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Alpha Synuclein: A therapeutic target and biomarker for Parkinson's Disease

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Alpha Synuclein: A Therapeutic Target and Biomarker for Parkinson's Disease

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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Table of Contents

List of Tables.....	iii
List of Figures	iv
List of Abstract.....	v
Main Body.....	1
a. Introduction.....	1
i. PD and Alpha-Synuclein.....	1
ii. The pathological role of alpha synuclein.....	3
iii. The α -Syn Explanation for PD selectivity.....	4
iv. Alpha Synuclein in PD Progression.....	6
v. Current therapeutic strategies for PD targeted alpha synuclein.....	7
vi. The major issue in AD treatment.....	8
1. Attacking the Brain.....	8
2. Diagnosis.....	8
vii. Other disoriented protein in PD.....	9
b. The foundation of our research in alpha synuclein related PD project.....	10
c. Methods.....	13
i. Creating Standards.....	13
ii. Alpha-Syn, Tau standards.....	14
iii. Testing Against Human PD.....	14
d. Results.....	16

e. Discussion and Conclusion.....	20
References.....	22

List of Tables

Table 1: the reactivity of each antibody on alpha-synuclein.....10

List of Figures

Figure 1: the pKa of each antibody reacting with alpha-synuclein.....	11
Figure 2: Western Blot and Silver Stain for alpha-synuclein aggregation.....	12
Figure 3: DOT Blot results for Standards.....	16
Figure 4: Standard Curve created from standards.....	17
Figure 5: Results for 1H5 antibody.....	18
Figure 6: Results for 2A4 antibody.....	19

Abstract

Parkinson's Disease is the second most common neurodegenerative disease after Alzheimer's Disease, and the most common motor disorder. In fact, nearly a third of those with AD have PD. A major hallmark of PD is the appearance of Lewy Bodies, proteinaceous cytoplasm inclusions, that build up and spread throughout the CNS in a prion like fashion. The major component of these Lewy Bodies is a protein called alpha-synuclein. Alpha-syn is a 14kDa protein made of 140 amino acids and found in the presynaptic ends of CNS neurons, acting as a chaperone and regulator. The protein is also found in the rest of the body besides the liver, most importantly in the blood. It has been shown in various studies that targeting alpha-syn can slow down or mitigate the effects of PD. It has also been shown that alpha-syn levels in the blood significantly increase in PD patients compared to non-PD patients. This makes alpha-syn a good biomarker for both diagnosis and potential treatment. However, being able to quickly identify alpha-syn is paramount for quick and efficient diagnosis. In this experiment, I use antibodies, 1H5 and 2A4, to quickly identify relative amounts of alpha-syn in human PD blood plasma. I also used the DOT Blot apparatus as a quick throughput method of antibody staining. The results showed that these monoclonal antibodies combined with using a DOT Blot technique could be used for clinical diagnosis or treatment with proper treatment.

Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease, coming only after Alzheimer's Disease (AD). It is also the most common movement disorder (1), with 30% of those effected also suffer from AD. Though being able to hit most age groups, it hits those above the age of 70 the most, affecting 1.087% of people ages 70-79 and 1.903% of people over 80 (2). PD affects a person's motor and non-motor functions. The motor function impairments include: tremor, gait and balance impairment, unstable posture, bradykinesia, and rigidity (3). Several non-motor functions include: constipation, rapid eye movement, sleep impairment, autonomic impairment, and cognitive decline (4). The major hallmark feature of PD is the accumulation of alpha-synuclein (α -syn) resulting in the creation of Lewy-bodies and Lewy neurites, proteinaceous cytoplasm inclusions (5). Current approaches to PD therapy are to find ways to delay and diminish symptoms as well as delay the progression of the disease (6). Of the various parts of PD to target to reach these goals, α -syn seems to be the most promising.

PD and alpha synuclein

PD is named after James Parkinson who first described and reported the disease with symptoms in 1817 (7). The exact pathological cause was not known until Fritz Jacob Heinrich Lewy disclosed a protein formed a structure called Lewy body. Lewy-bodies were discovered by their namesake Fritz Jacob Heinrich Lewy in 1912 during his study of what was then called Paralysis Agitans and we now call Parkinson's Disease. It was found that these eosinophilic inclusion bodies in the neurons of certain brain nuclei were pathologic to the disease, later named

Lewy-bodies. (8). Over 80 years later, in 1996-7, the study of chromosome 4q21-23 found a point mutation that later led to the identification of the gene that encode alpha synuclein (SNCA) (9). The same year, the link between α -syn and PD was established with the finding that it was a major component of LBs and LNs (10). While in the years since α -syn has been studied tremendously, the full extent of its involvement remains to be found.

