

Review

Potential Modes of Intercellular Alpha-Synuclein Transmission

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Abstract: Intracellular aggregates of the alpha-synuclein protein result in cell loss and dysfunction in Parkinson's disease and atypical Parkinsonism, such as multiple system atrophy and dementia with Lewy bodies. Each of these neurodegenerative conditions, known collectively as alpha-synucleinopathies, may be characterized by a different suite of molecular triggers that initiate pathogenesis. The mechanisms whereby alpha-synuclein aggregates mediate cytotoxicity also remain to be fully elucidated. However, recent studies have implicated the cell-to-cell spread of alpha-synuclein as the major mode of disease propagation between brain regions during disease progression. Here, we review the current evidence for different modes of alpha-synuclein cellular release, movement and uptake, including exocytosis, exosomes, tunneling nanotubes, glymphatic flow and endocytosis. A more detailed understanding of the major modes by which alpha-synuclein pathology spreads throughout the brain may provide new targets for therapies that halt the progression of disease.

Keywords: alpha-synuclein; Parkinsons disease; dementia with Lewy bodies; multiple system atrophy; tunnelling nanotube; glymphatic system; cell-to-cell spread

1. Alpha synuclein

Alpha synuclein (α -syn) is a 140 amino acid, soluble protein found predominantly within the central nervous system (CNS), and is enriched in the peripheral nervous system and circulating erythrocytes (Barbour et al., 2008; Theillet et al., 2016; Weinreb et al., 1996). The normal role of α -syn remains unclear, however high concentrations of the protein exist within neuronal presynaptic terminals, indicating potential function(s) in synaptic transmission (Cheng, Vivacqua and Yu, 2011). Multiple specific roles have been proposed for α -syn, including SNARE protein regulation, production of dopamine and regulation of synaptic vesicle recycling (Burre et al. 2010; Perez et al., 2002; Larsen et al., 2006; Yavich et al., 2004; Yavich, Jäkälä and Tanila, 2006). Contention exists as to the normal cellular conformation of α -syn. Recombinant α -syn exists as an unfolded monomer, whereas recent evidence shows that extraction from eukaryotic sources, such as erythrocytes or human brain tissue, produced α -syn multimers, mostly tetramers, with α -helical content (Bartels, Choi and Selkoe, 2011; Jakes, Spillantini and Goedert, 1994; Luth et al., 2015). Burre et al and Theillet et al., provide evidence that α -syn normally exists largely as an unfolded monomer in the free cytosolic form whilst adopting an α -helical conformation once bound or interacting with lipid membranes (Burré et al., 2013; Theillet et al., 2016).

2. Alpha-Synuclein in Disease

Intracellular inclusion bodies comprised primarily of misfolded and aggregated α -syn are the neuropathological hallmark of a group of neurodegenerative diseases with overlapping complex clinical phenotypes. These are known collectively as α -synucleinopathies and include Parkinson's disease (PD), multiple system atrophy (MSA) and dementia with Lewy bodies (DLB) (McCann et al., 2014). PD patients develop characteristic motor dysfunction such as bradykinesia, tremor, rigidity and postural instability which are collectively known as Parkinsonism. As the disease progresses patients often develop depression, anxiety and dementia in advanced stages (Sveinbjornsdottir, 2016; Menza, Robertson-Hoffman and Bonapace, 1993). DLB patients present with Parkinsonism and cognitive impairment such as hallucinations, fluctuations in alertness and short-term memory with a relatively rapid progression and decline (McKeith et al., 2004). MSA is classified into two subcategories, based on α -syn distribution, pattern of spread, neurodegeneration and clinical phenotypes. MSA-P (Parkinson's) patients typically display a combination of parkinsonian-like symptoms, such as tremors and bradykinesia, whilst MSA-C (cerebellar ataxia) cases present with signs of ataxia and gait disturbance (Longo, Fanciulli and Wenning, 2015; Ubhi, Low and Masliah, 2011). Both forms also display autonomic dysfunction in addition to other symptoms. α -Synucleinopathies exhibit overlap with regard to brain regions affected, with all three disorders displaying neuronal loss, particularly of dopaminergic neurons, within the substantia nigra in the midbrain as well as the basal ganglia (Braak et al., 2003; Ubhi, Low and Masliah, 2011). Differences in disease duration also exist between the three diseases, with MSA patients surviving for around 8 years after symptoms appear, whilst PD and DLB typically survive more than 10 years (Marttila and Rhine, 1991; Wenning and Stefanova, 2009; Williams et al., 2006)

