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Development and Application of Novel Conformation-Specific Monoclonal Antibodies for Parkinson's Disease Pathology.

Nour Khaled Majbour

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Development and Application of Novel Conformation-Specific Monoclonal Antibodies for Parkinson’s Disease Pathology

Nour Khaled Majbour

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences in Pharmacology and Toxicology

Under the direction of Professor Omar M.A El-Agnaf

June 2014
Declaration of Original Work

I, Nour Khaled Majbour, the undersigned, a graduate student at the United Arab Emirates University (UAEU) and the author of the thesis/dissertation titled “Development and Application of Novel Conformation-Specific Monoclonal Antibodies for Parkinson’s Disease Pathology”, hereby solemnly declare that this thesis/dissertation is an original research work done and prepared by me under the guidance of Prof. Omar MA El-Agnaf, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or similar title at this or any other university. The materials borrowed from other sources and included in my thesis/dissertation have been properly cited and acknowledged.

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Dedication

To My Parents
Abstract

An abundance of genetic, biochemical and histopathological evidence suggests that the formation of α-synuclein (α-syn) protein deposits is an important, and likely seminal, step in the development of several important neurodegenerative diseases, the so-called synucleinopathies, including Parkinson's Disease (PD), dementia with Lewy bodies and multiple-system atrophy. Despite decades of intensive research, to date, the only accepted diagnosis for PD is based on clinical criteria. α-Syn conversion from soluble monomers into oligomers or fibrils represents a crucial step in synucleinopathies pathogenesis; however, the exact mechanism underlying the cellular aggregation has not been fully elucidated. A growing body of evidence suggests that α-syn soluble oligomers, rather than mature fibrils, are the neurotoxic species. A lack of biomarkers for the early diagnosis of PD is a major obstacle to providing early disease-modifying therapies. Toward this aim, we developed and thoroughly characterized novel conformation-specific monoclonal antibodies that specifically recognize α-syn pathology. Importantly, our antibodies were able to detect different assemblies of α-syn aggregates, ranging from soluble oligomers to mature insoluble fibrils. Considering the critical pathogenic role of α-syn soluble aggregates, our novel antibodies might provide important diagnostic and therapeutic opportunities.

Next, we developed new immunoassays to quantify different species of α-syn (total α-syn, α-syn oligomers and p-S129-α-syn) levels in cerebrospinal fluid (CSF) from PD patients and age-matched healthy controls. Combining the measurements of different α-syn species in CSF, we found a clear differential CSF pattern between PD
and controls. Our results validated the usefulness of combining multiple CSF biomarkers in improving PD diagnostic accuracy and prognostic evaluation.
ملخص

تشير الكثير من الدراسات الجينية والكيميائية إلى أن تراكم البروتين "الفا سيتوكلين" يلعب دورًا حيويًا في الكثير من الأمراض المرتبطة بتلف الخلايا العصبية مثل مرض باركنسون و خرف الشيخوخة المرتبط بأجسام لوتي. وعلى الرغم من أن العلمي المكلف في مجال مرض باركنسون إلا أنه إلى يومنا هذا لا توجد أدوات تشخيص دقيقة أو علامات بيولوجية يمكن الاعتماد عليها للحصول على تشخيص مبكر للإصابة، لنتظر الصورة المرئية هي الوسيلة الوحيدة المعتمدة لتشخيص مرض باركنسون. الجدير بالذكر أن تحوّل البروتين "الفا سيتوكلين" من مونومرات قابلة للذوبان إلى أوليوغرات أو ألياف غير قابلة للذوبان يعد خطوة حاسمة في الآلية الإمراضية لمرض باركنسون. حيث إن هذه الوفيات البروتينية ساهمت في الخلايا العصبية الدوبامينرجية و تؤدي إلى اضطرابات الحركة المعروفة لدى مرضى باركنسون.

في هذا البحث، أُولًا لاحذر بتطوير أجسام مضادة استثنائية قادرة على التعرف بشكل نوعي على المكونات المرمزية للسلل الرعشي، الأمر الذي يساهم إيجابيًا في مجال تشخيص وعلاج مرض باركنسون وأمراض عصبية أخرى ذات الصلة، ثانياً، فننا يتمظوف هذه الأجسام المضادة لتطوير تقنية ELISA قادرة على قياس مستويات أشكال مختلفة من بروتين "الفا سيتوكلين" وأهمها الأشكال المرمزية من البروتين في سوائل الجسم الحيوية. أخيرًا، بواسطة اختبارات ELISA في عينات من السائل الدماغي الشوكي من مرضى بنكسيس ومرضى باركنسون.

خلصت هذه الدراسة إلى أن الجمع بين قياسات الأشكال المختلفة من بروتين "الفا سيتوكلين" في سوائل الجسم الحيوية ذو أهمية بالغة في تشخيص مرض باركنسون وتحسين دقة تمييز مرضى باركنسون عن نظراهم الأصحاء.
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CHAPTER ONE: Introduction
1.1 Parkinson’s disease

Neurodegenerative diseases are the leading cause for disability in the world and one of the biggest burdens on our societies. Parkinson’s Disease (PD) is the second most common neurodegenerative disease after Alzheimer’s Disease (AD), and PD is a chronic progressive irreversible disorder. PD was first described by James Parkinson, an English physician, in his essay “Shaking Palsy” over 100 years ago. Although in less than three years PD will enter its next century, many aspects of the disease remain to be elucidated.

PD affects mainly a region within the midbrain known as the substantia nigra (SN) which is the main center of the dopaminergic neurons. Dopamine (DA), a neurotransmitter that is mainly involved in the control of the movement in our bodies is synthesized within dopaminergic neurons. Degeneration of these neurons results in reduced levels of DA. The clinical symptoms of PD manifest when degeneration affects 75% of these neurons. The cardinal symptoms of PD are: 1) Resting tremor: shaking that is mainly seen in the hands and jaw, 2) Rigidity: patient finds it difficult to move his limbs, 3) Bradykinesia: slowness in movement and 4) Postural instability.

In 1912, while Frederick Lewy was examining the brains of people with PD, he noticed abnormal microscopic structures which are now known by Lewy bodies (LBs). LBs and Lewy neurites (LNs) are the neuropathological hallmark of PD and dementia with Lewy bodies (DLB). LBs are eosinophilic intracytoplasmic inclusions that are mainly composed of the protein named alpha-synuclein (α-syn).
1.2 Epidemiology

PD is the second most common neurodegenerative disease. The social and economic burden imposed by PD is significantly increasing as populations age. In 2007, Dorsey et al., expected that the number of people affected by PD will rise to 9.3 million in 2030 (Dorsey et al., 2007). In 2009, Muangpaisan et al., concluded in his systematic review that PD prevalence and incidence is slightly lower in Asian countries as compared to Western countries (Muangpaisan, Hori, & Brayne, 2009). However no significant difference was seen in the occurrence of PD between Asians from different ethnic groups (L. C. Tan et al., 2004). In 2009, Yamawaki et al., reported that the occurrence and risk of developing PD has remained stable over 25 years (Yamawaki, Kusumi, Kowa & Nakashima, 2009). Nevertheless the burden of PD in Asia is significantly increasing as a result of increased life expectancy and population ageing.

A recent study conducted in United States estimated that approximately 630,000 people in US were diagnosed with PD in 2010, and this number is most likely to double by 2040, while the national economic burden of PD exceeded $14.4 billion in 2010 (Kowal, Dall, Chakrabarti, Storm, & Jain, 2013).

The question still remains whether prevalence rates vary between males and females. Some studies reported a higher prevalence of PD in men than in women (Benito-León et al., 2004; Benito-León et al., 2003; Fall et al., 1996; Mayeux et al., 1995), whereas other studies found no significant differences in PD prevalence between men and women (de Lau et al., 2004; de Rijk et al., 1995; de Rijk et al., 1997; Morgante et al., 1992). The reason for higher risk of PD in men than women
is mostly explained by the difference in estrogens levels; however this explanation is still being debated (Cardona-Rossinyol, Mir, Carballo-Miralles, Lladó, & Olmos, 2013; Morissette, Al Sweidi, Callier, & Di Paolo, 2008; Saunders-Pullman, 2003).

1.3 Neuropathology

PD can be sporadic or inherited, and in both cases it is accompanied by degeneration of neurons in the pars compacta of the SN which projects to putamen. This mainly results in a disruption of the nigrostriatal dopaminergic system. It is mostly difficult to diagnose PD at early stages due to the compensatory mechanism of the surviving dopaminergic neurons. Once the loss exceeds 75%, this mechanism fails to compensate and the clinical symptoms of PD start to appear. Remarkably, the midbrain remains significantly unaffected, which is helpful in the differential diagnosis of PD from other related parkinsonism disorders like progressive supranuclear palsy and multiple system atrophy (MSA).

1.3.1 Lewy bodies and Lewy neurites.

LBs and LNs are the most common neuropathology of PD, and mainly but not exclusively exist in the SN. LBs and LNs are also present in other brain regions like dorsal motor nucleus of the vagus, the nucleus basalis of Meynert, and the locus coeruleus. LBs are mainly intracellular spherical assemblies with dense eosinophilic core surrounding a pale halo, while LNs are long filaments that shares the same composition. Brain sections rich with LBs and LNs from patients with confirmed DLB were strongly stained with antibodies directed against α-syn but
not beta-synuclein (β-syn) or gamma-synuclein (γ-syn) (Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998; Spillantini et al., 1997). These sections also stained positive for ubiquitin, but with less in intensity and number, suggesting that α-syn is not the only component of LBs and LN\(_6\), but the most predominant one.

![Fig. 1.1](image)

**Fig. 1.1** Lewy bodies and Lewy neurites in post-mortem brain
Substantia nigra from patients with PD (A-F) immunostained for α-syn (in brown).

### 1.3.2 Mitochondria dysfunction

Mitochondria are unique structures with a double lipid bilayer membrane and are found in all eukaryotic cells. These organelles generate energy in the form of adenosine triphosphate (ATP) through the electron transport chain. Mitochondria plays essential roles in maintaining calcium hemostasis and mediating programmed cell death “apoptosis”. The electron transport chain is composed of five complexes, deficiency or inhibition of any of these complexes leads to disruption of this chain resulting in mitochondrial dysfunction and free radicals.
amounts of ATP, they are rich in mitochondria. The first piece of evidence that linked mitochondrial dysfunction with PD is the neurotoxin MPTP ((1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine). MPTP causes parkinsonian symptoms similar to those seen in PD patients by inhibiting Complex 1 in electron transport chain thus decreasing ATP levels and increasing ROS production. The link between dopaminergic neurons susceptibility and mitochondrial dysfunction is still a subject of debate. DA is synthesized in the cytosol and then uptaken into the presynaptic vesicles through vesicular monoamine transporter2 in an ATP-dependent manner. Therefore, any decrease in ATP levels due to mitochondrial dysfunction will impair this mechanism inducing SN degeneration. Furthermore, DA metabolism by itself generates ROS stress, making DA neurons more vulnerable to environmental toxins or mutations that increase DA turnover (S. J. Lee, 2003). Put together, these facts suggest that mitochondrial dysfunction is a potential cause of PD.

1.4 Aetiology of PD

Despite the fact that PD was first diagnosed a century ago, till date the main causes for this disease remain undefined. All studies point to the involvement of both environmental and genetic factors in the etiology of PD.

1.4.1 Genetic factors
Tremendous progress toward understanding the genetic basis of PD has been achieved in the past fifteen years. The first clue that genetic factors may be involved in PD pathology came in the 1890s, when Gowers described the clustering of PD within families (Eadie, Scott, Lees, & Woodward, 2012; Gowers, 1893). It is clear now that PD is a genetically heterogeneous disorder with at least 28 distinct chromosomal regions involved. Familial PD accounts only for 10% of all PD cases (B. Thomas & Beal, 2007), and the remaining 90% is characterized as sporadic PD. The aetiology of sporadic PD is still not clear, but it is thought to be triggered by the synergy of environmental and genetic factors. Familial PD can be classified as autosomal dominant and autosomal recessive.

1.4.1.1 Autosomal dominant forms of PD

1.4.1.2.1 PARK1 - SNCA

The α-syn gene, SNCA, coding for α-syn protein, was the first gene to be linked to autosomal dominant PD. It was first identified in a large Italian-American family with autosomal dominant early-onset PD, the Contursi kindred (Golbe, Di Iorio, Bonavita, Miller, & Duvoisin, 1990). The disease in this family locus was linked to chromosome 4q21-q23 and was later confirmed by the identification of a missense mutation, i.e. the replacement of alanine at position 53 with threonine, (A53T), in exon 4 of the gene (Polymeropoulos et al., 1997). The same mutation was also found in three Greek families, who originate from the Peloponesos in Southern Greece (Polymeropoulos, et al., 1997). The clinical and pathological features of the individuals with the A53T mutation in the SNCA are very similar to those with idiopathic PD, including a response to levodopa and the
presence of LBs in the SN. However, PD in these individuals is associated with earlier onset, more severe phenotype, and faster disease progression than that observed in patients with idiopathic PD (Fuchs et al., 2007; Ross et al., 2008).

A second missense mutation in exon 3 of SNCA, namely the replacement of alanine at position 30 with proline, (A30P), was found in a German family (Krüger et al., 1998). In individuals with the A30P mutation, the age of onset of the disease is in their 50s, which is later compared to the age of onset of the disease in A53T individuals.

In 2004, a third point mutation was identified in a Spanish family. The replacement of glutamic acid at position 46 with lysine, (E46K), has been associated with autosomal dominant PD (Zarranz et al., 2004).

Two another novel missense mutations have been recently identified in this gene in PD patients. The first mutation was in exon 4 of SNCA encoding a histidine-to glutamine Substitution (H50Q) was identified in an English female patient at age 71 presented with tremor and was L-dopa responsive with negative family history (Proukakis et al., 2013) and in a Canadian patient with positive family history for parkinsonism and dementia (Appel-Cresswell et al., 2013).

The second mutation was reported in a British family with early onset PD causing a glycine to aspartic acid amino acid change (G51D). Juvenile parkinsonism, moderate response to L-dopa and rapid disease progression with death in less than 10 years from onset mostly are the main symptoms associated with G51D missense mutation (Kiely et al., 2013).
Duplications (Chartier-Harlin et al., 2004; Ibáñez et al., 2004) and triplications (A. B. Singleton, Farrer, & Bonifati, 2013) of the α-syn gene have also been reported, and it is now understood that the severity of familial PD depends on SNCA gene dosage and the α-syn expression levels (Ross, et al., 2008). This has led to the simple hypothesis of a dose relationship between α-synuclein levels and disease severity (A. Singleton & Gwinn-Hardy, 2004).

1.4.1.2.2 PARK5-UCHL1

Ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1); is a neuron-specific enzyme that cleaves carboxy-terminal peptide bond of polyubiquitine chain, thus suggesting a role as a ubiquitin-recycling enzyme (Leroy et al., 1998). Mutation of UCH-L1 was identified in a family of German descent in which PD was inherited in an autosomal dominant fashion. The affected members had a missense mutation (I93M) in UCH-L1 that reduces its catalytic activity. In vitro this missense mutation cause various protein metabolites to aggregate (E. K. Tan & Skipper, 2007).

1.4.1.2.3 PARK8-LRRK2

Mutations in the leucine-rich repeat kinase 2 (LRRK2) are the most common known causes of autosomal dominant PD. LRRK2 was identified in a large Japanese pedigree, named the Sagamihara family, in which PD is inherited in an autosomal dominant fashion (Funayama et al., 2002). So far, seven mutation have been identified in LRRK2 (N1437H, R1441C, R1441G, R1441H, Y1699C, G2019S, and I2020T) that are considered disease causing. G2019S is the most common mutation followed by R1441C. G2019S is very common among Arab
patients from North Africa (Hulihan et al., 2008) and Ashkenazi Jewish patients (Ozelius et al., 2006). Degeneration of the dopaminergic neurons in the SN and classical LBs are the common pathological features associated with LRRK2 mutations (Zhu et al., 2006).

1.4.1.2.4  PARK 17- Vacuolar protein sorting 35 (VPS35)

In 2011 Farrer and colleagues identified a missense mutation in the VPS35 gene where aspartic acid was substituted with asparagine (D620N), as a novel cause of autosomal dominant PD (Vilaríño-Güell et al., 2011). The mutation was reported in Swiss kindred presented with late-onset PD. In the same year, the same missense mutation was reported in an Austrian family with 16 affected individuals with age of onset around 53 years (Zimprich et al., 2011). VPS35 is a component of the retromer complex and mediates retrograde transport between endosomes and the trans-Golgi network. The brain pathology in carriers of this mutation remains unknown.

