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Published in:
Progress in Neurobiology

DOI:
10.1016/j.pneurobio.2011.11.003

2012

Link to publication

Citation for published version (APA):
Dunning, C., Reyes, J. F., Steiner, J., & Brundin, P. (2012). Can Parkinson's disease pathology be propagated from one neuron to another? *Progress in Neurobiology*, *97*, 205-219. https://doi.org/10.1016/j.pneurobio.2011.11.003

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Can Parkinson's disease pathology be propagated from one neuron to another?

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Abstract

Parkinson's disease is the second most prevalent neurodegenerative disease, yet despite this, very little is known about the underlying cellular mechanisms. Initially it was thought to be a disease primarily involving loss of dopaminergic neurons in the substantia nigra pars compacta. Recent studies, however, have focused on observations that aggregated α-synuclein protein, the major component of Lewy bodies, is found throughout the nervous system. It is speculated that misfolded α-synuclein transfers between cells in a prion-like manner, thereby mediating the spread of the neuropathology. In this review, we discuss the staging (according to Braak) of Parkinson pathology and the concept describing the disease progression from one region of the brain to the other. We highlight how α-synuclein might be responsible for the spread of the disease. We compare the idea of a prion-like mechanism contributing to Parkinson's disease to emerging concepts that other proteins participate in similar processes in other neurodegenerative diseases. We then examine the future implications of a critical role in disease pathogenesis of α-synuclein for the classification, diagnosis and treatment of Parkinson's disease in the future.

Keywords: Parkinson's disease, α-synuclein, cell-to-cell transfer, prion-like, neurodegenerative disease

Introduction

Behind Alzheimer's disease (AD), Parkinson's disease (PD) is the second most prevalent neurodegenerative disease, with a life time risk of development of 2% (Schapira, 2009). For several decades, PD was thought to involve a relatively simple neuropathological process primarily focused on the dopaminergic neurons in the substantia nigra pars compacta and resulting in the loss of dopaminergic neurotransmission in the striatum. This loss of dopamine is believed to underlie the majority of the classic motor symptoms of PD, including bradykinesia and rigidity. This view is beginning to change, however, due to a more complex symptomology and indeed more widespread neuropathology for PD coming to light in recent years.

In addition to loss of dopaminergic neurons in the substantia nigra pars compacta, PD is characterized neuropathologically by the presence of insoluble protein inclusions termed Lewy bodies and Lewy neurites, located in either the neuronal cell body or neuronal processes, respectively. The Lewy bodies, first described by Friedrich Lewy in 1912, are typically spherical globules, consisting of a dense core surrounded by a pale-stained halo of radiating filaments, whereas Lewy neurites display a thread-like structure (Forno, 1996). The major constituent of Lewy bodies and neurites is a misfolded version of the protein α-synuclein (αsyn). Whether the cellular effects of these protein inclusions are neuroprotective or neurotoxic is debated (Olanow et al., 2004a). Braak and colleagues have suggested that in the central nervous system Lewy bodies first emerge in the brain stem and olfactory bulb prior to the appearance of classic motor symptoms in PD. They

proposed that Lewy pathology then gradually spreads throughout the brain to involve eventually large parts of the central nervous system (Braak and Del Tredici, 2008). This staging concept gained support a few years ago from autopsies that were performed in PD patients who had received implants of embryonic brain tissue in the 1980-1990s. These studies showed that αsyn-immunoreactive Lewy pathology is not only present throughout the patients' own brains, but also eventually appears in the grafted neurons (Kordower et al., 2008a; 2008b; Kurowska et al., 2011; Li et al., 2010; 2008). These grafted neurons are so young (10-15 years beyond embryonic stage) that it appears very unlikely that they have developed aggregates through an independent cell-autonomous process. Several cellular mechanisms, including neuroinflammation, oxidative stress and lack of appropriate neurotrophic support were hypothesized to underlie the appearance of Lewy pathology in the grafted neurons (Brundin et al., 2008). However, the idea that has gained the greatest support is that the aggregates were the result of transfer of protein, either native or altered, from host brain cells to grafted neurons. In this review, we recount the literature surrounding Braak's staging model. We discuss in detail whether the major component of Lewy pathology, i.e. αsyn, can transfer from cell to cell whereby it recruits and aggregates protein in the recipient cells in a prion-like manner. In addition, we review the role of intercellular transfer in the spread of other neurodegenerative disease. Finally, we address the relevance of αsyn toxicity and prion-like propagation for the future of PD research.

Braak's staging concept for Parkinson's disease

Braak and colleagues, who examined brains from people with confirmed neurodegenerative disease diagnoses, made several key observations with respect to the development of PD neuropathology. In brain sections derived from autopsied PD patients, they noted an apparent correlation between the amount of insoluble αsyn deposition, its location, and the stage of the disease (Braak et al., 2002). These findings bore resemblance to their prior observations in AD brains, where the distribution of tau-containing neurofibrillary tangles is coupled to the clinical disease stage and follows a stereotypic pattern in all patients (Braak et al., 1993). In the case of PD, the extent of Lewy pathologies in the whole PD brain appeared to increase, and, importantly, their localizations seemed to progress in a largely caudo-rostral direction over time. Based on these initial observations, Braak and colleagues performed anatomical neuropathological studies in order to characterize the cerebral progression of αsyn-positive Lewy bodies and neurites as the disease process continued. Furthermore, they established an association between Lewy pathology and clinical symptoms (Braak et al., 2004; 2003; 2002). They proposed that the neuropathological profile of PD develops in a characteristic, nonrandom manner (Braak et al., 2003). In analogy to the Braak staging for AD (Braak et al., 1993), they described six stages of neuropathology that represent "pre-symptomatic" (i.e. before onset of motor symptoms in this context) and "symptomatic" phases (Braak et al., 2002).

In stage 1, the first Lewy pathology appears in locations outside the substantia nigra pars compacta. These initial αsyn-immunoreactive deposits

are found within the dorsal motor nucleus of the vagal nerve and the olfactory bulb. Later, in stage 2, the Lewy bodies and neurites are more widespread within the medulla, including monoaminergic areas such as the lower raphe nuclei and the locus coeruleus. Notably, during these early stages the individuals do not exhibit any noticeable motor symptoms (Braak et al., 2004). In stage 3, the Lewy pathology progresses caudo-rostrally from the brain stem and rostral-caudally from the olfactory areas but is still mainly subcortical. The αsyn-positive deposits are typically detected within the midbrain and basal forebrain, and later enter into the substantia nigra pars compacta. In stage 4, cell loss within the substantia nigra pars compacta is apparent. The αsyn immunoreactivity is evident in the cerebral cortex, specifically within the anteromedial temporal mesocortex. Neuropathology present at this time may lead the individual to display the first symptoms consistent with parkinsonism. Finally, in end stages 5 and 6, few neuromelanin-positive cells of the substantia nigra remain while αsyn-positive pathology begins to invade the neocortex. At these stages, the motor symptoms are severe and cognitive dysfunction becomes apparent (Braak et al., 2003; Duda et al., 2000; Halliday and McCann, 2010; Hawkes et al., 2010). Furthermore, Braak and colleagues suggested that projection neurons with long and thin axons with no or limited myelination were especially vulnerable to the deposition of αsyn pathology, suggesting that the progressive pathology described above might spread via these neuronal pathways (Braak et al., 2003), affecting more sensitive neurons prior to reaching the less vulnerable cells.

Based on the location of the first appearances of Lewy bodies and neurites and the involvement of progressively greater areas of the nervous system Braak and colleagues proposed the “dual-hit” theory. According to this hypothesis an unknown pathogen enters via the 1) respiratory pathway, through the nasal passages, and 2) gastric pathway, due to swallowing of saliva containing nasal secretions (Hawkes et al., 2009; 2007). The toxic pathogen, suggested to potentially be viral according to Braak and coworkers, is likely capable of crossing the epithelial and mucus membranes thereby affecting nearby neural structures. For the respiratory pathway, the neurotropic pathogen might then enter the olfactory bulb and be transported in an anterograde direction to temporal structures, while retrograde mechanisms transmit the pathogen along the vagal nerve to the medullary vagal dorsal motor nucleus (Braak et al., 2004; Hawkes et al., 2009; 2007).