In addition to neuronal loss, α -synucleinopathies each display characteristic inclusions, with the predominant types in PD and DLB known as the Lewy body (LB) and the Lewy neurite (LN). LBs and LNs both share similarities, such as their composition of filamentous fibrillar α -syn, ubiquitin and neurofilament and are found in surviving neurons upon autopsy (Spillantini et al., 1997; Engelender, 2008, Braak et al., 1999; Galloway, Mulvihill and Perry, 1992). However, these inclusions have distinctly different morphologies, with LBs being largely spherical whilst LNs have rod- or snake-like morphologies (Braak et al., 1999). Work by Kanazawa et al., using 3D reconstruction of confocal images revealed that both LB and LN share the same structural components of an inner filamentous α -syn and ubiquitin layer coated by neurofilament (Kanazawa et al., 2008), also finding LB/LN intermediary like structures sharing the same characteristics. This suggests LNs may be an earlier LB stage, thus explaining the abundance of these inclusions together in affected brain regions. MSA inclusion bodies, termed glial cytoplasmic inclusions (GCIs), share similarities to LBs and LNs, containing filamentous α -syn and ubiquitin, however, also contain α B-crystallin and tau (Miki et al., 2010; Tu et al., 1998). GCIs have a triangular or sickle-like morphology (Miki et al., 2010; Grazia Spillantini et al., 1998) and occur predominantly in oligodendrocytes (Ahmed et al., 2012). Although oligodendrocyte lineages are known to express α -syn mRNA at low levels both normally and in MSA, α -syn is expressed primarily in neurons, implicating uptake as the main source of α -syn in GCIs (Asi et al., 2014; Djelloul et al., 2015).

Although the majority of cases of α -synucleinopathy are sporadic, rare missense mutations in the α -syn gene (SNCA) have been found to cause familial PD phenotypes and α -syn pathology with some variants also displaying pathology similar to MSA (Lesage et al., 2013; Polymeropoulos, 1997). Gene duplications and triplications have also been reported resulting in increased α -syn synthesis and PD phenotypes (Chartier-Harlin et al., 2004). Furthermore, genome wide association studies have highlighted SNCA as one of the strongest risk loci for developing PD, strengthening the link to α -syn as a causative agent in α -synucleinopathies (Simón-Sánchez et al., 2009; Nalls et al., 2014; Satake et al., 2009). The intracellular aggregation of α -syn can be triggered by a wide range of stimuli, including raised metal ion concentrations, oxidative stress and post-translational modifications, such as phosphorylation at Serine 129 and Serine 87 or other modifications such as nitration (Rcom et al, 2016; Fujiwara et al., 2002; Reynolds et al., 2007). Phosphorylation at Serine 129 can be associated with an insoluble, oligomeric β -sheet conformation and has been shown to be the

dominant modification in MSA and DLB (Cookson, 2009; Anderson et al., 2006)). Oligomeric α -syn is then thought to aggregate into fibrils, the characteristic form of α -syn observed in diseases such as PD. These fibrils together with a multitude of other proteins form the pathological inclusion bodies. The central hydrophobic region of α -syn termed non-A β Component (NAC), comprising residues 61-95, is the region susceptible to β -sheet formation (Ueda et al., 1993). It has been described recently that α -syn can form strains in vitro other than fibrils which display different binding, penetration and toxic characteristics as well as a different structure, such as α -syn ribbons (Bousset et al., 2013). Whilst ribbons have not yet been detected in humans, various strains of oligomers have been observed (Gai et al., 2003; Pountney et al., 2004). These different strain types may account for some of the differences observed in different α -synucleinopathies, such as inclusion body morphology or cell types affected.

3. Alpha-synuclein and disease spread

The current model of tissue spread of α -syn pathology, devised by Braak et al., in 2003 proposed that LB in sporadic PD originate within the olfactory bulb and medulla oblongata and later spread to the substantia nigra, midbrain and basal ganglia (Braak et al., 2003). In MSA-P, degeneration occurs within the nigrostriatal region due to appearance of α -syn in the substantia nigra and putamen, later spreading to the frontal cortex (Paviour et al., 2007). MSA-C has recently been proposed to have a 4-phase stage pathology, whereby it begins in the cerebellar subcortical white matter and olivo-cerebellar fibres within the medulla, later spreading to the basal ganglia, neocortex, amygdala and hippocampus (Brettschneider et al., 2016). A spatial relationship between GCIs and myelinated axons was also observed, revealing that GCIs occur largely in myelinating oligodendrocytes. Consensus guidelines of the DLB Consortium aim to classify DLB pathology via LB presence and density in specific brain regions in conjunction with the presence of Alzheimer's-like degeneration. Typical DLB classifications have intermediate to high LB load within the neocortical regions with an intermediate Alzheimer's-like degeneration (McKeith et al., 2005). The presence of Alzheimer's pathology is also part of the diagnostic criteria for DLB. Experiments with in-vivo animal models have revealed the cell-to-cell transmission of pathological α -syn. Different synthetic strains (oligomers, ribbons & fibrils) are observed to have variable pathogenicity and uptake when systemically or locally administered into mice (Peelaerts et al., 2015). Luk et al. (2012) showed that single intrastriatal inoculation of preformed fibrils into non-transgenic mice brains caused LB appearance and PD-like symptoms (Luk et al., 2012). Using longitudinal in vivo multiphoton imaging, Ostenberg et al. (2015) demonstrated that inoculation of synthetic α -syn fibrils induced aggregation, inclusion formation and subsequent death of a large proportion of neurons harboring inclusions (Osterberg et al., 2015). Other studies have shown similar results, with α -syn spread also found to be a potential factor leading to further inclusion body development (Paumier et al., 2015; Sacino et al., 2013; Rey et al., 2016).

