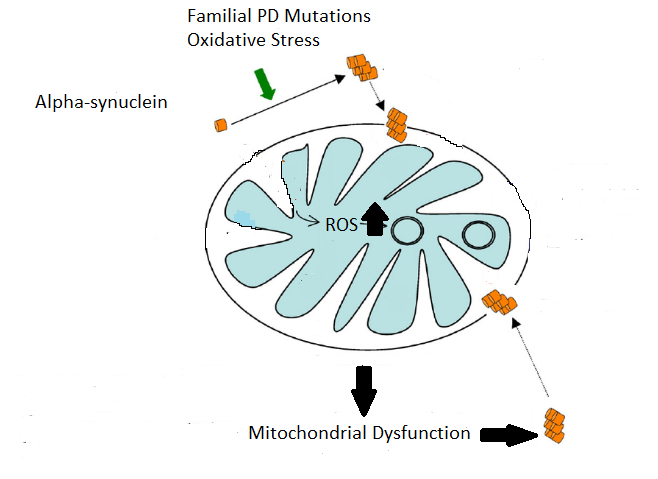
**Relating the structure of aggregated alpha-synuclein in Parkinsonian neurons to its preferential binding on the outer mitochondrial membrane**

1. **Introduction**

Parkinson’s disease is a progressive disorder of the nervous system that affects the human body through the occurrence of motor and non-motor symptoms. Many researchers and scientists concur that the incidence of Parkinson’s disease has increased over the past thirty years. Parkinson’s disease is on the rise because of the inability to effectively recognize and treat the disease. Parkinson’s disease does not have a simple diagnostic test and therefore is diagnosed solely on the basis of clinical non-motor, depression, memory problems, and hallucinations, and motor symptoms, trembling, problems with gait, and balance.

These symptoms arise due to the degeneration of dopaminergic neurons that release dopamine (Jankovic, 2008). Dopamine is a neurotransmitter that functions in a multi-dimensional manner. Its multifaceted effect can be observed in the variety of symptoms produced, ranging from non-motor to motor complications. Dopamine is transported into the synaptic cleft by a vesicle where alpha-synuclein, a cytoplasmic protein, resides (Burre et al., 2010). Native alpha synuclein resides in the presynaptic terminal at high frequencies to assist in the formation of vesicles around a neurotransmitter. The formation of vesicles aids in the process of exocytosis. In its natively unfolded form alpha-synuclein occurs as a soluble, in cytosol, tetramer which resists aggregation (Bartels et al., 2011).

In Parkinsonian neurons, alpha-synuclein aggregates due to oxidative stress. The aggregation of alpha-synuclein prevents it from functioning as it does in its native state. Aggregated alpha-synuclein significantly impacts the neurotoxicity of dopaminergenic neurons in the substantia nigra because of its ability to aggregate on the lipid membranes of organelles. One of the reasons alpha-synuclein is able to preferentially bind to lipid membranes is because of aggregated alpha-synuclein’s ability to change from a soluble form to an insoluble form (Bandopadhyay, 2016). The change in its physical interaction with other molecules enables it to permeate the hydrophobic portion of the outer mitochondrial membrane, resulting in mitochondrial stress (loss of mitochondrial membrane potential).

**Fig 1:** Aggregated alpha synuclein is able to permeate the outer mitochondrial membrane causing mitochondrial dysfunction. Aggregated alpha-synuclein associated with PD occurs mainly due to familial PD mutations and oxidative stress on the cell. This causes the overproduction of alpha-synuclein which is enhanced to bind to the hydrophobic portions of the mitochondrial membrane. Once aggregated alpha-synuclein binds, this causes a dysregulation of mitochondrial activity by increasing the levels of reactive oxygen species (ROS). Homeostatic mechanisms in the cell combat this by apoptosis because the neuron is heavily dependent on mitochondrial activity for energy (Bueler, 2009).

Figure 1 is an in-depth depiction of the pathway in which aggregated alpha-synuclein exacerbates mitochondrial dysfunction to trigger a positive feedback mechanism. Mitochondrial dysfunction increases levels of alpha-synuclein to become aggregated.

