DEVELOPMENT OF A SMALL ALPHA-SYNUCLEIN-KNOCKDOWN PEPTIDE AS A POTENTIAL THERAPY FOR PARKINSON'S DISEASE

by

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Abstract

Accumulating evidence supports the premise that reducing α-synuclein levels may be an effective and specific therapy for Parkinson's disease. To date, a clinically applicable α-synuclein reducing therapeutic strategy has yet to be developed. To remedy this, I developed a blood brain barrier and plasma membrane-permeable α-synuclein knockdown peptide that may have therapeutic potentials. By modifying a peptide-based method that was recently developed in our lab to rapidly and reversibly decrease the levels of endogenous proteins, I developed an α-synuclein knockdown peptide, Tat-βsyn-degron, and tested its specificity and efficacy in reducing the level of α-synuclein and its neuroprotective efficacy in well-characterized cellular and animal models of Parkinson's disease. I found that the peptide effectively reduced the level of α-synuclein via proteasomal degradation both in cell cultures and in freely moving animals. More importantly, the peptide-induced α-synuclein reduction was associated with a significant decrease in parkinsonian toxin-induced neuronal damage and motor impairment in an animal model of Parkinson's disease. These results suggest that targeted degradation of α-synuclein using the Tat-βsyn-degron peptide represents a novel, specific and effective therapeutic strategy for reversing a core cellular mechanism contributing to Parkinson's disease.

Preface

Major parts of the results in Chapter 3 have been published in:

1) A research article:

   Fan X*, **Jin WY***, Lu J, Wang J, Wang YT (2014b) Rapid and reversible knockdown of endogenous proteins by peptide-directed lysosomal degradation. Nature neuroscience 17:471-480. **(*Co-first author)**

   I constructed many of the plasmids in the study and worked with Dr. Xuelai Fan to finish the in vivo part of the project. Dr. Xuelai Fan finished most of the in vitro and part of the in vivo work and wrote the manuscript.

2) A review paper:

   Fan X, **Jin WY**, Wang YT (2014a) The NMDA receptor complex: a multifunctional machine at the glutamatergic synapse. Frontiers in cellular neuroscience 8:160.

   Under the guidance of my supervisor Dr. Yu Tian Wang, Dr. Xuelai Fan and I co-wrote this review paper.

3) An international patent:

   Name: Peptide directed protein knockdown

   Inventors: Yu Tian Wang, Xuelai Fan, **Wu Yang Jin**

   Application No.: PCT/CA2013/050741

   Publication No.: WO2014047741 A1

   Under the guidance of Dr. Wang, Dr. Xuelai Fan and I each produced some of the data for the patent application.

4) A scientific conference presentation:

Jin WY, Fan X, Lu J, Wang YT (2014) Peptide-mediated degradation of a death-inducing kinase as a novel therapy for stroke. Canadian National Proteomics Network meeting, Montreal.

I presented the data both in a poster presentation session and in an oral presentation session.

Manuscript reporting the major parts of the results presented in Chapter 4 and 5 has been submitted to a peer-reviewed journal:

Jin WY*, Fan X*****, Zhou L, Dai C, Gibbs E, He W, Li H, Wu X, Hill A, Girling K, Leavitt B, Cashman N, Liu L, Lu J, Dong Z, Wang YT. Development of a small α-synuclein-knockdown peptide as a potential PD therapy. Scientific reports. In revision. (***Co-first author**)

I conceived the ideas, designed the experiments, conducted most of the *in vitro* and part of the *in vivo* work, and performed all the data analysis. Dr. Xuelai Fan conceived the ideas, designed the experiments and conducted part of the *in vitro* work. Limin Zhou, Chunfang Dai, Wenting He, Hongjie Li and Xiaobin Wu from Dr. Zhifang Dong's lab in the Children Hospital of Chongqing Medical University conducted the rotarod behavioral test and did the immunostaining of the mouse brain tissues. Dr. Ebrima Gibbs from Dr. Neil Cashman's lab at University of British Columbia (UBC) conducted the Biacore binding assay. Austin Hill from Dr. Blair Leavitt's lab at UBC assisted in blinded neuron counting in slices. Kimberly Girling assisted in neuronal culture. Dr. Jie Lu assisted in plasmid construction and experiment design. Dr. Lidong Liu assisted in peptide synthesis and experiment design. My supervisor Dr. Yu Tian Wang conceived the ideas,

designed the experiments, supervised the whole project and wrote the manuscript with me.

Results in Chapter 6 have not been published. For the results in Fig. 14, I conceived the idea, designed the experiments, conducted the peptide administration, sliced the fresh brains, and performed all the data analysis; Dr. Shujun Lin from the Centre for High-Throughput Phenogenomics at UBC did the MALDI mass spectrometer imaging; Dr. Zhifang Dong in the Children Hospital of Chongqing Medical University conducted the *in vivo* recording experiment; My supervisor Dr. Yu Tian Wang conceived the idea, designed the experiments and supervised the whole project. For the results in Fig. 15, I conceived the idea, designed the experiments and constructed the plasmids for GST-fused proteins; Dr. Horacio Bach's lab at UBC is still conducting the phage display screening; My supervisor Dr. Yu Tian Wang conceived the idea, designed the experiments and supervised the whole project.

All experimental protocols in this thesis were approved by the UBC Animal Care Committee and the Chongqing Medical University Animal Care Committee, and the methods were carried out in accordance with the approved guidelines and regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used. At UBC, the protocol number for the rat MCAo ischemia surgery is #A12-0023 and the protocol number for the slice and primary culture is #A13-0139. My UBC animal care certificates are: 4449-10, RBH-1130-10, RSx-018-11, RA-44-11.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BBB – Blood brain barrier

CCA - Common carotid artery

CMA - Chaperone-mediated autophagy

CRISPR - Clustered regularly-interspaced short palindromic repeats

CSF - Cerebral spinal fluid

CTM - Chaperon-mediated autophagy targeting motif

DAPK1 - Death-associated protein kinase 1

DIV - Days *in vitro*

DMEM - Dulbecco's Modified Eagle's Medium

ECA - External carotid artery

EDC - N-ethyl-N'-(dimethylaminopropyl) carbodiide

EF1α - Elongation factor 1α

ES cell - Embryonic stem cell

FBS - Fetal Bovine Serum

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GBA - Glucocerebrosidase

GFAP - Glial fibrillary acidic protein

H&E - Hematoxylin (Mayer’s) and Eosin Y

HEK 293 cell - Human embryonic kidney 293 cell

hsc70 - Heat shock cognate protein 70

hsp90 - Heat shock protein 90

ICA - Internal carotid artery

i.n. - Intranasal

i.p. – Intraperitoneal

iPS cell - Induced pluripotent stem cell

i.v. – Intravenous

LAMP-2A - Lysosome-associated membrane protein type 2A

Lys-hsc70 - Lysosome-specific form of hsc70

LRRK2 - Leucine-rich repeat kinase 2

LTD – Long term depression

MALDI - Matrix-assisted laser desorption ionization

MAO-B - Monoamine oxidase B

MAPT - Microtubule-associated protein tau

MCAo - Middle cerebral arterial occlusion

MPP+ - 1-methyl-4-phenylpyridinium

MPPP - 1-methyl-4-phenyl-4-propionoxypiperidine

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride

MSC - Mesenchymal stem cell

NHS - N-hydroxysuccinimide

OCT3 - Organic cation transporter 3

PB - Paired-burst

PBS - Phosphate buffered saline

PD - Parkinson’s disease

PEG - Polyethylene glycol

PEP - Post-encephalitic Parkinsonism

PET - Positron emission tomography

PFA - Paraformalhyde

protac - Proteolysis targeting chimera

PVDF - Immobilon-PTM polyvynilidene fluoride

ROS - Reactive oxygen species

RU - Resonance units

RVG - Rabies virus glycoprotein

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

shRNA - Short hairpin RNA

siRNA - Small interference RNA

TALEN - Transcription-activator-like effector nuclease

TBST - Tris-buffered saline containing 0.1% Tween-20

TH - Tyrosine hydroxylase

TTC - 2,3,5-triphenyltetrazolium chloride

WT – Wild type

YFP - Yellow fluorescent protein

List of Plasmids

α-synuclein

FLAG-βsyn

FLAG-βsyn-degron

FLAG-βsynN-degron

HA-β-synuclein

HA-γ-synuclein

pcDNA3.0

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Frist of all, I would like to thank my supervisor Dr. Yu Tian Wang for offering me the precious opportunity to study in his lab and giving me the best research guidance he can give. Dr. Wang's vision, passion and deep understanding of scientific research have significantly influenced my career as a junior neuroscientist in the past six years. Every time I felt depressed about my results, it was Dr. Wang's encouragement and guidance that gave me enormous passion to move on. I sincerely thank all my committee members, Dr. Matthew Farrer, Dr. Lynn Raymond and Dr. Neil Cashman, for providing me with valuable suggestions for my PhD project. I also would like to thank all my colleagues and collaborators, especially Dr. Zhifang Dong, Dr. Ebrima Gibbs, Mr. Austin Hill, Dr. Jie Lu, Dr. Lidong Liu, Dr. Steven Connor and Ms. Yuping Li, for offering me plenty of help both in the lab and in my daily life. And special thanks to my labmate, Dr. Xuelai Fan, for being a fantastic collaborator and friend.

I thank all the organizations and generous donors that provided me with various scholarships: UBC Four Year Fellowship, UBC College for Interdisciplinary Studies Graduate Award, UBC International Partial Tuition Scholarship, Chinese Government Award for Outstanding Self-financed Students Abroad, Millie and Ralph Drabinsky Graduate Scholarship in Medicine, Harry and Florence Dennison Fellowship in Medical Research, Vancouver Coastal Health Research Institute Top Graduating Doctoral Student Award. I also thank the Canadian National Proteomics Network and the British Columbia Proteomics Network for providing me with travel awards.

Last but not least, I would offer my deepest gratitude to all my family members, especially my parents, my wife and my sister, for unconditional love and support.

To my parents and wife

For helping me become the best I can be

Chapter 1: Introduction

1.1 Overview of Parkinson's Disease

Parkinson's disease (PD) is one of the major degenerative disorders of the central nervous system. It was first described in 1817 by a British surgeon, James Parkinson, in his work *An Essay on the Shaking Palsy* (Parkinson, 1817), which established PD as a recognized medical condition. PD usually begins in mid to late life and its prevalence rises with age. It affects over 1% of the population over the age of 60, and 4-5% of the population over the age of 85 (Trinh and Farrer, 2013; Reeve et al., 2014). The mean age of onset of PD is 70 years (Trinh and Farrer, 2013), although about 4% of all PD cases, classified as young-onset PD, develop symptoms before the age of 50 (Trinh and Farrer, 2013; Ferguson et al., 2015). According to statistics from the US Parkinson's Disease Foundation, 7-10 million people worldwide are now affected by PD, regardless of race and culture; medication costs for an individual person with PD average $2,500 per year, and therapeutic surgery can cost up to $100,000 per patient. Therefore, PD has placed a heavy burden on numerous families, as well as the health care system in general.

The pathological hallmark of PD are cytoplasmic inclusions called Lewy bodies which form in dopaminergic neurons of the substantia nigra, due to abnormal aggregates of a protein called α-synuclein (Luk et al., 2012b; Recasens et al., 2014). Accumulating evidence suggests that there is a significant loss of dopaminergic neurons in the substantia nigra pars compacta of patients with PD (Goedert et al., 2013), which causes severe motor disorders such as tremors, rigidity, bradykinesia and impaired balance (Xia and Mao, 2012; Muller et al., 2013), and nonmotor disorders such as dementia and

sleep dysfunction (Chaudhuri and Schapira, 2009; McDowell and Chesselet, 2012; Muller et al., 2013). To date, there is no effective cure for PD, and current clinical treatments like deep brain stimulation, levodopa and dopamine agonists provide limited relief of symptoms while contributing to a range of undesirable complications and side effects (Fasano et al., 2012; Garcia-Ruiz et al., 2014; Bastide et al., 2015). Therefore, there is an urgent need for directed therapies that target the core cellular pathologies of PD, to reduce off target effects while improving efficacy in treatment of this devastating disease.

Although the cellular mechanisms contributing to PD lack full characterization and remain controversial, a general consensus is emerging within the scientific community that PD is a multifactorial disease, caused by both environmental and genetic factors (Trinh and Farrer, 2013; Goldman, 2014). Both are discussed in detail below.

1.1.1 Environmental Factors

Recent estimates suggest that about 90% cases of PD are idiopathic or sporadic, without any positive family history (Klein and Westenberger, 2012). Initial evidence suggesting that environmental factors could contribute to PD emerged when an epidemic of post-encephalitic parkinsonism (PEP) broke out in late 1910s (Trinh and Farrer, 2013). Due to the presence of oligoclonal IgG bands in the cerebral spinal fluid (CSF) of suspected cases and the finding of chronic active lesions in the brain tissue at autopsy (Casals et al., 1998), many people suggested that PEP was caused by viral infection that triggered dopaminergic neuron degeneration in the substantia nigra. However, identity of the virus responsible for PEP has yet to be determined and the

causes and risk factors contributing to PEP remain undefined (Casals et al., 1998; McCall et al., 2001).

However, a growing body of literature supporting links between PD and various other environmental factors, such as toxins (e.g. MPTP, 6-OHDA) (Jackson-Lewis and Przedborski, 2007; Simola et al., 2007), insecticides (e.g. rotenone) (Cannon et al., 2009; Goldman, 2014), herbicides (e.g. paraquat) (Berry et al., 2010; Tanner et al., 2011), fungicides (e.g. maneb, ziram) (Fitzmaurice et al., 2014), organic compounds (e.g. polychlorinated biphenyls) (Hatcher-Martin et al., 2012), chemical solvents (e.g. trichloroethylene, perchloroethylene, carbon tetrachloride) (Goldman et al., 2012) and metals (e.g. Fe, Cu, Mn, Hg) (Shribman et al., 2013; Dusek et al., 2015) continued to accumulate. Generally, these factors interfere with various aspects of normal physiological processes in dopaminergic neurons, such as mitochondria function (Exner et al., 2012), reactive oxygen species (ROS) production (Drechsel and Patel, 2008; Dias et al., 2013) and α-synuclein aggregation (Yamin et al., 2003), and thus contribute to the pathogenesis of PD. Among those, the most well-characterized risk factor for PD is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), a synthetic neurotoxin that has been extensively characterized in various models of PD (Jackson-Lewis and Przedborski, 2007).

MPTP was first discovered in 1976 by accident when a 23-year-old man in Maryland, USA, started exhibiting PD symptoms soon after self-injecting a synthetic opioid drug called 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (Tropini, 2007). Further analysis of the synthetic drug showed that MPTP was the major impurity generated during the synthesis of MPPP. Later, many research laboratories independently

confirmed that MPTP is a parkinsonian toxin that is able to selectively kill dopaminergic neurons in the substiantia nigra pars compacta and induce many features of PD (Jackson-Lewis and Przedborski, 2007; Meredith and Rademacher, 2011; Blesa et al., 2012; Khan, 2015). Studies showed that MPTP is able to freely cross the blood brain barrier (BBB) and the plasma membrane of neurons (Blesa et al., 2012). Once it enters the brain, it is metabolized into 1-methyl-4-phenylpyridinium (MPP+) by monoamine oxidase B (MAO-B) in astrocytes (Blesa et al., 2012). After MPP+ is released from astrocytes by organic cation transporter 3 (OCT3), it is taken up by the dopamine transporter on dopaminergic neuron membranes after which it inhibits complex I of the electron transfer chain in mitochondria (Blesa et al., 2012). As a result, stressed mitochondria decrease ATP production and generate ROS that activate cell death signaling pathways. These eventually result in the degeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in a PD-like illness. The toxicity of MPTP now has been well characterized in plenty of *in vitro* and *in vivo* models of PD. One of the most widely used models in PD research is the mouse MPTP model, due to its reliability, reproducibility, and ease of induction (Jackson-Lewis and Przedborski, 2007). The MPTP model is based on normal wild type (WT) C57BL/6 mice and thus does not require complicated genetic engineering of the mouse gene. Meanwhile, this model is not technically challenging and does not require invasive animal surgery or specialised equipment, such as stereotaxic frame. Systemic injection (e.g. intraperitoneal (i.p.) injection) of MPTP into C57BL/6 mice has been shown to produce reliable and reproducible lesion of the nigrostriatal dopaminergic pathway in less than a month, which leads to several major PD phenotypes, such as dopaminergic neuronal

loss in the substantia nigra, motor deficit and cognitive disorders (Jackson-Lewis and Przedborski, 2007; Yabuki et al., 2014; Lauretti et al., 2016). Therefore, I chose to use the MPTP mouse PD model for my *in vivo* experiments.

The relationship between environmental factors and PD is gaining in relevance and importance as environment pollution is escalating to unprecedented levels globally. Thus, further investigation of the environmental etiology of PD is necessary if we are to gain a fuller understanding of not only the risks in our community, but also preventative and treatment strategies going forward.

1.1.2 Genetic Factors

Evidence supporting a genetic basis for PD was lacking until the A53T mutation in α-synuclein was reported in 1997 as a PD risk factor in an Italian family (Polymeropoulos et al., 1997). Since then, multiple PD-associated genetic mutations have been identified that differentially interfere with various aspects of normal physiological processes (Trinh and Farrer, 2013; Lin and Farrer, 2014). Several of the most well characterized PD mutations are listed below:

1. α-synuclein: Aggregated α-synuclein protein is a key component of Lewy bodies, a hallmark of PD (Luk et al., 2012b; Recasens et al., 2014). Five PD-associated single mutations in the α-synuclein gene have been identified so far, which include: A30P (Kruger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Appel-Cresswell et al., 2013), G51D (Lesage et al., 2013), and A53T (Polymeropoulos et al., 1997). Multiplication (duplication and triplication) of the α-synuclein gene has also been found in some PD

patients (Singleton et al., 2003; Chartier-Harlin et al., 2004). All of these mutations/multiplications are linked with dominantly inherited late-onset PD (Trinh and Farrer, 2013) but their frequencies in PD patients are relatively low (Klein and Westenberger, 2012).

2. Leucine-rich repeat kinase 2 (LRRK2): Mutations in LRRK2 are the most common genetic cause for PD. Seven missense mutations (R1437H, R1441H, R1441G, R1441C, Y1699C, G2019S, I2020T) have been identified in LRRK2 so far and they are all linked with dominantly inherited late-onset PD (Trinh and Farrer, 2013). It is noteworthy to mention that the most frequent LRRK2 mutation, G2019S, is responsible for 1-2% of sporadic PD cases and 2-5% of familial PD cases (Lesage and Brice, 2009). Furthermore, LRRK2 G2019S was surprisingly found to be present in 18.3% of PD cases in Ashkenazi Jews (Ozelius et al., 2006) and 41% of PD cases in North African families (Lesage et al., 2005), suggesting the important role of LRRK2 mutations in the pathogenesis of PD. Although some evidence suggests that LRRK2 plays multiple biological roles in synaptic vesicle endocytosis, neurite length, synaptic transmission, and autophagy (Cookson, 2012), the detailed mechanisms by which the LRRK2 mutations contribute to the pathogenesis of PD is still not fully understood.

3. PINK1, Parkin and DJ-1: PINK1 and Parkin normally contribute to clearance of damaged mitochondria. PINK1 is a cytoplasmic protein kinase that accumulates on the outer membrane of mitochondria and activates Parkin upon mitochondria damage (Pickrell and Youle, 2015). Parkin, an E3 ubiquitin ligase, then ubiquitinates the outer membrane of mitochondria to induce selective mitophagy (Pickrell and Youle, 2015). Meanwhile, DJ-1 is a redox sensor for oxidative stress and its expression is increased in

response to cellular stressors (Ariga et al., 2013). DJ-1 both protects neurons against oxidative stress and facilitates maintenance of mitochondria integrity (Moore et al., 2005; Xiong et al., 2009; Ottolini et al., 2013), although the detailed molecular mechanisms have yet to be fully elucidated. Numerous mutations have been identified in PINK1, Parkin and DJ-1, which are all linked with juvenile and early-onset recessively inherited PD (Trinh and Farrer, 2013; Lin and Farrer, 2014). Mutations in Parkin are the most common genetic causes for early-onset PD, accounting for 77% of sporadic PD cases with an age at onset of 20 years or younger (Lucking et al., 2000). Loss-of-function mutations in PINK1, Parkin, and DJ-1 affect mitochondria biogenesis and autophagy induction, and have indistinguishable clinical phenotypes of PD (Klein and Westenberger, 2012; Trinh and Farrer, 2013).

4. VPS35 and DNAJC: VPS35 and DNAJC are both involved in endosomal receptor-sorting/recycling processes in the cell. VPS35 is a subunit of retromer, which is responsible for retrograde transport of proteins from endosomes to the trans-Golgi network (Zimprich et al., 2011), whereas DNAJC regulates the dynamics of clathrin coats on early endosomes (Vilarino-Guell et al., 2014). Their link with PD pathogenesis was not discovered until recently. Several mutations of VPS35 and DNAJC have been identified, including VPS35 (D620N) (Vilarino-Guell et al., 2011; Zimprich et al., 2011), DNAJC13 (N855S) (Vilarino-Guell et al., 2014) and DNAJC6 (splice site c.801−2 A>G mutation and homozygous truncating mutation p.Q734X) (Edvardson et al., 2012; Koroglu et al., 2013). It is interesting to note that the VPS35 and DNAJC13 mutations are linked with dominant late-onset PD (Vilarino-Guell et al., 2011; Zimprich et al., 2011; Vilarino-Guell et al., 2014), while the DNAJC6 mutations are linked with recessive early-

onset PD (Edvardson et al., 2012; Koroglu et al., 2013). Compared with other PD-associated mutations that are mentioned above, considerably less is known about the pathogenic mutations of both VPS35 and DNAJC. Further investigation is required to elucidate the molecular mechanisms by which these mutations contribute to neurodegeneration in PD patients.

5. ATP13A2 and ATP6AP2: The autophagy-lysosomal pathway removes abnormal proteins within cells and is thus vital for normal cellular functions. In addition to LRRK2 G2019S (Orenstein et al., 2013), mutations in several other proteins have also been linked with lysosomal dysfunction in PD, such as ATP13A2 and ATP6AP2, two types of ATPases that are found on the lysosome membrane. Altered splicing of ATP6AP2 has been observed in X-linked parkinsonism (Korvatska et al., 2013; Gupta et al., 2015) and a variety of mutations in ATP13A2 have been linked with juvenile and early-onset recessively inherited parkinsonism (Trinh and Farrer, 2013).

In addition to the aforementioned genes, studies in recent years have also implicated many other risk factors for PD, such as eukaryotic translation initiation factor EIF4G1 (Chartier-Harlin et al., 2011), microtubule-associated protein tau (MAPT) (Pankratz et al., 2012) and glucocerebrosidase (GBA) (Schapira, 2015). Further investigation in this field will undoubtedly reveal more genes which contribute to PD.

1.2 Current Clinical Approaches for Parkinson's Disease

Unfortunately, currently available treatments for PD have limited therapeutic efficacy and fail to target the core molecular pathologies contributing to the disease. Current

therapeutic approaches provide modest symptom relief only, and their use is complicated by a range of undesirable side effects. Several commonly-used clinical medications and therapies for PD are discussed below.

1.2.1 Levodopa and Dopamine Agonists

Loss of dopaminergic neurons in the substantia nigra of PD patients leads to insufficient dopamine signaling in the brain. Systemic administration of dopamine is not effective in patients because dopamine cannot cross the BBB to enter the brain (Ahlskog, 2014). Therefore, the dopamine precursor, levodopa, was introduced to the market in the 1960s and since has been the most effective and widely used medication for PD (Contin and Martinelli, 2010). Levodopa is orally bioavailable and can cross the BBB through the large neutral amino acid carrier system (Contin and Martinelli, 2010). Once inside the cell, levodopa is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase (Contin and Martinelli, 2010). Since levodopa is easy to break down by enzymes in the peripheral system of the body, it is often administered together with enzyme inhibitors, such as aromatic L-amino acid decarboxylase inhibitor (e.g. carbidopa, benserazide) (Iwaki et al., 2015) and catechol-O-methyltransferase inhibitor (e.g. entacapone) (Ahlskog, 2014), to allow a higher proportion of peripheral levodopa to enter the brain. Use of the enzyme inhibitors dramatically decreases the required levodopa dose in PD patients which in turn reduces levodopa-associated side effects. Inhibitor of MAO-B, an enzyme that breaks down dopamine in brain cells, is also often used with levodopa to prolong levodopa's effects (Ahlskog, 2014).

Unfortunately, although levodopa has been shown to dramatically ameliorate the motor symptoms of PD (Olanow et al., 2014), it cannot halt the degeneration of dopaminergic neurons and thus does not stop or slow down the progression of PD. After long-term use, the patient's response to levodopa declines and dose adjustment is often necessary. In terms of side effects, use of levodopa in human patients can cause plenty of side effects such as vomiting, nausea and dyskinesia (Ahlskog, 2014). Furthermore, many non-motor symptoms of PD, such as cognitive dysfunction, are not responsive to levodopa treatments (Sprenger and Poewe, 2013).

In addition to levodopa, dopamine agonists are another class of drugs that are also widely used as a PD medication. While levodopa needs to be converted to dopamine to have effects, dopamine agonists directly target dopamine receptors to mimic the effects of endogenous dopamine. Unfortunately, dopamine agonists are less effective at reducing motor symptoms relative to levodopa (Group et al., 2014) while recapitulating many of the same side effects such as vomiting, nausea and daytime sleepiness (Sprenger and Poewe, 2013). The serious concern about the off-target effects of dopamine agonists has been supported by clinical evidence showing that dopamine agonists pergolide and cabergoline are associated with an increased risk of cardiac valve disease in PD patients by acting at 5-hydroxytryptamine 2B receptors in heart valves (Antonini and Poewe, 2007; Schade et al., 2007; Zanettini et al., 2007). Furthermore, similar to levodopa, dopamine agonists do not halt or reduce the progression of PD. Meanwhile, many non-motor symptoms of PD, such as cognitive symptoms, are nonresponsive to dopamine agonists (Sprenger and Poewe, 2013).

1.2.2 Deep Brain Stimulation

Deep brain stimulation is a neurological procedure that involves delivering electric stimulation to selected areas of the brain. It blocks abnormal nerve signals that generate tremors and other PD symptoms and is only used to treat pharmacologically nonresponsive patients (Moro et al., 2016). The deep brain stimulation system has three components: 1) a stimulating electrode (also called a lead) that is inserted through a tiny hole in the skull and implanted into select areas of the brain; 2) a battery-powered neuro-stimulator that is implanted under the skin near the collarbone, in the chest or over the abdomen; 3) and an insulated extension wire that is implanted under the skin and connects the simulator and the electrode. Once implanted, electric stimulation generated from the neurostimulator is sent to the electrode via the extension wire to block nerve signals in targeted brain areas. Deep brain stimulation is mostly performed bilaterally in the patient's brain (Petraglia et al., 2016), but several studies have also suggested that unilateral simulation is sufficient to improve PD symptoms in some PD patients, such as aged patients who may be unable to withstand the unfavorable side effects and risks of bilateral procedures (Germano et al., 2004; Slowinski et al., 2007; Shemisa et al., 2011; Shenai et al., 2015). Deep brain stimulation has been widely used in treatment-resistant movement and affective disorders (Kringelbach et al., 2007), such as major depression (Morishita et al., 2014), chronic pain (Boccard et al., 2013) and essential tremor (Zhang et al., 2010), and was approved by the US FDA for treatment of PD in 2002. Although the physiological mechanisms responsible for symptom alleviation following deep brain stimulation remain unknown, long-term studies have shown that stimulation of either the subthalamic nucleus or the globus pallidus internus results

in significant improvement of motor features of PD (Moro et al., 2010; Castrioto et al., 2011), and stimulation of the thalamic ventralis intermedius results in better management of tremor symptoms of PD (Pahwa et al., 2006). Some non-motor symptoms of PD, such as sleep disorder and cognitive dysfunction, may also be improved by deep brain stimulation, possibly due to either the direct effects of the stimulation or reduced drug dose after motor symptom improvement (Fasano et al., 2012).

However, deep brain stimulation cannot stop the degeneration of dopaminergic neurons in the substantia nigra. It only induces symptomatic improvements and can only be applied in certain groups of patients (Lang and Widner, 2002; Machado et al., 2012). Patient's eligibility for deep brain stimulation is determined by rigorous and standardized evaluation in specialised surgical movement disorder centres (Moro et al., 2016). PD patients with depression, dementia or severe illness are often excluded from such surgical treatment (Lang and Widner, 2002). Deep brain stimulation also causes various complications and side effects in patients, such as infection, intracranial bleeding, cognitive decline and impaired decision-making (Frank et al., 2007; Witt et al., 2013; Bang Henriksen et al., 2016). The deep brain stimulation procedure involves invasive brain surgery which requires extensive post-surgery monitoring, making this approach particularly expensive.

1.2.3 Stem Cell Transplantation

Since PD is caused by loss of nigral dopaminergic neurons which extend their axons into the striatum, transplantation of stem cells to the brain seems to be a plausible way to repair the damaged central nervous system. Stem cells can be transplanted into either the substantia nigra or the striatum to restore dopamine signaling (Zhu et al., 2009), but the striatum is often the preferred place for cell transplantation since it is the primary site of dopamine loss in PD (Wijeyekoon and Barker, 2009). These transplanted stem cells can migrate from the striatum to the substantia nigra and potentially improve PD symptoms (Zhu et al., 2009). The feasibility of stem cell transplantation therapy has been demonstrated in several clinical trials of human fetal mesencephalic tissue transplantation into the striatum of PD patients (Lindvall, 2015). The first transplantation work in human patients was conducted in 1987 (Lindvall, 2015), but many conflicting results have been reported from various clinical trials since then.