What is known is that α -syn is a 14kDa sized protein, consisting of 140 amino acids and 3 domains: Its N-terminal alpha helix domain that binds lipids, an acidic C-terminal tail, and a non-amyloid-component domain in-between the other two. It is expressed in the presynaptic sites of neurotransmitters in the central nervous system (CNS) and is involved in the regulation of vesicle docking, fusion, and neurotransmitter release (11). Despite how widespread it is in the CNS; its pathology only affects certain vulnerable sites. Besides the CNS, α -syn is also seen in red blood cells (RBCs) (12). It is very structurally flexible (13), existing in a dynamic in-between of monomeric and oligomeric states and can change conformation depending on the binding partner.

In the presynaptic area, α -syn is a chaperone and controls exocytosis through its management of vesicle pools and traffic. A mutation in the SNCA gene coding for α -syn affects the functionality of SNAP receptor (SNARE) proteins, which regulate the activity of SNAP proteins (14). The DA (dopamine) active transporter is another target for α -syn. Normally when interacting with a lipid layer under physiological conditions, the alpha synuclein forms an alpha helix that doesn't cross it. However, under specific circumstances, α -syn oligomers can form pores that dissipate the membrane potential, dysregulating ion gradients (15). α -syn also possesses a polar C terminal that can interact with the hydrophobic region of a separate denatured protein, allowing it to act as a chaperone (16). The chaperone ability is also due to its

ability to aggregate, with a truncated C terminal increasing aggregation at the cost of its chaperone ability. Despite its important role in ion gradients and chaperones, double knockouts of α -syn (and beta-syn) are entirely survivable in test mice, not impairing mice brain function or survival. However, dopamine level decrease by around 20% and caused selective changes in 2 small synaptic signaling proteins, 14-3-3 proteins, and complexins. This suggests that while the synucleins are not needed for basic neurotransmitter machinery or release, they may influence long term maintenance and regulation of presynaptic function (17). For pathology, it's the unnecessary accumulation of alpha synuclein rather than its absence that poses a problem. In studies on rodents, it was found that overexpression of the α -syn gene caused neuronal loss and the formation of LD-like inclusions. (18). Errors in assembly of this protein can be mitigated by subcellular and intercellular correction mechanisms, however enough error buildup over time leads not to a singular issue but to a cascade of intercellular neurodegenerative errors (19).

The pathological role of alpha synuclein

As mentioned before, it was found that α -syn played a role in PD development due to them being a part of Lewy-Bodies. Some of the environmental and genetic factors for PD also seem to be the conditions for α -syn toxicity (20). These include: neuroinflammation, mitochondrial dysfunctions, oxidative stress, point mutations and multiplications. Other factors can include: overexpression, failure in molecular cleavage, pH changes, oxidation stress, and mitochondrial overwork. Oxidized α -syn could result from Oxidized derivatives of DA that leads to a decrease in fibril formation but increases the accumulation of proto-fibrils. The normally monomeric soluble protein become oligomeric forming oligomers that combine to form small proto-fibrils, that aggregate to fully fibrils which form LBs (13). Concentration is also a key

point. α -syn is normally “naturally unfolded” but still requires partially folded intermediates. These intermediates are unstable and easily reversed except in high concentrations which stabilizes this conformation.

Some post-translational modification such as the aforementioned truncation of the C-terminus, tyrosine nitration (Tyr125) and phosphorylation on the Serine 129 are seen in aggregated α -syn. (21, 22). In fact, the Serine 129 phosphorylation is thought to be a dominant form of α -syn in LBs (23). Aging is also a factor, as aging shows a decline in proteolytic mechanisms, allowing used α -syn to build up (24). The ubiquitin–proteasome (UP) system and the lysosomal autophagy system (LAS) work together in the healthy brain to keep α -syn homeostasis (25). Failure in these and other compensatory mechanisms can begin the cascade to overproduction and aggregation. The aggregation itself may inhibit those homeostatic systems, while also reducing the chaperoning of misfolded proteins, creating a cycle of neurodegenerative pathways (26).

