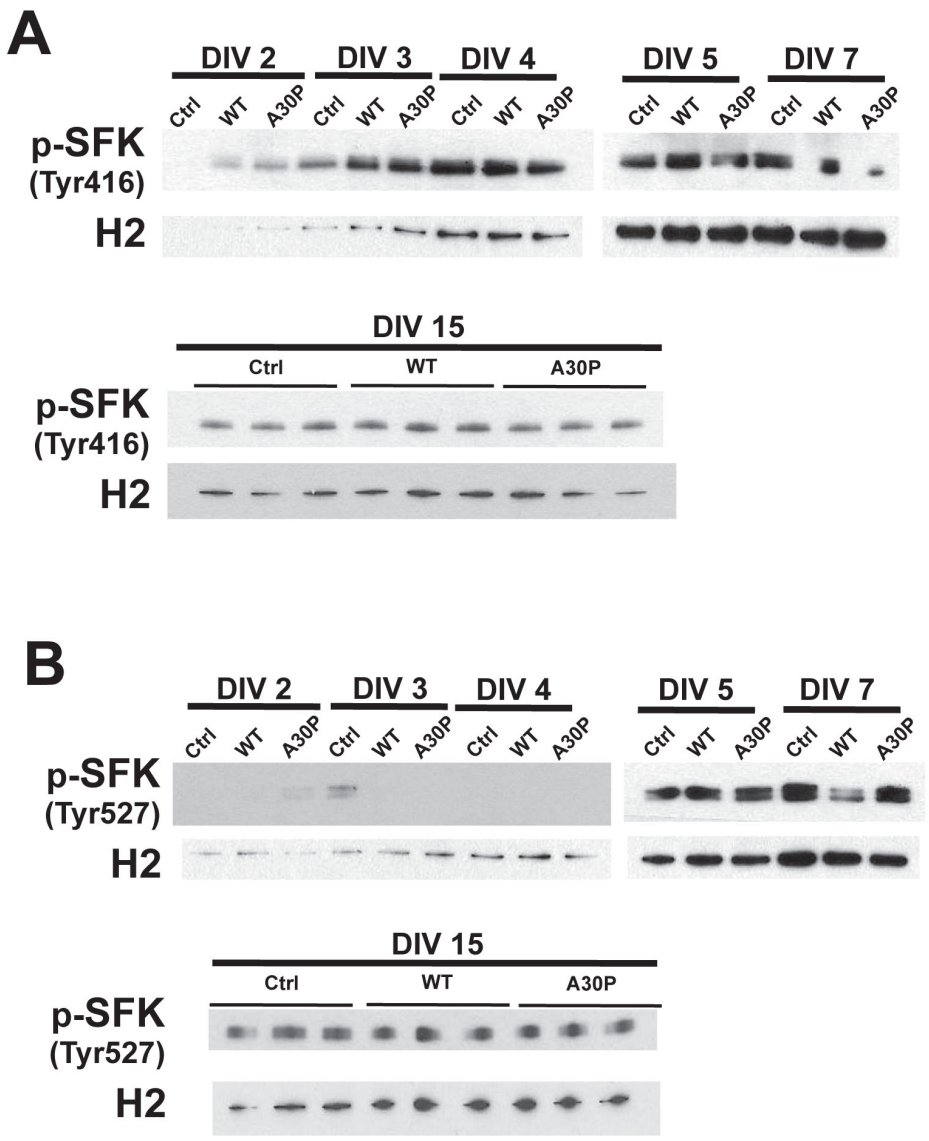

Fig.14. SFKs phosphorylation was changed in lentiviral α-Syn infected rat cortical neurons.
Rat primary cortical neurons(E18) were infected with lentiviral WT or A30P α-Syn at DIV1 and harvested from postindection day (DIV 2) 1 to day 14 (DIV 15). Interestingly, there was slight changes in Tyr416 phosphorylation of SFKs in both WT and A30P infected groups at early time potins(Ctrl, empty lentiviral vector-infected group; W, WT α-Syn lentivirus infected; A30P, A30P α-Syn lentivirus infected; p-SFK(Tyr416), phospho-SFKs at Tyr416; p-SFK(Tyr527), phopsho-SFKs at Tyr527; H2: Kinesin heavy chain for protein loading control; DIV 2, postinfection day 1; DIV3, postinfection day 2; DIV4, postinfection day 3; DIV 6,postinfection day 5; DIV 15, postinfection day 14).

Evaluation of *in vitro* src family kinase autophosphorylation and kinase activity affected by alpha-synuclein

α-Syn was shown to colocalize with SFKs in SFKs-overexpressing cells (Nakamura T et al, 2001) and got phosphorylataed by recombinant SFKs *in vitro* (Nakamura T et al, 2001; Ellis CE et al, 2001), implying that α-Syn transiently interacts with SFKs, serving as a substrate. But it is still unclear what effects mutant α-Syn exhibits on SFKs activation associated event such as autophosphorylation and kinase activity. SFKs activation is regulated by two distinct intramolecular interactions mediated by SH2 and SH3 domains. SH2 domain interacts with C-terminal phosphor-Tyr527, leading to closed conformation called "inactive state". SH3 domain interacts with SH2-kinase linker also called poly-proline linker, keeping the kinase in closed form. However, disrupting these two intramolecular interactions by competitive activators that resemble the domains interacting with either SH2 or SH3 was reported to induce SFKs activation (reviewed in Roskosi R Jr, 2004 and 2005). Based on this idea, we analyzed the composition of α-Syn amino acid sequence and found C-terminally located domains such as exon 5 and exon 6 contain homologous regions with SFKs regulatory domains: at the C-terminus of α-Syn, exon 5 contains some domains similar to SH2-kinase linker (Fig.16A, 108-113, P-X-L), poly-proline region (Fig.16A, 117-120, PXXP), and C-terminal phosphorylable Tyr125 and exon 6 has two C-terminal phosphorylable Tyr133 and Tyr136 (Fig.16A). Here, we hypothesized α-Syn may activate SFKs by facilitating SFKs autophosphorylation, which may be mediated by the α-Syn C-terminus. To evaluate the hypothesis, we tested C-terminally truncated α-Syn constructs on SFKs autophosphorylation and the kinase activity in vitro. This assay helped us determine the effect of α-Syn on SFKs activation and further identify critical domains involved in SFKs activation.

