MGluR5 modulation effect on protein degradation in Parkinson's disease

by

Viktoria Xing

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of

Master of Science

in

Neuroscience

Carleton University
Ottawa, Ontario

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease that is characterized by motor symptoms and dysregulation of the dopaminergic system. One of the main hallmarks of PD is abnormal alpha-synuclein (α-syn) aggregation that is the main component of Lewy bodies, the formation of which leads to oxidative stress, excitotoxicity and eventual cell death. In out experiment α-syn fibrils and A53T adenovirus will be added to SH-SY5Y cells to mimic PD conditions of protein aggregation and overexpression. In this thesis investigates the possibility of clearing α-syn fibrils from SH-SY5Y cells using an allosteric mGluR5 inhibitor, CTEP. We found that CTEP contributed to significant elimination of α-syn fibrils form SH-SY5Y cells. Assessment of AKT/mTOR pathway has shown that CTEP action is at least in part mTOR dependent. Additionally, we found a significant upregulation of autophagy system components, such as Beclin1, Atg5-Atg12 complex, Atg7, Atg101 and Atg3, and significant downregulation of inhibitory pULK1 in response to CTEP administration. We conclude that CTEP is a useful pharmacological agent that could be used against the abnormal α-syn aggregation.

Acknowledgements

I would like to express my gratitude to all members of Hayley and Biggar labs. I wish to include a special thank you to Teresa Fortin and Zach Dwyer whose advice and support allowed for such a prompt completion of this thesis. Also my deepest gratitude goes to my committee members for their valuable feedback and advice. Finally, I would like to express a special thank you to my supervisors Dr. Shawn Hayley and Dr. Kyle Biggar for their guidance, patience and overall positive attitude.

Table of Contents

Abbreviations

6-OHDA	6-hydroxydopamine; monoamine neurotoxin
AKT	Protein Kinase K, serine/threonine-specific protein kinase
ANOVA	Analysis of Variance
A-syn	Alpha synuclein
Atg	Autophagy
AV	Adeno virus
CK1/2	Casein kinases
CTEP	(2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine)
DA	Dopamine
DBS	Deep brain stimulation
GFP	Green fluorescence protein
HSP	Heat shock protein, chaperone
IF	Immunofluorescence
LC3 I/II	Microtubule-associated proteins 1A/1B light chain 3A
L-Dopa	Levodopa
LRRK2	Leucine-rich repeat kinase 2
mGluR1/5	Metabotropic Glutamate Receptors 1/5
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR	Mammalian target of rapamycin
p70s6K	Ribosomal protein S6 kinase beta-1
PD	Parkinson's disease
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLK2	Polo-like kinase 2
PP2A	Protein phosphatase 2A
RA	Retinoic acid
ROS	Reactive Oxygen Species
SNpc	Substantia nigra pars compacta
ULK1	Unc-51 like autophagy activating kinase;
VTA	Ventral tegmental area
WB	Western blot

List of Tables

List of Illustrations

Introduction

1.1 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease with a mean age of onset of 60 years old, affecting about five million people worldwide (Dorsey et al., 2007). The three primary motor symptoms that comprise PD include unilateral tremors, gait disturbance and bradykinesia (Li et al., 2008). Dementia is not very prevalent among PD patients, but cognitive impairment often becomes more apparent as the disease progresses (Hoogland et al., 2017). Additionally, non-motor symptoms include depression, visual hallucinations, sleep disturbances and olfactory changes are often comorbid with PD (Zarow et al., 2003; Cheng et al., 1991; Thannickal et al., 2007; Kertelge et al., 2010). Hence, PD is a complex disease that likely involves pathology in multiple systems.

The motor dysfunctions that characterize PD are believed to stem from on the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and the corresponding loss of their downstream terminals in the striatum. Dopaminergic neurons appear to be particularly vulnerable to mitochondrial damage and autophagy dysfunction (Chen et al, 2019; Sato et al., 2018). While mitochondrial dysregulation is associated with oxidative stress and diminished energetic supply to the cell, the inhibition of autophagy observed affects the ability of the cell to manage misfolded proteins that are characteristic of PD pathology. Indeed, histopathological proteinaceous inclusions referred to as Lewy bodies are comprised of abnormal often mutated α-synuclein (α-syn) protein also characterize PD patient brains (Sato et al., 2018). In addition to autophagy,

chaperone and proteasome systems may also be affected in PD (Petrucelli et al., 2002; Greene et al., 2003). Components such as cholecystokinin, met-enkephalin and substance P that activate the dopaminergic system also show a substantial loss in the SNpc and ventral tegmental area (VTA) in PD brains (Studier and Javoy-Agid, 1985; Llorens-Cortes et al., 1984; Tenovuo et at, 1984).

The dopaminergic system is not the only system that shows abnormality in PD. Mood disturbances in PD are associated with dysfunction in dorsal raphe nucleus and locus coeruleus, where serotonergic and noradrenergic systems are dysregulated (Zarow et al., 2003). In fact, about 50% of PD patients report visual hallucinations that may be caused by abnormal serotonergic activity. Likewise, there have been reports of substantial loss of locus coeruleus neuros in PD that could contribute to disturbances of mood. Cheng et al. also reported an increase of serotonin receptors in temporal cortex, the area selectively affected by protein inclusions of PD (Cheng et al., 1991). Another PD symptom is sleep dysregulation, in particular, excessive sleepiness during the day. The condition may be attributed to the brain stem and hypothalamic damage that leads to loss of hypocretin, a hormone that regulates sleep-wake patterns (Thannickal et al., 2007). Interestingly, some of the early signs of PD onset include impaired sense of smell and color discrimination. These impairments are associated with idiopathic PD and have a strong genetic component (Kertelge et al., 2010).

It is suggested that pathological changes in PD begin in the dorsal motor nucleus of the glossopharyngeal and vagus nerves, progress into lower brainstem nuclei and eventually move upwards into the cerebral cortex (Braak et al., 2003). According to this back-forward progression of pathology, the particular symptoms emerge in a time-

dependent manner and are associated with specific damage to progressively more brain regions. Resting tremors in PD have been associated with increased metabolism in the thalamus, pons and premotor cortex, which indicates dysregulation in thalamic inhibition and resulting failure to stop movements. Rigidity, on the other hand, is caused by substantia nigra dopaminergic cell loss that results in cortical inhibition through over-activation of striatal GABAergic neurons which in turn inhibit thalamus. The reduced dopaminergic signal from the substantia nigra acts through the indirect loop and results in failure to initiate movement (Benrheimer et al., 1973; Braak et al., 1996). Rigidity and akinesia are also associated with dysregulation of the noradrenergic system and a more substantial neuronal loss in locus coeruleus. Interestingly, locus coeruleus damage also correlates with dementia onset in PD (Chan-Palay and Asan, 1989). Posture and gait abnormalities may be attributed to cholinergic signaling disruption in the pedunculopontine tegmental nucleus. The projections from pedunculopontine tegmental nucleus reach to the brain regions participating in the motor loop such as the thalamus, SNpc, globus pallidus and subthalamic nucleus (Coles et al., 1989; Hallanger et al., 1987).

The main mechanisms of pathogenesis are thought to be (1) oxidative stress, (2) excitotoxicity, (3) mitochondrial damage, (4) inflammation and (5) abnormal protein aggregation (Dawson and Dawson, 2003). It has been suggested that dopamine metabolism could be the source of oxidative stress, but this does not explain the death of non-dopaminergic neurons in PD and persistent survival of some of the nigrostriatal neurons. Additionally, dopamine metabolism produces hydrogen peroxide as a by-product, not the more reactive free radicals such as superoxide (Schapira et al., 2008).

Oxidative stress goes hand in hand with mitochondrial damage because under normal conditions mitochondrial electron carriers and acceptors produce reactive oxygen species (ROS), but this process is balanced by endogenous antioxidants. However, as the mitochondrial function becomes dysregulated, ROS are produced at higher rates which can result in increased damage to the cell (Andreyev et al., 2005). Mitochondrial dysfunction appears in both sporadic and idiopathic PD. In the case of sporadic PD, environmental toxins such as paraquat and rotenone play a role as mitochondrial complex I inhibitors (Manning-Bog et al., 2002; Sherer et al., 2003).

Another hallmark of PD pathology, as already briefly mentioned, is abnormal protein aggregation. A-syn is the main culprit when it comes to Lewy body formation, spherical filamentous inclusions that are found in the brainstem and cortex of PD patients (Braak et al., 2003). Abnormal protein formations are present in both sporadic and idiopathic PD and genetic mutation of α-syn is thought to inhibit mitochondrial function (Hu and Wang, 2016). The inhibitory effect of α-syn on mitochondrial function is protein concentration dependent, meaning that high levels of a-syn overexpression are cytotoxic (Liu et al., 2009). Additionally, α-syn appears to interact with mitochondrial inhibitory toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as supported by data showing that when α-syn was deleted from experimental animals they showed resistance to MPTP damage (Fornai et al., 2005).

One substantial contributor to neurodegeneration in PD is excitotoxicity that is marked by high levels of glutamate in extracellular space and subsequent calcium overload (Ambrosi et al., 2014). It is possible that exaggerated glutamate levels are a natural cellular response to mitochondrial damage, oxidative stress, as well as protein

aggregation. Unfortunately, glutamatergic response creates more stress to already damaged cell which eventually leads to neural degeneration (Donget al., 2009). Some positive effects in managing PD pathology were achieved by modulation of NMDA and AMPA receptors in animal models (Steece-Collier et al., 2000; Löschmann et al., 1991). However, NMDA and AMPA inhibition has a very modest and sometimes short-lived benefit in treating motor symptoms in human PD patients (Johnson et al., 2009).

PD inflammatory state is marked by the increase of circulating proinflammatory cytokines such as TNF-α, which may be found in the SNpc, as well as in cerebrospinal fluid (Hunot and Hirsch, 2003). Microglial activation in SNpc is another feature commonly observed in PD postmortem brains (Ferreira and Ramero-Ramos, 2018). However, it is still unclear if inflammation is the cause of pathogenesis or the secondary result of subsequent neuronal death (Barcia et al., 2004).

PD has a substantial environmental component which makes the cause of pathology largely difficult to determine. Several decades of research established some links between environmental chemicals and PD (Obeso et al., 2017). Consumption of well-water that is contaminated with heavy metals and pesticides, as well as exposure to industrial solvents is now considered to be risk factors for the development of PD (Gatto et al., 2009; Gorell et al., 1998; Tanner et al., 2011; Lock et al., 2013).

Genetic abnormalities account for a smaller proportion of cases (5%-10%). The most common causal mutation is glycine replacement with serine at 2019 position (G2019S) of PARK8 gene that codes for leucine-rich repeat kinase 2 (LRRK2). It accounts for about 30% of genetically linked cases and is autosomal dominant, which means that some of the PD cases are heritable (Thaler et al., 2009).

A point mutation in PARK1/4 is another culprit in PD pathology, with duplication and triplication being known to play a role in the age of PD onset. The duplicated form can lead to the onset of PD in the patient's 50s, and triplication can cause an early onset of PD at around 30 years of age (Fuchs et al., 2007). Histologically, PARK mutation leads to overexpression of the α-syn protein, which was demonstrated to be toxic in high concentrations (Spillantini et al., 1997). Additionally a missense mutation in the position 53 with Ala to Thr (A53T) substitution was identified in abnormal α-syn. Mutated α-syn induces cytotoxicity through Lewy body formation, microtubular fibrillization and abnormal protein aggregation that leads to widespread neurodegeneration (Daher et al., 2012).

It is clear that PD is a complex neurodegenerative condition with many contributing factors that could either be present genetically or accumulate throughout the life span of an individual. PD is difficult to study and even more complicated to manage in its pathological state. <u>In this thesis we are going to focus on α-syn aggregation and potential pharmacological possibilities to manage abnormal protein aggregation</u>.

1.2 Alpha-synuclein

A-syn is a 14 kDa presynaptic protein that contains three domains: an α-helical N-terminal domain, a central hydrophobic non-A β component (NAC) domain, and a C-terminal domain that is proline rich and highly acidic (Hoyer et al., 2004). The NAC domain is important for α-syn aggregation, as even minor deletions within this region reduced amyloidogenic ability of α-syn (Bartels et al., 2010).

The function of α-syn is largely unknown. It has been proposed to act as a soluble N-ethylmaleimide-sensitive factor attachment protein receptors complex (SNARE-complex) chaperone that maintains the confirmation of vesicle tethering proteins that participate in the process of vesicular docking to the presynaptic membrane (Burre et al., 2010; Baker and Hughson, 2016). Under physiological conditions, α-syn presents in a monomeric soluble form and binds to negatively charged phospholipids. Unfortunately, the monomeric form of the protein is not the most stable and is prone to abnormal aggregation (Burre et al., 2013). Bartels et al. observed α-syn protein purified from erythrocytes in α-helical tetrameric was a more stable form. Such tetrameric forms are thought to be resistant to aggregation under physiological conditions. However, it is not clear which of the α-syn confirmations contribute to α-syn aggregation during protein overexpression and cytosolic overload (Bartels et al., 2011).

Misfolded α-syn can affect cell function in several ways. Firstly, the abnormal conformation of the protein triggers its release into the extracellular space where α-syn can influence synaptic activity. Adamczyk et al. have found that extracellular alpha-synuclein inhibits the dopamine transporter through the action of nitric oxide affecting presynaptic terminal activity (Adamczyk et al., 2006). Secondly, under pathological conditions, α-syn has the ability to enhance Ca^{2+} influx to the intracellular space (Adamczyk and Strosznajder, 2006). Both PD and Alzheimer's disease (AD) are marked by an excess of intracellular Ca2+ that leads to excitotoxicity. It has been shown that increased influx of calcium triggers phosphorylation of essential cellular proteins, which in turn leads to apoptosis (Pierrot et al., 2004).

A-syn appears to have prion qualities, which means that even a small amount of misfolded protein can turn otherwise normal α-syn into a pathological one. Indeed, Li et al. have shown the possibility of host-to-graft transfer of misfolded α-syn, wherein mesencephalic grafts tested positive for the presence of Lewy bodies after contact with brain tissue taken from PD patients (Li et al., 2008). Misfolded α-syn has also shown seeding activity that ensures cell to cell spread of the aggregated protein form. It is hypothesized that seeding is the way misfolded proteins spread to anatomically adjacent brain regions (Guo and Lee, 2014). It appears that polymerization of α-syn is concentration dependent, which means that overexpression of the protein inevitably leads to aggregation (Giasson et al., 1999).