1.4.1.2.5  PARK 18- Eukaryotic translation initiation factor 4 gamma 1 (EIF4G1)

Missense mutation (R150H) was identified in the EIF4G1, which is implicated in causing autosomal dominant PD by a genome-wide linkage approach in a large French family. Affected members of EIF4G1 display with variable clinical and pathological symptoms that mostly resemble idiopathic, late-onset Lewy body PD (Chartier-Harlin et al., 2011).
1.4.1.2 Autosomal recessive, early-onset typical parkinsonism

1.4.1.2.1 PARK2: Parkin

In 1998, Kitada et al., was the first to describe the mutations in the newly identified gene parkin (PARK2) that is associated with autosomal recessive early-onset PD (Kitada et al., 1998). Since then, a number of mutations have been identified in PARK2 that account for up to 10% of early onset PD cases. The clinical phenotype of PARK2 mutations is usually indistinguishable from early onset idiopathic PD, with slower response to levodopa and frequent late motor complications. It was thought that PARK2 patients lack LBs and neurofibrillary tangle pathology; however α-syn immunopositive inclusions existence in the pedunculopontine nucleus was reported in some recent studies (Farrer et al., 2001; Gouider-Khouja et al., 2003; Mori et al., 1998; Pramstaller et al., 2005). Neurofibrillary tangles have also been reported in some cases with PARK2 mutations (Mori, et al., 1998).

1.4.1.2.2 PARK6- PINK1

In 2001, Valente et al., reported linkage to chromosome 1p35-36 in a large Italian family, the Marsala kindred, in which PD was inherited in an autosomal recessive fashion. PINK1 codes for a protein kinase that is localized in mitochondria and is related to cellular stress and response (Valente et al., 2001). Studies of highly conserved Drosophila orthologs of parkin and PINK1 suggest that PINK1 and Parkin participate in a pathway that influences the integrity of flight muscle, sperm, and a subset of dopaminergic neurons in the brain. A prominent and an early feature in fly tissues is mitochondrial dysfunction linked to
Parkin and PINK1 mutants (Clark et al., 2006; Greene et al., 2003). Hence the parkin and PINK1 proteins are functionally linked. Together, they are important to tag damaged mitochondria for degradation by autophagy (Narendra et al., 2010). The clinical features of PINK1-linked parkinsonism include the typical signs of PD apart from an early age of onset (32-48 years), slow progression of symptoms and an early occurrence of levodopa-associated dyskinesia (Valente et al., 2004).

1.4.1.2.3 PARK7- DJ-1

PD patients with DJ-1 mutation have an early onset between the ages of 20-40 years. Although the function of DJ-1 is still under investigation, however it has been proposed to play a protective role in the mitochondria by reducing oxidative stress generated during the inhibition of the electron transport chain (Abou-Sleiman, Healy, Quinn, Lees, & Wood, 2003; Blackinton et al., 2009; Canet-Avilés et al., 2004; Trempe & Fon, 2013). It has been also implicated in multiple cellular processes, including oxidative stress response, protein quality control, anti-apoptotic signaling, transcriptional regulation and translational control (da Costa, 2007). Several studies have indicated that DJ-1 may also be involved in other age-related neurodegenerative disorders like Alzheimer disease (Neumann et al., 2004).

1.4.1.3 Autosomal recessive, juvenile typical parkinsonism

Several genes with recessive mutation were linked to very early onset (juvenile) PD with other clinical signs in addition to parkinsonism. PARK9 (Kufor–Rakeb syndrome), is characterized by juvenile, levodopa-responsive parkinsonism and dementia and is caused by recessive mutations in the ATPase
type 13A2 (ATP13A2) gene (Bras, Verloes, Schneider, Mole, & Guerreiro, 2012), which encodes a lysosomal membrane transporter (Bras, et al., 2012).

Recessive mutations in PARK14 (phospholipase A2, group VI (PLA2G6)) gene were later identified in patients with levodopa-responsive parkinsonism, pyramidal signs and cognitive features, with onset in early adulthood (Paisán-Ruiz et al., 2012). Pathological analysis of brains of patients with PLA2G6 mutations revealed widespread LBs (Paisán-Ruiz, et al., 2012).

Mutations in PARK15, F-box only protein 7 gene (FBXO7), also causes a recessive form of juvenile parkinsonism with pyramidal disturbances (T. Zhao et al., 2013). The brain pathology in patients with FBXO7 mutations remains unknown. However, Zhao et al., reported FBXO7 immunoreactivity in the LBs of typical PD, and in glial cytoplasmic inclusions of multiple system atrophy, suggesting an involvement of α-syn in the pathogenesis of the common forms of synucleinopathies (T. Zhao, et al., 2013).

During the past year, mutations in two other genes, DNAJC6 and SYNJ1, were identified as a rare cause of autosomal recessive, juvenile parkinsonism. DNAJC6 mutations were initially reported in a Palestinian family (Edvardson et al., 2012), and later in a Turkish family (Köroğlu, Baysal, Cetinkaya, Karasoy, & Tolun, 2013), while SYNJ1 mutation was identified independently in two families of Iranian and Italian origins (Krebs et al., 2013; Quadri et al., 2013). Both genes are implicated to play roles in the post-endocytic recycling of synaptic vesicles.
<table>
<thead>
<tr>
<th>Symbol</th>
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<th>Inheritance</th>
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1.4.2 Environmental factors

It was in 1877 that Jean-Martin Charcot, a French neurologist known as “the founder of modern neurology”, pointed out that PD is not a family disease. Since then, there is accumulating evidence that exposure to some environmental agents contribute to the pathogenesis of PD. Experimental, epidemiological and animal models have helped in our understanding of the mechanisms through which these neurotoxicants act.

1.4.2.1 MPTP (1,4.2.1-Methyl-4-pheny-1,2,3,6-tetrahydropyridin)

In order to claim that a certain toxicant is a potential risk factor for PD, exposure to this toxicant should produce the well-known features that characterize PD. Unlike other environmental toxicants that can cause brain damage and result in symptoms that resemble PD, MPTP targets selectively the neurons involved in PD pathology. This makes MPTP one of most dangerous neurotoxicants but on the other hand, MPTP has been used to develop animal models that substantially helped in understanding the disease pathogenesis. The toxic effects of MPTP in PD pathogenesis was first discovered in 1983 when a group of drug abusers administered meperidine analogues contaminated with MPTP (Calne & Langston, 1983). These individuals developed the typical clinical symptoms of parkinsonism and responded fairly to the treatment with levodopa (Ballard, Tetrud, & Langston, 1985). Again Langston et al., in 1999 reported that examination of the brains of three self-administered drug abusers who developed Parkinsonism revealed moderate to severe depletion of pigmented nerve cells in the substantia nigra, thus building more evidence of the role of MPTP in idiopathic PD. Although LBs were
absent in the patients brains, the reason behind that was not clear (Langston et al., 1999). MPTP is a lipid-soluble molecule which facilitates its penetration through the blood brain barrier. Once it enters the brain, MPTP is readily oxidized through MAO-B that is produced in astrocytes and serotonergic neurons into MPP⁺, the toxically active form of MPTP (Chiba, Trevor, & Castagnoli, 1985). MPP⁺ is easily up-taken by the plasma membrane dopamine transporter (DAT) into the dopaminergic neurons. Accumulation of MPP⁺ in the dopaminergic neurons is the leading cause of the nigrostriatal damage. This was further supported by the observation of Gainetdinov et al., that dopaminergic neurons lacking DAT are resistant to MPTP toxicity (Gainetdinov, Fumagalli, Jones, & Caron, 1997) while Sanghera et al., indicated that there is a positive correlation between DAT mRNA levels and DA neurons susceptibility to MPTP (Sanghera, Manaye, McMahon, Sonsalla, & German, 1997). Within dopaminergic neurons, part of the MPP⁺ is taken through vesicular monoamine transporter 2 into the synaptic vesicles while the free MPP⁺ will accumulate in the mitochondria. Free MPP⁺ that accumulates in the mitochondria is the main player in triggering neurotoxicity. It has been proposed that trapping MPP⁺ in the synaptic vesicles protects against MPP⁺ toxicity and this hypothesis was further supported by the fact that vesicular monoamine transporter 2 heterozygote knockout mice showed a greater susceptibility to MPTP neurotoxicity (Takahashi et al., 1997). Free MPP⁺ accumulated in the mitochondria leads to disruption of the respiratory chain by inhibiting complex I, resulting in two main cytotoxic consequences: decreased ATP production (Chiba, et al., 1985) and increased ROS generation (Cleeter, Cooper, & Schapira, 1992).
1.4.2.2 **Pesticides.**

Clinical and experimental evidence has implicated the role of paraquat (bipyridyl), organochlorine, organophosphate and rotenoid compounds that are mainly found in pesticides and herbicides in the aetiology of PD. Epidemiological studies have shown that individuals in the rural environments especially farming workers are at higher risks of developing PD. In 1987, Sanchez was the first to address paraquat as a neurotoxicant that contributes to PD pathogenesis (Sanchez-Ramos, Hefti, & Weiner, 1987) and the fact that paraquat closely resembles MPTP in its chemical structure built a stronger evidence (Snyder & D'Amato, 1985).

Interestingly, both *in vitro* and *in vivo* studies showed that paraquat is capable of stimulating α-syn aggregation, a multi-step process that highlights the disease initiation and progression (Manning-Bog et al., 2002). Other additional classes of pesticides that are to be considered as potential neurotoxicants are organophosphate and rotenoid compounds. Rotenone is widely used in broad-spectrum pesticides and is a highly lipophilic compound that readily crosses the BBB. Similar to MPTP, rotenone is a classic complex I inhibitor, making it a potential mitochondrial poison. However, unlike MPTP, rotenone accumulates in the dopaminergic neurons independently of DAT (Uversky, 2004).

1.4.2.3 **Heavy Metals.**

Although the exact etiology of PD is still to be convincingly established, many epidemiological studies reported that long term exposure to heavy metals like iron, manganese, copper, mercury, lead and aluminum is a risk factor for PD. Although exposure to these metals results in parkinsonian symptoms, however,
this doesn't necessarily imply on the pathophysiology of the disease. Interestingly, patients exhibiting parkinsonism due to manganese exposure did not respond to L-DOPA treatment and unlike idiopathic PD patients, midbrain region was not affected (Guilarte, 2010; Pal, Samii, & Calne, 1999).

Several studies have shown that the level of iron is significantly elevated in the SN of PD patients. However, the link between iron accumulation and inducing PD pathology is not completely explained. It is hypothesized that iron acts on several levels causing dopaminergic degeneration in the SN. Considering that iron is a redox-active metal, it is capable of catalyzing the formation of ROS inducing neuronal degeneration. As PD etiology involves the fibrilization of α-syn to form toxic aggregates, Uversky et al., demonstrated that low concentrations of some metals including iron, can directly facilitate α-syn aggregation (Uversky, Li, & Fink, 2001b).

1.4.2.4 Neuroprotective Agents.

The first evidence on the existence of neuroprotective substances in the environment was illustrated by Baumann et al., in study where he concluded that tobacco smokers are at lower risk of developing PD (Baumann, Jameson, McKean, Haack, & Weisberg, 1980). Consistent with this finding, further consecutive studies (for example, Grandinetti et al., 1994; Hellenbrand et al., 1997; Gorell et al., 1999) showed lower incidence of PD in tobacco smokers in a dose-dependent pattern. The first problem that scientists faced was in identifying which of the tobacco constituents protect against neuronal damage knowing that tobacco contains hundreds of chemicals. The main focus was on nicotine that acted as a
neuroprotective but not neurorestorative agent in animal models. More light remains to be shed on the mechanism by which nicotine prevents nigrostriatal degeneration as that can greatly contribute to the development of novel therapies for PD. It has been suggested that nicotine action is mediated mainly through specific striatal nicotinic subtypes receptors that modulate dopamine release and thus dopaminergic neuron function (F. M. Zhou, Wilson, & Dani, 2002). Studies done on PD patients as well as animal models showed that nicotine is not able to improve the motor deficits associated with PD; rather it can halt disease progression (Bordia, Campos, Huang, & Quik, 2008; Quik et al., 2007). In 2013 Quik et al., showed that further to nicotine’s role in nigrostriatal prevention, it can also reduce dyskinesia associated with long term L-dopa treatment (Quik, Mallela, Ly, & Zhang, 2013). More investigation needs to be done about the role of nicotine as a potential candidate in developing therapeutic strategies for PD.

Several recent studies provided an evidence that caffeine intake is associated with improvement in motor activity, through neuroprotection and neurorestoration by thropic proteins (Airavaara, Voutilainen, Wang, & Hoffer, 2012; J. F. Chen & Chern, 2011; Postuma et al., 2012). Initiating PD in rats followed by direct administration of caffeine after one week of disease onset prevented the loss of nigral dopaminergic neurons, indicating a role for caffeine in delaying neuronal degeneration (Sonsalla et al., 2012). Moreover, caffeine has been shown to down regulate neuroinflammatory responses and nitric oxide production, which protects against many pathological processes including cell death (Salvemini, Kim, &
Mollace, 2013; Tsutsui et al., 2004; Yadav, Gupta, Srivastava, Srivastava, & Singh, 2012).

1.5 Synuclein Family

Synuclein family has been under spotlight for a long while now, since α-syn was found to play a major role in PD pathogenesis and that mutated α-syn was associated with rare familial cases of early-onset PD. In 1988, Maroteaux et al. was the first to isolate and identify α-syn from *Torpedo californica* using antisera against purified cholinergic synaptic vesicles. The name synuclein emerged from the protein localization; in the nuclear envelope of the neurons and in the presynaptic nerve terminals (Maroteaux, Campanelli, & Scheller, 1988).

Synucleins are small, soluble proteins highly expressed by neuronal cells. However, synuclein family is less expressed in other tissues such as heart, lung, Pancreas, Kidney and liver (Ltic et al., 2004). Synuclein family consists of three distinct members that are exclusively produced in vertebrates: α-, β- and γ-syn. α-Syn and β-syn are primarily expressed in the brain tissues while γ-syn is primarily found in the peripheral nervous system and retina. α-Syn over-expression has been implicated in neurodegenerative disorders such as PD, DLB and multiple system atrophy (MSA); however, γ-syn over-expression is correlated with breast cancer progression (Bruening et al., 2000). On the structural level, all synucleins consist of highly conserved amino-terminal domain where repetitive presence of the amino acid motif KTKEGV is seen, and a less-conserved carboxy-terminal domain that is rich in acidic residues. Synucleins are natively unfolded proteins, meaning that under natural physiological conditions, these proteins have a linear structure as...
random coils. Understanding the exact normal cellular function of this family and its contribution to human diseases attracts the attention of many researchers.

1.5.1 α-Syn

α-Syn is a pre-synaptic neuronal protein and its aggregation and dysfunction is linked to a number of neurodegenerative disorders named as “synucleinopathies”. Antibodies against α-syn labeled both synapses and nuclei, leading to the naming of synuclein (Maroteaux, et al., 1988). Synucleinopathies mainly refer to PD, dementia with Lewy bodies (DLB) and MSA. α-Syn is 140 amino-acid protein that is highly abundant in the brain and can also be found in red blood cells (Barbour et al., 2008), plasma, cerebrospinal fluid (CSF) (El-Agnaf et al., 2003) and saliva (Devic et al., 2011). α-Syn is encoded by SNCA gene, in which point mutations and gene multiplications are identified in families with autosomal-dominant forms of PD (Hardy, Lewis, Revesz, Lees, & Paisan-Ruiz, 2009). The protein is natively unfolded; however, it is capable of forming an α-helical structure upon binding to negatively charged lipids like cellular phospholipids. Structures rich in β-sheets are formed as well under prolonged incubation of α-syn. Three main domains define α-syn protein: (1) amino-terminus (N-terminal) that includes residues (1-60) and are responsible for α-syn propensity to form α-helical structures, (2) the central hydrophobic region (NAC: non-Aβ component), which includes residues (61-95) and confers the β-sheet potential, (3) a carboxy terminus (C-terminal), which is negatively charged and unlike NAC region, C-terminal is prone to stay unstructured (Hoyer, Cherny, Subramaniam, & Jovin, 2004; Maroteaux & Scheller, 1991) (Fig. 1.2).
The precise function of this protein and the mechanism by which it provokes neuronal cell death is not fully understood. Several lines of evidence through studies on different model organisms, suggest that α-syn is a major player in vesicle trafficking, synaptic plasticity and neurotransmitter release.

Abeliovich et al. showed in his study using α-syn knockout mice, that α-syn deficiency did not comprise the DA neuronal morphology, suggesting that α-syn is neither essential for neuronal development nor for pre-synaptic terminals development. On the other hand, mice exhibited an increase of DA release at nigrostratal terminals in response to electrical paired stimuli and a reduction in striatal DA content, suggesting that α-syn is an essential, activity-dependent negative regulator of DA neurotransmission (Abeliovich et al., 2000).

