공학석사학위논문

Characterization of Alpha-Synuclein Aggregation Pathways Induced by Oxidized and Unoxidized Dopamine in Physiological pH

생리학적 pH에서 산화환원된 도파민에 의한 알파-시뉴클린 단백질의 응집 과정

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Abstract

Selective loss of dopaminergic neurons in brain is a hallmark of Parkinson's disease. Major species that is thought to induce the cytotoxicity is α-synuclein protein, which changes biochemically during the onset of Parkinson's disease, including its oxidation under oxidative stress and aggregation. Many recent studies have focused on dopamine-mediated oxidation of α-synuclein due to the specific susceptibility of dopamine-generating cell; however, the relationship is still not well understood. I investigated how oxidized and un-oxidized dopamine affect α-synuclein oxidation and aggregation. Dopamine is known to be stable in acidic pH but oxidized in higher pH. The pH of the vesicle storing dopamine stably and the pH of the cytosol of dopaminergic neurons, with high level of dopamine oxidation and α-synuclein modification during the pathogenesis of Parkinson's disease, were mimicked in this *in vitro* studies to give a close account to what may actually occur in physiological conditions. Four distinct aggregation pathways were found. Un-oxidized dopamine under acidic pH condition mainly induced short fibrillar forms of α-synuclein, while oxidized dopamine under neutral pH condition induced soluble oligomeric forms of α-synuclein. When only α-synuclein is present without dopamine, short fibrils as well as amorphous aggregates were discovered for acidic pH while extensive fibrillation occurred in neutral pH. Furthermore, covalent dopamine adducts on α-synuclein was observed when treated with oxidized dopamine.

Keywords: α-Synuclein, Parkinson's disease, Dopamine, pH-mediated oxidative stress, Aggregation pathway, Dopamine-α-synuclein covalent adduct

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1. Introduction

Parkinson's disease is the second most common neurodegenerative disorder that affects more than 1.5 million people in the United States alone over the age of 65 (Ruf et al., 2008). Major symptoms of the disease are resting tremor, rigidity in muscles, bradykinesia, and gait disturbance. Although the exact cause of Parkinson's disease is still yet to be defined, it has been known to be pathologically characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies in the midbrain region of the substantia nigra. About 50% of dopaminergic neurons are being lost from the substantia nigra pars compacta to the striatum and Lewy bodies are found in surviving neurons (Venda et al., 2010). Lewy bodies are intracytoplasmic inclusions composed largely of the α-synuclein amyloid fibrils. Different paths of α-synuclein oligomerization can lead to soluble oligomers / protofibrils, insoluble fibrils comprised mostly of β-pleated sheets, and accumulation of these fibrils into Lewy bodies (Sidhu et al., 2004).

The aggregation of α-synuclein is, therefore, implicated in impairing neuronal function and cellular apoptosis in the development of Parkinson's disease. α-Synuclein is a soluble, acidic protein of a small size that is expressed mostly at presynaptic terminals in the central nervous system with functions related to vesicle trafficking. However under a pathological condition, α-synuclein can induce cytotoxicity via abnormal aggregation or interaction with other ligands such as dopamine and metal ions (Recchia et al., 2004).

Recent study has proposed that dopaminergic neuronal loss in Parkinson's disease is caused by combinations of multiple oxidative insults on various cellular targets, among which are mitochondrial dysfunction, dopamine oxidation and the impairment of protection system against oxidative stress (Venda et al., 2010). In animal models and patients suffering from Parkinson's disease, biological markers related to reactive oxygen species and oxidative stress were found (Kim and Kang, 2005). Oxidative stress has long been implicated in other neurodegenerative diseases such as Alzheimer's disease, dementia with Lewy bodies, and Huntington's disease as well (Uversky et al., 2002). Dopamine, a key neurotransmitter in the dopaminergic neurons, is readily oxidized and also generates reactive oxygen species such as superoxides, hydrogen peroxide, and free radicals, which may cause covalent modifications on proteins or damage cells (Leong et al., 2009). Synergistic interaction of α-synuclein and dopamine has been found to cause toxicity in HEK293 cells. Moreover, oxidative products of dopamine have been suggested to interact with α-synuclein and induce formation of soluble α-synuclein oligomers or protofibrils. Many research now suggest that these oligomeric intermediates of α-synuclein are the ones responsible for pathogenic cytotoxicity, rather than fibrils which are found to be relatively inert (Nakaso et al., 2013).

α-Synuclein is ubiquitously expressed in many brain regions at high levels, including substantia nigra, and this accounts up to approximately 0.1% of total proteins in brain. High expression of α-synuclein has been known to be the main cause of protofibril and Lewy body formations in Parkinson's disease. However, increasing evidence

suggests that selective loss of dopaminergic neurons is primarily linked to dopamine itself. This is because the substantia nigra neurons express much less α-synuclein compared to other brain regions and it is these neurons that are specifically vulnerable in Parkinson's disease (Sidhu et al., 2004). Therefore, concerted relation in dopamine, dopamine-mediated oxidative stress, and α-synuclein aggregation are the key features in promoting neurodegeneration in Parkinson's disease.

In this thesis, dopamine under two pH conditions which mimic those of vesicle and cytosol in the dopaminergic neurons were incubated with α-synuclein to characterize their different oxidation pattern and aggregation pathways. The hypothesis is that dopamine remains un-oxidized and stable in the acidic condition of the vesicle, but oxidized in the cytosolic pH and, hence, causes covalent modification on α-synuclein. Such modification would cause different α-synuclein aggregation patterns, such as stabilization of toxic protofibrils or oligomers instead of fibril formation.

2. Theoretical Background

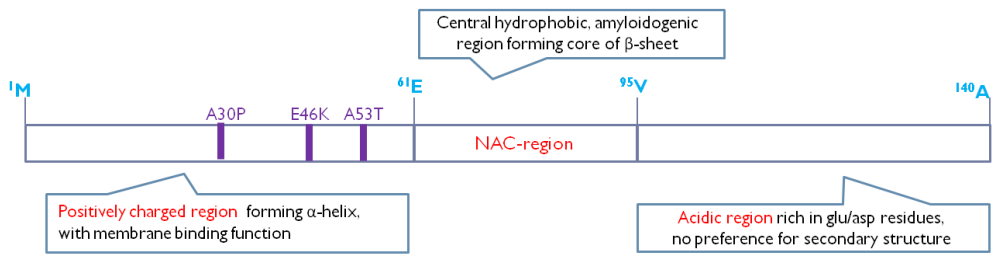
2.1 Structure and function of α-synuclein

α-Synuclein is a small protein of 140 amino acid residues (14 kDa) that is also characterized by its extreme heat resistance and high acidity (Recchia et al., 2004). Human α-synuclein is originally identified as a precursor of the non-Aβ component peptide in amyloid plaques from the brains with Alzheimer's disease and in the electric organ of the ray *Torpedo californica* (Sidhu et al., 2004). It is a highly conserved member of the synuclein family along with β-synuclein and γ-synuclein that are all structurally similar and abundantly expressed in various regions of the brain. However, only α-synuclein is implicated in promoting Parkinson's disease and is in close proximity to synaptic vesicles at presynaptic terminals.

Under physiological conditions, α-synuclein belongs to a group of intrinsically disordered proteins with little or no defined secondary structure in aqueous solutions (Uversky et al., 2002). Its structure looks similar to random coils. Yet, it may adopt specific secondary structures of α-helix or β-sheet conformation under specific conditions or interaction with different ligands such as phospholipid or metals (Recchia et al., 2004).

The overall sequence of α-synuclein can be divided into 3 distinct domains (Fig. 1). The highly conserved amino-terminal domain is comprised of residues 1-60 with six

imperfect repeats of KTKEGV consensus sequence (Dusa et al., 2006). The functional importance of these repeats is unknown. Although disordered in aqueous solution, the amino-terminal of α-synuclein adopts α-helix conformations upon binding to negatively charged phospholipids. This indicates that α-synuclein is associated with membrane for specific functions under physiological circumstances. This domain also has three loci for missense mutations of A53T, A30P and E46K that have been commonly found on early-onset in the familial form of Parkinson's disease (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004). The central hydrophobic domain of α-synuclein, consisting of residues 61-95, is the non-Aβ component (NAC) found in amyloid plaque of Alzheimer's disease. Such highly amyloidogenic domain contributes to α-synuclein aggregation via a conformational change from disordered random coil to β-sheet configuration (Recchia et al., 2004). Hydrophobicity is thought to play a role in sheet-sheet interaction. Lastly, the carboxyl-terminal domain of residues 96-140 is highly acidic, containing many glutamate and aspartate residues (Sidhu et al., 2004). It has no distinct propensity for secondary structures, unlike other domains, but is known to be responsible for chaperone-like activity of α-synuclein. Carboxyl-terminal truncated from α-synuclein has been detected in Lewy bodies and thus regarded to be significant for α-synuclein fibrillation in neurodegenerative diseases (Recchia et al., 2004).



1MDVFMKGLSK10 11AKEGVVAAAE20 21KTKQGVAEAA30 31GKTKEGVLYV40

41GSKTKEGVVH50 51GVATVAEKTK60 61EQVTNVGGAV70 71VTGVTAVAQK80

81TVEGAGSIAA90 91ATGFVKKDQL100 101GKNEEGAPQE110 111GILEDMPVDP120

121DNEAYEMPSE130 131EGYQDYEPEA140

Figure 1. (a) Schematic representation of α-synuclein domains (b) Human α-synuclein sequence. Underlined, N-terminal sequence; Dash-underlined, NAC domain sequence; Double-underlined, C-terminal sequence

Although still under discussion, α-synuclein's known physiological functions include regulation of vesicle trafficking in presynaptic neurons. α-Synuclein is an inhibitor of phospholipase D_2, a transmembrane enzyme, which hydrolyzes phosphatidylcholine to phosphatidic acid, and promoting secretory vesicle production from membranes. α-Synuclein can also bind vesicles directly and regulate vesicle budding or turnover (Glaser et al., 2005).