This dual-hit theory is supported by several neuropathological and clinical observations, which will be discussed in more detail in a later section. Briefly, people commonly lose their sense of smell during early stages of PD, even prior to the appearance of motor symptoms or being diagnosed with PD (Müller et al., 2002). In cases already exhibiting motor symptoms and clinically diagnosed with PD, numerous Lewy pathologies were identified within the olfactory bulb, especially within the anterior olfactory nucleus. In this area, LB-related neuronal loss has been positively correlated with disease duration (Daniel and Hawkes, 1992; Pearce et al., 1995). Furthermore, an association between olfactory dysfunction, REM sleep behavior disorder (RBD), and early Braak staging has been reported in imaging studies (Stiasny-Kolter et al,

Brain 2005). Finally, gastrointestinal disturbances, including constipation, are also present in early PD and can precede diagnosis (Abbott et al., 2001). Independent researchers identified enteric plexus Lewy pathology in colon biopsies from people with PD, we introduce the literature concerning the αsyn protein and later we describe how the protein might transfer from cell to cell, leading to negative cellular consequences.

α-Synuclein

αSyn has long been thought of as a small, intrinsically unstructured neuronal protein, enriched in presynaptic nerve terminals. Although the native function is poorly understood, it appears to play a role in regulating synaptic vesicle recycling (Burré et al., 2010; Chandra et al., 2004). Predominately cytosolic, αsyn contains a hydrophobic non-amyloid component (NAC) region that is thought to be responsible for aggregation (Trexler and Rhoades, 2010; Uversky, 2003). Recent results have challenged the concept of an unfolded protein; with previous results largely born out of studies using bacterially-expressed recombinant protein and thus now believed to be artifactual. These new results suggest that when endogenous αsyn is purified from both neuronal and non-neuronal sources under non-denaturing conditions, it is a folded tetramer (Bartels et al., 2011). The tetramer was less likely to form aggregates when compared to unfolded monomers, suggesting that this tetramer must first be disrupted, providing αsyn monomers, for αsyn to misfold.

Mutations of the αsyn gene (*PARK1*/*SNCA*) were first identified in autosomal dominant PD in 1997 (Polymeropoulos et al., 1997), this was soon followed by the discovery that αsyn is a major component of Lewy bodies (Spillantini et al., 1997). To date, three missense mutations (Ala53Thr, Ala30Pro, Glu46Lys) have been identified in the αsyn gene. They cause autosomal dominant forms of PD, with no mutations being present in sporadic cases of PD or healthy individuals (Krüger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004). A number of genome wide association studies (GWAS) have also highlighted the role of αsyn in PD, with variants of the *SNCA* gene increasing an individuals' susceptibility to PD (Edwards et al., 2010; Satake et al., 2009; Simón-Sánchez et al., 2009). Moreover, duplications and triplications of *SNCA* have been identified in families with autosomal dominant PD (Chartier-Harlin et al., 2004; Fuchs et al., 2008; Singleton et al., 2003). Along with duplications, genetic variation in both the promoter and 3′ region of *SNCA* have been found to increase susceptibility to PD, particularly to the sporadic form of the disease (Farrer et al., 2001a; Fuchs et al., 2008; Pals et al., 2004; Tan et al., 2004). Mutations in these domains of *SNCA* have recently been shown to affect αsyn levels in the blood and brain (Mata et al., 2010). It is also worth noting that levels of αsyn increase with age (Chu and Kordower, 2007; Li et al., 2004;), an effect not seen with β-synuclein (Ohtake et al., 2004; Lincoln et al., 1999), a non-pathogenic analogue of αsyn. As age remains the biggest risk factor for developing PD (Collier et al., 2011), taken together with the genetic studies mentioned above, in particular the multiplications of the αsyn gene, it suggests that heightened levels of αsyn are deleterious. These increases may in fact predispose an individual to PD. While the effects these

genetic variations have on αsyn are not fully understood, and in turn, what effect mutated αsyn plays in the pathogenesis of PD, it is known that misfolded and post-translationally modified αsyn is present in Lewy bodies (Li et al., 2008; Neumann et al., 2002), and that misfolded αsyn has neurotoxic properties (Emmanouilidou et al., 2010b; Luk et al., 2009a; Nonaka et al., 2010).

Mechanisms of α-synuclein transfer

It has been known for several years that αsyn is secreted and can be detected in cerebrospinal fluid, plasma and saliva, as well as in the media of cultured cells (El-Agnaf et al., 2003; Jang et al., 2010; Lee et al., 2005;). However, the exact pathway that leads to secretion of αsyn is still not fully understood. As the experimental evidence grows to support the theory that αsyn does in fact transfer from one cell to another, elucidating the mechanisms (depicted in Figure 1) by which this occurs should be a priority. Below we discuss recent discoveries supporting a prion-like spread of αsyn and recount the literature surrounding intercellular spread of key proteins involved in the pathogenesis of other neurodegenerative diseases.

Release from cells

Exocytosis

Early experiments looking at secretion of αsyn suggested that vesicular exocytosis was involved in transporting the protein out of the cell. In SH-SY5Y human neuroblastoma cells overexpressing αsyn, a small percentage of freshly translated αsyn was packaged into the lumen of vesicles and rapidly

secreted from cells. This secretion of αsyn was inhibited by low temperature but not by treatment with the ER/Golgi secretory pathway inhibitor BFA, suggesting that the classic ER/Golgi exocytic pathway was not being utilized for αsyn secretion (Lee et al., 2005). Perhaps important for the pathology of PD, the intravesicular form of αsyn was found to be more prone to aggregation, and αsyn secretion was enhanced when cells were subjected to various stress conditions, such as proteasomal and mitochondrial inhibition, along with the induction of protein misfolding (Jang et al., 2010; Lee et al., 2005).

Exosomes

Exosomes are small (30-100 nm in diameter) membrane vesicles of endocytic origin. They have been found to contain mRNA, miRNA and protein. Originally exosomes were believed to be involved in the removal of unwanted protein from cells (Record et al., 2011; Simons and Raposo, 2009). Current opinion suggests they play a variety of roles including signaling in immune cells (Lässer et al., 2011), and having virus-like properties that allow gene regulation in the recipient cell (Schorey and Bhatnagar, 2008; Simons and Raposo, 2009).

Exosomes have previously been shown to be involved in the secretion of prion protein from cultured cells, and indeed, the secreted prion was able to act as a seed for prion propagation in an uninfected cell (Fevrier et al., 2004; Vella et al., 2007). Recently it was found that αsyn is also secreted via exosomes in SH-SY5Y cells (Alvarez-Erviti et al., 2011; Emmanouilidou et al.,

2010a). These findings highlight that αsyn is transferred between cells via exosomes and that transmission is enhanced under conditions associated with PD pathology, including aggregated αsyn and lysosomal dysfunction.

Whilst confirming that αsyn is indeed secreted via exosomes, Emmanouilidou and colleagues also ruled out the possibility of passive diffusion as the source of extracellular αsyn, as they found that αsyn release to be dependent on intracellular levels of calcium. These data are indicative of an endosomal pathway, and indeed αsyn was detected not only in exosomes but also in the membrane and lumen of membrane vesicles that they identified as endosomes (Emmanouilidou et al., 2010a). The authors determined that medium from cells secreting αsyn was found to be toxic when added to differentiated SH-SY5Y and cultured cortical neurons; however they did not detect any αsyn taken up by these cells. By contrast, proliferating SH-SY5Y cells exhibited evidence of αsyn internalization.

Another study reported that exosome-mediated release of αsyn was enhanced in cells in which the authors induced lysosomal dysfunction (Alvarez-Erviti et al., 2011). While the authors were unable to detect clear evidence of αsyn aggregates, they suggested that the amount of insoluble aggregates increased when lysosomes were inhibited. Furthermore, extracellular αsyn from these cells was transmissible to both mitotic and differentiated SH-SY5Y cells. The idea that aggregates are more transmissible may support evidence that reducing the degree of

oligomerization reduces the toxicity of αsyn delivered to the extracellular space (Danzer et al., 2011; Emmanouilidou et al., 2010a).

Having been implicated in not only intercellular transfer of αsyn, but also other neurodegenerative disease-causing proteins, it is clear that the role of exosomes warrants further investigation in this context. The exosome field is still in its infancy. In relation to αsyn, several questions need to be addressed, including why is αsyn packaged into exosomes; are αsyn-containing exosomes targeted to other cells; how might αsyn leave exosomes and enter the cytosol of the recipient cell?

Uptake by cells

Central to the hypothesis that αsyn spreads via a prion-like mechanism is that the extracellular protein must gain access to the cytosol of a recipient cell, and once there, recruit and induce aggregation of endogenous protein. This process raises several questions, such as are αsyn-containing vesicles targeted to recipient cells, and if so how? Are different types of neural cells, or even selective neurons, more or less prone to take up αsyn? By what mechanism does extracellular αsyn enter the recipient cell? How does this αsyn then recruit and induce aggregation in the new host cell?