Several recent studies have proposed pathological α -syn to behave in a prion-like manner. Prions are classified based on their transmissibility as well as their ability to interact with and convert healthy proteins (PrP^C) into the pathological form (PrP^{Sc}) (Kovacs and Budka, 2008; Goedert, 2015). Pathological α -syn displays many of these characteristics. The NAC region of α -syn contributes to stabilizing the β -sheet conformational change (Iwai, 2000; Theillet et al., 2016; Rodriguez et al., 2015). α -Syn fibrils have been shown to be insoluble, and persist in the extracellular environment, thus aiding transmissibility (Cookson, 2009). Additionally, recent work has shown that certain α -syn species can operate in a prion-like manner to auto-catalyze misfolding and aggregation, thereby resulting in neurotoxicity and neurodegeneration (Goedert, 2015). Radford et al. (2015) reported glial inclusion body formation in mice injected with dispersed GCI material. Recasens et al. (2014) showed that human LB enriched fractions were able to induce PD-like pathology in macaque monkeys (Recasens et al., 2014). Rey et al. (2016) also demonstrated trans-neuronal transport in mice injected with α -syn (Rey et al., 2016). Prusiner et al. (2015) discovered that MSA CNS homogenates can cause misfolding, inclusion formation and neurodegeneration in mice expressing the human familial PD α -syn mutant A53T, leading to MSA

being termed a prion-like disease (Prusiner et al., 2015). This discovery led to speculation that α -syn in MSA is of a different strain than that of PD or DLB due to the presentation of prion characteristics with the inoculation of MSA homogenates and the absence of these with PD and DLB homogenates. This may also explain the presence of inclusion bodies in and degeneration of oligodendrocytes observed in MSA. Curiously, aggregated α -syn was observed primarily in neurons rather than reproducing the mostly glial inclusions observed in MSA tissue (Cykowski et al., 2015). Interestingly, homogenates from PD and DLB patients failed to induce significant neurodegeneration or α -syn pathology in the same study. This raises the question of the identity of the pathogenic α -syn biochemical species in the different α -synucleinopathies and whether other proteins, in conjunction with α -syn are required for it to become prion-like.

Evidence for α -syn spread has also been observed in humans who received tissue grafts. Li et al. (2008) was the first reported case in which 2 subjects with PD developed LB pathology in grafted cells over a decade following the procedure (Li et al., 2008). Likewise Kordower et al. (2008) revealed a similar result, in which an individual developed LBs in engrafted striatal neurons 14 years following tissue graft (Kordower et al., 2008). Other works such as by Ahn et al. (2012) produced a similar report in which fetal grafts developed PD pathology in addition to gliosis (Ahn et al., 2012). These results give further credence to the proposition that pathological α -syn can transmit to healthy cells and promote further α -syn aggregation similar to prion proteins. These studies highlight the potential for extracellular α -syn to be taken up by CNS cells and cause neurodegeneration in a prion-like manner. Although routes of α -syn spread and uptake are still poorly understood, recent studies have begun to clarify how this may occur. The current review will discuss potential routes of pathological α -syn spread, including exosomal transport, tunneling nanotubes, microglia and the glymphatic system.