2.2 Aggregation pathways of α-synuclein induced by different pathological factors

The α-synuclein pathological aggregation pathway can be divided into two major types: on-pathway and off-pathway (Fig. 2). On-pathway is characterized by the formation of partially folded intermediates with exposed hydrophobic clusters for intermolecular interaction, followed by the formation of beta-sheet rich-oligomers that act as nuclei for nucleation-elongation type process. The oligomers then convert to protofibril intermediates and finally, lead to fibrillation (Uversky et al., 2001[a]). Off-pathway oligomer that does not consist of beta-sheet structure can be formed by independent structural changes from the monomer, but its nature is unclear due to variations in conformations and properties (Ehrnhoefer et al., 2008).

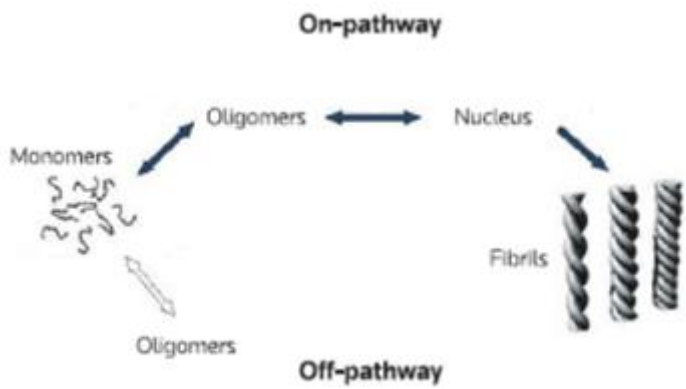


Figure 2. Schematic representation of α-synuclein aggregation via on- and off-pathway (Madine and Middleton, 2009)

Originally, α-synuclein fibril was thought to be the main cytotoxic species in Parkinson's disease, but increasing evidence suggests that oligomer or protofibril itself induces more cytotoxicity. However, whether the cytotoxic oligomer is on-pathway or off-pathway remains unclear, due to difficulties in separating coexisting species of different oligomers (Giehm et al., 2011).

Many factors have been discussed in previous studies that promote various aggregation pathways of α-synuclein, such as overexpression of the protein, mutation at specific positions, and oxidative stress (Recchia et al., 2004). Overexpression of α-synuclein via triplication of the SNCA gene was found in autosomal dominant form of Parkinson's disease (Singleton et al., 2003). Inducing overexpression in murine models for 6 months led to cytoplasmic inclusions and dystrophic neurites in dopaminergic neurons similar to those found in patients with Parkinson's disease. Loss of 30~80% of dopaminergic neurons and 40~50% of dopamine levels along with significant motor impairment progressed over time (Kirik et al., 2002). Higher level of α-synuclein not only increases the rate of aggregation by itself, but also relates to other pathological factors such as increased mutant form of α-synuclein and more oxidative stress in the dopaminergic neurons.

Another pathological factor found in the autosomal dominant form of Parkinson's disease is mutations at specific residues of α-synuclein. Ala53Thr, Ala30Pro and Glu46Lys substitutions in families of independent cases with Parkinson's disease suggest genetic causes for the disease (Polymeropoulos et al., 1997; Kruger et al.,

1998; Zarranz et al., 2004). These point mutations do not change the global structure of α-synuclein but induce more subtle changes. Ala30Pro reduces the propensity to form helical structure, Ala53Thr slightly enhances extended conformations around the mutation site, and Glu46Lys enhances the interaction between the amino- and the carboxyl- terminals of the protein. Ala30Pro mutation induces oligomerization, while Ala53Thr and Glu46Lys enhance fibrillation of α-synuclein. However, whether these mutations are the exact causes for the disease is still controversial, since new evidence indicates loss of dopaminergic neurons is higher in oligomer-forming Glu35Lys and Glu57Lys mutants than fibril-forming Ala53Thr mutant (Breydo et al., 2012).

Oxidative stress as the main cause for Parkinson's disease is also being supported by increasing evidence. Increased metal concentration has drawn much attention as one of the causes for oxidative stress that modifies α-synuclein in Parkinson's diseases. Moreover, an epidemiological study has pointed out occupational exposure to single or combinations of metals such as manganese, copper, lead, and iron as a risk factor for Parkinson's disease (Gorell et al., 1999). Several groups have reported binding of metal ions to α-synuclein and their effects on α-synuclein structural changes leading to its aggregation. Among many Group I, II, III and transition metals from periodic table at their various transition states, Al(III), Fe(III), Cu(II) and Mn(II) have the strongest effects on the conformational changes of the protein. They favor formation of partially folded intermediate that leads to fibrillation, via binding to the negatively charged carboxyl-terminal of α-synuclein (Uversky et al., 2001[b]). Now it is known that several metal ions can bind α-synuclein at μM-mM range concentrations non-specificially on

carboxyl terminal or on specific sites of amino terminal. Among the metals with the strongest effect, Cu(II) and Fe(III) binding was found to be able to catalyze the production of hydrogen peroxide and hydroxyl radical, which exert oxidative stress on α-synuclein. The stress effects include fragmentation of the protein, oxidation of specific residues and formation of intermolecular dityrosine bridges. The role of metal ions in exerting oxidative stress is further supported by application of antioxidants such as Vitamin E which attenuated the effect (Li et al., 2011; Binolfi et al., 2012).

2.3 α-Synuclein regulates dopamine homeostasis

α-Synuclein, expressed widely in brain but selectively affecting dopaminergic neurons in Parkinson's disease, has brought attention to the α-synuclein-dopamine relationship for a pathological cause. The general physiological roles of α-synuclein suggest that it may be responsible for dopamine synthesis and metabolism (Sidhu et al., 2004). Dopamine synthesis involves the amino acid tyrosine conversion into L-DOPA by tyrosine hydroxylase (TH), followed by decarboxylation into dopamine by amino acid decarboxylase (AADC) in the cytosol of the dopaminergic neurons (Fig. 3). α-Synuclein overexpression reduces the activity of the TH promoter, hence decreasing expression of the enzyme (Gao et al., 2007). Furthermore, α-synuclein has been shown to bind to TH and AADC to prevent phosphorylation-mediated activation of the enzymes while increasing the activity of protein phosphatase 2A (PP2A), which in turn, inhibits TH and AADC through dephosphorylation (Perez et al., 2002; Peng et al., 2005; Tehranian et al., 2006). Such loss of activities for dopamine synthesizing enzymes may account for the lowered level of dopamine in Parkinson's disease.

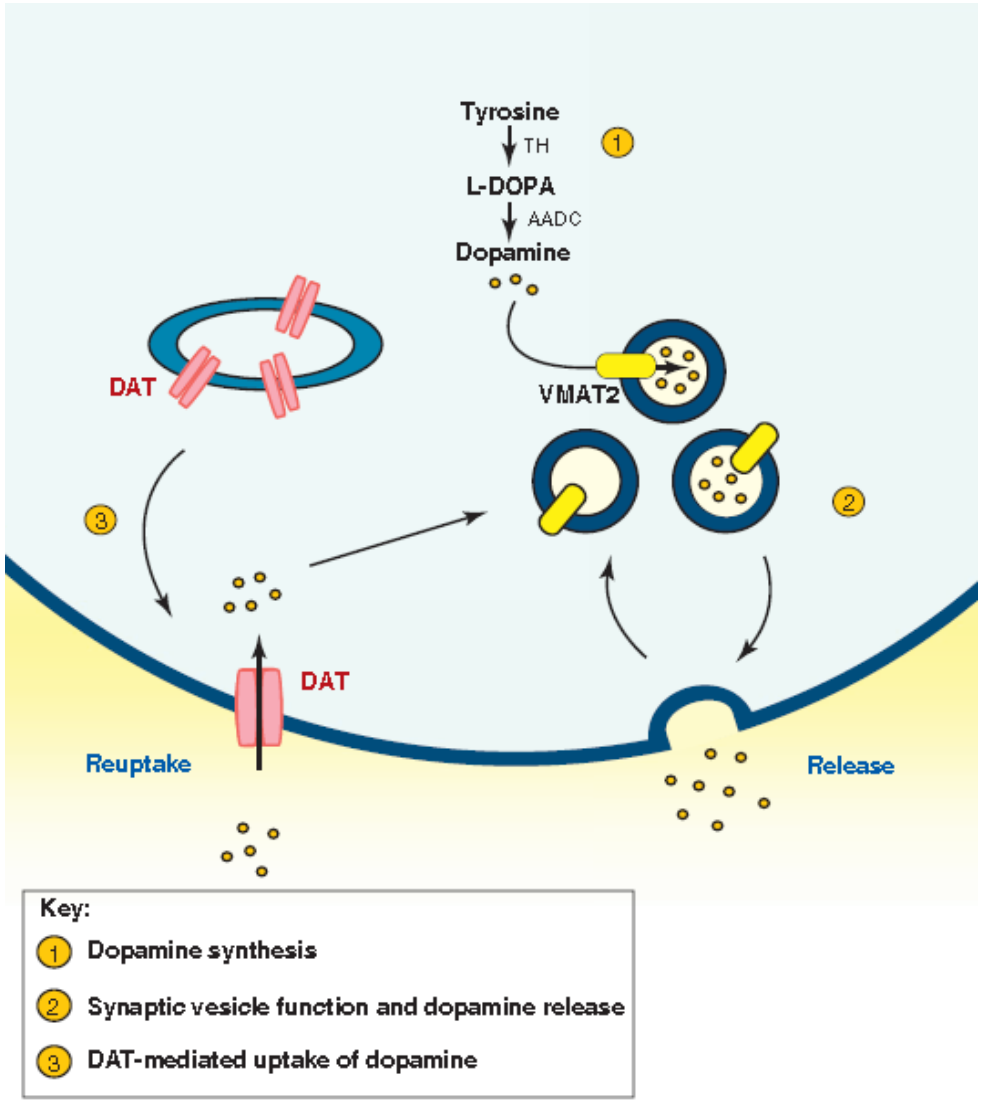


Figure 3. Dopamine synthesis, storage, release into synapse and uptake for recycle in the presynaptic terminal. TH, tyrosine hydroxylase; AADC, amino acid decarboxylase; VMAT2, vesicular monoamine transporter 2; DAT, dopamine transporter (Venda et al., 2010).