Direct cell-to-cell transmission

While the section above focused on αsyn secreted within a vesicle, it is also possible that direct cell-to-cell transmission of αsyn could occur. It has recently been suggested that αsyn can transfer trans-synaptically from one

neuron to another at axonal terminals. Heat shock protein 70 (Hsp70) is associated with extracellular αsyn in this paradigm, and when it is overexpressed it appears to reduce αsyn oligomerization (Danzer et al., 2011). Hsp70 is also secreted from several cell types in response to stress (De Maio, 2011), as well as being present in exosomes (Lancaster and Febbraio, 2005). Thus it might play a role in chaperoning a number of aggregation-prone proteins in the extracellular space. Currently it is not clear, however, if Hsp70 reduces oligomerization of αsyn intracellularly or extracellularly.

Intercellular transfer of αsyn could also occur between neighboring neurons via tunneling nanotubes (TNTs). Indeed this has been shown to occur for prion protein (PrP) (Gousset et al., 2009). TNTs are long thin extensions comprising F-actin between 50-200 nm in diameter and often the length of several cells. They are involved in the intercellular transfer of organelles, as well as vesicles of endocytic origin and cytoplasmic molecules. Taken together, TNTs have been proposed to play a role in signaling between cells (Gerdes et al., 2007; Gerdes and Carvalho, 2008; Onfelt et al., 2005; Rustom et al., 2004). What was highlighted in the work of Gousset and colleagues was PrP could efficiently transfer along TNTs, not only from one neuron to another, but also from bone marrow-derived dendritic cells to primary neurons. Direct cell-to-cell transmission overcomes many obstacles other forms of transport are faced with, and if TNTs form between neurons *in vivo*, this process could mediate the spread of αsyn and explain how neuropathology spreads in accordance with Braak's staging concept.

Endocytosis

It is well established that recombinant αsyn, when added to cultured cells, can be taken up by the recipient cell (Danzer et al., 2009; 2007; Luk et al., 2009a; Nonaka et al., 2010; Waxman and Giasson, 2010), and thus may model a situation when the cellular contents of a lysed cell are released into the extracellular space. Typically, however, the amount of recombinant protein added to the culture medium is quite high, and in some cases, it is necessary to promote uptake through the addition of lipid based agents (Nonaka et al., 2010). Therefore the physiological relevance of this type of experimental model system can be questioned. On top of this, αsyn from a lysed cell is likely to be unprotected and thus susceptible to degradation by matrix metalloproteinase 3 or other extracellular enzymes (Choi et al., 2011; Sung et al., 2005). It was recently shown however, that recombinant αsyn does not require lipid-based agents to enter neurons (Volpicelli-Daley et al., 2011). Through a series of elegant experiments, these authors showed seeding of endogenous αsyn by pre-formed fibrils and that as a consequence of this, neurons displayed a loss of synaptic proteins, reduction in neuronal excitability and connectivity and eventual death. It was also noted that uptake and seeding was enhanced in neurons that had been maintained in culture for longer periods, possibly due to an increased amount of αsyn in older neurons.

Cellular entry of extracellular αsyn also appears to be affected by its assembly state, with monomeric protein able to interact with membranes and lipids and enter via passive diffusion (Ahn et al., 2006; Auluck et al., 2010; Lee et al.,

2008). For oligomeric and fibrillar species, however, uptake appears dependent on the assembly of oligomers. By adding different species of oligomers to cultured cells, Danzer and colleagues found that not all oligomers could enter the cell, with some instead causing increases of intracellular calcium and cell death via an interaction with the membrane. Other species entered cells and increased intracellular aggregation of αsyn (Danzer et al., 2007). Interestingly, the species that entered cells were comprised primarily of higher order oligomers, rather than monomers, again highlighting the importance of oligomerization/aggregation in cell-to-cell transfer.

Moreover, the uptake of oligomeric species is reduced at low temperatures and when dynamin inhibitors are applied to cells, suggesting a classical endocytic mechanism (Hansen et al., 2011; Lee et al., 2008). Further supporting this is the association of αsyn with GTPases Rab5a and Rab7 (Desplats et al., 2009), and the observation that removal of endocytic receptors with trypsin mitigates uptake of extracellular αsyn (Sung et al., 2001). The endocytic pathway of uptake might have evolved as a protective mechanism to reduce extracellular levels of toxic αsyn. This mechanism, of course, requires that the recipient cell is healthy and able to process the aggregated protein via normal proteolysis or the lysosomal pathway. In a healthy individual, this may well be the case, but dysfunctions of both autophagy and lysosomal functions have been implicated as pathogenetic mechanisms in PD. Thus when these pathways are defective and unable to clear the newly imported protein, the entry of αsyn from the extracellular

space will lead to an even greater build-up of protein (Xilouri and Stefanis, 2011). If αsyn is imported via an endocytic pathway, however, it will most likely be retained within the lumen of a vesicle. This point raises the question of how αsyn might leave the vesicle and facilitate recruitment and misfolding of endogenous αsyn? The answer to this question is not certain, but could be related to perturbations in autophagy. αSyn can directly impair autophagy by inhibiting Rab1 (Winslow et al., 2010). It has also been suggested that in cells with lysosomal dysfunction there is abnormal permeabilisation of lysosomal membranes and breakdown of lysosomes (Dehay et al., 2010). Protein aggregates might also disrupt lipid bilayers, and therefore vesicles containing aggregates are more prone to disruption, releasing their cargo into the cytosolic space (Crouch et al., 2008; Jayasinghe and Langen, 2007). Therefore, one can envisage a vicious cycle where the presence of excess αsyn, especially in aggregated forms, in the cytosol causes impairment of autophagy, in turn leading to disruption of endocytic vesicles and release of additional αsyn into the cytoplasm.

Seeding

The next stage in the pathogenic spread of αsyn is the recruitment of endogenous protein from the host cell by the newly imported protein. This permissive templating or seeding action by the newly introduced protein induces aggregation of host cell proteins that would presumably only aggregate in the presence of the seed. Thus, seeding perpetuates the cycle of misfolding and spread of αsyn akin to the actions of prion proteins (Aguzzi and Rajendran, 2009; Brundin et al., 2010; Krammer et al., 2009). The

majority of groups looking at permissive templating of imported αsyn thus far have utilized artificial systems, including recombinant protein and/or lipid based protocols to facilitate entry of the oligomers or seeds into the recipient cell (Danzer et al., 2009; Luk et al., 2009b; Nonaka et al., 2010; Waxman and Giasson, 2010). While these studies advance the understanding of the field, their physiological relevance is under question. As more review articles, authored by us and others (Angot et al., 2010; Angot and Brundin, 2009; Lee et al., 2011b; Steiner et al., 2011), than original articles have been published, this section will focus on the most recent reports where the authors have reported seeding in *in vitro* models and in neurons grafted into animal models in conditions featuring natively derived proteins.

We recently demonstrated for the first time that αsyn that had been released from co-cultured cells could be taken up and act as a seed for aggregation in the recipient cell (Hansen et al., 2011). Using differentiated SY-SH5Y cells, a small amount of imported αsyn-GFP was detected surrounded by αsyn-DsRed (derived from recipient cell), providing clear evidence of imported αsyn acting as a seed for propagating αsyn aggregation in the recipient cell (Figure 2). In some cases the αsyn-DsRed that had been taken up was present in Thioflavin S-positive aggregates. As is the case for previously mentioned studies that report the aggregation of αsyn, it is unclear if this aggregation occurs before or after the protein has transferred from one cell to the next.

To date, two groups have examined *in vivo* seeding of αsyn occurring in animal models analogous to the human transplantation patients, i.e. grafting

of nigral cells into the striatum. Although we demonstrated intercellular transfer of huαsyn in a mouse model of genetic huαsyn overexpression, in our initial study we did not observe subsequent seeding (Hansen et al., 2011). Kordower and colleagues recently demonstrated that embryonic dopaminergic neurons grafted into the striatum of rats engineered to express huαsyn contain αsyn transferred from the host to the graft, which they postulate then forms aggregates in the recipient cell (Kordower et al., 2011). In this study, the process of transfer is quite clear. However, only a small amount of protein taken up by the graft was reported to fail to digest with proteinase K (indicative of insoluble aggregates), and these data were not actually shown in the paper. Consequently, it is not clear to what degree aggregation and/or seeding is occurring. The authors suggest the short time period of their experiment (5 weeks post grafting) might limit their detection of large aggregates in recipient cells (Kordower et al., 2011).