4. Extracellular α -syn, secretion and uptake

Several studies have demonstrated the existence of α -syn in the extracellular environment. El-Agnaf et al. (2006) demonstrated higher levels of oligomeric α -syn in the cerebrospinal fluid (CSF) and blood plasma of PD and DLB patients compared to healthy patients, suggesting that this may be due to release of the protein via cell death or other systems (El-Agnaf et al., 2006). Later works have shown trans-synaptic release of oligomeric α -syn at neuron terminals mediated by Hsp70, later spreading to neighboring neurons, following the Braak model of spread and possibly explaining the spread of LBs in grafted neurons (Danzer et al., 2011). This was expanded upon by Fontaine et al., showing that Hsp70 in conjunction with its co-chaperone DnaJ also regulates the release of α -syn from synapse (Fontaine et al., 2016). This suggests that systems exist within the CNS to control and combat formation and spread of pathological α -syn oligomers, and that these regulatory systems are overloaded or non-functional in α -synucleinopathies. Peelaerts et al. (2015) explored the efficiency of extracellular spread of different α -syn strains comprising of oligomers, fibrils and ribbons in vivo, revealing that oligomers spread most efficiently (Peelaerts et al., 2015). In addition, dopaminergic neurons were observed to take up and enact trans-synaptic transport of all α -syn types. Whilst free extracellular α -syn movement may explain neuron-neuron spread to neighboring neurons it cannot explain the occurrence of GCIs in MSA as oligodendrocytes are not connected to neuronal synapses or cases where α -syn pathology occurs in disconnected brain regions.

Neurons have another secretory mechanism at their disposal allowing for the release of cargo in the form of exosomes. Exosomes are 50-100nm vesicles that facilitate intercellular communication by transporting specific proteins (e.g. Heat shock proteins) or RNA (e.g. mRNA & miRNA) (Valadi et al., 2007; Gibbings et al., 2009; Lancaster and Febbraio, 2005). Composed of raft lipids such as cholesterol, exosome formation can occur in one of two ways. They can be directly shed from the plasma membrane, in which the cellular membrane forms a small vesicular bubble on its extracellular surface with the cargo of interest inside before budding off the main cell (Beaudoin and Grondin, 1991). Alternatively, exosomes can be formed inside the multi-vesicular body, which then fuses with the cell membrane in an exocytic fashion thereby releasing cargo vesicles into the extracellular environment (Denzer et al., 2000). An example of this is oligodendrocytes releasing

neuroprotective exosomes which support neuronal metabolism once internalized (Fitzner et al., 2011; Frühbeis et al., 2013; Frühbeis et al., 2013). This process is not exclusive to oligodendrocytes as astrocytes and neurons facilitate bi-directional transfer of mitochondria in order to support neuronal homeostasis via micro-vesicles (Davis et al., 2014; Hayakawa et al., 2016). Other actions of exosomes include toxic material export as well as their potential role as a mechanism of intercellular α -syn spread.

As discussed previously, the normal function of α -syn is thought to be related to vesicle recycling within the presynaptic terminal involving direct interaction with lipids, whereas, neuronal exosomes are produced via MVB-plasma membrane fusion or shedding of the neuronal membrane at the synapse (Von Bartheld and Altick, 2011). Work by Danzer et al., revealed that α -syn oligomers can utilize exosomes in cell lines as well as primary neuronal cells and that not only do host cells expel α -syn oligomers via exosomes, but that α -syn containing exosomes are more readily taken up via endocytosis than α -syn fibrils (Danzer et al., 2012). Oligomers occurred both on the inner and outer membrane of the expelled exosomes and dysfunction of autophagy reduced degradation of oligomers and increased exosomal release (Danzer et al., 2012; Alvarez-Erviti et al., 2011). Calcium has also been implicated in exosomal α -syn release in PD, with increased calcium leading to increased release of oligomer-containing exosomes (Emmanouilidou et al., 2010). As described previously, the glial α -syn deposits observed in MSA are likely derived from an external source. Selective accumulation of endogenous α -syn occurred in primary astrocyte/oligodendrocyte co-cultures (Radford et al., 2015) and cargo transport between neurons and oligodendrocytes and astrocytes has been demonstrated (Frohlich et al., 2014). α -syn was found in vesicular structures (40nm) in astrocytic endfeet in post-mortem MSA patient tissue with long disease duration, while α -syn pathology is observed both in astrocytes and Bergmann glia in α -synucleinopathies (Nakamura et al., 2016; Braak, Sastre and Del Tredici, 2007; Piao et al., 2003).