- *In vitro* SFKs autophosphorylation affected by α-Syn: Effects of mutant α-Syn on src family kinases autophosphorylation was evaluated by nonradioactive autophosphorylation assay. As described above, recombinant Fyn (Millipore/Upstate) was coincubated with either WT or A30P full length α-Syn (provided by Dr.Julia M.George) *in vitro*. Since no other proteins were added in the reaction tubes, this experiment helped us exclude unknown negative regulation on SFKs autophosphorylation induced by α-Syn. The reaction mixture was added 6X sample buffer to stop *in vitro* autophosphorylation, and run SDS-PAGE for immunoblot analysis. The autophosphorylation level was detected by phospho-SFKs Tyr416 specific antibody and visualized by ECL.

In the presence of either WT or A30P full length α-Syn, recombinant Fyn showed significant increase in Tyr416 autophosphorylation as the α-Syn concentration increases (Fig.15A). However, both WT and A30P showed similar degree of increasing effect on Fyn autophosphorylation, which suggests both WT and A30P share the domains activating SFKs. As suggested, WT and A30P show 100% homology in exon 5 and exon 6, supporting that C-terminally located exon 5 or exon 6 may be critical region for directly inducing SFKs autophosphorylation. However, β-Syn, which was used as a negative control due to its C-terminal difference from α-Syn, also resulted in increased autophosphorylation of SFKs (data not shown). Despite C-terminal variation between α-and β-Syn, we found there are C-terminal tyrosine residues in β-Syn, which resemble tyrosine residues within exon 6 of α-Syn.

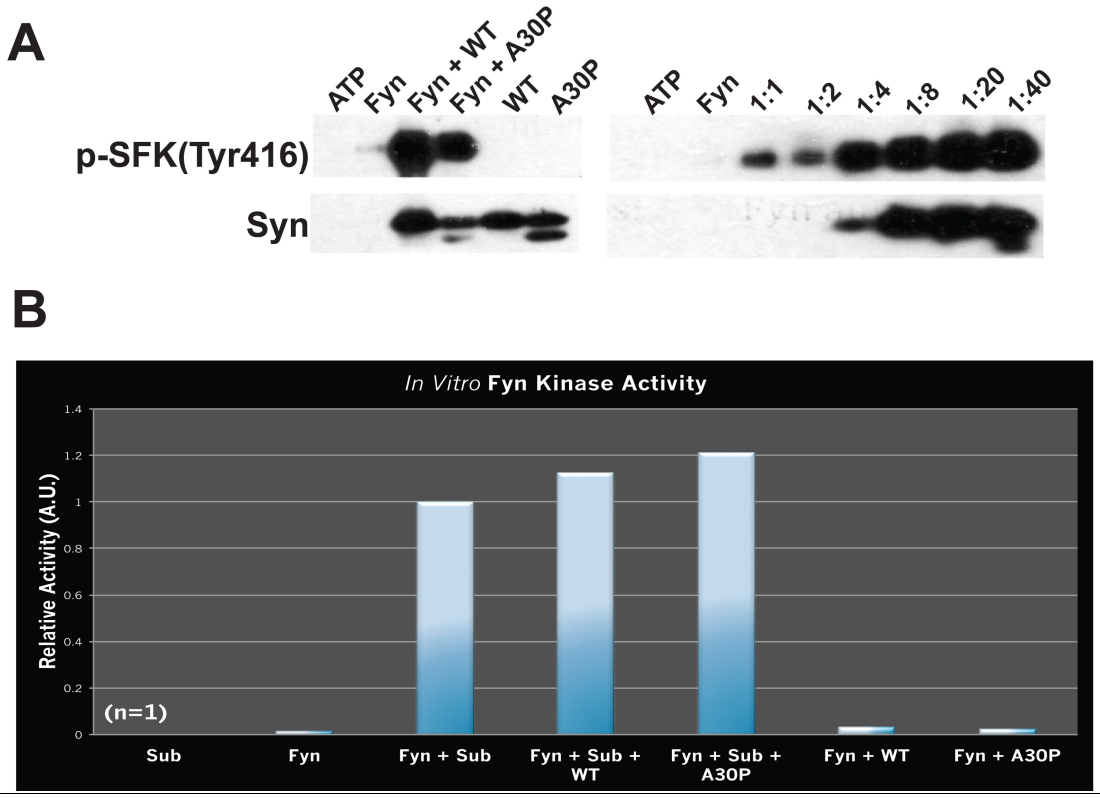

Fig.15. á-Syn increases Fyn autophosphorylation and kinase activity *in vitro*.

To determine role of α-Syn on SFKs activation, we tested effect of α-Syn on recombinant Fyn autophosphorylation and kinase activity *in vitro*. (A) Recombinant 25nM Fyn was incubated with 100uM ATP in the presence or absence of either 10uM WT or 10uM A30P α-Syn (A, Left). Interestingly, in the presence of WT or A30P α-Syn, Fyn autophosphorylation was dramatically enhanced, implying α-Syn increases kinase activity. Even lower ratio of α-Syn (1:1~1:20) showed dramatic increase in p-SFK (Tyr416)(A, Right). The elevation was similarly detected in A30P-coincubated Fyn (data not shown)(ATP: 100uM ATP alone; Fyn: 25nM Fyn+100uM ATP; Fyn+WT: 25nM Fyn+10uM WT+100uM ATP; Fyn+A30P: 25nM Fyn +10uM A30P+100uM ATP; WT: 10uM WT + 100uM ATP; 10uM A30P: 10uM A30P + 100uM ATP; 1:n: Fyn:WT = 1:n).