Using a similar approach, we have recently observed both intercellular transfer and seeding to occur in naïve neurons grafted into the striatum of rats overexpressing huαsyn in the striatal terminals (Angot et al., 2011, submitted). We found transfer of huαsyn from host terminals to grafted cells to be a frequent event (in one condition, 23% of grafted cells expressed huαsyn puncta). In a few rare instances, we discovered imported huαsyn surrounded by a large core of rat αsyn derived from the grafted neuron, providing strong evidence for seeding (Angot et al., 2011, submitted).

As we have explained above, the hypothesis regarding prion-like properties of αsyn is relatively new and not extensively studied. The papers discussed earlier in this review shed little light on the prerequisites for imported αsyn to cause seeding and aggregation. For example, what intracellular compartments must imported αsyn access to cause seeding? What is the minimum required concentration of imported and endogenous αsyn for aggregate formation to take place? What chaperones can prevent seeding? How do cellular protein degradation systems interplay with a seeding/aggregation process? It should also be pointed out that in the majority of studies, transfer and seeding events are quite rapid compared to the slow spread in human transplant studies that sparked interest in the prion-like hypothesis. While the rapid transfer might be explained by the artificial nature of the experiments involving, e.g., overexpression of protein and the use of transgenic animals. Furthermore, in the human brain (in contrast to cell cultures) aberrant protein is likely to be cleared either from the extracellular space by e.g. microglia, or from the recipient cell by endogenous proteolytic pathways. This then raises yet more questions as to the events leading to the tipping point where αsyn is misfolded and is able to cause aggregation on a scale large enough to cause pathology in the human brain.

However, these are not trivial research questions. For comparison, it has been nearly 30 years since the identification of prions (Prusiner, 1982), and the mechanism by which PrP converts from wildtype to prion forming has yet to be fully elucidated (Colby and Prusiner, 2011). Even though it is early days for research on the prion-like properties of αsyn, the papers discussed above

demonstrate *in vivo* transmission of αsyn and possible seeding, giving further weight to the hypothesis that a prion-like mechanism contributes to PD pathogenesis.

What causes aggregation of α-synuclein, and what are the consequences to the cell?

Assuming that αsyn, in particular, aggregated αsyn, is neurotoxic, what elements of the protein cause this toxicity? Does αsyn exert its toxic effects extracellularly, or only after uptake by the host cell? Unfortunately the answers to these questions remain a mystery. It is often assumed that the host of cellular dysfunctions associated with sporadic PD occurs as a result of Lewy pathology, however the mechanisms behind the assumed negative effects of αsyn aggregates remain unknown. Notably, it has been claimed that in autosomal recessive juvenile onset PD due to mutations in the *parkin* gene, nigral neurodegeneration occurs in the absence of Lewy pathology, leading to controversial suggestions that the two are not inexorably linked (Farrer et al., 2001b; van de Warrenburg et al., 2001).

Many groups have shown that αsyn is toxic when added to the medium of cultured cells (see review by Lee 2007). What causes this neurotoxicity is, however, not clear and the aggregation state (oligomeric, protofibril or fibril) of the toxic species is also debated (Oueslati et al., 2010; Volles and Lansbury, 2003). It has been suggested αsyn can disrupt the outer cell membrane (Volles et al., 2001). Another possibility is that αsyn triggers a neuroinflammatory response, demonstrated in cultured microglia and

astrocytes (Klegeris et al., 2008; 2006; Lee et al., 2010b; Su et al., 2008; Zhang et al., 2005). Along with exerting its effects extracellularly, intracellular αsyn oligomers and aggregates are proposed to interact with lipid membranes and lead to a host of cellular dysfunctions (Auluck et al., 2010; Su et al., 2010).

As mentioned earlier, post-translationally modified αsyn is present in Lewy bodies and neurites. These modifications include truncated and ubiquinated αsyn, and also phosphorylation which perhaps is the most studied modification (Oueslati et al., 2010). Leucine-rich repeat kinase 2 (LRRK-2) has been identified as a crucial protein in the pathology of PD and a recent paper highlights a direct role in transmission and aggregation of αsyn (Kondo et al., 2011). This report follows previous work that has shown kinase inhibitors and phosphatase activity are protective in different models of PD (Lee et al., 2011a; 2010a). Based on results from a model where they overexpress both αsyn and LRRK-2, Kondo and colleagues suggest that, alternatively, LRRK-2 enhances aggregation of αsyn, and that αsyn transmission from one cell to another was also increased. Whether transmission was a direct result of LRRK-2 or the increase in aggregation is not clear.

The section above has demonstrated that αsyn is capable of intercellular transfer, and that transferred αsyn appears capable of inducing aggregation both *in vivo* and *in vitro*. This prion-like phenomenon is likely to occur for other

proteins in neurodegenerative diseases, suggesting common mechanisms might be involved.

Prion like behavior in other neurodegenerative disease

In addition to αsyn, several other proteins associated with neurodegenerative diseases have been suggested to have a prion-like behavior. Like αsyn, these proteins have a hydrophobic region that is prone to aggregation (Figure 3). In this section we briefly highlight the role that intercellular transfer of these proteins plays in the pathogenesis of neurodegenerative disease, highlighting mechanisms that are analogous to those discussed for αsyn.

Alzheimer's disease – Tau & Amyloid β

Tau

In solution, the tau protein is highly soluble with limited secondary structure and dominated by random coil structures (Schweers et al., 1994). Despite this structure, tau is capable of aggregating into protein inclusions. Such aggregates emerge in selected regions of the human brain and characterize over 20 neurodegenerative tauopathies, including AD (reviewed by Lee et al., 2001). Of direct relevance to this review are recent studies suggesting that the pathogenesis of tauopathies includes a prion-like behavior of misfolded tau. Tau proteins have been identified to be secreted into the extracellular space (Blennow et al., 2010) and Frost and colleagues have now demonstrated that exogenous truncated tau inclusions can promote endogenous tau aggregation by entering cells via endocytic mechanisms (Frost et al., 2009).

These experiments were confirmed by expressing a fluorescently tagged tau fragment of the molecule in a neuronal cell line. Small round YFP-positive structures were identified and these tau inclusions were able to cross the cell membrane. In the new cells they could induce tau aggregation in neurons expressing either YFP- or mCherry-labeled full-length tau. When cell lines expressing tau with different labels were co-cultured, intracellular protein aggregates of dual colors were identified, suggesting that a transfer of full-length tau proteins had occurred (Frost et al., 2009). Interestingly, another study using different isoforms of tau in different cells showed that seeding tau aggregation is isoform specific, a finding that is consistent with specific tau isoforms aggregating in the various tauopathies (Nonaka et al., 2010).

Not only is transfer and seeding of tau possible in cultured cells, but intercellular transmission of tau was also recently shown to occur in experimental animals. Clavaguera and colleagues utilized a mouse model of tauopathy to examine whether tau might act in a prion-like manner *in vivo* (Clavaguera et al., 2009). They showed that intracerebral injections of tau extracts derived from mice expressing a mutant form of tau promoted tau inclusion formation in neurons and glia in mice expressing human wild-type tau proteins (Allen et al., 2002). These inclusions spread in a time- and region-dependent manner to areas of the brain anatomically connected to the injection site. These experiments indicate that tau can induce aggregation and propagate via a prion-like manner, as shown for αsyn.

Amyloid Beta

Amyloid plaques are also a hallmark of AD and, similar to tau, they are present in an increasing number of brain regions as the disease progresses (reviewed by Thal et al., 2006). This coupling between amyloid plaque distribution and symptomatic progression led researchers nearly three decades ago to first hypothesize that a prion-like mechanism was involved in AD (reviewed by Schnabel, 2011). The distribution of amyloid plaques in the AD brain at different disease stages has also been described by Braak and colleagues (Braak et al., 1993). The amyloid plaques are composed of Aβ, which is derived from a much larger protein known as the amyloid precursor protein after cleavage by several secretases (for review please see Zhang et al., 2011).

Analogous to long term studies of beta-amyloidosis in primates (Ridley et al., 2005), Meyer-Luehmann and colleagues injected human AD brain extracts or Aβ extracts from aged mice (APP23) into the brains of young, presymptomatic APP23 mice to investigate whether Aβ deposition can propagate in a prion-like manner (Meyer-Luehmann et al., 2006). They showed that extracts derived from human AD brain and from aged mouse brain served as seeds to promote the formation of Aβ pathology in young transgenic mice. As expected, induced Aβ deposits were found within the injected areas. Notably, some Aβ deposits were found in areas outside of the injection site, a result suggestive of a spread of the Aβ pathology to adjacent brain regions. Similar results have also been recently reported using mice expressing human wild type APP proteins (Morales et al., 2011). These mice would not normally develop AD, yet after injection with AD brain, hallmark plaques were observed

to spread throughout the brain. Of particular interest in this study was the time frame for plaque development. Plaques were first visible 285 days post injection, and increased in number up until sacrifice at day 585, providing clear evidence of a progressive prion-like transmission of Aβ (Morales et al., 2011).