It is well known that lack of the synaptic co-chaperone cysteine-string protein alpha (CSPα) results in a severe progressive neurodegeneration, but an interesting observation was that transgenic expression of α-syn ameliorated the neurodegeneration and the motor impairment seen in CSPα knockout mice. Chandra et al., also showed that transgenic α-syn expression helps reverse the inhibition of SNARE complex assembly due to CSPα deletion (Chandra, Gallardo, Fernández-Chacón, Schlüter, & Südhof, 2005). Hereby, there is a growing body of knowledge that α-syn has a physiological role as natural neuroprotective protein at the synapse.
1.5.2 β-Syn

Another member of synuclein family is β-syn (phosphoneuroprotein-14, PNP-14), encoded by SNCB gene, and located at chromosome 5. β-Syn is a 134 amino acid protein sharing 78% homology with α-syn (Beyer et al., 2010). Similar to α-syn, β-syn is a natively unfolded protein that co-localize in presynaptic terminals (Maroteaux et al., 1988). Unlike α-syn, it lacks 11 hydrophobic amino acid residues in its central region that correspond to the 72-84 residues of α-syn, resulting in non-amyloidogenic homologue of α-syn (Fig. 1.2). There is a growing evidence that β-syn can be a natural inhibitor for α-syn aggregation. Rockenstein et al., pointed out that a critical balance in the ratio of β-syn to α-syn should be maintained for the normal function of the brain and that this ratio is altered in DLB patients (Rockenstein et al., 2001). While wild type β-syn fails to fibrilize, mutant β-syn where NAC domain is added was able to confer aggregation properties similar to α-syn (Hashimoto, Rockenstein, Mante, Mallory, & Masliah, 2001). These results were consistent with another study where β-syn was exposed to assembly conditions for 6 weeks but still did not polymerize ascertaining the importance of the hydrophobic region for the protein aggregation (Giasson, Murray, Trojanowski, & Lee, 2001). Behavioral assessment of transgenic (tg) mice expressing human α-syn solely and tg mice co-expressing human α-syn and human β-syn supported the idea that β-syn can act as a natural negative regulator for α-syn aggregation (Hashimoto, et al., 2001). While motor dysfunction was clearly seen in tg mice expressing human α-syn only, significant amelioration was observed in tg mice co-expressing human α-syn and human β-syn (Hashimoto, et al., 2001).
Furthermore, fewer pathological inclusions were seen in bigenic mice compared to human α-syn tg mice. As a further confirmation, cells over expressing human α-syn and transfected with β-syn didn’t display any inclusions suggesting that β-syn can block α-syn aggregation (Hashimoto, et al., 2001). Taken together, these studies suggest the possibility of using the non-amyloidgenic properties of β-syn in developing novel treatments for PD.

1.5.3 γ-Syn

Unlike α-syn, γ-syn is not clearly involved in neurodegenerative diseases. However, γ-syn has been implicated in advanced breast carcinomas (Wu et al., 2007) and other malignancies, including ovarian (Bruening, et al., 2000), gastric (Yanagawa et al., 2004), esophagus (C. Q. Zhou et al., 2003), liver (W. Zhao et al., 2006), and pancreatic (Hibi et al., 2009) cancers. The γ-syn gene, SNCG, is located on chromosome 10q23 and encodes a 127 amino acid protein. γ-syn gene which is also known as “Breast cancer -specific gene 1” (BCSG1), is barely present in normal or benign breast lesions, whereas it is highly abundant in advanced infiltrating breast cancers suggesting that BCSG1 can be useful as a marker of breast cancer progression (Ji et al., 1997). The highest homology between this protein and the other two members of this family is seen in the amino-terminal region (Fig. 1.2). Several studies showed that γ-syn has a different pattern of expression; it is expressed in the early stages of axonal outgrowth and maintained throughout life, suggesting that γ-syn is important for maintaining the integrity of the neurofilament network (Buchman et al., 1998). Unlike α-syn, γ-syn has a weak tendency to form amyloid fibrils (Giasson, et al., 2001).
Fig. 1.2 The human synuclein family.
The bars represent the different types of synucleins. The different amino acid residues are represented at the bottom. The N-terminal (amphipathic region), the hydrophobic NAC domain and the C-terminal (hydrophilic acidic tail) are separated by vertical dashes and clearly indicated by different colors. The percentages under each domain represent the degree of amino acid identity as compared to α-syn.

Adapted

1.6 α-Syn post-translational modifications

α-Syn is subjected to several post-translational modifications such as phosphorylation, oxidation, nitrosylation, truncation and ubiquitination. However, whether these post-translational modifications act to enhance or inhibit α-syn neurotoxicity remains to be understood. A better understanding of the role of α-syn post-translational modifications will help to elucidate the exact role of α-syn in the pathogenesis of PD, paving the way to develop new diagnostic and therapeutic strategies for synucleinopathies. Although all these modifications have been studied, the main attention is focused on phosphorylation. Iwatsubo and colleagues presented the first evidence of α-syn phosphorylation at Ser129 through immunohistochemical and biochemical studies which was later supported by other studies. Antibodies generated specifically against S129-phosphorylated α-syn (p-
S129-α-syn), were able to stain LBs from brains of patients who died with PD, MSA or DLB (Anderson et al., 2006; Fujiwara et al., 2002; Okochi et al., 2000).

Other studies demonstrated that α-syn can be phosphorylated at multiple sites. Paleologou et al., showed that α-syn is phosphorylated at Ser87 in vivo and in LBs in synucleinopathies (Paleologou et al., 2010), while Feany and his colleagues proved that α-syn can also be phosphorylated at Tyr125 (L. Chen et al., 2009).

In human brains, 90% of total α-syn found in LBs is phosphorylated at Ser129, raising an important question whether α-syn phosphorylation promotes or protects against neurotoxicity? Some studies reported that over-expression of PLKs, which phosphorylates α-syn specifically at Ser129, in the nematode (C. elegans) suppressed neurotoxicity and protected against dopaminergic cell loss (Kuwahara, Tonegawa, Ito, Mitani, & Iwatsubo, 2012). In rat models, where S129A or S129D mutation were induced, which mimics the absence of phosphorylation or mimics the phosphorylation respectively, an increase in α-syn toxicity through up-regulation of beta-sheet rich, proteinase-K resistant aggregates formation was observed (Gorbatyuk et al., 2008). More findings that support the hypothesis that phosphorylation of α-syn is protective, Mbefo et al., showed that PLK2, which is a member of Polo-Like Kinases and a major contributor to α-syn phosphorylation, is protective against α-syn toxicity in vitro and in vivo (Mbefo et al., 2010). On the other hand, Chen et al., showed in transgenic drosophila models that phosphorylation at Ser129 is a crucial step in enhancing α-syn neurotoxicity through the formation of α-syn soluble oligomers but not α-syn mature inclusions
Biochemical examination of LBs revealed the presence of full-length α-syn as well as different species of truncated α-syn with molecular weight ranging from 10-15kDa (Anderson, et al., 2006; Baba et al., 1998). Interestingly, truncated α-syn was found in the brains of patients as well as the brains of healthy controls, suggesting that α-syn truncation takes place even under normal physiological conditions. Interestingly, a marked difference in the amount of truncated α-syn in synucleinopathies patients compared to controls was found (Beyer et al., 2004). Beyer et al. showed that there was a twofold increase in truncated α-syn expression in DLB compared to controls, and a threefold increase in DLB compared to AD patients (Beyer, et al., 2004). Truncated α-syn was found to be abundant in the brains of PD and DLB patients, suggesting that truncated α-syn may play a normal physiological role as well as a pathological one (W. Li et al., 2005). SH-SY5Y cells exhibited more vulnerability to oxidative stress when full length α-syn and truncated α-syn were co-overexpressed (Liu et al., 2005). Accumulation of truncated α-syn was significantly high in the brains of tg mice expressing human A53T mutation which is found in some cases of familial PD and linked to early onset of the disease (Michell et al., 2007). Taken all together, these data suggest that α-syn truncation forms highly amyloidgenic fragments that accelerate or seed α-syn aggregation. Light was mostly shed on C-terminal truncated α-syn as it exhibited a higher propensity to fibrilize in comparison with WT full length α-syn. Extensive research was done in this area that supported this hypothesis. Hoyer et
reported by *in vitro* studies that truncated α-syn specifically C-terminal residues 109-140 promoted aggregation presumably through nucleation formation (Hoyer, et al., 2004).

Immunohistochemical studies have shown that tyrosine nitration in LBs is a common feature of synucleinopathies (Dekker, Abello, Wisasta, & Bischoff, 2012). Giasson *et al.*, was the first to provide a strong evidence of nitrated α-syn accumulation in LBs via the use of specific monoclonal antibodies for the nitrated form of α-syn, demonstrating that nitration affects α-syn solubility and promotes its aggregation (Giasson *et al.*, 2000).

**1.7 α-Syn Aggregation**

The findings that (a) fibrillar α-syn is the major component of LBs, and (b) the A30P, E46K and A53T forms of α-syn, which are associated with autosomal dominantly inherited forms of PD, have higher rates of self-oligomerization compared to WT α-syn (El-Agnaf Curran, et al., 1998; Zarranz, et al., 2004), have led to the speculation that the aggregation of α-syn has a seminal role in PD. As a result extensive efforts have been put into the elucidation of the mechanisms responsible for the polymerization and aggregation of α-syn.

In vitro studies have shown that α-syn, the wt and the A53T, A30P and E46K mutated forms, are able to self-aggregate and form fibrils which structurally resemble those isolated from LBs and LNs (El-Agnaf, Curran, et al., 1998; Zarranz, et al., 2004). Interestingly, the mutated forms of α-syn, especially the ones with the A53T and E46K mutations (Conway, Harper, & Lansbury, 1998; El-
Agnaf, Curran, et al., 1998; Zarranz, et al., 2004), were shown to have higher aggregation rates, possibly because these mutations disrupt the α-helical structure of the protein in the N-terminal region. In fact, studies done on aged α-syn solutions have demonstrated that the aggregation of the protein is accompanied by a conformational transition from random coil or α-helical structure to cross β-pleated sheet (El-Agnaf, Jakes, et al., 1998; Serpell, Berriman, Jakes, Goedert, & Crowther, 2000).

The fibrillation process of α-syn has been suggested to occur via oligomers and then protofibril formation, which in turn associate to produce mature filaments (Conway, et al., 1998). α-Syn aggregation is a nucleation dependent process and this process can be divided into three phases (Wood et al., 1999):

1. Initial phase during which the nuclei are formed.

2. Elongation phase, characterized by the exponential growth of the fibrils.

3. The Equilibrium phase, during which the protein in solution is equilibrium with the protein in fibrillar form (Uversky et al., 2002). This phase is associated with a dramatic conformational change from random coil to predominantly β-pleated sheet (Conway, Harper, & Lansbury, 2000). The β-sheets then proceeds through the formation of several altered-sized oligomers to form protofibrils (intermediate stage) before maturing into long strands and then LBs (Goldberg & Lansbury, 2000). It has been shown that the fibrillation rate is concentration dependent, increasing with higher protein concentration and pH-dependent, increasing by a decrease in pH (Uversky, Li, & Fink, 2001a), temperature dependent, increasing
with increasing temperature (Hashimoto et al., 1998) and the presence of metals (Kanda, Bishop, Eglitis, Yang, & Mouradian, 2000).

1.8 α-Syn Aggregation and Toxicity

As described above, there are numerous findings suggesting that α-syn aggregation plays a central role in the pathogenesis of synucleinopathies. First, the main components of LBs are filaments of α-syn (Spillantini et al., 1998), and in vitro studies have shown that recombinant α-syn can form inclusions that resemble LBS (Hashimoto, et al., 1998; Narhi et al., 1999).

Second, five missense α-syn mutations (see also section 1.4.1) have been linked to rare forms of early-onset familial PD (Appel-Cresswell, et al., 2013; Krüger, et al., 1998; Polymeropoulos, et al., 1997; Proukakis, et al., 2013; Zarranz, et al., 2004), and they have been shown to increase α-syn aggregation in vitro (Conway, et al., 2000; El-Agnaf, Curran, et al., 1998; Narhi, et al., 1999). It has been shown that mutant α-syn contain more β-sheet structure than the wt protein (El-Agnaf, Jakes, et al., 1998). Third, oxidative damage to α-syn makes the protein more prone to aggregation (Esteves, Arduino, Swerdlow, Oliveira, & Cardoso, 2009; Kanda, et al., 2000).

In an effort to elucidate the toxic effect of the aggregated forms of α-syn on different cell lines in vitro, El-Agnaf et al., reported that fresh and aged NAC (1-18) and NAC (1-35) added directly to cultures of SH-SY5Y cells were toxic, with the aged NAC being more toxic than the fresh, suggesting that the cytotoxicity is aggregation-dependent (El-Agnaf, Jakes, et al., 1998).
In vitro studies on the effect of α-syn overexpression have been carried out using EK293 and SK-N-SH cells. Overexpressing α-syn induced toxicity in both cell lines in a dose-dependent manner, and the mutations (A53T and A30P) increased cell toxicity; however, the A53T mutant α-syn form was more toxic to the cells (Ostrerova et al., 1999).

Toxic effects of aggregated α-syn were observed in many conducted in vivo studies. For example, Masliah and co-workers have used transgenic mice overexpressing human α-syn. Their results showed loss of dopaminergic terminals in the basal ganglia in addition to motor impairments. Moreover, formation of both cytoplasmic inclusions and electron-dense intranuclear deposits was seen through ultrastructural analysis. These inclusions were composed of granular aggregated α-syn (Masliah et al., 2000).

Furthermore, many studies have shown that overexpression of human α-syn in transgenic flies and mice is accompanied by neuronal dysfunction and loss of synaptic terminals and/or neurons and the formation of lesions similar to those found in PD brain (Feany & Bender, 2000; Kahle, Neumann, Ozmen, & Haass, 2000; Martin, 2007; Martin et al., 2006; Mizuno, Fujikake, Wada, & Nagai, 2010; Siebert et al., 2010; van der Putten et al., 2000). These animals, also, progressively develop motor abnormalities.

Many recent findings support the notion that α-syn spreads in a prion-like fashion, playing a crucial role in PD propagation and pathogenesis. α-Syn was thought first to be an exclusively intracellular protein, however the detection of α-
syn in biological fluids, such as blood plasma and CSF has challenged this idea (Borghi et al., 2000; El-Agnaf, Salem, et al., 2003). It has been found that α-syn is released in the culture medium of neuronal cells under normal or induced expression (Danzer et al., 2011; El-Agnaf, Salem, et al., 2003; Emmanouilidou et al., 2010; H. J. Lee, Patel, & Lee, 2005; Sung et al., 2005), but whether released α-syn has physiological function is still unknown. This hypothesis was supported by the discovery that PD patients subjected to striatal mesencephalic grafts transplantation, developed α-syn-positive and ubiquitin-positive LBs decade following transplantation (Kordower, Chu, Hauser, Freeman, & Olanow, 2008; J. Y. Li et al., 2008).

The mechanism of α-syn release has not been fully elucidated. Lee and colleagues showed that a portion of intracellular α-syn is constitutively secreted to the culture medium via endoplasmic reticulum/Golgi-independent exocytosis (H. J. Lee, et al., 2005). When applied to cultured neuronal cells, both oligomeric and fibrillar forms of α-syn were internalized via endocytosis and degraded by the lysosome (H. J. Lee et al., 2008).

It has been proposed that α-syn is directly transmitted from one cell to another via sequential exocytosis and endocytosis, forming Lewy-like inclusions in recipient cells (Desplats et al., 2009). Emmanouilidou et al., used an inducible neuroblastoma cell line that expresses α-syn and found that α-syn is secreted from neuronal cells in a calcium-dependent manner by exosomes and that soluble oligomeric and monomeric α-syn species were released in association with externalized membrane vesicles (Emmanouilidou, et al., 2010).
Cell to cell transmission has been also demonstrated in vivo. Desplats et al., showed that injection of mouse cortical neuronal stem cells in the hippocampus of transgenic mice overexpressing human α-syn, resulted in transfer of α-syn species from host neurons to grafted cells (Desplats, et al., 2009).

Hangen et al., demonstrated that neurons can internalize α-syn species regardless its aggregation state and that the aggregated form structure is conserved throughout the internalization process (Hansen et al., 2011). Furthermore, in the same study, transplantation of wt mouse embryonic mesencephalic grafts into the striatum of tg mice overexpressing human α-syn, has led to α-syn transmission from the host striatal tissue to the transplanted dopaminergic neurons (Hansen, et al., 2011).

Virginia Lee and colleagues showed that α-syn cell-to-cell transmission occurred after a single intrastriatal inoculation of synthetic α-syn fibrils in non-transgenic mice and that Parkinson's-like Lewy pathology was seen in anatomically interconnected regions (Luk et al., 2012).