(B) *In vitro* kinase activity of Fyn was tested in the presence or absence of WT(or A30P) α-Syn. 10nM recombinant Fyn was incubated in the presence of ^{32}P-ATP and 50uM polyE4Y1 substrate at 30°C, for 10min and reaction mixture was spotted on P81 paper, washed in 75mM phosphoric acid and read by scintillation counting method. Basal kinase activity of Fyn(Fyn+Sub) was plotted as "1(A.U.=arbitrary unit)" and background(Sub) was set "0". 10nM WT or 10nM A30P coincubation with Fyn slightly increases Fyn activity(12.3% or 21.1%, respectively) suggesting both WT and A30P α-Syn directly have an enhancing effect on Fyn activity in vitro (Fyn:0.014AU; Fyn+Sub:1AU; Fyn+Sub+WT:1.123AU; Fyn+Sub+A30P:1.211AU; Fyn+WT: 0.033AU; Fyn+A30P:0.024).

- *In vitro* SFKs activity affected by α-Syn: Kinase activity of Fyn was tested in the presence of α-Syn to determine whether α-Syn directly affects functionality of SFKs. Recombinant Fyn (10~25nM) was coincubated with either WT (10~1uM) or A30P in phosphorylation buffer as described in Chapter IV Materials and Methods. For kinase activity measurement, SFKs-specific substrate (0.1mg/ml PolyE4Y1, Sigma) was added to reaction tubes and the amount of ^{32}P-incorporated into SFKs substrate was read by scintillation counting method. β-Syn (provided Dr. Julia M. George) was used as a negative control.
As we see in Fig.15B, both WT and A30P which were incubated at 1:4 molar ratio (1:4=Fyn:α-Syn) slightly increased substrate phosphorylation, telling WT and A30P both directly enhance the kinase activity of Fyn (Fig.15B, WT: 1.123AU; A30P;1.211AU; Fyn: 1AU). However, a similar increment was also shown in Fyn + β-Syn + Sub group (data now shown) in my preliminary experiment, implying β-Syn also contains activation domains. Fig. 15 shows the experimental result in which α-Syn positively affects Fyn kinase activity.

- C-terminal domains of α-Syn affecting SFKs autophosphorylation: To identify specific domains of α-Syn critical for enhancing autophosphoryation and kinase activity of Fyn, we carried out *in vitro* kinase assay in the presence of C-terminally truncated α-Syn constructs. Recombinant Fyn was coincubated with different types of α-Syn truncation (Fig.16B, WT full length, WTΔExon5, WTΔExon6, A30P fulle length, or β-Syn full length) with or without substrates and kinase reaction started by addition of ^{32}P-ATP. After 10min coincubation, the reaction was stopped by addition of 6X sample buffer and the resultant was run SDS-PAGE, further visualized by ECL. The result was shown by autoradiography using Typhoon phosphorimager analysis software equipped in our laboratory. The gel was performed Coomassie staining to confirm that the protein loading of Fyn or α-Syn was equivalent between all lanes.

This experiment represented the kinase activity affected by each form of α-Syn *in vitro* and helped identify critical domains for SFKs activation. Fig.16C shows the result from *in vitro* kinase assay of recombinant Fyn coincubated with different truncation/mutation/isoform of synuclein. Interestingly, synuclein coincubation increased Fyn autophosphorylation (Fig.16C, around 59kDa, Fyn(arrow)) in all synuclein coincubated groups, indicating that all types of synucleins share particular domains activating Fyn *in vitro*. This implies that the activation effect of WT synuclein might be negatively regulated within cells, further leading to mutant A30P specific activation of SFKs. In addition to Fyn autophosphorylation, all types of synucleins except WTΔExon5 showed synuclein phosphorylation around 14kDa (Fig.16C, around 14kDa, Syn(arrow)), meaning Tyr125 located within exon 5 is the major phosphorylation site by Fyn. This observation correlates with the reports showing SFKs phosphorylates Tyr125 of α-Syn *in vitro (*Nakamura T et al, 2001; Ellis CE et al, 2001*)*. Interestingly, WTΔX5 showed less radioactivity in Fyn autophosphorylation than other synuclein coincubated groups (Fig.16C, WTdX5, Fyn), suggesting exon 5 may contain particular domain critical for facilitating SFKs autophosphorylation.

The result from kinase activity (Fig.16C, Fyn+Sub group) was too saturated to be quantitated, so it could not determine substrate phosphorylation affected by the presence of synucleins.