Several sources report that phosphorylation of α-syn contributes to its pathological aggregated confirmation (Saito et al., 2003; Fujiwara et al., 2002; Anderson et al., 2006). However, the issue is controversial as there are studies that found either no effect of α-syn phosphorylation on protein aggregation, or have actually seen α-syn phosphorylation contributing to inhibition of Lewy bodies' formation (Paleologou et al., 2008; McFarland et al., 2009). It is possible that the studies that did not find an effect of a-syn phosphorylation may be due to the methodological issues. These studies used point mutations to assess the role of phosphorylation site, but this does not account for the complexity of interaction between the studied phosphorylation site and other sites of modification. Additionally, different species may react differently to α-syn phosphorylation (Chen and Feany, 2005; McFarland et al., 2009).

The evidence for kinase participation in PD pathology has been steadily building over the years. Gain of function mutation of LRRK2 is a common genetic factor of PD.

This mutation promotes increased activity of LRRK2 and as a result hyperphosphorylation of α-syn (Qing et al., 2009). Polo-like kinase 2 (PLK2) is an α-syn specific kinase that has the ability to phosphorylate both the monomeric and fibrillary form of α-syn, thus contributing to α-syn confirmation at different stages of Lewy bodies formation (Mbefo et al., 2010; Waxman and Giasson, 2011). Casein kinases (CK1 and CK2) are also known for α-syn phosphorylation and both were shown to co-localize with Lewy bodies (Ryu et al., 2008; Waxman and Giasson, 2008). Interestingly, the levels of protein phosphatase 2A (PP2A) were reduced in PD, which is important since the PP2A B-subunit is particularly important in α-syn dephosphorylation. Indeed, PP2A increased activity has been linked to the alleviation α-syn aggregation in a PD mouse model (Lee et al., 2011).

1.3 Protein degradation in PD

Considering that abnormal α-syn aggregation may be one of the main contributors to DA neuron death in PD, it makes sense to look into ways of clearing the pathological aggregates. Since preventing α-syn aggregation may not be practical, as the protein is naturally prone to aggregate. Autophagy-lysosomal pathway may be a useful target that can aid in α-syn clearing.

Autophagy system role in PD

Eukaryotic cell have two main house keeping mechanisms, the ubiquitin-proteasome and the autophagy-lysosomal systems. The autophagy-lysosomal pathway is responsible for degradation of intracellular proteins and organelles, and it is a promising

target for therapeutics that aims at preventing excessive protein aggregation. Three main subtypes of autophagy-lysosomal pathways are macroautophagy, chaperone-mediated-autophagy, and microautophagy (Limanaqi et al., 2019). All tree subtypes lead to lysosomal degradation of autophagy targets by proteases, lipases and glycosidases in the presence of acidic pH inside the lysosome (Klionsky, 2007). During autophagy initiation ULK1, Atg101 and Atg13 are mobilized, followed by an Atg5 – Atg12 complex that associates with Atg16 forming an oligomerizing complex that determines LC3 lipidation sites. After phagophore expansion autophagosome (autophagic vacuole) is formed that then fuses with lysosome to breakdown autophagosome cargo (Oshumi, 2001).

Autophagy can be induced through cell stress, such as nutritional starvation or oxidative overload (Kuma et al., 2007; Filomeni et al., 2015). It has been shown that autophagy initiation takes place through mTOR pathway. Specifically, under physiological conditions, the mTORC1 (mTOR complex 1) inhibits autophagy by phosphorylation of Atg13 and ULK1, and a subsequent inhibition of autophagy initiating protein Beclin1 (Kamada et al., 2010). However, when mTORC1 inhibitor, rapamycin, is applied to the pathway autophagy activity increases (Pan et al., 2008).

Pathological assessment of PD patients' brain tissue revealed that autophagy vacuoles were either overproduced or not efficiently cleared in SNpc, an area where α-syn aggregation is considered to be responsible for dopaminergic neuron loss (Anglade et al., 1997). Independently, mTOR has been implicated in PD (Lan et al., 2017), and it is not clear if autophagy upregulation in PD patients SNpc is attributed to mTOR pathway dysregulation. However, mTOR upregulation and Atg7 depletion was observed in PD patients, along with increased level of LC3-II, an autophagosome marker (Crews et al.,

2010). In contrast, LAMP1, a lysosomal marker, appears to be downregulated in PD. This suggests that autophagy system may be responding to α-syn aggregation, but that the clearing of misfolded protein is not efficient (Dehay et al., 2010). In particular, α-syn mutants, A53T and A30P, tend to have a strong affinity for chaperon associated lysosomal adapter LAMP2A. However, mutated α-syn fails to internalize into the lysosome, impairing protein degradation process (Cuervo et al., 2004; Xilouri et al., 2009). The pathological fibrillar, form of α-syn shows the same pattern of high affinity for lysosomal adapter and similar failure to internalize, creating high volumes of cytosolic α-syn that is prone to further aggregation (Martinez-Vincente et al., 2008). Both cell culture and an α-syn transgenic mouse model have shown increased levels of α-syn associated with macroautophagy inhibition at the stage of autophagosome formation (Winslow et al., 2010). Interestingly, deletion of Atg7 and Atg5, both selectively and globally, caused presynaptic accumulation of α-syn and LRRK2. Moreover, increased dopaminergic cell death was observed in the case of selective depletion of Atg7 and Atg5 in the midbrain (Freidman et al., 2012).

When it comes to α-syn degradation, there seems to be some discrepancy between the wild type and mutated protein. Autophagy-lysosome system is mainly responsible for the wild type α-syn degradation (Cuervo et al., 2004; Martinez-Vicente et al., 2008). Whereas ubiquitin-proteosome system clears A53T and A30P α-syn forms (Yoshimoto et al., 2005). Interestingly, it has been demonstrated that chaperon-macroautophagy system is incapable of degrading mutant α-syn forms (Cuervo et al., 2004).

Overexpression or activation of autophagy demonstrated positive effects on toxin PD models. For example, Atg5 and Beclin-1 over-expression reduced nigrostriatal

damage in MPTP treated zebra fish (*Danio rerio*) (Hu et al., 2017; Song et al., 2014). Likewise, autophagy activation with mTOR inhibition by rapamycin was protective against rotenone and 6-OHDA induced motor dysfunction (Pan et al., 2009; Jiang et al., 2013). At the same time, the damage from rotenone and 6-OHDA treatments were much greater when the autophagy system was inhibited (He et al., 2018; Pal et al., 2016).

Despite the evolutionary conservation of autophagy and chaperon systems, their action appears to be different in different species which has to be kept in mind when trying to translate positive results of a certain therapy from the animal model to human patients. For example, mutated α-syn in mice is cleared by chaperon-macroautophagy system, which contradicts findings from rat and human studies (Mak et al., 2010).

Heat shock protein chaperone system role in PD

Heat shock proteins (HSPs) are a group of molecular chaperone proteins that protect the cell from pathological protein aggregation (Hartl et al., 2011). HSP key features are the presence of a conserved α-crystallin domain, as well as their ability to oligomerize to form a stable chaperone structure that allows HSPs to modify target protein conformation (Stamler et al., 2005). HSPs either return target proteins to their native confirmation or mark irreversibly denatured proteins for degradation. Cytosolic proteins that contain a pentapeptide targeting motif (KFERQ) are recognized by molecular chaperones (HSP70 and co-chaperones), bind to LAMP2A and are then translocated to the lysosomal surface (Arias and Cuervo, 2011). Heat shock transcription factor (HSF1) overexpression through mTOR inhibition with rapamycin leads to autophagy induction (Kumsta et al., 2017).

Chaperone and autophagy systems tend to not only work together, but also compensate each other's action. Chaperone mediated protein degradation tends to be upregulated when macroautophagy system is compromised (Kaushik et al., 2008). In contrast, when HSF1 is deleted from the cell autophagy response is upregulated (Dokladny et al., 2013).

Failure of the chaperone system leads to aggregation of insoluble protein inclusions such as amyloid plaques in Alzheimer's disease or Lewy bodies in PD (Irvine et al., 2008). HSP70 is particularly important in managing α-syn insoluble aggregates, as the chaperon was confirmed to bind α-syn fibrils (Gao et al., 2015).

1.4 PD Therapy

Current strategies

Presently used PD therapies do not stop pathological molecular pathways or prevent neurodegeneration. Current PD treatment could be described as a band-aid approach that attempts to manage symptoms and maintain the quality of life for as long as possible.

Levodopa (L-Dopa) is the most commonly used medication to manage PD. It is a precursor that is metabolized into dopamine. Levodopa has been largely successful in supplementing plummeting levels of dopamine and alleviating motor and some of the cognitive symptoms (Rascol et al., 2003). Unfortunately, the beneficial effect of levodopa wears off with time. After about two to three years of L-Dopa administration, patients start to develop motor regressions, such as increased tremors as well as more severe bradykinesia (Fahn et al., 2004). Additionally, patients may experience on-off periods of

opposite motor symptoms. “On” periods are characterized by dyskinesia, the onset of uncontrollable movements that are linked with high plasma concentration of L-Dopa. The “Off” period comes with akinesia or a failure to initiate movement and is linked with low plasma concentration of L-Dopa (Marsden and Parkes, 1976). There have been trials to reduce levodopa dosage in an attempt to alleviate complications. However, lower doses have reduced efficacy in alleviating motor symptoms (Rascol et al., 2003).

Deep brain stimulation (DBS) of subthalamic nucleus was reported to be beneficial in advanced cases of PD. DBS not only reduced tremors but also allowed patients to use smaller doses of levodopa, which reduced unwanted side effects (Limousin et al., 1998; deHemptinne et al., 2015). The mechanism of DBS action is not entirely understood. Despite high efficacy of the procedure, DBS has its own limitations. Firstly, DBS is a very invasive procedure that involves cranial surgery as well as implantation of an electrode, which may lead to infection and edema. Secondly, it takes several weeks to adjust electrical stimulation parameters for DBS to take effect. Finally, the effect of DBS, like L-DOPA, eventually wears off, as it does nothing to slow neuronal degeneration. Therefore a new, preferably pharmacological, approach is needed to manage PD (Lyons et al., 2004).

CTEP as a novel PD therapy

CTEP (2-chloro-4-[2-[2,5-dimethyl-1-[4-(trifluoromethoxy)phenyl]imidazole 4yl]ethynyl] pyridine) is a negative allosteric metabotropic glutamate receptor 5 (MGluR5) modulator. CTEP possesses high affinity for mGluR5 over other types of mGlu receptors, which creates highly desirable specificity. mGluR5 is a member of the

Gα protein-coupled receptor family and is highly expressed in the brain, striatum and cortex in particular (Shigemoto et al., 1993). Increased glutamatergic activity in PD that leads to excitotoxicity makes mGluR5 an interesting target that should be explored. mGluR5 modulation is particularly useful because inhibition of other members of glutamatergic system, NMDA and AMPA, usually leads to many harmful side-effects (Johnson et al., 2009).

CTEP was reported to cross the blood-brain barrier successfully and have a substantial half-life of 18 hours, which makes CTEP a robust pharmacological agent that could be taken orally with fewer side-effects than the current therapeutic strategies for PD (Lindeman et al., 2011).

Two animal models of Fragile X syndrome (FXS) and HD have shown positive outcomes after administration of CTEP. Chronic CTEP treatment of FMR1 KO mice reduced motor impairments as well as improved memory and cognition (Michalos et al., 2012). CTEP administration in z75Q mice reduced inhibitory S757 ULK1 phosphorylation, which promoted autophagy initiation and reduced pathological aggregates of huntingtin. Similarly to FXS mice, HD model showed decrease levels of mTOR activity due to CTEP inhibition of mGluR5.

Considering these findings, it may be useful to apply CTEP to PD model as there are obvious pathological correlations of protein aggregation, mTOR hyperactivity and autophagy inhibition.

Potential pathway of CTEP action

AKT is a Thr and Ser kinase that has many targets downstream including mTORC1 that are important for autophagy. AKT activity is initiated mainly through

growth factors in an PI3K-dependent matter. Among other phosphorylation sites, AKT activity can be enhanced by phosphorylation at S473 by mTORC2 (Liu et al., 2013). In neurons, activation of AKT and mTORC1 signaling is important for synaptic plasticity, affecting both long-term potentiation and long-term depression through its induction of localized protein synthesis.

AKT/mTORC1 activity was shown to be enhanced by activation of mGluR1/5. Indeed, increased levels of glutamate and mGluR5 activation resulted in not only hyperphosphorylation of mTOR, but also in more p70S6K1 phosphorylation, which in turn stimulates more mTOR phosphorylation through positive feedback loop (Hou and Klan, 2004; Switon et al., 2016). Exaggerated activity of AKT/mTOR is common in neurodegenerative conditions and PD in particular. As PD is marked by excitotoxicity, it is no surprise that high levels of glutamate trigger mGluR1/5, thus enhancing AKT/mTOR activity even further (An et al., 2003; Ambrosi et al., 2014). Increased mTOR activity can then phosphorylate ULK1 at ser754, inhibiting its ability to initiate autophagy, a crucial system in managing PD α-syn aggregation.

Research objectives

My hypothesis is that CTEP and rapamycin will promote significant elimination of α-syn fibrils from SH-SY5Y cells. A-syn clearing is likely takes place through autophagy system.

In this thesis we first wish to assess the impact of α-syn-driven accumulation of fibrils in cultured dopamine-like SH-SY5Y cells. Using this model, we then aim to determine whether CTEP promotes clearing of α-syn preformed fibrils from the SH-SY5Y cells. Using parallel administration of rapamycin with CTEP, we will also ascertain whether CTEP effects are mTOR dependent. If CTEP does clear the α-syn fibrils from the cells, we will assess the extent of autophagy and HSP chaperone systems involvement.