1.9 Animal Models of PD

To acquire a better understanding of PD pathogenesis and to develop better disease modifying therapeutic strategies, PD animal models can be of great assistance. The last two decades have witnessed a marked improvement in developing multiple models of PD with different mechanisms of insult. Modeling PD can be quite a challenge, as the “reasonable” model should be able to recapitulate the clinical and pathological features of PD (Chesselet, Fleming,
Mortazavi, & Meurers, 2008). PD models should develop dopaminergic degeneration progressively and age-dependently as PD begins mostly in late adulthood. Another major obstacle is that they should be able to reproduce the motor symptoms of PD like bradykinesia, rigidity and resting tremor and they should respond to DA replacement therapy as well. Among the widely used animal models of PD, pathogenic and etiologic models are the most common. Pathogenic models refer to the ones where DA depletion was induced through neurotoxins like reserpine, MPTP, rotenone and paraquat. Etiologic models are also known as disease gene-based models. They mimic PD by expressing specific mutations in genes associated with familial forms of PD.

1.9.1 Neurotoxin models

It was in 1970s that Ungerstedt reported the first model of PD in rats produced by an intracerebral injection of 6-hydroxydopamine (6-OHDA) (Ungerstedt & Arbuthnott, 1970). 6-OHDA was first isolated in 1950s and it affects the dopamine transport system and disrupts the electron transport chain of complex I in the mitochondria. It does not cross the blood brain barrier (BBB), which necessitates its direct injection into substantia nigra, medial forebrain bundle, and striatum (Perese, Ulman, Viola, Ewing, & Bankiewicz, 1989; J. Thomas et al., 1994). Although it causes a progressive neuronal degeneration in the substantia nigra and ventral tegmental complex, no LBs are formed (Berger, Przedborski, & Cadet, 1991; Sauer & Oertel, 1994). 6-OHDA is mainly used to Screen for therapies that may improve PD symptoms and to study cell death mechanisms. Ten years later, a group of young drug addicts displayed clinical symptoms that greatly resembled
PD due to administration of heroin analogous contaminated by MPTP, which triggered the researchers curiosity to test this compound in different animal species. MPTP administration was capable of reproducing most of the PD clinical and pathological features in monkeys (Chiueh et al., 1984; Crossman, Mitchell, & Sambrook, 1985; Doudet, Gross, Lebrun-Grandie, & Bioulac, 1985; Heikkila, Hess, & Duvoisin, 1984, 1985; Langston, Forno, Rebert, & Irwin, 1984; Nomoto, Jenner, & Marsden, 1985), and while dopaminergic degeneration was obviously seen in mice, rats showed resistance to MPTP effects (Chiueh, et al., 1984). MPTP model produces PD features by the same mechanism as 6-OHDA. The main advantage of MPTP model is that MPP+ selectively damages dopaminergic neurons. MPTP is highly lipophilic, thus it readily crosses the BBB after systemic administration. Subcutaneous and intravenous are the most common routes of administration (Przedborski et al., 2001) while unilateral intracarotid injection is used when only unilateral parkinsonism in required (Bankiewicz et al., 1986). In MPTP models, loss of DA neurons is dose dependent and inclusions are rarely seen, however Jin et al., reported that combining MPTP with proenecid (a pesticide) induces the formation of LBs-like (Jin et al., 2005).

Rotenone is a pesticide that is able to diffuse through all cellular membranes including the BBB. It strongly inhibits complex I in the electron transfer chain, leading mainly to nigrostriatal dopaminergic degeneration with α-syn positive inclusions similar to true LBs (Betarbet et al., 2000; Sherer, Kim, Betarbet, & Greenamyre, 2003; Testa, Sherer, & Greenamyre, 2005).

Paraquat is a herbicide similar to MPTP that crosses the BBB and destroy dopaminergic neurons in the substantia nigra. Paraquat is associated with the
formation of α-syn positive inclusion in the substantia nigra pars compacta over prolonged exposure (Brooks, Chadwick, Gelbard, Cory-Slechta, & Federoff, 1999; Manning-Bog, et al., 2002).

1.9.2 Transgenic Animals for PD

Genetic animal models of PD were developed mainly to overcome the flaws seen with the classical toxin-based models. Genetic mutations in PD represent only about 10% of all PD cases; however gene-based models can be very instructive in understanding not only PD pathogenesis but other neurodegenerative diseases as well.

The developed animal models succeeded in exhibiting most PD features like neuronal loss, neuropathology, neurochemical alteration and motor deficits (for details see above section 1.8).

1.10 α-Syn: a potential biomarker for PD

Several recent studies have explored the use of α-syn as a potential PD biomarker in human biological fluids. α-Syn was first detected in CSF using immunoprecipitation and immunoblotting with α-syn specific antibodies (Borghi, et al., 2000; EL-Agnaf, Salem, et al., 2003). Several studies have investigated the levels of total α-syn (t-α-syn) in CSF as measured with ELISA, but results were not conclusive. Some studies have shown a clear trend of lower CSF t-α-syn in PD and other synucleinopathies such as MSA and DLB (Hong et al., 2010; Mollenhauer et al., 2008; Mollenhauer et al., 2011; Tateno, Sakakibara, Kawai, Kishi, & Murano, 2012; Tokuda et al., 2006). On the other hand, a large overlap in
t-α-syn was observed between PD and other disease (Aerts, Esselink, Abdo, Bloem, & Verbeek, 2012; Noguchi-Shinohara et al., 2009; Ohrfelt et al., 2009; Parnetti et al., 2011). Collectively, these studies suggest that the use of t-α-syn alone as CSF biomarker for PD is not enough. Many factors might contribute to inconsistency between the groups including variability in patients’ characteristics, such as age, disease duration and disease severity. The discordance of the reported results might also be caused by methodological issues, including pre-analytical differences in collection, handling and storage of CSF samples, use of different antibodies that detect different species of α-syn, and variability among sources of recombinant α-syn protein used as the standard in the ELISA assays. Another critical factor is represented by blood contamination, which could cause substantial increases in CSF α-syn concentration (Hong, et al., 2010).

Substantial evidence suggests that soluble oligomers of amyloid proteins play a crucial role in the pathogenesis of neurodegenerative diseases. Several recent studies showed that α-syn oligomers (o-α-syn) are neurotoxic in vitro and in vivo (Winner et al., 2011; Xu et al., 2002). Paleologou et al., reported that the levels of o-α-syn are elevated in brain homogenates in PD and DLB compared with normal brains suggesting a role for o-α-syn in PD pathogenesis (Paleologou et al., 2009). Accordingly, detection of these species in CSF could represent a good biomarker of PD and related synucleinopathies (El-Agnaf, Walsh, & Allsop, 2003). In 2010, El-Agnaf and colleagues found that o-α-syn levels and o-α-syn: t-α-syn ratios were substantially higher in patients with PD including those with mild and early-stage disease than in individuals with other neurological disorders, or those who
underwent lumbar puncture for diagnostic purposes (Tokuda et al., 2010). CSF o-α-syn had a sensitivity of 75.0% and a specificity of 87.5% for PD, and the CSF o-α-syn: t-α-syn ratio had a sensitivity of 89.3% and a specificity of 90.6%. These findings have been confirmed in two independent reports (Park, Cheon, Bae, Kim, & Kim, 2011; Sierks et al., 2011).

LBs are formed mostly of post-translationally modified forms of α-syn. The modifications include phosphorylation, nitration and ubiquitination, and most of the accumulated α-syn in Lewy bodies is phosphorylated at Ser129 (pS129-α-syn). Whether pS129-α-syn promotes or protects against PD is unclear, however, pS129 α-syn levels can improve discrimination between PD and other forms of parkinsonism (Wang et al., 2012).

1.11 Objectives of the Project

Taken together, all of the genetic, pathological, biochemical and animal model data support the idea that α-syn aggregation plays a critical role in synucleinopathies. Therefore, developing methods that can detect the pathogenic forms of α-syn may provide diagnostic tools for an early diagnosis of PD. The aim of the project was to develop new immunoassays for measuring t-α-syn, o-α-syn and p-S129-α-syn species in human CSF as potential biomarkers for PD to provide an early diagnosis, monitor disease progression and demonstrate treatment efficacy.
CHAPTER TWO: Materials
2.1 Suppliers

Details of the suppliers of chemicals and equipment used are summarized in table 2.1.

<table>
<thead>
<tr>
<th>Short Name</th>
<th>Full Name and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcam</td>
<td>Abcam Inc. Kendall Square, Cambridge, UK.</td>
</tr>
<tr>
<td>Aldrich/Sigma</td>
<td>Sigma-Aldrich Co. Ltd., Dorset, UK.</td>
</tr>
<tr>
<td>BD Biosciences</td>
<td>Becton, Dickinson and Company, Franklin Lakes, USA.</td>
</tr>
<tr>
<td>Gilson</td>
<td>Gilson, Inc.3000 Parmenter Street, Middleton, WI, USA.</td>
</tr>
<tr>
<td>Invitrogen</td>
<td>Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, USA</td>
</tr>
<tr>
<td>Nunc</td>
<td>Nalgene, Nunc International, Life Technologies Ltd., Paisley, UK.</td>
</tr>
<tr>
<td>Pierce</td>
<td>Thermo Scientific Life Science Research Products, Rockford, IL, USA.</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology</td>
<td>Santa Cruz Biotechnology, Delaware Avenue, Santa Cruz, CA, U.S.A.</td>
</tr>
<tr>
<td>Jackson</td>
<td>Jackson ImmunoResearch, UK</td>
</tr>
<tr>
<td>IBL</td>
<td>Immuno-Biological Laboratories Co., Ltd, Japan</td>
</tr>
<tr>
<td>Millipore</td>
<td>Merck Millipore, Billerica, Massachusetts</td>
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<tr>
<td>SeraCare KPL</td>
<td>SeraCare Kirkegaard &amp; Perry Laboratories, Maryland, USA</td>
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<tr>
<td>Merck</td>
<td>Merck Sharp &amp; Dohme, USA</td>
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<tr>
<td>Gibco</td>
<td>Gibco, Life technologies, New York, USA</td>
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<tr>
<td>Hyclone</td>
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2.2 Materials and equipments

Details of the chemicals and equipment used are summarized in table 2.2.

<table>
<thead>
<tr>
<th>Materials/Equipment</th>
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</thead>
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<tr>
<td>Albumin from bovine serum ≥98% (agarose gel electrophoresis)</td>
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<tr>
<td>BCA Protein assay reagent kit</td>
<td>Pierce</td>
</tr>
<tr>
<td>Bovine serum Albumin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CDM4mAb Media</td>
<td>Hyclone</td>
</tr>
<tr>
<td>Cellytic M cell lysis reagent for cultured mammalian cells</td>
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</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Fetal bovine serum</td>
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<tr>
<td>Glutamax</td>
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<tr>
<td>Glutamax</td>
<td>Invitrogen</td>
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<td>HAT Media Supplement (50X) Hybri-Max</td>
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<tr>
<td>HT Media Supplement (50X) Hybri-Max</td>
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<tr>
<td>Isocovemod Dulbecco (IMDM)</td>
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<td>Isotyping Kit</td>
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<td>MaxiSorp™ 384 well black plates</td>
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<td>Phosphate buffered saline (PBS) tablets</td>
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<td>Sodium dodecylsulphate (SDS)</td>
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**Continued Table 2.2: Summary of chemicals/equipments**

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<td>Substrate</td>
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<td>TEMED</td>
<td>Invitrogen</td>
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<tr>
<td>Tissue culture Coverslips 25mm (plastic)</td>
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<tr>
<td>TMB substrate</td>
<td>KPL</td>
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<tr>
<td>β-mercaproethanol</td>
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**Table 2.3: Summary of antibodies used**

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<td>Anti-α- synuclein (Syn-1) mouse monoclonal antibody</td>
<td>BD Bioscience</td>
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<tr>
<td>Anti-α- synuclein (211) mouse monoclonal antibody</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Anti-α/β/γ- synuclein (FL-140) rabbit polyclonal antibody</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-β-syn (8) mouse monoclonal antibody</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-γ- synuclein (C-20) goat polyclonal antibody</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-alpha Synuclein (phospho S129) mAb (EP1536Y)</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-Tau (5E2) mouse monoclonal antibody</td>
<td>Millipore</td>
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<tr>
<td>Anti-human Amyloid beta (N) (82E1) mouse monoclonal antibody</td>
<td>IBL</td>
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<tr>
<td>Anti- Amylin (R10/99) mouse monoclonal antibody</td>
<td>Santa Cruz Biotechnology</td>
</tr>
</tbody>
</table>
2.3 Solutions and buffers

A. Phosphate Buffered Saline (PBS), pH 7.4

One PBS tablet was dissolved in 200 ml sterile distilled water (dH₂O) and then filtered through a 0.2 μm filter and stored at 4°C.

B. Phosphate buffered saline with 0.05% Tween-20 (PBST)

PBS with 0.05% Tween-20.

C. 200 mM NaHCO₃, pH = 9.6

4.2 g of NaHCO₃ was dissolved in about 230 ml of sterile water, and then pH was adjusted to 9.6 at RT with 3M NaOH. The final volume was made up to 250 ml with sterile water and then the buffer was filtered through a 0.2 μm filter and stored at 4°C.

D. RIPA buffer (50mM Tris, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS)

5 ml of 1M Tris pH= 8 was added to 90ml of sterile dH₂O, and 0.88 g of NaCl was dissolved followed by 0.5 g of sodium deoxycholate. Then 1 ml of NP-40 was added and 0.1g of SDS were added and the final volume was made up to 100 ml. The buffer was filtered through a 0.2 μm filter and stored at 4°C.

E. Artificial CSF (ACSF)

Solution A The following (5.19g NaCl, 0.134g KCl, 0.123g CaCl₂·H₂O, 0.0975g MgCl₂·6H₂O) were dissolved in 300 ml of sterile dH₂O and the buffer was filtered through 0.2 μm and then stored at 4°C.
Solution B

The following (0.128g Na2HPO4.7H2O, 0.016g NaH2PO4.H2O) were dissolved in 300 ml of sterile dH2O, and the buffer was filtered through 0.2 µm and then stored at 4°C.

Solution A and Solution B were mixed in equal proportions before use.
CHAPTER THREE: Development and Characterization of Conformation-Specific Monoclonal Antibodies for α-Synuclein Aggregates
3.1 Introduction

α-Syn is central to the pathogenesis of PD and other neurodegenerative diseases. Developing monoclonal antibodies capable of targeting pathological forms of α-syn is of great importance. Increasing evidence suggests that α-syn oligomers (o-α-syn) play a crucial role in the pathogenesis of PD (Dimant et al., 2013; el-Agnaf & Irvine, 2002; El-Agnaf, Walsh, et al., 2003; H. J. Lee, Khoşaghideh, Patel, & Lee, 2004; Paleologou, et al., 2009; Tokuda, et al., 2010; Winner, et al., 2011), and therefore, antibodies that can selectively target the pathogenic species of α-syn may open doors for developing effective diagnostic and therapeutic strategies for synucleinopathies. Toward this end, we developed highly selective mouse monoclonal antibodies (mAbs) that target the pathological species of α-syn. In this chapter, we present the methodology used to develop and characterize six novel conformation-specific mouse mAbs that specifically recognize α-syn pathological forms. Two more mouse mAbs and one sheep polyclonal antibody targeted against α-syn were also generated and characterized.
3.2 Methods

3.2.1 Preparation of immunogen

The immunogen employed for the development of the monoclonal antibodies was composed of aggregates of α-syn. α-Syn fibrils were generated using a solution of recombinant human α-syn at a concentration of 50 μM. α-Syn aggregation was induced by continuous mixing in a Thermomixer (Thermomixer compact, eppendorf) (800 rpm) at 37°C for 7 days and was monitored using thioflavin-S (Th-S) binding assay. After the fibrillation was complete, the aggregates were aliquoted into small aliquots and stored at -80°C until required.

3.2.2 Immunization

BALB/c female mice (6-8 weeks old) were immunized subcutaneously with α-syn aggregates. Each mouse received an initial immunization of 50 μg of α-syn aggregates in a solution that was mixed with Freund’s Complete Adjuvant (1:1 v/v). Following a 3-week interval, the initial immunization was followed by a booster immunization of 25 μg of antigen mixed with Freund’s Incomplete Adjuvant (1:1 v/v). On the 10th day after the booster immunization, blood was collected from the tail vein and antiserum was separated. Using the antisera, the antibody response was evaluated with an indirect ELISA. The mice selected for fusion were subjected to a final immunization of 150 μg of the immunogen at 3 days prior to fusion.