Another effect of α-synuclein on dopamine homeostasis may come from the regulation of vesicle trafficking. Dopamine synthesized in cytosol is immediately sequestered into preformed vesicles for storage via vesicular monoamine transporter 2 (VMAT2) protein (Fig. 3). When electrical nerve signal stimulates the presynaptic terminal, the vesicles dock to cell membrane to release dopamine to synaptic cleft. α-Synuclein is involved in the regulatory mechanism for vesicle availability in different pools, and docking and fusion to the cell membrane (Fig. 4). Suppression of α-synuclein in primary cultured hippocampal neurons decreased the reserve synaptic vesicle pool, and mice lacking α-synuclein showed an increased rate of refilling the releasable pool (Murphy et al., 2000; Yavich et al., 2004). Mice overexpressing α-synuclein have impaired synaptic vesicle exocytosis and reduced reclustering of vesicles after endocytosis, which is necessary for recycling of the vesicles (Yavich et al., 2004; Nemani et al., 2010). α-Synuclein mutations of Ala53Thr and Glu46Lys in hippocampal cultures also caused an inhibition of exocytosis similar to the one observed by overexpression of the protein (Nemani et al., 2010). Although these mechanisms were not confirmed directly on dopaminergic neurons, similarity between hippocampal and dopaminergic neurons suggests suppression of α-synuclein leads to more dopamine release into synaptic cleft, while overexpression or mutations reduce dopamine release and decrease the vesicle pool available for dopamine recycling (Venda et al., 2010). Therefore, this can cause insufficiency in sequestering cytosolic dopamine into the vesicles for stable storage.

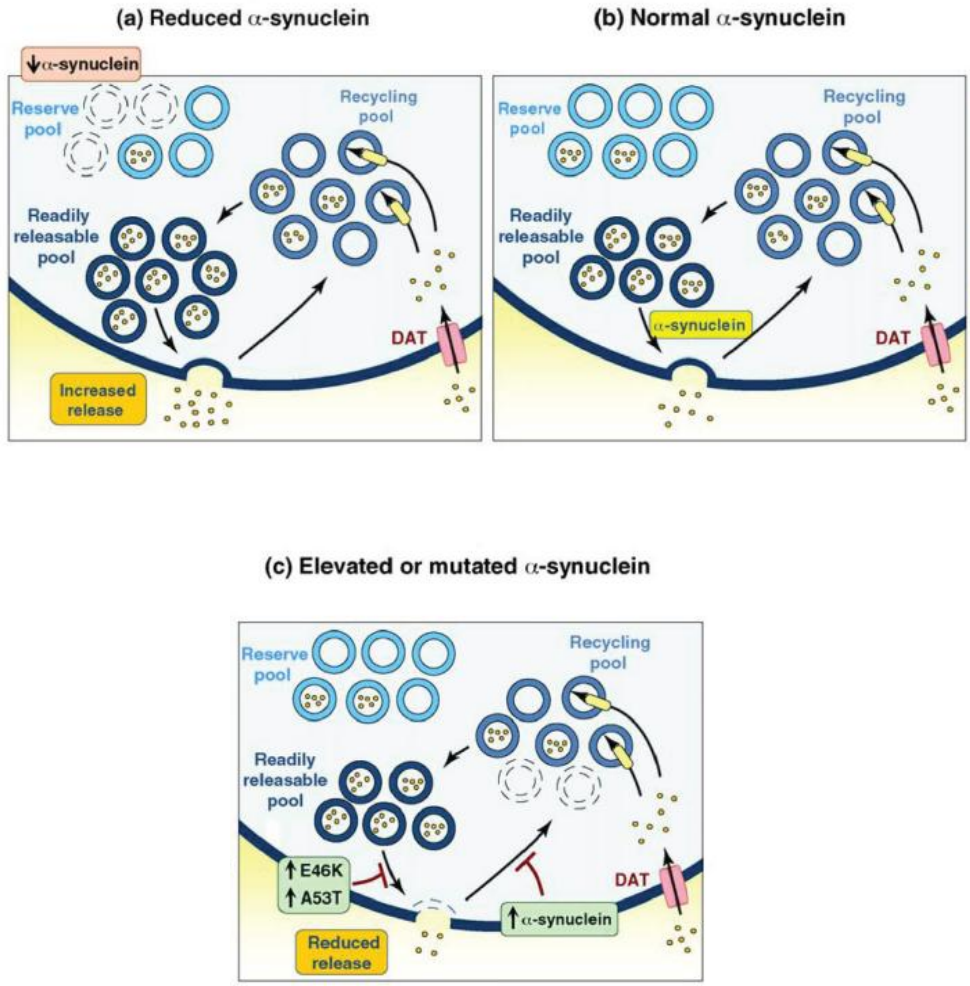


Figure 4. Effect of (a) reduced, (b) normal, (c) elevated or mutated α-synuclein on the vesicle trafficking in presynaptic terminal (Venda et al., 2010).

Dopamine released into the synaptic cleft is recycled by uptake via dopamine transporter (DAT) protein (Fig. 3). The non-Aβ component domain of α-synuclein binds directly to the carboxyl terminal of DAT, and the resulting α-synuclein-DAT complex mediates clustering of DAT in the membrane to accelerate dopamine uptake (Lee et al., 2001). This would increase the intracellular concentration of dopamine.

2.4 Dopamine-mediated oxidative stress

Dopamine can cause oxidative stress and cellular damage because it is known to be relatively unstable in the cytosol of dopaminergic neurons compared to the vesicle and thus undergo oxidation by two mechanisms. One is enzymatic reaction by monoamine oxidase (MAO) and alcohol dehydrogenase, which generates non-toxic 3,4-dihydroxy-phenylacetic acid (DOPAC) along with hydrogen peroxide. MAO replaces the amine group of dopamine with aldehyde in the presence of molecular oxygen, and the resulting aldehyde is oxidized to acid by the alcohol dehydrogenase with NAD^+ (Elsworth and Roth, 1997). Another mechanism is auto-oxidation under neutral pH, while acidic pH allows stable unoxidized dopamine. The first step of dopamine oxidation, creating dopamine-o-quinone, shows an oxidation peak at ~300mV in cyclic voltammetry traces in physiological saline, pH 7.4 condition. There is +50mV increase per unit pH decrease, indicating the reaction is much favored in higher pH (Sulzer and Zecca, 2000). This enables auto-oxidation of dopamine in the cytosol of dopaminergic neurons, with formation of dopamine-o-quinone, aminochrome, 5,6-dihydroxyindole and indole-5,6-quinone along the pathway, until relatively stable melanin is formed (Fig. 5).

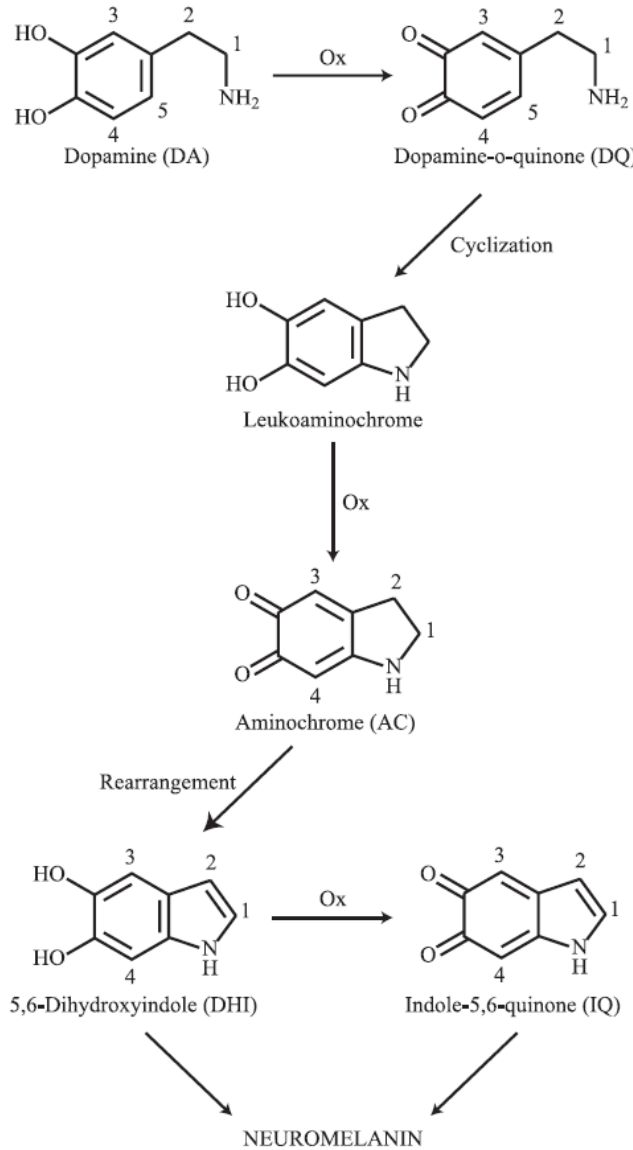


Figure 5. Dopamine oxidation pathway (Bisaglia et al., 2007)

Oxidation of dopamine has been implicated in many Parkinson's disease studies. Flavonoids, which have phenolic rings and can undergo auto-oxidation like dopamine, can inhibit fibrillation of α-synuclein and induce flavonoid-modified monomeric and oligomeric species of α-synuclein (Meng et al., 2009). Dopamine was shown to be able to stabilize toxic α-synuclein protofibril, which may be one mechanism of stabilizing on-pathway intermediate (Conway et al., 2001). On another study, dopamine promoted formation of non-fibrillar oligomer, which reflects the oligomer from off-pathway (Lee et al., 2011). These variations in the role of dopamine oxidation on α-synuclein aggregation pattern may arise from different conditions used in the studies, such as how the dopamine was oxidized, and the concentrations of dopamine and α-synuclein. It still remains elusive how the dopamine oxidation exerts cytotoxicity via changes in α-synuclein aggregation.