How α -syn enters target cells following exocytosis is still largely unknown. Uptake via clathrin-dynamin-1 mediated endocytosis has been shown to occur in neurons, oligodendrocytes and microglia *in vitro* (Figure 1). However, inhibition of this process in dynamin-deficient cells or blocking GTPases associated with this pathway did not completely inhibit α -syn entry (Reyes et al., 2014; Desplats et al., 2009; Liu et al., 2007; Lee et al., 2008; Konno et al., 2012). Recently Mao et al. (2016) discovered that lymphocyte activating gene-3 (LAG-3), a leukocyte immunoglobulin protein also found on the surface of neurons, may potentiate the entry of fibrillar α -syn via clathrin-dependent endocytosis (Mao et al., 2016). The results also show LAG-3 is an α -syn-specific binding site, with tau and amyloid proteins found to bind to it in a non-specific manner. Based on the work by Mao et al. (2016) LAG3 appears to be expressed exclusively on neurons in the brain, however this does not rule out the participation of similar immunoglobulin proteins on astrocytic, microglial or oligodendroglial surfaces. Recently TM9SF2, a nonspanin transmembrane protein found on endosomes, was implicated in α -syn spread and entry (Schimmöller et al., 1998; Wadman, 2016). As endosomes deliver molecules to the lysosome for degradation, endocytosis of α -syn oligomers may in turn provide a link to impaired lysosomal degradation. Indeed a variety of genetic factors linked to α -synucleinopathy are associated with the ineffective degradation of α -syn by lysosomes (Usenovic et al., 2012; Siebert, Sidransky and Westbroek, 2014).