3.2.3 Indirect ELISA for the assessment of the immune response of the hosts
A 96-well clear MaxiSorb plate (Nunc) was coated with 100 μl per well (70 ng/well) of α-syn aggregates in PBS and incubated overnight at 4°C. The following day, the plate was washed three times with PBST (PBS containing 0.05% Tween-20) and blocked with blocking buffer (PBST containing 2.25% gelatin) for 1 h at room temperature (RT). The plate was then washed three times with PBST, and 100 μl of serially diluted antiserum (the initial dilution was 1:100, followed by 10 dilutions) from the mice was added to the wells in duplicate. The plate was then incubated at RT for 1 h. Next, the plate was washed with PBST, goat anti-mouse IgG-HRP (1:20 K, Jackson) was added (100 μl/well), and the plate was incubated for 1 h at RT. The plate was then washed three times with PBST and incubated with 100 μl of TMB substrate (KPL) until the color developed. The reaction was stopped by adding 0.6 N H2SO4 (100 μl/well). The absorbance was measured at 450 nm using a VictorX3 microtiter plate reader. The hosts that exhibited a good immune response were selected for fusion.

### 3.2.4 Generation of mouse anti-α-syn hybridomas

Splenocytes from mice exhibiting high titers were fused with mouse myeloma cells (Sp2O-Ag14; American Type Culture Collection) at a 5:1 ratio using 50% polyethylene glycol (MW 4000; Merck). After fusion, the cells were seeded and cultured in 96-well plates at a density of 2.4 x 105 cells/well in IMDM (Gibco) selection media containing 2 mM Glutamax (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma), 50 μg/ml gentamycin (Sigma), 50 μM β- mercaptoethanol (Sigma) and 20% fetal bovine serum (HyClone) supplemented with HAT (1x, Sigma) and 6 x 103 macrophages (6 x 103 cells/well; freshly
isolated from 5-6-week-old BALB/c mice). Hybridomas positive for α-syn fibrils were identified by analyzing their supernatants with ELISA (see the following section).

3.2.5 Screening for positive clones using ELISA

To identify positive hybridomas, supernatants from the growing fused cells were screened using ELISA. A 96-well clear MaxiSorb plate (Nunc) was coated with 100 µl of α-syn aggregates in PBS (1 µg/ml) and incubated overnight at 4°C. The following day, the plate was washed three times with PBST and blocked with blocking buffer for 1 h at RT. The plate was then washed with PBST, and 100 µl of culture supernatant from the fused cells was added to each well. The plate was incubated at RT for 1 h and then washed three times with PBST prior to the addition of the secondary antibody. The plate was washed with PBST and then incubated with TMB substrate (100 µl/well; KPL) until the color developed appropriately. The reaction was stopped by adding 0.6 N H₂SO₄ (100 µl/well), and the absorbance was measured at 450 nm using a Victor3 1420 multi-label microtiter plate reader. The positive hybridomas were transferred into a 24-well plate and screened again to identify stable clones.

3.2.6 Sandwich ELISA for isotyping of positive hybridomas

A 96-well clear MaxiSorb plate (Nunc) was coated with 1:1 K anti-mouse heavy-chain antibodies (Isotyping Kit, Sigma) in PBS and incubated overnight at 4°C. The following day, the plate was washed 3 times with PBST and blocked with blocking buffer (400 µl/well) for 1 h at RT. The plate was then washed 3
times with PBST, culture supernatant from each clone was added to each well (100 μl/well), and the plate was then incubated for 1 h at RT. Next, the plate was washed and then incubated with secondary antibody for 1 h at RT. The plate was washed with PBST and incubated with TMB (100 μl/well; KPL) until the color had developed appropriately. The reaction was stopped by adding 0.6 N H₂SO₄ (100 μl/well), and the absorbance was measured at 450 nm using a Victor3 1420 multi-label microtiter plate reader. The IgG-positive clones were transferred into a 25 cm² flask and then subjected to single-cell cloning.

3.2.7 Single cell cloning

Cells were collected from the cultures of positive hybridoma clones and were counted and diluted in IMDM. Approximately 100 cells were taken and added to 20 ml of IMDM (Gibco) supplemented with Glutamax (2 mM, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Penstrep, Sigma), 50 μg/ml Gentamycin (Sigma), 50 μM β-mercaptoethanol (Sigma), 20% fetal bovine serum (Hyclone) and macrophages (6000 cells/well) that were freshly isolated from young BALB/c mice. The cells were mixed and plated (200 μl/well) in a 96-well tissue culture plate and incubated in 5% CO₂ at 37°C. The hybridomas were allowed to grow. The wells containing single cells were marked, the cells were allowed to grow to confluency, and the culture supernatant was then collected and screened using an indirect ELISA. The ELISA-positive cells were transferred into 24-well plates and screened again prior to transfer to 25 cm² flasks. The culture supernatant from the 25 cm² flasks was screened at least three times prior to the selection of stable clones.
3.2.8 Mass culture and purification of monoclonal antibody

The selected clones were cultured on a larger scale (mass culture). The clones were cultivated in CDM4mAb (Hyclone) supplemented with 2 mM Glutamax (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Penstrep, Sigma), 50 μg/ml Gentamycin (Sigma) and 50 μM beta-mercaptoethanol (Sigma). Once the cells were confluent and the color of the media had turned yellow, the culture supernatants were collected and stored at -20°C until required for use. The monoclonal antibodies were purified from the culture supernatants using protein G-agarose affinity chromatography. Protein G-agarose columns were prepared and equilibrated using 20-bed volumes of 20 mM phosphate buffer, pH 7.2. The culture supernatant (200 ml) was centrifuged at 1500 rpm for 10 min at 4°C. The supernatants were collected and passed 5-6 times through the columns at a flow rate of approximately 1 ml/min. The columns were then washed with 15-bed volumes of 20 mM phosphate buffer, pH 7.2, to remove unwanted proteins. The bound antibody was eluted using 50 mM glycine, pH 2.5, into 1.5 ml centrifuge tubes containing 50 μl of neutralization buffer (1 M Tris, pH 8.0). The purified antibody was finally dialyzed against PBS. The antibodies purified from different batches were pooled, and the concentration was determined using a BCA assay. These monoclonal antibodies were lyophilized prior to storage at -20°C in 100 μg aliquots until required for use.
3.2.9 Dot Blot

Monomeric and aggregated forms of α-, β- and γ-syn; tau; Abeta-42; Islet Amyloid Polypeptide (IAPP); or ABri were used for exploring the specificity of our mAbs. Each protein was spotted (50 ng) onto a nitrocellulose membrane and allowed to dry at RT for 1 h. The membranes were then incubated for 1 h at RT with 5% skimmed milk in PBST with gentle agitation. The blocking solution was then removed, and the membranes were incubated for 2 h at RT with the primary antibodies. The membranes were then washed three times with PBST and incubated with secondary antibodies – goat anti-mouse HRP (1:20 K) or goat anti-rabbit (1:20 K) as appropriate – for 1 h at RT. The membranes were then washed three times with PBST and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

3.2.10 Western Blotting

Samples of human-, mouse- and rat-brain lysates (15 μg), α-, β- or γ-syn (50 ng) were mixed with loading buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 0.02% bromophenol blue) and then separated on 15% SDS-PAGE gels. The separated proteins were transferred to 0.45 μm nitrocellulose membranes (Whatman GmbH-Germany) at 100 V for 45 min. The membranes were boiled for 5 min in PBS and then blocked for 1 h with 5% non-fat milk prepared in PBST prior to incubation with 10 ml of primary antibody (50 ng/ml) for 2 h at RT. The membranes were then washed 4 times with PBST followed by incubation with the
secondary antibody. The membranes were then washed 4 times with PBST, and immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) according to the manufacturer's instructions.

3.2.11 Immunohistochemistry

1Ten-micron, formalin-fixed, paraffin-embedded sections were cut from the anterior cingulate cortex of PD cases and aged-neurological control cases. The sections were de-waxed in xylene (2 x 100% washes) and rehydrated through descending concentrations of ethanol (2 x 100%, 1 x 95%, 1 x 70%, 1 x dH2O) followed by formic acid (90%) antigen retrieval for three min. The sections were washed in Tris buffer (pH 7.2) followed by washing in 50% ethanol containing 1% H2O2 to block endogenous peroxidase activity. After washing in Tris buffer, sections were blocked for 20 min in Tris buffer containing 10% normal horse serum. The sections were then incubated overnight at 4°C with the following antibodies: Syn-1 (1:200; BD), anti-p-S129-α-syn antibody (EP1536Y; 1:500; Abcam), Syn-F1 (1:10 K), Syn-F2 (1:5 K), Syn-O1 (1:10 K), Syn-O2 (1:5 K), Syn-O3 (1:5 K) or Syn-O4 (1:5 K). After three washes in Tris buffer, the sections were incubated with biotin-conjugated horse anti-mouse (1:200; Vector Laboratories) or goat anti-rabbit (1:200; Vector Laboratories) antisera, as appropriate, for 30 min at 37°C. Following three washes in Tris buffer, the sections were incubated in avidin-biotin complex (1:500; Vectastain Elite Kit; Vector Laboratories) for 30 min at room temperature followed by DAB

1 Immunohistochemistry was conducted by our collaborator Prof. Glenda Halliday, University of New South Wales, Sydney, Australia.
incubation (1 x 10 mg DAB tablet in 15 ml Tris buffer). The peroxidase reaction was initiated by adding 0.0047% H₂O₂ to the DAB solution. The development of a dark-brown reaction product was monitored by eye under a dissecting microscope and stopped using two quick washes in Tris buffer prior to a final wash in dH₂O. The sections were counter-stained with 0.5% cresyl violet and dehydrated through ascending concentrations of ethanol (1 x 70%, 1 x 95% and 2 x 100%) and cleared in xylene. The sections were cover-slipped using DPX water-free mounting media.

All tissue was visualized using a Zeiss Axioskop microscope (Munchen-Hallbergmoos, Germany), and 8-bit RGB images of DAB-stained sections were captured using a Zeiss AxioCam HRc camera and AxioVision 4.7 software. For figure production, no adjustments were made to RGB images except for minor adjustments to brightness and contrast with the levels command in Adobe Photoshop CS6 (San Jose, CA) to best represent the staining observed directly under the microscope.

3.2.12 Sandwich ELISA for testing specificity towards o-alpha-syn

A 384-well ELISA microplate (Nunc MaxiSorb, NUNC) was coated overnight by incubating the tested mAbs (0.1 µg/ml) in 200 mM NaHCO₃, pH 9.6 (50 µl/well), at 4°C. The plate was washed with PBST and incubated with blocking buffer (100 µl/well) for 2 h at 37°C. After washing, 50 µl of the samples for testing was added to each well (α-syn monomer, oligomer or protofibril solutions), and the plate was then incubated at 37°C for another 2.5 h. FL-140,
diluted in blocking buffer (1/1 K), was added after washing the plate 4 times with PBST, which was then incubated at 37°C for 2 h. The plate was washed and then incubated for 1 h at 37°C with 50 µl/well of goat anti-rabbit IgG HRP (Jackson) diluted to 1:15 K in blocking buffer. Bound HRP activity was assayed based on a chemiluminescent reaction using the enhanced chemiluminescent substrate SuperSignal ELISA Femto (Pierce), after which chemiluminescence in relative light units was immediately measured using a Victor3 1420 (Wallac) microplate reader.

3.3 Results

3.3.1 Antibody response in α-syn immunized mice

To raise specific monoclonal antibodies for α-syn pathologies, recombinant α-syn aggregates were employed as immunogens. Following booster immunizations, the mice were bled to assess antibody responses, and a high response following the second booster immunization was obtained (Fig. 3.1.1A-F). The reason for this high response maybe that aggregated proteins are generally more immunogenic than their monomeric forms (Qian et al., 2012). Prior to using mice for fusion, pre-fusion titers (after 1 month of rest) were performed to assess whether the antibody response remained elevated. When the titer value was reduced to at least half of the peak value, the antibody-producing splenocytes of the mice were isolated and fused with mouse myeloma cells to generate hybridomas that secrete antibodies.
Fig. 3.1: Antibody response from α-syn immunized mice
Titer check for hosts immunized with α-syn aggregates (A-F).
3.3.2 Selection of positive hybridoma clones

To identify the hybridomas that produce antibodies against α-syn aggregates, the hybridoma supernatants obtained from the fusion of mice splenocytes with mouse myeloma cells was assessed for binding to α-syn aggregates. For this purpose, an indirect ELISA was performed using the supernatant from the hybridomas as the primary antibody. A total of 1100 positive clones were obtained from the initial screening, but only 100 clones were selected for further characterization.

The isotypes of the antibodies selected for the ELISA positive clones were identified using an isotyping kit. Out of the 100 clones identified, only 33 parental clones were found to be of the IgG isotype, whereas the remaining clones were either IgM or a mixture of IgG and IgM isotypes. The clones secreting IgG antibodies were selected, passaged multiple times to identify stable clones and then subjected to single-cell cloning to achieve monoclonality. The single-cell clones obtained were transferred to 24-well plates, and their stability was assessed using ELISA. The stable clones were then cultivated in 25 cm² flasks for further characterization.

The specificity of the mAbs produced for α-syn aggregates was assessed using dot blots. Out of a total of 278 clones, 45 clones were found to be stable. Out of these 45 clones, only 6 stable clones (Syn-F1, Syn-F2, Syn-O1, Syn-O2, Syn-O3 and Syn-O4) were specific to α-syn aggregates. Two clones, 11D12 and 7A11, which reacted to both monomeric and aggregated forms of α-syn, were also
selected for further characterization. Four clones were found to be IgG1, three clones were IgG2a, and one clone was found to be of the IgG2b isotype (see Table 3.1). 11D12 and 7A11 detected monomeric, oligomeric and fibrillar forms of α-Syn with similar affinity (Fig. 3.2). However, Syn-F1 & 2 and Syn-O1-4 specifically detected α-syn aggregates but not α-syn monomers (Fig. 3.3). Syn-1 (anti-α-syn) mouse mAb (BD biosciences) or 211 (Santa Cruz) were employed as positive controls.

<table>
<thead>
<tr>
<th>Table 3.1 Generated Clones' Isotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone ID</td>
</tr>
<tr>
<td>Syn-F1</td>
</tr>
<tr>
<td>Syn-F2</td>
</tr>
<tr>
<td>Syn-O1</td>
</tr>
<tr>
<td>Syn-O2</td>
</tr>
</tbody>
</table>

Fig. 3.2: Specificity of anti-α-syn antibodies towards α-syn. 50 ng of recombinant full length monomeric, oligomeric and aggregated α-syn were spotted into nitrocellulose membranes and probed with the indicated antibodies.
3.3.3 Affinity of the generated mAbs

To determine the affinity of Syn-F1, Syn-F2, and Syn-O1-4 for α-syn aggregates, an inhibition ELISA was performed. The inhibition ELISA is an indirect ELISA used to assess whether the pre-incubation of the antibodies generated with α-syn fibrils or monomers inhibits the ability of these antibodies to detect coated α-syn fibrils. All six antibodies were shown to be highly selective for α-syn fibrils compared with monomers, as shown in Fig. 3.4 and Table 3.2. Syn-1, employed as a control, exhibited an almost equal reactivity toward both α-syn fibrils and monomers.

<table>
<thead>
<tr>
<th>mAb ID</th>
<th>IC50 (μM) for α-syn fibrils</th>
<th>IC50 (μM) for α-syn monomers</th>
<th>Fold affinity towards α-syn fibrils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syn-F1</td>
<td>0.00012</td>
<td>2.083</td>
<td>16558</td>
</tr>
<tr>
<td>Syn-F2</td>
<td>0.00118</td>
<td>3.707</td>
<td>3141</td>
</tr>
<tr>
<td>Syn-O1</td>
<td>0.00037</td>
<td>4.793</td>
<td>12737</td>
</tr>
<tr>
<td>Syn-O2</td>
<td>0.00012</td>
<td>3.283</td>
<td>27312</td>
</tr>
<tr>
<td>Syn-O3</td>
<td>0.00047</td>
<td>5.329</td>
<td>11129</td>
</tr>
<tr>
<td>Syn-O4</td>
<td>0.00052</td>
<td>3.605</td>
<td>6932</td>
</tr>
<tr>
<td>Syn-1</td>
<td>0.0021</td>
<td>0.001</td>
<td>0.476</td>
</tr>
</tbody>
</table>
Fig. 3.4: Affinity the generated mAbs.
Inhibition ELISA demonstrating the binding preferences of our mAbs. The commercially available antibody Syn-1 is also included. Our mAbs displayed a marked selectivity for α-syn aggregates (○) versus monomers (▲). Syn-1 had an equal preference for both forms.
3.3.4 Our conformation-specific mAbs do not cross-react with amyloid fibrils formed by other synucleins or unrelated amyloid proteins

To evaluate the specificity of our mAbs, monomers and fibrils of α-syn, tau protein, β-amyloid (Aβ), Islet Amyloid Polypeptide (IAPP) and ABri and fibrillar forms of β- or γ-syn were blotted onto a nitrocellulose membrane and probed with our antibodies with appropriate positive control antibodies. Surprisingly, the selected antibodies were shown to be exclusively specific for α-syn fibrils because they did not recognize amyloid fibrils formed by other proteins, including β- or γ-syn fibrils (Figs. 3.5, 6).