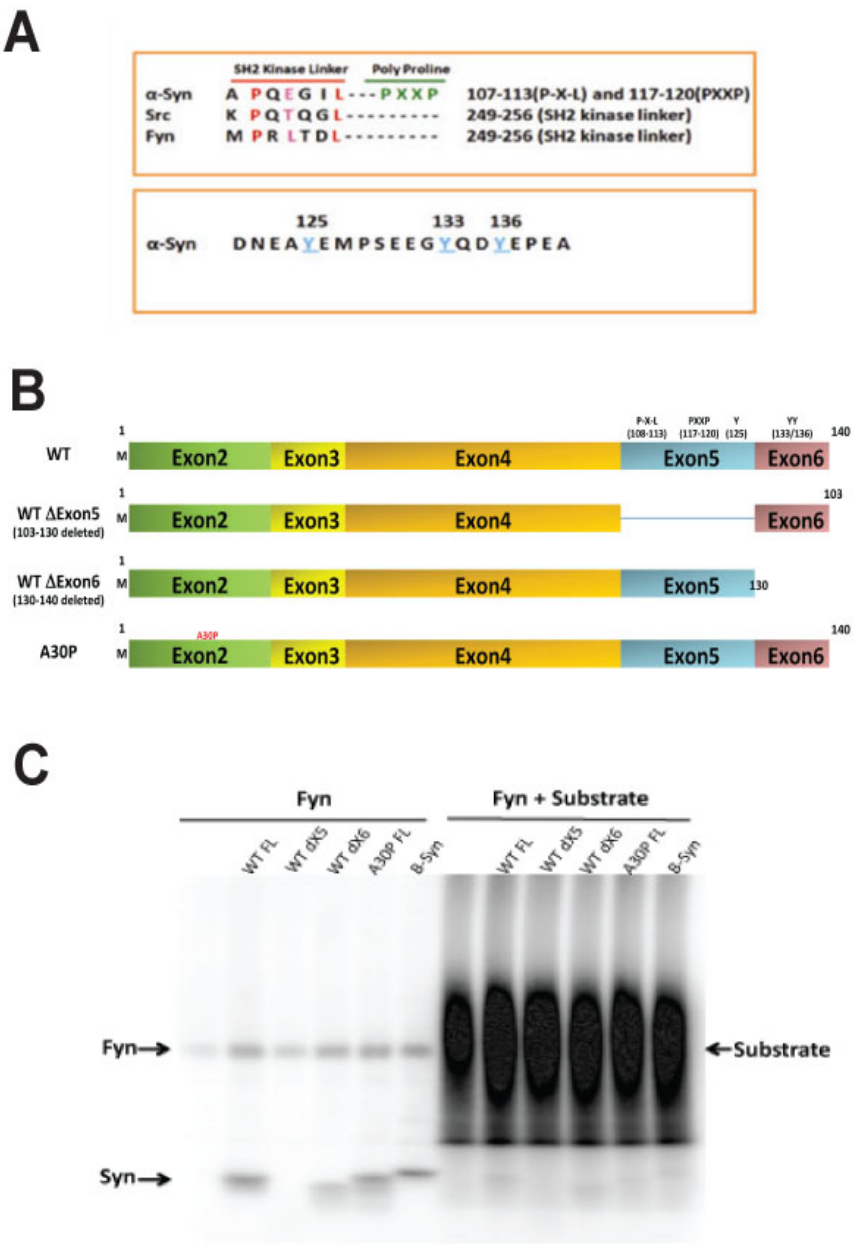

Fig.16. Effects of C-terminally truncated α-Syn on Fyn autophosphoryation and kinase activity *in vitro*.
(A) Candidate domains within α-Syn activating SFKs. (B) Schematic representation of truncated α-Syn proteins (provided from Dr.Julia M.George). (C) Recombinant Fyn was coincubated with different truncation/mutation/isoform of synucleins in the presence/absence of substrate. Interestingly, synucleins enhanced autophosphorylation of Fyn(Fyn, arrow) and also phosphorylated synucleins(Syn, arrow). However, the enhancing effect of synuclein was slightly decreased by exon 5 truncation. Substrate phosphorylation was too saturated to be quantitated(WT FL, WT α-Syn full length; WTdX5, WT α-Syn ΔExon5; WTdX6, WT α-Syn ΔExon6; A30P FL, A30P full length; β-Syn, β-Syn full length).

2. Results for specific aim 2

Rationale and hypothesis

Cytoplasmic dynein (CDyn) is a MT-based primary molecular motor for rFAT and form a complex containing two heavy chains (DHCs), two intermediate chains (DICs), four light intermediate chains (DLICs) and light chains (LCs) (Brill LB and Pfister KK, 2000). These subunits were found as phosphorylated forms *in vivo* (Brill LB and Pfister KK, 2000; Dillman JFr and Pfister KK, 1995), implying that their functionality may be linked to the degree of phosphorylation as regulated by the balance between kinases and phosphatases.

Interestingly, our preliminary studies using rat cortical neurons showed that rFAT activation by neurotrophins such as NGF or BDNF induced significant increase in the phosphorylation level of DIC, which migrates at >74kDa upon phosphorylation (Fig.10B, NGF/BDNF, upper band: phosphorylated DIC). This indicates there were certain kinases mediating the phosphorylation process, but they were not identified yet. As we learned from our vesicle motility assay using squid axoplasms, mutant α-Syn activates rFAT through the activities of SFKs and PKCμ. This brings an idea that SFKs and PKCμ may play a regulatory role in the activation of rFAT induced by neurotrophins in rat primary neurons. Further, we assumed that those kinase activities might contribute to increases in the phosphorylation of DIC during the rFAT activation. To address the hypothesis, we evaluated effects of fPD-linked mutant α-Syn on the molecular events of DIC using lentivirus infected rat primary neurons and also examined the regulatory pathway composed of SFKs and PKCμ on DIC phosphorylation during rFAT activation.