On one hand, some studies suggest that some of the transplanted neurons are able to survive in the PD patient's brain, as demonstrated by methods such as positron emission tomography (PET) imaging (Wenning et al., 1997; Piccini et al., 1999) and post-mortem histological analysis of patients' brains (Kordower et al., 2008; Li et al., 2008; Li et al., 2010b). For example, PET imaging demonstrated that ^{18}F-DOPA uptake and ^{11}C-raclopride binding to dopamine receptors were normalized in the grafted striatum of PD patients (Wenning et al., 1997; Piccini et al., 1999), suggesting successful integration of these stem cell-derived dopaminergic neurons into host neuronal circuits. Furthermore, studies have also shown that in the most successful cases, some patients are able to withdraw from levodopa treatment and exhibit major

recovery for several years after human fetal mesencephalic tissue transplantation surgery (Wenning et al., 1997; Hagell et al., 1999; Piccini et al., 1999; Brundin et al., 2000).

On the other hand, some clinical studies failed to confirm the beneficial effects following human fetal mesencephalic tissue transplantation in PD patients (Freed et al., 2001; Olanow et al., 2003), indicating substantial variability in this therapy's efficacy in treating PD. It was also surprising to find that dyskinesia, a common motor disorder observed after levodopa treatment, was also observed in a subgroup of PD patients following transplantation of human fetal mesencephalic tissues (Politis, 2010). Meanwhile, post-mortem analysis of several patients' brains revealed that a fraction of transplanted healthy neurons also developed Lewy body pathology after surviving in the brain for more than 10 years (Kordower et al., 2008; Li et al., 2008; Li et al., 2010b), suggesting that PD pathology can propagate from host to graft and that stem cell transplantation therapy may not be able to stop the progression of PD.

Considering that human fetal mesencephalic tissues are often obtained from cadaveric fetuses following spontaneous or elective abortions, stillbirth, or surgery, the amount of available tissue is severely limited and its quality is also difficult to standardize (Ishii and Eto, 2014). Therefore, it is unlikely that human fetal mesencephalic tissue can meet the huge demand of stem cell transplantation in large numbers of PD patients.

Human embryonic stem (ES) cells and human induced pluripotent stem (iPS) cells are now becoming two alternate and important sources of stem cell transplantation in

PD patients (Lindvall, 2015). ES cells are derived from the undifferentiated inner mass of a human blastocyst and iPS cells are reprogrammed from adult cells (Lindvall, 2015). ES or iPS cells can be converted into dopaminergic neuron precursors/neuroblasts in culture dishes and then transplanted into the striatum of PD patients. Although beneficial effects have been seen in animal models of PD (Grealish et al., 2014; Hallett et al., 2015), the therapeutic efficacy of these ES or iPS-derived dopaminergic neurons have not been well characterized in clinical trials. Additionally, several major challenges have to be resolved before these ES or iPS-derived dopaminergic neurons can be safely and effectively used in a cell replacement therapy for PD. First, it is important to demonstrate the potency of ES or iPS-derived dopaminergic neurons in animal models of PD before moving into clinical trials. These transplanted neurons should survive in large numbers, integrate into host neuronal circuits and then significantly improve PD symptoms. Second, ES or iPS-derived cells contain some potentially tumorigenic cells that might pose a risk to PD patients (Ben-David and Benvenisty, 2011; Lin and Ying, 2013). Therefore, a safe protocol should be developed to convert stem cells into dopaminergic neurons in high efficiency and to remove unwanted cells by cell sorting methods. Finally, since ES cells are derived from human blastocysts (Tachibana et al., 2013), ethical issues should be properly addressed.

The use of mesenchymal stem cells (MSCs) is another viable option for stem cell transplantation in PD patients (Glavaski-Joksimovic and Bohn, 2013). What differentiates MSCs from the aforementioned stem cells, which mainly work by replacing damaged dopaminergic neurons in the brain, is that transplanted MSCs are able to protect and regenerate the existing damaged dopaminergic neurons primarily by

secreting a variety of growth factors and cytokines, which create a favorable environment to facilitate intrinsic restorative processes and to induce immunomodulatory and anti-inflammatory effects in the PD brain (Joyce et al., 2010; Glavaski-Joksimovic and Bohn, 2013; Murphy et al., 2013). Compared with other stem cells, the obvious advantage of MSCs is that they can easily be obtained in large quantities. Bone marrow is the primary source, and MSCs can also be isolated from various adult and neonatal tissues, such as adipose tissue, blood, umbilical cord and cord blood (Glavaski-Joksimovic and Bohn, 2013). More importantly, MSCs seem to be relatively safe with low tumorigenic properties, as observed in a phase 1 clinical trial in PD patients (Venkataramana et al., 2010). The administration of MSCs to PD patients is also easier than that of all the aforementioned stem cells, because many studies have shown that MSCs can be delivered to the PD brain by intrastriatal transplantation, intravenous injection or even intranasal injection (Cova et al., 2010; Wang et al., 2010; Danielyan et al., 2011). After getting into the PD brain, these MSCs effectively protected nigral dopaminergic neurons from degeneration and improved motor functions in a rat 6-OHDA unilateral lesion model of PD (Cova et al., 2010; Wang et al., 2010; Danielyan et al., 2011). Nevertheless, in spite of all the exciting results that have been obtained from animal studies of PD, the reliability of MSC transplantation therapy in preventing the degeneration of nigral dopaminergic neurons and in improving both the motor and non-motor symptoms in large numbers of PD patients remains uncertain. Considerably more validation is required before MSC transplantation is validated as a clinically effective therapy for PD.

1.3 Role of α-synuclein in Parkinson's Disease

PD is a multifactorial neurodegenerative disorder (Trinh and Farrer, 2013; Goldman, 2014), and accumulating evidence suggests that α-synuclein is one of the major proteins responsible for the pathogenesis of PD (Ruiperez et al., 2010; Stefanis, 2012). α-synuclein belongs to the synuclein family that consists of α-, β- and γ-synuclein (Surguchov, 2013). It is a cytosolic protein primarily present in neural tissues (Stefanis, 2012). The physiological roles of α-synuclein inside the cell have not been fully characterized, but recent studies have suggested that α-synuclein proteins are involved in various physiological processes such as ER-Golgi vesicle trafficking, mitochondrial fragmentation and dopamine synthesis (Gao et al., 2007; Kamp et al., 2010; Thayanidhi et al., 2010). α-synuclein is also found at the presynaptic terminal of neurons, inhibiting neurotransmitter release and synaptic vesicle recycling. Overexpressing α-synuclein in neurons inhibits synaptic vesicle exocytosis and reduces the readily releasable and recycling synaptic vesicle pools, and thus reduces neurotransmitter release (Nemani et al., 2010; Venda et al., 2010; Busch et al., 2014). Meanwhile, deleting α-synuclein in neurons results in smaller reserve pool and larger readily releasable and recycling pools (Venda et al., 2010; Scott and Roy, 2012).

In PD, α-synuclein proteins aggregate into cytoplasmic inclusions called Lewy bodies in dopaminergic neurons of substantia nigra, which is a hallmark of the disease (Luk et al., 2012b; Recasens et al., 2014), and thus interfere with normal physiological processes inside the cell. α-synuclein occurs physiologically as a helically folded tetramer that resists aggregation (Bartels et al., 2011), but during PD pathogenesis, α-synuclein proteins destabilize and then form β-sheet-like pathological oligomers before

further aggregating into Lewy body fibrils (Breydo et al., 2012). Increasing evidence from recent studies shows that the pre-fibril α-synuclein oligomers, but not the mature aggregates, are toxic to neurons and disrupt cellular homeostasis (Winner et al., 2011; Colla et al., 2012; Roberts and Brown, 2015). Moreover, most of the α-synuclein mutations (A30P, E46K, H50Q and A53T) identified thus far and linked with familial PD, efficiently promote protein aggregation into toxic oligomers and fibrils, thereby promoting cell dysfunction in PD patients (Conway et al., 2000; Li et al., 2001; Fredenburg et al., 2007; Khalaf et al., 2014). It is also known that there is an age-dependent increase of α-synuclein in the human brain (Chu and Kordower, 2007; Xuan et al., 2011) and large-scale genomic studies in human subjects have confirmed a linkage between familial PD and duplication or triplication of the α-synuclein gene (Singleton et al., 2003; Chartier-Harlin et al., 2004). Overexpression of α-synuclein both *in vitro* and *in vivo* have been shown to impart various physiological abnormalities including vesicle trafficking blockage, mitochondria energy production failure and neurotransmitter reduction (Kamp et al., 2010; Diogenes et al., 2012; Scott and Roy, 2012). Suppression of α-synuclein protein levels using genetic manipulation methods significantly protected against parkinsonian toxin MPTP/MPP+ -induced toxicity and thereby reduced neuronal damage in both human dopaminergic neuroblastoma cells and free-moving animals (Hayashita-Kinoh et al., 2006; Fountaine and Wade-Martins, 2007; Wu et al., 2009; Thomas et al., 2011).

Studies in recent years also indicate that α-synuclein is central to the prion hypothesis of PD, which suggests that α-synucleinopathy can propagate from affected neurons to interconnected unaffected healthy neurons in the brain in a prion-like

manner (Recasens and Dehay, 2014). Data suggests that addition of exogenous α-synuclein preformed fibrils to primary neuronal cultures seeded the recruitment of endogenous α-synuclein into Lewy bodies and Lewy neurite–like aggregates (Volpicelli-Daley et al., 2014), and that a single intracerebral inoculation of synthetic α-synuclein fibrils led to cell-to-cell transmission of pathologic α-synuclein and Parkinson's-like Lewy pathology in anatomically interconnected regions in both transgenic PD mice and WT non-transgenic mice (Luk et al., 2012b; Luk et al., 2012a). Furthermore, in non-transgenic mice intrastriatally injected with α-synuclein preformed fibrils, blocking cell-to-cell spreading of pathologic α-synuclein using an α-synuclein monoclonal antibody effectively reduced Lewy bodies and Lewy neurite pathology, ameliorated substantia nigra dopaminergic neuron loss, and improved motor impairments (Tran et al., 2014). This further supports the vital role of α-synuclein in the pathogenesis of PD.

Notably, either chronic or acute depletion of α-synuclein in animals using genetic manipulation methods has minimal effects on normal brain function and animal behavior (Abeliovich et al., 2000; Cabin et al., 2002; McCormack et al., 2010; Zharikov et al., 2015), presumably due to functional compensation by β- or γ-synuclein. Therefore, selective knockdown of α-synuclein protein might be a good therapeutic treatment for PD.

1.4 α-synuclein Knockdown Methods

Although there is still no clinically-approved drug/technique for α-synuclein knockdown in PD patients, considerable efforts have been made in both culture and

animal models of PD to create new ways to reduce the level of this vital protein in brain cells. Researchers have targeted every step of the central dogma “from gene to RNA to protein”, resulting in a variety of α-synuclein knockdown techniques that will be discussed below.

1.4.1 Gene Knockout

The invention of gene knockout methods has revolutionized scientific research by offering a novel way to precisely inactivate the function of a particular gene/protein. Gene knockout can be achieved in many ways, such as homologous recombination, zinc finger nucleases, transcription-activator-like effector nucleases (TALENs), the clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 system and more recently the NgAgo-ssDNA system (Hall et al., 2009; Gaj et al., 2013; Sander and Joung, 2014; Gao et al., 2016). Many genes have been successfully knocked out in animal models in the past two decades, including α-synuclein. Interestingly, α-synuclein knockout mice are viable and fertile, exhibit intact brain architecture, and develop dopaminergic cell bodies, fibers, and synapses normally (Abeliovich et al., 2000). Nigrostriatal terminals of α-synuclein knockout mice display a standard pattern of dopamine discharge and reuptake in response to simple electrical stimulations (Abeliovich et al., 2000). Moreover, results from several independent studies suggest that these α-synuclein knockout mice are resistant to parkinsonian toxin MPTP-induced degeneration of dopaminergic neurons and loss of striatal dopamine, suggesting the great therapeutic potential of α-synuclein gene knockout in treating PD (Dauer et al., 2002; Drolet et al., 2004; Thomas et al., 2011). However, gene knockout is irreversible,

and the manipulation is often accomplished in embryonic stem cells of animals followed by *in vitro* selection and *in vivo* breeding (Hall et al., 2009), which limits its applicability to adult PD patients at this moment. Furthermore, the ethics of human genome editing are still under debate.

1.4.2 RNA Targeting

Until now, silencing α-synuclein RNA remains to be the most popular approach in knocking down α-synuclein protein in preclinical research, because it is effective, cheap and easy to design. Many effective RNA silencing methods have been invented, such as antisense oligonucleotide, small interference RNA (siRNA) and short hairpin RNA (shRNA), and all of these show great efficacy in reducing α-synuclein RNA and protein levels in various animal models, including rats, mice and even primates (Leng and Chuang, 2006; Lewis et al., 2008; McCormack et al., 2010; Cooper et al., 2014; Zharikov et al., 2015). For instance, expression of an α-synuclein-targeting shRNA in the substantia nigra of adult rats using an adeno-associated virus expression vector significantly reduced the level of endogenous α-synuclein protein in the brain and thereby substantially attenuated the degeneration of nigral dopaminergic neurons and the progressive motor deficits in a rat rotenone toxicity model of PD (Zharikov et al., 2015). Unfortunately, in spite of all the promising results in animal PD studies, none of these RNA targeting methods mentioned above has been applied to PD patients in the clinic, possibly due to the inability of RNA therapeutics to cross the BBB and the plasma membrane of neurons. RNA targeting materials are often delivered into the brain via invasive intracerebral injection or viral transfection (McCormack et al., 2010; Zharikov et

al., 2015), which might not be immediately practical for chronic treatments in human PD patients. To overcome this disadvantage, a new brain-targeting technology was invented recently, which effectively brought α-synuclein siRNA across both the BBB and the plasma membrane of neurons in free-moving animals. Briefly, a 29-amino-acid peptide derived from rabies virus glycoprotein (RVG) is capable of crossing the BBB and then binds to acetylcholine receptor-expressing neurons in the brain (Kumar et al., 2007). By adding 9 positively-charged arginine residues at the carboxyl terminus of the RVG peptide, the chimeric RVG-9R peptide is able to effectively bind and deliver negatively-charged siRNA to acetylcholine receptor-expressing neurons in the brain after systemic injection in free-moving animals (Kumar et al., 2007). To avoid the immunogenicity of siRNA during repeated injections in the body, siRNA can be loaded into dendritic cell-derived exosomes using electroporation following which the neuron-specific RVG peptide is expressed on the exosome surface ensuring effective brain targeting (Alvarez-Erviti et al., 2011). Using this technique, a recent study showed that an intravenous injection of α-synuclein siRNA-loaded RVG-exosomes effectively reduced the mRNA and protein levels of α-synuclein throughout the brain and also significantly decreased the intraneuronal α-synuclein aggregates in dopaminergic neurons in the substantia nigra in a transgenic mouse PD model that overexpressed the human phosphorylation-mimic S129D α-synuclein (Cooper et al., 2014). However, preparation of the RVG-exosome-siRNA complex still remains technically challenging and this method is restricted to acetylcholine receptor-expressing neurons in the brain. Moreover, siRNA-mediated protein knockdown still faces fundamental limitations including waiting for the natural clearance of α-synuclein protein in the cell and the lack

of tunable and rapid protein knockdown. Since α-synuclein is a stable protein with a long half-life, it may take a few weeks for RNA targeting materials to induce significant knockdown of α-synuclein protein in the brain (Lewis et al., 2008; McCormack et al., 2010), which significantly limits the clinical application of RNA targeting materials in rapid treatment of PD symptoms in human patients. Meanwhile, because human genome has plenty of similar DNA/RNA sequences and sometimes RNA targeting materials can knock down a sequence without perfect base pairing, the off-target effects of RNA targeting materials such as siRNA are a common problem during clinical development and may lead to various side effects in human patients (Fedorov et al., 2006; Jackson and Linsley, 2010; Caffrey et al., 2011).

1.4.3 Direct Protein Knockdown

Posttranslational control of protein abundance using clinical relevant methods is still difficult for the time being. As reviewed by Dr. Thomas Wandless, most of the current protein knockdown methods heavily rely on genetic engineering of the target protein (Rakhit et al., 2014). For instance, after delicate genetic engineering, the target protein was linked with a Tor1 adaptor and the proteasome was linked with an Fpr1 adaptor. Upon the addition of rapamycin, a drug that heterodimerizes Tor1 and Fpr1, the genetically modified target protein was delivered to the proteasome for efficient degradation (Janse et al., 2004; Rakhit et al., 2014). Although these genetic manipulation-based methods provide convenient and powerful ways to control the level of a particular protein, they are unsuitable for clinical application in human patients since they are not able to knock down unmodified endogenous proteins in the cell. Importantly,

there is a well characterized method that is able to degrade unmodified endogenous proteins without using genetic modifications. This method uses synthetic molecules called bifunctional proteolysis targeting chimeras (protacs), which can bind to both the target protein and a known E3 ligase. Adding protacs to the system brings the target protein to the E3 ligase and thereby efficiently induces the degradation of the target protein (Sakamoto et al., 2001; Schneekloth et al., 2004). However, the protacs method is largely limited to proteins with known small-molecule ligands, and the ligands should also be functionalized while retaining reasonable solubility and cell permeability (Rakhit et al., 2014). Therefore, broad application of the protacs method in knocking down pathogenic proteins seems untenable at this moment.

Immunotherapy has also been a popular method in the recent years to reduce the level of a particular pathogenic protein in the brain. They are highly selective to their drug targets and therefore off-target effects usually are not a big concern. Many studies have shown that passive immunization with α-synuclein antibodies attenuates α-synuclein pathology in the brain, prevents loss of dopaminergic neurons and improves behavioral deficits in various animal models of PD (Masliah et al., 2011; Games et al., 2014; Tran et al., 2014; Bergstrom et al., 2015). Nevertheless, the detailed mechanism of antibody-mediated α-synuclein clearance is not fully understood. It was demonstrated that systemically injected α-synuclein antibodies could cross the BBB, bind to cells that display α-synuclein accumulation and promote α-synuclein clearance via the intracellular lysosomal degradation pathway (Masliah et al., 2011), while another study by Bae *et al* showed that α-synuclein antibodies could effectively block cell-to-cell transmission of pathological α-synuclein aggregates in the extracellular space of the

brain and that this antibody-assisted clearance occurred mainly in microglia but not astrocytes or neurons (Bae et al., 2012). Bae *et al* also showed that the antibody-α-synuclein complex was taken up through the Fcγ receptor on the microglia surface and then was delivered to the lysosome for degradation (Bae et al., 2012). Based on these results, several companies are racing to develop the first antibody-based drug for reducing α-synuclein, such as NeuroPhage (candidate: NPT088; in pre-clinical stage), Prothena (candidate: PRX002; in phase 1 clinical trial) and Biogen (candidate: BIIB054; in phase 1 clinical trial). In addition, active immunization, which uses the body's own immune system to produce antibodies against α-synuclein, has also been tried by various researchers and pharmaceutical companies. A selected fragment or full-length α-synuclein protein was injected into the body to induce endogenous antibody production, which has been found to effectively reduce the aggregation of α-synuclein in the brain and ameliorate the degeneration of dopaminergic neurons in various animals models of PD (Masliah et al., 2005; Sanchez-Guajardo et al., 2013; Mandler et al., 2014; Mandler et al., 2015). As a result of these encouraging translational research findings, several α-synuclein vaccines have also been moved into clinical trials, such as the PD01 and PD03 vaccines from AFFiRiS (both are in phase 1 clinical trial). However, since the BBB is a natural barrier against large molecules such as antibodies, CNS delivery remains an issue as it is estimated that only 0.1-0.2% of peripherally administered antibody can gain access to the brain (Banks et al., 2007; George and Brundin, 2015). Therefore, despite all the exciting results from animal studies, at this moment it still remains unclear as to how effective these immunotherapies will be in

reducing the level of α-synuclein protein in the brain of PD patients. Clearly, a gap still exists between studies in animals and clinical trials in human patients.

1.5 Endogenous Protein Degradation Systems

The autophagy lysosome system and the ubiquitin proteasome system are the two most important cellular systems for degrading endogenous proteins. Since many human diseases, including some of the age-related neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease, are pathologically linked to gain-of-function mutations, and/or increased expression level of a particular protein (e.g. α-synuclein), artificially inducing the degradation of a particular pathogenic protein via either of the two protein degradation systems using a clinically tenable method might be a good strategy in treating various diseases. To make this possible, we must have a good understanding of the molecular mechanisms underlying both endogenous protein degradation systems. Utilizing these systems, we may ultimately be able to develop novel therapeutic tools which harness the power of endogenous protein degradation systems to selectively reduce pathogenic target proteins.

1.5.1 Through the Autophagy Lysosome System

The lysosome is a single-membrane organelle in the mammalian cell that is responsible for breaking down all kinds of biological molecules. It is called vacuole in plants, fungi and algae. Over 50 hydrolytic enzymes specific to a wide range of targets have been identified in the lysosome and they are activated by the highly acidic pH

(approximately 4.5-5.5) in the lysosome's interior (Mindell, 2012; Wang et al., 2015b). The degradation products, such as amino acids and nucleotides, are recycled back to the cytosol by specific transporters on the lysosomal membrane and then can be used as building blocks for synthesizing new biological molecules in the cell (Appelqvist et al., 2013; Schwake et al., 2013). The waste disposal and recycling functions executed by the lysosome are vital for the maintenance of a healthy cellular environment. And dysfunction of the lysosome, which is caused by mutations in lysosome-associated enzymes, has been linked with various human diseases, such as lysosome storage disease, PD, and Alzheimer's disease (Dehay et al., 2013; Klein and Futerman, 2013; Gowrishankar et al., 2015).

Autophagy is an orderly cellular process in eukaryotic cells that takes cytoplasmic constituents to the lysosome for destruction and recycling. It is highly regulated by over 30 autophagy related genes and this self-degradative process is quite complex (Glick et al., 2010; Feng et al., 2014). Autophagy is highly conserved from yeast to mammals, both morphologically and with regard to the protein constituents that make up the core autophagy machinery (Feng et al., 2014). There are three well characterized forms of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). They are different from each other in target selectivity and in the organization of their degradation complexes.

Macroautophagy is the most prevalent form of autophagy, characterized by a distinctive double-membrane structure called autophagosome (Feng et al., 2014). Macroautophagy starts with a double-membrane open structure called phagophore, which sequesters the cargo, further expands and ultimately closes to become the

autophagosome (Eskelinen and Saftig, 2009; Feng et al., 2014). Afterwards, the autophagosome fuses with the lysosome so that the cargo is finally delivered for destruction and recycling. The phagophore assembly site, where the nucleation of the phagophore initiates, has been well characterized in yeast. It has been shown that various autophagy related genes have participated in this phagophore biogenesis process and almost every membrane compartment in the yeast has contributed to the formation of the phagophore membrane, including ER, Golgi, mitochondria and the plasma membrane (Geng and Klionsky, 2010; Shibutani and Yoshimori, 2014; Wang et al., 2014b). However, an equivalent structure of the yeast's phagophore assembly site in mammalian cells remains uncharacterized (Feng et al., 2014). Evidence also shows that some autophagosomes can fuse with late endosomes or multivesicular bodies to form a transient structure called amphisome before they finally fuse with lysosomes (Sanchez-Wandelmer and Reggiori, 2013; Klionsky et al., 2014). In this way, the autophagosomes can receive lysosome constituents such as membrane proteins and proton pumps (Eskelinen and Saftig, 2009).

Meanwhile, microautophagy is a much simpler process, involving direct engulfment of cytoplasmic constituents by the lysosome (Mijaljica et al., 2011). Compared with macroautophagy, microautophagy is still poorly characterized in mammalian cells, as aside from the commonly used electron microscope technique, there is a lack of specific approaches to directly monitor the lysosome structure during the microautophagy process (Mijaljica et al., 2011). Therefore, despite sparse pieces of evidence, the mechanism underlying microautophagy and its regulation of cargo selection largely remain undefined (Li et al., 2012b; Hu et al., 2015). It is worth mentioning that

microautophay and macroautophagy can be both selective and non-selective. Non-selective autophagy occurs in the turnover of bulk cytoplasm, while selective autophagy occurs in the degradation of damaged/superfluous organelles (e.g. mitochondria and peroxisome) and invasive microbes (Feng et al., 2014).

The third form of autophagy, CMA, is a highly selective lysosome-associated degradation process that only occurs in mammalian cells (Feng et al., 2014). CMA only targets single proteins that have a KFERQ or a KFERQ-like motif (Dice, 1990). This motif is recognized by a cytoplasmic chaperon protein called heat shock cognate protein 70 (hsc70) (Cuervo and Wong, 2014). Complexed with other chaperons and co-chaperons, such as hsp90, hsp40, BAG-1,Hip and Hop, hsc70 takes the target protein to the surface of the lysosome, where the target protein binds to the cytosolic tail of a single span membrane protein, the lysosome-associated membrane protein type 2A (LAMP-2A) (Agarraberes and Dice, 2001). The mechanism underlying the binding between the target protein and LAMP-2A is not fully understood. It has been suggested the binding is mediated by electrostatic interaction, since replacement of 4 positively charged amino acids (KHHH) in the cytosolic tail of LAMP-2A by neutral alanines significantly impaired the CMA process in transfected Chinese hamster ovary cells (Cuervo and Dice, 2000). After binding with its target protein, LAMP-2A undergoes a conformational change from a monomeric protein to a large multimeric translocation complex at the lysosome membrane, which internalizes the target protein into the lysosome lumen for efficient degradation (Bandyopadhyay et al., 2008b; Cuervo and Wong, 2014). This translocation process requires the presence of a lysosome-specific form of hsc70 (lys-hsc70) in the lysosome lumen, which helps “drag” the target protein

across the lysosome membrane (Agarraberes et al., 1997; Cuervo et al., 1997). It is interesting to note that the target protein must be unfolded before the translocation process (Salvador et al., 2000), a unique step that is not observed in other forms of autophagy. After the target protein is translocated, the translocation complex quickly disassembles and the LAMP-2A protein goes back to its monomeric form so that it is able to bind new target proteins (Bandyopadhyay et al., 2008b). It has been suggested that different forms of LAMP-2A have distinct functions: the monomeric form of LAMP-2A is responsible for target protein binding and the multimeric form of LAMP-2A is responsible for target protein translocation (Cuervo and Wong, 2014). Supportive evidence has demonstrated that a radiolabeled CMA substrate, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was only detected in association with LAMP-2A monomers, but not high molecular weight complexes of LAMP-2A, while preventing the multimerization of LAMP-2A through mutating the transmembrane domain of LAMP-2A specifically abolished the translocation process without affecting the target protein's binding to LAMP-2A (Bandyopadhyay et al., 2008a). During CMA, LAMP-2A undergoes continuous cycles of multimerization and disassembly, and it is tightly regulated by a number of proteins. For instance, the lysosome-specific form of heat shock protein 90 (hsp90) in the lysosome lumen and glial fibrillary acidic protein (GFAP) on the cytosolic side of the lysosome membrane enhance the stability of the translocation complex whereas hsc70 and elongation factor 1α (EF1α) on the cytosolic side of the lysosome membrane facilitate the dissociation of LAMP-2A from the translocation complex (Bandyopadhyay et al., 2008b; Bandyopadhyay et al., 2010).

As discussed above, the molecular mechanisms underlying macroautophagy, microautophagy and CMA are distinct and non-redundant, however, crosstalk has been found among the three autophagy systems. For example, inhibiting CMA function by RNAi-mediated silencing of LAMP-2A upregulated macroautophagy levels in mouse fibroblast cultures (Massey et al., 2006), whereas in mouse embryonic fibroblasts deficient in Atg5, an autophagy-related protein required for autophagosome formation, blockage of macroautophagy led to upregulation of CMA (Kaushik et al., 2008). In summary, different forms of autophagy actively compensate for each other so that normal function of the autophagy-lysosome system can be maintained within the cell.

1.5.2 Through the Ubiquitin Proteasome System

The ubiquitin proteasome system is another important system in the cell for protein degradation. While the autophagy lysosome system mainly targets long-lived macromolecule complexes and organelles, the ubiquitin proteasome system mainly targets short-lived or abnormally folded proteins (Li et al., 2012b). In most cases, proteins are degraded in the proteasome through the following steps (Amm et al., 2014; Vilchez et al., 2014): 1) the ubiquitin-activating enzyme (E1) activates the glycine residue at the carboxyl tail of a ubiquitin in an ATP-dependent manner; 2) activated ubiquitin is transferred to a cysteine site of a ubiquitin-conjugating enzyme (E2) to yield an E2-ubiquitin thioester intermediate; 3) a ubiquitin ligase (E3) links the activated ubiquitin from E2 to a lysine residue of the target protein; 4) procedures 1 to 3 are repeated several times to add more ubiquitins to the primary ubiquitin on the target protein. Ubiquitin has 7 internal lysine residues in positions 6, 11, 27, 29, 33, 48, and 63,

and the polyubiquitin chain usually is linked through the internal lysine 48 residue (Kravtsova-Ivantsiv et al., 2013); 5) the target protein labelled with a linear chain of at least 4 ubiquitins is recognized, unfolded and degraded by the proteasome. The whole degradation process is selective, primarily regulated by over 600 E3 ubiquitin ligases that have been identified in the human genome (Berndsen and Wolberger, 2014; Mattiroli and Sixma, 2014). As shown by previous studies, the proteasome exists in many forms, but contains two major components, the 28-subunit core particle (CP, also known as the 20S particle, contains the proteolytic active sites) and a regulatory particle of 19–20 subunits, depending on the species (RP, also known as the 19S particle or PA700) (Finley, 2009). In the proteasome, the target protein is degraded into short peptides, which are then hydrolyzed rapidly to amino acids by cytosolic peptidases (Kisselev et al., 1999; Saric et al., 2004; Zanker and Chen, 2014), whereas ubiquitins are removed from the target protein by proteasome-associated deubiquitinating enzymes and then are recycled for use in other ubiquitination processes (Amerik and Hochstrasser, 2004; Lee et al., 2011).