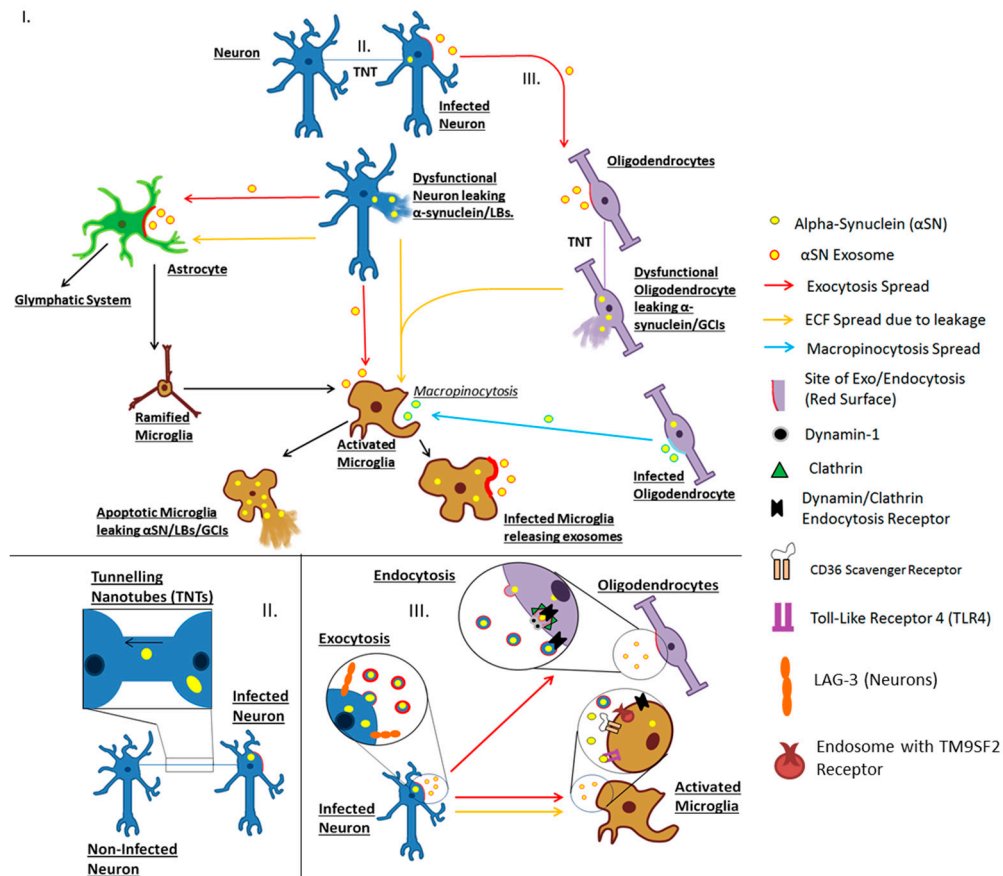


Figure 1. Hypothetical modes by which α -synuclein spreads in α -synucleinopathy disease. **I.** Release of α -synuclein/LBs via either exocytosis or membrane leakage due to apoptosis, necrosis or other damage. Astrocytes detect α -synuclein/LBs and signals for microglial recruitment by inflammatory factors. This also has the effect of activating microglia from the surveying ramified state to the phagocytic amoeboid phenotype (black arrow). Activation is also caused when ramified microglia detect α -synuclein either in exosomes or free floating in extra cellular matrix. Astrocytic activation can lead to dysregulation of glymphatic circulation via loss of aquaporin-4 polarization to endfeet, causing turbulent flow. Oligodendrocytes can take up α -synuclein containing exosomes from neurons via endocytosis. Oligodendrocytes transfer α -syn to other oligodendrocytes either via exocytosis or TNTs, eventually releasing α -synuclein/GCIs through apoptosis/necrosis for phagocytosis by microglia. Oligodendrocytes may also release α -syn in exosomes. Microglia can engulf exosomes via macropinocytosis. Microglia perform phagocytosis on free floating and exosome-associated α -synuclein. Microglia may then propagate spread either via exosomes or α -syn via cell death or membrane leakage. **II.** F-actin TNTs exchange material between cells. In this illustration, an aggregate-bearing neuron (right) is transferring α -synuclein/LBs to an adjacent neuron (left) via a thick TNT causing spread of the pathological protein/inclusion body. **III.** Transportation of α -synuclein via exo- and endocytosis. A neuron (blue), is undergoing exocytosis and the exosomes are travelling toward the oligodendrocyte (purple). Once close to the cell membrane of the oligodendrocyte, the exosomes may undergo different modes of endocytosis. One mode (top) involves fusing with the membrane of a target cell leading to transfer of cargo. An alternate mode involves dynamin and clathrin mediated endocytosis, whereby the exosome binds to the receptor (black) which triggers its entry. Clathrin (green triangles) surrounds the budding vesicle whilst dynamin (grey circles) pinches the membrane from the vesicle. This clathrin coated vesicle will then be fused with lysosomes for degradation. Microglia are shown activating clathrin-dynamin mediated endocytosis as well as microglial activation by CD36 scavenger receptor and TLRs. Neurons have LAG3 and TM9SF2 receptors on the surface which when bound by fibrillar α -syn, mediate clathrin dependent endocytosis.

Macropinocytosis is an actin-dependent mechanism whereby membrane ruffling allows entry of material into a variety of cell types *in vitro* and can be utilized by immune cells, such as microglia, to initiate lysosomal degradation. Heparan sulfate proteoglycans on the surface of cells have been shown to mediate the entry of α -syn fibrils via macropinocytosis (Holmes et al., 2013). Overall, current studies indicate that multiple mechanisms of exogenous α -syn cell entry exist. Further research is required to determine if other potential endocytic sites or receptors exist to facilitate α -syn entry into cells and whether therapeutic interventions can be developed to selectively inhibit uptake by healthy target cells.

5. Tunneling Nanotubes

Tunneling nanotubes (TNTs) are another mode of intercellular transfer, where F-actin interacts with the plasma membrane to form thin protrusions. Upon contacting and fusing with neighboring cells, TNTs function as a conduit to facilitate intercellular exchange and communication, with connections lasting up to several hours (Agnati and Fuxe, 2014; Gousset et al., 2009; Abounit et al., 2016). Currently, there are 2 classifications of TNTs described based on their length and thickness, each utilized by cells for different purposes. Thinner TNTs, composed solely of F-actin, are up to 15-60 μ m long with a diameter of 50-200nm thought to be used for bacterial and small molecule exchange (Gousset et al., 2009; Eugenin, Gaskill and Berman, 2009; Abounit et al., 2016). Thicker TNTs composed of microtubules in addition to F-actin, can have a length of 30-140 μ m with a diameter of >700nm accommodating the transportation of larger cargo, such as mitochondria, endosomes and vesicles (Eugenin, Gaskill and Berman, 2009; Abounit et al., 2016; Onfelt et al., 2004; Onfelt et al., 2006). TNTs usage have been between a variety of CNS cells such as neurons, astrocytes and dendritic cells *in vitro* (Zhu et al., 2005; Rustom et al., 2004; Onfelt et al., 2004). Sun et al., demonstrated TNTs between neurons and astrocytes, demonstrating that TNTs are not restricted to facilitating transport between cells of the same type (Sun et al., 2012).

There is evidence that pathogens, such as bacteria, HIV and the Prion Protein (PrP), have the ability to utilize TNTs to propagate into neighboring cells from an infected host *in vitro*, the spread of which could occur between cells of both similar and dissimilar cell type (Gousset et al., 2009; Eugenin, Gaskill and Berman, 2009). Recently, fibrillar α -syn was shown to be transported into lysosomes via TNTs in neuronal like cells *in vitro*. This fibrillar α -syn was found to induce the subsequent aggregation of cytosolic α -syn in the target cells (Abounit et al., 2016). As TNTs are able to facilitate material exchange between differing cell types, it may be possible that TNTs could also mediate spread of α -syn between different cell types in α -synucleinopathies, such as in MSA. Further investigation is required to elucidate the role of TNTs in intercellular α -syn transfer in α -synucleinopathies, although the small size and fragile nature of TNTs make their study *in vitro* and particularly *in vivo* technically challenging (McCoy-Simandle, Hanna and Cox, 2016).