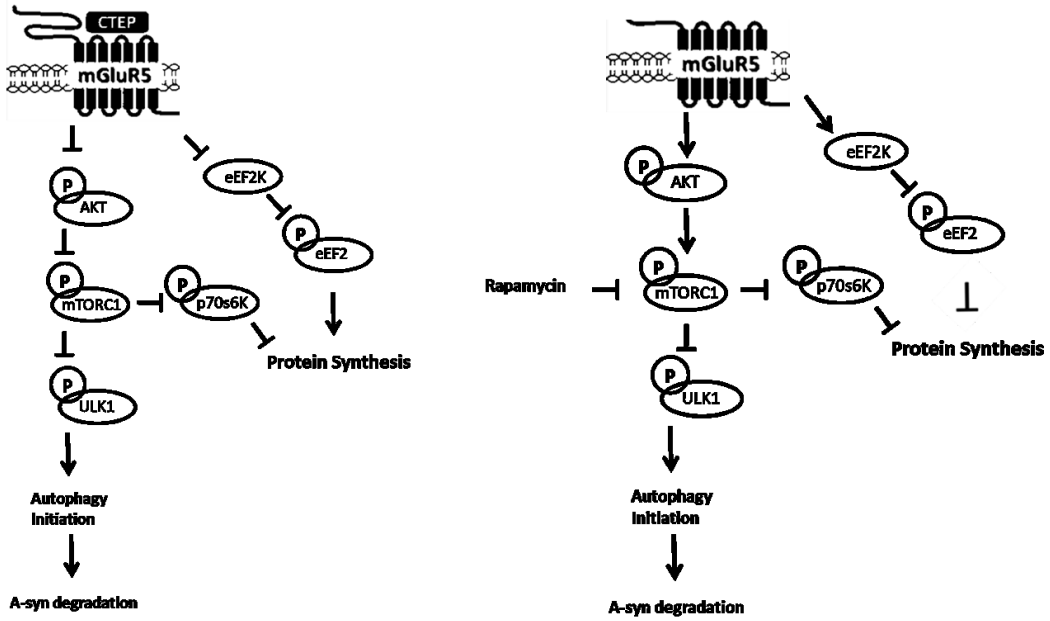

Figure 1 Suggested pathway for CTEP and rapamycin action

Methods

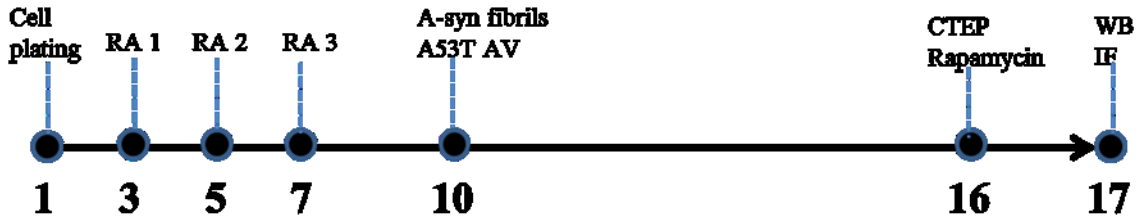

Figure 2 Experimental timeline. On days 3, 5 and 7 after plating the SH-SY5Y cells were treated with retinoic acid. A-syn fibrils and/or A53T AV were added on day 10. Then the incubation of 6 days took place. Treatment with CTEP and rapamycin was applied on day 16. SH-SY5Y cells were harvested after 24 hours.

Model selection

SH-SY5Y, human neuroblastoma, cells were selected for their DA neuron-like qualities and their robust survivability and steady growth. Cell culture was maintained by weekly passage. Purely neuronal culture was selected to determine CTEP effect without the participation of glial cells. A53T α-syn adenovirus was used to mimic genetic component of PD that is responsible for α-syn overexpression. Preformed α-syn fibrils were prepared from α-syn monomer (Proteos #R003) and added to experimental culture to mimic pathological α-syn aggregation.

Firstly, the concentration of α-syn fibrils was determined through visual assessment of immunofluorescence (IF) stained SH-SY5Y cells treated with α-syn fibrils at 1:100, 1:200, 1:400, 1:800, 1:1000, 1:2000, 1:5000, 1:10000 concentrations from stock solution of 5mg/mL. Secondly, the effective concentration of CTEP was determined through IF assessment of SH-SY5Y cells treated with α-syn fibrils at 1:100 concentration. CTEP was applied to cells at 0.1μM, 1μM, 10μM and 100μM

concentrations. Both α-syn fibrils and CTEP dose response assessments were conducted with the same timeline as the upcoming experiment (Fig. 2). Rapamycin concentration of 5μM was selected from literature (Lin et al., 2018). Additionally, prior to the experiment we have validated that SH-SY5Y cells do indeed express mGluR5 (Fig. 16 App.3).

Cell Culture

SH-SY5Y cells were plated at 10^4 cells/mL to 6-well plates for Western Blot, and at $5x10^3$ cells/mL to 96-well plate for IF. Cell growth media contained 10% of FBS and 1% of Pen-Strep (stock concentration: 10000-12000 units/mL). Cells were differentiated with three treatments of RA (10μM). In 48 hrs after the last RA treatment α-syn fibrils (1:200) and A53T AV (1 MOI) were added to fibril and AV treatment groups respectively. Cells were incubated with α-syn fibrils and AV for 6 days. On the 6th day, CTEP (10μM) and rapamycin (5μM) were added to their respective treatment groups. After 24 hrs incubation with CTEP and rapamycin, cells were either harvested and preserved in -80°C for WB, or fixed with 4% PFA for IF.

Immunofluorescence

SH-SY5Y cells were fixed with 4% PFA for 15 min. After 3 PBS washes cells were blocked in 2% BSA with 0.1% Triton-X diluted in PBS for 30 min. Cells were incubated with primary antibody (see Table 1) in 0.1% BSA solution for 1 hr. After 3 PBS washes, cells were incubated with secondary antibody in 0.1% BSA solution for 30 min. Cell were washed 3 times in PBS and imaged with ThermoFisher EVOS FL Auto 2

at x40 magnification. GFP labeled a-syn fibrils were counted by image-J particle counting option.

Western Blot

Protein from SH-SY5Y cells preserved in -80° was extracted using RIPA-like buffer (0.1% SDS, 1 mM Na ortho-vandate in 10 mM tris) with addition of EDTA-free protease inhibitors (Roche cOmplete). Samples were sonicated in the presence of the extraction buffer 5 cycles, 30 sec each. Protein content was quantified by Pierce's BCA Protein Assay Kit. Samples were diluted with loading buffer and preserved at -20°C. Samples were loaded into acrylamide mini-gels of appropriate concentration and run at 140V. Then gels were transferred onto MeOH activated PVDF membranes at 100V. Membranes were dried overnight and reverted with MeOH. To acquire normalized signal, membranes were stained with FastGreen. Membranes were blocked with 0.5% fish gelatin for 30 min, and then incubated with primary antibody (see Table 1) in 0.05% fish gelatin solution for 2 hours. After 4 TBS-T washes, membranes were incubated with secondary antibody in 0.5% fish gelatin solution for 1 hr. After 4xTBS-T 5 min and 2xTBS 5 min washes membranes were imaged with Licor Odyssey FC. Images were quantified with Image Studio Lite 5.2.

Statistics

One-way or Three-way ANOVAs were conducted as appropriate. Post hoc testing was completed using Tukey's pairwise comparison in SPSS.

Table 1 List of antibodies

Company	Antibody	Clone	Catalog #	Concentration	Use
Abcam	α-syn filament	MJFR-14-6-4-2	ab209538	1:1000	IF
BD Transduction Lab	α-syn	42/α-Synuclein	610786	1:2000	WB
Cell Signaling Tech	AKT	poly	9272	1:1000	WB
	mTOR	7C10	2983	1:1000	WB
	p-AKT (Ser473)	D9E	4060	1:1000	WB
	p-mTOR (Ser2448)	D9C2	5536	1:1000	WB
	p-p70S6 (Thr389)	poly	9205	1:1000	WB
	p-ULK1 (S757)	D1H4	5869	1:1000	WB
	ULK1	D8H5	8054	1:1000	WB
	Beclin-1	D40C5	3495	1:1000	WB
	Atg101	E1Z4W	13492	1:1000	WB
	Atg12	D88H11	4180	1:1000	WB
	Atg13	D4P1K	13273	1:1000	WB
	Atg16	D6D5	8089	1:1000	WB
	Atg3	poly	3415	1:1000	WB
	Atg5	D5F5U	12994	1:1000	WB
	Atg7	D12B11	8558	1:1000	WB
	LAMP1	D2D11	9091	1:1000	WB
	LC3	D3U4C	12741	1:1000	WB
	BiP	C50B12	3177	1:1000	WB
	Calnexin	C5C9	2679	1:1000	WB
	HSF1	poly	4356	1:1000	WB
	HSP40	C64B4	4871	1:1000	WB
	HSP60	D6F1	12165	1:1000	WB
	HSP70	poly	4872	1:1000	WB
	HSP90	C45G5	4877	1:1000	WB
	MGluR5	D6E7B	55920	1:1000	WB

Results

A-syn fibril model assessment

To assess CTEP effect on α-syn accumulation and overexpression, we firstly determined the working concentration of α-syn fibrils to be added to the SH-SY5Y cells, and secondly we determined effective CTEP concentration. A-syn fibrils concentration

was selected from dose response (Fig. 3). SH-SY5Y cells were imaged by Zeiss axio imager M2 and ThermoFisher EVOS FL Auto 2. Concentration of α-syn fibrils of 1:100 was selected for CTEP dose response (Fig. 4), and concentration of 1:200 was selected for experimental work (Fig. 5).

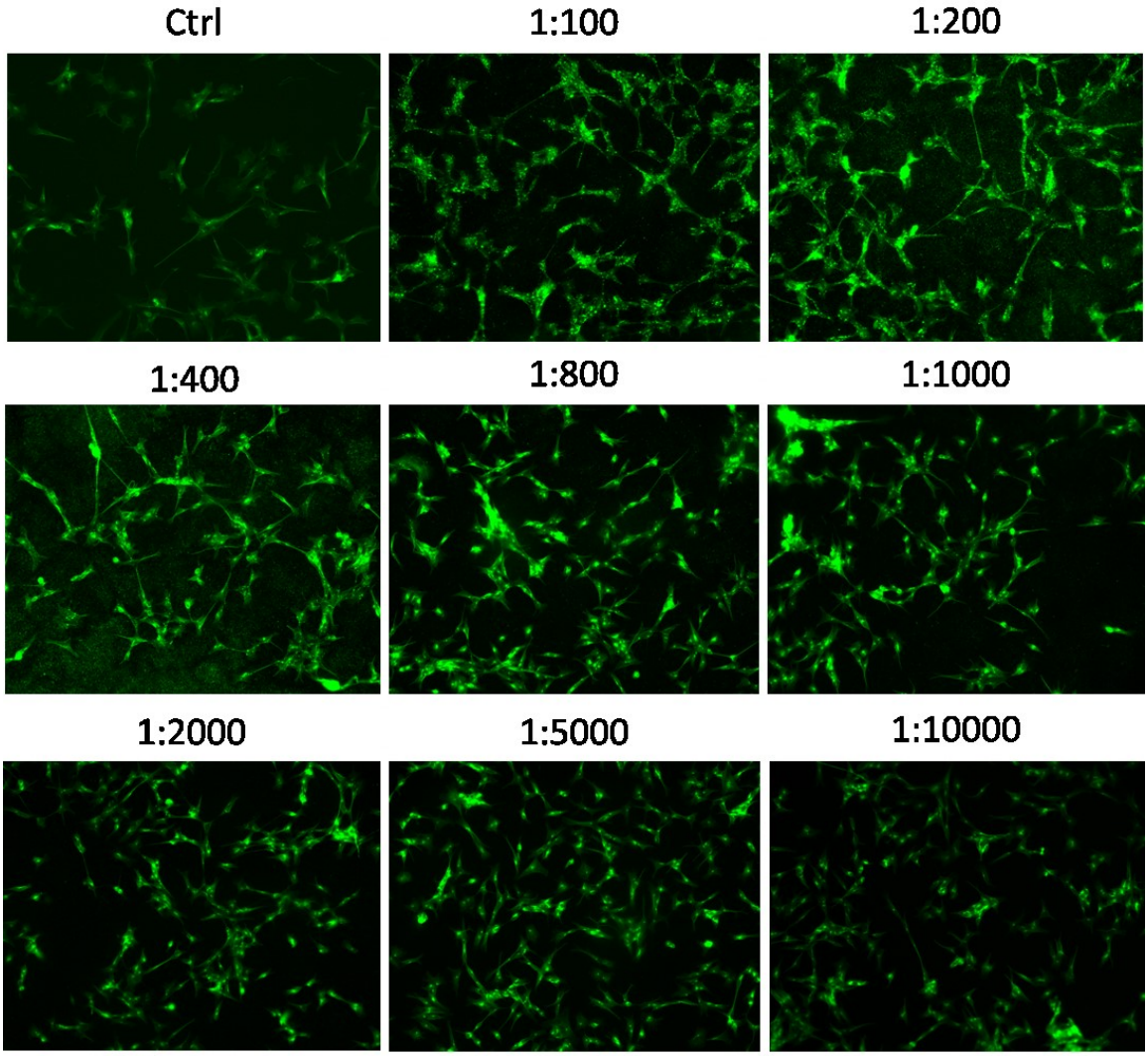

Figure 3 A-syn fibrils dose response in SH-SY5Y cells. Control depicts cells with no α-syn fibrils added, whereas the rest of the panels depict α-syn fibril concentration from 1:100 to 1:10000 (stock solution 5μg/μL). SH-SY5Y cells were incubated with α-syn fibrils for 7 days. Based on visual assessment the concentration of 1:200 was adopted as a working concentration for further experiments.

CTEP concentration was selected from dose response conducted with 0.1µM, 1µM, 10µM and 100µM concentrations (Fig. 4). There was no visual difference in α-syn fibrils elimination from SH-SY5Y cells between 10µM and 100µM. Therefore 10µM CTEP was used as a working concentration in further experimental work.

