Dissecting the potential molecular mechanisms underlying α-synuclein cell-to-cell transfer in Parkinson's disease

Elodie Angot and Patrik Brundin*

Neuronal Survival Unit, Wallenberg Neuroscience Center, Lund University, BMC A10, 221 84 Lund, Sweden

1. Parkinson's disease is a synucleinopathy

In PD, abnormal cytoplasmic protein inclusions develop in neurons indicating that, akin to several other neurodegenerative diseases, it is a proteinopathy. α-Syn, a 14 kDa protein, is the main component of these proteinaceous aggregates. They are named Lewy bodies (LB) or Lewy neurites (LN), if they are localized to the cell body or the processes, respectively. The α-syn is misfolded and assembled in aggregates in the LBs and LNs. Several lines of evidence support the notion that α-syn is central to the pathogenesis of PD and related "synucleinopathies". For example, three missense mutations in the α-syn gene [1] cause autosomal-dominant PD and di- and triplications [2] of the gene have been identified in families affected by parkinsonism syndromes.

2. Growing evidence for α-synuclein cell-to-cell transfer in Parkinson's disease progression

Due to the clear relevance of α-syn to PD pathogenesis, the spatio-temporal pattern by which LBs and LNs appear in the central nervous system is of great interest. Braak and collaborators have studied this in detail. They correlated the extent of the nervous system that is affected by LNs and LBs with clinical symptoms and proposed 6 neuropathological stages of PD [3,4]. During the early stages of the disease, called "presymptomatic phase" due to the absence of any motor disability, PD patients develop non-motor deficits, including olfaction impairment, vagal dysfunction and sleep disorder. Typically, motor symptoms (e.g. akinesia, rigidity and tremor) appear later, and cognitive functions decline in even more advanced stages. According to Braak's staging, the progression of symptoms could be due to spreading of aggregated α-syn along unmyelinated or poorly myelinated axonal pathways. Braak postulates that unknown events, e.g. viral infections, initiate the process of α-syn misfolding in the intestine and the olfactory system. According to Braak's suggestions, the misfolded protein then spreads from enteric nerves to the brainstem via the vagal nerve, and simultaneously to brain regions connected to the olfactory system. Several years after the initial trigger, the Lewy pathology reaches the midbrain, including the substantia nigra, and then slowly spreads to neocortical areas [5]. Misfolded α-syn-containing fibrils have been shown to cause aggregation of soluble, natively unfolded α-syn in the test tube [6]. Moreover, as mentioned earlier α-syn is the main protein component of LBs and LNs. Consequently, a "prion-like" propagation of misfolded α-syn has been suggested [7] within the brains of PD patients. Prion disorders, e.g. Creutzfeldt-Jacob disease, are transmissible neurodegenerative diseases. The pathogenic abnormal isoform of the prion protein (PrP*) is known to be the infectious agent that propagates itself by imposing its conformation onto the cellular prion protein (PrP) of the host [8]. The overriding idea is that misfolded α-syn (in analogy to PrP*) is transmitted from a LB-loaded donor cell to a "healthy" recipient cell and acts as a template for conversion of native, unfolded α-syn (in analogy to PrP) in the recipient cell into a β-sheet conformation. This process has...
3. Possible molecular mechanisms underlying cell-to-cell transfer of α-synuclein aggregates

Assuming that LB propagation within the central nervous system relies on direct cell-to-cell transmission, this process is likely to occur in three consecutive steps (Fig. 1). First, the release of misfolded α-syn from donor cells into the extracellular space. Second, recipient cells take up α-syn. Third, the misfolded α-syn template recruits native, unfolded α-syn from the cytosol of the recipient cell, giving rise to nucleation of pathogenic aggregates and eventually the formation of LBs.

3.1. Release of α-syn

Cells can clearly release α-syn into their surroundings. Low nanomolar concentrations of extracellular monomeric and oligomeric α-syn have been detected in cerebrospinal fluid (CSF) and plasma samples from both PD patients and controls by immunoprecipitation coupled to Western Blot analyses and ELISA methods [12]. Lee and collaborators have implicated exocytosis as a mechanism of α-syn release from cultured neuroblastoma cells overexpressing α-syn [13]. They found the release to be blocked by lowering the temperature, a commonly used approach to inhibit exocytosis. α-Syn has also been detected in the culture medium of rat primary cortical neurons [13], suggesting that α-syn release from neuroblastoma cells is not an artifact due to the overexpression of the protein. The observation that α-syn is present in the lumen of vesicles prepared from rat brain homogenates, rat embryonic cortical neurons and human neuroblastoma cells further supports the notion that vesicle-mediated exocytosis of α-syn can take place. These results were unexpected because α-syn lacks a signal peptide sequence. Interestingly, brefeldin A – a classical inhibitor of endoplasmic reticulum/Golgi-dependent secretion – is ineffective at blocking α-syn release [13], which indicates that α-syn exocytosis relies on an unconventional secretory pathway. Interestingly, intravesicular α-syn appears to be more prone to aggregation than the cytosolic protein [13]. Consequently, one can hypothesize that if vesicular α-syn that has undergone exocytosis succeeds in penetrating a recipient cell, it may be particularly effective as a seed that can initiate protein aggregation. Of course, in addition to exocytosis, release of pathogenic misfolded α-syn from dying cells could also contribute to the extracellular pool of α-syn in the brain.