6. Microglia

Microglia, the principal immune-phagocytic cells of the CNS, display some characteristics required by a potential mechanism of α -syn intercellular spread (Kettenmann et al., 2011). Under normal conditions, microglia exist in a resting state whereby they survey different brain regions for harmful or pathological molecules/proteins, detection of which causes a change from a resting to a phagocytic state (Jonas et al., 2012). This change is accompanied by a morphological change from ramified to amoebic, allowing microglia to perform phagocytosis for the breakdown of harmful agents (Jonas et al., 2012; Perry, Cunningham and Holmes, 2007). This activation is typically reinforced with release of pro-inflammatory factors, such as interleukin- β and tumor necrosis factor- α for the recruitment of additional microglia to the site to allow for effective clearance (Neumann, Kotter and Franklin, 2008).

Gliosis, the term used to describe activation of glial cells such as microglia and astrocytes, is a major pathological feature of MSA and to a lesser extent DLB and PD. Elevated microglial activation in areas corresponding to α -syn deposition and neurodegeneration has been observed in patients with PD, DLB and MSA via Positron Emission Tomography studies using a ligand to target

activated macrophages and has been replicated in neuropathological studies of α -synucleinopathies (Fellner and Stefanova, 2012; Politis, 2012). Whether this is just a correlate of neurodegeneration or actively contributes to disease pathogenesis is currently unknown, although there is some evidence to show that microglia may have an active role in α -synucleinopathies. Increased pro-inflammatory cytokines are observed in MSA cases (Kaufman et al., 2013; Wenning and Stefanova, 2009; Koga et al., 2015). Activation of microglia is observed in PD, however the inflammatory response is not as widespread as in MSA (Nagatsu and Sawada, 2005; Tufekci et al., 2012). Gliosis is also observed in DLB, and whilst gliosis also occurs in Alzheimer's disease, it can be distinguished based on areas affected (Higuchi et al., 2000). This difference in inflammatory profile may be related to different strains of α -syn as discussed earlier. Peelaerts et al., 2015 partially support this hypothesis, observing microglial activation to be most pronounced when exposed to fibrillar α -syn diffusing via the blood-brain barrier (Peelaerts et al., 2015).

Microglia have been observed *in vitro* to take up exosomes released by oligodendrocytes containing α -syn via macropinocytosis, but lack the ability to effectively degrade fibrillar α -syn (Fitzner et al., 2011). This inability to clear α -syn may be associated with impairment of the lysosomal pathway, the dominant mechanism associated with degradation after phagocytosis of α -syn and other pathological molecules. Previous studies have shown that lysosomal impairment is observed in PD, in both sporadic and familial cases, although this work has focused on neurons (Chu et al., 2009; Alvarez-Erviti et al., 2010). However, vaccination of transgenic mice was found to promote microglial clearance of α -syn (Masliah et al., 2005).

α -syn has also been found to utilize GM1 dependent lipid rafts by binding to the ganglioside to initiate receptor-mediated endocytosis in addition to macropinocytosis and clathrin-dynamin to enter microglia (Park et al., 2009; Liu et al., 2007; Lee et al., 2008). Other studies have shown that lipid rafts may mediate the localization of α -syn at the synapse (Fortin et al., 2004; Bar-On et al., 2008; Kubo et al., 2005). This may suggest another α -syn entrance pathway into cells in α -synucleinopathies relevant to neurons and microglial. α -Syn also functions as a chemo-attractant, recruiting microglia to damaged cells (Wang et al., 2015). Microglia are particularly sensitive to exogenous α -syn and it has been found to elicit pro-inflammatory and phagocytic responses *in vitro* via Toll-like receptor (TLR) dependent pathways, such as TLR4, in addition to scavenger receptors, like CD36 (Su et al., 2008; Stefanova et al., 2011; Kim et al., 2013; Roodveldt et al., 2013; Fellner et al., 2013; Tang and Le, 2015; Asai et al., 2015; Radford et al., 2015; Vieira et al., 2015). It is conceivable that α -syn could attract and activate microglia which fail to completely clear α -syn and potentially cause neurodegeneration themselves through an excessive pro-inflammatory response (Tang and Le, 2015).

Microglia have been shown to play an active role in the progression of multiple neurodegenerative diseases. Specifically, depleting microglia and blocking the synthesis of exosomes in mice has been shown to decrease tau deposition in nearby healthy cells (Asai et al., 2015). This suggests a potential synergy between microglia and exosomes in pathological spread in neurodegenerative conditions and is potentially an important mechanism of spread in α -synucleinopathies. In mice injected with pathological α -syn aggregates, microglial activation was observed to have spread further away from the site of injection over three weeks than the diffusion of α -syn aggregates (Radford et al., 2015). Whilst there is little literature regarding microglia and their use of TNTs, TNTs or other systems may be employed for intercellular communication. Macrophages have been shown to utilize both forms of TNT for material exchange and phagocytic clearance of bacterial species between themselves and other connected cells (Onfelt et al., 2004; Onfelt et al., 2006). Given the functional relationship between microglia and macrophages, it is possible that α -syn aggregates could use TNTs for intercellular spread to microglia. One important characteristic of microglial cells is their plasticity, allowing them to travel to distal brain regions. That plasticity, in combination with the inability of microglia to degrade pathological α -syn effectively, could make microglia an effective transport hub for α -syn during disease.