3. Materials and Methods

3.1 Materials

Human recombinant α-synuclein gene cloned in pRK172 was a kind gift from Professor Jin Ryoun Kim (Department of Chemical and Biological Engineering, Polytechnic Institute of New York University) and Professor Seung R. Paik (Department of Chemical and Biological Engineering, Seoul National University). For protease inhibition cocktail during lysis, phenylmethyl sulfonyl fluoride (PMSF) was purchased from Amresco, leupeptin from Sigma-Aldrich, lysozyme from Bio Basic Canada and DNase I from Biosesang. For α-synuclein purification, DEAE bead was purchased from GE Healthcare, S-200 bead from Sigma-Aldrich, and purification columns from Bio-Rad. SDS-PAGE analysis was performed using 15% bis-Tris gels and SDS-PAGE kit was obtained from Bio-Rad. Thioflavin T dye (ThT) was generously obtained from Professor Seung R. Paik. For UV and fluorescence measurements, quartz glass cell from Hellma was used. All other materials, including dopamine hydrochloride were purchased from Sigma-Aldrich. All chemicals used in this study were of analytical grade.

3.2 α-Synuclein expression and purification

Human recombinant α-synuclein gene cloned in plasmid pRK172 was expressed in *Escherichia coli* BL21 (DE3) cells. The cells were transformed with the plasmid and

cultured on a LB plate containing 0.1 mg/ml of amipicillin. A bacterial clone from the plate was pre-cultured overnight in 100 ml LB medium with ampicillin at 37 ℃ and 180rpm. Next day, 10 ml of the pre-culture was separately added to each of four 1 L LB/Amp media to final 4 L and incubated at 37 ℃ until OD_{600nm} reached 0.6~0.8. IPTG was added at this point with 0.8 mM working concentration to induce expression and further incubation was carried out until OD_{600nm} reached 1.3.

The cells were harvested by centrifugation at 3600rpm for 20 minutes, and lysed with lysis buffer containing 20 mM Tris/Cl pH 7.5, 0.1 M NaCl, 0.1 mM PMSF, 1 μg/ml leupeptin, 2 mM EDTA, 0.1 mg/ml lysozyme and 10 U/ml DNase while stirring at 4 ℃. The lysed cells were incubated at 37 ℃ with continuous shaking for 2 hours, heated at 100 ℃ for 20 minutes to denature proteins other than α-synuclein, centrifuged again at 4 ℃, 7000rpm for 20 minutes, and filtered with 0.22 μm syringe filter.

α-Synuclein was first purified with DEAE-anion exchange chromatography. The filtered protein from the previous step was loaded onto the DEAE column equilibrated beforehand with 20 mM Tris/Cl (pH 7.5) containing 0.1 M NaCl. Elution of α-synuclein was carried out with linear salt gradient between 0.1 M and 0.5 M NaCl in the equilibration buffer. α-Synuclein containing fractions were identified with OD_{280nm} and SDS-PAGE, and subjected to S-200 size-exclusion chromatography. The S-200 column was equilibrated with 20 mM Mes (pH 6.5) containing 2 mM EDTA and loaded with the fractions from the previous step. Eluted fractions from S-200 were

checked with OD_{280nm} and SDS-PAGE for the presence of pure α-synuclein and dialysed for buffer change (Paik et al., 1997).

The purified α-synuclein was quantified with bicinchoninic acid (BCA) protein assay kit (Thermo scientific) according to the manufacture's protocol and divided into aliquots of 1 $mg \cdot mL^{-1}$ in 20 mM Mes buffer (pH 6.5) for storage at -80 ℃.

3.3 Dopamine oxidation

100 μM of dopamine was incubated in 10 mM phosphate buffer with pH 5.8 or pH 7.4 for 125 hours at 37 ℃, 180rpm. Aliquots at various time intervals were subjected to UV absorbance scan between 600~190nm using a Varian Cary 50 UV spectrophotometer to examine dopamine oxidation (Graham, 1978).

3.4 Incubation of α-synuclein under different dopamine conditions

Sample mixtures containing 0.5 mg/mL α-synuclein in 10 mM phosphate buffer in the presence and absence of 100 μM dopamine were incubated at 37 ℃, 180rpm. The phosphate buffer was in two different pH of 5.8 and 7.4. The dopamine solution to be added to pH 7.4 α-synuclein sample was pre-incubated for 10 hours in pH 7.4 phosphate buffer to allow oxidation of dopamine.

3.5 Fibrillation studies using ThT assays

ThT fluorescence measurements were used to monitor α-synuclein amyloid fibril formation via Perkin-Elmer LS-55 luminescence spectrometer (Koo et al., 2013). Aliquots (20 µL) of the sample incubation mixtures were removed at various time points and added to a cuvette containing 20 µL of 25 µM ThT and 160 µL of 50 mM glycine buffer at pH 8.5. The solution was excited at 450 nm and the emission intensity at 482 nm was recorded and plotted against time.

3.6 Intrinsic fluorescence

Aliquots (20 µL) of the α-synuclein incubation sample mixtures were removed at various time points and mixed into a cuvette containing a 180 µL solution of 10 mM phosphate buffer at pH 5.8 and pH 7.4. Dityrosine fluorescence was measured with excitation at 320 nm and dopamine-α-synuclein adduct fluorescence was measured with excitation at 360nm. The emission was scanned between 300 and 500 nm. The wavelength and intensity at the maximum emission were recorded (Dusa et al., 2006).

3.7 SDS-PAGE

Aliquots (20 µL) of the incubation sample mixtures were removed at various time points and subjected to SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) following the standard procedures and silver-stained using Elpis Biotech

PowerStain™ Silver Stain kit according to the manufacturer's protocol.

3.8 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to visualize the α-synuclein fibrils, oligomers and/or amorphous aggregates present at different time points of the aggregation process. The α-synuclein samples (10 µL) were applied onto carbon-coated grids (200-mesh) for 1 min, negatively stained with (10 µL) 20% uranyl acetate for 1 min, washed twice with deionized water, and dried for 4-5 min. They were viewed with Carl Zeiss LIBRA 120 Energy-Filtering Transmission Electron Microscope (120 kv).

4. Results and Discussion

4.1 Oxidation of dopamine

Dopamine was incubated in 10mM phosphate buffer with pH 5.8 and 7.4 to mimic the pH physiological dopamine encounters in vesicle and cytosol of the dopaminergic neurons, respectively. UV absorbance spectra showed that the maximum absorbance peak at 280nm did not change significantly over the time course of measurement when dopamine was present in acidic pH 5.8, indicating no auto-oxidation occurred and the unoxidized dopamine remained stable (Fig. 6a). On the other hand, dopamine in pH 7.4 showed overall increases in the spectra, with diminished absorption maximum peak (Fig. 6b). The overall increase is attributable to light scattering caused by formation of melanin, which appear as black precipitates in the dopamine solution by naked eye (Bisaglia et al., 2007). Melanin is a relatively stable end product in the dopamine oxidation pathway, thus appearance of melanin is an indication of dopamine auto-oxidation in pH 7.4. Dopaminergic neurons with higher concentration of melanin-pigmentation were found to be more vulnerable to death than the non-pigmented neurons, which suggest a high level of oxidation has occurred in the neuronal cells (Hirsch et al., 1988).

Changes in the absorbance spectra for the oxidation of dopamine relative to the maximum peak of wavelength 280nm were also observed (Fig. 7). In pH 5.8, dopamine remains un-oxidized at least for 125 hours, shown by a constant level of

OD_{280nm} around 0.17. Under pH 7.4, however, OD_{280nm} progressively increases with the incubation time until 30 hours due to the effect of overall spectra increase, where it reaches the maximum OD_{280nm} of approximately 0.35. From these results, dopamine could be controlled for how much auto-oxidation it undergoes *in vitro* by controlling the pre-incubation time in the pH 5.8 and 7.4 before being added to α-synuclein. If oxidation is allowed over 30hours in pH 7.4, most dopamine would have converted into melanin, which is a stable end product of the dopamine oxidation pathway, and may not be able to exert oxidative stress. Therefore dopamine in pH 7.4 was pre-incubated for 10 hours where the OD_{280nm} is half the maximum, before being added to α-synuclein incubation.