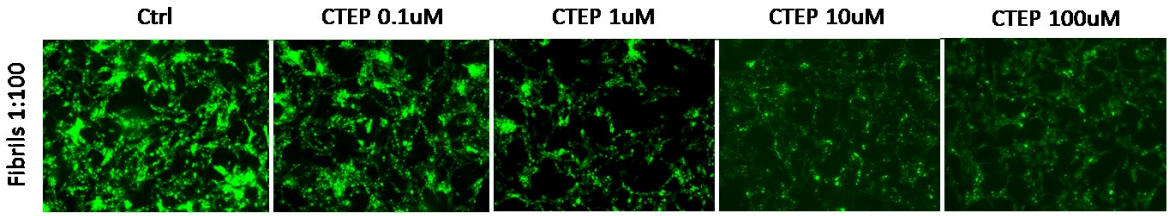

Figure 4 CTEP dose response for α-syn fibril elimination from SH-SY5Y cells. Control depicts SH-SY5Y cells with α-syn fibrils added at 1:100 concentration. SH-SY5Y cells were incubated with α-syn fibrils for 7 days. Then media containing CTEP was added and incubated for 24 hours. Based on visual assessment the concentration of 10µM was adopted as a working concentration for further experiments.

From preliminary data we have seen that CTEP is efficacious in α-syn fibrils elimination from SH-SY5Y cells. Based on that result we set up our experiment to test the reliability of CTEP effect on α-syn fibrils. Significant reduction of α-syn fibrils was repeatedly achieved by the application of CTEP. In addition to CTEP we used rapamycin that showed similar effect with regards to α-syn fibrils elimination.

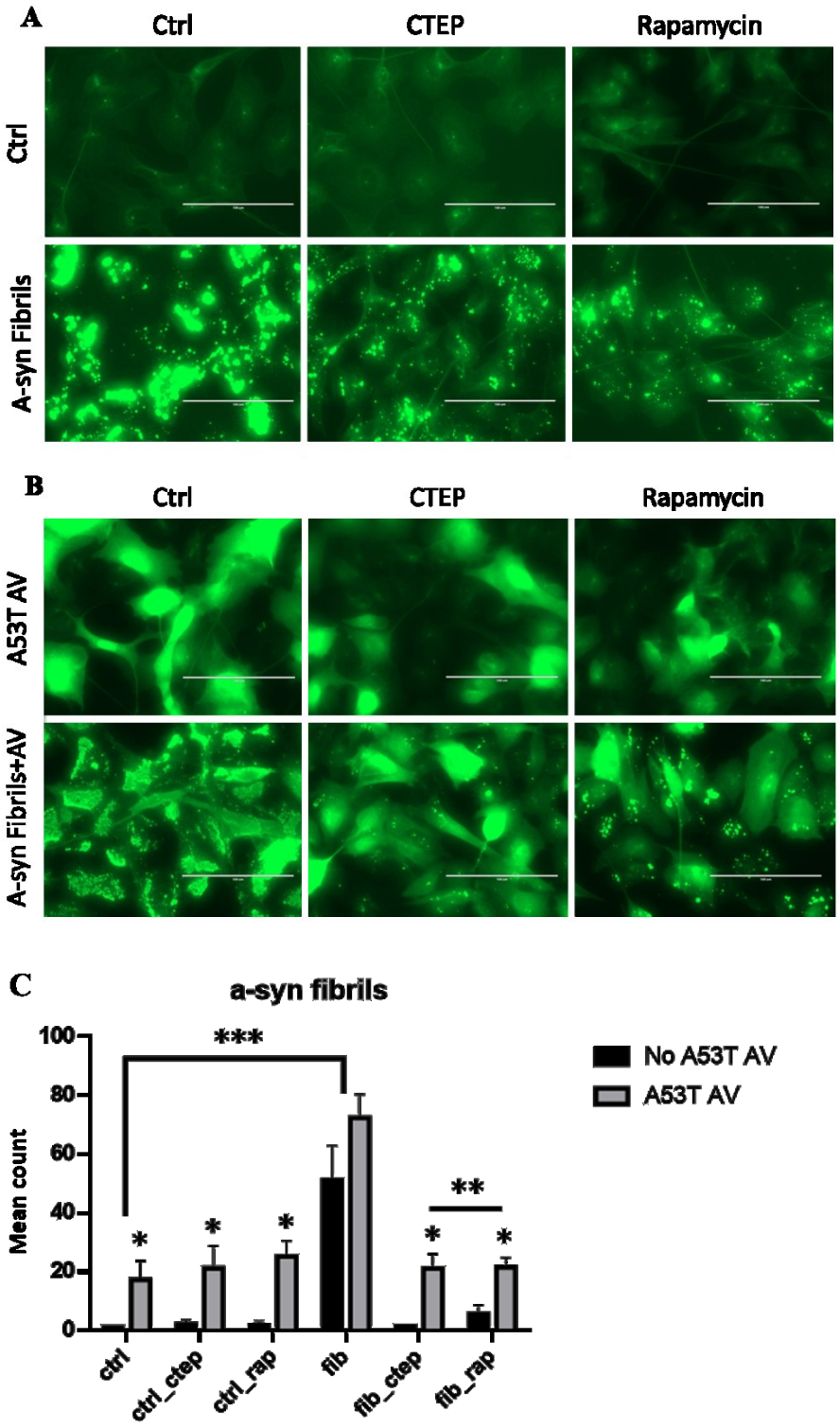

Figure 5 Immunofluorescence of α-syn fibrils and A53T AV + CTEP/ Rapamycin. **(A)** Control depicts SH-SY5Y cells with no α-syn fibrils added. A significant decline of α-syn particles in α-syn treated SH-SY5Y cells was observed in both CTEP and rapamycin treatments. **(B)** Control depicts SH-SY5Y cells with A53T

AV, but no α-syn fibrils added. There was no effect of CTEP or rapamycin on the endogenous α-syn overexpression produced by A53T AV. However, CTEP and rapamycin treatment resulted in significant decline of α-syn particles in α-syn treated SH-SY5Y cells. **(C)** Presented data is the mean result of 3 separate experiments with each condition carried out in triplicates in every experiment. (* $p<0.05$, difference between A53T AV and no A53T AV; ** $p<0.05$, difference with regard to α-syn fibrils; *** $p<0.05$, difference between control and α-syn fibrils.) Particle count was performed with ImageJ. Statistical analysis: SPSS. Tukey's pairwise comparison was used as a post hoc method. Scale bars: 100µm. All treatments and incubations were according to timeline (Figure 2).

In α-syn fibril levels the main effects were found for CTEP (F(1,18)= 124.663; p=0.000), and rapamycin (F(1,18)= 102.893; p=0.000) in non-viral group. Interactions were found between CTEP and α-syn fibrils (F(1,18)= 241.600; p=0.001), as well as between rapamycin and α-syn fibrils (F(1,18)= 212.450; p=0.000) in A53T AV group.

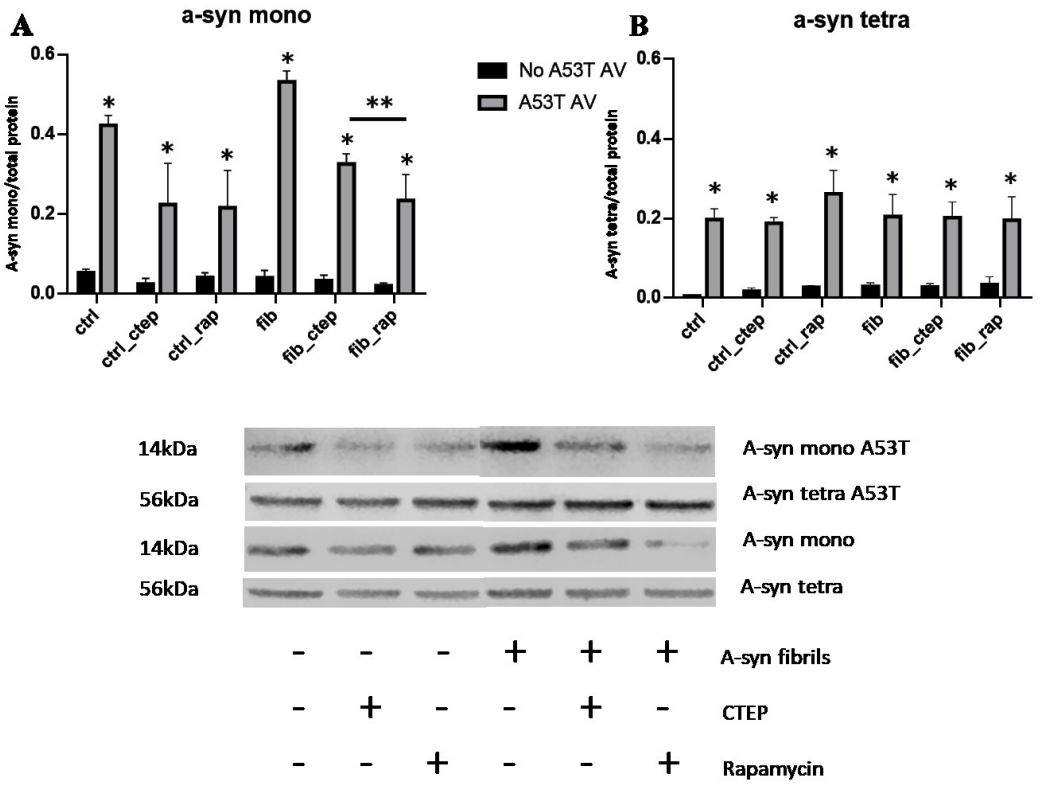

Figure 6 Western Blot A-syn Monomer and Tetramer with A-syn fibrils + A53T AV + CTEP/Rapamycin. **(A, B)** A53T AV treatment significantly increased the concentration of α-syn within the cells in all treatments. Both CTEP and rapamycin decreased the concentration of monomeric α-syn in the treatment group where both A53T AV and α-syn fibrils were combined. Presented data is the mean result of 3 separate experiments. All WB signals were normalized to total protein content. (* p<0.05, difference between A53T AV and no A53T AV; ** p<0.05, difference with regard to α-syn fibrils). Tukey's pairwise comparison was used as a post hoc method. All treatments and incubations were according to timeline (Figure 2).

The main effect was found in α-syn monomers when cells were treated with A53T AV for CTEP ($F(1,18)= 28.674$; $p=0.000$), for rapamycin ($F(1,18)=44.446$; $p=0.000$), and for α-syn fibrils ($F(1,18)=4.729$; $p=0.050$). No interaction was observed in α-syn monomer or tetramer. Significant difference was found between viral and non-viral groups. Tukey's pairwise comparison has shown significant increase of α-syn levels in A53T AV groups for both α-syn monomer and tetramer. Additionally, in comparison to α-syn tetramer, α-syn monomer has increased two-fold in viral control group and three-fold in viral fibril group. A-syn monomer was significantly reduced by the application of both CTEP and rapamycin in viral fibril group.

Effect of CTEP on mTOR/AKT Pathway

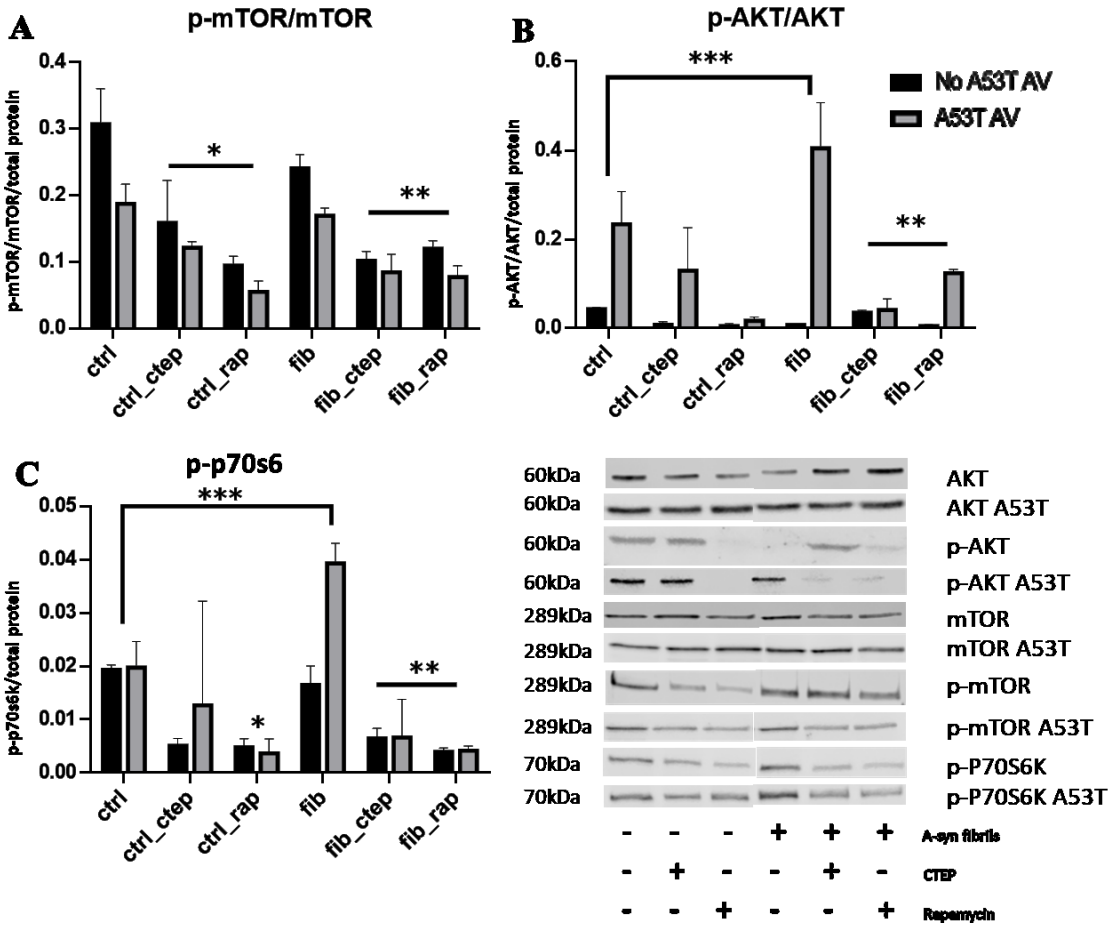

Figure 7 Western Blot mTOR and AKT phosphorylation ratio and p-p70s6K with A-syn fibrils + A53T AV + CTEP /Rapamycin. **(A,B,C)** CTEP and rapamycin appear to bring the protein level down when α-syn fibrils are combined with A53T AV. P-AKT and p-p70s6K levels were significantly increased with regards to control by the combination of α-syn fibrils and A53T AV. (* $p<0.05$, difference with regards to control; ** $p<0.05$, difference with regard to α-syn fibrils; *** $p<0.05$, difference between control and α-syn fibrils). Presented data is the mean result of 3 separate experiments. All WB signals were normalized to total protein content. Tukey's pairwise comparison was used as a post hoc method. All treatments and incubations were according to timeline (Figure 2).

p-mTOR/mTOR levels main effects were found for CTEP ($F(1,18)=46.339$; $p=0.000$) and rapamycin ($F(1,18)=62.399$; $p=0.000$) in non-viral group, and for CTEP

(F(1, 18)=42.969; p=0.000) and rapamycin (F(1,18)=94.852; p=0.000) in A53T AV group.