The α -Syn Explanation for PD selectivity

For reasons that still elude researchers, α -syn aggregation toxicity only effects some areas in the brain over others. α -syn also modulates DA metabolism as it reduces the phosphorylation state of tyrosine hydroxylase and stabilizes it in its inactive state (27). Lack of α -syn has been shown to lead to a decreased level of DA and DAT function, as well as a decrease DA striatal uptake (28, 29). This also decreases the number of TH-positive terminals and nigral DA cells (30). DA neuron sensitivity to α -syn toxicity depends on both the lack of DA metabolism support and the selective and intrinsic vulnerability of these neurons to excitotoxic challenges (insults originating from outside the body). During PD, dopaminergic neurons of substantia nigra pars

compacta (SNc) show selective neurodegeneration and cell death. These cells have an impairment of several basal ganglia functions and reduced DA levels in the striatum. SNc neurons show particular vulnerability to oxidative stress, particularly those in brain nuclei involved in arousal responses and ones in control of the sensorimotor networks, which is needed for surviving behaviors such as vigilance, escape, and attack (31). These neurons also display 2 particular factors make them vulnerable to excitotoxic insults. Firstly, these neurons are highly branched, good for special distributed networks, however it increases their mitochondrial stress. The second reason is that DA neurons have a spontaneous, autonomic pacemaker activity held together by calcium ion (Ca^{2+}) voltage channels oscillating Ca to a rhythm (32). This leads the neurons to have low internal stores of Ca^{2+} that needs to be strictly controlled for effective use in Calcium-mediated processes such as: intracellular stores of Ca^{2+} , promoting entry of Ca^{2+} into the mitochondria, the production of ATP, and oxidative phosphorylation (33, 34).

These events fulfill a bioenergetic need, without which potassium ion channels would need to compensate for them, which in turn would turn ongoing neuronal activity (35). These factors lead SNc cells to be particularly vulnerable to outside toxins, age, and genetic mutations, allowing them the chance to build up reactive oxygen species (ROS). ROS can particularly damage DNA and the mitochondria, to the point where mitophagy, and autophagy in general is damaged. This disrupts the UP and LAS systems mentioned earlier. A study found that mice with over expressed α -syn and deleted DJ-1 (another gene whose deletion was found to be linked with PD) showed increased levels of oxidized DA in the nigral neurons of mice with decreased lysosomal activity compared to mice with only deleted DJ-1 (36). α -syn disbalances or exasperates and already existing imbalance of homeostasis. This was shown experimentally where the over expression of an α -syn mutation created a new Ca^{2+} dependent pathway that led

to neuronal loss (37). Another study found that increasing the overexpression of wildtype α -syn created more, non-selective, pore-like channels associated with an increase in membrane conductance and cell death (38).

Alpha Synuclein in PD Progression

It is proposed that α -syn propagates from the peripheral nervous system (PNS) to the central nervous system (CNS) and spreads through cell-to-cell transmission (39). PD has 6 proposed stages based on the pattern of α -syn pathology. Stages 1 and 2 involve areas of lower thinking processes such as the caudal brainstem, olfactory system, and autonomic nervous system. Stages 3 and 4 affects areas like the substantia nigra (SN), creating a significant loss of dopaminergic neurons. Stages 5 and 6 is where the cortex is affected with subsequent extensive cortical involvement. (40). The precise mechanisms underlying the disease mechanisms are unknown and several studies have shown this model to not be universal with all sporadic PD or α -syn pathology. However, it does show that α -syn does indeed spread and hits both the PNS and CNS. α -syn may have a “prion-like” propagation mechanism, were neuron released aggregated α -syn are internalized by neighboring neurons and cause further misfolding and aggregation and the cycle continues and spreads (41). In vivo studies have shown evidence of this. In a study by *Luk et al.*, they injection α -syn preformed fibrils (PFFs) into the striatum of transgenic mice, which then showed the development of Lewy-Body pathology and nigrostriatal degeneration. It showed that even synthetic PFFs could lead to a Lewy pathology (42). Despite the evidence for prion like propagation, such as in the study Sorrentino *et al.* in which the spread of α -syn did not spread along a predictable path through the connectome like in prion diseases (43). The results being that there is significant evidence of prion-like spread of α -syn but it is not gospel.

Current therapeutic strategies for PD targeted alpha synuclein

Trying to specifically target the spreading α -syn is difficult since it also targets the endogenous α -syn and as seen above, some α -syn is still necessary for neuron function. One hopeful method to mitigate the spread of α -syn was to block its receptors. Cell surface heparan sulfate proteoglycans have been proposed to mediate alpha-syn uptake via endocytosis. So, inhibiting to damaging these proteoglycans can potentially slow the spread of α -syn (44). Heparin and chloral hydrate have both been shown to disrupt heparan sulfate proteoglycans, and in treated cell cultures have shown a decrease in α -syn endocytosis.