Prion proteins have also been effective in promoting prion disease via intraperitoneal injections (Prusiner, 2004). Eisele and colleagues tested whether Aβ extracts administration via intraperitoneal injections could induce amyloid deposition in the brain of young APP23 mice (Eisele et al., 2010). In line with their hypothesis, Aβ deposition was clearly observed within the anterior and entorhinal cortices as well as the hippocampus in a manner similar to aged transgenic APP23. Notably, no pathology was observed in mice injected with extracts of wild type mice or extracts from aged APP23 mice immuno-depleted of Aβ (Eisele et al., 2010).

Aβ peptides aggregate within the extracellular space as amyloid plaques and until recently, the mechanism of protein release was unknown. Several investigators have suggested that Aβ is associated with exosomes (Okabayashi and Kimura, 2010; Rajendran et al., 2006; Sharples et al., 2008; Sullivan et al., 2011). Not only have exosomes been suggested as a route of Aβ trafficking, but they have also been shown to contain secretases and be a site of APP cleavage (Sharples et al., 2008). Thus exosomes might play a role in trafficking and processing of APP to Aβ. These results are supported by

findings of exosomal proteins in amyloid plaques in AD brains (Rajendran et al., 2006).

As discussed later, it has recently been suggested that mutations in the gene encoding VPS35, a component of the retromer complex, leads to early-onset PD (Vilariño-Güell et al., 2011; Zimprich et al., 2011). This protein also appears to play in processing and trafficking APP. When this protein, and thus retromer transport, is disrupted, it leads to increased Aβ production and increase in total levels of APP within exosomes. (Sullivan et al., 2011). Clearly the role(s) of exosomes in AD are not yet fully explored. However, the fact that APP, Aβ and various secretases are located to exosomes and exosomal proteins are found in amyloid plaques suggest that exosomes might play a role in intercellular transfer of Aβ and spreading of the AD pathology throughout the brain.

Amyotrophic Lateral Sclerosis – Superoxide Dismutase

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disorder that causes the selective death of motor neurons within the cerebral cortex, brain stem and spinal cord, leading to muscle weakness and eventually paralysis (Chattopadhyay and Valentine, 2009). While the vast majority of ALS cases are sporadic, about 10% have been identified as familial with mutations in several genes including the copper-zinc superoxide dismutase gene (SOD1) and, most recently, the TAR DNA binding protein (Kabashi et al., 2008; Sreedharan et al., 2008).

SOD1 is an abundant soluble protein with enzymatic activity responsible for catalyzing the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen. To date, over 140 mutations associated with familial ALS have been reported within the SOD1 gene. These mutant proteins readily form protein inclusions that selectively accumulate within the neocortical and spinal cord motor neurons (Borchelt et al., 1994). Notably, expression of mutant SOD-1 in transgenic animals leads to protein inclusions and clinical symptoms analogous to those seen in ALS, supporting the notion that SOD1 inclusions are important in the pathogenesis of ALS (Crook and Housman, 2011).

The aggregation of the SOD1 protein into highly stable amyloid fibrils can promote the accumulation of normal wild type proteins, which suggests that it might possess prion-like qualities (Chattopadhyay and Valentine, 2009; Chiti and Dobson, 2006). SOD1 protein inclusions have been identified in cerebrospinal fluid of ALS patients and have been suggested to promote the spread of SOD1 pathology by affecting normal cells in other brain regions (Borchelt et al., 1994; Bruijn et al., 1998; 2004).

Recently, a series of cell culture experiments demonstrated that mutant SOD-1 aggregates can exhibit a prion-like behavior by propagating between cells (Münch et al., 2011). By expressing recombinant forms of mutant SOD-1 labeled with Dylight dyes, Münch and colleagues showed that SOD-1 aggregates were taken up by cultured neurons and internalized via endocytic vesicles using a mechanism that was independent of clathrin or dynamin but

required ATP. Due to the observed response to pharmacological challenge and the size of the vesicles, the authors confirmed that aggregated SOD-1 proteins utilized lipid raft-dependent macropinocytosis (Münch et al., 2011), a mechanism also used by a number of viruses (Mercer and Helenius, 2009).

To determine whether the SOD1 inclusions could transfer to other cells, cells expressing SOD1-GFP proteins were exposed to exogenous SOD-1 aggregates. Punctate inclusions formed that appeared to transfer to other generations as the cells underwent cell divisions. To confirm that protein inclusions were transferred from cell to cell, cells were inoculated with different exogenous, fluorescently-labeled SOD-1 aggregates, and populations were then mixed. Over time, co-localization was observed within aggregates of both cell types indicating that SOD-1 aggregates have a heritable phenotype and behave in a prion-like fashion (Münch et al., 2011).

Huntington's disease – Huntingtin

Huntington's disease (HD) is a progressive and neurodegenerative disease characterized by motor dysfunction, cognitive decline, personality changes and weight loss (Vonsattel et al., 2011). It is caused by an expansion of a CAG trinucleotide repeat (repeats >36) within the gene encoding the huntingtin protein. The mutation leads to neurodegeneration primarily in medium sized spiny striatal neurons, but also in other widespread brain areas including neocortex and hypothalamus. Notably, the expanded polyglutamine stretch causes huntingtin to form aggregates that are capable of seeding wild-type huntingtin proteins (CAG repeats<40) (Hoffner and Djian, 2002).

To determine whether exogenous polyglutamine aggregates can seed endogenous huntingtin proteins, Yang and colleagues synthesized polyglutamine peptides that were chemically aggregated and capable of penetrating the cell membranes. These aggregates, however, were shown not to be toxic to cells unless a nuclear localization sequence was added to the polyglutamine peptide (Yang et al., 2002). Their potential to act in a prion-like fashion was not explored further in these initial experiments.

In a subsequent study, Ren and colleagues incubated different cell types with fluorescently-tagged polyglutamine peptides and characterized the mechanism of entry into the cytoplasmic compartment (Ren et al., 2009). In agreement with the Yang study mentioned above, polyglutamine aggregates penetrated the outer cell membrane of several different cell types. However, Ren and colleagues focused on the ability of the imported polyglutamine peptides to trigger aggregation of endogenous wild type huntingtin, and the inability of the peptides to interact with other aggregation prone proteins. The aggregates formed by the imported polyglutamine peptides were not associated with endosomal compartments, but co-localized with chaperones (Hsp70, Ubiquitin) (Ren et al., 2009). Based on electron microscopic observations and the high hydrophobic nature of the polygluatmine peptides, Ren and colleagues suggest that the polyglutamine aggregates were capable of penetrating the outer cell membranes. Taken together, these experiments show that an aggregation-prone protein can also enter cells via a non-endocytic pathway and supports other observations which indicate that

protein aggregate formation occurs in a protein- and sequence-specific manner.

The studies shown above indicate that cell-to-cell transfer of proteins associated with neurodegenerative disease is not unique to αsyn. On top of the intercellular transfer, it appears that the transferred proteins are also able to induce protein misfolding in the recipient cell in a manner similar to prion disorders.

Identifying & Predicting Parkinson's disease

What causes PD?

In addition to mutations in *SNCA* and other genes mentioned later in this review, other causes of PD have been hypothesized. One theory suggests that a viral infection triggers development of PD. This theory is partly stimulated by rare findings in patients, who, after surviving encephalitis during the Spanish flu epidemic in 1918 developed a post-encephalitic parkinsonian syndrome. These patients initially responded favorably to antiparkinsonian dopamine replacement therapy (Sacks, 1983). Although their brains did not contain Lewy pathology, they exhibited degeneration in the nigrostriatal system (Jellinger, 2009b). Recent reports highlight a potential role of H5N1 infection ("bird flu") in the CNS. In experiments in mice, H5N1 infection can lead to microglia activation and αsyn phosphorylation and aggregation (Jang et al., 2009a). In these mice it was also noted that the virus moved from cell to cell, first being detected in the enteric and peripheral nervous system before moving to the CNS, perhaps mirroring the spread of the unknown pathogen

according to Braak's staging scheme. While no proof exists that a viral infection causes PD, microglia activation and protein aggregation have been noted not just for PD but also for AD and as such, viral infection remains a possible cause in the development of neurodegenerative disease (Jang et al., 2009b).