In p-AKT/AKT levels interactions were found between CTEP and α-syn fibrils (F(1,18)= 107.157; p=0.000); rapamycin and α-syn fibrils (F(1,18)= 33.840; p=0.000) in non-viral group. Additionally, interaction between CTEP and α-syn fibrils (F(1,18)= 11.565; p=0.005) was observed in A53T AV group.

p-p6s70K levels main effect was found for CTEP (F(1,18)= 118.912; p=0.000) and rapamycin (F(1,18)= 147.756, p=0.000) in non-viral group. Interaction was found between CTEP and α-syn fibrils (F(1,18)= 6.124; p=0.029) in A53T AV group.

Effect of CTEP on Autophagy Initiation

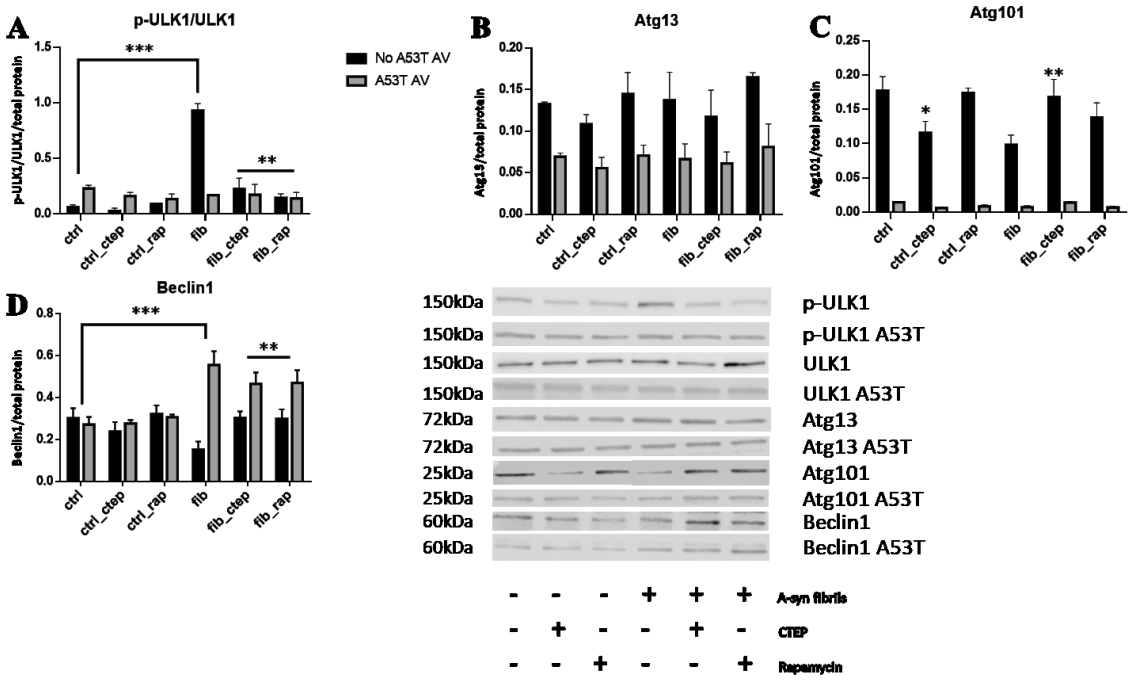

Figure 8 Western Blot Autophagy Initiation Proteins with A-syn fibrils + A53T AV + CTEP/Rapamycin. **(A)** The inhibitory phosphorylation of ULK1 was significantly increased in the α-syn fibril group. Both CTEP and rapamycin brought the p-ULK levels back to control. **(C,D)** Atg101 was downregulated in the A53T condition, however Beclin1 was upregulated when α-syn fibrils and A53T AV were combined. Additionally, atg101 was upregulated by CTEP alone in α-syn fibril condition. (* p<0.05, difference with

regards to control; ** $p<0.05$, difference with regard to α-syn fibrils; *** $p<0.05$, difference between control and α-syn fibrils). Presented data is the mean result of 3 separate experiments. All WB signals were normalized to total protein content. Tukey's pairwise comparison was used as a post hoc method. All treatments and incubations were according to timeline (Figure 2).

In p-ULK1/ULK1 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 127.354$; $p=0.000$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 188.414$; $p=0.000$) in non-viral group. No interactions of main effects were observed in A53T AV group. No interactions or main effects were found for Atg13 levels. In Atg101 levels interaction was observed between CTEP and α-syn fibrils ($F(1,18)= 37.185$; $p=0.000$) in non-viral group. Atg101 levels interactions were found between CTEP and a-syn fibrils ($F(1,18)= 48.635$; $p=0.000$); rapamycin and α-syn fibrils ($F(1,18)= 6.201$;$p=0.028$) in A53T AV group. Beclin1 levels interactions were found between CTEP and a-syn fibrils ($F(1,18)= 19.422$;$p=0.001$); rapamycin and α-syn fibrils ($F(1,18)= 6.579$;$p=0.025$) in non-viral group. Interaction was found between rapamycin and α-syn fibrils ($F(1,18)= 5.049$; $p=0.044$) in A53T AV group.

Effect of CTEP on Autophagy Elongation

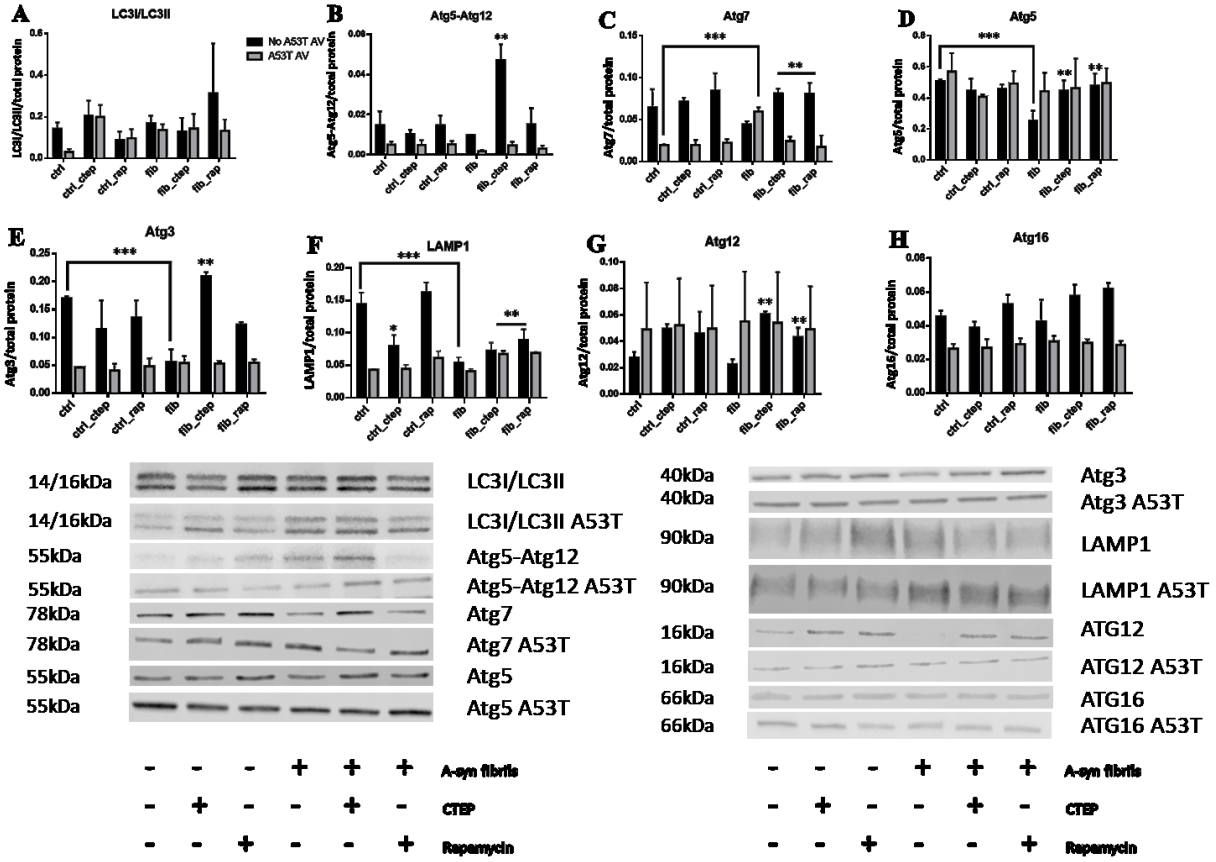

Figure 9 Western Blot Autophagy Elongation/Nucleation Proteins with A-syn fibrils + A53T AV + CTEP/Rapamycin. Atg3, Atg5, Atg7, Atg12 and Atg5-Atg12 complex were all upregulated by CTEP in α-syn fibril condition. LAMP1 levels were brought down by α-syn fibrils and then rescued by CTEP and rapamycin in α-syn fibrils condition. (* $p<0.05$, difference with regards to control; ** $p<0.05$, difference with regard to α-syn fibrils; *** $p<0.05$, difference between control and α-syn fibrils). Presented data is the mean result of 3 separate experiments. All WB signals were normalized to total protein content. Tukey's pairwise comparison was used as a post hoc method. All treatments and incubations were according to timeline (Figure 2).

In LC3I/LC3II levels interaction was found between CTEP and α-syn fibrils ($F(1,18)= 8.353$; $p=0.014$) in A53T AV group. No main effect or interaction was found in non-viral group. In Atg5-Atg12 complex levels interaction was found between CTEP and α-syn fibrils ($F(1,18)= 37.673$; $p=0.000$) in non-viral group. No main effect or interaction was found in A53T AV group. In Atg5 levels interactions were found between CTEP and

α-syn fibrils ($F(1,18)= 13.080$; $p=0.004$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 14.932$; $p=0.002$) in non-viral group. No interactions or main effects were observed in A53T AV group. In Atg12 levels the main effects were found for CTEP ($F(1,18)= 42.304$; $p=0.000$), and rapamycin ($F(1,18)= 18.040$; $p=0.001$) in non-viral group. No interactions of main effects were observed in A53T AV group. In Atg3 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 45.837$; $p=0.000$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 10.790$; $p=0.007$) in non-viral group. No interactions or main effects were observed in A53T AV group. In Atg16 levels interaction was found between CTEP and α-syn fibrils ($F(1,18)= 7.201$; $p=0.000$) in non-viral group. No main effect or interaction was found in A53T AV group. In Atg7 levels the main effects were found for CTEP ($F(1,18)= 7.491$; $p=0.018$), and rapamycin ($F(1,18)= 12.720$; $p=0.004$) in non-viral group. Interactions were found between CTEP and α-syn fibrils ($F(1,18)= 18.341$; $p=0.001$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 29.201$; $p=0.000$) in A53T AV group. In LAMP1 levels interaction was found between CTEP and α-syn fibrils ($F(1,18)= 22.124$; $p=0.001$) in non-viral group. Interaction was also found between CTEP and α-syn fibrils ($F(1,18)= 15.289$; $p=0.002$) in A53T AV group.

Discussion

Alpha-synuclein fibril model

Alpha-synuclein fibril model is highly relevant to PD pathology assessment as the model represents the protein aggregation that is an outcome of both genetic and environmental factors. Models that utilize genetic mutations, such as A53T, or environmental toxins, such as paraquat, may produce parkinsonism behaviorally; however, these models do not always create a-syn protein aggregation that is a major hallmark of PD.

Introduction of soluble a-syn fibrils to SH-SY5Y cells mimicked the exogenous α-syn that constitutes Lewy bodies prominent in PD brain; whereas, A53T AV introduction mimicked α-syn overexpression that is also common in PD. In general, α-syn is present within the cell in multimeric form. There appears to be a dynamic equilibrium between mono- and multimeric (alpha-helical) forms (Dettmer et al., 2015). Interestingly, it is the monomer that tends to be upregulated in pathological conditions. Due to its instability, the α-syn monomer could be the main culprit in abnormal protein aggregation in PD (Lashuel et al., 2013). In this study we have also seen that in comparison to α-syn tetramer, α-syn monomer has increased two-fold with the introduction of A53T AV and three-fold when the virus was combined with α-syn fibrils.

As expected the application of A53T AV significantly increased α-syn concentration within the cell as determined by both IF and WB. With the increased concentration even the more stable multimeric forms of α-syn begin to aggregate. IF has shown that, though the α-syn fibrils were not introduced, A53T AV alone created

localized α-syn aggregates (Fig. 15 App. 3). This confirms that abnormal aggregation of α-syn is concentration dependent (Giasson et al., 1999).

The effect of CTEP mTOR/AKT pathway

Based on the previous success of mGluR5 modulation by CTEP in protein aggregates clearing from Huntigton's disease model, we hypothesized that the drug could also be useful for PD model (Abd-Elrahman et al., 2017). Indeed, we have seen a significant decrease of filament form α-syn in both viral and non-viral groups treated by CTEP, as assessed by IF (Fig. 5 and 6). Rapamycin, a known mTORC1 inhibitor, had a very similar effect on α-syn fibrils elimination. Additionally, both CTEP and rapamycin significantly reduced the concentration of the monomeric α-syn from the group that was treated by both α-syn fibrils and A53T AV (Fig. 8). This suggests a that when the conditions are closest to pathological α-syn aggregation, in the presence of both exogenous and endogenous α-syn, CTEP and rapamycin contribute to α-syn stabilization into forms that are less prone to aggregation.