3.2. Entry of α-synuclein into cells

Whatever the mechanism involved in α-syn secretion, according to the direct cell-to-cell transmission model, extracellular α-syn has to gain access to the interior of the neighbouring cells in order to trigger a subsequent nucleation step. Monomeric α-syn can directly translocate through the plasma membrane of living cells [14]. Conserved 11-amino acid imperfect repeats in the α-syn sequence play a critical role in this translocation [14]. This is a rapid process that can be monitored within 5 min of cells being exposed to monomeric α-syn. It is neither sensitive to low temperature nor inhibited by cytochalasin D and brefeldin A, two classical inhibitors of endocytosis [14]. While passive membrane translocation may account for monomeric α-syn penetration into cells, a different mechanism is likely to operate when α-syn species with higher...
molecular weight enter cells. Exogenous fibrillar α-syn, prepared from recombinant human protein, is internalized by differentiated human neuroblastoma cells [15]. This uptake is slower and reaches a maximum level after more than 24h. It is significantly decreased at low temperature and when neuroblastoma cells are transfected with a dominant-negative mutant dynamin-1, suggesting an endocytosis-mediated pathway [15]. Most of the fibrillar α-syn in the neuroblastoma cells is detected in the vesicle fraction [15], which is consistent with endocytosis-mediated uptake. Finally, fibrillar α-syn internalization is inhibited by cells treatment with a non-specific protease [15]. This suggests that binding of α-syn to an unidentified receptor could be involved in this endocytic uptake. Similar results have been shown for oligomeric α-syn. These findings are in agreement with studies describing that rat neuronal progenitors can perform Rab5A-dependent endocytosis of α-syn [16]. In these experiments, exogenously added recombinant α-syn was detected in the cytosol of the progenitors. The cells exhibited microvilli and endocytic vesicles 30 min after α-syn stimulation. The α-syn that was internalized colocalized with GTP-binding Rab5A protein. Overexpression of a dominant-negative Rab5A was found to decrease α-syn endocytosis [16]. Interestingly, the α-syn that had undergone endocytosis formed intracytoplasmic granular deposits containing ubiquitin, synaptophysin and Tau [16], which all are constituents of LB in human synucleopathies. As rat neuronal progenitor cells do not express α-syn, these aggregates were derived entirely from exogenous α-syn. Consequently, it appears that α-syn is prone to misfolding and aggregation when internalized into cells in endocytic vesicles.

Exocytosis/endocytosis and passive membrane translocation are classical mechanisms for the transfer of proteins between cells. Regarding intercellular transmission of proteins in neurodegenerative proteinopathies, other less conventional processes have been suggested to contribute. For example, exosomes have been proposed to play a role in the spreading of disease-related proteins within the brain. Specifically, studies have suggested that exosomes may play a role in the spreading of PrPSc (in prion disease) and β-amyloid peptide (Aβ) that aggregate in extracellular deposits, named amyloid plaques, in Alzheimer’s disease (AD). Exosomes are small membrane vesicles derived from endocytic pathway and released from cells into the surrounding environment. They have been suggested to fuse with the outer membrane of recipient cells and deliver their exosomal content in the cytoplasm [17]. A wide range of cells secretes exosomes in vitro, including neurons and astrocytes [18]. In 2007, Vella and collaborators demonstrated that both PrPc and PrPSc are released in exosomes from a neuronal cell line. Moreover, exosomes derived from prion-infected neuronal cells initiated prion propagation in vitro in uninfected recipient cells and in vivo after inoculation in the left parietal region of healthy mice [18]. Moreover, a minute fraction of extracellular Aβ and released by neuroblastoma cells expressing a mutant for amyloid precursor protein (APP), has been detected in association with exosomes [19]. APP and some of its proteolytic fragments are also present within exosomes from differentiated neuroblastoma and primary neuronal cortical cells [20]. Whether α-syn can be associated with exosomes remains to be investigated, but its presence in biological fluids, as CSF and plasma, where exosomes have been identified [17], makes it a viable hypothesis.