Fig. 3.5: Specificity of generated mAbs to α-syn aggregates. 50 ng of both monomers (M) and fibrils (F) of α-syn, tau, IAPP or ABri were spotted onto nitrocellulose membranes. The membranes were probed with the indicated antibodies.

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Fig. 3.6: Specificity of our cmAbs to synuclien fibrils. 50 ng of both monomeric (M) or fibrils (F) of recombinant α-, β- and γ- were spotted onto nitrocellulose membranes. Then the membranes were probed with the indicated antibodies.
The six selected conformation-specific mAbs were also assessed for their cross reactivity with amyloid fibrils formed by different fragments of α-syn. Fibrils and monomers from full-length α-syn (1-140) and α-syn fragments, i.e., α-syn (1-122), NAC (61-95) and NAC (61-78), were tested. None of the six conformation-specific antibodies reacted with fibrils formed from any of the α-syn fragments; they recognized only the fibrils formed by the full-length α-syn (1-140), suggesting that a part of the epitope for these mAbs is within the C-terminal region (Fig. 3.7).

Fig. 3.7: Specificity of our mAbs towards the fibrils formed by full-length α-syn protein.
50 ng of both monomeric (M) and fibrils (F) of the full-length α-syn or α-syn fragments (1-122, 61-95 and 61-78) were spotted onto nitrocellulose membranes, then probed with the indicated antibodies.
3.3.5 11D12 and 7A11 mAbs do not cross-react with other synucleins

To explore the specificity of 11D12 and 7A11 for α-syn, human α-, β- and γ-syn were used for Western blotting. Western blotting revealed that both 11D12 and 7A11 were specific for α-syn, and no cross-reactivity against β- or γ-syn (Fig. 3.8) was observed.

Fig. 3.8: Specificity of anti-α-syn antibodies towards synuclein proteins. 50 ng of recombinant α-, β- and γ-syn were loaded on SDS gels and transferred to nitrocellulose membranes for western blotting. The membranes were then probed with indicated antibodies.
3.3.6 Specificity of 11D12 and 7A11 to α-syn

To investigate the specificity of 11D12 and 7A11 mAbs towards different α-syn species, including human, mouse and rat brain lysates were tested. Western blot analysis for 7A11 revealed a band about 14-kDa in all brain lysates representing full-length α-syn while the same band was detected only in the human brain lysate when the membrane was probed with 11D12 (Fig. 3.9)

![Image of Western blot analysis](image)

**Fig. 3.9**: Specificity of anti-α-syn antibodies towards α-syn homologs. 15 μg of human, mouse and rat brain lysates were loaded on SDS gels and transferred to nitrocellulose membranes for western blotting. The membranes were probed with indicated antibodies.
3.3.7 Cross-reactivity of 11D12 and 7A11 mAbs with modified forms of α-syn

To illustrate the binding specificity of these two mAbs to o-α-syn and post-translational modified forms of α-syn, monomeric, o-α-syn, p-S129-α-syn and nitrated α-syn were spotted onto nitrocellulose membranes. Dot blot analyses revealed that 11D12 recognized all three forms of α-syn, whereas 7A11 recognized only o-α-syn and nitrated α-syn with no cross-reactivity to pS129-α-syn (Fig. 3.10).

Fig. 3.10: Cross-reactivity of anti-α-syn antibodies with different species of α-syn. 50 ng of human monomeric, oligomeric, nitrated and p-Ser129 were spotted onto nitrocellulose membranes for dot blotting. The membranes were then probed with the indicated antibodies.
3.3.8 Immunostaining of α-syn pathology in human brain with our mAbs

In PD cases, Syn-1 labeled LBs and numerous LNs predominantly in deeper cortical layers of the anterior cingulate cortex, whereas anti-p-S129α-syn (EP1536Y) labeled a greater proportion of LBs; however, LNs were sparse. LBs labeled with EP1536Y were larger in size than those labeled with Syn-1 (Fig. 3.11A, B). Very faint synaptic synuclein-immunoreactivity was observed with EP1536Y. Syn-F1, Syn-F2, and Syn-O1-4 labeled a similar number of LBs of similar size and LNs as EP1536Y (Fig. 3.11C-H). Only very faintly immunoreactive synaptic syn was observed with our mAbs.

Fig. 3.11 Immunostaining of α-syn on human PD brain tissue.
Images showing LBs immunoreactive for Syn-1 (A), EP1536Y (B) and our six mAbs (C-H) in the anterior cingulate cortex from a case with PD. Sections are counterstained with Cresyl violet (blue). Scale bar
3.3.9 Development of specific sandwich ELISA for α-syn oligomers

The ELISA is based on a sandwich system in which the antibody for capturing is one of our novel conformation-specific mAbs and the antibody for detection is FL-140, a rabbit polyclonal anti-α-synuclein antibody (Santa Cruz). Serial dilutions of α-syn monomers, oligomers and protofibrils were used to evaluate this ELISA. α-α-Syn was prepared by mixing recombinant human α-syn with Gn Rb1 to induce the formation of soluble SDS-stable oligomers that were characterized by electron microscopy, Western blotting and gel filtration (Ardah et al., manuscript submitted for publication). For α-syn protofibril preparation, we used a protocol similar to that described by the Lee, V.M. research group (Norris et al., 2005). α-Syn oligomers and protofibrils were detected specifically by our ELISA, whereas the ELISA using Syn-1 for capture showed equal detection for all three forms of α-syn (Fig. 3.12).
Fig. 3.12: Characterization of our mAbs specificity and robustness in sandwich-ELISA system. Histograms shown are representative of 3 independent experiments using serial dilutions (500µM- 0.5µM) of recombinant α-syn monomers, oligomers and protofibrils. Measurements were taken in duplicate, and the results show the mean ± standard deviation for each point. Signal is displayed in relative light units (RLU).
3.3.10 Syn-140 Characterization

Syn-140 is a sheep polyclonal antibody raised against full-length human α-syn. Western blotting revealed that Syn-140 specifically detects α-syn of mouse, rat and human as well as p-S129- α-syn and nitrated α-syn.

Fig. 3.13: Specificity of Syn-140 towards synuclein proteins. 50 ng of recombinant α-, β- and γ-syn were loaded on SDS gels and transferred to nitrocellulose membranes for western blotting. The membranes were then probed with indicated antibodies.

Fig. 3.14: Specificity of Syn-140 towards α-syn homologs. 15 µg of human, mouse and rat brain lysates were loaded on SDS gels and transferred to nitrocellulose membranes for western blotting. The membranes were probed with indicated antibodies.
3.4 Discussion

α-Syn is the major component of LBs, one of the defining pathological hallmarks in the brains of patients with PD and DLB. Although the accumulation of α-syn is central to the development of synucleinopathies, the precise mechanism remains uncertain. The aggregation of α-syn takes place in a multi-step, nucleated polymerization process in which a conformational shift of the monomeric structure results in partially folded intermediate ‘oligomers’ that grow in size and eventually accumulate as insoluble amyloid fibrils in LBs. Of the different species of α-syn, intermediate oligomers/protofibrils are thought to be the major neurotoxic species. Therefore, developing therapeutic and diagnostic strategies that target these species is of paramount importance. Several hypotheses have been developed regarding the mechanism of the neuropathogenicity of α-syn oligomers. Some groups have reported that α-syn oligomers exhibit neurotoxic effects via the disruption of cellular membranes (Danzer et al., 2007; Winner, et al., 2011) or by neuroinflammatory mechanisms (Wilms et al., 2009). Recently, Masliah and colleagues reported that α-α-syn exacerbates synaptic degeneration by interfering with synaptic vesicles, leading to neuronal cell death (Rockenstein et al., 2014). However, the exact neuropathological mechanism of α-syn oligomers remains to be fully elucidated.

In 2003, Kayed et al. reported the development of A11, a polyclonal antibody raised against Aβ1-40. In vitro studies showed that A11 did not discriminate between oligomeric species from different amyloid proteins, including α-syn (Kayed et al., 2010). Another polyclonal antibody described by El-Agnaf et al.
Fila-1, which specifically recognizes α-syn aggregates, was used to detect α-α-syn in lysates from post-mortem DLB brains using indirect ELISA (Paleologou, et al., 2009). Moreover, a human single-chain antibody fragment that binds specifically to α-α-syn has also been developed and was reported to inhibit α-syn aggregation and oligomer-induced toxicity (Emadi, Barkhordarian, Wang, Schulz, & Sierks, 2007). More recently, Ingelsson and colleagues reported the development of two mouse mAbs with high selectivity toward large oligomers of α-syn only (Fagerqvist et al., 2013).

We used preparations of α-syn aggregates to immunize female BALB/C mice, thereby producing mAbs with high selectivity toward α-syn oligomers/fibrils. Furthermore, two mouse monoclonal antibodies equally reactive to different species of α-syn were also generated. All of our mAbs were thoroughly characterized using multiple methods, including immunoblotting, inhibition ELISA, sandwich ELISA and immunohistochemistry. Next, we employed our mAbs to develop specific and sensitive ELISA assays for the rapid and accurate quantification of α-syn oligomeric species.

Although several hybridomas secreting mAbs specific for α-syn oligomers/fibrils were isolated, only the clones that generated IgG isotype antibodies were selected. Considering the multivalent architecture of IgM antibodies, their potential therapeutic use is compromised. In contrast, the simple divalent structure of IgG antibodies is more suitable for future development as diagnostic or therapeutic tools.
In contrast with previously reported oligomer-specific antibodies, our novel mAbs do not cross-react with aggregated forms of other amyloid proteins, such as tau, Aβ, amylin or IAPP, and interestingly, they do not even recognize aggregates formed by other synuclein proteins, such as β- and γ-syn.

Prior to characterization, all mAbs were purified using protein G agarose beads. Using immunoblotting, in which the proteins were blotted onto nitrocellulose membranes, our conformation-specific antibodies (Syn-F1-2 and Syn-O1-4) were shown to be highly selective for α-syn aggregates, whereas 11D12 and 7A11 were shown to be specific for α-syn.

Consistent with our dot blot results, inhibition ELISA analyses, in which α-syn monomers and soluble aggregates were in solution, showed that our conformation-specific mAbs exhibited much higher affinity for α-syn soluble aggregates than for monomers. Our mAbs showed very strong affinity for 0-α-syn compared with other reported anti-0-α-syn (Fagerqvist et al., 2013). The affinity was estimated as the molar concentration of free antibody required to achieve 50% of the maximal binding calculated using binding curves.

The most important question was whether our novel mAbs could detect native pathological species of α-syn in LBs. Immunohistochemistry was performed using our mAbs on post-mortem brain tissue from patients with PD. Interestingly, our mAbs clearly stained LBs and both punctate and neuritic structures only. In a comparison of the staining patterns between our novel mAbs and commercial mAbs (Syn-1 and anti-phosphorylated Ser129 α-syn), our mAbs showed a lack of
cross-reactivity with synaptic α-syn, confirming their specificity toward α-syn pathology.

Additionally, to further investigate the specificity of our mAbs toward α-syn aggregates, our conformation-specific mAbs were used as capturing antibodies in sandwich ELISAs. Signals were detectable only from soluble α-syn aggregates, confirming our aforementioned results.

Interestingly, our research group has recently shown that Syn-O1-4 recognizes both soluble and mature fibrils, whereas Syn-F1 and Syn-F2 recognize α-syn amyloid fibrils preferentially compared with oligomers (Vaikath et al., manuscript submitted for publication).
CHAPTER FOUR: Developing Novel Sandwich-ELISAs for Measuring α-Synuclein Species in Biological Fluids
4.1 Introduction

PD is the most common movement disorder; however, its diagnosis is primarily based on motor-related clinical criteria. PD is characterized by a large time-gap between the beginning of the neurodegenerative processes and the onset of clinical neurological manifestations (Jankovic, 2008). The natural history of the disease includes an initial asymptomatic stage followed by a long pre-motor phase; finally, when the classical motor symptoms appear, the majority of nigral dopaminergic neurons have already degenerated (Tolosa, Gaig, Santamaria, & Compta, 2009). Therefore, there is an urgent need for the development of biomarkers for PD diagnosis and prognosis. Toward this aim, recent intensive investigations have attempted to identify biomarker(s) to enable the early diagnosis of PD, preferably during the pre-motor phase. Molecular changes in the brain are reflected in CSF composition, and this makes CSF an ideal source of reliable biomarkers (Anoop, Singh, Jacob, & Maji, 2010).

α-Syn has been consistently detected in biological fluids, such as human blood plasma and CSF (Borghi, et al., 2000; El-Agnaf, Salem, et al., 2003; Mollenhauer, et al., 2008). Recently, several studies have explored the potential use of CSF t-α-syn as a putative biomarker for PD. A clear trend of lower CSF total α-syn (t-α-syn) levels in PD and other synucleinopathies has been consistently reported, although a large overlap between PD and control groups has been noted (Aerts, et al., 2012; Hong, et al., 2010; Mollenhauer, et al., 2011; Noguchi-Shinohara, et al., 2009; Parnetti, et al., 2011; Parnetti, Farotti, et al., 2014; Spies, Melis, Sjögren, Rikkert, & Verbeek, 2009; Tateno, et al., 2012; Tokuda, et al., 2010; Tokuda, et
al., 2006). However, all of these studies noted that t-α-syn levels alone could not discriminate patients with synucleinopathies from normal individuals or those with other neurodegenerative diseases.

In contrast, combining the measurements of different species of α-syn may improve the discrimination between PD and other neurodegenerative diseases (Parnetti, Farotti, et al., 2014; Tokuda, et al., 2010; Wang, et al., 2012). Soluble α-syn oligomers (o-α-syn) have been increasingly linked to synaptic and neuronal degeneration (Rockenstein, et al., 2014). It has been shown that soluble o-α-syn is elevated in brain homogenates from PD and DLB cases compared with controls (Paleologou, et al., 2009; Sharon et al., 2003). The exact mechanism by which α-syn oligomers cause neuronal cell death is not fully elucidated. Masliah and co-workers reported in a recent study that o-α-syn exacerbates synaptic and neuronal degeneration in vivo (Rockenstein, et al., 2014). Interestingly, several independent studies showed that o-α-syn levels were significantly higher in PD patients compared with other neurological disorders (Park, et al., 2011; Parnetti, Chiasserini, et al., 2014; Sierks, et al., 2011). CSF o-α-syn levels and o-α-syn/t-α-syn ratios were found to be substantially higher in patients with PD, including those with mild and early-stage disease, compared with individuals with other neurological disorders or those who underwent lumbar puncture for diagnostic purposes (Tokuda, et al., 2010). CSF o-α-syn shows a sensitivity of 75.0% and a specificity of 87.5% for PD, and the CSF o-α-syn/t-α-syn ratio shows a sensitivity of 89.3% and a specificity of 90.6%. These findings have been confirmed in two independent reports (Park, et al., 2011; Sierks, et al., 2011).
Wang et al. measured p-S129-α-syn and t-α-syn levels in fresh CSF samples from a large cohort (~600 samples) of patients with varying types of parkinsonism, including PD, MSA and PSP and healthy individuals. CSF p-S129-α-syn levels showed a positive correlation with PD severity, and the combination of CSF t-α-syn and p-S129-α-syn improved the discrimination between PD and other forms of parkinsonism (Wang, et al., 2012). Taken together, these reports strongly suggest that o-α-syn and p-S129-α-syn are potential candidates as CSF biomarkers.

In this study, we developed specific and sensitive ELISA assays using our novel conformation-specific mAbs for measuring CSF o-α-syn. Moreover, we also developed a new ELISA for measuring CSF t-α-syn and a third ELISA for measuring CSF p-S129-α-syn in. In addition, the classical AD CSF biomarkers total-tau (t-tau), phosphorylated-tau (p-tau) and Amyloid-beta 42 (Aβ-42) were included in our study.

4.2 Methods

4.2.1 Development of ELISA for measuring total α-syn in human CSF

A 384-well ELISA microplate (Nunc MaxiSorb, NUNC) was coated in an overnight incubation at 4°C with 0.1 μg/ml sheep polyclonal anti-α-syn antibody (Syn-140) in 200 mM NaHCO₃, pH 9.6 (50 μl/well). The plate was washed with PBST and incubated with blocking buffer (100 μl/well) for 2 h at 37°C. After washing, 50 μl of the CSF samples (thawed on ice; Tween-20 was added to a final concentration of 0.05%) was added to each well, and the plate was then incubated at 37°C for an additional 2.5 h. 11D12 diluted in blocking buffer (0.2 μg/ml) was
added, and the plate was incubated at 37°C for 2 h. The plate was washed and then incubated for 1 h at 37°C with donkey anti-mouse IgG HRP (Jackson; 50 µl/well) diluted in blocking buffer (1:20 K). After washing, the plate was incubated with 50 µl/well of SuperSignal ELISA Femto Enhanced Chemiluminescent Substrate (Pierce). The chemiluminescence, in relative light units, was measured immediately using a Victor3 1420 (Wallac) microplate reader. The samples were screened in a blinded fashion and tested randomly. The case and control samples were run on a single plate to avoid plate-to-plate variations, and the results were confirmed using at least two independent experiments. A series of internal controls were also run to check for run-to-run variations.