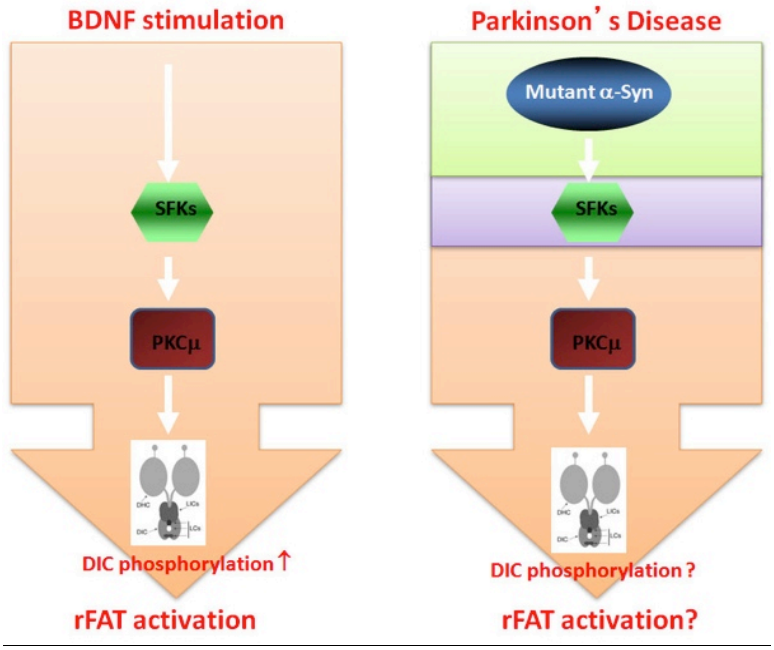

Fig.17. Schematic representation of hypothesis in specific aim 3.
Left diagram represents that BDNF-induced rFAT activation involves DIC phosphorylation mediated by SFKs and PKCμ. Right diagram reveals proposed mechanism of mutant α-Syn-induced rFAT activation, mediated by SFKs and PKCμ.

Determination of role of src family kinases and protein kinase C mu in molecular shift of cytoplasmic dynein in neurotrophin stimulated rat cortical neurons

To induce molecular alterations of CDyn in the context of rFAT activation, we stimulated rat cortical neurons with BDNF (20ng/ml) for 20min, which is already established method to detect molecular modification of DIC (Fig.10, in Chapter II Background studies). This allowed us to detect activated pTrkA/B (Fig.18) indicating activation of retrograde transport of neurotrophin. To determine the role of SFKs and PKCμ in the molecular modification of CDyn during rFAT activation, we blocked SFKs or PKCμ activity using SU6656 and Gö6976 inhibitors, respectively. Cell lysates was run through 7.5%-16% Tris-Glycine gradient gel which allowed us to detect even small molecular shifts caused by posttranslational modification as shown in Fig.18 (Vehicle vs BDNF). The proteins separated in the gel were transferred to PVDF and then probed with DIC 74.1 antibody recognizing DIC isoforms I and II. However, the DIC antibody only represented DIC-II (Fig.18, Vehicle) corresponding to 74kDa, and there was no significant DIC-I (72kDa) detected in cultured neurons at DIV1 (Fig.18). DIC immunoreactivity was visualized by ECL.

As we see in Fig.18, BDNF stimulation induced molecular shift of DIC shown as doublet bands (Fig.18, BDNF). The upper band indicates DIC phosphorylated by neurotrophin stimulation, thus migrating at higher molecular weight (>74kDa). However, pretreatment with Gö6976 or SU6656 dramatically inhibited the BDNF-induced molecular shift, indicating that a pathway involving PKCμ and SFKs mediates the phosphorylation of DIC during rFAT activation. This result suggests that PKCμ and SFKs may underlie regulatory pathway for DIC phosphorylation during rFAT activation in the context of neurotrophin transport and possibly fPD pathogenesis as well.

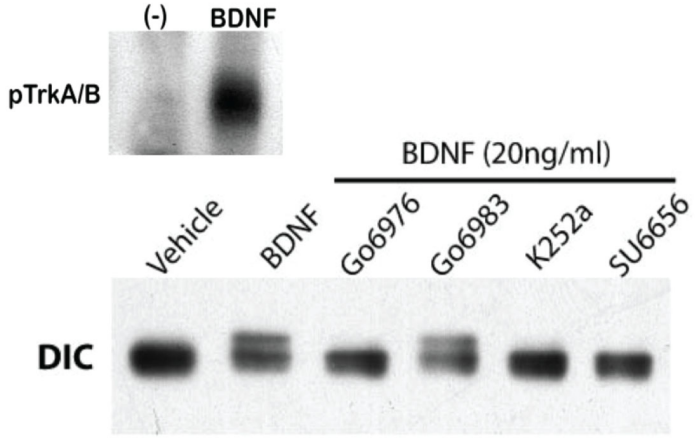

Fig.18. SFKs and PKCμ mediate BDNF-induced DIC shift in rat cortical neurons.
A) Increased Trk A/B phosphorylation indicates activation of rFAT-dependent BDNF signaling in rat cortical neurons. (B) BDNF induced DIC shift was prevented by treatment of Go6976(PKCμ-specific inbhitor) or SU6656(SFKs inhibitor). (Ctrl: vehicle alone; BDNF:Brain-derived neurotrophic factor; Go6983:general PKC inhibitor; K252a:Trk inhibitor).

Analysis of molecular shift of cytoplasmic dynein in lentiviral alpha-synuclein infected rat cortical neurons

To evaluate effects of mutant α-Syn on functionality-associated molecular modification of CDyn, we analyzed the molecular shift of DIC in lentiviral α-Syn infected rat cortical neurons. As described in Chapter IV Materials and Methods, rat primary cortical neurons were infected with either lentiviral α-Syn (WT or A30P) or control vector at DIV1 for 24hr. Transgene expression was confirmed by fluorescence microscopy (DsRed) or immunoblot analysis using DsRed and α-Syn antibodies. Upon 80~90% lentivirus infectivity which was confirmed by the presence of red fluorescent proteins (DsRed), neurons in the time course between postinfection Day1 (DIV2)-Day14 (DIV15) were collected. And cell lysates at particular time points showing overexpression of lentiviral synuclein proteins were analyzed for DIC molecular shift. As a positive control for phosphorylated DIC, cell lysates from rat primary cortical neurons (DIV1) stimulated with BDNF (20ng/ml) for 20min was run in the same gel with other samples. This procedure helped us evaluate effects of mutant α-Syn on posttranslational modification of CDyn, which might be a critical component underlying rFAT activation during PD pathogenesis.