In some uncommon cases, beyond the aforementioned homogeneous lysine 48 linkage, a variety of other polyubiquitin chain linkages have also been shown to induce the degradation of the target protein, such as the homogeneous linkages of lysine 29, 11, 27 and 6 residues, the heterogeneous linkages of different lysine residues, and even the multiply branched (forked) linkages of several ubiquitins (Kravtsova-Ivantsiv et al., 2013; Amm et al., 2014). Additionally, although monoubiquitination is often associated with protein regulation, such as internalization and endosomal sorting of a plasma membrane protein (Haglund and Dikic, 2012; Baker et al., 2013; Tanno and

Komada, 2013), it has also been shown to induce the proteasomal degradation of certain target proteins (Boutet et al., 2007; Boutet et al., 2010; Carvallo et al., 2010; Dimova et al., 2012). Sometimes, ubiquitin labeling on residues other than lysine (e.g. cysteine, serine or threonine) is also sufficient to serve as a proteasome-dependent degradation signal (Kravtsova-Ivantsiv et al., 2013; Amm et al., 2014). Taken together, these findings suggest that ubiquitination of cellular proteins for proteasomal degradation can be done in a highly diverse manner.

Interestingly, people have identified a few peptide sequences that can serve as a signal for proteasomal degradation, such as the PEST sequence (Ramakrishna et al., 2011; Li et al., 2012a) , the “destruction box” motif from cyclin (Yang et al., 2013) and the 4-amino-acid degron sequence (RRRG) (Bonger et al., 2011). Among them, the RRRG degron sequence is particularly interesting because it is the shortest proteasomal degradation signal identified so far. When the RRRG degron sequence was fused directly to the carboxyl terminus of yellow fluorescent protein (YFP), it was sufficient to lower YFP fluorescence to the background level observed in untransduced NIH-3T3 cells (Bonger et al., 2011). Since the RRRG peptide is quite easy to synthesize using a commercially available chemical synthesizer, it may become a powerful tool in regulating the level of a target protein directly at the protein level.

1.6 Rationale and Hypothesis

Taken together, PD is a major neurodegenerative disorder that currently lacks a clinically relevant treatment that can directly target the disease-causing processes.

Current clinical approaches, like deep brain stimulation and pharmacological treatments with levodopa and dopamine agonists, only relieve symptoms. The efficacy of these treatments is largely limited by their undesirable complications and side effects (Lundqvist, 2007; Groiss et al., 2009; Karlsborg et al., 2010b). Accumulating evidence supports the theory that nigral dopaminergic neuronal loss, along with the main symptoms of PD, are at least partly associated with α-synuclein protein aggregation in the cytoplasm of these neurons (Cookson, 2009; Stefanis, 2012). Indeed, knockdown of α-synuclein using genetic manipulations, such as siRNAs, has shown protection of dopaminergic neurons from the effects of parkinsonian toxin MPP+ or MPTP-induced cell death, both *in vitro* and *in vivo* (Hayashita-Kinoh et al., 2006; Klivenyi et al., 2006; Fountaine and Wade-Martins, 2007; McCormack et al., 2010). The clinical translation of siRNA into an efficient PD therapy has however been hindered, in part, due to the limited ability of siRNA to cross the BBB and the plasma membrane of neurons in affected areas of the brain. Although several recent studies suggest that this may be partially improved by coupling siRNA with a brain delivery vehicle (Kumar et al., 2007; Alvarez-Erviti et al., 2011; Cooper et al., 2014), there remains an urgent need for developing new α-synuclein knockdown strategies that are more practical for therapeutic use in human patients. *Therefore, I propose to develop a clinically relevant technology that uses short, BBB and plasma membrane-permeant synthetic peptides to rapidly reduce endogenous proteins via lysosomal or proteasomal degradation.*

As shown in **Fig. 1.1**, the proposed targeting peptide is composed of three domains: 1) the plasma membrane transduction domain Tat, which is capable of delivering peptides across both the BBB and the plasma membrane of neurons following systemic

administration in freely moving animals (Hill et al., 2012; Fan et al., 2014b); 2) the protein binding domain that is able to specifically bind to the target protein with high affinity; and 3) the lysosomal or proteasomal targeting domain that can direct the peptide-protein complex to the lysosome or proteasome for degradation.

Based on this peptide design, and in collaboration with my colleague Dr. Xuelai Fan, I first designed two targeting peptides for death-associated protein kinase 1 (DAPK1) and PSD-95, respectively. These two synthetic peptides were linked with a lysosomal degradation signal and effectively knocked down their target proteins in cultured neurons. These results were later published in Nature Neuroscience (Fan et al., 2014b; I was one of the two co-first authors). We were the first to show that an endogenous protein can be rapidly and reversibly knocked down using a clinically applicable peptide. Furthermore, the peptide-mediated protein knockdown technology we developed is versatile. By changing the protein binding sequence in the middle of the targeting peptide, in theory, we should be able to knock down any protein of interest. Therefore, I propose to further optimize this technology and use it to develop an α-synuclein knockdown peptide as a potential PD therapeutic. ***I hypothesize that by using a small synthetic peptide that is both BBB and plasma membrane-permeable, I will be able to effectively knock down endogenous α-synuclein in the brain and therefore rescue the dopaminergic neuronal loss and behavioral deficits in an animal model of PD.***

The main objectives of the present thesis research include the following:

1) Investigate the peptide-mediated protein knockdown technology in freely moving animals. To become a therapeutic drug for PD patients, a molecule has to be able to modulate the level of endogenous α-synuclein protein *in vivo*, which remains technically challenging at this time. Therefore, after Dr. Xuelai Fan finished the *in vitro* part of her project, I collaborated with her and tested the peptide-mediated protein knockdown technology *in vivo*. To this end, we apply the DAPK1 targeting peptide in a well-characterised rat model of focal ischemia to see whether the peptide can effectively knock down activated DAPK1 in the brain and thus protect against ischemia-induced brain infarct.
2) Develop a small α-synuclein knockdown peptide. The α-synuclein knockdown efficacy, efficiency and specificity of the peptide are thoroughly investigated in cultured cells.
3) Investigate the therapeutic efficacy of the α-synuclein knockdown peptide in PD models. The α-synuclein knockdown peptide is investigated in both cell culture and animal models of PD to see whether it can significantly knock down α-synuclein and thus protect dopaminergic neurons against parkinsonian toxin-induced damage.

The present thesis research is expected to lead to a novel technology to manipulate endogenous protein levels both *in vitro* and *in vivo*, and a novel peptide that can rapidly reduce the level of endogenous α-synuclein in the brain. Not only will the new technology become a powerful tool to research protein functions, but will also significantly contribute to the development of novel therapeutics for various diseases. Additionally, by knocking down endogenous α-synuclein, one of the most important

proteins associated with PD pathology, the α-synuclein knockdown peptide may become a novel disease-modifying treatment for PD that directly targets the core pathological processes driving the disease.

Figure 1

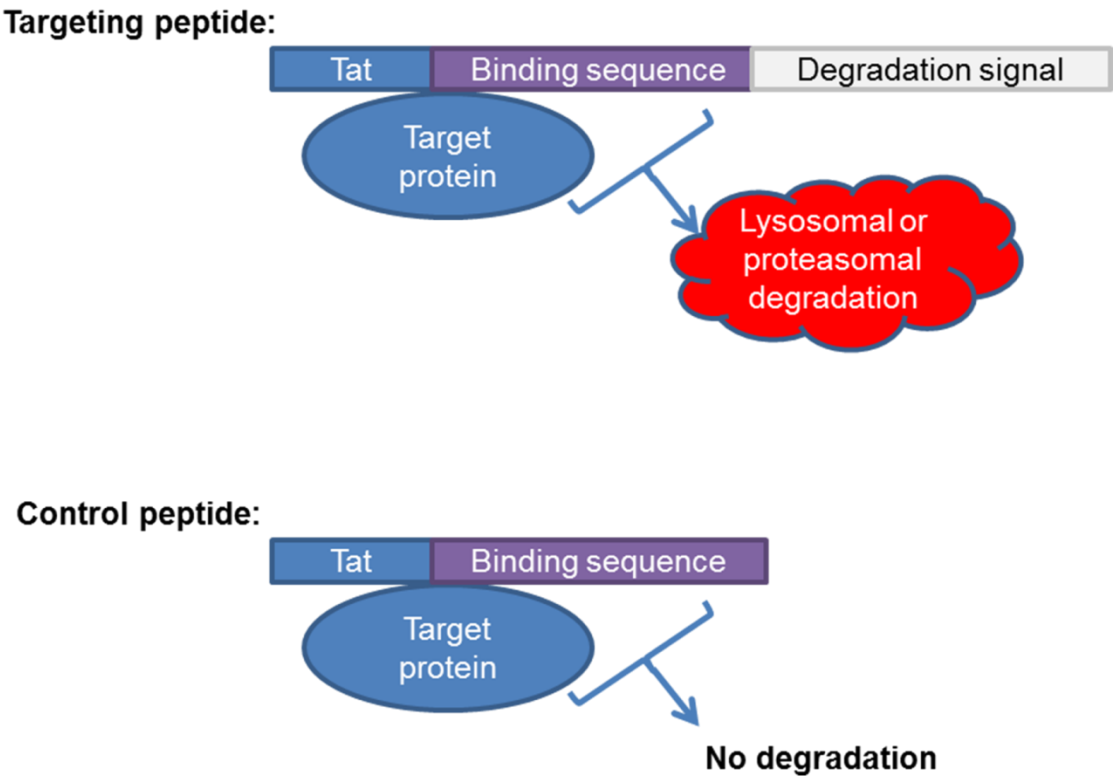

Targeting peptide:
Tat
Binding sequence
Degradation signal
Target protein
Lysosomal or proteasomal degradation
Control peptide:
Tat
Binding sequence
Target protein
No degradation

Figure 1. Schematic of the peptide-mediated protein knockdown technology

Schematic illustration of the targeting peptide design. The targeting peptide has three domains: 1) the Tat transduction domain that enables the peptide to penetrate the BBB and cell membranes, 2) the binding sequence that will bind to the target protein with high affinity, and 3) the degradation signal that targets the peptide-protein complex to the lysosome or proteasome for degradation. In contrast, the control peptide lacks the degradation signal, and hence while it can bind to the target protein, it cannot direct the peptide-protein complex to the lysosome or proteasome for degradation.

Chapter 2: Methods and Materials

2.1 Cell Culture

2.1.1 HEK 293 Cell Culture and Plasmid Transfection

Human Embryonic Kidney 293 (HEK 293) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, D6429) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, 12483020). When HEK 293 cells achieved 90% confluence, plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, 11668019) according to manufacturer's instruction. Total plasmid transfection amount in every group was made equal by supplementing the pcDNA3.0 empty vector. HEK 293 cells were then maintained in the 37°C incubator with 95% O_2 and 5% CO_2 for 48hrs before being used in experiments.

2.1.2 Primary Neuron Culture and Drug Treatment

Rat primary neuron cultures were prepared from embryos of pregnant Sprague-Dawley rats (E18). Briefly, the cortical tissue or ventral midbrain tissue was isolated into ice cold HBSS (Invitrogen, 14170-112) and then digested with 0.25% trypsin-EDTA at 37°C for 30 min. After washing with warm DMEM (supplemented with 10% FBS) three times, neurons were suspended in neuron culture media (2% B-27 supplement (Invitrogen, 17504-044) and 0.5 mM GlutaMax supplement (Invitrogen, 35050-061) in Neurobasal Media (Invitrogen, 21103-049)) and dissociated by trituration using varying sizes of pipettes. Neurons were then centrifuged, and the pellet was re-suspended in culture media, washed twice with culture media, and plated on the poly-D-lysine-coated

plates. Neuron culture was maintained in the 37°C incubator with 95% O_2 and 5% CO_2. The morning after culturing, 2/3 of the neuron culture media was replaced with fresh neuron culture media. Media was then replaced every 3-4 days. Rat primary cortical neuron culture was used on 14 days *in vitro* (DIV) and rat primary ventral midbrain neuron culture was used on 3 DIV.

Mouse primary cortical neuron cultures were prepared from postnatal FVB-N mice (P0-P1). Briefly, the cortical tissue was isolated into ice cold HBSS and then digested with 0.25% trypsin-EDTA at 37°C for 30min. After washing with warm DMEM (supplemented with 10%FBS) three times, neurons were suspended in plating media (Neurobasal Media, B27, GlutaMax and antibiotic-antimycotic (Life Technologies, 15240-062)) with DNAse1 (Sigma, D4527) and dissociated by trituration using varying sizes of pipettes. Cells were then centrifuged, and the pellet was re-suspended in plating media (no DNAse), washed twice with plating media, and plated on the poly-D-lysine-coated plates. Neuron culture was maintained in the 37°C incubator with 95% O_2 and 5% CO_2. The morning after culture, 2/3 of the plating media was replaced with fresh media. Cells were then fed every 3-4 days and the neuron culture was used on 9-11 DIV.

Tat-βsyn-degron and Tat-βsyn peptides were first dissolved in sterile water as 25 mM stock solutions and then diluted directly in the neurobasal culture media to make the desired working concentration. 20 mM MPP+ iodide stock solution was made freshly each time and diluted in the neurobasal culture media directly to make the desired working concentration. For 48hrs treatment, neuron culture media containing MPP+ and peptides were replaced every 24hrs.

2.2 Plasmid Engineering

2.2.1 Oligonucleotide Annealing

The FLAG-βsyn-degron and FLAG-βsynN-degron peptide minigenes were constructed by oligonucleotide annealing. The sequence information of both peptide minigenes is shown in **Table 7** in section **2.2.6**. Briefly, after the sense and anti-sense oligonucleotides of a minigene were chemically synthesized in Integrated DNA Technologies (IDT), they were reconstituted with nuclease-free duplex buffer (IDT, 11-01-03-01) to a final concentration of 1μg/ul. 5ul of each oligonucleotide solution (1ug/ul) and 10ul duplex buffer were mixed together in a clean tube to make the total volume 20ul. The tube was heated at 65 degree in a water bath for 5min, and then slowly cooled down below 37℃ (~30-60min). The sense and antisense oligonucleotides will form duplex during this process. The mixture was mixed with 30ul ddH_2O, 5ul 3M sodium acetate (pH 5.2) and 150ul 100% ethanol, and then was quickly frozen at -80℃ for 1 hour. The tube was centrifuged at 4℃ for 20min. Supernatant was carefully removed and the DNA pellet was air-dried and subsequently dissolved in 60ul nuclease-free ddH_2O. To verify annealing, 1ul of the DNA solution was subject to electrophoresis on a 1% agarose DNA gel at 100V for 5 min.

For both FLAG-βsyn-degron and FLAG-βsynN-degron peptide minigenes, a HindIII digestion site was added to the 5’ end and a NotI digestion site was added to the 3’ end during oligonucleotide synthesis in IDT.

2.2.2 PCR Amplification

HA-β-synuclein and HA-γ-synuclein were amplified from a rat cDNA library using PCR. The primers for HA-β-synuclein were:

HA-β-synuclein_Forward (BamHI):

5’-
CGGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGGACGTGTTCATGAAG
GGCCTGTCCATG-3’

HA-β-synuclein_Reverse (NotI):

5’-AAGGAAAAAAGCGGCCGCTTACGCCTCTGGCTCGTATTCCTGATATTCCTC-3’

The primers for HA-γ-synuclein were:

HA-γ-synuclein_Forward (BamHI):

5’-
CGGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGGACGTCTTCAAGAAA
GGCTTCTCCATT-3’

HA-γ-synuclein _Reverse (NotI):

5’-AAGGAAAAAAGCGGCCGCCTAGTCTCCTCCACTCTTGGCCTCTTCGCCCTC-3’

The PCR mixture was prepared as follows:

Table 1. PCR Mixture Preparation

Component	25ul Reaction Volume
2X KAPA HiFi HotStart ReadyMix	12.5ul
Forward Primer (125ng/ul)	1.25ul
Reverse Primer (125ng/ul)	1.25ul
cDNA	1ul
PCR-grade water	9ul

PCR was performed with the following cycling protocol:

Table 2. PCR Cycling Protocol

Step	Temperature	Duration	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	98 ℃	20 sec	35
Annealing	55-75 ℃ (5℃ below the Tm of primers)	15 sec	
Extension	72℃	60 sec/kb	
Final extension	72℃	1 min/kb	1

To verify PCR, 2ul of the PCR product was subject to electrophoresis on a 1% agarose DNA gel at 100V for 10 min. PCR product was purified using QIAquick PCR Purification Kit (QIAGEN, 28106).

2.2.3 Double Digestion, Purification and Ligation

Annealed oligonucleotides, PCR products or pcDNA3.0 mammalian expression vector was double digested using corresponding FastDigest restriction enzymes (Thermo Fisher Scientific). Digestion was performed with the following protocol:

Table 3. Double Enzymatic Digestion Protocol

Component	50ul Reaction Volume
1st Restriction Enzyme	2.5ul
2nd Restriction Enzyme	2.5ul
10× Double Digestion Buffer	5ul
DNA	~2.5ug in 30ul volume
Nuclease-free water	10ul

Double digestion was performed for 30min-60min according to manufacturer's instruction. Digested DNA products were then purified using QIAquick PCR Purification Kit.

Digested DNA products were ligated into pcDNA3.0 mammalian expression vector that was digested with same restriction enzymes as the DNA products. Ligation was performed with the following protocol:

Table 4. DNA Ligation Protocol

Component	20ul Reaction Volume
Digested DNA products	10ul
Digested pcDNA3.0 vector	1ul
10× Ligation Buffer	2ul
T4 ligase (NEB, M0202S)	1ul
Nuclease-free water	6ul

The mixture was maintained at 22℃ for 2h.

2.2.4 Plasmid Identification, Amplification, Purification and Storage

10ul ligation mixture was mixed with 50ul DH5α E.Coli competent cells for 30min on ice. Competent cells were heat shocked at 37℃ for 1 min and then placed back on ice for another 2 min. Next, competent cells were mixed with 200ul LB solution and then shaked at 37℃ at 250-280rpm for 45 min before they were cultured on LB plates with appropriate antibiotics (ampicillin or kanamycin) at 37℃ for 15-18 hours. Then, each individual E.Coli colony was hand-picked and transferred to a tube containing 3ml liquid LB solution and appropriate antibiotics (ampicillin or kanamycin). The LB solution was shaked in the tube at 37℃ at 250-280rpm for 15-18 hours.

To screen for positive plasmid constructs after ligation, plasmid from LB solution was purified using QIAprep Spin Miniprep Kit (QIAGEN, 27106) according to manufacturer's instruction. Sequences of the purified plasmids were verified by DNA sequencing at

UBC. Only plasmids with the right DNA sequence were kept for further use. The LB solution containing the right plasmid was kept for plasmid amplification.

To amplify the plasmid to a large amount, 250ul of the aforementioned LB solution with amplified E.Coli was added to 250ml fresh LB solution with appropriate antibiotics (ampicillin or kanamycin). The solution was shaked at 37℃ at 250-280rpm for 15-18 hours. A large amount of the plasmid was purified using a plasmid Maxi Kit (QIAGEN, 12163) according to manufacturer's instruction. Purified plasmid was dissolved in nuclease-free water solution and stored at -20℃ for further use.

2.2.5 Site-directed Mutagenesis

The FLAG-βsyn peptide minigene was constructed by mutating the CGA residues (corresponding to the first arginine residue in the "RRRG" degron peptide sequence) into the stop codon TGA on the FLAG-βsyn-degron plasmid.

The primers for the site-directed mutagenesis were:

Stopatβsyn_Forward: TATTTGGTTGGTTGACGACGAGGCT

Stopatβsyn_Reverse: AGCCTCGTCGTCAACCAACCAAATA

The PCR mixture was prepared as follows:

Table 5. PCR Mixture Preparation for Point Mutation

Component	25ul Reaction Volume
2X KAPA HiFi HotStart ReadyMix	12.5ul
Forward Primer (125ng/ul)	1.25ul
Reverse Primer (125ng/ul)	1.25ul
FLAG-βsyn-degron plasmid template	1ul (50ng)
PCR-grade water	9ul

PCR was performed with the following cycling protocol:

Table 6. PCR Cycling Protocol for Point Mutation

Step	Temperature	Duration	Cycles
Initial denaturation	95℃	3 min	1
Denaturation	98 ℃	20 sec	15
Annealing	55℃	15 sec	
Extension	72℃	60 sec/kb	
Final extension	72℃	1 min/kb	1

After PCR, the mixture was treated with 1ul DpnI FastDigest enzyme (Thermo Fisher, FD1703) at 37℃ for 1 hour to digest all the original templates. Next, the PCR mixture was transformed into DH5α E.Coli competent cells as shown in section **2.2.4**, followed by screening and plasmid identification.

2.2.6 Plasmid Sequence Information

Human α-synuclein plasmid was a generous gift from Dr. Hong Qing from Beijing Institute of Technology, China. HA-β-synuclein and HA-γ-synuclein were amplified from a rat cDNA library as shown in section **2.2.2** and then inserted into pcDNA3.0 mammalian expression vector.

The sequences for the α-synuclein targeting peptide minigenes are shown below.

Table 7. Plasmid Sequence Information

Name	Vector	Restriction sites	Translated peptide sequence	DNA sequence
FLAG-βsyn-degron	pcDNA3.0 mammalian expression vector	HindIII at 5'; NotI at 3'	DYKDDDDKR TKSGVYLVG RRRG	ATGGACTACAAG GACGACGATGAC AAGCGTACTAAAT CTGGTGTTTATTT GGTTGGTCGACG ACGAGGCTAA
FLAG-βsynN-degron	pcDNA3.0 mammalian expression vector	HindIII at 5'; NotI at 3'	DYKDDDDKG VLYVGSKTRR RRG	ATGGACTACAAG GACGACGATGAC AAGGGGGTGCTG TACGTGGGGAGC AAGACGAGGCGA CGACGAGGCTAA

Name	Vector	Restriction sites	Translated peptide sequence	DNA sequence
FLAG-βsyn	pcDNA3.0 mammalian expression vector	HindIII at 5'; NotI at 3'	DYKDDDDKR TKSGVYLVG	ATGGACTACAAG GACGACGATGAC AAGCGTACTAAAT CTGGTGTT TATTTGGTTGGTT GACGACGAGGCT AA

2.3 Peptide Synthesis, Storage and Administration

2.3.1 Synthesis, Storage and Administration

Tat-GluN2B, Tat-GluN2BCTM, Tat-βsyn-degron and Tat-βsyn peptides were chemically synthesized by GL Biochem (Shanghai, China). Tat peptide was synthesized in our lab using the Prelude peptide synthesizer (Protein Technologies Inc.). Dry peptide powder was kept at -80℃ for long-term storage. For *in vitro* use, Tat-βsyn-degron and Tat-βsyn peptides were first dissolved in sterile water as 25 mM stock solutions and then diluted directly in the neurobasal culture media to make the desired working concentration. For *in vivo* use in mice, Tat-βsyn-degron and Tat-βsyn peptides were directly dissolved in sterile saline and then administered into mice by i.p. injection. For *in*

vivo use in rats, Tat-GluN2B and Tat-GluN2BCTM peptides were directly dissolved in sterile saline and then administered into rats by intravenous (i.v.) injection.

2.3.2 Peptide Sequence Information

The sequences of all the peptides used in this study are shown below. All these peptides were chemically synthesized using natural L-amino acids.

Table 8. Peptide Sequence Information

Peptide Name	Sequence	Purity (Confirmed by HPLC)
Tat-GluN2BCTM	YGRKKRRQRRRKKNRNKLRRQHSY KFERQKILDQRFFE	>95%
Tat-GluN2B	YGRKKRRQRRRKKNRNKLRRQHSY	>95%
Tat-βsyn-degron	YGRKKRRQRRRRTKSGVYLVGRRRG	>95%
Tat-βsyn	YGRKKRRQRRRRTKSGVYLVG	>95%
Tat	YGRKKRRQRRR	>95%

2.4 Biacore Peptide-Protein Binding Assay

Biacore experiments were performed using a Biacore 3000 instrument (GE Healthcare Biosciences, Upsala, Sweden) and HBS running buffer, pH 7.4, containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20. A research-grade CM5 sensor chip was activated with a mixture containing equal molar amounts of EDC (N-ethyl-N'-(dimethylaminopropyl) carbodiide) and NHS (N-hydroxysuccinimide).

Purified recombinant human α-synuclein (N-terminal histidine tagged, Sigma, S7820-500UG), diluted in sodium acetate pH3.1, was then injected and covalently coupled to a flow cell of the sensor chip surface by amide bonding. Residual unreacted sites were blocked with ethanolamine. A reference surface, to account for non-specific binding, was similarly generated by activating and blocking an adjacent flow cell. Approximately 1500 resonance units (RU), equivalent to a surface concentration of 1500 pg/mm^2 of α-synuclein, was immobilized.

To ascertain the binding interaction between α-synuclein targeting peptides and α-synuclein, synthetic Tat-βsyn-degron peptide or control Tat peptide was serially diluted and sequentially injected over the active surface containing immobilized α-synuclein and the reference surface for 3 minutes at a flow rate of 30μl/minute. The peptides were then allowed to dissociate for 3 minutes during which time HBS running buffer was injected. The resultant sensorgrams were double-referenced by subtracting out the binding on the reference surface and the response from the HBS blank buffer control. Binding response report points were collected 20 seconds into the dissociation phase of the interaction at time 200 seconds, which represents a stable binding response and excludes bulk refractive index changes and nonspecific binding.

2.5 Processing of Cell and Tissue Samples

2.5.1 Protein Concentration Determination

Cultured cells or brain tissues were lysed on ice in the lysis buffer for 1 hour. Cell lysis buffer contained 0.5% Triton X-100, 0.5% deoxycholic acid, and 1× protease and phosphatase inhibitor cocktail (Thermo Scientific, 78442) in sterile phosphate buffered

saline (PBS, pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4). The lysis solution was then centrifuged at 14,000 rpm for 10 min at 4°C. Cell debris was removed and the supernatant was transferred to a clean tube.

Protein concentrations were determined using a BCA protein assay kit (DC Protein Assay Kit, Bio-Rad, 5000112) according to manufacturer's instruction. Briefly, 10mg/ml BSA solution was serially diluted using lysis buffer to yield protein standards with the following concentrations: 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.312mg/ml. 10ul protein standards or protein samples were mixed with 100ul Reagent A and 890ul Reagent B. The mixture was rested at room temperature for 15-30min to allow color to develop and subsequently measured at 750nm in a Biochrom Ultrospec UV/Vis Spectrophotometer (Fisher Scientific, BC80710008). Protein concentrations of the samples were calculated according to the standard curve.

2.5.2 Immunoblotting Analysis

Equal amount of protein samples were mixed with 4× sample buffer (50% Glycerol, 125 mM pH 6.8 Tris-HCl, 4% SDS, 0.08% bromophenol blue, and 5% β-mercaptoethanol), boiled at 100°C for 5 min, and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the electrophoresis system from Bio-Rad (Bio-Rad, 1658000). Proteins were then transferred to Immobilon-PTM polyvynilidene fluoride (PVDF) membranes (Bio-Rad, 162-0177) using the electrophoretic transfer system from Bio-Rad (Bio-Rad, 1703989). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20

(TBST, pH 7.4, containing 50mM Tris-HCl, 150mM NaCl, and 0.1% Tween-20) for 1 hr at room temperature, and then incubated overnight at 4°C with primary antibody. After washing 3× 5 min in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit IgG, PerkinElmer, NEF812001EA, 1:5000 dilution; Goat anti-mouse IgG, PerkinElmer, NEF822001EA, 1:5000 dilution; Goat anti-rat IgG, Amersham, NA935, 1:5000 dilution) for 1 hr at room temperature. After another three washes with TBST, protein was visualized in the Bio-Rad Imager (Bio-Rad ChemiDoc MP System, 1708280) using ECL Western blotting substrate (Pierce, 32016). The band density of each protein was quantified by the Bio-Rad Quantity One software and the relative optical density was analyzed relative to the loading control β-actin on the same membrane.

2.5.3 Synaptosomal Fractionation

Synaptosomes from cultured cortical neurons were isolated according to a previous literature (Hahn et al., 2009). Briefly, after culture media were aspirated from the dishes, cortical neurons were homogenized on ice in a homogenization buffer that contained 1 mM $NaHCO_3$, 1mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1× protease and phosphatase inhibitor in 0.32M sucrose solution. The total homogenate was centrifuged at 1,400g for 15min at 4°C. The pellet was discarded and the supernatant was further centrifuged at 16,000g for 20min at 4°C. Then, the pellet, which contained the synaptosome fraction, and the supernatant, which contained the cytoplasmic fraction, were harvested separately and subject to immunoblotting analysis.

2.5.4 List of Primary Antibodies Used for Immunoblotting

All the primary antibodies used in this study for immunoblotting are shown below.