However, it's the genes that make α -syn, so a good place to strike would be the genetic and protein machinery. RNA interference (RNAi) can be used to silence genes by targeting the α -syn messenger RNA (mRNA). Studies by Sapru *et al.*, and Lewis *et al.*, showed that using shRNA and siRNA (types of RNAi) in the brains of rats reduced the numbers of α -syn (45, 46). A major setback in the use of RNAi has been the amount of and method of delivery of these gene silencers. Studies of rats and non-human primates have shown that reducing α -syn by over 90% causes nigrostriatal system degeneration (47).

While interfering with the mRNA machinery is one approach, interference with the gene itself is another. A study found that using Beta-2 adrenoreceptors (B2AR) agonists could interfere with α -syn transcription via altering histone deacetylase (HDAC) activity at the α -syn gene promoter and enhancer regions. These changes were also neuroprotective in the cell lines and rodent models (48). In a Norwegian asthma study, the use of B2AR agonists reduced lifetime risk of PD, whereas the B2AR antagonist had the opposite effect.

The major issue in AD treatment

Attacking the Brain

The brain has been infamous in how difficult it is to send treatments to it, mainly due to its incredible Blood Brain Barrier (BBB). This barrier keeps out all but select chemicals, including beneficial substances. However, there is more than one route to enter the brain, in this case the Nasal Route (49). It bypasses the BBB, sidestepping its problems, but it may not be the most efficient method (50). Internasal sprays, for example, get at least partially absorbed by the pulmonary system before going into the brain. However, there are still advantages with intranasal delivery: rapid onset of action due to fast delivery, less systemic delivery enhances neurotherapeutic effects. Several of the disadvantages are restricted volume and mass of the drug, and the absorption pathway can vary depending on physiology and chemistry (51). In a study by Change et al., they used the protein Pep-1 (P-Mito) conjugated with mitochondria to intranasally insert into rodents for PD treatment. The intranasal delivery worked, showing a significant improvement in rotational and locomotive behaviors (52).

Diagnosis

Treatment is all well and good but if the disease has progressed to the point where it is easily noticeable, then it has already done irreparable damage. Diagnosing for PD is paramount to obtaining effective treatment. One of the best ways to find a disease early, with a higher degree of certainty, is the use of biomarkers. Biomarkers are reproducible signs that “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (53). Going back to the sections on alpha-syn, its abundance, aggregated form during PD, and heightened levels make it a good target for its use as a biomarker. Multiple studies have found

that the protein is heightened in PD patients compared to non-PD patients, including in blood plasma. (54) (55). Other prominent proteins in tauopathies such as tau and Abeta have been suggested, but alpha-syn is more specific for Parkinson's Disease compared to those. Having a protein isn't enough however. In order to be relevant, the biomarker must be easily identified and with a timely manner. Antibodies are naturally talented at attaching to specific proteins and can easily be attached to some sort of secondary marker to track them. There are also several different stains and measuring techniques that can be used to identify alpha-syn from a blood sample, such as Western Blot, Silver Stain, DOT-blot, etc.

Other disoriented protein in PD

Tauopathies represent the most common forms of dementia, and show an increased prevalence of certain proteins than can be considered biomarkers. While there is no definitive biomarker yet, it has been shown that Tau and Abeta proteins, in excess, are signs of tauopathies. The excess of alpha-synuclein is a further indicator of specifically Parkinson's Disease. Previous work in this lab have labeled several antibodies as being also to correctly identify alpha-synuclein in blood plasma: 2A4, 2C6, and 1H5. In this experiment, with the use of DOT-Blotting techniques, I propose that 1H5 can be an effector indicator of alpha-syn in blood plasma, able to correctly show the difference in concentrations between Non-PD and PD patients. The use of DOT-Blot will show how this can be done in a quick simple but effective method.

The foundation of our research in alpha synuclein related PD project

Much of my work was precluded by the already completed similar work made by the lab I was in. There they did thermodynamic studies on 3 potential biomarkers, the 2A4 and 1H5 that I worked on for this project, and another antibody 2C6. There were a series of tests between the full line of alpha-synuclein (the full 140 amino acid string and not sections of the protein) and the three proteins. 2A4, being the standard, seemed to come out the best in terms of number of binding sites (2.847) and pKa (4.3). 2C6 on the other hand had 10 binding sites and a pKa of 3. 1H5 had 0.1 binding sites and a pKa of 3.13. (Figure and table 1).