Fig.19 represents the experimental results showing lentiviral α-Syn effects on the phosphorylation-induced molecular shift of DIC. Basically, negative control group (Fig.19, (-) of DIV7), which was not infected with lentivirus represented two distinct bands of DIC, of which the lower band corresponds to 72kDa (Fig.19, (-), lower band) and upper band indicates 74kDa (Fig.19, (-), upper band). Similarly, all other groups including control, WT and A30P infected neurons also showed the same two molecular weights (Fig.19, Ctrl, WT and A30P) as shown in negative control (Fig.19, (-)). Since the DIC antibody used in this research recognizes two isoforms DIC-I (72kDa) and –II (74kDa), we had to determine their identity of DIC bands (Fig.19, DIV7 and DIV15) by comparing with two DIC bands shown in BDNF stimulated neurons. When we ran all lysates in the same gel, it turned out the upper bands (74kDa) in (-),

Ctrl, WT and A30P at postinfection day 6 (Fig.19, DIV7) migrates at same MWs of lower band (74kDa) in BDNF stimulated neurons (Fig.18, BDNF, lower band), indicating that they are DIC-isoform II (DIC-II, 74kDa). Thus, the lower band shown in Fig.19 was determined as DIC isoform-I (DIC-I, 72kDa). However, BDNF stimulated neurons (Fig.18, BDNF) revealed another higher MWs (>74kDa) of DIC which corresponds to phosphorylated form of DIC (Fig.18, BDNF, upper band; Fig.10B, BDNF, upper band).

As DIV progresses, the ratio between DIC-I and –II was changed in all groups. Although there were still two DIC bands (Fig.19, DIV15) which are migrating at the exactly same MWs shown in DIV7 groups (Fig.19, DIV7), DIC-II (Fig.19, DIV15, upper band, 74kDa) level was slightly lower than DIC-I (Fig.19, DIV15, lower band, 72kDa), telling that there was no significant increase in the phosphorylation level of DIC-II in synuclein infected groups. Instead, this result showed us that there might be development regulation of DIC isoforms expression in these neurons, which was not significantly affected by overexpressed synucleins. Further speculation with regard to the negative result of synuclein effect on DIC phosphorylation will be discussed in Chapter VI.

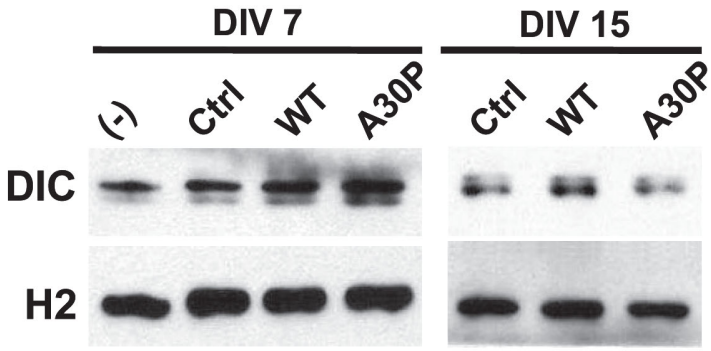

Fig.19. Effects of α-Syn on molecular shift of DIC in lentiviral synuclein infected rat cortical neurons. Change in the migration pattern of DIC caused by phosphorylation was analyzed through immunoblot. At DIV 7 (postinfection day 6), there were two distinct bands detected in non-infected group ((-) of DIV 7) that indicates DIC-I (72kDa,lower) and DIC-II (74kDa,upper). Also, all other groups including Ctrl, WT, and A30P (DIV7) showed similar pattern of DIC doublets. However, none of experimental groups showed higher molecular weight than DIC-II, corresponding to phosphorylated DIC thus migrating higher MWs (>74kDa) which was shown in BDNF-stimulated neurons (Fig.18, BDNF, upper band). At DIV 15 (postinfection day 14), DIC-II (74kDa, upper) decreased in all groups, indicating there was developmental downregulation in DIC-II expression but no molecular shift of phosphorylated DIC was detected. H2 shows their equivalent loading of proteins. (DIC: Dynein intermediate chain; DIC-I: DIC isoform 1, 72kDa; DIC-II: DIC isoform 2, 74kDa; H2: Kinesin heavy chain; (-): non-infected rat cortical neurons; Ctrl: empty vector-infected; WT: WT-synuclein-infected; A30P: A30P-synuclein-infected)

VI. DISCUSSIONS

In Aim 1

Different levels of α-Syn transgene expression: We have used lentiviral synuclein infected rat cortical neurons to evaluate synuclein effects on endogenous SFKs. On postinfection day 3, α-Syn transgene appears in both WT and A30P groups (Fig.13), however the protein level was not equivalent. It was not clear whether the differential expression is due to difference in the rate of protein expression between WT and A30P, or due to difference in the protein stability between those two groups. As we detected the protein level of DsRed through immunoblot analysis, both WT and A30P groups showed similar level of DsRed protein expression (Data not shown), meaning that the rate of transgene expression was not significantly different between WT and A30P infected groups. Based on the observation, it is thought that A30P synuclein, which is known to have higher propensity to form aggregates than WT (reviewed in Cookson MR, 2005) might be more stable than WT upon overexpression in neurons, resulting in higher level of proteins detected in A30P group. The transgene expression produces abnormally higher amounts than physiologically accepted concentration, as a result, giving a physical burden, which might be toxic to neurons. Assuming that neurons were trying to clear the excessive amount of synuclein proteins, A30P, which has higher propensity to aggregate, might be more stable than WT from attacks by degrading enzymes, therefore remaining at higher level than WT.

To evaluate WT or A30P synuclein effects at equal concentration, further lentivirus titration needs to be done for determining adjusted titer showing equivalent amount of synuclein proteins in WT and A30P groups. It will help us measure the cell biological effects caused by transgene expression of either WT or A30P comparatively.