Table 9. List of Primary Antibodies Used for Immunoblotting

Antibody Name	Company	Catalogue No.	Host	Dilution
Anti-DAPK1	Sigma	D1319	Rabbit polyclonal IgG	1:1000
Anti-phospho-DAPK1 (pSer^{308})	Sigma	D4941	Mouse monoclonal IgG	1:1000
Anti-β-actin	Abcam	ab8227	Rabbit polyclonal IgG	1:1000
Anti-α-synuclein	BD Transduction Laboratories	610786	Mouse monoclonal IgG	1:1000
Anti-HA	Roche	118674231001	Rat monoclonal IgG	1:1000
Anti-GAPDH	Abcam	ab9485	Rabbit polyclonal IgG	1:1000
Anti-synapsin	Synaptic Systems	106001	Mouse monoclonal IgG	1:1000
Anti-$GABA_A$ β2/3 receptor	Millipore	05-474	Mouse monoclonal IgG	1:1000
Anti-HSP90	BD Transduction Laboratories	610418	Mouse monoclonal IgG	1:1000

Antibody Name	Company	Catalogue No.	Host	Dilution
Anti-14-3-3	Millipore	06-511	Rabbit polyclonal IgG	1:1000
Anti-tyrosine hydroxylase	BD Transduction Laboratories	612300	Mouse monoclonal IgG	1:1000

2.6 Histology and Immunochemistry Analysis

2.6.1 Fresh Brain Slicing for MALDI Mass Spectrometry Imaging

After the rat was sacrificed, fresh rat brain was taken out from the head, snap frozen on the dry ice, mounted on a Leica cryostat using Tissue-Tek CRYO-OCT compound (Fisher Scientific, 14-373-65) and then sliced into 30 um sections. After overlaid with a Mass Spectrometry matrix on the surface, the whole brain slice was scanned two-dimensionally at 100 µm resolution using matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging at UBC Centre for High-Throughput Phenogenomics. Presence of a peptide signal was indicated by a pseudo-green dot in the image of the brain slice. The full-length peptide and its degradation fragments can be simultaneously detected in the same brain slice.

2.6.2 2,3,5-triphenyltetrazolium chloride (TTC) Staining of Fresh Brain Tissues

Rat was euthanized by an i.p. overdose of 2.5 g/kg urethane (Sigma, U2500). Fresh brain was taken out from the skull, rinsed with ice-cold PBS, and then sliced into 2mm sections from rostral to caudal using blades in a brain mold. About seven sections were

expected from each rat. Brain sections were then transferred to a 2% TTC (Sigma, T8877-25G) solution and kept in the dark at room temperature for 15-30min to allow color to develop. Healthy brain tissues would gradually turn red but infarct areas would remain white. Each brain section was photographed using a digital camera. White infarct tissues and the contralateral healthy tissues were dissected out from the brain sections and subject to further immunoblotting analysis.

2.6.3 Paraformalhyde (PFA) Fixation of Animal Brains

Rat or mouse was anesthetized by an i.p. injection of 1.5 g/kg urethane. Right after the animal became unresponsive, the abdomen was carefully opened and a needle that was connected with a perfusion pump was directly inserted into the protrusion of left ventricle. A cut in the atrium was made to allow blood to flow out of the body. Animal was first perfused with 0.9% saline until the fluid coming out of the atrium became clear. Then, animal was perfused with same amount of 4% PFA solution. Brain was taken out afterwards and fixed at 4% PFA in a falcon tube for another 24 hrs at 4°C before being transferred to 30% sucrose/PBS solution for cryoprotection at 4°C. Once the brain has sunk to the bottom of the sucrose/PBS solution, it was taken out and then frozen at -80°C. Frozen brain was mounted on a Leica cryostat using Tissue-Tek CRYO-OCT compound and sliced into 30 um sections. Brain slices were kept in PBS at 4°C.

2.6.4 Hematoxylin (Mayer's) and Eosin Y (H&E) Staining

PFA-fixed rat brain was sliced into 30um coronal sections, which were then mounted and dried on glass slides (Fisher Scientific, 12-550-15). Slides were stained 0.1%

Hematoxylin solution (Mayer's) (Sigma, MHS1) for 15 min in the dark, rinsed under tap water for 5 min and then dipped 12 times into 0.5% Eosin Y (Sigma, E4009-5G) solution in 95% ethanol. Slides were washed in distilled water until the eosin stopped streaking. Slides were then dehydrated in 50% and 70% ethanol sequentially by dipping into each solution 10 times. Finally, Slides were equilibrated in 95% ethanol for 30s and 100% ethanol for 1min, cleared with xylene for 2×1min, and mounted with coverslips using Permount (Fisher Scientific, SP15-500) mounting medium.

2.6.5 Fluoro-Jade B Staining

30um PFA-fixed brain slices were placed in a chamber, washed with PBS for 3×10min, equilibrated in 100% ethanol for 3min, and then sequentially re-hydrated in 70% ethanol for 1min and distilled water for 1min. To decrease background staining, brain slices were soaked in 0.06% $KMnO_4$ solution with gentle agitation for 15min and then washed with distilled water for 1min. Brain slices were stained in 0.001% fresh Fluoro-Jade B (Millipore, AG310) solution in 0.1% acetic acid for 40min with gentle agitation, washed with distilled water for 3×10min, and then mounted on glass slides in the dark room. After overnight drying, brain slices were cleared in xylene for 3×2min and mounted with coverslips using Permount mounting medium.

Fluoro-Jade-positive neurons in the rat hippocampus were imaged with the Zeiss Axio Observer D1 microscope at 10× and the neuron numbers were automatically counted by Image J particle analyzer.

2.6.6 DAPK1 Staining in Brain Slices

PFA-fixed rat brains were sliced into 30 µm sections using a Leica cryostat. After the brain slices were washed with PBS for 3×10min with gentle agitation, they were blocked and permeabilized using a PBS solution containing 1% BSA and 0.2% Triton X-100 for 30 min at room temperature. The brain slices were washed with 0.5% BSA in PBS for 3×10min and then incubated with anti-DAPK1 antibody (1:100 dilution in 0.5% BSA in PBS; Sigma, D1319) for 3 days at 4°C with constant agitation. The brain slices were washed in PBS for 3×10min at room temperature and then incubated with Alexa Fluor 488 (1:1000 dilution in 0.5% BSA in PBS; ThermoFisher Scientific, A-11034) overnight at 4°C with gentle agitation. Brain slices were washed in PBS for 3×10min and then mounted on glass slides in the dark room. After overnight drying, brain slices were mounted with coverslips using Fluoromount-G slide mounting media (SouthernBiotech, 0100-01). DAPK1 immunofluorescence was visualized under Zeiss Axio Observer D1 microscope.

2.6.7 Tyrosine Hydroxylase (TH) Staining in Cultured Neurons

Cultured ventral midbrain neurons on coverslips were rinsed 4× with ice-cold PBS, 2min each time, and fixed with 4% PFA for 1 hr at 37°C. Neurons were then washed 3× 5min in PBS with gentle agitation, and subsequently incubated in 0.25% TritonX-100/PBS for 5min at room temperature with gentle shaking. Next, neurons were washed 1× in PBS for 5min, and then incubated for 30min at 37°C in 10%BSA/PBS without agitation to block non-specific staining. To label TH, neurons were incubated in primary TH antibody (1:100 dilution in 3% BSA/PBS; BD Transduction Laboratories, 612300) at

4°C for 5 days without agitation. Neurons were then washed 6× 2min in PBS, and incubated in Alexa Fluor 488 (1:500 dilution in 3% BSA/PBS; ThermoFisher, A-11034) for 45min at 37°C without agitation. Next, neurons were washed 6× 2min in PBS, mounted on glass slides with Fluoromount-G slide mounting media and stored at room temperature overnight to dry. TH-positive neurons were visualized under Zeiss Axio Observer D1 microscope.

2.6.8 TH Staining in Brain Slices

PFA-fixed mouse brains were sliced into 30 µm sections using a Leica cryostat. After the brain slices were washed with PBS for 3×10min with gentle agitation, they were blocked and permeabilized using a PBS solution containing 1% BSA and 0.2% Triton X-100 for 30 min at room temperature. The brain slices were washed with 0.5% BSA in PBS for 3×10min and then incubated with anti-TH antibody (1:800 dilution in 0.5% BSA in PBS; BD Transduction Laboratories, 612300) for 3 days at 4°C with constant agitation. The brain slices were then washed in PBS for 3×10min at room temperature. TH-positive neurons in the substantia nigra and TH-positive neuronal terminals in the striatum were stained using the anti-mouse Ig HRP detection kit (BD Transduction Laboratories, 551011) according to manufacturer's instruction. Visible color was directly developed on the brain slices. Stained brain slices were dehydrated through 4 changes of alcohol solution (95%, 95%, 100% and 100%) for 5 min each and mounted on glass slides. After overnight drying, brain slices were cleared in xylene for 3×2min and mounted with coverslips using Permount mounting medium. TH staining in both striatum and substantia nigra were visualized under Zeiss Axio Observer D1 microscope.

2.6.9 List of Primary Antibodies Used for Immunochemistry

All the primary antibodies used in this study for immunochemistry are shown below.

Table 10. List of Primary Antibodies Used for Immunochemistry

Antibody Name	Company	Catalogue No.	Host	Dilution
Anti-DAPK1	Sigma	D1319	Rabbit polyclonal IgG	1:100
Anti-TH	BD Transduction Laboratories	612300	Mouse monoclonal IgG	1:100 in cultured neurons; 1:800 in brain slices

2.7 TH-positive Neuron Death Analysis

2.7.1 Densitometric Analysis of Striatal TH Staining

The optical density of the TH-positive neuronal terminal staining in the mouse dorsolateral striatum where dopaminergic inputs from the substantia nigra pars compacta were received (Antzoulatos et al., 2011; Macdonald and Monchi, 2011) was quantified using NIH Image J software. The optical density from the overlying corpus callosum was used as a background (Antzoulatos et al., 2011) and subtracted from every measurement in the striatum. Briefly, a circle was placed in the dorsolateral striatum, and optical density was read by Image J software. Then, the circle was moved to the overlying corpus callosum and the second optical density was read. The adjusted optical density was calculated by subtracting the value in the corpus callosum from

every measurement in the striatum. Finally, the optical density in the experimental group was normalized to the value from the control group.

2.7.2 TH-positive Neuron Counting in Ventral Midbrain Cultures

TH-positive neurons in ventral midbrain cultures were imaged with the Zeiss Axio Observer D1 microscope at 20× and 10 fields of view per coverslip were randomly selected and counted. Imaging and counting for TH-positive cells were performed by an experimenter blinded to the treatment conditions.

2.7.3 TH-positive Neuron Counting in Brain Slices

Mouse substantia nigra was sliced into a series of 30μm sections (from rostral to caudal), and one in every six sections was stained with anti-TH antibody. The substantia nigra pars compacta was outlined using TH-positive neurons between identifiable landmarks (Baquet et al., 2009) from bregma -2.92mm to -3.64mm. Identifiable TH-positive neurons in three stained sections within this distance from each animal were manually counted by an experimenter blinded to the treatment conditions under the Zeiss Axio Observer D1 microscope at 40×, and the total number of TH-positive neurons in this region was estimated using the following equation: N=counted number × 6.

2.8 Animal Models

2.8.1 Rat MCAo Stroke Model

The Middle Cerebral Arterial occlusion (MCAo) experiments were performed in the UBC ARU animal surgery room according to UBC animal protocol A12-0023. The protocol was approved by the University of British Columbia Animal Care Committee and all the methods were carried out in accordance with the approved guidelines and regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used. All surgery procedures were performed using sterile techniques.

Briefly, male Sprague-Dawley rats (280-320g) purchased from Charles River were housed in plastic cages with free access to food and water and maintained in a temperature-controlled room (21°C) with a 12/12hrs light/dark cycle. On the day of the surgery, animals were weighed and then anesthetized with isoflurane (PPC, DIN 02237518) using a precision vaporizer (4% for anesthesia induction and 2% for anesthesia maintenance during the surgery). Once reflexes such as toe pinch had been lost and the animals became fully anesthetized, they would be placed ventral side up on a sterile surgical drape on the surgical table. Animals' eyes were covered with Refresh Lacri-Lube eye gel to relieve dryness and the body temperature was maintained at 36.8°C using a regulated heating pad place under the surgical drape. Hair on the ventral surface of the neck was removed and the surface was sequentially cleaned with Hibitane skin cleanser and 70% ethanol 3 times. 1 ml of 0.25% marcaine (Hospira, DIN 02305909) was injected at the incision site as a local analgesia. Next, a midline incision was made in the hair-removed surface of the ventral neck, and the right common carotid

artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were clearly exposed. Two branches of the ECA, the superior thyroid artery and the occipital artery, were cauterized and cut using the Bovie Low Temperature Cautery (Bovie, AA90) to prevent bleeding and also to allow more operation space. The pterygopalatine artery branch on the ICA was also tied off to facilitate insertion of the occluder. A tight ligation was made at the distal end of ECA to prevent bleeding. After ICA and CCA were clamped with aneurysm clips, a small hole was cut in the middle of the exposed ECA and an occluder (Beijing Cinontech Co. Ltd., 2838-A4) was subsequently inserted into ECA, which was secured by a loose ligation at the proximal end of ECA. ECA was completely cut off in the middle so that the occluder could be flipped over and inserted along the ICA. Aneurysm clips on ICA and CCA were removed and the occluder was further inserted along ICA until a small resistance was felt, which indicated that the occluder had blocked the origin of right MCA. The ligation at the proximal end of ECA was then tightly fastened to hold the occluder in position. MCAo was maintained for 60min. After that, the occluder was withdrawn from the artery to induce blood reperfusion. Incisions were closed with 4.0 sutures and animals were recovered in a clean cage with heat support. Animal behaviors were tested at this time point and animals without any deficit in the left part of the body were abandoned and then sacrificed. One hour after reperfusion, animals were injected with saline or peptide via a single i.v. injection. The brains were harvested at 6 hrs after peptide treatment for further analysis.

2.8.2 Mouse MPTP Model of Parkinson's Disease

The experiments were performed in our collaborator, Dr. Zhifang Dong's laboratory in the Children Hospital of Chongqing Medical University. The protocol was approved by the Chongqing Medical University Animal Care Committee and all the methods were carried out in accordance with the approved guidelines and regulations. All efforts were made to minimize animal suffering and to reduce the number of animals used.

The experiments were performed according to a well-characterized protocol in the research field (Jackson-Lewis and Przedborski, 2007). Briefly, male C57BL/6 mice (20-25g) purchased from Charles River (Beijing office, China) were housed in plastic cages with free access to food and water and maintained in a temperature-controlled room (21°C) with a 12/12hrs light/dark cycle. After resting in the cages for a few days, mice received i.p. injection of 30mg/kg parkinsonian toxin MPTP hydrochloride (Sigma, M0896) once a day for 5 days to induce dopaminergic neuron death in the substantia nigra, while the control mice received equal volumes of saline injection. 6 μmol/kg Tat-βsyn-degron peptide or control Tat-βsyn peptide was i.p. injected into the MPTP-treated mice every 12hrs from the first day of MPTP injection until 7 days after the last injection of MPTP. All groups of mice then underwent a rotarod test before they were sacrificed.

2.9 Rotarod Behavioral Test

The rotarod test was performed as previously described, with modifications (Heldermon et al., 2007). Briefly, 6 days after the last MPTP injection, all mice received 4 rounds of training on the rotarod (Stoelting Co.). In the first two rounds of training, the rotarod was maintained at constant speed of 20 rpm for 3 min. In the second two rounds

of training, the rotarod reversed rotation direction every 3 turns at the constant speed of 20 rpm for 3 min. 24hrs after the last round of training, all groups of mice received formal rotarod testing in which the rotarod reversed rotation direction every 3 turns at the constant speed of 20 rpm. Mice were tested 10 times at 20 min intervals, and the time that they remained on the rotarod during each test was recorded. Maximum test time (cut-off limit) was 300s. The motor performance of the mouse was expressed as the latency to fall off the rotarod.

2.10 Intranasal Administration of Synthetic Peptides

The intranasal administration was conducted in male Sprague-Dawley rats (250-300g) according to an instructional video from Charles River (Machholz et al., 2012). Briefly, rats were gently restrained and their heads were held in the upright position. 5 µl peptide solution was intranasally administered into both nostrils each time, with an interval of 1 min between each administration to allow full absorption of the solution.

Chapter 3: Investigating the Peptide-mediated Protein Knockdown Technology in Freely Moving Animals

3.1 Introduction

An *in vivo* system is fundamentally different from an *in vitro* system. The presence of the BBB and the plasma membrane of neurons make it extremely difficult for functional molecules to reach brain cells after systemic administration. Meanwhile, the bioavailability of a functional molecule in the brain is also largely limited by enzymatic digestion in the serum after systemic administration. Therefore, it will be a great scientific advance if we can successfully apply our peptide-mediated protein knockdown technology in freely moving animals. To this end, my colleague Dr. Xuelai Fan and I chose the well-characterized DAPK1 targeting peptide for the *in vivo* test, which has been shown in Xuelai's preliminary results to knock down endogenous DAPK1 protein in rat primary cortical neuron cultures in a rapid and reversible manner. (The major part of the data has recently been published in Nature Neuroscience and I shared the co-first authorship with Dr. Xuelai Fan; Fan et al., 2014b). DAPK1 is a calcium/calmodulin-regulated serine/threonine protein kinase and plays important roles in various disease conditions, such as ischemia, cancer and Alzheimer's disease (Tu et al., 2010; Fan et al., 2014a; Kim et al., 2014; Zhao et al., 2015). The catalytic activity of DAPK1 is controlled by its autophosphorylation at serine 308. Calcineurin-mediated dephosphorylation of serine 308 allows DAPK1 to bind to calmodulin, thereby activating DAPK1 and its downstream pathways (Tu et al., 2010).

As shown in **Fig. 2**, the DAPK1 targeting peptide, Tat-GluN2BCTM, is composed of three domains: 1) the plasma membrane transduction domain Tat, which is capable of delivering peptides across both the BBB and the plasma membrane of neurons following systemic administration in freely moving animals (Hill et al., 2012; Fan et al., 2014b); 2) the 13-amino-acid protein binding domain GluN2B that is derived from amino acids 1292-1304 of the GluN2B subunit of NMDA receptors; GluN2B has been shown to specifically bind to activated but not inactive DAPK1 with high affinity (Tu et al., 2010); and 3) the lysosomal targeting domain, CMA targeting motif (CTM), that can take the peptide-protein complex to the lysosome for degradation. The CTM we used here is a combination of the three most well-characterized CMA sequences: KFERQ, QKILD and QRFFE (Uversky and Witt, 2011). We hypothesize that such design will significantly enhance the recognition of the CTM-containing peptide by hsc70. In contrast, the control peptide, Tat-GluN2B, containing the GluN2B binding sequence but not the CTM, while having the ability to interact with DAPK1, cannot target the peptide-protein complex to the lysosome for degradation.

We propose to test the DAPK1 targeting peptide in a well-established stroke model in rat: MCAo focal ischemia model (Taghibiglou et al., 2009; Tu et al., 2010; Fan et al., 2014b). A previous study by Tu et al. showed that ischemia can induce activation of DAPK1, which then binds to the GluN2B subunit of NMDA receptors and potentiates NMDA receptor-mediated excitotoxicity by enhancing its channel conductance (Tu et al., 2010). Interestingly, genetic deletion of the DAPK1 gene significantly protected against ischemia-induced neuronal damage and behavioral deficits in a mouse model of focal ischemia (Tu et al., 2010). Therefore, we hypothesize that knocking down DAPK1 using

our targeting peptide Tat-GluN2BCTM may also induce robust neuroprotection against ischemia-induced brain damage in a rat MCAo model of focal ischemia.

3.2 Results

3.2.1 Targeting Peptide Facilitated Knockdown of Endogenous DAPK1 *in vivo*

As shown in **Fig. 3a**, to measure the Tat-GluN2BCTM peptide's efficacy of DAPK1 knockdown using both immunoblotting and immunocytochemistry, we used a relatively mild (60 min unilateral occlusion) ischemic insult to induce activation of endogenous DAPK1 in the brain. Rats subjected to 60 min of MCAo were i.v. injected with either saline, Tat-GluN2BCTM or Tat-GluN2B 1 hr after reperfusion. TTC staining of transverse brain sections from saline-treated rats revealed that unilateral MCAo reliably induced ischemic brain damage, mostly in the ipsilateral striatum (**Fig. 3b**). As DAPK1 is activated by MCAo-induced ischemic stimulation (Tu et al., 2010) and the Tat-GluN2BCTM knockdown of DAPK1 is DAPK1-activation dependent (Fan et al., 2014b), we reasoned that DAPK1 would be maximally activated and degraded in regions most affected by ischemic insult. To determine ischemia-induced knockdown of DAPK1 by Tat-GluN2BCTM, we excised tissues from both MCAo-challenged and contralateral sides of the striatum and nearby cortex (**Fig. 3c**) and immunoblotted for DAPK1. Whereas Tat-GluN2B (10 mg/kg, i.v.) produced no obvious change in the levels of DAPK1 in the brain tissues of either ischemic or contralateral side, Tat-GluN2BCTM (10 mg/kg, i.v.) resulted in a significant reduction in DAPK1 only in the ischemic side of the brain: DAPK1 was reduced to 43.3% of that on the contralateral side (**Fig. 3d**; $P < 0.001$,

n = 3 individual experiments; two-tailed Student's t-test). Given that the collected tissues included some non-infarct areas (**Fig. 3c**), the actual efficiency of targeting peptide–mediated knockdown would be expected to be even greater.

To further assess region-specific DAPK1 degradation in a more direct manner, we used immunohistochemistry to probe for DAPK1 in transverse brain sections (**Fig. 3e**, right). As expected, DAPK1 knockdown was specific to stroke-damaged areas, as visualized with hematoxylin and eosin (H&E) staining (**Fig. 3e**, left) of the adjacent sections. To our knowledge, this is the first evidence for such a disease-specific protein knockdown in intact animals.

3.2.2 DAPK1 Knockdown Is Neuroprotective against Stroke-induced Brain Damage

Next, we measured the functional consequences of DAPK1 knockdown in ischemic rats. As shown in **Fig. 3e**, the specific knockdown of DAPK1 by Tat-GluN2BCTM was associated with a much more significant reduction of the infarct area, in comparison with that of Tat-GluN2B, which partially decreased ischemic damage by uncoupling DAPK1 from the GluN2B receptor signaling complex, as previously reported (Tu et al., 2010). We further confirmed the more prominent neuroprotective effects of Tat-GluN2BCTM–mediated DAPK1 knockdown by quantifying numbers of degenerating neurons in both striatum and cortex. For this purpose, we used Fluorojade B, a common stain that labels degenerating cells; it is suitable for assessment of cellular injury following MCAo (Taghibiglou et al., 2009; Tu et al., 2010; Li et al., 2015). Consistent with the results of

H&E staining (**Fig. 3e**, left), Fluorojade B staining showed that while both Tat-GluN2B and Tat-GluN2BCTM significantly reduced the numbers of degenerating neurons in both striatum and cortical areas, the neuroprotective effect of the latter was significantly more prominent (**Fig. 3f**). Thus, it appears that knocking down active DAPK1 (by Tat-GluN2BCTM) is much more neuroprotective than just simply uncoupling the GluN2B-DAPK1 association (by Tat-GluN2B).

3.3 Discussion

Taken together, these results demonstrate that systemically injected DAPK1 targeting peptide Tat-GluN2BCTM is able to cross the BBB and the plasma membrane of neurons to knock down activated DAPK1 in the brain, and thereby effectively protect against ischemia-induced brain damage in a rat MCAo model of focal ischemia. These results provide proof-of-concept evidence for the feasibility of applying our targeting peptide–based protein knockdown strategy *in vivo*. The peptide-mediated protein knockdown is both rapid and robust, as more than 50% reduction of DAPK1 level was found 6 hours after a single i.v. injection of the targeting peptide. Furthermore, the peptide-mediated DAPK1 knockdown was found to only localize in the ischemia infarct area (**Fig. 3e**), suggesting the high selectivity of this targeting peptide-based strategy in controlling endogenous protein levels. It is noteworthy to mention that this peptide-mediated protein knockdown technology can be generalized to potentially degrade any protein of interest. By changing the protein binding sequence in the middle of the targeting peptide, we might be able to create selective targeting peptides against other important proteins (e.g. α-synuclein) that have been difficult to inhibit or knock down

using a clinically relevant approach. Therefore, the broad application of this peptide-mediated protein knockdown technology may significantly contribute to the development of novel peptide therapeutics for a large number of human diseases, such as PD.

Figure 2

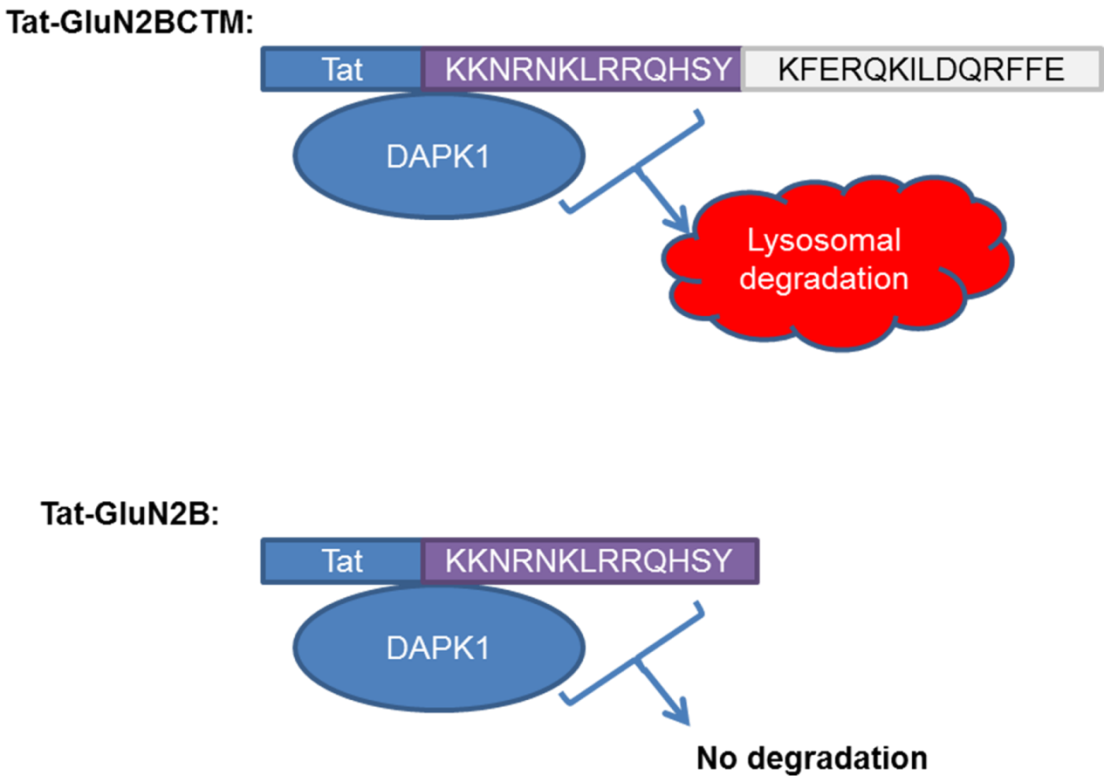
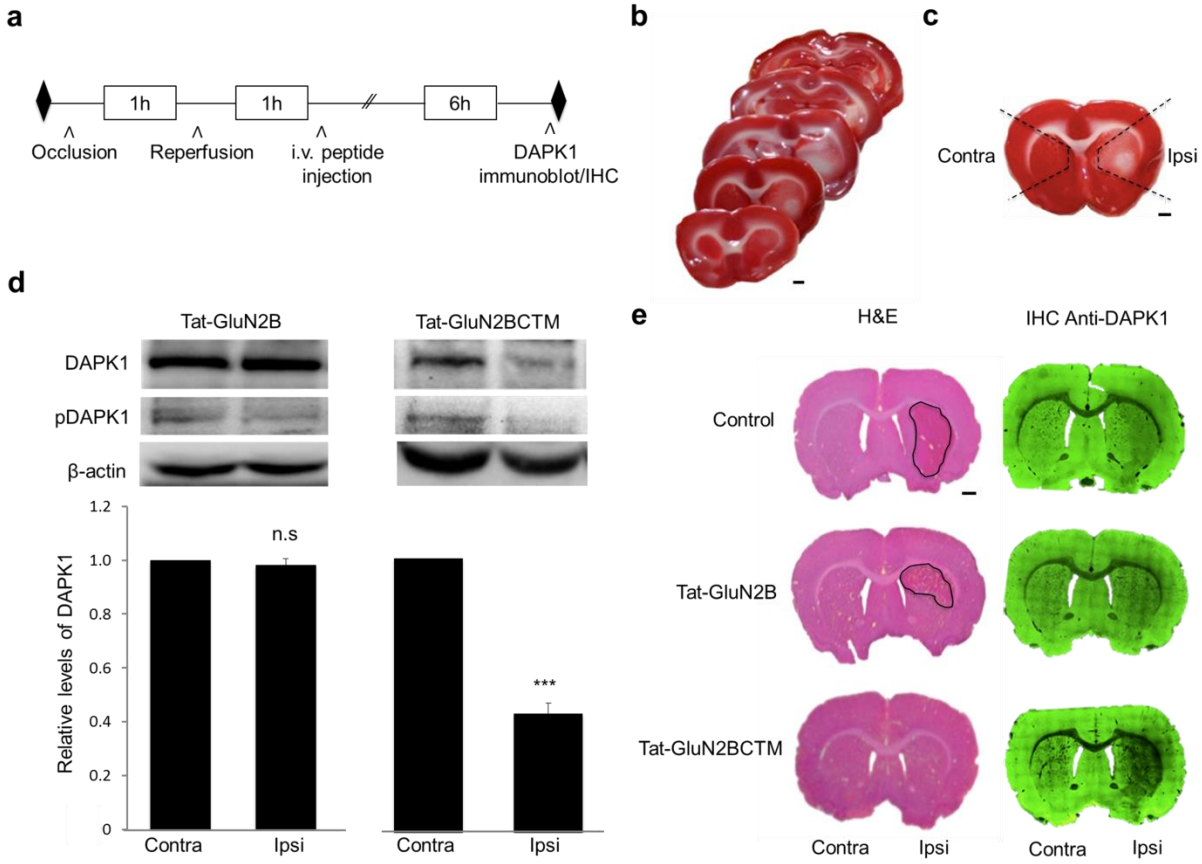

Tat-GluN2BCTM:
Tat
KKNRNKLRRQHSY
KFERQKILDQRFFE
DAPK1
Lysosomal degradation
Tat-GluN2B:
Tat
KKNRNKLRRQHSY
DAPK1
No degradation

Figure 2. Schematic illustration of the DAPK1 targeting peptide design

The DAPK1 targeting peptide has three domains: 1) the Tat transduction domain that enables the peptide to penetrate the BBB and the cell membranes, 2) the 13-amino-acid binding sequence derived from GluN2B subunit of NMDA receptor that will bind to activated but not inactive DAPK1 with high affinity, and 3) the degradation signal that targets the peptide-DAPK1 protein complex to the lysosome for degradation. In contrast, the control peptide Tat-GluN2B lacks the degradation signal, and hence while it can bind to the target protein, it cannot direct the complex to the lysosome for degradation.