Toxins, in particular pesticides, have also been implicated in the development of PD. The fact that pesticides have been widely implicated in PD has led to numerous epidemiological studies suggesting that those living in rural areas are more susceptible to developing PD (Berry et al., 2010; Brown et al., 2006). Other studies, however, have concluded that those living in rural areas are no more likely to develop PD than their city counterparts (Chen et al., 2009; Walker et al., 2010). The study of pesticides, in particular Paraquat and Rotenone, as causative agents of PD gained significant attention when it was discovered that they were toxicologically or structurally similar to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). MPTP was discovered to cause Parkinsonism about three decades ago when a group of people injected the drug as a contaminant of synthetically produced opioid (Davis et al., 1979; Langston and Ballard, 1983;). The active metabolite, MPP^+ (1-methyl-4-phenylpyridinium) inhibits not only Complex I of the mitochondrial respiratory chain (Krueger et al., 1990), but also uptake of dopamine by synaptic vesicles, leading to degeneration of dopaminergic neurons of the substantia nigra pars compacta (Lotharius and O'Malley, 2000). Inhibition of vesicular uptake leads to accumulation of cytosolic dopamine, which is known to undergo oxidation, leading to increased reactive oxygen species production

and subsequent cell death (Lotharius and Brundin, 2002; Lotharius and O'Malley, 2000).

Based on the above observations, one obvious question arises: do toxins and viruses lead to immediate neuronal death in PD, or do they induce cellular dysfunction that several years later causes neurodegeneration? As a number of cellular defects have been linked to PD pathogenesis, and these dysfunctions, oxidative stress in particular, increases as we age, it must be asked whether these cellular events play a major role in PD pathogenesis. Age remains the greatest risk factor for PD (Collier et al., 2011), so it is clear that during aging, our cells display a greater degree of dysfunction. This dysfunction results in a greater cell stress, which in turn places greater energy demand on the cells. In attempting to keep up with increasing energy demands, there might be an increase in oxidative stress and a repeating cycle of dysfunction and oxidative stress escalates. It is this increase in energy demand that may explain why dopaminergic neurons of the substantia nigra pars compacta are the primary site of PD pathogenesis, with nigrostriatal dopaminergic neurons estimated to form in the region of 40,000 synapses. This is 10-fold more than those of the dopaminergic neurons located in the adjacent ventral tegmental area and which undergo degeneration in PD later and to a lesser degree than nigral neurons (Moss and Bolam, 2010).

Approximately half the cases with early (before the age of 40 years) onset PD are associated with genetic causes of PD. While sporadic onset PD has no confirmed causes of origin, and typically occurs later in life recent studies

have highlighted possible genetic risk factors (Satake et al., 2009; Simón-Sánchez et al., 2009). Genes encoding the mitochondrial proteins PINK1 and Parkin are implicated in recessive (or early onset) parkinsonism, while *SNCA* and *LRRK2* are the two genes more commonly associated with dominant or sporadic PD (Cookson and Bandmann, 2010). Recently another gene has been implicated in late onset, autosomal dominant PD. Exome analysis revealed mutations in the gene encoding VPS35 in two separate families with a history of late onset autosomal dominant PD (Vilariño-Güell et al., 2011; Zimprich et al., 2011). As mentioned earlier, VPS35 is a component of the retromer complex, which mediates retrograde transport between endosomes and the trans-Golgi network and has also been implicated in AD (Muhammad et al., 2008; Small et al., 2005; Sullivan et al., 2011). With increased sensitivity and improved GWAS technology, it is likely further PD candidate genes will be identified. Recent reports suggest at least 11 risk loci for PD (International Parkinson Disease Genomics Consortium, 2011). The genes associated with these loci have yet to be fully characterized. The various genes outlined by GWAS and those mutations discussed above raise the possibility that there are at least two separate pathways leading to PD (Cookson and Bandmann, 2010).

Parkinson's disease: One disease or many?

It is one thing to understand how αsyn propagates through the body and how this spread leads to the pathology of PD, but it is a completely different matter to translate this to the broader area of diagnostics and treatment. Perhaps the most pressing question in this area is, does everyone diagnosed with PD

actually have the same disease? In recent years, particularly with the identification of disease-causing genes, scientists have begun to question whether PD is actually a single disease (Langston, 2006; Obeso et al., 2010; Selikhova et al., 2009; Williams-Gray et al., 2009).

At present dementia with Lewy bodies (DLB) is classified as a separate disease to PD. As for PD, a diagnosis of DLB is confirmed by Parkinsonism and the presence of αsyn-positive Lewy bodies; however, these lesions appear more abundantly in the cerebral cortex (Langston, 2006; Weisman and McKeith, 2007). Due to the similarities in symptoms, it has been argued that they are indeed the same disorder, just with different points of origin (Langston, 2006). So while DLB and PD may actually be the same disorder, it is also possible to split classical PD up into a number of sub-groups; Early disease onset, Tremor dominant, Non-tremor dominant and Rapid disease progression without dementia (Selikhova et al., 2009). Depending on which sub-group a patient fell into, the time of onset of falls and hallucinations along with length of life after diagnosis were found to differ. The non-tremor group also displayed higher pathological grading of cortical Lewy bodies, perhaps more akin to DLB. These observations raise several interesting questions. For example, are these sub-groups representing differing points of origin for PD, or a different class of PD? Do those patients that fall outside the classic Braak staging scheme represent a different class of PD?

When does Parkinson's disease start?

By the time clinical diagnosis is made, based on bradykinesia and at least one other symptom including rest tremor, rigidity and postural instability, it is estimated that loss of dopaminergic neurons in some areas of the substantia nigra is as high as 70% (Fearnley and Lees, 1991). Braak and others have proposed that not just dopaminergic neurons are affected in PD, but glutamatergic, cholinergic, serotonergic and adrenergic neurons along with the autonomic nervous system are affected too. Therefore it is hardly surprising that PD patients present with a host of non-motor symptoms, with more than 90% of patients presenting with these at some stage during the disease (Shulman et al., 2001).

PD likely has a significant preclinical period, with conservative estimates putting this in the order of 5-6 years (Hawkes et al., 2010; Savica et al., 2010; Schapira and Obeso, 2006). It is becoming increasingly evident, however, that there are a host of non-motor symptoms, including autonomic dysfunction, sensory problems, sleep disorders and neuropsychiatric symptoms(Poewe, 2006; Reichmann, 2010), some of which may precede the onset of motor symptoms by 20 years or more (Savica et al., 2010). While suffering from any number of these non-motor symptoms does not facilitate the development of PD, they could offer vital clues in identifying at risk patients, and perhaps even propose the possibility of treating patients long before the onset of the classic PD motor symptoms. In order to do this, it is necessary to develop methods to identify those at risk of developing PD and to identify objective biomarkers that track progression of the disease. Clearly, non-motor symptoms might be

useful for both this tasks. Given the great range of non-motor symptoms, only three of the more frequently occurring non-motor symptoms are discussed below.

REM sleep behavior disorder

It was perhaps as early as 1817 when James Parkinson was first describing PD that sleep dysfunction, potentially RBD, was mentioned as a symptom (Langston, 2006). In his monograph, Parkinson writes, ”Sleep becomes much disturbed. The tremulous motion of the limbs occur during sleep, and augment until they awaken the patient, and frequently with much agitation and alarm” (Parkinson, 2002). RBD is characterized by excessive bursts of physical activity, including crying out and kicking in association with dream content (Gagnon et al., 2006). Studies indicate that somewhere in the order of 1/3 of PD patients also suffer from RBD (Gagnon et al., 2006), however some reports suggest this number may be as high as 60% (Gagnon et al., 2002). A retrospective study on 44 patients with RBD revealed that 45% developed a neurological disorder, with 9 cases of PD recorded (Iranzo et al., 2006). In these patients, it appears that the pons is involved (Langston, 2006), with RBD developing somewhere in the order of 13 years before PD (Schenck et al., 1996).

Olfactory dysfunction

In the earliest stages of PD, Lewy bodies are predominantly found in the olfactory bulb (Braak et al., 2003; Braak and Del Tredici, 2008) and some studies show olfactory dysfunction in all PD patients (Müller et al., 2002).

Olfactory dysfunction is of particular interest in prediction of PD, mainly due to the ease of testing using smelling tests (Doty et al., 1984; Hummel et al., 1997). These tests have the benefit of being non-invasive, inexpensive and easy to perform. While these tests are not perfect, and the lead time of detecting loss of olfaction appears to be only 4 years before classic disease onset, there is evidence to suggest olfaction can predict PD (Ponsen et al., 2004; Ross et al., 2008). The ease of these smell tests makes them perfect to use in combination with another test, such as sleep analysis to monitor RBD, in identifying at risk patients (Stiasny-Kolster et al., 2005).