Effects of WT overexpression: As we see in Fig 14A, at the beginning of synuclein transgene expression, both WT and A30P groups showed similar responses in SFKs phosphorylation at Tyr416. It is predicted, in terms of familial PD, WT overexpression might mimic the WT duplication/triplication which are implicated in fPD. WT multiplication is expected to produce abnormally high amount of synuclein proteins therefore leading to molecular crowding effect within cells, which will eventually promote synuclein aggregation as well as alterations in the functionality of synuclein proteins during fPD progression. As a negative control, we have used control group infected with empty vector (lentivirus without synuclein gene encoded). Also, non-infected group was added in our experimental design, which represented no significant difference from responses in empty vector-infected group.

SFKs phosphorylation during time course progression: To evaluate synuclein effects on endogenous SFKs activation in mammalian cells, we infected rat cortical neurons with synuclein lentiviruses and analyzed time course responses of SFKs phosphorylation.

At DIV 2 and 3 (postinfection day 1 and 2, respectively), we detected significant increase of phospho-SFK Tyr416 in both WT and A30P groups even at which it was relatively too early to detect strong overexpression of lentiviral synuclein proteins. This might explain why both WT and A30P lentiviral synucleins were capable of facilitating SFKs autophosphorylation even before the amount of overexpressed synuclein proteins reached at maximal concentration. However, as DIV progresses (DIV4~DIV15), the phosphorylation level between all groups including control, WT and A30P became similar, suggesting a possibility that there might be negative regulation on abnormally upregulated SFKs phosphorylation at Tyr416. In other way, it would be possible that overexpressed WT or A30P could not affect SFKs phosphorylation due to molecular crowding effect, which prevented synuclein monomers from interacting with SFKs. Also, it is suspected whether there was failure in the successful anterograde

transport of overexpressed synuclein proteins to the synaptic terminals, which might result in negative false result. Since early time points showed lower levels of synuclein proteins, these cells might be able to represent proper cell biological effects, however, which could be no longer seen because of spatial limitation of overexpressed transgene products. Consistent with this, our preliminary data on A30P effects in squid axoplasm showed slight decrease in aFAT (Fig.8), suggesting the pathogenic mutant form of synuclein might interrupt anterograde fast axonal transport, which would result in unsuccessful localization of newly translated synuclein proteins. This might lead to a lack of significant effects on SFKs phosphorylation despite successful transgene overexpression of lentiviral synucleins within cell bodies. In addition, it was too difficult to detect any changes of SFKs phosphorylation at Tyr416 since the basal phosphorylation level of SFKs (Tyr416) in control groups became stronger as DIV progresses. The phospho-SFKs antibodies we used in this research recognizes all subfamily members of SFKs, therefore it was difficult to identify which specific member was getting more phosphorylated by the presence of synucleins. Another challenge was that small increase in SFKs phosphorylation induced by synucleins would not be well quantitated using ECL method. As an alternative, we have tried to quantitate the SFKs phosphorylation affected by synucleins through immunoblot analysis using fluorescent dye-conjugated secondary antibodies. Unfortunately, this approach did not produce successful result because the signal from phospho-SFKs Tyr416 as well as phospho-SFKs Tyr527 antibodies was too low to quantitiate phospho-SFKs signals without enzymatically enhanced chemiluminescence. However, the *in vitro* autophosphorylation assay helped us to determine whether pathogenic synuclein elevates SFKs autophosphorylation *in vitro*. This method will be also useful to identify specific SFKs members, by evaluating *in vitro* autophosphorylation of individual SFKs members, which are commercially available in the presence of synuclein.

Effects of WT and A30P synucleins on Fyn autophosphorylation *in vitro*: When recombinant Fyn was coincubated with either WT or A30P *in vitro*, there was significant elevation in the autophosphorylation of Fyn, indicating that both WT and A30P are capable of enhancing Fyn activity. As we analyzed, both WT and A30P contain particular domains within exon 5 and exon 6, which are homologous to SFKs regulatory domains (Schematically drawn in Fig.16). Specifically, exon 5 contains two potential activation domains described as PXL (SH2 kinase linker) and PXXP (poly-proline) and a phosphorylatable tyrosine residue (Tyr125). Furthermore, within their exon 6 there are two phosphorylatable tyrosine residues called Tyr 133 and Tyr 136. These particular motifs present in WT and A30P might play a role of activation by interrupting inhibitory intramolecular interactions of SFKs, and leading to kinase activation.

Surprisingly, β-Syn also showed significant increase in the autophosphorylation of Fyn and substrate phosphorylation (Fig.16). We analyzed the C-terminal sequence of β-Syn, finding it containing two C-terminal tyrosine residues homologous with those tyrosine residues (Tyr125, Tyr133, Tyr136) located at the C-terminus of α-Syn. On the other hand, we could not find homologous sequences within middle or N-terminal domains of β-Syn that were similar to SFKs regulatory domains. This also suggests that C-terminal tyrosines at synuclein isoform resembling Y125 (exon 5), Y133(exon 6), or Y136(exon 6) of α-Syn may be able to enhance Fyn autophosphorylation.

Although both WT and A30P showed increase in Fyn autophosphorylation *in vitro*, it is thought that there might be further regulatory factors besides WT or A30P synucleins within squid axoplasms, by which rFAT might exhibit A30P-specific activation and SFKs might be phosphorylated. A30P mutation has been known to interrupt synuclein binding with lipids, thus releasing synuclein proteins into cytosolic compartment. This might release more free synuclein proteins into the cytosolic compartment in which synuclein is spatially apart from all cell and molecular environments that used to regulate the function of synuclein in normal condition. Further study to identify interacting partners of WT or A30P

will be helpful to investigate the mechanisms underlying A30P-specific activation of rFAT and SFKs as shown in squid axoplasm study.