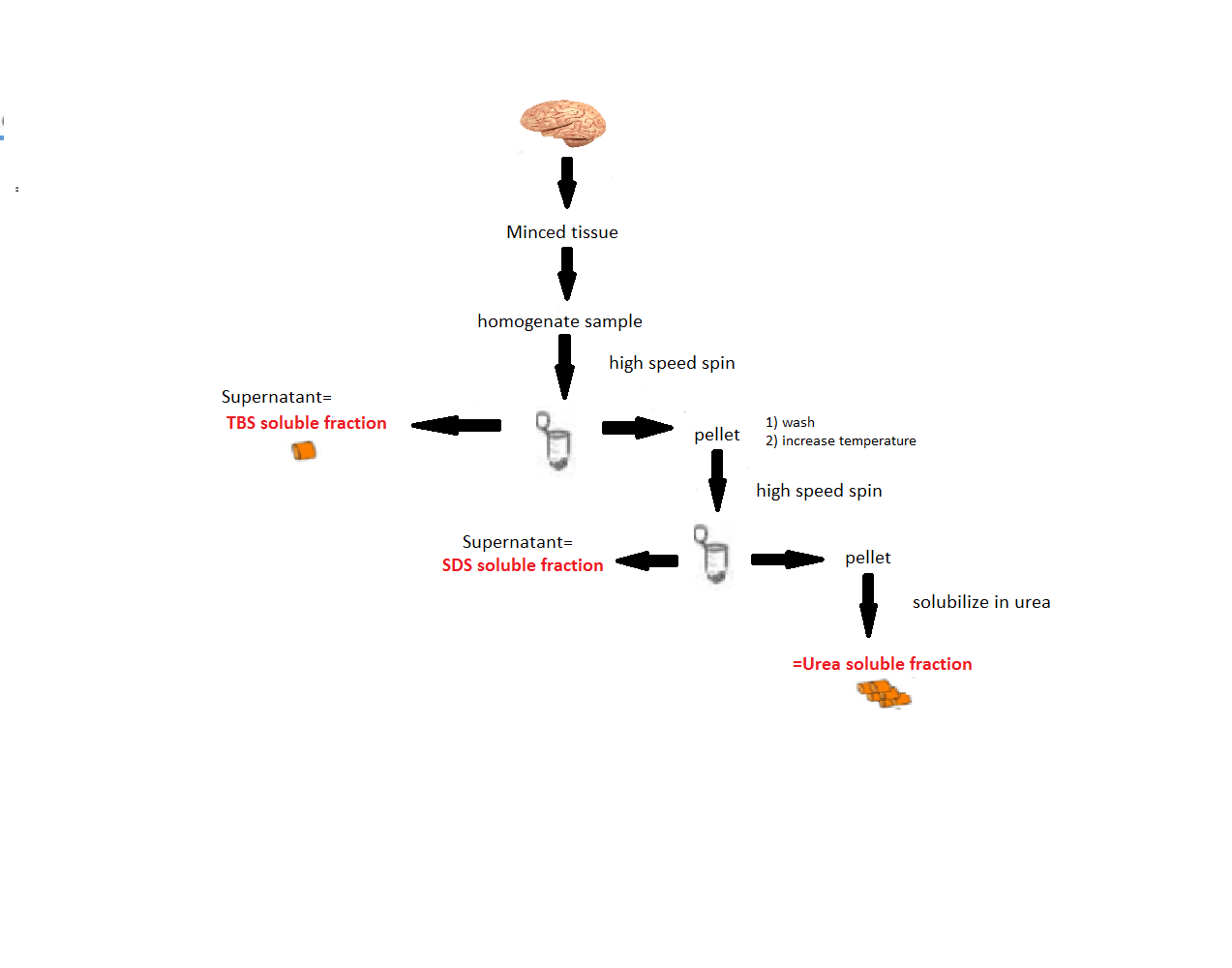
Many researchers conclude that the aggregated form of alpha-synuclein typify many neurodegenerative diseases such as Parkinson’s disease (Lashuel et al., 2013). Although, the observations above lend a hand in determining the function of aggregated alpha synuclein, a more detailed understanding is necessary. Native state alpha-synuclein resists aggregation due to its stable tetramer formation (Bartels et al., 2011). Therefore, the only method of aggregation is for the protein to be modified or amino acid level changed. Subsequently, supporting evidence suggests that the physical characteristics of alpha-synuclein change from a soluble form to an insoluble form (Bandopadhyay, 2016). The differences highlighted above prove that there could be potential differences at the amino acid level of aggregated alpha-synuclein in comparison to native alpha-synuclein.