4.2.2 Development of ELISA for measuring phosphorylated S129 α-syn in human CSF.

A 384-well ELISA microplate (Nunc MaxiSorb, Nunc) was coated for overnight incubation at 4°C with Syn-140 (0.5 µg/ml) in 200 mM NaHCO₃, pH 9.6 (50 µl/well). The plate was washed with PBST and incubated with blocking buffer (100 µl/well) for 2 h at 37°C. After washing, 50 µl of the CSF samples (thawed on ice; Tween-20 was added to a final concentration of 0.05%) was added to each well, and the plate was then incubated at 37°C for another 2.5 h. Rabbit monoclonal anti-p-S129-α-syn antibody (EP1536Y; Abcam) diluted in blocking buffer (1:2 K) was added, and the plate was incubated at 37°C for 2 h. The plate was washed and then incubated for 1 h at 37°C with goat anti-rabbit IgG HRP (Jackson; 50 µl/well) diluted in blocking buffer (1:10 K). After washing, the plate was incubated with 50 µl/well of SuperSignal ELISA Femto Enhanced
Chemiluminescent Substrate (Pierce). The chemiluminescence, in relative light units, was measured immediately using a Victor3 1420 (Wallac) microplate reader. The samples were screened in a blinded fashion and tested randomly. The case and control samples were run on a single plate to avoid plate-to-plate variations, and the results were confirmed using at least two independent experiments. A series of internal controls were also run to check for run-to-run variations.

4.2.3 Development of ELISA for measuring oligomeric α-syn in human CSF

A 384-well ELISA microplate (Nunc MaxiSorb, Nunc) was coated by overnight incubation at 4°C with Syn-O2 (0.2 μg/ml) in 200 mM NaHCO₃, pH 9.6 (50 μl/well). The plate was washed with PBST and incubated with blocking buffer (100 μl/well) for 2 h at 37°C. After washing with PBST, 50 μl of the CSF samples (thawed on ice; Tween-20 was added to a final concentration of 0.05%) was added to each well, and the plate was then incubated at 37°C for another 2.5 h. FL-140, diluted in blocking buffer (1/1 K), was added, and the plate was incubated at 37°C for 2 h. The plate was washed and then incubated for 1 h at 37°C with goat anti-rabbit IgG HRP (Jackson; 50 μl/well) diluted in blocking buffer (1:15 K). After washing, the plate was incubated with 50 μl/well SuperSignal ELISA Femto Enhanced Chemiluminescent Substrate (Pierce). The chemiluminescence, in relative light units, was measured immediately using a Victor3 1420 (Wallac) microplate reader. The samples were screened in a blinded fashion and tested randomly. The case and control samples were run on a single plate to avoid plate-to-plate variations, and the results were confirmed using at least two independent
experiments. A series of internal controls were also run to check for run-to-run variations.

4.2.4 Transgenic mice description

Mouse brains and microdialysis fluid were obtained from our collaborator Dr. Louise Buur Vesterager (Lundbeck, Denmark).

Approximately 7 kb of the mouse α-synuclein promoter was combined with human α-synuclein cDNA and the SV40 poly-adenylation sequence in a construct used to establish transgenic mouse lines by pronuclear microinjection into oocytes from C57Bl6xD2 hybrids. The subsequent transgenic lines were bred into C57Bl6 mice for at least nine generations. Non-transgenic mice from the same colony served as controls. Expression of the transgene was assessed by Northern blotting, in situ hybridization and Western blotting (Syn-1) using standard methods (Westerlund et al., 2008). KO mice were created by replacing a 400 bp genomic fragment containing the translocation start with a neocassette. WT mice in which the fragment was not knocked out were also used as controls.

4.2.5 Brain lysates preparation

Frozen brain samples (1 g) from Tg, WT and KO mice were homogenized in 5 ml of CelLytic buffer comprising mild detergent, bicine and 150 mM NaCl (Sigma-Aldrich) and containing a cocktail of protease inhibitors including AEBSF, aprotinin, E-64, EDTA and leupeptin (Pierce) and then centrifuged at 3000 g for 30 min. The supernatant was collected, and the total protein concentration was
measured. All samples were adjusted to 1 mg/ml and then stored at -80°C. The samples were thawed on ice directly prior to analysis.

4.2.6 AD CSF biomarkers

Concentrations of t-tau, p-tau and Aβ-42 in CSF were determined using the sandwich ELISAs Innotest™ β-Amyloid (1-42), Innotest hTAU-Ag and Innotest Phosphotau (181P); Innogenetics. Assay performance was monitored using pools of surplus CSF specimens. For this purpose, multiple pools at varying concentrations were included in 7-18 runs. The interassay CVs obtained were 11.3% for Aβ-42, 9.3% for t-tau and 9.4% for p-tau (Mulder et al., 2010).

4.2.7 Preparation of the standards for the oligomeric ELISA

Using an SDS gel, recombinant α-syn purity was estimated to be > 95%. Ginsenoside Rb1 (Gn Rb1) stock solutions (10 mM) were prepared in 100% DMSO, and the final amount of DMSO in the sample solutions was 1%. Samples of 25 μM α-syn in PBS were aged alone or with Gn Rb1 at a molar ratio of 4:1 (CMC: α-syn molar ratio). The samples were placed in 1.5 ml sterile polypropylene tubes, drops of mineral oil were added to prevent sample evaporation, and the tubes were then sealed with parafilm and incubated at 37°C for 5 days with continuous shaking at 800 rpm in a Thermomixer (Eppendorf). α-α-Syn was then purified using FPLC (Ardah et al., submitted for publication).
4.2.8 **Study population**

The human CSF samples were collected by our collaborator Dr. Wilma van de Berg (VU University Medical Center, Amsterdam) as described recently (*van Dijk et al., 2014*). In this study, we included 49 patients with PD that attended the outpatient clinic for movement disorders at the VU University Medical Center between September 2008 and February 2011, and 49 self-declared healthy controls who were recruited via an advertisement in the periodical of the Dutch Parkinson Foundation. All patients with PD fulfilled the United Kingdom Parkinson’s Disease Society Brain Bank clinical diagnostic criteria (Hughes, Daniel, Kilford, & Lees, 1992). Patients were included only if they were able to understand the study aims and procedures. Mini-Mental State Examinations (MMSE) and/or neuropsychological assessments of the patients did not indicate dementia. In the controls, individuals with dementia based on the Cambridge Cognitive Examination Scale (Roth et al., 1986) were excluded. Patients and controls underwent a standardized clinical assessment that included their medical history and a neurological examination. Disease duration was defined as the time period starting from the first motor symptom until the time of lumbar puncture. The severity of parkinsonism and disease stage in the ‘on’ state were rated using the UPDRS-III (Fahn, 1987) and the modified Hoehn and Yahr classification (H&Y; Jankovic et al., 1990), respectively. The study was approved by the local ethics committee of the VU University Medical Center, and all participants provided written, informed consent.
CSF fluid was obtained by lumbar puncture and collected in polypropylene collection tubes. CSF was routinely assayed for cell counts, centrifuged at 1800 g at 4°C for 10 min, aliquoted and stored at -80°C within 2 h, consistent with published guidelines (Teunissen et al., 2009). All the samples were screened in a blinded fashion and were tested randomly.

4.2.9 Data Analysis

Statistical analyses were performed using GraphPad Prism (version 5.0) software and IBM SPSS software 21. Continuous variables were described using medians and ranges because the data distributions were skewed. Correlations were calculated using Spearman’s Rho(r). Mann-Whitney U-tests were used for comparisons between the two diagnostic groups (p < 0.05). The accuracy of the diagnostic value of the biomarkers was assessed based on the area under the curve (AUC) of the receiver operating characteristic (ROC) curve (Eusebi, 2013; Hajian-Tilaki, 2013; Robin et al., 2011). Cut-off values were calculated using sensitivity and specificity values that maximized Youden’s index (sensitivity + specificity – 1). All CSF samples with > 500 erythrocytes/μl were excluded from further analysis because traces of blood may influence CSF α-syn levels (Barbour, et al., 2008; Hong, et al., 2010).
4.3 Results

4.3.1 Development of ELISAs for measuring α-syn species in human CSF

Total-ELISA

We developed, validated and optimized a sandwich-type ELISA for measuring t-α-syn levels in human CSF. Assay conditions were first optimized using different concentrations of the capture sheep anti-α-syn antibody (Syn-140). Based on its sensitivity and background signal, 0.1 µg/ml of the capture antibody was selected for subsequent assays (see below). The detection limit of the assay was as low as 50 pg/ml, which is 20-fold less than the concentration detected in human CSF (Fig. 4.1A). The standard curve for the ELISA assay was constructed using different concentrations of recombinant human α-syn in solution. Serial dilutions of recombinant human β-syn and γ-syn were also included as negative controls (Fig. 4.1B). To exclude the possibility of our assay exhibiting disparate day-to-day performance, signal variability was examined over several days (> 50 plates analyzed thus far). Our assay displayed a high degree of reproducibility with a day-to-day (plate-to-plate) CV < 10% (Fig. 4.2A). Next, we confirmed that our assay permitted the direct quantification of native α-syn in biological samples such as human CSF and Tg mouse-brain lysates. α-Syn was detected in Tg mice and human CSF, whereas no signal was observed in WT or knock-out (KO) lysates (Fig. 4.2B). Moreover, recovery rates were determined by spiking recombinant α-syn into human CSF. Our assay consistently featured a mean recovery rate > 90% (Fig. 4.2C).
Fig. 4.1: Characterization of total-ELISA protocol. (A) Histograms representing the optimization of the capture antibody (Syn-140) concentration. (B) Standard curves displaying the specificities and sensitivities for α- (◆), β- (■) and γ-syn (▲) in total-ELISA we used.
Fig. 4.2: Characterization of total-ELISA reproducibility, specificity and robustness.
(A) Data shown are representative of 3 independent experiments using serial dilutions of recombinant α-syn freshly prepared over 3 non-consecutive days. Measurements were taken in duplicate, and the results show the mean ± standard deviation for each point. (B) Antibody specificity determination using murine brain lysates and human CSF. Tg, WT and KO brains were homogenized in CelLytic reagent, containing EDTA and protease inhibitors, and lysates were diluted to 5μg/ml. (C) Assessment of α-syn recovery rate in human CSF. The recovery rates were calculated from measured α-syn concentrations relative to spiked amounts.
**Phospho-ELISA**

For p-S129-α-syn-specific ELISA, we followed a similar protocol for optimizing with t-α-syn, where anti-Syn-140 was used as the capture antibody and rabbit monoclonal anti-p-S129-α-syn antibody (EP1536Y) was used as the reporter. To address the issue of specificity, serial dilutions of recombinant human p-S129-α-syn and α-syn were tested, and signal was detected only with the anti-p-S129-α-syn (Fig. 3A). Using similar criteria (sensitivity, reproducibility and background signal), optimal conditions were adopted for the assay (Fig. 4.3B). Sensitivity was as low as 20 pg/ml, and a standard curve with an $R^2$ of 0.999 was generated (Fig. 4.3B). Coefficients of variation (CVs) were calculated by repeated measurements on non-consecutive days, and the day-to-day (plate-to-plate) variability in signal was $< 15\%$ (Fig. 4.4A). To explore the assay suitability for quantifying p-S129-α-syn in biological fluids, multiple concentrations of recombinant human p-S129-α-syn were spiked into human CSF. The mean recovery rates were between 95 and 97% (Fig. 4.4B).
Fig. 4.3: Characterization of phospho-ELISA protocol. (A) Standard curves displaying the specificities and sensitivities for p-S129-α-syn (●) and α-syn (■). (B) Histograms representing the optimization of the capture antibody (Syn-140) concentration.
Fig. 4.4: Characterization of phospho-ELISA reproducibility and robustness.

(A) Examination of Interassay variability. Measurements were taken in duplicate, and the results show the mean ± standard deviation for each point. (B) Assessment of α-syn recovery rate in human CSF. Recovery rates were calculated from measured α-syn concentrations relative to spiked amounts.
Oligomeric-ELISA

Following the complete characterization of our mAbs using monomeric and aggregated α-syn protein (for details, see Section 3.2), we developed and optimized a sensitive and specific ELISA for detecting o-α-syn in human CSF. Similarly, our oligomeric ELISA is based on a conventional sandwich ELISA system, where o-α-syn is captured using one of our highly specific anti-o-α-syn mAbs (Syn-O2; see Section 3.2.8). We focused on the pair Syn-O2/FL-140, which consistently generated more robust signals than other pairs (see Section 3.2.8). Multiple conditions were tested to assess assay performance. Serial dilutions of recombinant human o-α-syn were run in duplicate. The signal detected for o-α-syn increased with increasing concentrations of Syn-O2 (0.5 μg/ml compared with 0.2 μg/ml or 0.1 μg/ml; Fig. 4.5A). However, the assay sensitivity did not improve with coating at 0.5 μg/ml compared with 0.2 μg/ml (see Fig. 4.5A). Based on optimal sensitivity, specificity, reproducibility and background signal results, we selected 0.2 μg/ml for coating. Our assay specificity toward o-α-syn was further validated because no signal was detected when β- or γ-syn oligomers were used (Fig. 4.5B). The intra-assay and inter-assay precision was < 10% (based on an examination of over 100 plates; Fig. 4.6A). To assess the specificity and sensitivity of our oligomeric-ELISA for o-α-syn in biological samples, brain lysates and microdialysis fluid from young Tg mice that over-expressed human α-syn were used, and WT and KO mice were also included as negative controls. As anticipated, o-α-syn levels were significantly greater in Tg mice compared with WT, whereas no signal was detected in KO mice (Fig. 4.6B). In parallel, our assay quantified the levels of o-α-syn in human CSF successfully (Fig. 4.6B).
Next, we spiked increasing amounts of recombinant o-α-syn into human CSF samples. Interestingly, the average recovery rates were > 95% (Fig. 4.6C). We estimated that the lower limit of detection of recombinant o-α-syn using this ELISA was as low as 10 pg/ml based on the initial concentration of the protein (5 ng/ml). Taken together, the results show that our oligomeric-ELISA is specific, sensitive and reproducible, and it is thus suitable for the quantification of o-α-syn in biological samples.
Fig. 4.5: Characterization of oligomeric-ELISA protocol. (A) Histograms representing the optimization of the capture antibody (Syn-O2) concentration. (B) Standard curves displaying the specificities and sensitivities for α- (●), β- (■) and γ-syn (▲) oligomers.
Fig. 4.6: Characterization of total-ELISA reproducibility, specificity and robustness.

(A) Assessment of Inter assay variability. Measurements were taken in duplicate, and the results show the mean ± standard deviation for each point.

(B) Antibody specificity determination using murine brain lysates and human CSF. Tg, WT and KO brains were homogenized in Cellytic reagent, containing EDTA and protease inhibitors, and lysates were diluted to 5μg/ml.

(C) Assessment of α-syn recovery rate in human CSF. Recovery rates were
4.3.2 Descriptive analysis

Patient demographic data and clinical features are listed in Table 4.1. Subjects with PD and healthy controls (HC) were matched for age but not for gender ($p = 0.002$). Because no significant differences between PD and control groups were found with respect to gender, we decided not to correct for gender.

<table>
<thead>
<tr>
<th>Table 4.1: Demographic data and clinical features for HC and PD.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>HC (n=48)</td>
</tr>
<tr>
<td>PD (n=46)</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>62.9 ± 7.2</td>
</tr>
<tr>
<td>62.8 ± 10.1</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>32 (66.6%)</td>
</tr>
<tr>
<td>18 (33.3%)</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>16 (33.3%)</td>
</tr>
<tr>
<td>28 (61%)</td>
</tr>
<tr>
<td>Blood cells per microliter</td>
</tr>
<tr>
<td>37.3 ± 89; 0-490</td>
</tr>
<tr>
<td>35.6 ± 100; 0-499</td>
</tr>
<tr>
<td>Disease duration (years)</td>
</tr>
<tr>
<td>5.6 ± 5.2</td>
</tr>
<tr>
<td>Hoehn and Yahr stage</td>
</tr>
<tr>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>UPDRS-III (motor score)</td>
</tr>
<tr>
<td>21.8 ± 7.9</td>
</tr>
<tr>
<td>MMSE score</td>
</tr>
<tr>
<td>29 ± 1.1</td>
</tr>
<tr>
<td>28.5 ± 1.5</td>
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</tbody>
</table>

4.3.3 CSF biomarkers in diagnostic groups

Consistent with our previous reports (Parnetti, et al., 2011; Tokuda, et al., 2006), CSF t-α-syn levels were significantly decreased in PD patients compared with HC ($p < 0.0001$; Fig. 4.7A). However, the concentrations of CSF o-α-syn and p-S129-α-syn were significantly greater in PD patients compared with HC ($p < 0.0001$, $p < 0.01$, respectively), with considerable overlap noted between the two groups for p-S129-α-syn (Fig. 4.7B and C). Interestingly, the o-α-syn/t-α-syn ratio (%) substantially improved the discrimination between PD and HC ($p < 0.0001$) by reducing the overlap between the two groups (Fig. 4.7E). The p-S129-α-syn/t-α-
syn ratio (%) was also significantly and consistently greater in the PD group (p < 0.0001; Fig. 4.7D).