In Aim 2

Developmental regulation of DIC isoforms expression in rat primary neurons: Expression of DIC isoforms is well known to be developmentally regulated by neurotrophins (Brill II LB and Pfister KK, 2000; Pfister KK et al, 1996; Salata MW et al, 2001). It is predicted that neurons might need to modify machineries required for FAT to transport a variety of different molecules in timely manner, therefore undergoing developmentally regulated expression of different subsets of molecular motors. Right after birth, it was shown that DIC-II (74kDa) is the major expressed DIC in rat neurons and DIC-I (72kDa) levels become elevated as the neurons age (Brill II LB and Pfister KK, 2000). However, expression level of DIC-II was not consistent during development because three different DIC-II isoforms (A,B, and C) were expressed along different time courses (Brill II LB and Pfister KK, 2000). This report correlates with our observation in neurons at DIV1 and DIV7, in which no significant DIC-I (72kDa) was detected in neurons at DIV1 but DIC-I appeared in the neurons at DIV 7 (Fig.19, DIV7, lower band, corresponding to 72kDa). At DIV 15, it was also shown that DIC-II level was slightly lower than DIC-I (Fig.19, DIV15), correlating with the investigation showing DIC-II level decreases around postnatal day 20 (Brill II LB and Pfister KK, 2000). However, we could not see any effect induced by lentivirally expressed synucleins on the developmental regulation of DIC isoforms expression, meaning that synuclein overexpression had no effect on the developmental regulation of DIC expression. Furthermore, synuclein infected neurons did not show relevant MW shift (>74kDa) corresponding to phosphorylated DIC which is seen as an upper band in BDNF stimulated neurons (Fig.18, BDNF, upper band). Although there was no significant phosphorylated DIC-II detected migrating at >74kDa in

synuclein infected neurons, we still need to determine whether there is other DIC isoforms getting phosphorylated since neurons are expressing total 5 different types of DIC isoforms (DIC-IA, IB, IIA, IIB, and IIC). To specifically detect phosphorylation level of all DIC isoforms, time course study using metabolic labeling (described in Brill II LB and Pfister KK, 2000; Pfister KK et al, 1996; Salata MW et al, 2001) of synuclein infected rat primary neurons will be required. This method will be better suited to analyze all forms of DIC phosphorylated and to quantitate the phosphorylation level of DIC than the current DIC shift assay. For a future direction, it will be also desirable to use primary neuronal culture from transgenic animals expressing the A30P mutation instead of using lentiviral infection. Primary neurons using transgenic animals are expected to be better for reducing complexity caused by developmental regulation of DIC isoforms expression shown in lentivirus infected neurons. Neuronal culture from transgenic animals do not require 72hr postinfection which is necessary for lentiviral transgene expression, thus allowing us to evaluate synuclein effects at early time points when the complexity of DIC isoforms expression does not appear yet. We anticipate that the directions suggested here will be helpful to understand the role of pathogenic α-Syn in the molecular events underlying the activation of rFAT during early stages of Parkinson's disease.

VII. CONCLUSIONS

Although genetic alterations such as autosomal dominant mutations or duplications of the α-Syn gene are implicated in fPD, mechanisms underlying the pathogenesis are not clearly understood. Given that select dopaminergic neurons undergo dying-back neurodegeneration during PD progression, we hypothesized mutations linked to fPD might be associated with FAT impairment, which is often a critical pathogenic component in dying-back neurodegenerative diseases. To understand the pathogenic role of mutant α-Syn indying-back process, we started by evaluating α-Syn effects on fast axonal transport in isolated squid axoplasm, which allowed us to show that α-Syn abnormally activates rFAT through a mechanisms mediated by the SFKs-PKCμ pathway. Based on these observations, we aimed to evaluate α-Syn effects on molecular events underlying the activation of SFKs-PKCμ and eventually in changing rFAT. Results of specific aim 1 showed that fPD-linked A30P synuclein dramatically increases SFKs autophosphorylation at Tyr416 in squid axoplasm, indicating A30P induces abnormal activation of rFAT by enhancing kinase activity of SFKs. There was also slight increase of SFKs autophosphorylation in lentiviral α-Syn infected rat primary neurons at early time course, but the effect was not apparent at later DIVs, implying there might be unknown compensatory effect within synuclein overexpressing neurons. In parallel, we also examined *in vitro* SFKs autophosphorylation in the presence of α-Syn, aiming to determine the role of α-Syn on Fyn in terms of autophosphorylation and kinase activity. There was surprising increase in Fyn autophosphorylation induced by α-Syn *in vitro* as well as 10~20% increased kinase activity. Furthermore, our study suggested that particular domains within exon 5 and exon 6 of α-Syn which are homologous to SFKs regulatory domains might play a role as an activator for SFKs. All the results obtained in aim 1 indicated that α-Syn could activate SFKs by facilitating autosphorphorylation, which might be critical step of α-Syn in the activation of rFAT. In aim

2, we evaluated effects of α-Syn on the molecular events associated with rFAT activation and also determined role of SFKs and PKCμ during rFAT activation in rat primary neurons. As a results, we found SFKs and PKCμ are critical mediators for DIC phosphorylation occurring in the neurotrophin induced rFAT activation. However, we could not find evidence showing that α-Syn overexpression affected DIC phosphorylation in terms of rFAT activation, which was due to methodological limitations in identifying phosphorylated DIC. We concluded that SFKs and PKCμ were key regulators mediating the phosphorylation of DIC during neurotrohpin induced rFAT activation. However, their regulatory role for DIC phosphorylation in the context of PD pathogenesis needs to be further investigated.

The observations in this research suggest that SFKs and PKCμ are critical mediators for rFAT in cellular system, and both WT and A30P α-Syn contribute to activation of SFKs by increasing their autophosphorylation and kinase activity, This pathway might be tightly regulated in a cell biological system depending on the genetic pathogenicity of α-Syn. We expect that understanding the role of pathogenic forms of α-Syn in SFKs activation and consequent changes in rFAT will lead us to know the molecular mechanisms underlying early events in the pathogenesis of PD more clearly.

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