Tunnelling nanotubes (TNTs) have also been proposed to be involved in intercellular propagation of PrPSc [21]. TNTs are actin-containing membrane bridges between cells, that allow intercellular vesicle transfer [22]. Actin-mediated motors have been suggested to play underpin this transport. Transfer of endogenous and exogenous PrPSc has been reported to take place via TNTs between infected and naive mouse neuronal cells. Strikingly, interconnection between infected and naive cells through TNTs is required for PrPSc propagation in this model, strongly suggesting that TNT-mediated transport of PrPSc is an important mechanism in PrPSc spread between neurons. Furthermore, PrPSc transfer has also been observed between bone-marrow dendritic cells and primary neurons linked by TNTs. These observations suggest that TNTs could be involved in PrPSc propagation both between neurons in the brain and from the lymphoid system to the peripheral nervous system. TNTs have not yet been implicated in other neurodegenerative diseases, but studies of TNTs in the spreading of other misfolded pathogenic proteins, e.g. α-syn, could lead to interesting findings.

3.3. Permissive templating and seeding

To fulfil the requirements for a prion-like propagation model, internalized α-syn template must initiate the nucleation process by converting α-syn derived from the recipient cell into misfolded pathogenic form. Already in 1999, Wood and collaborators studied α-syn aggregation in bacteria and suggested that it may be “a nucleation-dependent process that can be seeded by aggregated α-syn functioning as nuclei” [6]. Danzer and collaborators recently suggested that α-syn can trigger a seeding process in cultured primary cortical neurons exposed to a specific type of α-syn oligomers [23,24]. They reported that the distribution of cytoplasmic α-syn changed from homogenous to punctuate, once cells had been invaded by these α-syn oligomers.

4. Monomers, oligomers, fibrils: Which one is the best “seeder”?

The association of two α-syn monomers into one dimer is the initial step in α-syn aggregation. This can eventually lead to the formation of insoluble fibrillar deposits, via soluble oligomers and protofibrils. All these α-syn species coexist in a highly dynamic equilibrium. Depending on its aggregation state, α-syn is likely to display different levels of seeding activity. By analogy, other biological activities of α-syn are highly dependent on its aggregation state. For example, a body of evidence suggests that soluble oligomers, rather than final insoluble fibrils, are the toxic species [25,26]. In an effort to provide in vivo data, a recent study designed α-syn variants to have a reduced propensity to fibrillize. Indeed, these α-syn variants form increased amounts of soluble oligomers and induce more neurotoxicity in worms and flies than wild-type protein [27]. Regulation of microglial phagocytosis is another illustration of divergence in biological function between different α-syn species: Extracellular monomeric α-syn is reported to increase microglial phagocytosis while aggregated α-syn inhibits this process [28]. For α-syn to be a good “seeder”, it must readily be released from donor cells, effectively internalized by recipient cells and prone to initiate the nucleation process. The nature of the α-syn species released from human neuroblastoma cells by exocytosis depends on the level of α-syn expression [13]. Thus, only monomeric forms are secreted when the expression level is low, whereas both monomers and aggregates are released when the cells express high amounts of α-syn. However, the physiological relevance of these findings remains to be demonstrated. The observations that monomeric and oligomeric α-syn can be detected in human cerebrospinal fluid and plasma [12] also argues for the idea that small α-syn assemblies are released into the extracellular space.

Following release into the extracellular space, α-syn must be internalized if it is to act as an efficient seed. A recent study examined cellular mechanisms of uptake of different α-syn forms: Monomers can directly cross the plasma membrane, while fibrils and oligomers may be internalized through an endocytic pathway [15]. This divergence in internalization process for monomeric and aggregated forms has also been documented for other “seeding-competent” proteins related to neurodegenerative
diseases. Comparisons between these proteins and α-syn may be very relevant. Tau is another such "aggregation-prone" protein. It makes up filamentous intracellular inclusions in several neurodegenerative diseases classified as tauopathies, including AD and frontotemporal dementia [29]. During AD progression, hyperphosphorylated Tau inclusions first appear in entorhinal cortex neurons, and later reach hippocampus and neocortex [30]. The identification of mutations in tau gene that cause familial forms of frontotemporal dementia [31] has shown that tau protein dysfunction is sufficient to induce neurodegeneration. Extracellular Tau aggregates, but not monomers, have been found to be internalized by cultured murine neuronal precursor cells [32]. An endocytosis-mediated pathway is likely to be involved as the Tau aggregates that are taken up from the culture medium are colocalised with dextran, a classical marker for endocytosis. Interestingly, these imported Tau aggregates can trigger fibrillization of intracellular Tau. Moreover, Tau aggregates generated within cultured cells have been shown to transfer to other cells within the same culture dish [32]. In a similar vein, α-syn oligomers, but not fibrils, are internalized by human neuroblastoma cells. Inhibition of endocytosis not only inhibits the entry of α-syn oligomers into the cells, but also significantly decreases α-syn oligomer-associated cell death [33].