Figure 3


a
1h
1h
6h
Occlusion
Reperfusion
i.v. peptide injection
DAPK1 immunoblot/IHC
b
c
Contra
Ipsi
d
Tat-GluN2B
Tat-GluN2BCTM
DAPK1
pDAPK1
β-actin
Relative levels of DAPK1
n.s
***
Contra
Ipsi
Contra
Ipsi
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H&E
IHC Anti-DAPK1
Control
Tat-GluN2B
Tat-GluN2BCTM
Contra
Ipsi
Contra
Ipsi

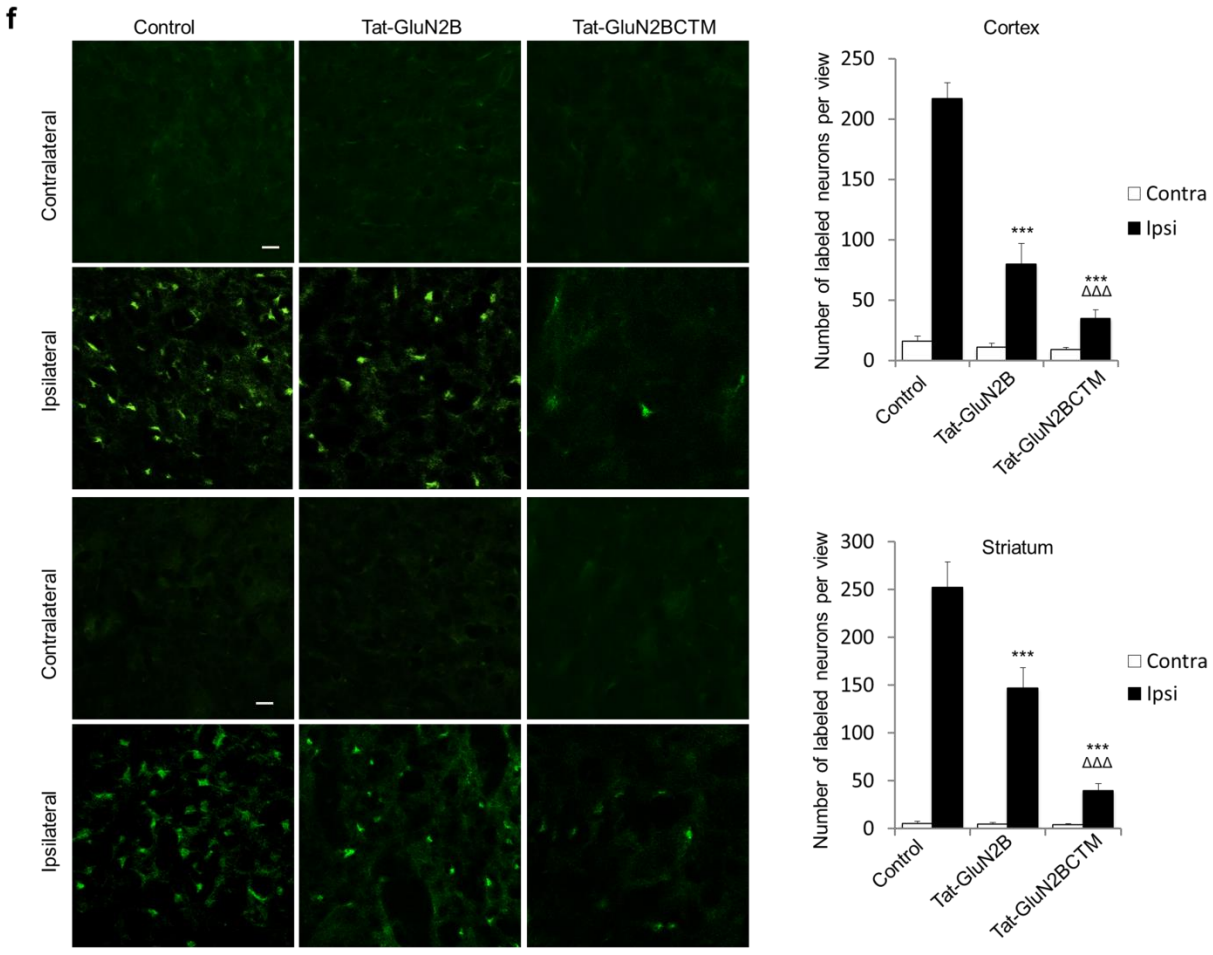

f
Control
Tat-GluN2B
Tat-GluN2BCTM
Contralateral
Ipsilateral
Contralateral
Ipsilateral
Cortex
Number of labeled neurons per view
250
200
150
100
50
0
Contra
Ipsi
***
***
ΔΔΔ
Control
Tat-GluN2B
Tat-GluN2BCTM
Striatum
Number of labeled neurons per view
300
250
200
150
100
50
0
Contra
Ipsi
***
***
ΔΔΔ
Control
Tat-GluN2B
Tat-GluN2BCTM

Figure 3. Tat-GluN2BCTM specifically knocks down DAPK1 in ischemic brain areas and reduces neuronal damage in the MCAo model of focal ischemia in rats

(**a**) Timeline of tissue collection for analysis of DAPK1 degradation in rats; IHC, immunohistochemistry. (**b**) TTC staining of a series of transverse brain sections showed reliable damage in the ipsilateral side following unilateral MCAo. (**c**) Black dashed lines on a section stained as in **b** represent brain areas removed for immunoblotting. (**d**) Immunoblots demonstrated specific DAPK1 knockdown in the infarct (Ipsi) but not contralateral (Contra) side following application of Tat-GluN2BCTM (10 mg/kg, i.v.; N = 3; $t(4) = 14.459$, $P < 0.001$) but not Tat-GluN2B (10 mg/kg, i.v.; N = 3; $t(4) = 0.739$, $P = 0.501$). β-actin was used as a loading control; two-tailed Student's *t*-test ***$P < 0.001$; n.s., not significant. (**e**) H&E (left) and immunohistochemical DAPK1 (right) staining of adjacent brain sections. Compared with those in saline (top) and Tat-GluN2B–treated (middle) controls, Tat-GluN2BCTM treatment (bottom) selectively reduced infarct area (left) and DAPK1 levels (right) ipsilaterally. Black outlining (left) delineates infarct areas as visualized with H&E staining. Green immunofluorescence (right) indicates high levels of DAPK1, and reduction of DAPK1 levels leads to reduction of green immunofluorescence intensity, as shown by the gray areas in the slices. (**f**) Left, brain sections stained with Fluorojade B in rats injected with saline (N = 6), Tat-GluN2B (N = 5) or Tat-GluN2BCTM (N = 5) after treatment as shown in **a**. Right, quantification of cellular damage by counting the number of Fluorojade B–positive cells in each 640 × 640 pixel image at 10× magnification. Tat-GluN2BCTM (10 mg/kg) displayed more prominent neuroprotection in the cortex ($P < 0.001$) and striatum ($P < 0.001$) as compared to Tat-GluN2B (10 mg/kg). Cortex: $H(2) = 41.235$; $P < 0.001$; striatum: $H(2) =$

38.808; $P < 0.001$. Kruskal-Wallis ANOVA on ranks with Dunn's *post hoc*; bars represent relative means ± S.E.M., ***$P < 0.001$ compared with the control. ΔΔΔ$P < 0.001$ for post-hoc comparison between the Tat-GluN2B group and the Tat-GluN2BCTM group. Scale bars 1 mm in **b**,**c**,**e**, 20 μm in **f.** *Data from this figure was a collaborative effort between Dr. Xuelai Fan and me. I did the animal surgery, peptide injection and tissue collection, and Dr. Fan did the immunoblotting, staining and data analysis. Figure Source: Fan et al., 2014b, Nature Neuroscience.*

Chapter 4: Development of a Small α-synuclein Knockdown Peptide, Tat-βsyn-degron

4.1 Introduction

Having confirmed the applicability of the peptide-mediated protein knockdown technology both *in vitro* and *in vivo*, I then tested the applicability of this technology to develop a clinically applicable targeting peptide for α-synuclein, a key protein in the pathogenesis of PD. Proteins can be degraded by targeting to either lysosomal or proteasomal degradation pathways in the cell, but depending on pathological conditions, lysosomes, proteasomes, or both can become compromised in PD (McNaught et al., 2003; Tofaris et al., 2003; Alvarez-Erviti et al., 2010; Dehay et al., 2010; Murphy et al., 2014). Thus, it would be beneficial to have a protein degradation system that can target either the lysosome or the proteasome. Therefore, I modified the peptide-based method that we have developed to rapidly and reversibly decrease the levels of endogenous proteins by directing them for degradation (Fan et al., 2014b). In the previous work, Dr. Xuelai Fan and I used a CTM lysosomal targeting signal to direct protein of interest into the lysosome for degradation (Fan et al., 2014b). Here I investigate whether I can also use a proteasomal targeting signal, instead of a CTM lysosomal targeting signal, to target α-synuclein to proteasomes for degradation.

4.2 Results

4.2.1 Designing the α-synuclein Knockdown Peptide, Tat-βsyn-degron

As shown in **Fig. 4**, the proposed α-synuclein targeting peptide (Tat-βsyn-degron) is composed of three domains: 1) the plasma membrane transduction domain Tat, which

is capable of delivering peptides across both the BBB and the plasma membrane of neurons following systemic administration in freely moving animals (Hill et al., 2012; Fan et al., 2014b); 2) the α-synuclein-binding domain βsyn, derived from amino acids 36-45 of β-synuclein that has recently been shown to specifically bind to α-synuclein with high affinity (Shaltiel-Karyo et al., 2010); and 3) the proteasomal targeting domain degron, a 4 amino-acid peptide signal that has recently been shown to efficiently direct its tagged proteins to proteasomes for degradation (Bonger et al., 2011).

4.2.2 Testing the α-synuclein Knockdown Efficacy of the Targeting Peptide in HEK 293 Cells

A recent study suggests that the amino acids 36-45 of β-synuclein (βsyn) can specifically bind to α-synuclein with high affinity (Shaltiel-Karyo et al., 2010). Briefly, the molecular mapping of β-synuclein fragments binding to α-synuclein was performed using the peptide array technology. Decamer peptides corresponding to overlapping sequences of the full length, 134 amino acids, β-synuclein protein were synthesized on a cellulose membrane matrix. Purified histidine-tagged α-synuclein was then overlaid onto the membrane, which led to identification of the amino acids 36-45 of β-synuclein (βsyn) as the strongest binding partner for α-synuclein. The study by Shaltiel-Karyo et al. also showed that βsyn not only binds to α-synuclein with high affinity, but also effectively inhibits its oligomerization both *in vitro* and *in vivo*.

As illustrated in **Fig. 5a**, to determine the efficacy and specificity of this sequence fragment as the binding domain of our α-synuclein targeting peptide, I first constructed two FLAG-tagged targeting peptide mini-genes (FLAG-βsynN-degron and FLAG-βsyn-

degron), that encode either natural or reverse amino acid sequences between 36-45 of β-synuclein (which have been shown to have a similar binding affinity for α-synuclein in an *in vitro* binding assay in a previous study (Shaltiel-Karyo et al., 2010)), along with the degron targeting signal. A control mini-gene encoding FLAG-βsyn without degron was also constructed. HEK 293 cells were co-transfected with a human α-synuclein plasmid and one of these mini-genes. As predicted, co-transfection of FLAG-βsynN-degron or FLAG-βsyn-degron (**Fig. 5b** and **5c**), but not the control FLAG-βsyn peptide (**Fig. 5d**), resulted in a robust reduction in recombinant α-synuclein levels in a dose-dependent manner. This suggests that the targeting peptides, when co-expressed with α-synuclein, are sufficient to bind to α-synuclein and target it for proteasomal degradation. Since FLAG-βsyn-degron appears to have higher α-synuclein knockdown efficacy in comparison with FLAG-βsynN-degron (**Fig. 5b** and **5c**), possibly due to its enhanced stability (Guichard et al., 1994), I chose to use the βsyn-degron as the α-synuclein targeting peptide to knock down endogenous α-synuclein for all the following experiments. The FLAG-βsyn-degron induced knockdown is target-specific, because this knockdown was not associated with a detectable change in β-actin levels (**Fig. 5b** and **5c**) and more importantly it was only selective to α-synuclein, but not β- or γ-synuclein which are the two other members of the synuclein protein family (**Fig. 6**).

4.2.3 Binding between α-synuclein and the Tat-βsyn-degron Peptide

The binding between the Tat-βsyn-degron peptide and α-synuclein was investigated using a Biacore peptide-protein binding assay. The Tat-βsyn-degron peptide was chemically synthesized, along with the tat control peptide. Purified recombinant α-

synuclein protein was immobilized on a Biacore chip, and two-fold serial dilutions (0.20μM, 0.39μM, 0.78μM, 1.56μM, 3.13μM, 6.25μM, 12.50μM) of the synthetic peptides (Tat-βsyn-degron or Tat control), in duplicate, were sequentially injected over the surface of the chip. Peptide-protein binding responses were recorded over time. As shown in **Fig. 7**, the synthetic Tat-βsyn-degron peptide displayed robust binding with α-synuclein in a dose-dependent manner, while the control Tat peptide displayed little binding with α-synuclein. Therefore, these results strongly support the physical interaction between α-synuclein and the synthetic Tat-βsyn-degron peptide.

4.2.4 Knocking down Endogenous α-synuclein *in vitro* Using the Tat-βsyn-degron Peptide

I next examined the efficacy of the targeting peptide in decreasing endogenous α-synuclein in neurons *in situ*, using chemically synthesized, membrane-permeant α-synuclein targeting peptide Tat-βsyn-degron, along with the control Tat-βsyn peptide, as illustrated in **Fig. 4**. As shown in **Fig. 8a**, bath application of Tat-βsyn-degron, but not the control Tat-βsyn, for 24hrs produced a dose-dependent reduction of endogenous α-synuclein in cortical neuron cultures. The Tat-βsyn-degron induced knockdown is mediated by proteasomal degradation, as it could be fully prevented by the presence of the proteasomal inhibitor MG132 (10 μM; 24hrs). Additionally, the knockdown is time dependent, reaching peak reduction within 24hrs (**Fig. 8b**). The peptide-mediated knockdown is specific to α-synuclein, as it did not affect the levels of several other neuronal proteins surveyed in the same treated cultures, including transmembrane protein $GABA_A$ receptor β2/3 subunits, intracellular protein HSP90, and a known α-synuclein binding protein 14-3-3 (**Fig. 8c-e**).

Since over-expression of α-synuclein has been shown to cause synaptic dysfunction in various studies (Nemani et al., 2010; Venda et al., 2010; Busch et al., 2014), knocking down α-synuclein in the synaptosome using the Tat-βsyn-degron targeting peptide might be beneficial for restoring normal synaptic transmission in PD patients. Therefore, cortical neuron cultures were treated with 25 μM of Tat-βsyn-degron, or the control Tat-βsyn peptide, for 24hrs, and then the cytosolic and synaptosomal fractions were isolated using a centrifuge method. As shown in **Fig. 9**, bath application of Tat-βsyn-degron, but not the control Tat-βsyn peptide, induced significant knockdown of α-synuclein in both cytosolic and synaptosomal fractions of the neuronal lysate.

4.3 Discussion

Taken together, these results demonstrate that I have developed a robust targeting peptide for knocking down α-synuclein *in vitro*. The Tat-βsyn-degron is efficient, effective and specific to α-synuclein, making it a great drug candidate for further characterization and development. Since TAT is a membrane permeable sequence with no selectivity, it was expected to see significant knockdown of endogenous α-synuclein in both cytosolic and synaptosomal fractions of cultured cortical neurons. With only 25 amino acids, Tat-βsyn-degron is simple in structure and small in size, making it unlikely to cause obvious immune responses during repeated administrations in PD patients. Meanwhile, because of the simple composition of the peptide, chemical synthesis of Tat-βsyn-degron is also relatively easy and cheap. Thus, with all these drug-like properties, the Tat-βsyn-degron peptide may become a potential peptide therapeutic for the treatment for PD.

I am also aware that poor serum stability of peptides has been a major concern in the peptide drug development field for a long time. Peptides with promising therapeutic value often have serum half-lives of only minutes to hours, largely limiting their clinical applications (Santi et al., 2012). Interestingly, the results in this Chapter showed that a single dose of the Tat-βsyn-degron peptide could knock down α-synuclein for more than 24 hours (**Fig. 8b**), suggesting the long-lasting effect of the Tat-βsyn-degron peptide. Although I haven't determined the half-life of the Tat-βsyn-degron peptide in the serum, I think its long-lasting knockdown efficacy might be partially due to the slow turnover of the target protein, α-synuclein. Previous studies have suggested that α-synuclein is a stable protein with a long half-life (~24 hours) (Li et al., 2004; Rott et al., 2011). Therefore, once α-synuclein is effectively degraded by a peptide treatment, it takes a while (24 hours in this case) for the cell to recover the α-synuclein protein level through *de novo* synthesis. This further emphasizes the advantage of my targeting peptides over traditional therapeutic peptides. Previously, therapeutic effects are present only when the functional peptide physically exists in the cell and interacts with its drug target. However, in my case, even when the targeting peptide has been entirely degraded in the cell, its knockdown effects are prolonged until *de novo* protein synthesis regenerates the target protein.

In summary, Tat-βsyn-degron seems to be a novel peptide with robust efficacy in knocking down endogenous α-synuclein *in vitro*. Further investigation of the therapeutic potential of this peptide in various PD models might produce many interesting and also important results.

Figure 4

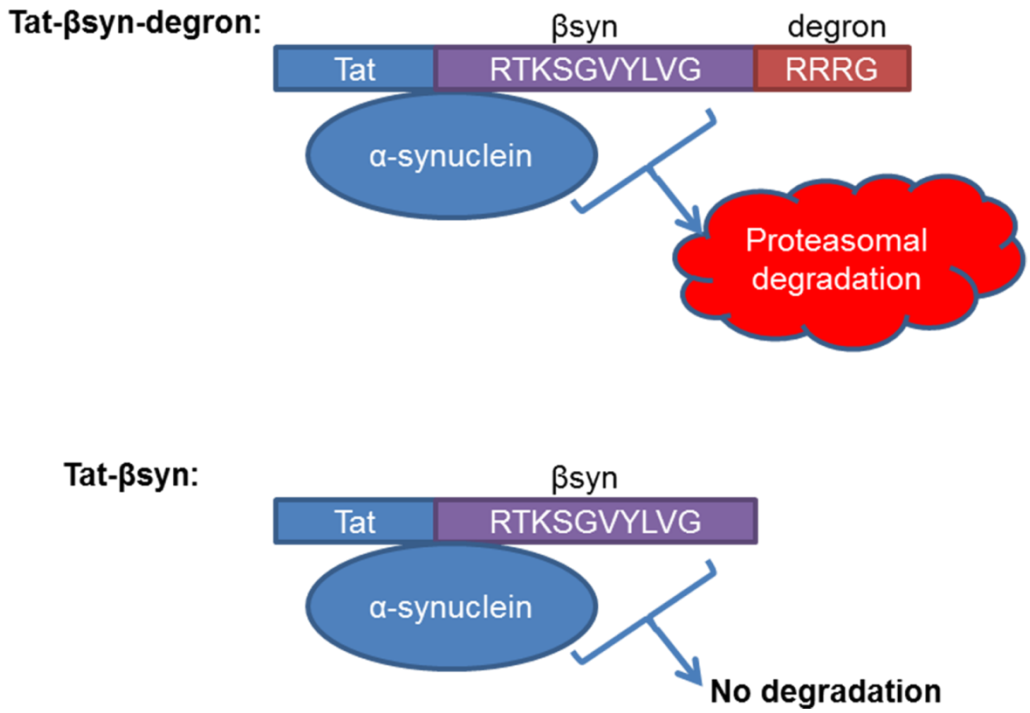

Tat-βsyn-degron:
βsyn
degron
Tat
RTKSGVYLVG
RRRG
α-synuclein
Proteasomal degradation
Tat-βsyn:
βsyn
Tat
RTKSGVYLVG
α-synuclein
No degradation

Figure 4. Schematic illustration of the Tat-βsyn-degron peptide design

The α-synuclein targeting peptide Tat-βsyn-degron has three domains: 1) the Tat transduction domain that enables the peptide to penetrate the BBB and the cell membranes, 2) the α-synuclein binding domain derived from β-synuclein, and 3) the degron sequence that targets the peptide-protein complex to the proteasome for degradation. In contrast, the Tat-βsyn control peptide lacks the proteasomal targeting signal, and hence while it can bind to α-synuclein, it cannot direct the peptide-protein complex to the proteasome for degradation.

Figure 5

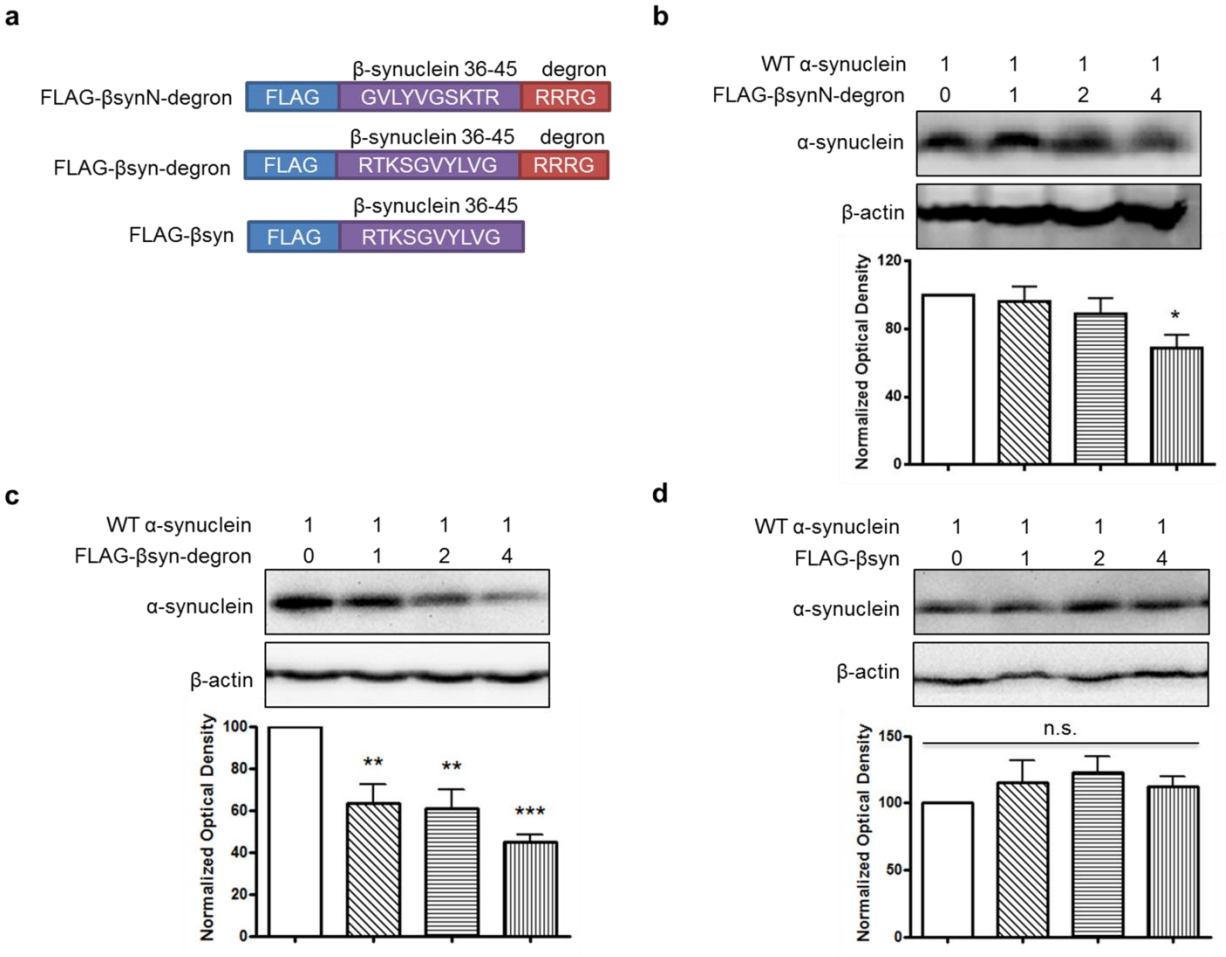

a
β-synuclein 36-45
degron
FLAG-βsynN-degron
FLAG
GVLYVGSKTR
RRRG
β-synuclein 36-45
degron
FLAG-βsyn-degron
FLAG
RTKSGVYLVG
RRRG
β-synuclein 36-45
FLAG-βsyn
FLAG
RTKSGVYLVG
b
WT α-synuclein 1 1 1 1
FLAG-βsynN-degron 0 1 2 4
α-synuclein
β-actin
Normalized Optical Density
120
80
40
0
*
c
WT α-synuclein 1 1 1 1
FLAG-βsyn-degron 0 1 2 4
α-synuclein
β-actin
Normalized Optical Density
100
80
60
40
20
0
**
**
***
d
WT α-synuclein 1 1 1 1
FLAG-βsyn 0 1 2 4
α-synuclein
β-actin
n.s.
Normalized Optical Density
150
100
50
0

Figure 5. The design of α-synuclein knockdown mini-genes and demonstration of knockdown efficacy in HEK 293 cells

(**a**) Schematic illustration of the mini-gene constructs encoding FLAG-βsynN-degron (βsynN: natural amino acid sequence between 36-45 of β-synuclein), FLAG-βsyn-degron (βsyn: reversed β-synuclein 36-45) or FLAG-βsyn. (**b-d**) Immunoblots sequentially probing for α-synuclein and β-actin (as loading and specificity controls) showing that expression of FLAG-βsynN-degron (**b**; N=9; $F(3,32)=3.43$; $P<0.05$) or FLAG-βsyn-degron (**c**; N=5; $F(3,16)=13.18$; $P<0.001$), but not control FLAG-βsyn (**d**; N=4; $F(3,12)=0.73$; $P=0.55$), induced a dose-dependent reduction in the levels of human α-synuclein co-expressed in HEK 293 cells. Note that FLAG-βsyn-degron appears to have a better efficacy in reducing α-synuclein. Transfection ratios of the plasmids are shown on the top. Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with the control. n.s. denotes not significant.