The use of rapamycin was mainly to assess the effect of mTOR inhibition on the α-syn fibril in vitro model, and how the effects correlate with mGluR5 inhibition by CTEP. Indeed, we have seen similarities in CTEP and rapamycin action, not only in α-syn fibrils elimination, but in fluctuating levels of autophagy and HSP system components. This suggests that CTEP action may be at least partially mTOR dependent. Further assessment of mTOR/AKT pathway has shown that both CTEP and rapamycin have similar effects on mTOR and AKT activity. Even though the mTOR activity was not upregulated by α-syn fibrils and A53T AV combination, the activity of mTOR was

still brought down below control levels by both CTEP and rapamycin (Fig. 9A). AKT activity, on the other hand, was significantly upregulated by the combination of α-syn fibrils and A53T AV, and subsequently brought down by both CTEP and rapamycin (Fig. 9B). Interestingly, the phosphorylation of the direct target of mTOR, p6s70K, was dramatically increased when α-syn fibrils and A53T AV were applied to SH-SY5Y cells, and again the phosphorylation of p6s70K was reduced by both CTEP and rapamycin (Fig. 9C). Upregulation of p-p6s70K tells us about the increased mTOR activity even though p-mTOR/mTOR ratio did not appear higher on WB test. Additionally, increased p6s70K phosphorylation leads to upregulation in protein synthesis which is very useful for neuronal plasticity under physiological conditions. However, when a cell is already overwhelmed by a combination of ER stress, mitochondrial depletion and excitotoxicity, additional protein synthesis may further the damage leading to cell death (Switon et al., 2016).

All of the above suggests that CTEP acts through mTOR/AKT pathway (Fig.1). Recently there have been reports on another drug that works through mTOR/AKT inhibition. Metformin has shown efficacy in PD, AD and HD, namely the conditions that are associated with abnormal protein aggregation (Rotermund et al., 2018). This is additional evidence that targeting mTOR and AKT, whether directly or through upstream targets, could be efficacious in the prevention of abnormal protein aggregation.

CTEP Effect on Autophagy Initiation

Next we were interested in autophagy system changes due to α-syn accumulation and overexpression, as there have been reports of autophagy dysregulation in PD (Cuervo

et al., 2004; Xilouri et al., 2009). Exaggerated mTOR activity in PD was associated with autophagy inhibition through phosphorylation of ULK1 (S757). In this study we observed an almost ten-fold increase of the inhibitory ULK1 phosphorylation when fibrils alone were present (Fig. 10A). Both CTEP and rapamycin reduced the inhibitory ULK1 phosphorylation (S757) levels in non-viral condition. Atg101 was brought back to control levels by CTEP, but not rapamycin in both viral and non-viral condition (Fig. 10C). Beclin1, a major component of autophagy system that was reported to be depleted in PD, was brought back to control levels by both CTEP and rapamycin (Fig. 10D) (Song et al., 2014). This suggests that both CTEP and rapamycin have an important role in restoring autophagy initiation that can potentially be inhibited by α-syn aggregation and overexpression.

CTEP Effect on Autophagy Elongation/Nucleation

LC3I/II and Atg5-Atg12 are the two important complexes that participate in autophagosome formation. LC3I and II ratio was not affected by CTEP or rapamycin in α-syn fibril conditions (Fig. 11A). Interestingly, Atg5 and Atg12 levels were upregulated by both CTEP and rapamycin (Fig. 11D,G). However, Atg5-Atg12 complex was increased by CTEP, but not rapamycin (Fig.11B). This effect may be due to enzymatic activity of Atg3, levels of which are also brought up by CTEP, but not rapamycin (Fig. 11E). Another enzyme, Atg7, which participates in LC3 and Atg5-Atg12 complex formation, was normalized to control levels by both CTEP and rapamycin in viral and non-viral groups (Fig. 11C). Lysosomal marker LAMP1 was upregulated in comparison to control by CTEP and rapamycin in A53T AV group (Fig. 11F). The upregulation of

LAMP1 is particularly important because it shows that protein degradation actually took place. As mentioned before, in some cases of synucleopathies the autophagy system was functional. However the lysosomal markers were low which suggests that the protein degradation process failed at the very last step (Dehay et al., 2010). Additionally, mutated or misfolded α-syn has a high affinity for lysosomal adaptor LAMP2. However, mutated or misfolded α-syn fails to internalize, clogging the lysosomal degradation system (Cuervo et al., 2004; Xilouri et al., 2009). This means that during α-syn overload, the cell will not only experience low expression of lysosome, but also its malfunction.

These findings confirm that CTEP is efficacious in restoring autophagy system's function as shown in Huntington's disease model (Abd-Elrahman et al., 2017). Rapamycin was also previously known to induce autophagy, so it is not surprising that the action of both inhibitors have similar outcomes (Pan et al., 2009).

Over all we can conclude that mGluR5 modulation by CTEP is a useful therapeutic method to eliminate aggregated α-syn from the cell. Autophagy system has been successfully induced in our *in vitro* α-syn fibril model by a single treatment of CTEP. Of course it is worth to consider that the current model didn't perfectly reproduce the expected hallmarks of PD pathology. For example, mTOR activity was not increased to the expected extend. Some of the effects were present in non-viral groups, but were extinguished when A53T AV was applied. In general cell groups that were treated by A53T AV had greater variability in protein levels as determined by WB. Potential disadvantage of the study is that it utilizes neuron-like neuroblastoma cells, and that it does not take into consideration the participation of glial cells in PD pathology. To have a

clearer picture of CTEP action, it may be useful to repeat the autophagy system assessment in primary DA neurons co-cultured with microglia.

References

Adamczyk A, Kazmierczak A, Strosznajder JB (2006) a-Synuclein and its neurotoxic fragment inhibit dopamine uptake into rat striatal synaptosomes. Relationship to nitric oxide. Neurochem Int 49:407–412.

Abd-Elrahman K, Hamilton A, Hutchinson SR,Liu F, Russell RC, Ferguson SG (2017) mGluR5 antagonism increases autophagy and prevents disease progression in the zQ175 mouse model of Huntington's disease. Sci. Signal.;10:6387.

Adamczyk A and Strosznajder JB (2006) Alpha-synuclein potentiates Ca2+ in£ux through voltage-dependent Ca2+ channels. Molecular Neurosci; 17(18): 1883-1886.

Ambrosi G, Cerri S, Blandini F (2014) A further update on the role of excitotoxicity in the pathogenesis of Parkinson's disease. J Neural Transm;121(8):849-59.

An WL, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Iqbal IG, Winblad B, and Pei JJ (2003) Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. Am. J. Pathol. 163, 591–607.

Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M (2006). Phosphorylation of Ser-129 is the dominant pathological modifi cation of alpha-synuclein in familial and sporadic Lewy body disease. J. Biol. Chem. 281; 29739 – 29752

Andreyev AY, Kushnareva YE, Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc.) 70, 200–214.

Anglade P, Vyas S, Javoy-Agid F, Herrero MT, Michel PP, Marquez J, Mouatt-Prigent A, Ruberg M, Hirsch EC, Agid Y. Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. Histol Histopathol. 1997; 12 (1):25–31.

Arias E, Cuervo AM. Chaperone-mediated autophagy in protein quality control. Curr Opin Cell Biol. 2011; 23(2):184–9.

Baker RW, Hughson FM (2016) Chaperoning SNARE assembly and disassembly. Nat Rev Mol Cell Biol. 17(8): 465–479.

Barcia C, Sanchez BA, Fernandez-Villalba E (2004) Evidence of active microglia in substantia nigra pars compacta of parkinsonian monkeys 1 year after MPTP exposure. Glia. 46: 402–409.

Bartels T, Ahlstrom LS, Leftin A, Kamp F, Haass C, Brown MF, Beyer K. (2010) The N-terminus of the intrinsically disordered protein alpha-synuclein triggers membrane binding and helix folding. Biophys. J. 99 , 2116 – 2124.

Bartels T, Choi JG, Selkoe DJ (2011) a-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature 477: 107–110.

Bernheimer H, Birkmayer W, Hornykiewicz O (1973) Brain dopamine and the syndromes of Parkinson and Huntington: Clinical, morphological and neurochemical correlations. J Neurol Sci 20: 415–455

Braak H, Braak E, Yilmazer D, de Vos RA, Jansen EN, Bohl J (1996) Pattern of brain destruction in Parkinson's and Alzheimer's diseases. J Neural Transm (Vienna) 103(4):455-90.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN and Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24: 197–211.

Burre J (2010) a-Synuclein promotes SNARE-complex assembly in vivo and in vitro. Science 329: 1663–1667.

Burre J, Vivona S, Diao J, Sharma M, Brunger AT, Sudhof TC (2013) Properties of native brain a-synuclein. Nature 498:E4-E7.

Chan-Palay V and Asan E (1989) Alterations in catecholamine neurons of the locus coeruleus in senile dementia of Alzheimer type and in Parkinson's disease with and without dementia and depression. I. Comp. Neural. 287,373-392.

Cheng AVT, Ferrier IN, Morris CM (1991) Cortical serotonin-S2 receptor binding in Lewy body dementia, Alzheimer's and Parkinson's diseases. J Neurol Sci 106: 50–55.

Chen L and Feany MB (2005) Alpha-synuclein phosphorylationcontrols neurotoxicity and inclusion formation in a Drosophila model of Parkinson disease. Nat. Neurosci.;8 :657 – 663.

Chen C, Turnbull DM, Reeve AK (2019) Mitochondrial Dysfunction in Parkinson's Disease—Cause or Consequence? Biology (Basel).8(2): 38

Coles SK, lies JF, Nicolopoulos-Stournaras S (1989) The mesencephalic centre controlling locomotion in the rat. Neuroscience 28, 149-157.

Crews L, Spencer B, Desplats P, Patrick C, Paulino A, Rockenstein E, Hansen L, Adame A,Galasko D, Masliah E (2010) Selective molecular alterations in the autophagy pathway

in patients with Lewy body disease and in models of alpha-synucleinopathy. PLoS One.;5(2):e9313

Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D (2004) Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science; 305 (5688):1292–1295.

Daher JP, Pletnikova O, Biskup S, Musso A, Gellhaar A, Galter D, Troncoso JC, Lee MK, Dawson TM (2012) Neurodegenerative phenotypes in an A53T a-synuclein transgenic mouse model are independent of LRRK2. Human Molecular Genetics 21(11): 2420–2431.

Dawson TM and Dawson VL (2003) Molecular pathways of neurodegeneration in Parkinson's disease. Science 302: 819–822.

de Hemptinne C, Swann NC, Ostrem JL, Ryapolova-Webb ES, San Luciano M, Galifianakis NB, Starr PB (2015) Therapeutic deep brain stimulation reduces cortical phase-amplitude coupling in Parkinson's disease. Nat Neurosci. 18(5):779-86.

Dehay B, Bove J, Rodriguez-Muela N, Perier C, Recasens A, Boya P, Vila M (2010) Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci.; 30 (37):12535–12544.

Dettmer U, Newman AJ, Soldner F, Luth ES, Kim NC, von Saucken VE, Sanderson JB, Jaenisch R, Bartels T, Selkoe D (2015) Parkinson-causing α-synuclein missense mutations shift native tetramers to monomers as a mechanism for disease initiation. Nat Comm: 6:Article number: 7314

Dokladny K, Zuhl MN, Mandell M, Bhattacharya D, Schneider S, Deretic V, Moseley PL (2013) Regulatory coordination between two major intracellular homeostatic systems: heat shock response and autophagy. J Biol Chem; 288:14959-72.

Dong X, Wang Y, Qin Z (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharm Sinica; 30: 379–387.

Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, Marshall FJ, Ravina BM, Schifitto G, Siderowf A (2007) Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. Neurology 68: 384–386.

Fahn S, Shoulson I, Kieburtz K (2004) Levodopa and the progression of Parkinson's disease. N Engl J Med 351:2498–2508.

Ferreira SA and Romero-Ramos M (2018) Microglia Response During Parkinson's Disease: Alpha-Synuclein Intervention. Front Cell Neurosci.;12:247

Filomeni G, De Zio D, Cecconi F (2015) Oxidative stress and autophagy: The clash between damage and metabolic needs. Cell Death Dier., 22, 377–388.

Fornai F, Schlüter OM, Lenzi P, Gesi M, Ruffoli R (2005) Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin–proteasome system and α-synuclein. Proc. Natl Acad. Sci. USA 102, 3413–3418.

Friedman LG, Lachenmayer ML, Wang J, He L, Poulose SM, Komatsu M, Holstein GR, Yue Z (2012) Disrupted Autophagy Leads to Dopaminergic Axon and Dendrite Degeneration and Promotes Presynaptic Accumulation of alpha-Synuclein and LRRK2 in the Brain. J Neurosci.; 32 (22):7585–7593.

Fuchs J, Nilsson C, Kachergus J, Munz M, Larsson EM, Schule B, Langston JW, Middleton FA, Ross OA, Hulihan M (2007) Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication. Neurology 68:916-922.

Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T (2002) Alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat. Cell Biol. 4 , 160 – 164.

Gao X, Carroni M, Nussbaum-Krammer C, Mogk A, Nillegoda NB, Szlachcic A, Guilbride DL, Saibil HR, Mayer MP, and Bukau B (2015) Human Hsp70 disaggregase reverses Parkinson's-linked -synuclein amyloid fibrils. Mol. Cell 59, 781–793

Gatto NM, Cockburn M, Bronstein J, Manthripragada AD, Ritz B (2009) Well-water consumption and Parkinson's disease in rural California. Environ. Health Perspect 117: 1912–1918

Giasson BI, Uryu K, Trojanowski JQ, and Lee VM (1999). Mutant and wild type human a-synucleins assemble into elongated filaments with distinct morphologies in vitro. J. Biol. Chem. 274: 7619–7622.

Gorell JM, Johnson CC, Rybicki BA, Peterson EL, Richardson RJ (1998) The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology 50: 1346–1350.

Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ (2003) Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci 100(7):4078-83.

Guo JL, Lee VM (2014) Cell-to-cell transmission of pathogenic proteins in neurodegenerative diseases. Nat. Med. 20: 130–138.

Hallanger AE, Levey AL, Lee HJ, Rye DB, and Wainer BH (1987) The origins of cholinergic and other subcortical afferents to the thalamus in the rat. J. Camp. Neural. 262, 105-124

Hartl FU, Bracher A, and Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. Nature 475, 324–332.

He X, Yuan W, Li Z, Hou Y, Liu F, Feng J (2018) 6-Hydroxydopamine induces autophagic flux dysfunction by impairing transcription factor EB activation and lysosomal function in dopaminergic neurons and SH-SY5Y cells. Toxicol. Lett. 2018, 283, 58–68.

Hoogland J, Boel JA, de Bie RMA (2017) Mild cognitive impairment as a risk factor for Parkinson's disease dementia. Mov Disord. 32(7):1056-1065.

Hou L, and Klann E (2004) Activation of the phosphoinositide 3-kinase-Aktmammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. J. Neurosci. 24, 6352–6361.

Hoyer W, Cherny D, Subramaniam V, Jovin TM (2004) Impact of the acidic C-terminal region comprising amino acids 109-140 on alpha-synuclein aggregation in vitro. Biochemistry.:28;43(51):16233-42.

Hu Q, Wang G (2016) Mitochondrial dysfunction in Parkinson's disease.Translational Neurodegeneration 5, Article number:14

Hu ZY, Chen B, Zhang JP, Ma YY (2017) Up-regulation of autophagy-related gene 5 (ATG5) protects dopaminergic neurons in a zebrafish model of Parkinson's disease. J. Biol. Chem., 292, 18062–18074.

Hunot S, Hirsch EC (2003) Neuroinflammatory processes in Parkinson's disease. Ann Neurol. 53(Suppl 3):S49–S58.

Irvine GB, El-Agnaf OM, Shankar GM, and Walsh DM (2008) Protein aggregation in the brain: The molecular basis for Alzheimer's and Parkinson's diseases. Mol. Med. 14, 451–464

Jiang J, Jiang J, Zuo Y, Gu Z (2013) Rapamycin protects the mitochondria against oxidative stress and apoptosis in a rat model of Parkinson's disease. Int. J. Mol. Med. 2013, 31, 825–832.

Johnson KA, ConnPJ, Nisvender CM (2009) Glutamate receptors as therapeutic targets in Parkinson's disease. CNS Neur Dis Drug Targ.; 8(6):475-491.

Kamada Y, Yoshino K, Kondo C, Kawamata T, Oshiro N, Yonezawa K, Ohsumi Y (2010) mTor directly controls the Atg1 kinase complex to regulate autophagy. Mol. Cell. Biol., 30, 1049–1058.

Kaushik S, Massey AC, Mizushima N, Cuervo AM. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. Mol Biol Cell. 2008; 19 (5):2179–2192.

Kertelge L, Bruggemann N, Schmidt A, Tadic V, Wisse C, Dankert S, Drude L (2010) Impaired Sense of Smell and Color Discrimination in Monogenic and Idiopathic Parkinson's Disease. Movement Disorders 25:2665-2669.

Klionsky DJ. (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol.; 8 (11):931–937.

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima . (2007) The role of autophagy during the early neonatal starvation period. Nature.; 432 (7020):1032–1036.

Kumsta C, Chang JT, Schmalz J, Hansen M (2017) Hormetic heat stress and HSF-1 induce autophagy to improve survival and proteostasis in C. elegans. Nat. Comm.; 8:14337.

Lan A, Chen J, Zhao Y, Chai Z, Hu Y (2017) mTOR Signaling in Parkinson's Disease. Neuromol Med ;19:1–10.

Lashuel HA, Overk CR, Oueslati A, Masliah E (2013) The many faces of α-synuclein: from structure and toxicity to therapeutic target. Nat Rev Neurosci.;14(1): 38–48.

Lee KW, Chen W, Junn E, Im JY, Grosso H, Sonsalla PK, Feng X, Ray N, Fernandez JR, Chao Y (2011) Enhanced phosphatase activity attenuates alpha-synucleinopathy in a mouse model. J. Neurosci.;31: 6963 – 6971.

Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, Lashley T, Quinn NP, Rehncrona S, Bjorklund A (2008) Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat. Med. 14: 501–503.

Limanaqi F, Biagioni F, Busceti CL, Ryskalin L, Soldani P, Frati A, Fornai F (2019) Cell Clearing Systems Bridging Neuro-Immunity and Synaptic Plasticity. Int. J. Mol. Sci., 20, 2197

Limousin P, Krack P, Pollak P (1998). Electrical stimulation of the subthalamic nucleus in advanced Parkinson's disease. N Engl J Med 339: 1105–11.

Lin X, Han L, Weng J, Wang K, Chen T (2018) Rapamycin inhibits proliferation and induces autophagy in human neuroblastoma cells. Biosci Rep:38 (6): BSR20181822

Lindemann L, Jaeschke G, Michalon A, Vieira E, Honer M, Spooren W, et al. CTEP: a novel, potent, long-acting, and orally bioavailable metabotropic glutamate receptor 5 inhibitor. J Pharmacol Exp Ther. 2011;339:474–86

Liorens-Cortes C, Javoy-Agid F, Agid Y, Taquet H, Schwartz J C (1984) Enkephalinergic markers in substantia nigra and caudate nucleus from parkinsonian subjects. J Neurochem. 43, 874-877.

Liu G, Zhang C, Yin J (2009) alpha-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity. Neurosci Lett. 454:187–192.

Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, Liu D, Wan L, Zhai B, Yu Y (2013). Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis. Nat. Cell Biol. 15, 1340–1350.

Lock EA, Zhang J, Checkoway H (2013) Solvents and Parkinson disease: A systematic review of toxicological and epidemiological evidence. Toxicology and Applied Pharmacology 266(3): 345–355.

Löschmann PA, Lange KW, Kunow M, Rettig KJ, Jähnig P, Honoré T, Turski L, Wachtel H, Jenner P, Marsden CD (1991) Synergism of the AMPA-antagonist NBQX and the NMDA-antagonist CPP with L-dopa in models of Parkinson's disease. J Neural Transm Park Dis Dement Sect 3:203–213.

Mak SK, McCormack AL, Manning-Bog AB, Cuervo AM, DiMonte DA (2010) Lysosomal degradation of alpha-synuclein in vivo. J Biol Chem.; 285 (18):13621–13629.

Manning-Bog AB, McCormack AL, Li J, Uversky VN, Fink AL, Di Monte DA (2002)The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: paraquat and alpha-synuclein. J Biol Chem. 277(3):1641-4.

Marsden CD, Parkes JD (1976) "ON-OFF" Effects in patients with Parkinson's disease on chronic levodopa therapy. The Lancet. 292-296.

Martinez-Vicente M, Talloczy Z, Kaushik S, Massey AC, Mazzulli J, Mosharov EV, Hodara R, Fredenburg R, Wu DC, Follenzi A, Dauer W, Przedborski S, Ischiropoulos H, Lansbury PT, Sulzer D, Cuervo AM (2008) Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. J Clin Invest.; 118 (2):777–788.

Mbefo MK, Paleologou KE, Boucharaba A, Oueslati A, Schell H, Fournier M, Olschewski D, Yin G, Zweckstetter M, Masliah E (2010) Phosphorylation of synucleins by members of the Polo-like kinase family. J. Biol. Chem.;285:2807 – 2822.

McFarland NR, Fan Z, Xu K, Schwarzschild MA, Feany MB, Hyman BT, McLean PJ (2009) Alpha-Synuclein S129 phosphorylation mutants do not alter nigrostriatal toxicity in a rat model of Parkinson disease. J. Neuropathol. Exp. Neurol. 68;515 – 524.

McLean PJ, Kawamata H, Shariff S, Hewett J, Sharma N, Ueda K, Breakefield XO, Hyman BT (2002) Torsin A and heat shock proteins act as molecular chaperones: suppression of α-synuclein aggregation J. Neurochem.; 83:846-854

Michalon A, Sidorov M, Ballard TM, Ozmen L, Spooren W, Wettstein JG, Jaeschke G, Bear MF, Lindemann L (2012) Chronic Pharmacological mGlu5 Inhibition Corrects Fragile X in Adult Mice. Neuron;74:49–56.

Obeso JA, Stamelou M, Goetz CG, Poewe W, Lang AE, Weintraub D, Burn D, Halliday GM, Bezard E, Przedborski S (2017) Past, Present, and Future of Parkinson's Disease: A Special Essay on the 200th Anniversary of the Shaking Palsy. Movement Disorders 32(9): 1265-1310.

Ohsumi Y. (2001) Molecular dissection of autophagy: two ubiquitin-like systems. Nat Rev Mol Cell Biol.; 2 (3):211–216.

Pal R, Bajaj L, Sharma J, Palmieri M, Di Ronza A, Lotfi P, Chaudhury A, Neilson J, Sardiello M, Rodney GG (2016) NADPH oxidase promotes Parkinsonian phenotypes by impairing autophagic flux in an mTORC1-independent fashion in a cellular model of Parkinson's disease. Sci. Rep., 6, 22866.

Paleologou KE, Schmid AW, Rospigliosi CC, Kim HY, Lamberto GR, Fredenburg RA, Lansbury PT, Fernandez CO, Eliezer D, Zweckstetter M (2008) Phosphorylation

at Ser-129 but not the phosphomimics S129E/D inhibits the fibrillation of alpha-synuclein. J. Biol. Chem. 283 , 16895 – 16905.

Pan T, Kondo S, Zhu W, Xie W, Jankovic J, Le W (2008) Neuroprotection of rapamycin in lactacystin induced neurodegeneration via autophagy enhancement. Neurobiol Dis.; 32 (1):16–25

Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W (2009) Rapamycin protects against rotenone-induced apoptosis through autophagy induction. Neuroscience 2009, 164, 541–551.

Petrucelli L, O'Farrell C, Lockhart PJ, Baptista M, Kehoe K, Vink L, Choi P, Wolozin B, Farrer M, Hardy J, Cookson MR (2002) Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron. 36(6):1007-19.

Pierrot N, Ghisdal P, Caumont AS, Octave JN (2004) Intraneuronal amyloidbeta 1-42 production triggered by sustained increase of cytosolic calcium concentration induces neuronal death. J Neurochem 88:1140–1150.

Qing H, Wong W, McGeer EG, McGeer PL (2009) Lrrk2 phosphorylates alpha synuclein at serine 129: Parkinson disease implications. Biochem. Biophys. Res. Commun.;387:149 – 152.

Rascol O, Payoux P, Ory F, Ferreira JJ, Brefel-Courbon C, Montastruc J (2004) Limitations of Current Parkinson's Disease. TherapyAnn Neurol 53(3):S3–S15.

Rotermund C, Machetanz G, Fitzgerald JC (2018) The Therapeutic Potential of Metformin in Neurodegenerative Diseases. Front Endocrinol; 9: 400.

Ryu MY, Kim DW, Arima K, Mouradian MM, Kim SU, Lee G (2008) Localization of CKII beta subunits in Lewy bodies of Parkinson ' s disease. J. Neurol. Sci.;266:9 – 12.

Saito Y, Kawashima A, Ruberu NN, Fujiwara H, Koyama S, Sawabe M, Arai T, Nagura H, Yamanouchi H, Hasegawa M (2003) Accumulation of phosphorylated alpha-synuclein in aging human brain. J. Neuropathol. Exp. Neurol.;62:644 – 654.

Sato S, Uchihara T, Fukuda T, Noda S, Kondo H, Saiki S (2018) Loss of autophagy in dopaminergic neurons causes Lewy pathology and motor dysfunction in aged mice.Sci. Rep.8, 2813

Schapira AH (2008) The clinical relevance of levodopa toxicity in the treatment of Parkinson's disease. Mov Disord. 23(Suppl 3):S515–S520.

Sherer TB, Kim JH, Betarbet R, Greenamyre JT (2003) Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. Exp Neurol.179(1):9-16.

Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, Mizuno N (1993). Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. Neurosci Lett.;163:53–7.

Song JX, Lu JH, Liu LF, Chen LL, Durairajan SS, Yue Z, Zhang HQ, Li M (2014) HMGB1 is involved in autophagy inhibition caused by SNCA/alpha-synuclein overexpression: A

process modulated by the natural autophagy inducer corynoxine B. Autophagy, 10, 144–154.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies.Nature 388:839-840.

Steece-Collier K, Chambers LK, Jaw-Tsai SS, Menniti FS, Greenamyre JT (2000) Antiparkinsonian actions of CP-101,606, an antagonist of NR2B subunit-containing N-methyl-d-aspartate receptors. Exp Neurol 163:239–243

Stamler R, Kappé G, Boelens W, and Slingsby C (2005) Wrapping the alpha-crystallin domain fold in a chaperone assembly. J. Mol. Biol. 353, 68–79

Studier J M and Javoy-Agid F (1985) Cholecystokinin octapeptide immunoreactivity distribution in human brain, Ann. NY Acad. Sci. 448, 656-659.

Switon K, Kotulska K, Janusz-Kaminska A, Zmorzynska J, Jaworski J (2016) Molecular neurobiology of mTOR. Neuroscience 341:112–153.

Tanner CM, Kamel F, Ross GW, Hoppin JA, Goldman SM, Korell M, Marras C, Bhudhikanok GS, Kasten M, Chade AR (2011) Rotenone, paraquat, and Parkinson's disease. Environ. Health Perspect 119: 866–872.

Tenovuo O, Rinne JK, and Viljanen MK (1984) Substance P immunoreactivity in post-mortem parkinsonian brain. Brain Res. 303, 113-116.

Thaler A, Ash E, Gan-Or Z, Orr-Urtreger A, Giladi N (2009) The LRRK2 G2019S mutation as the cause of Parkinson's disease in Ashkenazi Jews. J. Neural Transm 116(11): 1473–1482.

Thannickal TC, Lai YY, Siegel JM (2007) Hypocretin (orexin) cell loss in Parkinson's disease. Brain 130: 1586–95.