Table 1: Thermodynamic Studies on the Interaction of a-syn1-140 and different antibodies

Reaction	Number of binding Site	Binding constant	Molar enthalpy	Entropy contribution	Gibbs energy (at 37 °C)
	n	Ka (M ⁻¹)	ΔH (kJ/mol)	-TΔS (kJ/mol)	ΔG (kJ/mol)
FL+2A4	2.847	2.01E+04	-0.139	-25.41	-25.55
FL+1H5	0.1	1.37E+03	-1.507	-17.13	-18.63
FL+2C6	10	1.00E+03	-1.127	-16.69	-17.81

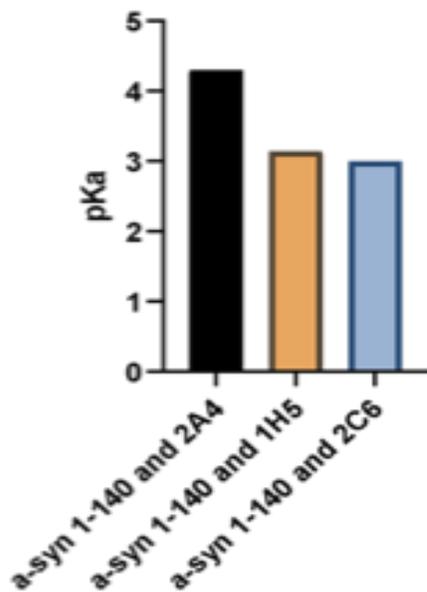


Figure 1: pKa of the reactions of full line alpha-syn and antibodies.

Beforehand both western blots and silver stains were performed on segments of alpha-syn. The Western showed that for a faction consisting of amino-acids 1-65 may promote, and 15-140 definitely promote aggregation. The silver staining also showed aggregation results: 15-65 can promote aggregation and with better function, while 15-140 shared the results of the western blot that it definitely promotes aggregation. However, there were several issues with this result. There was no 15-140 as control and 2A4 seemed to recognize leaner epitopes. There was also using 2A4 to measure aggregated full line alpha-syn with fragments as well as the aggregation of fragments. (Figure 2).

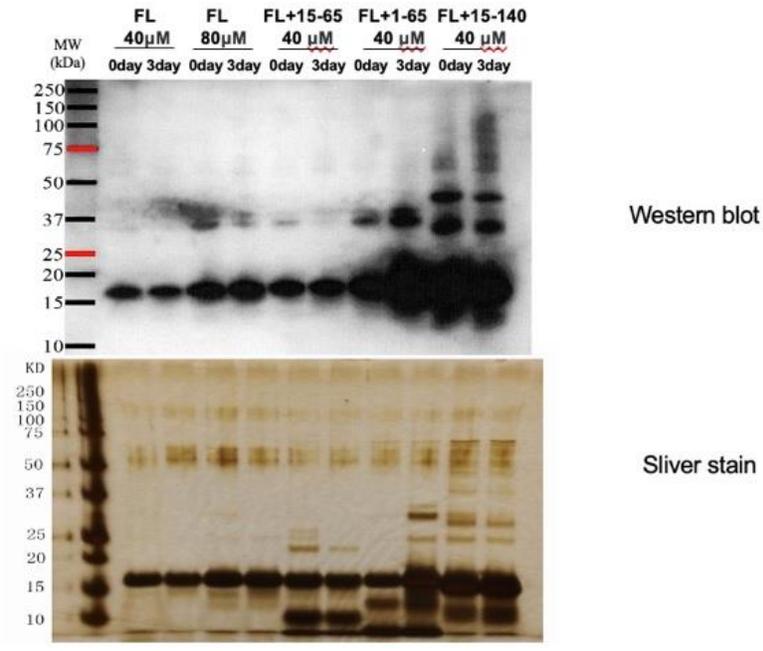


Figure 2: Western Blot and Silver Stain for Recombinant Human α -synuclein fragments aggregated with full-length α -synuclein (3 days)

Methods

Creating Standards

The first step was to create a standard to compare each sample's concentrations of ABeta; Tau; and Alpha-syn proteins.

For ABeta, it had a concentration of 250 pg/uL and each well could hold 50 ng/well without having detection issues. In 8 tubes, 480 uL of the buffer, PBS, in a 1328x dilution solution. The first tube was given 960 uL of ABeta solution with the same dilution, while the other seven were given 480 uL. Half of the first tube was mixed and then taken out via pipet. This solution in the pipet was then mixed in to the second tube, and after mixture 480 uL were then extracted. This repeated until seventh tube, serially diluting each tube. The eighth tube was just PBS, to have a baseline to compare to the other solution.