Figure 6

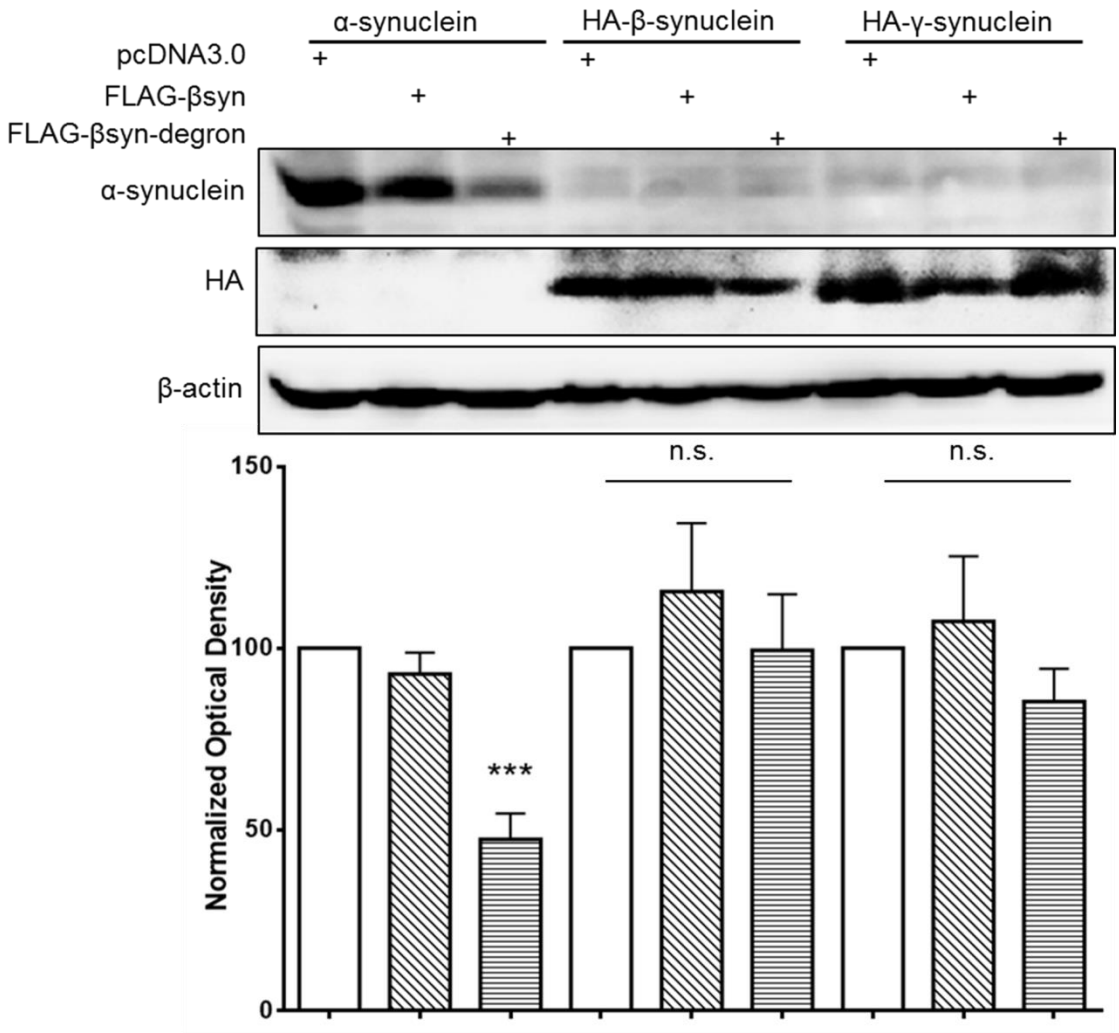

α-synuclein
HA-β-synuclein
HA-γ-synuclein
pcDNA3.0
FLAG-βsyn
FLAG-βsyn-degron
α-synuclein
HA
β-actin
n.s.
n.s.
Normalized Optical Density
150
100
50
0
***

Figure 6. FLAG-βsyn-degron mediated knockdown is α-synuclein specific

Immunoblots sequentially probing for synuclein and β-actin showing that when co-transfected at 4:1 ratio in the HEK 293 cells, FLAG-βsyn-degron, not FLAG-βsyn, specifically reduces the level of α-synuclein (N=7; F(2,18)=28.51; *P*<0.001), but not the levels of HA-β-synuclein (N=7; F(2,18)=0.42; *P*=0.66) or HA-γ-synuclein (N=7; F(2,18)=0.93, *P*=0.41). Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. ***P<0.001 compared with the control. n.s. denotes not significant.

Figure 7

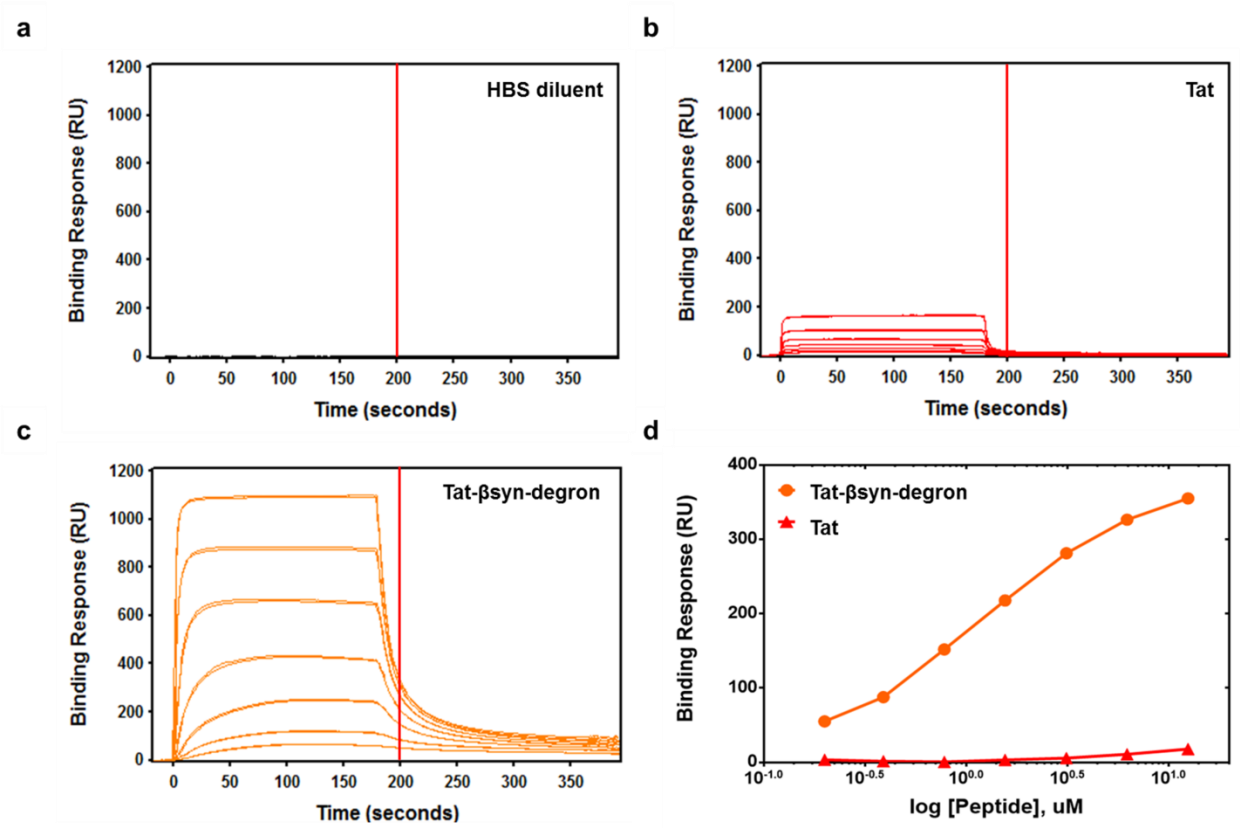

a
HBS diluent
Binding Response (RU)
Time (seconds)
b
Tat
Binding Response (RU)
Time (seconds)
c
Tat-βsyn-degron
Binding Response (RU)
Time (seconds)
d
Tat-βsyn-degron
Tat
Binding Response (RU)
log [Peptide], uM

Figure 7. Biacore peptide-protein binding assay

(**a-d**). Representative sensorgrams demonstrating the binding responses of HBS blank buffer control (**a**), the synthetic Tat peptide (**b**), or the synthetic Tat-βsyn-degron peptide (**c**) to α-synuclein. Two-fold serial dilutions (0.20μM, 0.39μM, 0.78μM, 1.56μM, 3.13μM, 6.25μM, 12.50μM) of peptides, in duplicate, were sequentially injected over immobilized purified recombinant human α-synuclein for 3 minutes, followed by a dissociation phase during which HBS buffer was flowed over the surface. Sensorgrams, depicting binding responses over time, were double-referenced by subtracting out the binding on the reference surface and the response from the HBS blank buffer control. Peptide - α-synuclein binding response report points were collected 20 seconds into the dissociation phase at time 200 seconds (as indicated by the vertical lines in the figures), to exclude bulk refractive index changes and nonspecific binding. (**d**) Graphing of peptide - α-synuclein binding response versus peptide concentration showing that synthetic Tat-βsyn-degron peptide displayed robust binding with α-synuclein in a dose-dependent manner (0.20μM, 0.39μM, 0.78μM, 1.56μM, 3.13μM, 6.25μM, 12.50μM), while the control Tat peptide displayed little binding with α-synuclein. *Data in this figure were generated by Dr. Ebrima Gibbs in our collaboration lab (Dr. Neil Cashman's lab at UBC).*

Figure 8

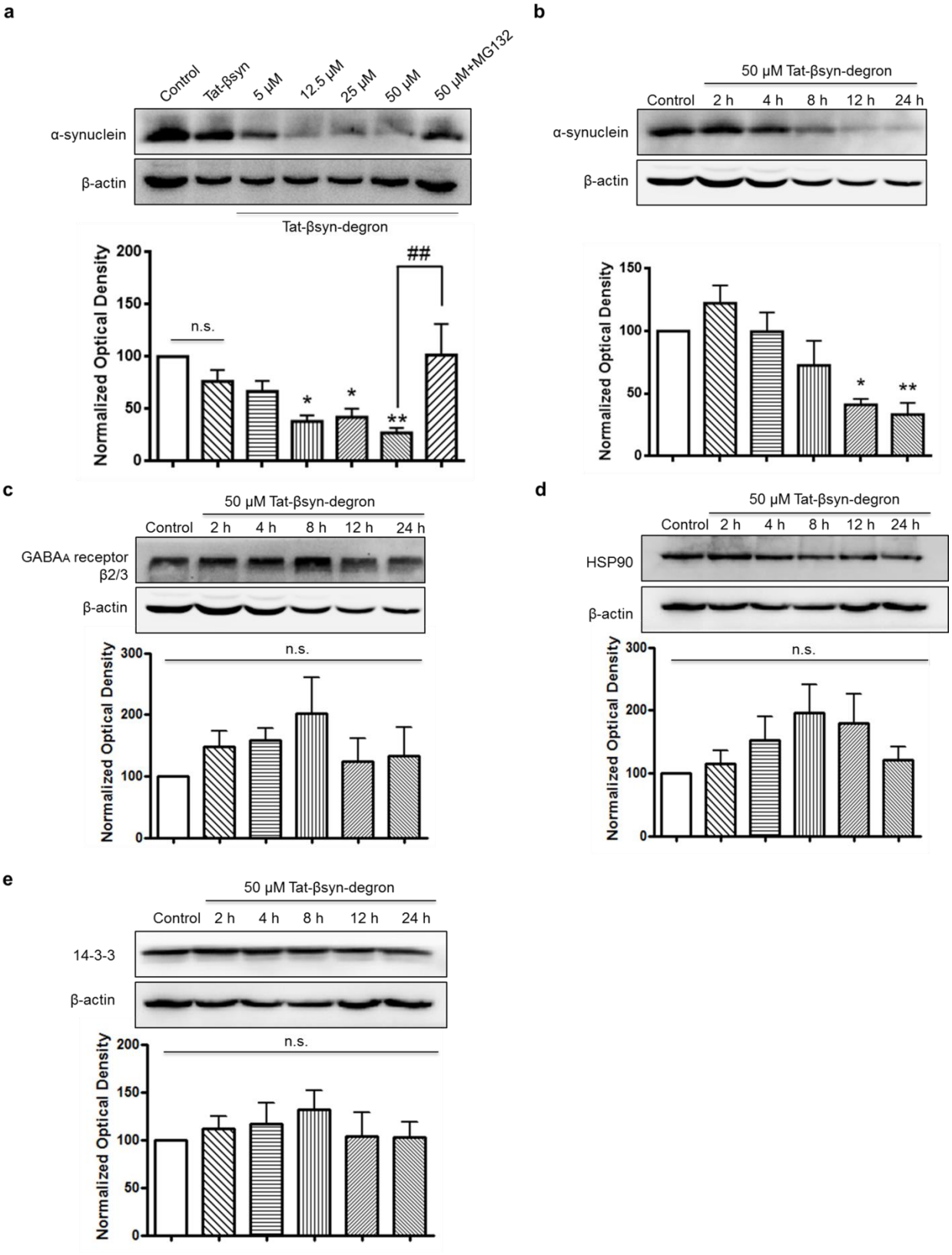

a
Control
Tat-βsyn
5 μM
12.5 μM
25 μM
50 μM
50 μM+MG132
α-synuclein
β-actin
Tat-βsyn-degron
Normalized Optical Density
n.s.
##
b
50 μM Tat-βsyn-degron
Control
2 h
4 h
8 h
12 h
24 h
α-synuclein
β-actin
Normalized Optical Density
c
GABAA receptor β2/3
β-actin
n.s.
d
HSP90
β-actin
n.s.
e
14-3-3
β-actin
n.s.

Figure 8. Tat-βsyn-degron peptide dose- and time-dependently knocks down α-synuclein and does not significantly affect the levels of several other cellular proteins in cortical neuron cultures

(**a**) Immunoblots showing that bath application of the membrane permeant synthetic peptide Tat-βsyn-degron at various concentrations for 24hrs induced a robust reduction of endogenous α-synuclein protein levels in a dose-dependent manner (5-50 μM; N=9; $F(6,56)=5.15$; $P<0.001$); this was prevented in the presence of the proteasome inhibitor MG132 (Tukey's HSD *post hoc* test: 50 μM Tat-βsyn-degron + 10μM MG132; ##$P<0.01$, compared with the 50 μM Tat-βsyn-degron group; N=9). In contrast, bath application of the control Tat-βsyn had no effect (50 μM, 24 hrs; Tukey's HSD *post hoc* test: $P=0.87$ compared with the control; N=9). (**b**) Bath application of Tat-βsyn-degron (50 μM) induced a time-dependent knockdown of endogenous α-synuclein in primary cortical cultures (N=8; $F(5,42)=8.06$; $P<0.001$). (**c-e**) Tat-βsyn-degron at high dose (50 μM; 24hrs) did not affect the expression levels of several un-related proteins in cortical cultures. These proteins include transmembrane protein β2/3 subunits of the $GABA_A$ receptor (**c**; N=3; $F(5,12)=0.90$; $P=0.51$), cytosol chaperone protein HSP90 (**d;** N=6; $F(5,30)=1.33$; $P=0.28$), and 14-3-3, a known α-synuclein binding protein (**e**; N=6; $F(5,30)=0.43$; $P=0.82$). Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Tukey's HSD *post hoc* test. *$P<0.05$ and **$P<0.01$ compared with the control. n.s. denotes not significant. *Fig. 8a and 8b were generated by Dr. Xuelai Fan.*

Figure 9

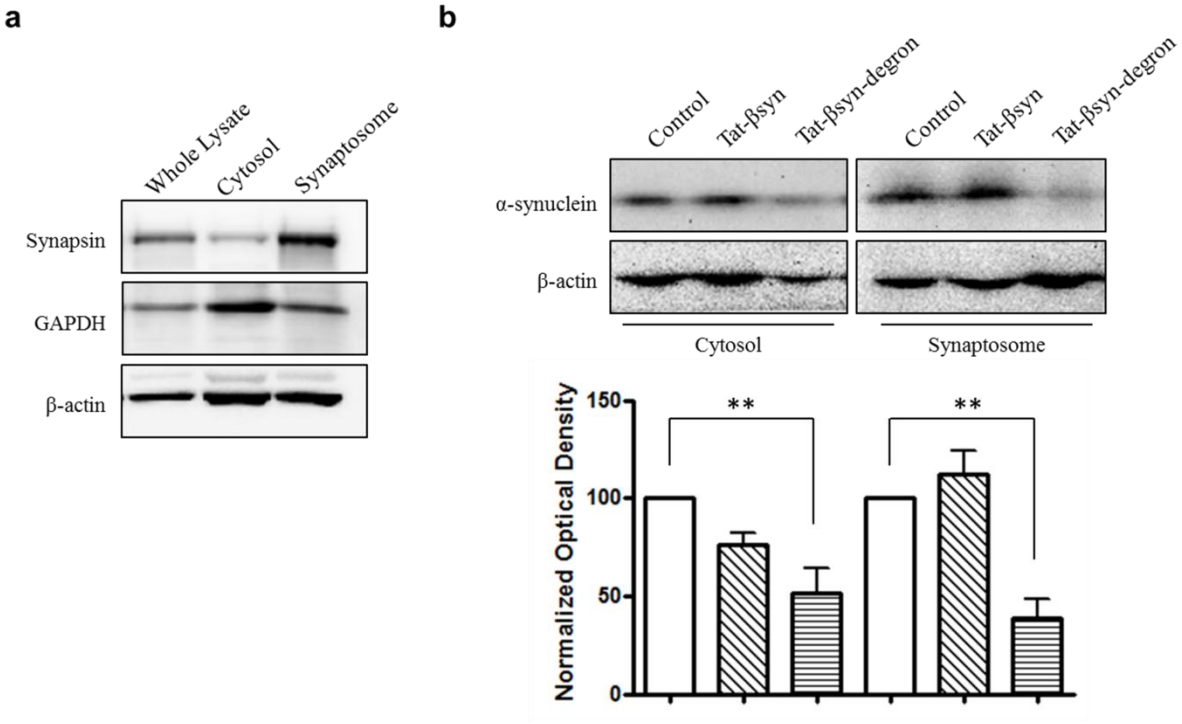

a
Whole Lysate
Cytosol
Synaptosome
Synapsin
GAPDH
β-actin
b
Control
Tat-βsyn
Tat-βsyn-degron
Control
Tat-βsyn
Tat-βsyn-degron
α-synuclein
β-actin
Cytosol
Synaptosome
Normalized Optical Density
150
100
50
0
**
**

Figure 9. Tat-βsyn-degron effectively knocks down endogenous α-synuclein in both cytosolic and synaptosomal fractions

(**a**) A representative immunoblot showing successful fractionation of both cytosol and synaptosome from the whole neuronal lysate. The synaptic marker, synapsin, was enriched in the synaptosomal fraction, while the cytosolic marker, GAPDH, was enriched in the cytosolic fraction. (**b**) Immunoblots (above) and quantification bar graphs (below) showing that bath application of 25 μM Tat-βsyn-degron peptide, but not the control Tat-βsyn peptide, for 24 hours induced significant knockdown of endogenous α-synuclein in both cytosol (N=7; $F(2,18)=8.56$; $P<0.01$) and synaptosome (N=7; $F(2,18)=18.25$; $P<0.001$) of cultured rat cortical neurons. Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. $^{**}P<0.01$ compared with the control.

Chapter 5: Therapeutic Efficacy of the α-synuclein Knockdown Peptide in Parkinson's Disease Models

5.1 Introduction

Having confirmed the efficacy and specificity of the Tat-βsyn-degron peptide in knocking down endogenous α-synuclein in neuronal cultures, I next tested the therapeutic potential of the Tat-βsyn-degron peptide in various PD models. As discussed in the introduction, one of the most widely used models in PD research is the MPTP mouse model of PD, because it has proved to be easy, reliable, reproducible, and fast (Jackson-Lewis and Przedborski, 2007). MPTP is able to freely cross the BBB and the plasma membrane of neurons after systemic injection (Blesa et al., 2012). Both acute and chronic treatments of MPTP have been shown to selectively kill dopaminergic neurons in the substantia nigra and induce behavioral deficits in C57BL/6 mice (Jackson-Lewis and Przedborski, 2007; Meredith and Rademacher, 2011). Once MPTP enters the brain, however, it has to be metabolized into MPP+ first by monoamine oxidase B in astrocytes to exert its toxicity (Blesa et al., 2012). As a result, for *in vitro* studies of PD, it is often suggested that treatment of MPP+, instead of MPTP, is sufficient to selectively and reliably kill dopaminergic neurons in ventral midbrain cultures (Aime et al., 2015; Wang et al., 2015a). Importantly, it has been demonstrated in many studies that knocking down α-synuclein protein using genetic manipulation methods significantly protects against parkinsonian toxin MPP+ or MPTP -induced cell death in both human dopaminergic neuroblastoma cells and free-moving animals (Hayashita-Kinoh et al., 2006; Fountaine and Wade-Martins, 2007; Wu et al., 2009;

Thomas et al., 2011). Therefore, my clinically relevant targeting peptide method may also induce robust neuroprotection in MPP+/MPTP –based PD models.

5.2 Results

5.2.1 Therapeutic Efficacy of Tat-βsyn-degron in the MPP+ *in vitro* Model of Parkinson's Disease

I first examined the ability of Tat-βsyn-degron in protecting dopaminergic neurons against MPP+ toxicity using a well-characterized *in vitro* model of PD (Tonges et al., 2012). As shown in **Fig. 10**, MPP+ treatment (20 µM; 48hrs) induced dramatic death of dopaminergic neurons in rat primary cultures of the ventral midbrain. This was demonstrated by the significant decrease in the level of TH, a dopaminergic neuronal marker protein, (**Fig. 10a** and **10c**) and TH-positive neurons (**Fig. 10d**). Bath application of Tat-βsyn-degron peptide (25µM; 48hrs), but not the control Tat-βsyn peptide (25 µM; 48hrs), induced a robust reduction in endogenous α-synuclein protein levels (**Fig. 10a** and **10b**). Importantly, the reduction of α-synuclein almost fully protected dopaminergic neurons from MPP+ induced neurotoxicity, as shown by the rescue of TH protein level (**Fig. 10a** and **10c**) and TH-positive neurons (**Fig. 10d**) in the culture dishes.

5.2.2 Therapeutic Efficacy of Tat-βsyn-degron in the Mouse MPTP Model of Parkinson's Disease

5.2.2.1 Tat-βsyn-degron-facilitated α-synuclein Knockdown Is Protective against MPTP-induced Neurotoxicity

To further demonstrate the therapeutic potential of the peptide-mediated knockdown of α-synuclein in freely moving animals, I then utilized the Tat-βsyn-degron peptide in a mouse parkinsonian toxicity model of PD (Jackson-Lewis and Przedborski, 2007). Since the efficacy of the Tat-βsyn-degron peptide has not been investigated in knocking down endogenous α-synuclein from mouse sources, I bath applied Tat-βsyn-degron in mouse primary cortical neuronal cultures. As shown in **Fig. 11**, a single treatment of the Tat-βsyn-degron peptide (25μM) resulted in robust knockdown of endogenous α-synuclein in the cultures starting from 8 hours post-treatment until 24 hours later, which was consistent with the dramatic results from rat primary cortical neuron cultures (**Fig. 8b**).

Then, I moved on to test the Tat-βsyn-degron peptide *in vivo*. C57BL/6 mice were i.p. injected with 30mg/kg parkinsonian toxin MPTP (or saline as control), once per day for 5 consecutive days, to induce dopaminergic neuron damage. The effects of MPTP administration on mouse rotarod performance and damage to dopaminergic neurons were then analyzed 1 week after the last injection of MPTP. To determine the effect of α-synuclein knockdown in protecting dopaminergic neurons against MPTP, the Tat-βsyn-degron peptide or its control Tat-βsyn (6 μmol/kg; i.p.) was used in some animals twice a day for 12 days, beginning the first day of MPTP injection. As shown in **Fig. 12**, the Tat-βsyn-degron peptide, not the Tat-βsyn peptide, induced significant α-synuclein degradation in both substantia nigra-containing ventral midbrain (**Fig. 12a** and **12b**) and

striatum (**Fig. 12d** and **12e**). The striatum is a region that receives dopaminergic neuronal projections from the substantia nigra and is also deeply affected in PD (Macdonald and Monchi, 2011). Consistent with the specific neurotoxic effects of MPTP on dopaminergic neurons, in mice receiving MPTP injection alone there was a significant loss of protein TH in both dopaminergic neurons in the substantia nigra-containing ventral midbrain (**Fig. 12a** and **12c**) and dopaminergic neuronal terminals in the striatum (**Fig. 12d** and **12f**). This was demonstrated by quantitative immunoblotting analysis of TH protein levels. As expected, the specific knockdown of α-synuclein by Tat-βsyn-degron protected against MPTP-induced dopaminergic neuronal injury (**Fig. 12c** and **12f**).

The neuroprotective effects of Tat-βsyn-degron peptide were further supported by immunohistochemical analysis. In mice receiving only MPTP injections, there was a significant loss of TH-positive neurons in the substantia nigra pars compacta as revealed by blinded neuron counting (**Fig. 13a** and **13b**) and TH-positive neuronal terminals in the striatum as quantified with densitometric analysis (**Fig. 13c** and **13d**). As expected, the MPTP-induced dopaminergic neuronal damage was largely protected by the Tat-βsyn-degron peptide, but not the Tat-βsyn peptide (**Fig. 13a-d**).

5.2.2.2 Tat-βsyn-degron-facilitated α-synuclein Knockdown Is Protective against MPTP-induced Behavioral Deficits

The motor function of these mice was tested by the rotarod test using a protocol modified from a previous study (Heldermon et al., 2007). Consistent with the dramatic effects of α-synuclein knockdown and its protection of dopaminergic neurons from

MPTP-induced neurotoxicity, the rotarod behavioral test revealed that the Tat-βsyn-degron peptide also significantly rescued the MPTP-induced motor deficits (**Fig. 13e**). Interestingly, treatment with Tat-βsyn, while producing a small, non-significant decrease in MPTP-induced neuronal damage (**Fig. 12** and **Fig. 13a-d**), also reduced motor deficits in mice (**Fig. 13e**).

5.3 Discussion

Taken together, these results demonstrate robust neuroprotective efficacy of Tat-βsyn-degron in protecting dopaminergic neurons against MPP+/MPTP-induced toxicity, suggesting Tat-βsyn-degron might become a potential therapeutic for effective treatment of PD. Since TAT is a membrane permeable sequence with no tissue selectivity, it was not surprising to find that Tat-βsyn-degron effectively knocked down endogenous α-synuclein in both straitum and substantia nigra-containing ventral midbrain. The α-synuclein plasmid I used for HEK cell transfection has a human origin, the primary neuron cultures used in **Chapter 4** of this thesis were all prepared from rat embryos, and the *in vivo* study in this chapter was conducted in C57BL/6 mice. It is interesting to find that the Tat-βsyn-degron peptide was able to knock down α-synuclein from all these sources. This might be because α-synuclein is a conserved protein with high similarity among different species. For example, sequence alignment using PubMed's BLAST suggests that rat α-synuclein is 95% identical to human α-synuclein with only 7 amino acid mismatches (data not shown). Although I haven't identified the Tat-βsyn-degron peptide's binding site in α-synuclein, I suspect it might be the same in all these species.

Notably, another interesting finding is that the control peptide, Tat-βsyn, while having little effect on α-synuclein protein levels (**Fig. 12**), induced similar protective effects with Tat-βsyn-degron in the mouse rotarod behavioral test (**Fig. 13e**). The detailed mechanism is not fully understood, and I suspect it might be due to Tat-βsyn's ability to inhibit α-synuclein aggregation inside the neuron (Shaltiel-Karyo et al., 2010). This point will be further discussed in section **6.3.2** in **Chapter 6**.