1. **Experiment**

In order to determine potential differences at the primary protein level, the first step is to isolate the different forms of alpha-synuclein. A piece of basal ganglia is used as the starting to point because of the high density of dopaminergenic neurons in this region. A series of centrifugation steps separates fractions containing different forms of alpha-synuclein that are apparent in the Parkinsonian brain. Once these fractions are obtained, a Western blot experiment is used to differentiate the forms of alpha-synuclein attained from the centrifugation steps, specifically, native versus aggregated. Following the identification these forms, a protease, such as trypsin, is introduced into the fraction with aggregated alpha-synuclein. The purpose of adding a protease is to cleave the peptide chain into reasonable chunks of amino acids. For this experiment, trypsin will cleave the peptide chain at every positive amino acid. A 2D-SDS polyacrylamide gel electrophoresis (PAGE) will allow for the identification of any differences in amino acids between native alpha synuclein and aggregated alpha synuclein.

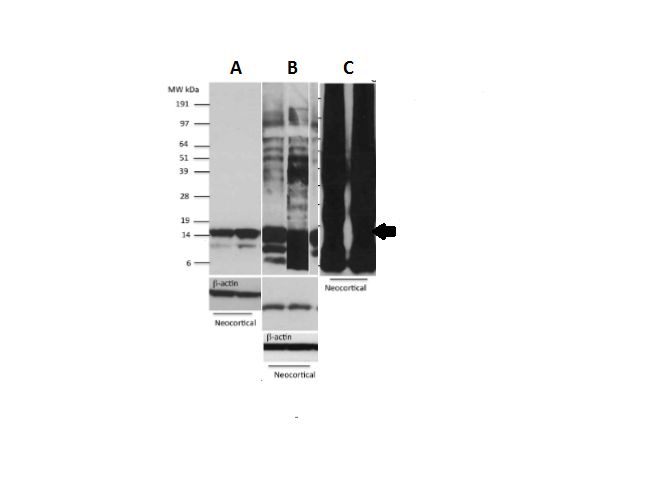
Part A: Extraction of AAS & Immunoblotting

Bandopadhyay (2016) outline a procedure that allows for the extraction of aggregated alpha synuclein from basal ganglia tissue in the form of a urea-soluble fraction. In this procedure, a tris-buffered Saline (TBS) solution is prepared to provide a buffered solution for the minced basal ganglia. A protease, protein that degrades other proteins, inhibitors and phosphatase inhibitors are introduced along with the TBS solution to prevent fragmentation of the alpha synuclein.

**Fig. 2:** Basal ganglia tissue is extracted from a Parkinsonian patient and minced. The minced components are homogenized and centrifuged to produce a supernatant, TBS-soluble fraction, and a pellet. To prevent the existence of the TBS-soluble solution to be present in the pellet, it is washed and the temperature is increased. Upon addition of sodium dodecyl sulfate (SDS), a detergent which disrupts noncovalent bonding, the pellet is centrifuged. This process yields a supernatant, known as the SDS-soluble fraction and a pellet. The pellet is solubilized in urea, a protein denaturant that breaks disulfide bonds and increases solubility of insoluble compounds (Hummon, 2007).

Once the three different fractions are present, a Western blot test is used to confirm the presence of the different forms of alpha-synuclein from the above procedure. A western blot is an analytical technique used in many molecular biological experiments to identify specific proteins. A western blot utilizes the molecular weight of the protein on a gel electrophoresis plate. Once the proteins are separated, a primary antibody known as anti-alpha-synuclein antibody is introduced to identify the presence of the protein, alpha-synuclein. A secondary antibody which is fluorescent is introduced to bind to the primary antibody. The function of the secondary antibody is to fluoresce under an X-ray. The secondary antibody makes visible the location of the protein of interesting on the gel electrophoresis plate.