At the same time, the dot blotting equipment (Bio-dot microfiltration apparatus, 170 6545, BIO-RAD) was set up. A piece of nitrocellulose (NC) membrane was wetted in PBS and then placed on the dot blot apparatus with the vacuum turned on. PBST was used to wash the membrane one time. Using the first two rows of eight columns, 200 uL of ABeta solution was transferred from tube to dot blot apparatus: the first tube's solution was transferred to the first column, the second tube's solution to the second column, and so on for all 8 columns. This was repeated for the second column. The dot blot apparatus was ran for 5 minutes before being turned off. The NC membrane was then taken from the dot blot apparatus and placed in 0.2% I-Block with 0.05% I

tween-20 for 30 minutes at room temperature with shaking, then incubated with primary antibody (R22W) at a 1:2000 dilution all at room temperature. This was followed up by 3 3-minute washings of the NC membrane with 1xPBST solution. After that the more 0.2% I-Block with 0.05% I tween-20 was added as well as a 1:1000 dilution of secondary antibody (rabbit) that was shaken for 45 minutes at room temperature. A second washing cycle of the NC membrane, same as the first.

For detection, solutions A and B were mixed in a 1:1 ration and pipetted on to the NC membrane in an even manner, which was then covered for 5 minutes. The NC membrane with the blot was then taken to a dark room machine where it was imaged.

Alpha-Syn, Tau standards

The standard making was the same as for ABeta above, with the only differences being the dilution of protein in PBS and primary and secondary antibodies. For alpha-syn, the concentration was a 771.2X dilution, primary antibody was 2A4 and secondary was Mouse HRP. For Tau protein, it was a 400X dilution, Ta antibody for the primary antibody and rabbit HRP for the secondary.

Testing Against Human PD

Testing the samples involved using 3 NC membranes, each designated a protein and each membrane split in half for a primary antibody to test that protein with. Each half had 3 rows of 8

samples, 1 row of 5 samples, and 1 row of controlled solution. The three proteins with their primary antibodies were: human alpha-syn (2A4, 1H5), tau (tau antibody and p-tau antibody), and ABeta (22WR and R42). The plasma samples were prepared with Glycine, Tris-HCl, and PBS before being used in the dot blot apparatus. Once the samples were loaded on to the dot blot apparatus, the rest of the procedure was similar to what was used for the standards, including which secondary antibody was used for which protein. After the use of the dot blot a dark room machine was used as well as a dark room for 10 seconds, 1 minute, 5 minutes, and 30 minutes.

Results

The results for the alpha-syn antibodies appeared the clearest in comparison to the other two. The standards for the two antibodies -1H5 and 2A4- were most clear in the ECG machine at their two highest concentrations for both of them, and for the 4 highest concentrations for the 2A4 standard.

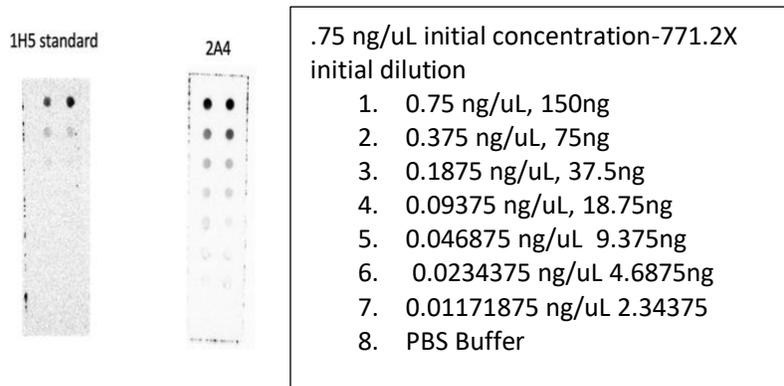


Figure 3: The standards and the concentration of protein in each well for 1H5 an 2A4.