Figure 10

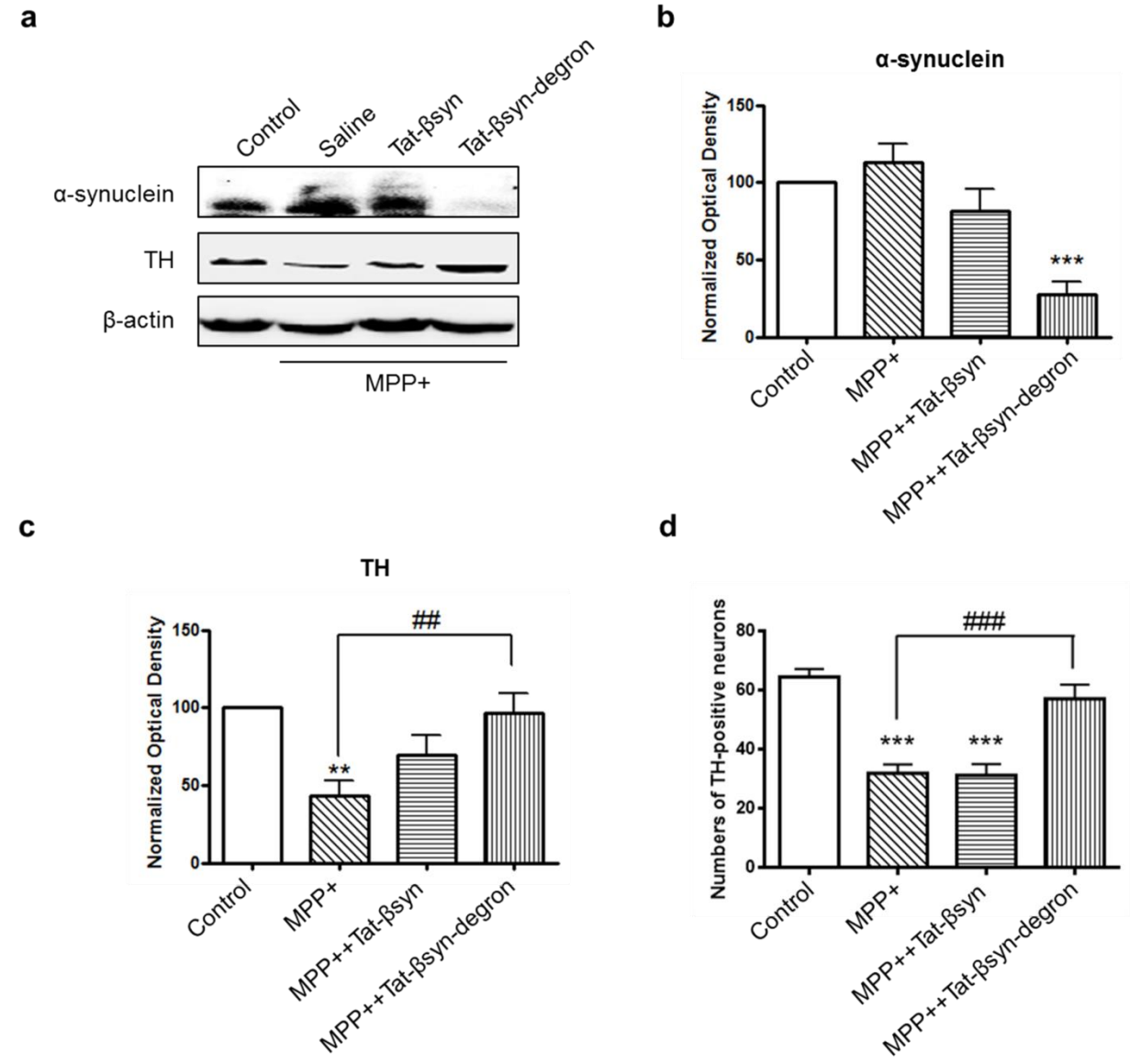

a
Control
Saline
Tat-βsyn
Tat-βsyn-degron
α-synuclein
TH
β-actin
MPP+
b
α-synuclein
Normalized Optical Density
150
100
50
0
***
Control
MPP+
MPP++Tat-βsyn
MPP++Tat-βsyn-degron
c
TH
Normalized Optical Density
150
100
50
0
##
**
Control
MPP+
MPP++Tat-βsyn
MPP++Tat-βsyn-degron
d
Numbers of TH-positive neurons
80
60
40
20
0
###
***
***
Control
MPP+
MPP++Tat-βsyn
MPP++Tat-βsyn-degron

Figure 10. Tat-βsyn-degron peptide protects against parkinsonian toxin induced neuronal damage in rat ventral midbrain cultures

(**a-c**) immunoblotting and (**d**) immunocytochemical staining followed by blinded TH-positive neuron counting showing that bath application (25 μM; 48hrs) of Tat-βsyn-degron, but not Tat-βsyn, significantly reduced the level of endogenous α-synuclein (**a** and **b**; N=8; $F(3,28)=13.58$; $P<0.001$), and prevented MPP+ (20 μM; 48hrs)-induced TH-positive neuronal damage as demonstrated by the loss of TH protein (**a** and **c**; N=8; $F(3,28)=6.99$; $P<0.01$; Bonferroni *post hoc* test: MPP+ + Tat-βsyn-degron *vs* MPP+: ##$P<0.01$) and by the decreased numbers of TH-positive neurons (**d**; N=8; $F(3,28)=22.03$; $P<0.001$; Bonferroni *post hoc* test: MPP+ + Tat-βsyn-degron *vs* MPP+: ###$P<0.001$). Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. **$P<0.01$ and ***$P<0.001$ compared with the control. *TH-positive neuron staining and counting in Fig. 10d was done by Dr. Xuelai Fan in a blinded manner.*

Figure 11

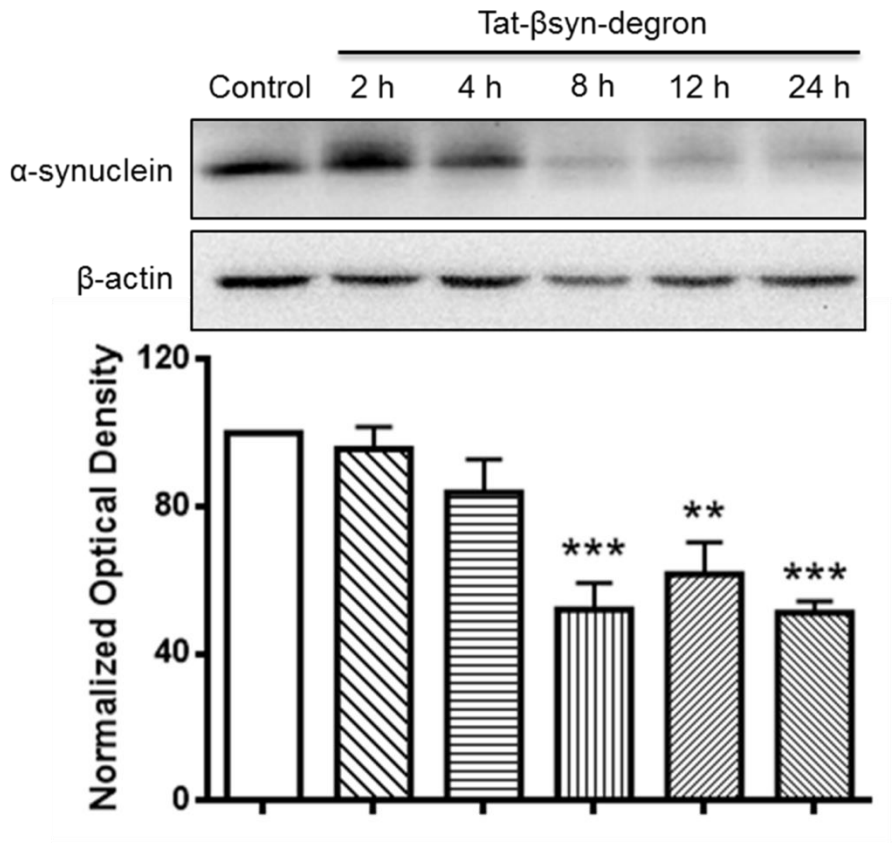

Tat-βsyn-degron
Control
2 h
4 h
8 h
12 h
24 h
α-synuclein
β-actin
Normalized Optical Density
120
80
40
0
***
**
***

Figure 11. Bath application of Tat-βsyn-degron also induces robust knockdown of endogenous α-synuclein in mouse primary cortical cultures

Immunoblots showing that bath application of the membrane permeant synthetic peptide Tat-βsyn-degron (25 uM) induced a robust reduction of endogenous α-synuclein protein levels in a time-dependent manner (2-24 hours; N=6; $F(5,30)=11.29$; $P<0.001$). The statistical difference between groups was determined by one-way ANOVA, followed by Tukey's HSD *post hoc* test. $^{**}P<0.01$ and $^{***}P<0.001$ compared with the control.

Figure 12

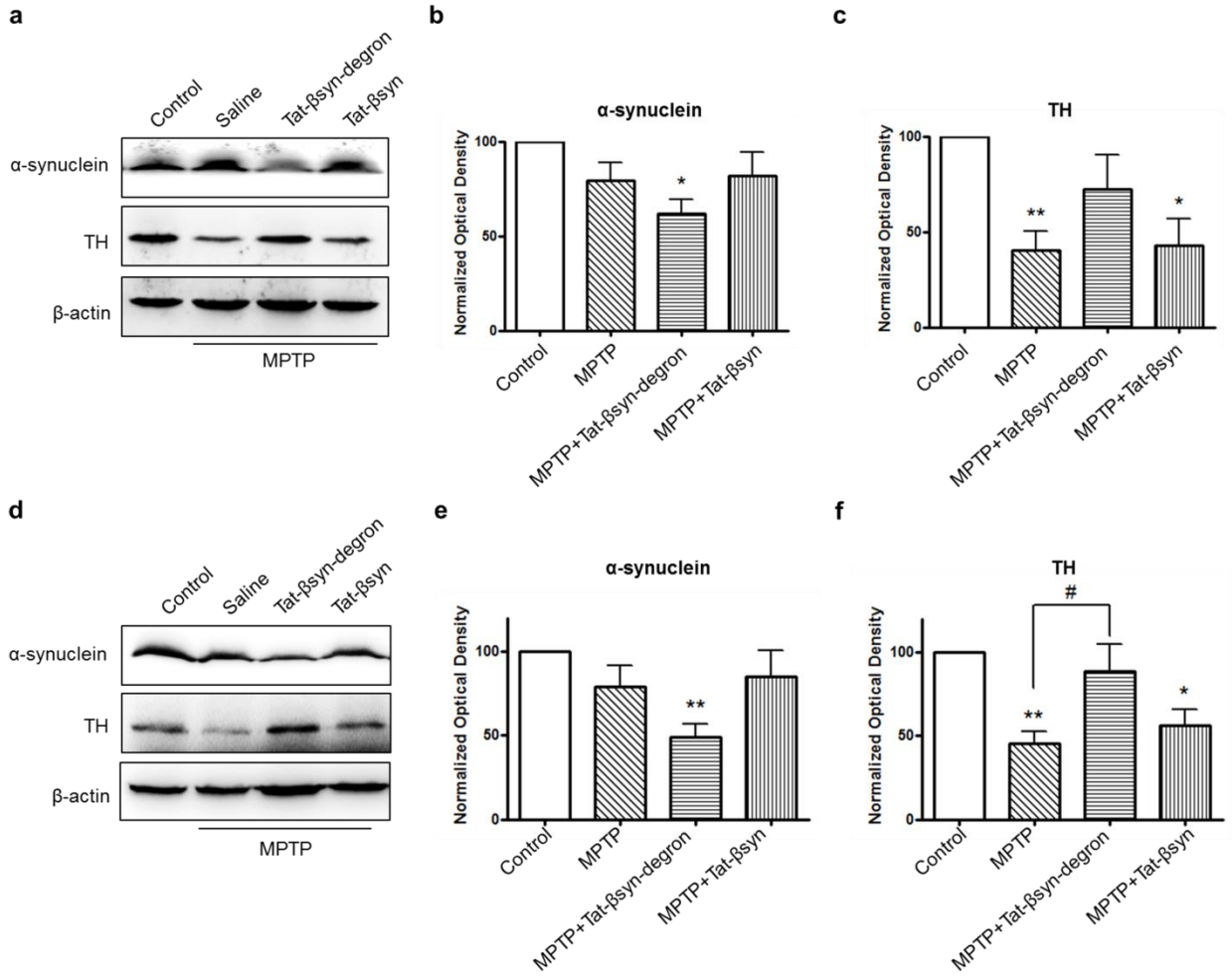

a
Control
Saline
Tat-βsyn-degron
Tat-βsyn
α-synuclein
TH
β-actin
MPTP
b
α-synuclein
Normalized Optical Density
100
50
0
*
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn
c
TH
Normalized Optical Density
100
50
0
**
*
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn
d
Control
Saline
Tat-βsyn-degron
Tat-βsyn
α-synuclein
TH
β-actin
MPTP
e
α-synuclein
Normalized Optical Density
100
50
0
**
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn
f
TH
#
Normalized Optical Density
100
50
0
**
*
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn

Figure 12. Tat-βsyn-degron peptide-mediated knockdown of α-synuclein protects against parkinsonian toxin MPTP-induced TH protein decrease

(**a-f**) Mice received i.p. injections of MPTP (30mg/kg) or same volumes of saline once a day for 5 days, along with Tat-βsyn-degron or its control Tat-βsyn (6 μmol/kg; i.p.) twice a day for 12 days. Brain tissues were collected for immunoblotting for α-synuclein and TH immediately after behavioral assessments (see **Fig. 13e**) on day 12. Tat-βsyn-degron, but not Tat-βsyn, significantly reduced α-synuclein in the substantia nigra-containing ventral midbrain (**a** and **b**; N=10; $F(3,36)=3.24$; $P<0.05$) and the striatum (**d** and **e**; N=11; $F(3,40)=4.12$; $P<0.05$), and significantly reduced the MPTP-induced decrease in the level of TH protein in both ventral midbrain (**a** and **c**; N=10; $F(3,36)=5.23$; $P<0.01$) and striatum (**d** and **f**; N=9; $F(3,32)=6.70$; $P<0.01$; Bonferroni *post hoc* test: MPTP+Tat-βsyn-degron *vs* MPTP: #$P<0.05$). Data are presented as mean±S.E.M. The statistical difference between groups was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. *$P<0.05$ and **$P<0.01$ compared with the control. *Animal treatments and sample preparation in this figure were done in our collaboration lab (Dr. Zhifang Dong's lab in the Children Hospital of Chongqing Medical University) and I am responsible for the data analysis.*

Figure 13

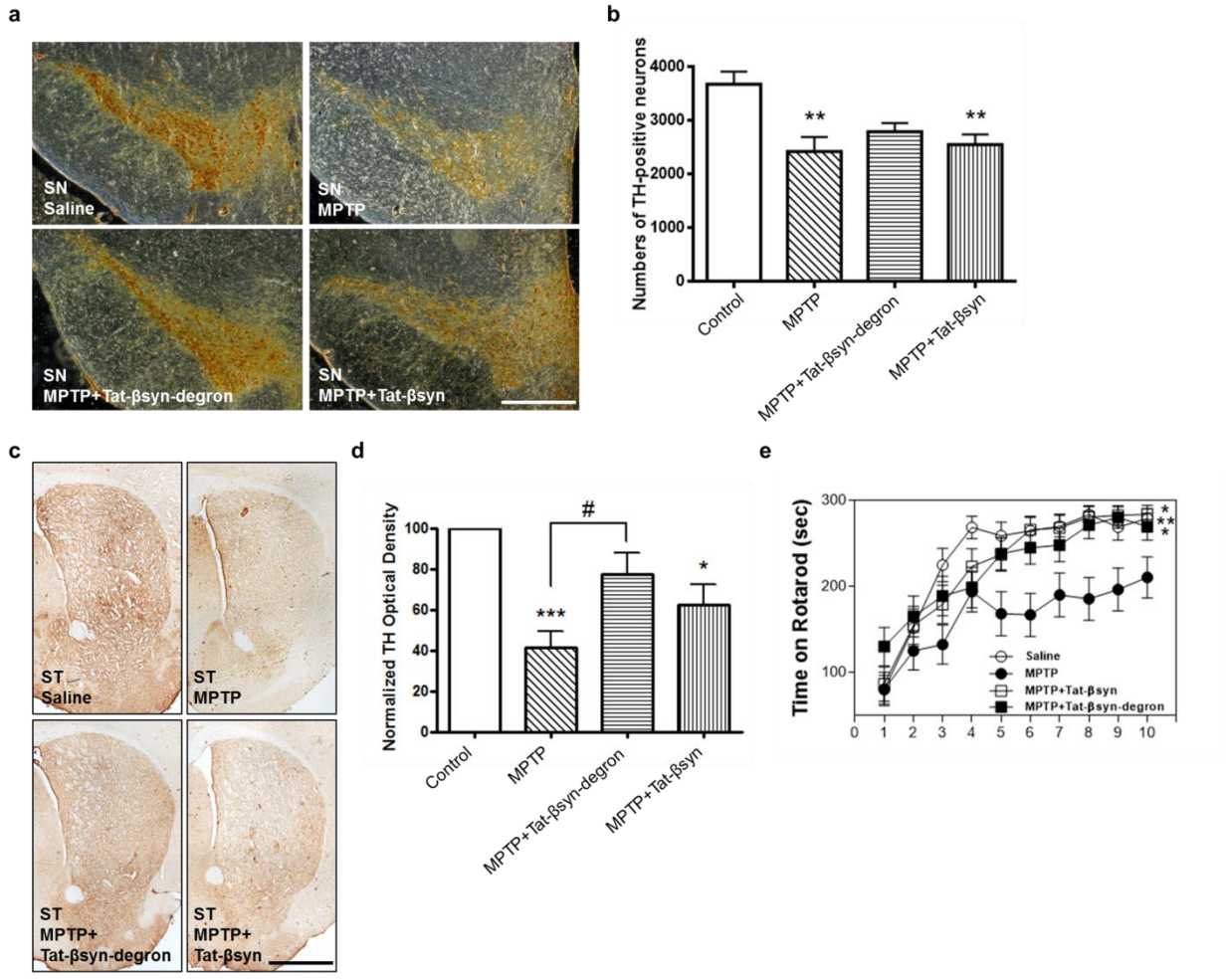

a
SN
Saline
SN
MPTP
SN
MPTP+Tat-βsyn-degron
SN
MPTP+Tat-βsyn
b
Numbers of TH-positive neurons
4000
3000
2000
1000
0
**
**
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn
c
ST
Saline
ST
MPTP
ST
MPTP+
Tat-βsyn-degron
ST
MPTP+
Tat-βsyn
d
Normalized TH Optical Density
100
80
60
40
20
0
#
***
*
Control
MPTP
MPTP+Tat-βsyn-degron
MPTP+Tat-βsyn
e
Time on Rotarod (sec)
300
200
100
0 1 2 3 4 5 6 7 8 9 10
Saline
MPTP
MPTP+Tat-βsyn
MPTP+Tat-βsyn-degron

Figure 13. Tat-βsyn-degron peptide-mediated knockdown of α-synuclein protects against parkinsonian toxin MPTP-induced dopaminergic neuronal damage and behavioral deficits in mice

(**a-e**) Mice received i.p. injections of MPTP (30mg/kg) or same volumes of saline once a day for 5 days, along with Tat-βsyn-degron or its control Tat-βsyn (6 μmol/kg; i.p.) twice a day for 12 days. Brain tissues were collected for immunohistochemical staining of TH (**a** and **c**) immediately after behavioral assessments (**e**) on day 12. (**a-d**) Tat-βsyn-degron, but not its control Tat-βsyn, protected against MPTP-induced decrease in the number of TH-positive dopaminergic neurons in the substantia nigra (SN) pars compacta (**b**; N=7 for the Tat-βsyn-degron group and N=8 for the other three groups; $F(3,27)=6.75$; $P<0.01$; Bonferroni *post hoc* test: MPTP+Tat-βsyn-degron *vs* control: $P=0.06$) and the density of TH-positive dopaminergic neuronal terminals in the striatum (ST; **d**; N=6 for all groups; $F(3,20)=8.58$; $P<0.01$; Bonferroni *post hoc* test: MPTP+Tat-βsyn-degron *vs* MPTP: #$P<0.05$). (**e**) Rotarod motor behavioral tests revealed that mice treated with chronic MPTP (MPTP; N=28) showed significantly shorter latency in falling off the rotarod compared with mice receiving saline control (Saline; N=28), and that the MPTP-induced motor deficits were significantly reduced by Tat-βsyn-degron (MPTP+Tat-βsyn-degron; N=28) and by Tat-βsyn (MPTP+Tat-βsyn; N=28) (two-way ANOVA, $F(3,108)=4.94$; $P<0.01$; Bonferroni *post hoc* test: compared with the MPTP group: saline, **$P<0.01$; MPTP+Tat-βsyn: *$P<0.05$; MPTP+Tat-βsyn-degron: *$P<0.05$). All data are presented as mean±S.E.M. The statistical difference between groups in **b** and **d** was determined by one-way ANOVA, followed by Bonferroni *post hoc* test. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared with the control. Scale bars 0.5 mm in (**a**)

and 1 mm in (**c**). *The animal behavioral tests and the brain slice staining were performed in our collaboration lab (Dr. Zhifang Dong's lab in the Children Hospital of Chongqing Medical University). I was responsible for the neuron counting in Fig. 13b, the optical density quantification in Fig. 13d, and the data analysis in Fig. 13e.*

Chapter 6: Discussion and Conclusion

6.1 Overall Significance

Together, these results suggest that I have developed a powerful technology to effectively knock down endogenous proteins of interest in freely moving animals using targeting peptides. The technology was first demonstrated in a rat model of focal ischemia, where a systemically-injected DAPK1 targeting peptide effectively knocked down endogenous DAPK1 in the brain and thereby protected against ischemia-induced brain damage. The technology is generalizable and can be used for developing selective targeting peptides for other target proteins too. Using this peptide-mediated protein knockdown technology, I developed an α-synuclein targeting peptide called Tat-βsyn-degron, which decreased α-synuclein levels with high efficiency, efficacy and specificity and thereby protected dopaminergic neurons from MPP+ induced neurotoxicity in a cell culture model of PD. Furthermore, the results also showed the efficacy of the Tat-βsyn-degron peptide in crossing the BBB and entering dopaminergic neurons in the brain to knock down endogenous α-synuclein, and in reducing MPTP-induced neuronal death in the substantia nigra and behavioral deficits following i.p. administrations in a mouse MPTP toxicity model of PD. Overall, my study not only validates α-synuclein as a therapeutic target upon which novel therapeutics can be developed for treating PD (Cookson, 2009; Stefanis, 2012), but also provides proof-of-concept evidence that the Tat-βsyn-degron peptide developed in this study may represent a disease-modifying therapeutic for PD.

6.2 Favorable Characteristics of the Tat-βsyn-degron Peptide

6.2.1 Novelty

The α-synuclein knockdown peptide (Tat-βsyn-degron) is innovative in several aspects: first, by knocking down α-synuclein, one of the major disease-causing molecules, the peptide directly targets one of the root causes of PD, and can be expected to stop or slow down the progression of the disease. This approach is in stark contrast to most of the PD therapeutic strategies currently used in clinics. Since deep brain stimulation and pharmacological treatment do not directly target the disease-causing processes, at best they are symptom-relieving and cannot stop or slow down the progression of the disease (Lundqvist, 2007; Groiss et al., 2009; Karlsborg et al., 2010a). Second, the Tat-peptide-mediated knockdown method also has clear advantages over other α-synuclein knockdown technologies, such as anti-sense RNA and siRNA. For instance, although siRNA-mediated knockdown of α-synuclein has also been proved to be effective in various models of PD (Fountaine and Wade-Martins, 2007; Liu et al., 2014), their clinical applications are hindered by an inability to cross the BBB and the plasma membrane of neurons. The delivery of siRNAs to the brain is mainly accomplished by an invasive intracerebral injection or viral infection, which may not be clinically practical for therapeutic use in human patients. Several recent studies suggest that delivery of siRNA to the brain by a non-invasive systemic injection may be achieved by coupling siRNA with brain delivery vehicles such as RVG-9R peptide or RVG-9R peptide-coated exosome (Kumar et al., 2007; Alvarez-Erviti et al., 2011; Cooper et al., 2014). These techniques are restricted to acetylcholine receptor-expressing neurons in the brain and remain technically challenging. However, using the

Tat-mediated protein transduction mechanism, my peptide-based method is much simpler and more effective at entering neurons in the brain following non-invasive systemic administration. The effectiveness was clearly demonstrated by the high efficacy of the peptide at knocking down α-synuclein in the brain and thereby protecting substantia nigra dopaminergic neurons from the parkinsonian toxin-induced toxicity following peripheral administration (**Fig. 12** and **13**). Additionally, the peptide-mediated knockdown has a clear temporal advantage over siRNA-mediated knockdown. α-synuclein is a very stable protein with a long half-life (Li et al., 2004) and it may take a few weeks for siRNAs to induce a significant reduction of endogenous α-synuclein protein level in the brain (McCormack et al., 2010), whereas by hijacking the endogenous proteasomal degradation system in the cell, the Tat-βsyn-degron peptide produced a rapid and robust degradation of α-synuclein protein within a few hours (**Fig. 8**).

6.2.2 Specificity

High target specificity is vital for the safe application of a potential drug candidate in human patients. It means fewer unfavorable side effects and better patient acceptance. When we intend to specifically knock down a target protein using our targeting peptides, the potential off-target effects are always a critical concern and have to be properly addressed. My results in primary neuron cultures clearly showed that the Tat-βsyn-degron peptide effectively knocked down its target protein, α-synuclein, without affecting several other important proteins in the cell, such as transmembrane protein $GABA_A$ receptor β2/3 subunits, intracellular protein HSP90, and a known α-synuclein binding

protein 14-3-3 (**Fig. 8**). I also determined the specificity of the α-synuclein targeting peptide by comparing its effect in lowering the levels of α-synuclein relative to the alternative β- or γ-synuclein proteins. As shown in **Fig. 6**, I found that expression of FLAG-βsyn-degron specifically reduced the expression level of co-transfected α-synuclein, but had little effect on the expression level of co-transfected β- or γ-synuclein. Given the high similarity among these synuclein isoforms, these results demonstrated a high level of specificity of the Tat-βsyn-degron peptide towards its protein target, α-synuclein.

A minor concern is that lowering an important protein in the cell may inevitably lead to some changes in protein homeostasis. Some proteins may be upregulated or downregulated to compensate for the loss of α-synuclein. In the future, a broader proteomics approach, such as Mass Spectrometry, may be necessary to further characterize the specificity of the Tat-βsyn-degron peptide and also the secondary effects of peptide-mediated α-synuclein knockdown. To this end, primary cultured neurons may be treated with a single dose of the Tat-βsyn-degron peptide or control peptide, and the cell lysates are then subject to Mass Spectrometry analysis. Any changes of protein levels observed shortly after peptide treatment (0-6 hours) may be interpreted as off-target effects of the Tat-βsyn-degron peptide and any changes observed afterwards (6-48 hours) may be interpreted as secondary changes in the cellular protein network.

6.2.3 Clinical Applicability

Right now, more than 60 peptidergic therapeutics have been approved by the US FDA and many more are in clinical trials and pre-clinical development (Fosgerau and Hoffmann, 2015). One advantage of the targeting peptides is that they are practical for clinical application. The targeting peptide strategy is very simple and does not rely on complicated genetic engineering of either the targeting peptides or the target proteins. The targeting peptides can be directly synthesized in a chemical synthesizer using the well established solid-phase synthesis method. They are membrane permeable and are also highly soluble in aqueous solution. Linked with the Tat membrane permeant sequence, the targeting peptides can readily cross the BBB and the plasma membrane of neurons without the need to use viral infection for delivery to the brain. This is supported by the *in vivo* results presented in this thesis, showing that Tat-βsyn-degron effectively knocked down endogenous α-synuclein in the mouse brain following systemic injections (**Fig. 12**). It's worth mentioning that the safety of the Tat sequence and its efficacy in reaching brain cells have been well demonstrated in a recent phase 2 clinical trial in human subjects (Hill et al., 2012). Since there is still no disease-modifying treatment in the field of PD, I hope that my Tat-βsyn-degron peptide may become the first peptide therapeutic that is able to slow down or even stop the progression of the disease.

Safety is a critical concern for any substance moving into clinical trials. Numerous compounds have failed at phase 1 clinical trials that evaluate the toxicity of the compounds in human subjects. However, peptides, especially unmodified, naturally-occurring peptide sequences composed of natural L-amino acids, may be relatively safe

compared with unnatural small molecules, because peptides are metabolized into non-toxic amino acids by various peptidases in the body and thus may have limited toxicity and side effects. Furthermore, despite previous assumptions that lowering the level of an important cellular protein such as α-synuclein may result in various detrimental consequences, it is surprising to find that α-synuclein knockout mice are viable and fertile, exhibit intact brain architecture, and have normal development of dopaminergic cell bodies, fibers, and synapses (Abeliovich et al., 2000), presumably due to functional compensation by β- and γ-synuclein. Therefore, I suspect that knocking down α-synuclein using the Tat-βsyn-degron peptide may not induce overt side effects and toxicity in animals. This was supported by our observations in C57BL/6 mice which received chronic Tat-βsyn-degron injections for 12 days. During the experiments, no overt abnormalities or unexpected death were observed in these mice, and furthermore chronic Tat-βsyn-degron injections induced profound neuroprotection against MPTP-induced brain damage and behavioral deficits (**Fig. 12** and **13**). This strongly indicates the safety and efficacy of the Tat-βsyn-degron peptide in treating PD symptoms in animals.

6.3 Potential Limitations and Future Directions

6.3.1 Investigating the Efficacy of Tat-βsyn-degron in a Different Animal Model of Parkinson's Disease

In the present thesis research, I tested the therapeutic effects of the Tat-βsyn-degron peptide in a commonly used MPTP toxin model of PD (Jackson-Lewis and Przedborski,

2007). This well characterized mouse model has proved to be easy, reliable, reproducible, and fast, and has since become an invaluable tool in the animal PD research (Jackson-Lewis and Przedborski, 2007). However, the major drawbacks of the MPTP toxin model of PD are the absence of Lewy body pathology in most cases and the lack of progressive age-dependent neurodegeneration in the brain (Beal, 2010). To further confirm the neuroprotective efficacy of the Tat-βsyn-degron peptide, it will be necessary to test the peptide in another well characterized animal model of PD. One option is using the transgenic mouse models of PD. Although sometimes difficult to establish and time-consuming, they have been popular choices for use in scientific research of PD (Beal, 2010; Chesselet et al., 2012). One of the most well characterized transgenic models of PD is the Thy-1 α-synuclein overexpression mouse model developed by Dr. Eliezer Masliah in University of California, San Diego (Chesselet et al., 2012). This mouse line was created based on clinical findings in PD patients that α-synuclein overexpression (due to duplication and triplication of the α-synuclein gene) has been found to be responsible for causing familial PD (Singleton et al., 2003; Chartier-Harlin et al., 2004). In this model, full-length, human, wild-type α-synuclein is overexpressed under the Thy-1 promoter, and this mouse line reproduces many features of sporadic PD, including progressive changes in dopamine release and striatal content, α-synuclein pathology, deficits in motor and non-motor functions that are affected in pre-manifest and manifest phases of PD, inflammation, and biochemical and molecular changes similar to those observed in PD (Chesselet et al., 2012). However, a drawback of this model is that it is a lengthy process to establish this mouse PD model. Many PD-like features do not occur in the Thy-1 α-synuclein overexpression mice until

many months after birth (Chesselet et al., 2012). Furthermore, even when examined at 22 months of age, Thy-1 α-synuclein overexpression mice did not show any reduction in the number of TH-positive neurons, the classical marker for dopaminergic neurons, in the substantia nigra pars compacta (Chesselet et al., 2012).

Another option is the fibril propagation model recently developed by Dr. Virginia Lee at University of Pennsylvania. It is based on the prion hypothesis of α-synuclein, which suggests that pathological α-synuclein can be secreted from the affected neuron and then spreads to unaffected regions in the brain to initiate Lewy body pathology (Recasens and Dehay, 2014). This is supported by recent research findings showing that a single intracerebral inoculation of synthetic α-synuclein fibrils led to cell-to-cell transmission of pathologic α-synuclein and Parkinson's-like Lewy pathology in anatomically interconnected regions in both transgenic PD mice and WT non-transgenic mice (Luk et al., 2012b; Luk et al., 2012a). This new PD model provides researchers with new insights into the molecular mechanisms underlying PD pathogenesis. However, synuclein propagation in the whole mouse brain only takes a few months (Luk et al., 2012b; Luk et al., 2012a), but in human patients it is a lengthy process (over 10 years) and only occurs in a limited number of neurons (Kordower et al., 2008; Li et al., 2008; Li et al., 2010b). Therefore, definitive evidence supporting the prion-like behavior of α-synuclein in human PD patients is still lacking and more investigation has to be conducted to fill the knowledge gap.