7. Glymphatic Clearance

There is evidence to suggest that the glymphatic system, facilitated by astrocytes, may also be involved as a mechanism of spread in α -synucleinopathies. The glymphatic system is analogous to the lymphatic system and operates to circulate nutrients and remove waste products via exchange of interstitial fluid with cerebrospinal fluid (CSF). CSF flows into the perivascular space before being exchanged with interstitial fluid in the brain parenchyma. This process is facilitated by bulk flow due to pressure differentials and aquaporin-4 transporters on the endfeet of astrocytes which enclose the perivascular space (Jessen et al., 2015). While α -syn clearance by the glymphatic system has yet to be determined, other aggregation-prone proteins, such as amyloid beta and tau, are cleared by the glymphatic system in models of Alzheimer's disease and Traumatic brain injury (Iliff et al., 2014; Iliff et al., 2012). Additionally, phosphorylated α -syn has been found in the endfeet of astrocytes in a subset of MSA patients upon autopsy. While a recent study showed negative correlation between α -syn deposition and astrocytic aquaporin-4 expression in the temporal neocortex of PD patients (Hoshi et al., 2016). This correlates with studies demonstrating that extracellular α -syn induces reactive astrogliosis (Radford et al., 2015). Lower levels of α -syn in the CSF of patients with α -synucleinopathies compared to neurologically normal and Alzheimer's patients has been observed which may indicate an impairment in α -syn clearance via the glymphatic system (Gao et al., 2015). Investigating the clearance of α -syn via the glymphatic system in various *in vivo* models of α -synucleinopathies, combined with new functional glymphatic neuroimaging techniques in patients will help to assess any contributions of the glymphatic system to the pathophysiology occurring in alpha-synucleinopathies (Lundgaard et al., 2016).

8. Summary and Concluding Remarks

Currently, exosome transport is the most widely investigated mode of α -syn spread. There are however still many factors relating to exosomal spread that are unknown, such as whether α -syn transport via exosomes is limited to neuron-neuron or if it is also utilized by other cell types. The multitude of routes of exosome-mediated cellular uptake renders therapeutic intervention a challenging target, whereby a candidate molecule would need to prevent entry via diverse mechanisms. Gaps in knowledge also exist regarding TNTs. Evidence of transportation has been shown to exist *in vitro*, however its role in α -synucleinopathies *in vivo* is still unclear. The role of the immune response of both astrocytes and microglia is also contentious whether this is primarily protective or degenerative. There is evidence of microglia propagating and enhancing the spread of tissue pathology in diseases such as Alzheimer's disease, however further work on microglia is required concerning α -synucleinopathies.

Although multiple potential modes exist of α -syn spread in the brain, the pattern of spread in α -synucleinopathies is consistent between individual cases. Currently, α -syn spread according to the Braak staging scheme is predicted to occur between anatomically connected regions, forming a pattern based on α -syn spreading largely to neighboring regions. Yet vehicles such as exosomes and microglia have capabilities as long distance transporters to allow for spread to distal regions. *In vivo*, each of the mechanisms would be expected to occur either in stages or in concert. For example, gliosis involving increased activation of microglia and astrocytes may impair processes such as the glymphatic system. Thus, interactions between different modes of α -syn spread will further complicate successful therapeutic intervention, as multiple pathways may need to be targeted. Overall, future studies will need to investigate approaches to inhibiting the various potentially important mechanisms of α -syn spread in disease models which may identify novel targets for α -synucleinopathy therapies.

Abbreviations:

α -syn- Alpha Synuclein

CNS- Central Nervous System

CSF- Cerebrospinal Fluid

DLB- Dementia with Lewy Bodies

GCI- Glial Cytoplasmic Inclusions

MSA- Multiple System Atrophy
 PD- Parkinson's disease
 TNTs- Tunneling Nanotubes
 TLR – Toll-Like Receptor
 LAG-3 – Lymphocyte Activating Gene 3
 LN – Lewy Neurite
 NAC - Non-A β Component of Alzheimer's Disease Amyloid

Acknowledgments: The authors gratefully acknowledge the financial assistance of the Menzies Health Institute Queensland and Macquarie University.

Conflicts of Interest: The authors declare no conflict of interest.

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