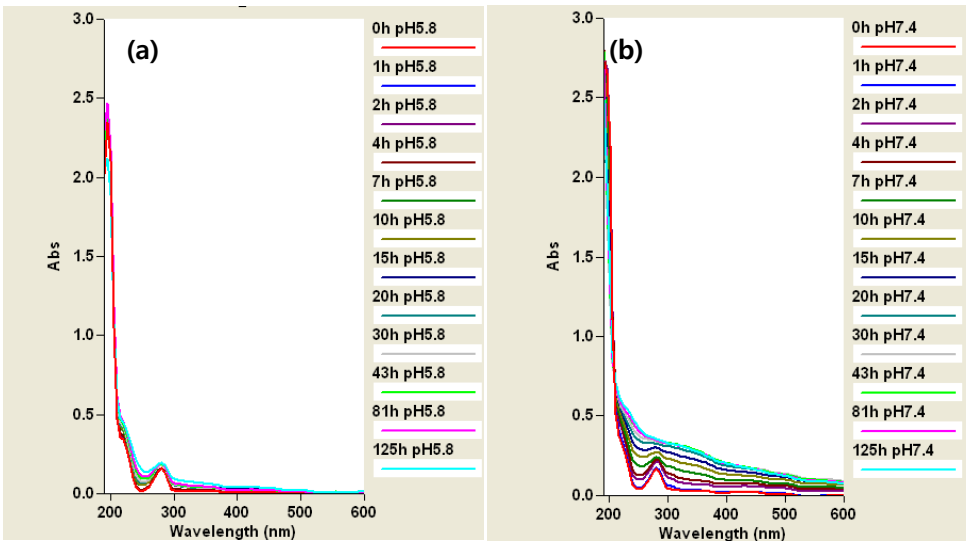


Figure 6. Oxidation of dopamine monitored by changes in UV absorbance in (a) pH 5.8 and (b) pH 7.4

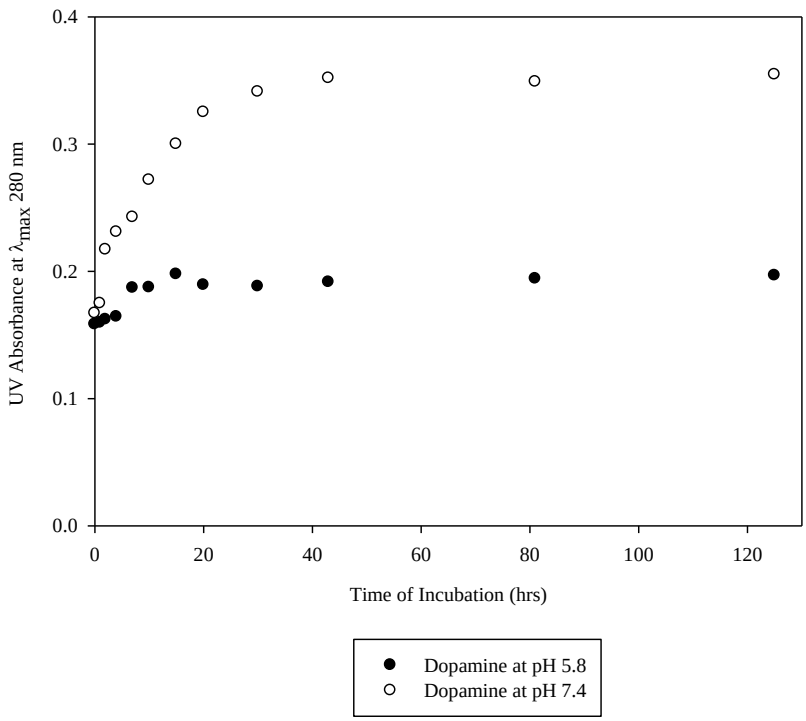


Figure 7. Changes in oxidation of dopamine observed by OD_{280nm} as a function of incubation time

4.2 Fibrillation kinetics of α-synuclein

A time-dependent ThT fluorescence assay was performed to monitor the fibrillation kinetics of different α-synuclein samples, as ThT dye binds specifically to β-sheet rich fibrils (Fig. 8). A typical nucleation-dependent α-synuclein aggregation kinetics show sigmoidal curves, characterizing initial phase as a lag phase for nucleation, including rate-determining critical nucleus formation, mid-phase as an elongation phase for fibril growth on preformed nuclei, and final plateau as depletion of soluble monomers and intermediates (Dusa et al., 2006). Figure 8 shows the changes in the ThT fluorescence intensity for α-synuclein alone and α-synuclein in presence of dopamine, each under pH 5.8 or 7.4 conditions during fibrillation. Under the acidic pH of 5.8, the fibrillation kinetics data indicate that there is a clear increase in the ThT intensity for α-synuclein in presence of dopamine compared to α-synuclein alone. α-Synuclein with dopamine formed fibrils more slowly until the end of nucleation phase (37 hrs) and the beginning of elongation phase (55 hrs). However, as the incubation time further progressed, the fibrillation rate of α-synuclein in presence of dopamine significantly increased to a higher level than that of α-synuclein only. Overall, both samples showed a moderate increase of nucleation, elongation, and a plateau for fibrillation.

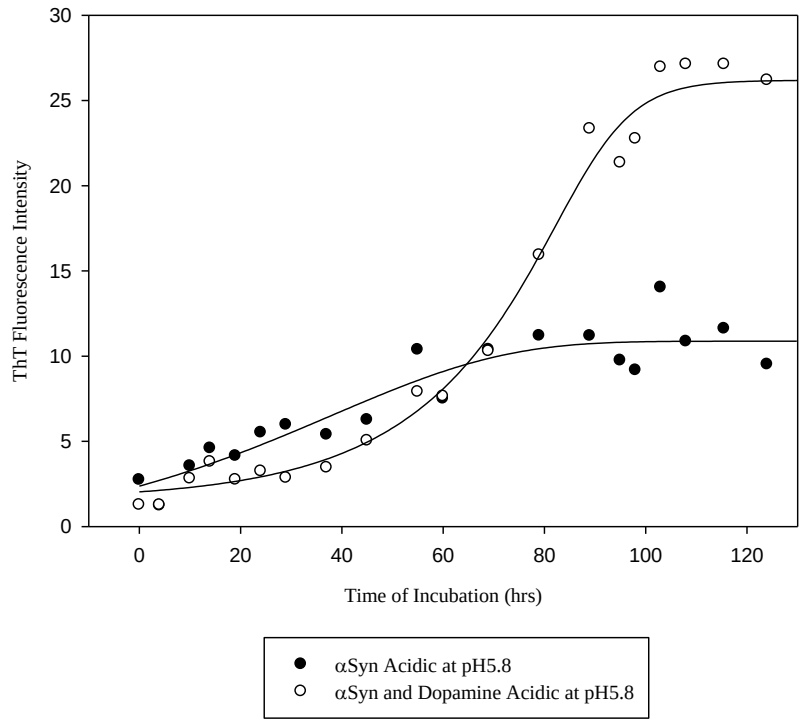


Figure 8. ThT fluorescence intensity for α-synuclein in the absence and presence of dopamine in pH 5.8

In neutral pH 7.4, a slow nucleation was observed until 80 hours of incubation time for α-synuclein alone (Fig. 9). A typical sigmoidal curve was observed after 80 hours, corresponding to the α-synuclein nucleation-dependent fibrillation kinetics. Under the same pH in presence of dopamine, overall constant ThT intensity was observed, with only a minor increase at the beginning of the incubation. Until the sample with α-synuclein alone reached plateau after 120 hours of incubation, α-synuclein with dopamine remained significantly low in terms of ThT fluorescence. Thus, the kinetics assay presented a strong fibrillation inhibition on α-synuclein incubated with dopamine in pH 7.4, due to the dopamine oxidation effect.

It is interesting to note that the oxidized and un-oxidized dopamine showed the opposite effects in terms of fibrillation of α-synuclein. The oxidized dopamine suppressed fibrillation, possibly by oxidation-mediated covalent modifications on the protein. The un-oxidized dopamine, on the contrary, enhanced the fibrillation, likely due to non-covalent interaction with the protein.

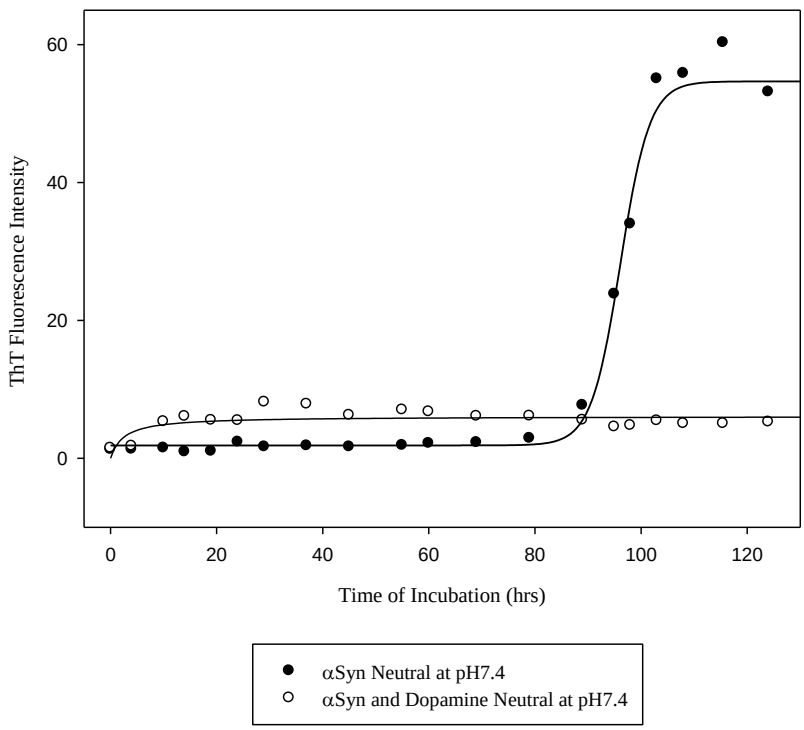


Figure 9. ThT fluorescence intensity for α-synuclein in the absence and presence of dopamine in pH 7.4

To ascertain that dopamine itself does not interfere with the ThT fluorescence measurement over the course of incubation, the ThT fluorescence intensity was measured in the absence of α-synuclein as a control (Table 1). In pH 5.8, the fluorescence intensity for dopamine was 0.86 at the beginning of incubation and 0.75 after 125 hours of the incubation time. In pH 7.4 they were measured approximately as 1.7 and 1.8 at the beginning and at 125 hours of incubation, respectively. Therefore, such negligible change by dopamine itself in both pH indicate that the ThT fluorescence increases observed in figure 8 and 9 are only due to fibrillation of α-synuclein during the incubation periods.