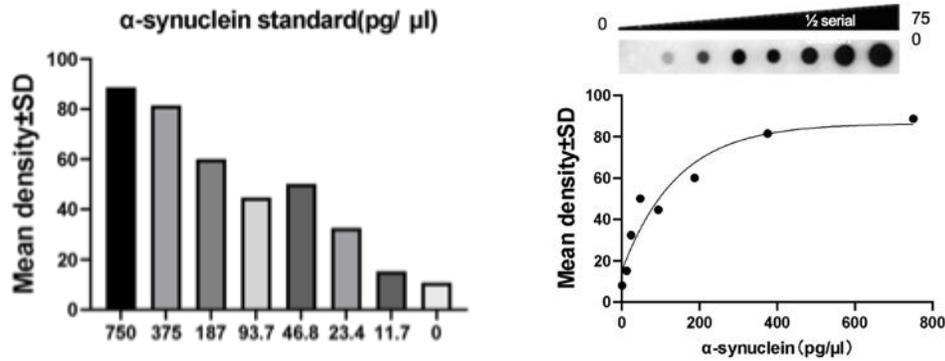


Figure 4: 2A4 dot-blot using aggregated α -synuclein as controls. 2A4 antibody dot-blot against a dilution series of aggregated α -synuclein (α -synuclein1-140 bind 15-65) as control. 2A4 binds specifically to aggregated α -synuclein in a dose-dependent manner.

The ECG scans showed little response with only the standard for the IH5 However, the Dark room showed clearer results. I used the time intervals of 10 seconds and 5 minutes, however only the former was readable. Afterword's, from that the mean density of shown samples compared with the severity of PD was graphed.

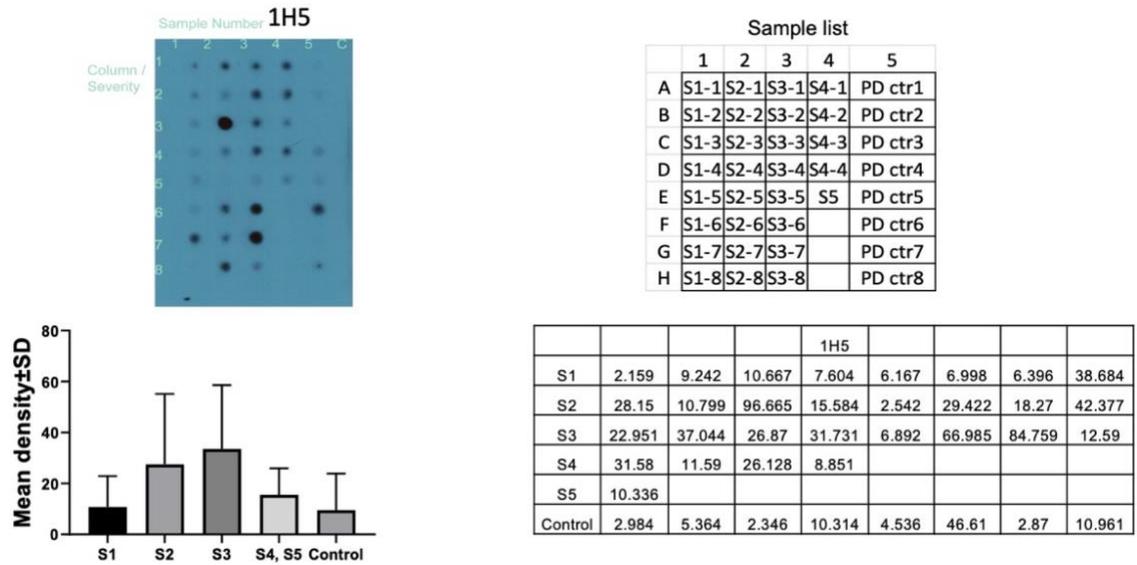


Figure 5: **Specificity of 1H5 as shown by dot-blot loaded with PD Patient’s plasma.** PD Patient’s plasma samples were treated with Glycine(pH2.5) diluted at 1:10, incubated for 20 minutes, then further diluted at 1:10 with Tris-HCl (pH8.0) to adjust pH back to 7.0. Samples were loaded onto Nitrocellulose membranes at final dilution of 1:100 (200 µl/dot). Membranes were then probed with 1H5 (1:3000) to detect α-synuclein.

For 2A4, it had clear results on the ECG machine, and with that, a similar density to PD severity graph was made.