Uryu K, Richter-Landsberg C, Welch W, Sun E, Goldbaum O, Norris EH, Pham CT, Yazawa I, Trojanowski JQ (2006) Convergence of heat shock protein 90 with ubiquitin in filamentous alpha-synuclein inclusions of alpha-synucleinopathies. Am. J. Pathol.;168: 947-961

Waxman EA and Giasson BI (2008) Specifi city and regulation of casein kinase-mediated phosphorylation of alpha-synuclein. J. Neuropathol. Exp. Neurol.;67:402 – 416.

Waxman EA and Giasson BI (2011) Characterization of kinases involved in the phosphorylation of aggregated alpha-synuclein.J. Neurosci. Res.;89:231 – 247.

Winslow AR, Chen CW, Corrochano S, Acevedo-Arozena A, Gordon DE, Peden AA, Lichtenberg M, Menzies FM, Ravikumar B, Imarisio S, Brown S, O'Kane CJ, Rubinsztein DC (2010) Alpha-Synuclein impairs macroautophagy: implications for Parkinson's disease. J Cell Biol.; 190 (6):1023–1037

Xilouri M, Vogiatzi T, Vekrellis K, Park D, Stefanis L (2009) Abberant alpha-synuclein confers toxicity to neurons in part through inhibition of chaperone-mediated autophagy. PLoS One.; 4(5):e5515

Yoshimoto Y, Nakaso K, Nakashima K. (2005) L-dopa and dopamine enhance the formation of aggregates under proteasome inhibition in PC12 cells. FEBS Lett.; 579 (5):1197–1202.

Zarow C, Lyness SA, Mortimer JA, Chui HC (2003) Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 60: 337–41.

Appendix 1 HSP System

HSP system consists of molecular chaperons that are relevant for PD as HSP40, HSP70 and HSP90 were reported to be co-localized with Lewy bodies (McLean et al., 2002; Uryu et al., 2006). Heat shock protein chaperon system works in parallel with autophagy protein degradation. Two systems complement each other's action. Namely, when autophagy are depleted, heat shock proteins are upregulated, and vice versa, when HSPs are inhibited, Atg proteins are upregulated (Kaushik et al., 2008). Indeed, in this study HSPs were generally upregulated by α-syn fibril treatment which coincided with downregulation of autophagy components.

The general trend of CTEP action is HSP downregulation, particularly when α-syn fibrils and A53T AV are involved. This effect may be due CTEP action towards upregulation of autophagy components. This is another indication that CTEP acts through autophagy system rather than through other protein degradation systems.

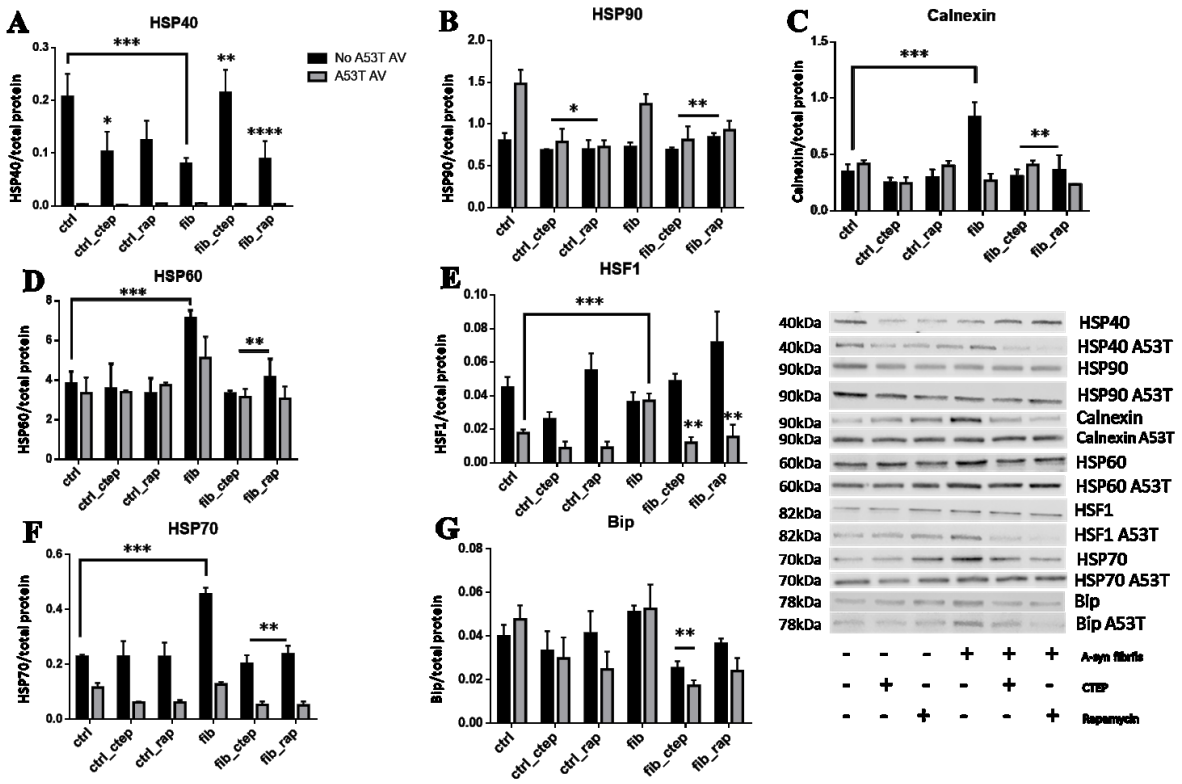

Figure 10 Appendix 1 Western Blot Heat Shock Chaperons with A-syn fibrils + A53T AV + CTEP/Rapamycin. **(A)** HSP40 was downregulated by α-syn fibrils, and then rescued by CTEP, but not rapamycin. **(B,E)** HSP90 and HSF1 were downregulated by CTEP and rapamycin when α-syn fibrils and A53T AV were combined. **(C,F)** HSP70 and Calnexin were downregulated by CTEP and rapamycin in α-syn fibril condition. **(D,G)** HSP60 and Bip were downregulated by CTEP in fibril alone and in fibril/virus combination treatment groups. (* $p<0.05$, difference with regards to control; ** $p<0.05$, difference with regard to α-syn fibrils; *** $p<0.05$, difference between control and α-syn fibrils; **** $p<0.05$, difference with regard to α-syn fibrils+CTEP). Presented data is the mean result of 3 separate experiments. All WB signals were normalized to total protein content. Tukey's pairwise comparison was used as a post hoc method. All treatments and incubations were according to timeline (Figure 2).

Statistical results

In HSP40 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 32.592$; $p=0.000$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 4.728$; $p=0.050$) in non-viral group. No interactions of main effects were observed in A53T AV group.

In HSP60 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 16.087$; $p=0.002$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 7.781$; $p=0.016$) in non-viral group. Interactions were also found between CTEP and α-syn fibrils ($F(1,18)= 7.542$; $p=0.016$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 11.301$; $p=0.006$) in A53T AV group.

In HSP70 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 34.379$; $p=0.000$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 25.100$; $p=0.000$) in non-viral group. The main effects were found for CTEP ($F(1,18)= 85.551$; $p=0.000$), and rapamycin ($F(1,18)= 88.030$; $p=0.000$) in A53T AV group.
In HSP90 levels interaction was also found between rapamycin and α-syn fibrils ($F(1,18)= 37.589$; $p=0.000$) in A53T AV group. No interactions or main effects were observed in non-viral group.

In Bip levels interaction was also found between rapamycin and α-syn fibrils ($F(1,18)= 6.849$; $p=0.023$) in non-viral group. The main effects were found for CTEP ($F(1,18)= 34.906$; $p=0.000$), and rapamycin ($F(1,18)= 32.728$; $p=0.000$) in A53T AV group.

In Calnexin1 levels interactions were found between CTEP and α-syn fibrils ($F(1,18)= 16.303$; $p=0.002$), as well as between rapamycin and α-syn fibrils ($F(1,18)= 15.784$; $p=0.002$) in non-viral group. Interaction was also found between CTEP and α-syn fibrils ($F(1,18)= 37.589$; $p=0.000$) in A53T AV group.

Appendix 2 Additional Figures

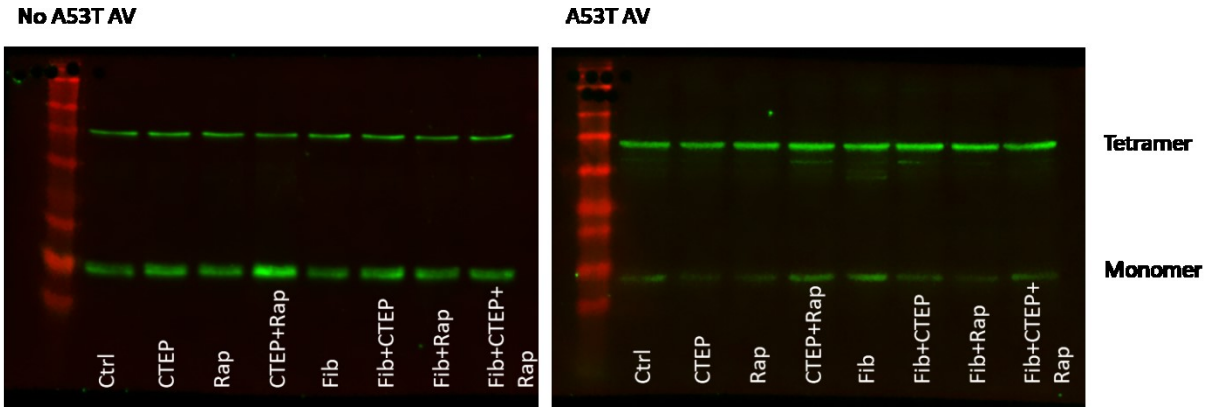

Figure 11 Appendix 3 A-syn WB on 15% acrylamide gel showing α-syn monomer and tetramer in viral and non-viral conditions. All treatments and incubations were according to timeline (Figure 2).

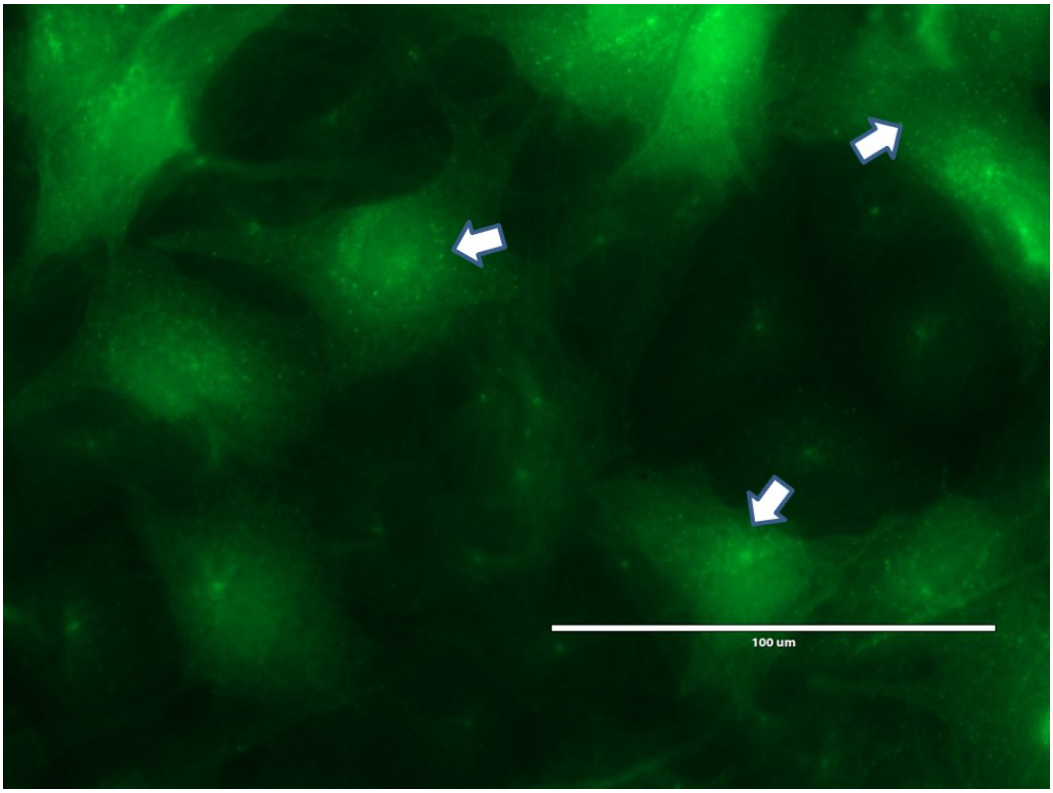

Figure 12 Appendix 3 IF A53T AV treated SH-SY5Y cells punctate α-syn aggregates. All treatments and incubations were according to timeline (Figure 2).

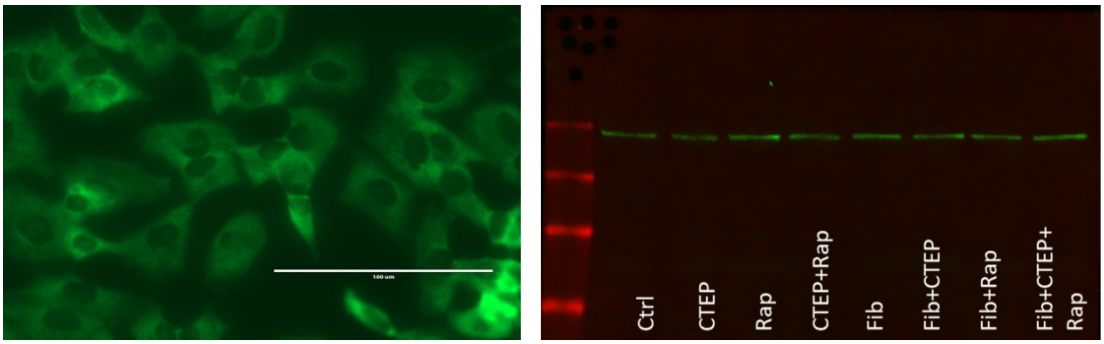

Figure 13 Appendix 3 MGluR5 expression by SH-SY5Y cells as determined by IF and WB (5% acrylamide gel). All treatments and incubations were according to timeline (Figure 2).