Time of Incubation (hrs)	Fluorescence Intensity for Dopamine	
	pH 5.8	pH 7.4
0	0.86	1.72
125	0.75	1.84

Table 1. ThT fluorescence changes by dopamine alone

4.3 Oligomer analysis

In order to find out if any oligomeric intermediate of α-synuclein was formed during the course of incubation, SDS-PAGE and TEM images were taken for analysis. Aliquots of the incubation samples were taken at 10, 80, 95 and 105 hours. At each hour, the four samples were in different phases as shown in Table 2.

Sample	Time of Incubation (hrs)			
	10	80	95	105
α-synuclein, pH 5.8	Lag	Late Elongation	Plateau	Plateau
α-synuclein, pH 7.4	Lag	Early Elongation	Middle Elongation	Plateau
α-synuclein + dopamine, pH 5.8	Lag	Middle Elongation	Late Elongation	Plateau
α-synuclein + dopamine, pH 7.4	N/A	N/A	N/A	N/A

Table 2. Fibrillation phases at each time the aliquots were taken for SDS-PAGE analysis. α-Synuclein with dopamine in pH 7.4 is not applicable because no fibrillation occurred during the incubation period.

α-Synuclein with dopamine in pH 5.8 shows oligomeric bands in SDS-PAGE from 80 hrs when it is in the middle of elongation phase and the bands persist even after the fibrillation reaches a plateau at 105 hours, showing on-pathway oligomers (Fig. 10). Relatively short length fibrils were shown by TEM (Fig. 11c). This is similar with α-synuclein in the same pH but without dopamine, which develops dimeric bands from 80 hours (Fig. 10). However, there is no oligomer larger than the dimer in this case, which could be explained by the formation of amorphous aggregates instead shown in TEM images (Fig. 11a). In a previous study, α-synuclein in millimolar concentrations and a low pH of 4 was found to form large amorphous aggregates (Hoyer et al., 2002). The formation of large amount of amorphous aggregates may account for why less ThT fluorescence is observed for α-synuclein in pH 5.8 without dopamine than with dopamine.

At 10 hours of incubation, all the samples were mostly monomers and showed no sign of oligomers except α-synuclein with dopamine in pH 7.4, which starts to develop SDS-resistant dimers and trimers as indicated by the weak bands at molecular weight (MW) of 36 and 50 kDa, respectively (Fig. 10). They do not occur on the exact multiples of α-synuclein monomer MW (14.4 kDa) because they have distinct conformations, which hinder their movement in the gel. The dimer and trimer have become more distinct gradually at 80, 95 and 105 hours. This indicates that dopamine oxidation in pH 7.4 began to inhibit fibrillation of α-synuclein by inducing oligomers instead at an early stage of incubation and throughout. The oligomer formed is an off-pathway oligomer, as no significant β-sheet structure was observed that is known to be

detected by the ThT fluorescence (Fig 9. and Fig 11d) (Wu et al., 2009). On the contrary, α-synuclein without dopamine in pH 7.4 undergoes extensive fibrillation, and no oligomer was observed at all (Fig. 9 and Fig. 10). TEM images show a large amount of long fibrils (Fig. 11b).

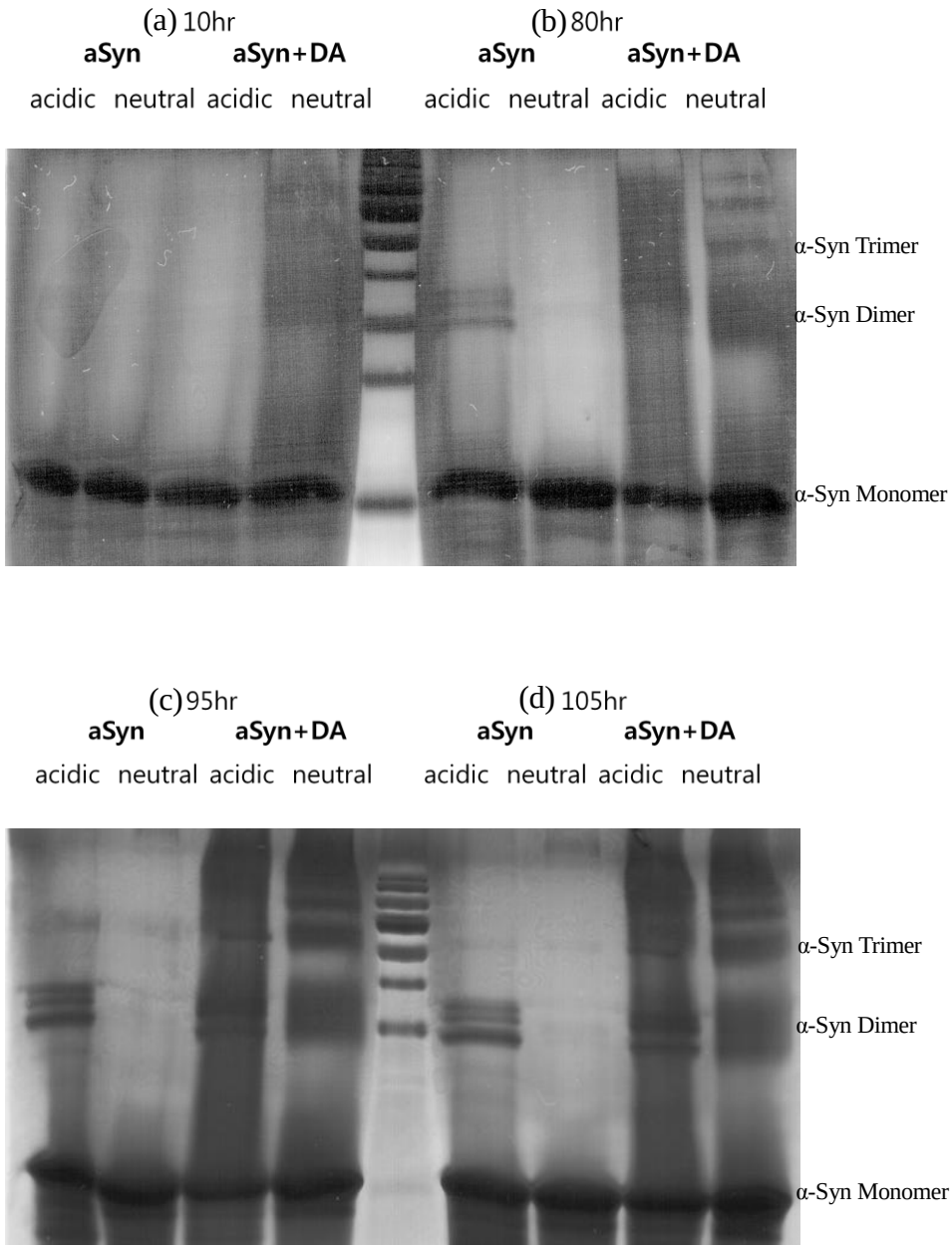


Figure 10. SDS-PAGE analysis on the oligomerization of α-synuclein samples taken at (a) 10 hrs, (b) 80 hrs, (c) 95 hrs and (d) 105 hrs. aSyn, α-synuclein; DA, dopamine