Not only does the precise form which the α-syn has adopted play a role for its ability to enter into cells, but it also appears to affect its seeding activity. Danzer and collaborators generated three different heterogeneous preparations of oligomers [23]. They characterized their propensity to trigger nucleation when added to cultures of human neuroblastoma cells overexpressing mutant α-syn. Whereas the first preparation did not induce aggregation, treatment with the second α-syn oligomer preparation led to some formation of aggregates containing both exogenous and mutated α-syn inside cells. The third type of oligomers caused extensive aggregation, and the aggregates contained both exogenously added α-syn and α-syn derived from the cultured neurons [24]. A similar link between oligomer structure (di- versus tetramer) and nucleation-inducing property has been recently dissected at the molecular level for α-syn [34]. The nucleation rate increases with the number of monomers present in the α-syn assembly. These findings appear highly relevant to in vivo experiments discussed further below, reporting different patterns of induced amyloidosis after injection of α-syn–containing brain extracts from two different transgenic mice [35]. These structure-based differences in seeding activity could be another feature shared by neurodegenerative diseases-related proteins and prion proteins, whose strain-dependent infectivity is known to correlate with distinct conformations [36].

5. Is protein seeding a common mechanism for neurodegenerative diseases?

The molecular mechanisms involved in nucleation triggered by an exogenously added protein have also been studied in some detail for proteins containing an expanded polyglutamine (polyQ) stretch. Expansion of CAG repeats in huntingtin (Htt) gene leads to the dominantly inherited neurodegenerative disorder Huntington’s disease. The polyQ tract in mutant Htt leads to generation of cytoplasmic and intranuclear protein inclusions. Ren and collaborators recently reported that fibrillar polyQ peptide aggregates, exogenously added to the culture medium of mammalian cells expressing exon 1 Htt with an expanded polyQ tract, are internalized in cells by direct penetration of the outer membrane. Once inside, these PolyQ aggregates induce aggregation of the endogenously expressed exon 1 Htt protein in the cytoplasm. Furthermore, the acquired aggregation pattern persists in subsequent generations of dividing cells, suggesting that the induced aggregates are themselves able to act as seeds when split into the cytoplasm of two daughter cells.

Further evidence that a seeding-nucleation process can contribute to the spreading of neuropathology in a slowly progressing neurodegenerative disease has been obtained in AD models. Specifically, the seeding activity of Aβ [35,37] and Tau [38] has been demonstrated in cell and animal models. Meyer-Luehmann and collaborators used a transgenic APP mouse line that normally develops β-amyloidosis in the hippocampus around 10 months of age. After intrahippocampal injection of brain extracts from AD patients or old APP transgenic mice, amyloid deposits developed in young APP transgenic hosts. Interestingly, these were not limited to the hippocampus, but also progressively developed at some distance from the injections in the entorhinal cortex and the corpus callosum [35]. Different patterns of amyloid deposition are observed after injection of brain extracts from two different transgenic mice: compact and punctate Aβ lesions for one and more diffuse and filamentous deposits for the other [35]. Minute amounts of Aβ-containing extract are sufficient to induce amyloid deposition [37]. Similar results have been reported for tauopathies. Mice expressing the human wild-type Tau protein have received intracerebral injections of brain extracts from other transgenic mice, carrying a pathogenic mutation of human tau gene. As soon as 6 months after injection, the hosts, that normally do not develop any tauopathy, display filamentous inclusions of wild-type human Tau that spread from the injection site to further brain regions, in a time-dependent manner [38].

6. Concluding remarks

The data presented in this review support the hypothesis that cell-to-cell transfer coupled to a seeding activity is a common pathogenic mechanism for several neurodegenerative diseases. Molecules that can interfere with this process (without disrupting vital cell functions) either at the level of exit from the donor cell or at the level of entry into the recipient cell or even targeting the seeding step, could open doors to new disease-modifying therapies. These could have a tremendous impact on the lives of patients who currently have to rely on symptomatic therapies that, at best, give partial relief without affecting the inexorable deterioration of brain functions.

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Conflict of interests

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