![Fig. 4.7: Scatterplots of CSF biomarkers values observed in PD and HC. Individual values of the levels of t-α-syn (A), o-α-syn (B), p-S129-α-syn (C), the % ratio of o-α-syn/t-α-syn (D) and the % ratio p-S129-α-syn/t-α-syn (E) in CSF from patients with PD (solid circles) and HC (solid triangle). Median values are indicated with a horizontal line. Significant differences are indicated with * (p < 0.05), ** (p < 0.01) or *** (p < 0.001).]
The mean CSF t-tau, p-tau and Abeta-42 concentrations did not vary significantly between PD and HC groups ($p > 0.05$; Fig. 4.8A, B and C). All CSF biomarker values are summarized in Table 4.2.

Fig. 4.8: Scatterplots of CSF biomarkers values observed in PD and HC. Individual values of the level of t-tau (A), p-tau (B), Aβ-42 (C) and the ratio of t-αu/t-α-syn (D) in CSF from patients with PD (solid circles) and HC (solid triangles). Median values are indicated with a horizontal line. Significant differences are indicated with * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).
Table 4.2 CSF biomarkers in HC and PD

<table>
<thead>
<tr>
<th>CSF Biomarker</th>
<th>HC</th>
<th>PD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-α-syn (ng/ml)</td>
<td>1.64 (1.38-2.28)</td>
<td>1.34 (1.22-1.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>o-α-syn (pg/ml)</td>
<td>57 (36-107)</td>
<td>116 (76-170)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>p-S129-α-syn (pg/ml)</td>
<td>222 (181-275)</td>
<td>262 (207-296)</td>
<td>0.01</td>
</tr>
<tr>
<td>t-tau (pg/ml)</td>
<td>229 (162-272)</td>
<td>190 (158-274)</td>
<td>0.6</td>
</tr>
<tr>
<td>p-tau (pg/ml)</td>
<td>42 (30-50)</td>
<td>40 (30-51)</td>
<td>1.0</td>
</tr>
<tr>
<td>Aβ42 (pg/ml)</td>
<td>996 (878-1153)</td>
<td>967 (794-1077)</td>
<td>0.1</td>
</tr>
<tr>
<td>o-α-syn/t-α-syn ratio (%)</td>
<td>3.5 (2.3-6.2)</td>
<td>8.9 (5.3-12.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>p-S129-α-syn/t-α-syn ratio (%)</td>
<td>13.7 (9.2-19)</td>
<td>19 (16-23)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>t-tau/t-α-syn ratio (%)</td>
<td>11 (9-18)</td>
<td>15 (12-18)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

4.3.4 Correlation analysis for CSF biomarkers

Interestingly, a significant positive correlation between t-α-syn and t-tau was found within the PD group (p < 0.0001, r = 0.5), i.e., higher t-tau levels were associated with higher α-syn levels (Fig. 4.9A). Similarly, a significant positive correlation was noted between t-α-syn and p-tau within the PD group (p < 0.0001, r = 0.6; Fig. 4.9B).
Fig. 4.9: Correlation of t-α-syn with AD biomarkers. t-α-Syn correlations with t-tau (A) p-tau (B) and Aβ-42 (C). All correlations were calculated with Spearman's rho ($p < 0.05$).
In contrast, o-α-syn and p-S129-α-syn levels did not correlate with any of AD CSF biomarkers (t-tau, p-tau or Abeta-42) within the PD group (Fig. 4.10A-F).

**Fig. 4.10:** Correlation of o-α-syn and p-S129-α-syn with AD biomarkers. o-α-Syn correlations with t-tau (A) p-tau (C) and Aβ-42 (E). p-S129-α-Syn correlations with t-tau (B) p-tau (D) and Aβ-42 (F). All correlations were calculated with Spearman’s rho (p < 0.05).
4.3.5 Correlation analysis between CSF biomarkers and clinical parameters

An inverse significant correlation between t-α-syn and MMSE scores was observed ($p = 0.003, r = 0.3$; Fig. 4.11D). In contrast, t-α-syn did not correlate with disease duration, H&Y stage or UPDRS scores within the PD group (Fig. 4.11A-C).

![Fig. 4.11: t-α-Syn correlations with clinical parameters. Correlations of t-α-syn with disease duration (A), H&Y stage (B) UPDRS scale (C) and MMSE scores (D) in PD group. All correlations were calculated with Spearman’s rho ($p < 0.05$).]
A weak but significant negative correlation was noted between o-α-syn and disease duration and H&Y stage ($p = 0.025$, $r = 0.3$ and $p = 0.025$, $r = 0.24$, respectively; Fig. 4.12A and B). o-α-Syn levels did not correlate with UPDRS or MMSE scores (Fig. 4.12C and D).

**Fig. 4.12:** o-α-Syn correlations with clinical parameters. Correlations of o-α-syn with disease duration (A), H&Y grade (B), UPDRS scale (C) and MMSE scores (D) in PD group. All correlations were calculated with Spearman's rho ($p < 0.05$).
p-S129-α-Syn did not correlate with disease duration, H&Y stage or UPDRS scores within the PD group (Fig. 4.13 A-D).

Fig. 4.13: p-S129-α-Syn correlations with clinical parameters. Correlations of p-S129-α-Syn with disease duration (A), H&Y grade (B), UPDRS scale (C) and MMSE scores (D) in PD group. All correlations were calculated with Spearman’s rho (p < 0.05).
4.3.6 ROC analysis

ROC analyses showed a sensitivity of 0.72 and a specificity of 0.63 for t-α-syn alone (AUC = 0.71). o-α-Syn exhibited a sensitivity of 0.74 and a specificity of 0.65 (AUC = 0.79), whereas p-S129-α-Syn exhibited a sensitivity of 0.60 and a specificity of 0.65 (AUC = 0.67). A combination of p-S129-α-syn with t-α-syn performed better than p-S129-α-syn alone, showing a sensitivity for detecting PD patients versus HC of 0.81 and a specificity of 0.56 (AUC = 0.75). Overall, the o-α-syn/t-α-syn ratio (%) achieved the best diagnostic performance, with a sensitivity of 0.81 and a specificity of 0.65 (AUC = 0.83; Fig. 4.14).

Fig. 4.14 Use of receiver operating characteristic (ROC) curves for the levels of α-syn species in CSF. ROC curves based on logistic regression analyses for the classification of PD patients versus control subjects based on various predictors and combination of predictors.
1.12 Discussion

To date, the only accepted clinical diagnostic criteria for PD are based on motor symptoms. However, a substantial degeneration of dopaminergic neurons in the SN precedes the manifestation of motor symptoms, making the establishment of an early diagnosis for PD during the pre-motor phase quite a challenge. Determining the mechanisms underlying the loss of the nigral dopaminergic neurons and the development of dementia in some PD cases represents another challenge to this field. Establishing accurate tests based on chemical analyses of blood or CSF is of great importance for both diagnostic accuracy and prognostic evaluation. In this study, we describe the development of specific, sensitive and reliable ELISA assays to detect different forms of α-syn, and we then demonstrated their utility in quantifying α-syn species in CSF from PD and age-matched healthy controls.

Our new ELISA assays provide multiple improvements over other reported immunoassays. First, our ELISA design using 384-well plates is perfectly compatible for accommodating multiple replicates even with limited a sample volume (50 µl/well). Second, the enhanced sensitivity shown by our assays validates their suitability for the analysis of human CSF specimens. Our assays were shown to be highly target specific based on several methods, including specificity validation of the employed antibodies to their respective antigens, using brain and blood products from genetically modified mice (Tg, WT, KO) and monitoring assay precisions. Our ELISAs are also highly robust because recovery rates from spike experiments were > 90%.
Tokuda et al. previously described the development of an ELISA for quantifying o-α-syn in human CSF using the same mAb for capture and (a biotinylated form) for detection when directed against an α-syn epitope (Tokuda, et al., 2006). Using this ELISA design, only o-α-syn species were quantified because they possess multiple epitopes for recognition by capture and detection antibodies. No signal was detected for the monomeric-α-syn because the only available epitope was already occupied by the capture antibody, precluding its binding to the detection antibody. However, heterophilic antibodies may interfere in this assay format, leading to false-positive signals (Sehlin et al., 2010). Heterophilic antibodies are polyreactive antibodies that recognize antibodies from the same species and that may crosslink the capture antibody to the detection antibody. Our new oligomeric-ELISA has addressed this shortcoming by using our novel conformation-specific mAb (Syn-O₂), which selectively distinguishes and captures o-α-syn, and a rabbit polyclonal antibody (FL-140) for detection, thus permitting the precise quantification of o-α-syn in biological samples.

Our results for t-α-syn are consistent with other reported studies that showed decreased levels of CSF t-α-syn levels in PD patients compared with controls (Hall et al., 2012; Hong, et al., 2010; Kang et al., 2013; Mollenhauer, et al., 2011; Mollenhauer et al., 2013; Parnetti, et al., 2011; Parnetti, Farotti, et al., 2014; Tokuda, et al., 2006; van Dijk, et al., 2014). Several research groups have attributed the observed reduction in t-α-syn in CSF to cellular mechanisms leading to α-syn sequestration in LBs (Stewart et al., 2014; Tokuda, et al., 2006). However, results showing a lack of correlation between CSF α-syn levels and
disease severity and the existence of a direct association between LB features and disease progression have challenged this hypothesis. Pathological dysfunction in the neurons secreting α-syn may also explain the reduction in CSF α-syn levels. Zhang and colleagues suggested that CSF α-syn depletion is most likely due to compensatory mechanisms (Stewart, et al., 2014). Under pathological conditions, a portion of α-syn is converted into toxic aggregates that are eventually sequestered in LBs. The reduction in intracellular α-syn levels shifts the normal homeostasis of functional α-syn between intra- and extra-cellular compartments. This shift leads to a depletion of α-syn in CSF and the accumulation of synaptic α-syn (Stewart, et al., 2014).

The UPDRS is the most commonly used clinical scale to provide an efficient and flexible assessment of motor symptoms in PD patients and to monitor the degree of resultant disability. However, thus far, no strong linear relationship has been established between UPDRS scores and the progressive nigrostriatal degeneration in PD, which may underlie the absence of a correlation between CSF t-α-syn levels and UPDRS scores. CSF biomarkers mirror changes within the brain as an entire unit, whereas UPDRS scores primarily reflect changes in the nigrostriatal dopaminergic system. In addition, compensatory responses in PD may further confound the correlation between CSF biomarkers and the severity of PD motor symptoms (Shi et al., 2011). Moreover, DA replacement therapy provided to PD patients enhances motor function while showing little or no effect on CSF protein concentrations (Hong, et al., 2010; Shi, et al., 2011).
In 2000, Mattila et al. reported, for the first time, a direct association between dementia and LBs in the cortex, i.e., the greater the burden of LBs, the worse the cognitive function in PD patients (Mattila, Rinne, Helenius, Dickson, & Röyttä, 2000). Subsequently, several other studies reported similar findings (Hurtig et al., 2000; Kövari et al., 2003). Similarly, given the proposed role of α-syn in synaptic transmission (Cheng, Vivacqua, & Yu, 2011), it is possible that altered α-syn levels play a critical role in cognitive impairment. However, the association between t-α-syn levels and cognitive impairment is not yet fully understood. The inverse correlation between t-α-syn and MMSE scores, although weak, is statistically significant and may be interpreted using the previously described hypothesis in which α-syn sequestration in LBs results in increased α-syn accumulation at synapses (Stewart, et al., 2014).

One of the most interesting findings in our study is the significant increase in CSF o-α-syn levels in PD, consistent with our previous studies using an oligomeric-specific ELISA based on the use of the same mAb for capturing and detection (El-Agnaf et al., 2006). Interestingly, the o-α-syn/t-α-syn ratio (%) further improved discrimination between PD and controls, emphasizing the usefulness of combining several CSF biomarkers for differential diagnosis of clinically overlapping parkinsonian diseases.

The overlap of both pathological and clinical features in patients with synucleinopathies and tauopathies dictates the necessity of combining different biomarkers to achieve an accurate differential diagnosis. Several lines of evidence support a link between the pathogenic roles of α-syn and tau in neurodegenerative
disorders. For example, a co-existence of α-syn and tau pathology has been detected in brains of patients with AD, PD and DLB (Vekrellis, Xilouri, Emmanouilidou, Rideout, & Stefanis, 2011). Moreover, several in vitro and in vivo studies investigated interactions among α-syn, tau and Aβ proteins, showing that these proteins promote each other’s aggregation and polymerization, leading to neuronal degeneration and worsening cognitive impairment (Clinton, Blurton-Jones, Myczek, Trojanowski, & LaFerla, 2010; Giasson et al., 2003; Jellinger, 2011, 2012; Waxman & Giasson, 2011). These data further explain the positive correlation between CSF t-α-syn and tau in the PD group, indicating that both biomarkers respond similarly to neurodegeneration. This finding is consistent with a study by Toledo et al., who found a positive correlation between CSF t-α-syn and tau levels in PD, AD and controls (Toledo, Korff, Shaw, Trojanowski, & Zhang, 2013). Interestingly, combining both CSF t-α-syn and tau levels improved their diagnostic value substantially, as illustrated by the ROC analyses. However, an understanding of the exact association between CSF tau and α-syn species is more complex and goes beyond being simply a secondary response to neuronal damage.

LBs are formed primarily from post-transnationally modified forms of α-syn. One of these modifications is α-syn phosphorylation at Ser129, which accounts for approximately 90% of accumulated α-syn in LBs. Although p-S129-α-syn may be critical for PD pathogenesis, it remains an open question whether p-S129-α-syn promotes or protects against α-syn aggregates' toxicity. Thus far, only one report has shown a weak positive correlation between p-S129-α-syn and UPDRS and that
a combination of CSF t-α-syn and p-S129-α-syn improved the discrimination of PD from other forms of parkinsonism (Wang, et al., 2012). Here, we have shown an increase in p-S129-α-syn in the PD group compared with controls. ROC analysis revealed a reasonable diagnostic sensitivity and specificity in differentiating PD from controls based on p-S129-α-syn levels alone. However, the combination of CSF t-α-syn and p-S129-α-syn levels further improved the discriminative power.
1.13 Conclusion and Future Perspectives

The most important feature of our mAbs is their ability to detect different assemblies of α-syn aggregates ranging from soluble oligomers to mature insoluble fibrils. Given this unique feature, our mAbs viewed as anti-pathogenic α-syn antibodies that recognize a specific conformation present in early aggregates and that is maintained in mature fibrils. Considering the critical pathogenic role of α-syn soluble aggregates, our novel antibodies may provide substantial diagnostic and therapeutic opportunities. Using our novel mAbs, we have developed ELISAs to measure t-α-syn, o-α-syn and p-S129-α-syn species in human CSF. Combining measurements of different α-syn species in CSF, we observed marked differential CSF patterns between PD and controls. Our results validated the usefulness of combining multiple CSF biomarkers in improving PD diagnostic accuracy and prognosis.

There is a need for novel therapeutics against PD, as traditional therapies only alleviate the symptoms of PD. α-Syn is an attractive target as it has a central role in the pathogenesis of PD and given the specificity of our mAbs towards α-syn pathology, they might be of potential use for future therapy and could also serve to identify reliable biomarkers for PD and other related disorders.

Biomarkers that can aid diagnose PD at its pre-motor phase, help physicians to monitor how PD patients progress with this condition and assess drug effectiveness are critical to develop new disease-modifying treatments. Furthermore, good biomarkers could be used to predict the risk that a given patient will develop adverse drug effects. In our study, we showed the potential of some
CSF biomarkers that can be promising diagnostic tools for PD diagnosis and prognosis. Validation of our assays is critical step that may aid in the translation of PD CSF biomarkers into clinical practice. The major limitation for the discovery of PD biomarkers is the lack of longitudinal studies; hopefully in the near future we can validate our ELISA assays in such study. Furthermore, we strongly believe that combined measurement of several CSF biomarkers in large cohorts will have great impact on both early diagnosis and prediction of disease progression. More importantly, future studies on CSF biomarkers of PD might help to reveal the underlying mechanisms behind the disease.


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