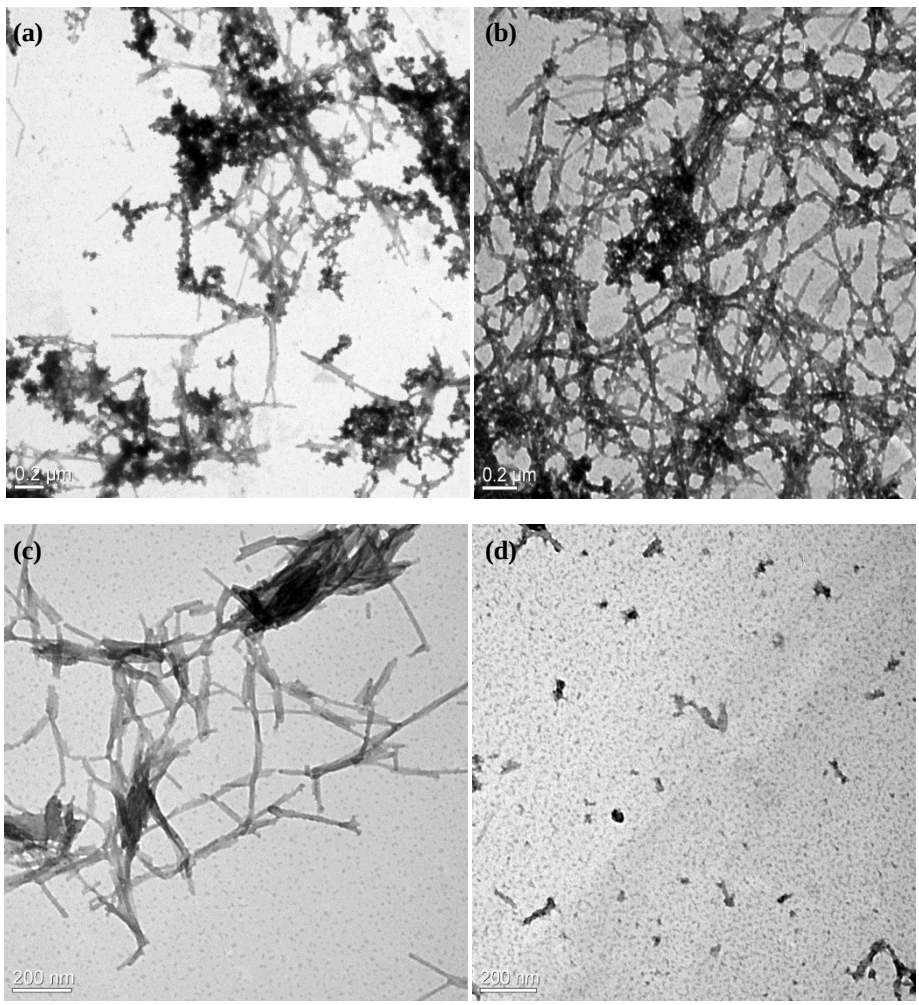
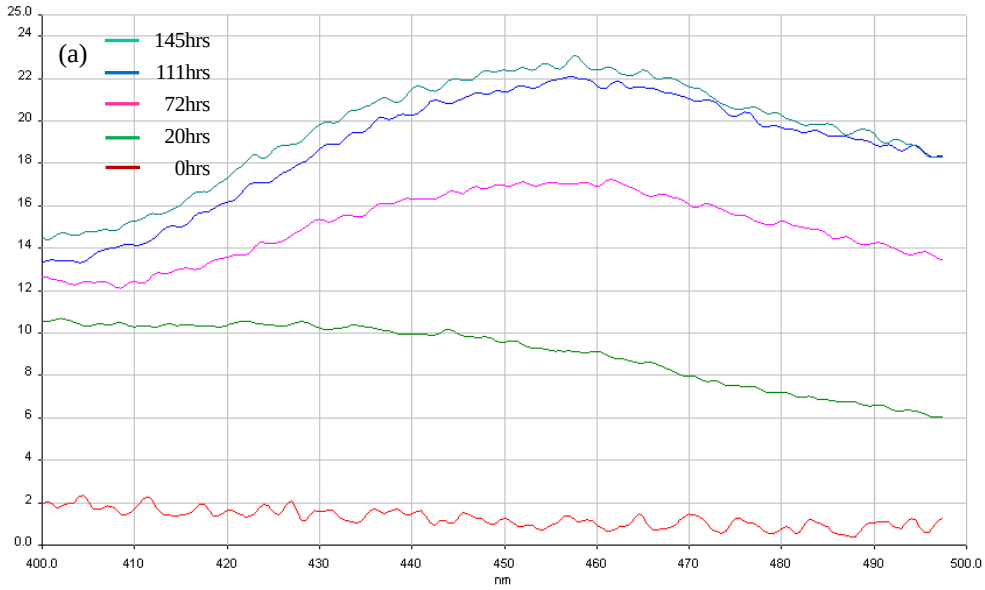
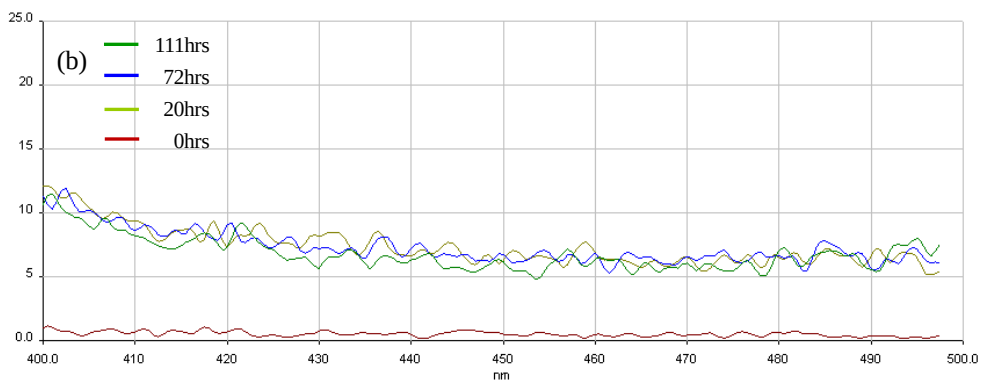


Figure 11. Transmission Electron Micrograph images of (a) α-synuclein in pH 5.8, (b) α-synuclein in pH 7.4, (c) α-synuclein with dopamine in pH 5.8, and (d) α-synuclein with dopamine in pH 7.4

4.4 Covalent modification of α-synuclein by oxidized dopamine

Oxidative stress is thought to either induce intermolecular dityrosine bridges between α-synuclein molecules through tyrosyl radical formation, or covalently attached dopamine-synuclein adducts (Conway et al., 2001; Krishnan et al., 2003). Both covalent modifications on α-synuclein can be tested via intrinsic fluorescence, with excitation at 320nm and emission at 420nm for the dityrosine bridge, and excitation at 360nm and emission at 465nm for the dopamine adduct approximately. The fluorescence showed no characteristic peak for dityrosine bridge, but peaks were observed for dopamine-synuclein adduct for the α-synuclein with dopamine sample in pH 7.4 (Fig. 12a). The adduct amount increased over the incubation time and seemed to reach a maximum after 145 hours (Fig. 13). However, other α-synuclein samples did not show any significant peaks representative of either the dityrosine bridge or the dopamine-synuclein adduct (Fig. 12b, 12c, and 12d).


(a)
145hrs
111hrs
72hrs
20hrs
0hrs
25.0
24
22
20
18
16
14
12
10
8
6
4
2
0.0
400.0
410
420
430
440
450
460
470
480
490
500.0
nm


(b)
111hrs
72hrs
20hrs
0hrs
25.0
20
15
10
5
0.0
400.0
410
420
430
440
450
460
470
480
490
500.0
nm

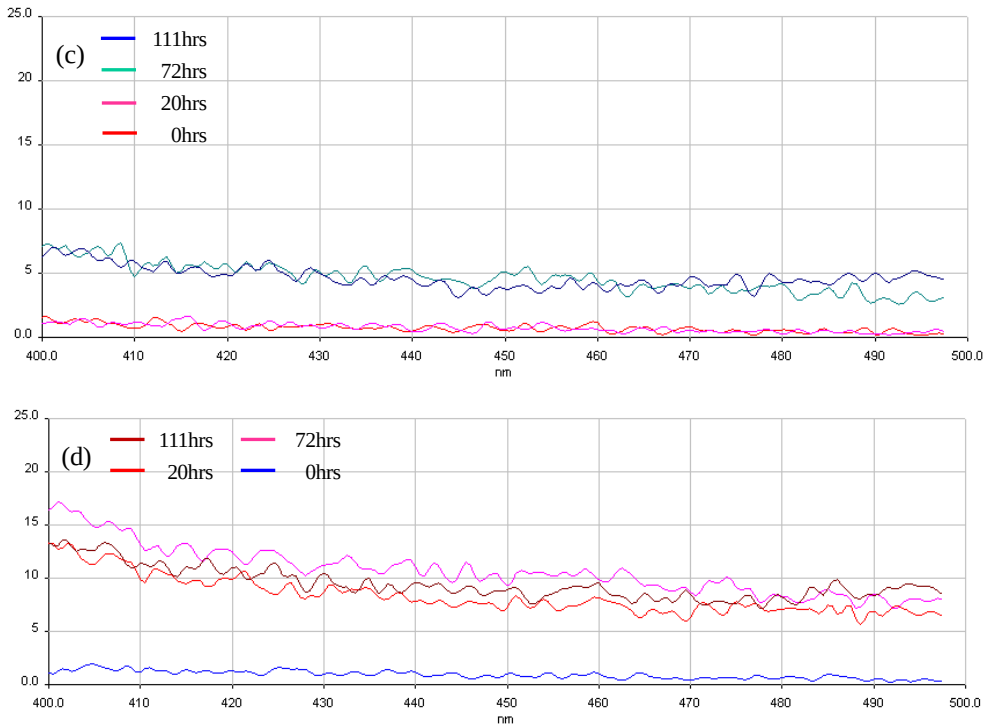


Figure 12. (a) Fluorescence spectra showing dopamine-synuclein adduct peaks at 457nm for α-synuclein with dopamine in pH 7.4; (b), (c) and (d) Fluorescence spectra for α-synuclein in pH 5.8, α-synuclein in pH 7.4, and α-synuclein with dopamine in pH 5.8 respectively.