Constipation

Another common symptom described by James Parkinson and one which is predicted to predate motor symptoms by at least a decade is constipation (Abbott et al., 2001; Savica et al., 2009). The exact cause of constipation in PD is not clear; however, according to Braak's hypothesis, the first Lewy bodies occur in the enteric plexuses, which could explain the loss of gut motility in PD patients (Braak et al., 2006). Recent studies suggest that the enteric nervous system is severely affected by PD (reviewed by Lebouvier et al., 2009) and indeed colonic biopsies show presence of αsyn in PD patients, yet this staining is absent in control groups (Shannon et al., 2011). A study born out of the Honolulu Heart Project identified that those men who reported less than one bowel movement a day were four times more likely to develop PD, while 25% of these individuals displayed incidental Lewy body pathology compared to only 6.5% of non-constipated individuals (Petrovitch et al., 2009).

What the above symptoms present us with, along with others such as depression, visual changes and somnolence, is not a checklist for preclinical PD, but a host of symptoms that may indicate an underlying problem. Of the three symptoms outlined above, constipation and olfactory dysfunction are very easy to determine clinically, while a proper diagnosis of RBD requires more extensive testing in a sleep laboratory. While age remains the biggest risk factor in developing PD (Collier et al., 2011), non-motor symptoms may prove the way for identifying at risk patients before more in-depth tests are performed.

Current & future therapies

As discussed above, PD manifests with a range of symptoms, many pre-motor, but diagnosis is not made before the onset of motor symptoms. By this time, a significant number of dopaminergic neurons in the substantia nigra pars compacta have already been lost. It is this loss of dopaminergic neurons that has been the mainstay of PD therapy for the past 40 years via L-dopa therapy. Levodopa (LD) was first introduced as a therapy in the late 1960s and proved highly effective at reducing symptoms in nearly all PD patients and increasing the time patients could enjoy independent activities (Olanow et al., 2004b; Rascol et al., 2011; Schapira et al., 2009). Despite this, treatment with LD has its drawbacks, with a high proportion of patients developing motor complications within 2 years of use, and in extreme cases, dyskinesia can completely negate the therapeutic benefit of the drug (Poewe et al., 2010). As highlighted in this review, αsyn may play a major role in the pathogenesis of PD and so it is worth examining ways that we can control its deleterious

effects. With our current understanding on the spread of αsyn, there are a number of potential areas that could be targeted to halt the prion-like spread (Brundin and Olsson, 2011).

Increase in the expression of αsyn has been suggested to be one contributing factor to the development of aggregates, and thus, if expression can be regulated, either by controlling its transcription or via knock-down approaches, it could reduce the amount of protein aggregation. Until we have a better understanding of the normal function αsyn plays in the cell, however, this approach is likely to have its limitations. So while controlling the levels of αsyn may prove difficult, and indeed deleterious in itself, it may be possible to stop the aggregation, which appears to be unique to the disease state, and less likely to play a normal physiological role. Approaches explored in this area include promoting the cellular defenses, either by enhancing the protein folding machinery, or improving clearance of misfolded proteins (Xilouri and Stefanis, 2011), using small peptides to inhibit or dissolve aggregates (Amijee et al., 2009), or with the recent discovery of αsyn existing as a tetramer, future research may focus on ways to stabilize this structure and thus prevent aggregation (Bartels et al., 2011).

The final potential αsyn target is in its intercellular transmission via a prion-like mechanism. Whether this inhibition is possible either by preventing its release from the cell, or its uptake by a new cell remains to be seen. As discussed above, several theories abound on how exactly αsyn is secreted and transferred between cells. While the *in vitro* data is compelling, more research

is required to elucidate the full mechanisms *in vivo*. Perhaps the area of most interest from a therapeutic perspective is: how is extracellular αsyn taken up by the host cell? Is it part of a general mechanism for cell communication or is it a specific process where only certain cells are targeted? If one could identify the contributing factors, inhibitory peptides used to block uptake are just one potential therapy. While there are many avenues that can be investigated that could led to future therapeutics, not least those looking at reducing neuroinflammation and oxidative stress, the prion-like spread of αsyn is a highly desirable target, and coupled with better screening, presents an opportunity to target the spread of disease before motor symptoms develop.

Conclusion

Despite the ongoing controversy over Braak's staging concept, the evidence is mounting that αsyn transfers between cells, and that in doing so in a prion-like manner, could well represent the pathogenic mechanism behind PD. Debate is still open over whether Lewy bodies are neurotoxic or neuroprotective; however, two major points mentioned in this review point to the former. Aggregates of αsyn transfer to neurons and while it would evolutionarily make sense for a cell to remove unwanted aggregated protein if it was then going to be taken up by immune cells such as microglia, it makes little sense to transfer it to a non-dividing neuron. However, perhaps the most compelling piece of evidence that αsyn is driving the pathogenesis of PD is the fact that it appears to recruit and cause further aggregation of endogenous αsyn once entered into the host cell.

The literature discussed in this review provides compelling evidence that αsyn does in fact transfer from cell to cell in a prion like manner and might indeed promote spread of PD. In what may prove to be a valuable research tool to study these mechanisms, prion-like acceleration of synucleinopathy was recently demonstrated for the first time in a transgenic mouse model of PD (Mougenot et al., 2011). The prion-like transfer does not appear unique for αsyn though, as it is present in many other neurodegenerative diseases. Further investigations into the prion-like spread of disease-associated proteins are necessary. The potential benefits of this research, in terms of new diagnostics and therapies, are far reaching, with neurodegenerative disease an increasing burden on our society.

Conflict of Interest

PB is a co-founding member of Neurprotex AB, a biotech company that has α-synuclein transfer as one of its therapeutic targets. The other authors declare no conflict of interest.

Acknowledgements

We wish to thank Ann-Louise Bergström (H. Lundbeck A/S, Valby, Denmark) for providing the image of cell-to-cell transfer in SH-SY5Y cells. Our work is supported by Michael J. Fox Foundation for Parkinson's Research; Swedish Brain Foundation; Swedish Parkinson Foundation; Söderbergs Foundation; Swedish Research Council, including the Linnaeus grant Bagadilico; Anna-Lisa Rosenberg Foundation; European Research Area Network of European Funding for Neuroscience Research Program MIPROTRAN; Human Frontier

Science Program; a European Research Council Advanced Award and a private donation from Mr Sven Astell. JAS and PB are active in the Strong Research Environment Multipark (Multidisciplinary research in Parkinson's disease at Lund University), and JAS is a recipient of a personal fellowship from this program. None of these funding sources determined or contributed to the content of this review.