6.3.2 Other Features of Parkinson's Disease That Can Be Investigated

PD is primarily a chronic and progressive motor disorder, characterized by features such as tremors, rigidity, bradykinesia and impaired balance (Xia and Mao, 2012; Muller et al., 2013). That's why I used the rotarod test to measure the motor behavior of MPTP-treated mice in the present study. However, this may not be sufficient to support the therapeutic potential of the Tat-βsyn-degron peptide for PD treatment, because a number of non-motor symptoms, such as dementia, gastrointestinal dysfunction, olfactory deficit and sleep disorder, have also been observed in both human patients and animal models of PD (Chaudhuri and Schapira, 2009; McDowell and Chesselet, 2012; Bichler et al., 2013; Muller et al., 2013; McDowell et al., 2014; Zhang et al., 2015). Since levodopa, the most effective anti-PD drug so far in the clinic, mainly ameliorates motor symptoms of PD (Olanow et al., 2014), but has little effect in controlling non-motor symptoms (Sprenger and Poewe, 2013), it is intriguing to test whether the Tat-βsyn-degron peptide used in this study may also protect against non-motor symptoms in animal models of PD. As aforementioned in section **6.3.1**, one of the best characterized animal models for studying non-motor symptoms of PD is the Thy-1 α-synuclein overexpression mouse model developed by Dr. Eliezer Masliah in University of California, San Diego (Chesselet et al., 2012). These mice reproduce multiple motor and non-motor deficits observed in pre-manifest PD, such as motor disorder, olfactory deficit, colonic motor alteration, disrupted circadian rhythm, cognitive deficit and increase anxiety (Chesselet et al., 2012). In the future, it may be beneficial to use this particular mouse line to have a comprehensive evaluation of the protective efficacy of the Tat-βsyn-degron peptide in PD.

6.3.3 The Ubiquitin-proteasome System in Parkinson's Disease and Its Relationship to the Degron-mediated Proteasomal Degradation

The Tat-βsyn-degron peptide used in this study is based on the RRRG degron proteasomal degradation signal. However, decline of the proteasome function has been observed in PD patients (McNaught et al., 2003; Tofaris et al., 2003) and mutations in the genes encoding enzymes of the ubiquitin-proteasome system, such as parkin and ubiquitin C-terminal hydrolase L1 (Gong and Leznik, 2007; Dawson and Dawson, 2010), have been shown to associate with neurodegeneration in some familial forms of PD. These may somehow compromise the therapeutic efficacy of the Tat-βsyn-degron peptide in certain PD patients. Meanwhile, lysosomes can also become compromised in certain PD patients (Alvarez-Erviti et al., 2010; Dehay et al., 2010; Murphy et al., 2014). Therefore, depending on the disease condition of PD patients, it would be beneficial to have a protein degradation system that can target either the lysosome or the proteasome. As shown by our previous work (Fan et al., 2014b), the lysosomal degradation signal-linked Tat-βsyn-CTM peptide that we have developed previously is also able to efficiently target α-synuclein for degradation via the lysosome.

Since degron is a newly identified proteasomal degradation signal (Bonger et al., 2011), it is still not clear whether it induces protein removal via the ubiquitin dependent or independent proteasomal degradation pathway. Further investigating the mechanism underlying the RRRG-mediated protein degradation pathway not only will reveal more details about this interesting phenomenon, but also may help us get a better idea in drug target selection and targeting peptide design.

6.3.4 Choosing a Different Control Peptide

In the present thesis research, it is interesting to find that the control peptide, Tat-βsyn, also has some mild neuroprotective effects in decreasing toxin-induced neuronal damage and behavioral deficits in PD models (see results in **Chapter 5**). I think this might be due to the intrinsic anti-α-synuclein aggregation property of the βsyn sequence, because a previous study had shown that by inhibiting α-synucein oligomerization, the βsyn sequence effectively protected against α-synucein overexpression-induced motor deficits in a Drosophila model of PD (Shaltiel-Karyo et al., 2010). Although α-synuclein aggregation, a pathological hallmark of PD, is not commonly observed in the MPTP mouse models, previous studies had reported that chronic MPTP infusions could induce α-synuclein oligomerization in mice (Fornai et al., 2005; Shioda et al., 2014). Evidence also suggested that it might be such oligomers that produced most pathological actions of α-synuclein, including neuronal death and motor dysfunction (Fornai et al., 2005; Shioda et al., 2014). Given my results indicating that the Tat-βsyn peptide can reduce motor deficits without lowering α-synuclein levels (**Fig. 12** and **13**), I propose that Tat-βsyn, by inhibiting α-synuclein oligomerization (Shaltiel-Karyo et al., 2010) (without affecting the amount of α-synuclein), may also be effective in reducing PD-associated motor deficits as long as a sufficient number of dopaminergic neurons remain functional. This effect, however, may not be long lasting as it appears insufficient in stopping the PD-associated degeneration of dopaminergic neurons. In contrast, Tat-βsyn-degron has an obvious advantage since it not only rescues the motor deficits, but also increases the survival of dopaminergic neurons. This is not surprising given that in comparison with Tat-βsyn, the Tat-βsyn-degron peptide not only acts as an interference peptide

inhibiting the oligomerization of α-synuclein, but also acts as a proteasomal targeting peptide reducing the levels of α-synuclein in neurons. Since α-synuclein is over-expressed under certain pathological conditions of PD (Singleton et al., 2003; Chartier-Harlin et al., 2004) and these up-regulated proteins can interfere with many physiological processes, such as ER-to-Golgi transport (Thayanidhi et al., 2010), synaptic transmission (Nemani et al., 2010), and mitochondria function and morphology (Devi et al., 2008; Kamp et al., 2010), robustly knocking down the over-expressed α-synuclein using Tat-βsyn-degron peptide may have better neuroprotective efficacy in restoring normal cellular functions in the PD brain than simply inhibiting the formation of toxic α-synuclein oligomers using the Tat-βsyn peptide. In the future, a different control peptide, such as a non-functional scramble peptide, may be considered for follow-up studies.

6.3.5 Further Characterization of βsyn as the Binding Sequence

In the present study, I used amino acids 36-45 of β-synuclein (βsyn) (Shaltiel-Karyo et al., 2010) as the binding sequence of the α-synuclein targeting peptide Tat-βsyn-degron, which showed robust efficacy in knocking down endogenous α-synuclein both *in vitro* and *in vivo*. Nevertheless, more characterization has to be done to confirm βsyn as the optimal binding sequence for α-synuclein: 1) the binding affinity between Tat-βsyn-degron and α-synuclein has to be determined to see whether it is in the low nanomolar range; 2) the binding selectivity of βsyn against α-synuclein has to be determined using a broader proteomics approach, such as Mass Spectrometry, to

ensure minimal off target effects; 3) the binding site of βsyn on α-synuclein may also have to be determined to see whether it is the optimal sequence to target.

6.3.6 Enhancing Peptide Stability

The clinical development of peptides is largely limited by their poor serum stability, as they are prone to enzymatic digestion in the body. It is unfortunate that most peptides with promising therapeutic effects often have serum half-lives of only minutes to hours (Santi et al., 2012). Therefore, various approaches have been tried to modify a peptide to make it more stable.

1) Retro-inversion: Retro-inversion is a process of reversing the primary sequence of a peptide and then replacing the natural L-amino acids with unnatural D-amino acids. In this way, although the peptides bonds are inverted, a retro-inverso peptide will still mimic the original L-peptide sequence by having a similar side chain topology (Guichard et al., 1994). Abundant evidence has shown that retro inverso peptides are biologically active, and also have great stability *in vivo* due to their high resistance to proteolysis in the body (Shaltiel-Karyo et al., 2010; Parthsarathy et al., 2013; Wei et al., 2014). However, retro-inversion does not work for every peptide (Li et al., 2010a), and can even cause severe cellular toxicity under certain conditions (Holm et al., 2011). This modification has to be tested on a case by case basis.
2) End modifications: Chemically synthesized peptides have free N- and C- terminals, and capping the ends of a peptide has been suggested to enhance its serum

stability. The most widely used end modifications are N-terminal acetylation and C-terminal amidation (Stromstedt et al., 2009; Nguyen et al., 2010; Shaltiel-Karyo et al., 2010). After modifications, both ends of a peptide become uncharged. As a result, a drawback of these end modifications is that by reducing the overall charge of a modified peptide, they might decrease the overall solubility of the peptide in water-based solutions. Therefore, it is necessary to re-evaluate the properties of a peptide after end modifications.

3) PEGylation: Peptide PEGylation is a process of covalently linking a large molecule called polyethylene glycol (PEG) to a small peptide (Hamley, 2014). As systemically-administered small peptides are often cleared by the kidney that filters substances according to size, it has been well demonstrated in previous studies that the addition of PEG dramatically increases the size of a small peptide and thus prevents the premature renal clearance of small peptides (Baumann et al., 2014; Benincasa et al., 2015). Meanwhile, the globular structure of PEG also acts as a shield to effectively protect its conjugated cargo against enzymatic digestion (Park et al., 2010). As a result, the half-life of a small peptide in the body may be dramatically extended by PEGylation. Since PEG is generally considered as non-toxic and non-immunogenic, quite a few PEGylated drugs have already been approved by the US FDA (Swierczewska et al., 2015). However, in spite of the promising progress, the PEGylation technique is mainly limited to the modification of large proteins. For small peptides that are less than 30 amino acids, unfortunately the large size of a conjugated PEG may sterically hinder their biological activities (Cully, 2015). Since the Tat-βsyn-degron peptide is a small

peptide with only 25 amino acids, it remains uncertain whether PEGylation will enhance its half-life and at the same time retain the peptide's bioactivity.

4) Cyclization: Peptide cyclization, a process of making ring-shape structures within a linear peptide, is another popular alternative to make a peptide more stable (White and Yudin, 2011). In contrast to linear peptides, cyclic peptides can be more resistant to protease digestion (Howell et al., 2014) and sometimes are even orally bioavailable (Wang et al., 2014a). However, since the Tat-βsyn-degron peptide is a linear peptide with a simple structure, cyclizing the peptide may completely change its structure and thus abolish its binding with the target protein, α-synuclein.

Since peptides are metabolized into non-toxic amino acids by various peptidases in the body, peptides, especially unmodified, naturally-occurring peptide sequences composed of natural L-amino acids, may have limited toxicity and side effects in human patients. Therefore, to overcome the poor serum stability problem of peptides, besides peptide modification, another potential alternative is giving a large dose of peptide (which is much higher than the minimal effective dose) to the patient so that once a day injection in patients is sufficient for maintaining the peptide effect for the whole day.

6.3.7 Peptide Delivery

In the present thesis research, to deliver a peptide to the brain, I used i.v. injection for the Tat-GluN2B-CTM peptide and i.p. injection for the Tat-βsyn-degron peptide. For acute diseases such as ischemia, several i.v. injections of peptide drugs seem to be

acceptable in human patients. However, for chronic diseases such as PD, repeated i.p. injections of peptide drugs may cause too much discomfort or even pain. Therefore, to move the Tat-βsyn-degron peptide into clinical trials, I may consider a better delivery method for the peptide. Intranasal (i.n.) administration may be a good idea, because it is non-invasive, not painful and easy to handle. Additionally, evidence has shown that intranasally administered biomolecules can bypass the BBB to directly target the central nervous system, and a variety of peptides, proteins and even gene vectors have already been successfully delivered to the brain using the intranasal administration method (Aly and Waszczak, 2015). In our lab, I have also tested an intranasal administration protocol for delivering a Tat-fused peptide (Tat-GluA2$_{3Y}$) to the brain. As shown in **Fig. 14**, both i.v. administered and i.n. administered Tat-GluA2$_{3Y}$ peptide efficiently entered the whole rat brain, as demonstrated by matrix-assisted laser desorption ionization (MALDI) mass spectrometry imaging that scanned the whole brain slice to detect the presence of the Tat-GluA2$_{3Y}$ peptide signal. Interestingly, an i.n. administration of 5mg/kg Tat-GluA2$_{3Y}$ peptide resulted in strong peptide signals in the whole brain slice 1.5 hours post-injection, and the signal intensity was comparable to that from the rat brain i.v. injected with 10mg/kg Tat-GluA2$_{3Y}$ peptide. This result strongly suggests that compared with i.v. administration, i.n. administration may lead to better bioavailability of a peptide in the brain. Since the Tat-GluA2$_{3Y}$ peptide has been well characterized as a blocker for long term depression (LTD) (Collingridge et al., 2010; Dias et al., 2012; Dong et al., 2012; Dong et al., 2015), function of the intranasally administered Tat-GluA2$_{3Y}$ peptide was also investigated in intact animals. As shown in **Fig. 14k**, a paired-burst (PB) stimulation protocol induced hippocampus LTD in anesthetized rats, and this LTD

was effectively prevented by an i.n. administration of the Tat-GluA2$_{3Y}$ peptide but not the scramble control 1 hour before the baseline recording. This suggests that once in the brain, the i.n. administered peptide is still functional. Therefore, based on these promising preliminary data, intranasal administration definitely should be considered in the future for repeatedly delivering peptides *in vivo* for treatment of chronic brain disorders, such as PD.

6.3.8 Large-scale Screening for Binding Sequences

Finding a selective and high-affinity binding sequence is vital for constructing a good targeting peptide. In the present thesis research, the binding sequences for Tat-GluN2BCTM and Tat-βsyn-degron are derived from known protein-protein interaction information from previous studies (Shaltiel-Karyo et al., 2010; Tu et al., 2010). However, for some target proteins with little information of well characterized interacting proteins, finding a good binding sequence may require more than a literature search. Therefore, it will be beneficial to set up a high throughput platform to screen for binding partners for a select target protein. I am working with Dr. Horacio Bach at UBC to create a binding peptide discovery platform using the phage display technique (**Fig. 15**). Briefly, a large peptide library containing several millions of 12-amino-acid peptides with distinct sequences is inserted into the phage genome and then the short peptides are individually expressed on the surface of the phages. The gene of the target protein is cloned and then inserted into a GST vector. After expression and purification, the GST-fused target protein or the GST control is overlaid onto the phages and the short peptide with highest binding affinity will be subsequently selected. Using this method, we may

also screen for a selective binding sequence for a mutated or post-translationally modified (such as phosphorylated) target protein. To do this, we may first add the WT protein to the phages to remove all the phages with binding peptides, and then add the mutated or post-translationally modified target protein to the phages that are left from the 1st screen. If this is successful, we may be able to find a unique peptide sequence that specifically interacts with the non-WT protein rather than the WT protein. As a result, we may be able to selectively knock down the non-WT disease protein using our peptide-mediated protein knockdown technology. As many mutated proteins still do not have a specific inhibitor, and as the RNA silencing technique cannot differentiate a post-translationally modified target protein from a WT protein, our strategy might substantially contribute to a rapid development of target-specific therapeutics for various human diseases.

6.3.9 Designing a Clinical Trial for Parkinson's Disease

If the Tat-βsyn-degron peptide successfully finishes all the pre-clinical development, designing a good clinical trial is necessary to properly evaluate its neuroprotective efficacy in PD patients. Since most PD patients start exhibiting motor deficits after losing at least 50% of dopaminergic neurons in the substantia nigra (Cheng et al., 2010), it may be too late to treat patients with the Tat-βsyn-degron peptide at this stage. Unfortunately, right now early diagnosis of PD is still difficult and a good biomarker for PD has yet to be established. Nevertheless, accumulating evidence suggests that increased oligomeric α-synuclein level in the CSF or blood plasma may be a good early indicator of this devastating disease (Foulds et al., 2013; Majbour et al., 2016).

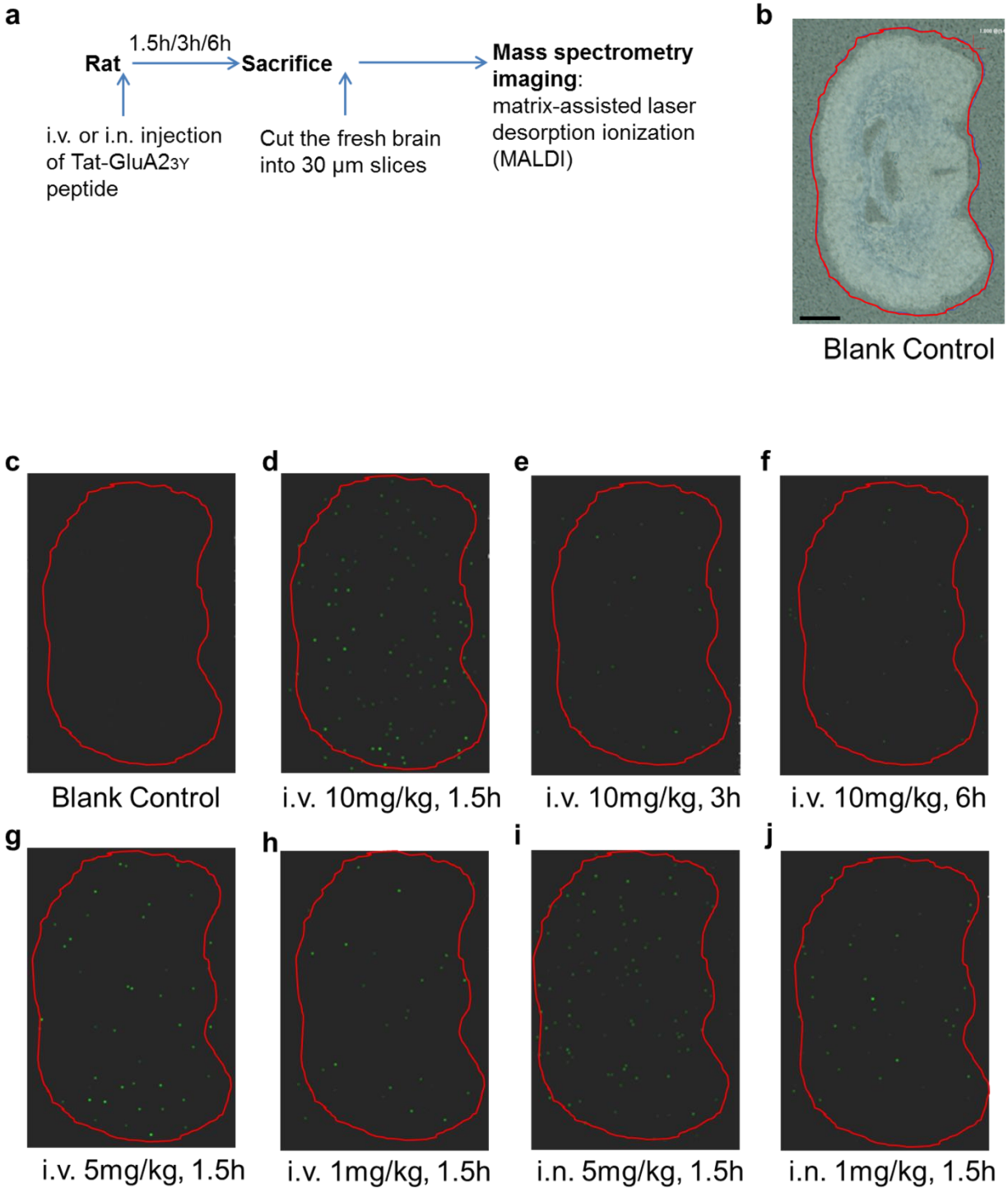
Therefore, it may be possible to administer the Tat-βsyn-degron peptide chronically in early PD patients long before any manifestation of the clinical symptoms, which may effectively slow down or even stop the degeneration of nigral dopaminergic neurons at an early stage. A variety of clinical measurements, such as oligomeric α-synuclein level in the CSF/blood plasma, motor function, gastrointestinal function, olfactory function and cognitive capability may be used as the readout of the peptide's therapeutic efficacy. Endpoint of the clinical trial will be slowed or even halted progression of the disease in human patients, including reduced oligomeric α-synuclein level in the CSF/blood plasma and improved motor and non-motor functions.

6.4 Conclusion

Taken together, this α-synuclein knockdown peptide Tat-βsyn-degron may represent a new, effective, and clinically practical therapeutic treatment for PD. A recent phase 2 clinical trial has already demonstrated that a Tat-fused short peptide is not only safe, but therapeutically effective in protecting neurons against ischemic damage in humans (Hill et al., 2012). I hope that the Tat-βsyn-degron peptide may also have the potential to be quickly translated to the clinic as the first effective disease-modifying treatment that directly targets the disease-causing processes of PD. Due to the versatility of the peptide-mediated protein knockdown method, I can theoretically target any cellular proteins by simply changing the protein-binding sequence of the targeting peptide. Since many human diseases, including some of the age-related neurodegenerative diseases such as Alzheimer's disease and Huntington's disease, are pathologically linked to gain-of-function mutations, and/or increased expression level of a particular

protein, the present thesis research can be expected to spur the development of novel therapeutics for human diseases beyond PD.

Figure 14


a
Rat
1.5h/3h/6h
Sacrifice
Mass spectrometry imaging: matrix-assisted laser desorption ionization (MALDI)
i.v. or i.n. injection of Tat-GluA2 3Y peptide
Cut the fresh brain into 30 μm slices
b
Blank Control
c
Blank Control
d
i.v. 10mg/kg, 1.5h
e
i.v. 10mg/kg, 3h
f
i.v. 10mg/kg, 6h
g
i.v. 5mg/kg, 1.5h
h
i.v. 1mg/kg, 1.5h
i
i.n. 5mg/kg, 1.5h
j
i.n. 1mg/kg, 1.5h

k

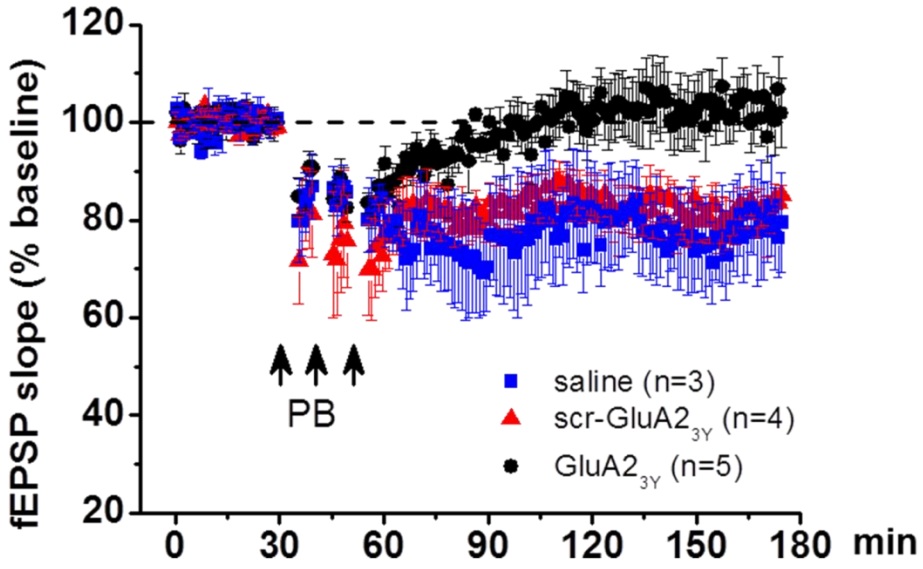

fEPSP slope (% baseline)
120
100
80
60
40
20
0
30
60
90
120
150
180
min
PB
saline (n=3)
scr-GluA2$_{3Y}$ (n=4)
GluA2$_{3Y}$ (n=5)

Figure 14. Intranasally-administered Tat-GluA2$_{3Y}$ peptide enters the brain and blocks the paired-burst stimulation-induced LTD in the hippocampus of Sprague-Dawley rats

(**a**) Illustration of the experimental procedures. (**b**) Freshly sliced brain slices were placed on clean slides for MALDI imaging. The brain slice of the blank control rat is shown in a bright field and its position is indicated by the red line. (**c-j**) Mass spectrum was recorded during the two-dimensional brain scanning. The Tat-GluA2$_{3Y}$ peptide signals were indicated as the pseudo-green dots in the brain slices. (**c**) No Tat-GluA2$_{3Y}$ peptide signal was detected in the brain slice of blank control rat without any peptide treatment. (**d-f**) The Tat-GluA2$_{3Y}$ peptide signals were detected in the whole brain slice of rat 1.5hrs after it received an i.v. injection of 10mg/kg peptide (**d**), and the signals decreased over time as measured 3hrs (**e**) and 6hrs (**f**) post-injection. The Tat-GluA2$_{3Y}$ peptide signal also showed a dose-dependent decrease of intensity in rats that received an i.v. injection of 10mg/kg (**d**), 5mg/kg (**g**), or 1mg/kg (**h**) peptide, measured 1.5hrs post-injection. (**i-j**) Strong Tat-GluA2$_{3Y}$ peptide signals were also detected in the brain slices of rats 1.5hrs after they received an intranasal injection of 5mg/kg (**i**) or 1mg/kg peptide (**j**). (**k**) Peptide or saline control was intranasally injected into rats 1hr before the baseline recording. Rats were anesthetized after the injection, and the hippocampus LTD was induced by 3 trains of PB stimulation (200 pairs of two-pulse bursts, one pair per second, 2.5-ms interpulse interval, 10-ms interburst interval), which was blocked by the Tat-GluA2$_{3Y}$ peptide but not the control scramble peptide Tat-scr-GluA2$_{3Y}$. Scale bar 2mm in **b**. *The peptide injection and fresh brain slicing were conducted by me. The MALDI mass spectrometer imaging (Fig. 14b-j) was conducted by Dr. Shujun Lin from*

the Centre for High-Throughput Phenogenomics at UBC. Fig. 14k was generated by Dr. Zhifang Dong in the Children Hospital of Chongqing Medical University.

Figure 15

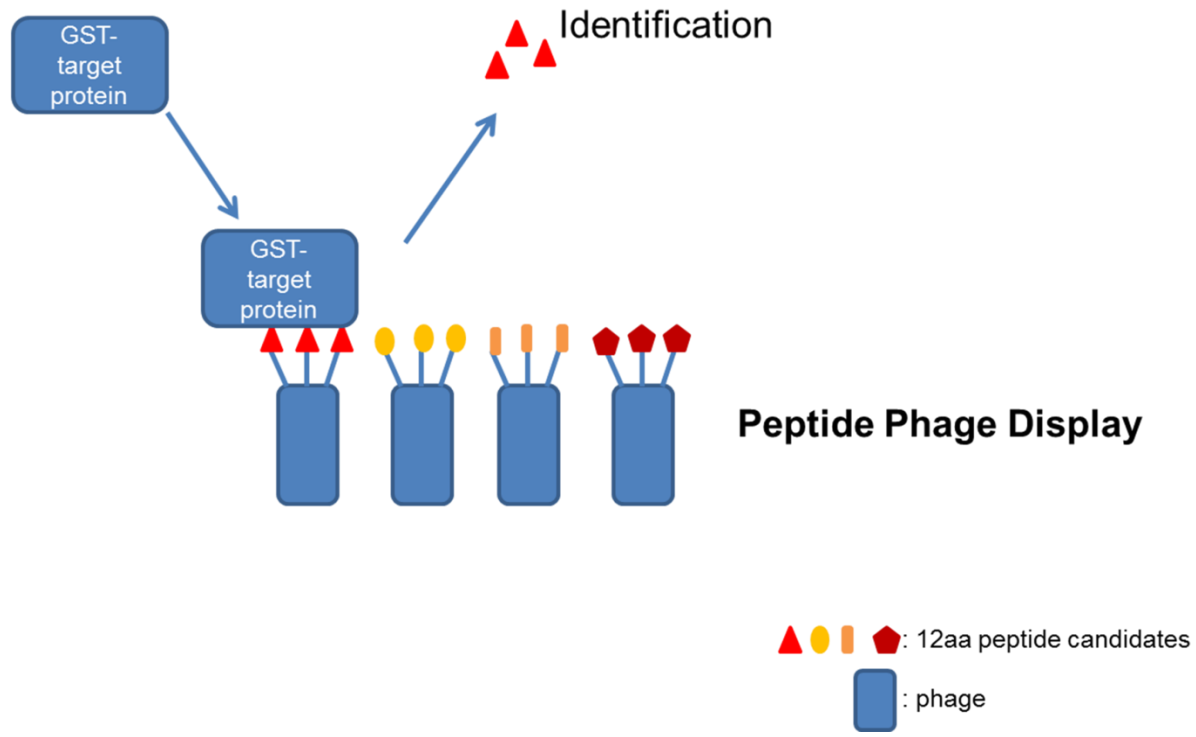

GST-
target
protein
Identification
GST-
target
protein
Peptide Phage Display
: 12aa peptide candidates
: phage

Figure 15. Schematic of the binding peptide discovery platform using phage display

A large peptide library with several millions of 12-amino-acid peptide candidates is expressed on the surface of the phages. Purified GST-fused target protein is overlaid onto the phages and the interacting peptide with the highest binding affinity will be identified from the library. Next, it will be used for constructing a robust targeting peptide for degrading the target protein. *I selected the target proteins and constructed the plasmids for GST-fused proteins. The peptide phage display experiment is being conducted at Dr. Horacio Bach's lab at UBC.*

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