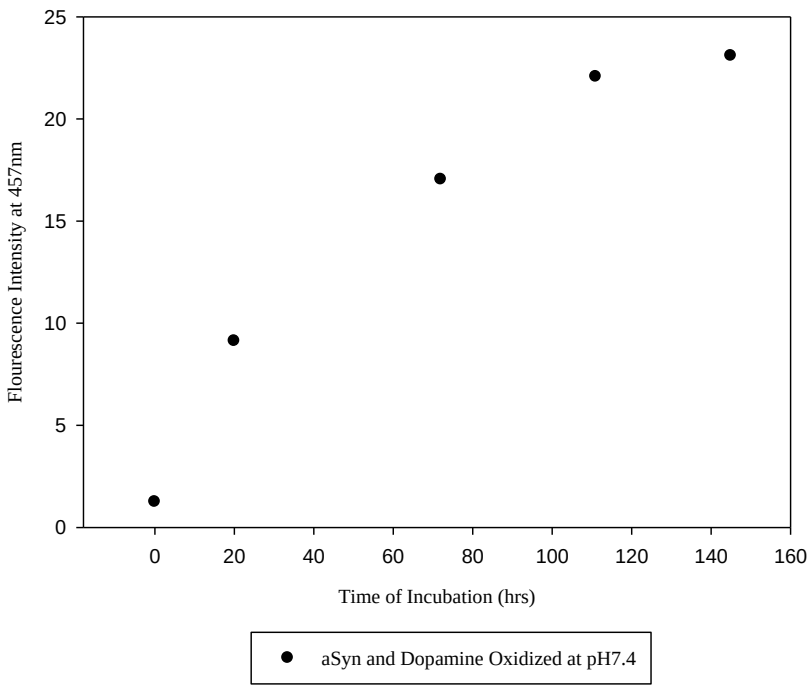


Figure 13. Formation of covalent dopamine-α-synuclein adduct monitored by fluorescence emission at 457nm as a function of incubation time

4.5 Overall discussion

In this study, I have shown that the pH changes alone can determine the oxidation state of dopamine and the α-synuclein aggregation pattern. This is important because pH is one of the major differences between the vesicle where dopamine is normally stored, and the cytosol where dopamine is found in high levels in pathological context.

α-Synuclein with unoxidized dopamine in acidic pH could develop short fibrils, and α-synuclein without dopamine in the same pH developed amorphous aggregate along with fibrils via stabilization of dimers. α-Synuclein without dopamine in neutral pH undergoes extensive fibrillation and no oligomer was seen, whereas with oxidized dopamine in neutral pH the major species was oligomers stabilized with covalent dopamine adduct (Fig. 14).

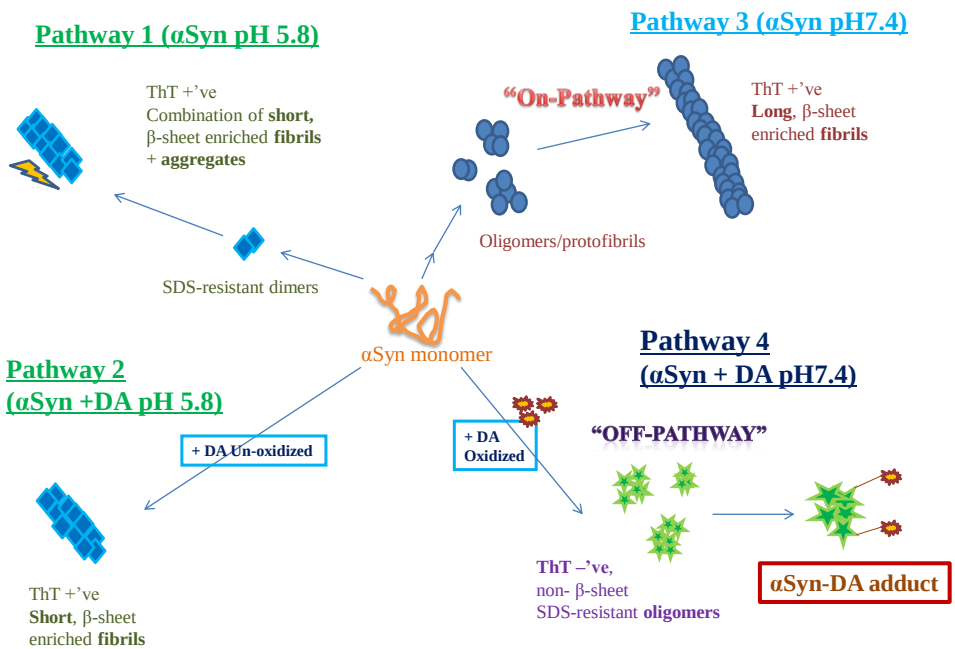


Figure 14. Proposed pathways of α-synuclein aggregation in the absence and presence of dopamine in acidic and neutral pH

However, there are other factors involved with the development of Parkinson's disease, including α-synuclein overexpression and mutation, the presence of nitrative and oxidative species such as peroxinitrite and hydrogen peroxide, high concentration of metals, alcohols and osmolytes, and most of all, aging of the patient (Souza et al., 2000; Recchia et al., 2004; Meng et al., 2009; Collier et al., 2011). It seems Parkinson's disease hardly develops due to a single factor but combination of the factors and such a diverse set of pathological causes make the Parkinson's disease study difficult. Combining these factors as well as *in vivo* cellular cytotoxic studies and animal models will provide a wealth of information on the pathological mechanism of the disease. This study also suggests that it would be meaningful to further investigate into the molecular mechanism of the cytotoxic dopamine and α-synuclein adduct formation and other structural changes under various conditions using more biophysical assays such as NMR or mass spectrometry.

Although it cannot be defined which of the various intermediates among the dopamine oxidation pathway is the most responsible one for oxidative damage on α-synuclein, there was a NMR study showing a time course of dopamine oxidation during which transient formation and degradation of each intermediate was observed. In this study, NMR peaks for 5,6-dihydroxyindole were observed in the absence of significant α-synuclein adduct, indicating that the precursors of 5,6-dihydroxyindole, dopamine-o-quinone and aminochrome, are not as reactive as expected toward α-synuclein but prefer further oxidation into 5,6-dihydroxyindole. They suggested indole-5,6-quinone as the first and most to react with α-synuclein in measurable

amount because it is the one that appears most transiently and correlates with formation of the adduct (Bisaglia et al., 2007).

5. Conclusion

The relationship between the pH environment, dopamine oxidation state and the aggregation pattern of α-synuclein were analyzed extensively in this study. The significance of this *in vitro* study lies in mimicking the natural auto-oxidation of dopamine by the pH difference of the cytosol and vesicle in the dopaminergic neurons, which are selectively vulnerable in Parkinson's disease. This should allow more accurate understanding of the effect of naturally oxidized dopamine on the α-synuclein aggregation, compared to the effect of dopamine oxidized by extreme pH or oxidants. The vesicle pH was sufficient to keep dopamine unoxidized, while the cytosolic pH effectively oxidized dopamine. All assays were also performed at physiological concentrations of the samples and they were sufficient for the purpose of this study. Structural characterization and morphological studies of the resulting α-synuclein and dopamine oligomeric intermediates or fibrils performed using ThT fluorescence, SDS-PAGE analysis, and TEM assays revealed the distinct α-synuclein aggregation pathways for each condition. Distinct oligomerization pathway of α-synuclein, and hence inhibiting fibrillation, by the oxidized dopamine was also shown by the intrinsic fluorescence study. The α-synuclein and dopamine adducts resulted from dopamine auto-oxidation indicated that α-synuclein was covalently modified in a pathway distinct from forming dityrosine cross-links or other oxidative damages on α-synuclein. Understanding distinct aggregation pathways of α-synuclein in the presence and absence of dopamine in two physiological pH conditions and the subsequent inhibition of α-synuclein fibrils while promoting oligomers in presence of

oxidized dopamine is critical to understanding the pathophysiology of Parkinson's disease and similar neurodegenerative diseases.

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국문요약

도파민 생성 신경세포의 선택적 손실은 파킨슨병 환자의 뇌에서 발견되는 전형적인 특징중 하나이다. 알파-시뉴클린 단백질은 이런 세포독성에 주요 원인으로써 파킨슨병의 진행과정에서 발생하는 산화스트레스에 의해 산화되고 응집되는 등 다양한 생화학적 변화가 일어나게 된다. 최근의 여러 연구들은 이런 도파민 생성세포 특유의 파킨슨병에 대한 취약성 때문에 도파민에 의한 알파-시뉴클린의 변화에 집중하고 있지만 여전히 그 관계는 정확하게 밝혀지지 않았다. 본 연구에서는 산화된 도파민과 그렇지 않은 도파민이 어떻게 알파-시뉴클린의 산화와 응집에 영향을 주는지를 실험을 통해 조사하였다. 평소에 도파민 생성 신경세포에서 도파민을 안정적으로 저장하는 세포내 소기관인 소포의 산성 pH와 파킨슨병에서 상대적으로 도파민이 축적되는 것으로 알려진 세포기질의 중성 pH를 모사한 두 *in vitro* 조건에서 각각 도파민이 산화되는지를 관찰하였다. 또한 이렇게 산화된 도파민이 어떻게 실제 알파-시뉴클린의 생화학적 변화와 응집에 영향을 줄 수 있는지를 보았으며, 그 결과 산성/중성과 도파민의 유무에 따라 네가지의 뚜렷한 알파-시뉴클린 응집 경로가 발견되었다. 특히 산성에서는 도파민이 산화되지 않고 짧은 섬유 형태의 알파 시뉴클린을 유도하였으며, 중성에서는 도파민이 산화되면서 용해성 알파-시뉴클린 올리고머를 생산하였다. 도파민이 없는 경우 산성에서는 알파-시뉴클린이 기존의 짧은 섬유형과 비결정성 단백질 집합체로서 관찰되었고 중성에서는 긴 섬유형 중합체로 나타났다. 중성에서 산화한 도파민은 또한 알파-시뉴클린에 공유결합된 부가물로 나타났다.

주요어: 알파-시뉴클린, 파킨슨병, 도파민, pH에 의해 조절된 산화스트레스, 응집경로, 도파민-알파-시뉴클린 공유결합 부가물

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