After the Western blot procedure was completed, the three immunoblots, as seen in Figure 2, were observed by Bandopadhyay. The left hand side of the immunoblots represents the molecular weight (MW) of alpha synuclein.



**Fig. 3:** A representation of the Western blots of the neocortical brain completed by Bandopadhyay (2016). The neocortical sample was used for this experiment because this is where a large amount of the somas of neurons reside. Immunoblot A represents the TBS-soluble fraction. Immunblot B represents the TBS-SDS soluble fraction. Immunoblot C represents the urea soluble fraction. The arrow represents the location on the blot for native state of alpha-synuclein.

The Western blot of the urea soluble fraction has a large amount has a large area of stained region, ranging from 6 kilo Daltons to 191 kilo Daltons. The arrow to the right of the Western blot represents the molecular weight of native alpha-synuclein (~19 kDa). This confirms that the urea-soluble fraction has aggregated alpha-synuclein because of its elevated molecular weight. The SDS-soluble and TBS-soluble fraction do not contain aggregated alpha-synuclein because there is no presence of an increased weight of the alpha-synuclein recognized in these fractions. However, the SDS and TBS-soluble fractions possess the native form of alpha-synuclein.

Part B: 2D SDS-PAGE

The purpose of the 2D SDS-PAGE (SDS-polyacrylamide gel electrophoresis) is used to analyze amino acid mixtures from extracted cells. This technique uses two dimensions: (1) isoelectric focusing and (2) SDS-polyacrylamide gel electrophoresis. The urea-soluble fraction, the only fraction which possesses aggregated alpha-synuclein in the extraction procedure above, is used in this procedure. This fraction is treated with trypsin, a protease which cleaves peptide linkages at positive amino acid sites. Upon separation, this fraction is poured into a manufactured isoelectric focusing strip, to be separated by isoelectric point. The isoelectric focusing strip contains a pH gradient range (sometimes from 3-7 pH). The sides of the strip have opposite electrodes, anode or cathode. Positively charged amino acids, amino acids that have an isoelectric point (pI) lower than the pH will move towards the cathode, a negatively charged electrode. Subsequently, an amino acid that has a pI greater than the pH will move towards the anode, a positively charged electrode. But, there is a potential for two different amino acids to have similar isoelectric points, therefore, preventing any separation between them on the isoelectric focusing strip. In the second dimension, the strip is applied on a polyacrylamide gel. The proteins will be separated on the gel are separated via molecular weight (MW). This allows for a distinction between two amino acids which have similar isoelectric points (Vigneswara, 2013).

1. **Discussion**

If my experiment goes well, I hope to pinpoint the change in primary protein structure on the alpha synuclein protein that promotes aggregation. These changes should affect alpha-synuclein mobility. From these results, I am able to make conclusions that could be rash, but attempt to explain its enhanced ability to bind to lipid membranes. Although this would be the ideal scenario, several problems could occur during the implementation of my experiment(s). My experiment looks at the putative changes in amino acid sequence rather than the secondary or tertiary sequence. Covalent/noncovalent interactions at higher folding interactions could be the cause of the change from a soluble to an insoluble protein. An example of this could be a modified folding pattern that flips the hydrophilic side chains on the outside to the inside of the chain, thereby allowing the hydrophobic regions to interact with other molecules.

One of the reasons I did not take this approach is because alpha synuclein is a 14 kD (140 amino acid protein). In its native state, it exists as a tetramer. There is no evidence to suggest that increased aggregation of alpha synuclein contributes to interactions between separate alpha- synuclein subunits. Subsequently, that in turn these interactions somehow increase the insolubility of the alpha synuclein protein.

If the experiment was extended the addition of an NMR Spectroscopy could prove beneficial in determining the secondary or tertiary structure of the protein. However, NMR Spectroscopy also has limitations. NMR Spectroscopy is mainly used to study large macromolecular components therefore the addition of protein crystallography might be useful in determining a more atomic resolution of the higher folding structures.

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