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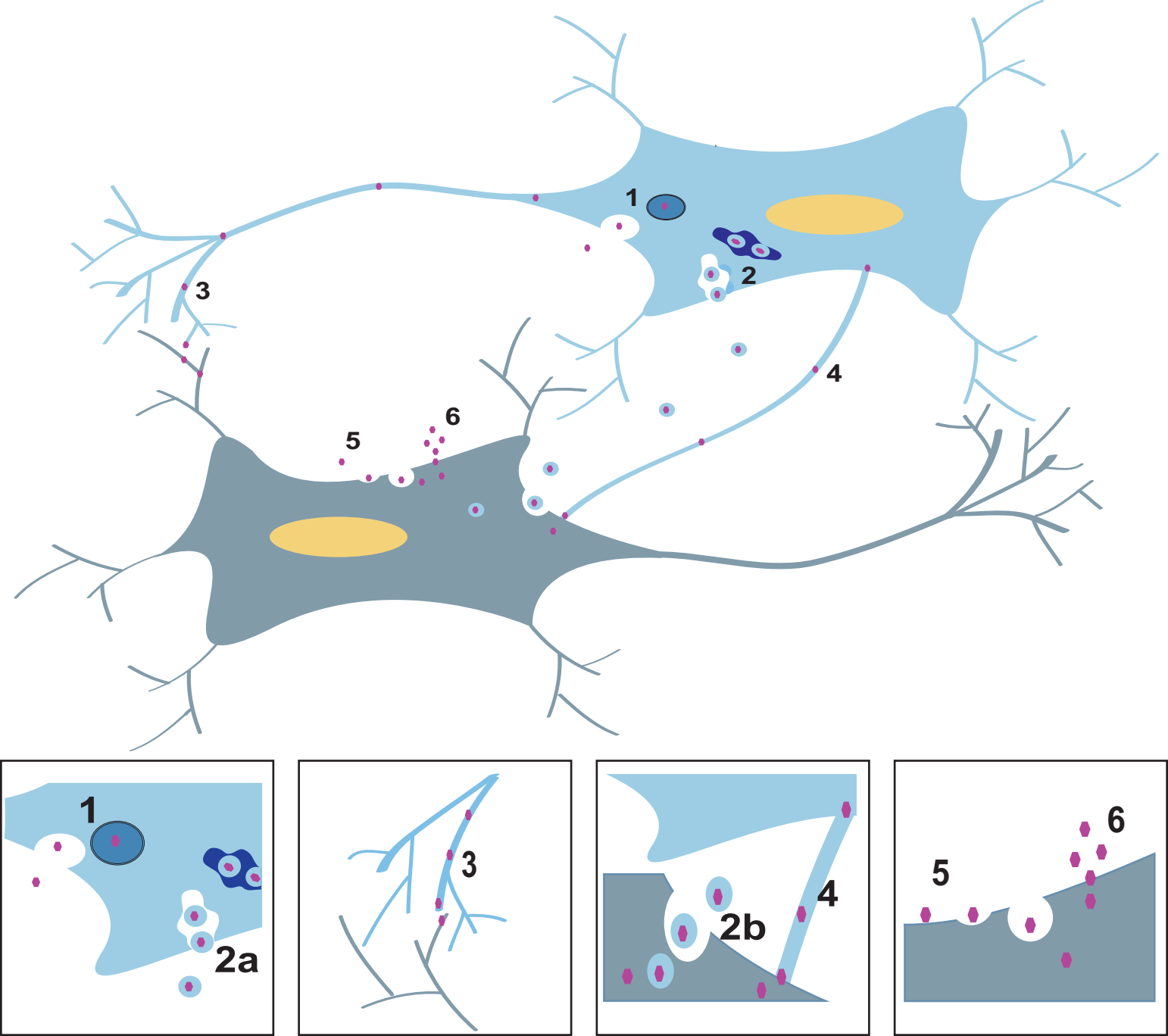
Figure 1: Mechanisms of α-synuclein cell-to-cell transfer.
Schematic depicting the various ways α-synuclein (purple) has been proposed to transfer between cells. α-Synuclein has been found in both exocytotic vesicles (1) and exosomes (2a & 2b). Direct cell-to-cell transmission is proposed to occur Trans-synaptically (3) and while not yet shown for α-synuclein, Tunneling nanotubes (4) have been shown to transfer prion from one cell to another. How α-synuclein is taken up by the host cell is less clear, with both Endocytosis (5) and Passive diffusion (6) shown to occur.


1
2
3
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2a
3
2b
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Figure 2. α-Synuclein transfers from cell to cell and seeds aggregates in the recipient cell.
Representative confocal Z-projection showing a double-labeled SH-SY5Y cell after 22 days of coculture of stable SH-SY5Y cell lines expressing αsyn fused to either GFP or DsRed. The DsRed-positive cell contains a transferred GFP punctum which colocalizes with the DsRed signal to form a double-labeled punctum. Image courtesy of Ann-Louise Bergström, H. Lundbeck A/S, Valby, Denmark.

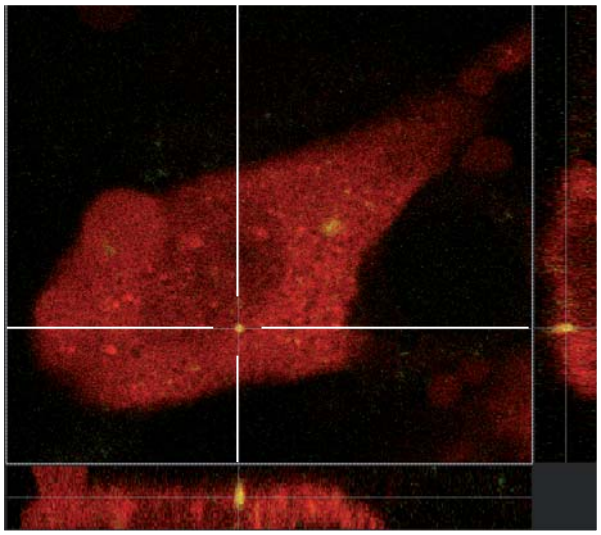
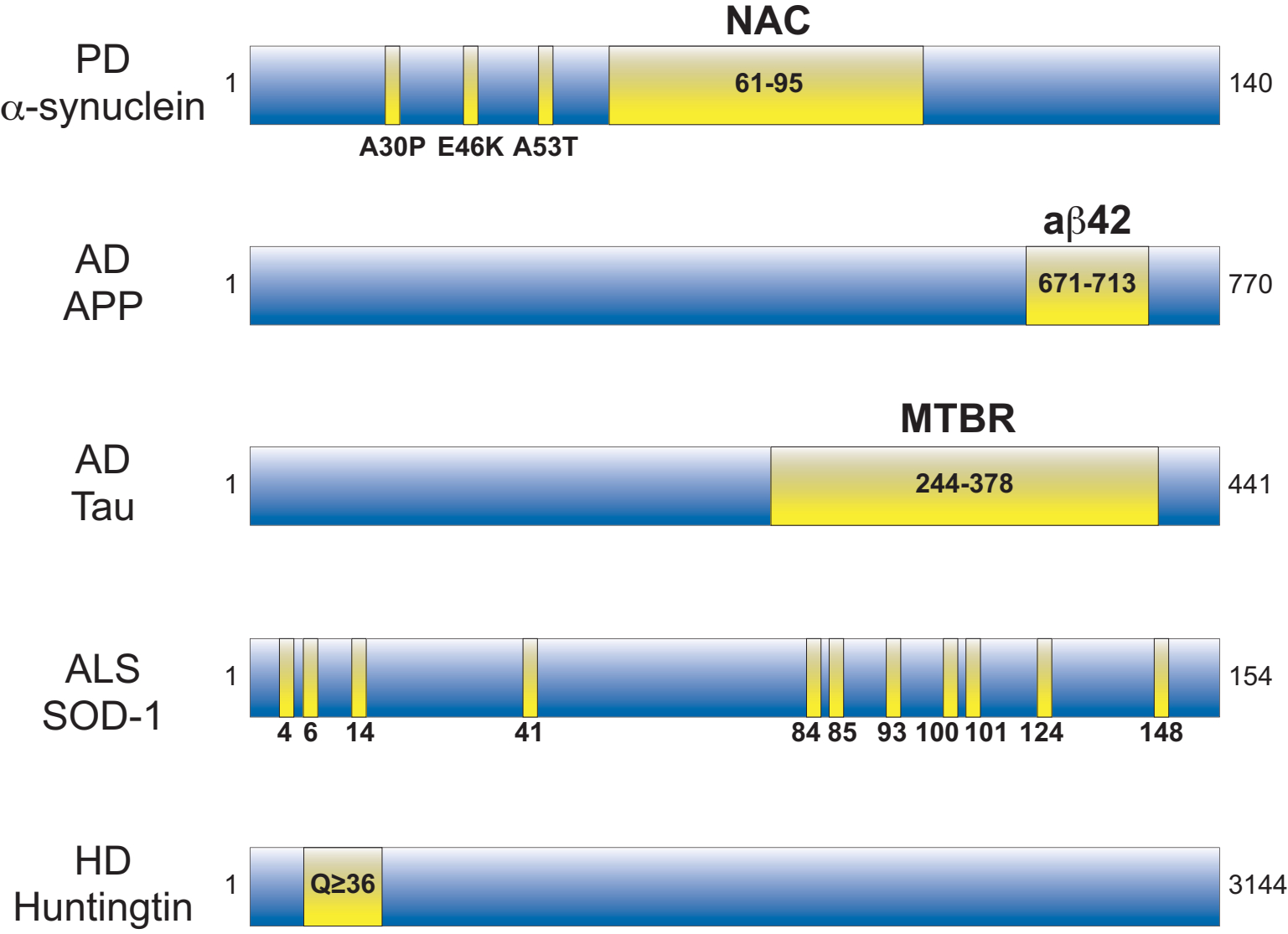


Figure 3. Schematic representation of the aggregation prone regions of neurodegenerative disease associated proteins.
Regions or mutations known to be involved in protein aggregation are highlighted for the five disease associated proteins discussed in this review. For tau, more than 30 mutations have been identified around the microtubule-binding region (MTBR) (Wolfe, 2009), while codons with mutations causing high to extreme aggregation are highlighted for SOD-1 (Prudencio et al., 2009). Proteins and highlighted regions are not drawn to scale.


NAC
PD
α-synuclein
1
61-95
140
A30P E46K A53T
aβ42
AD
APP
1
671-713
770
MTBR
AD
Tau
1
244-378
441
ALS
SOD-1
1
4 6 14 41 84 85 93 100 101 124 148
154
HD
Huntingtin
1
Q≥36
3144