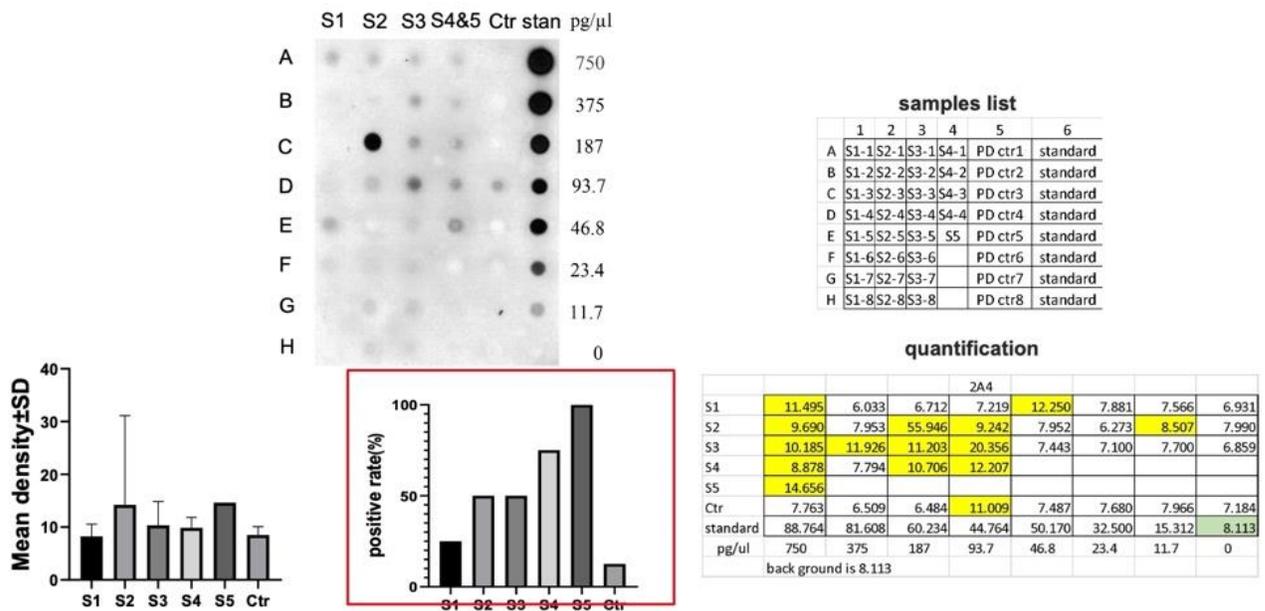


Figure 6: **Specificity of 2A4 as shown by dot-blot loaded with PD Patient’s plasma. PD Patient’s plasma samples were treated with Glycine(pH2.5) diluted at 1:10, incubated for 20 minutes, then further diluted at 1:10 with Tris-HCl (pH8.0) to adjust pH back to 7.0. Samples were loaded onto Nitrocellulose membranes at final dilution of 1:100 (200 µl/dot). Membranes were then probed with 2A4(1:3000) to detect α -synuclein. a-synuclein15-140 is used as standard.**

Discussion and Conclusion

The goal of this study was to determine a more optimal method for diagnosing Parkinson's Disease using plasma samples to search for alpha-syn. Despite the focus on alpha-syn in terms of neurons, the protein appears in all tissues apart from the liver, including blood (56). Multiple studies have shown that plasma concentrations of alpha-syn in plasma is significantly higher in PD patients than in non-PD patients (57) (58) (55). Plasma is preferred over other CSF as plasma can be collected in greater quantities and in an easier fashion. Plasma is also collected in routine clinical procedures, making it more likely someone would have a plasma sample than a CSF sample (59). The method used was Dot Blot, which has several advantages over other antibody detection assays such as ELISA. Dot Blot is a simpler, faster, and more robust method that doesn't require specialized equipment (60). Both the 2A4 and the IH5 showed promise in being able to accurately detect PD using the protein Alpha-Synuclein. While the ECG machine was not very clear the dark rooms results were, and if I were to continue, I would redo my attempt with the ECG machine if only to confirm results.

In a clinical setting the quick results allow for a more rapid testing, and thus the ability to get started on treatments. Diagnosis with Parkinson's Disease has typically been difficult despite advances, as symptoms can overlap with other neurodegenerative diseases (61). The ability to diagnose Parkinson's Disease premotor, before major symptoms occur, is paramount to the success of any treatment (62). Some prominent premotor symptoms are of smell loss and constipation (63). Being able to diagnose PD when its only constipation with a simple blood

sample and a few how dot blot test would most certainly improve the later life of these patients.

PD is also a highly varied disease, with factors such as: genetics, age of onset, clinical presentation, rate of progression, and treatment response (64).

At the moment, this experiment was with blood samples of patients that were already known to have PD, as well as compare the results of testing samples to a standard and a control. The next step would be to test it in animals that are known to have PD but have not shown any of the symptoms. If this 1H5 is in fact the better biomarker, then it would show aggregated alpha-syn before symptoms appear, also it should be tested against a non-PD animal.

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