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REVIEW ARTICLE

The cell biology of prion-like spread of protein aggregates: mechanisms and implication in neurodegeneration

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The misfolding and aggregation of specific proteins is a common hallmark of many neurodegenerative disorders, including highly prevalent illnesses such as Alzheimer's and Parkinson's diseases, as well as rarer disorders such as Huntington's and prion diseases. Among these, only prion diseases are 'infectious'. By seeding misfolding of the PrP^{C} (normal conformer prion protein) into PrP^{Sc} (abnormal disease-specific conformation of prion protein), prions spread from the periphery of the body to the central nervous system and can also be transmitted between individuals of the same or different species. However, recent exciting data suggest that the transmissibility of misfolded proteins within the brain is a property that goes way beyond the rare prion diseases. Evidence indicates that non-prion aggregates [tau, α-syn (α-synuclein), Aβ (amyloid-β) and Htt (huntingtin) aggregates] can also move between cells and seed the misfolding of their normal conformers. These findings have enormous implications. On the one hand they question the therapeutical use of transplants, and on the other they indicate that it may be possible to bring these diseases to an early arrest by preventing cell-to-cell transmission. To better understand the prion-like spread of these protein aggregates it is essential to identify the underlying cellular and molecular factors. In the present review we analyse and discuss the evidence supporting prion-like spreading of amyloidogenic proteins, especially focusing on the cellular and molecular mechanisms and their significance.

Key words: amyloid-β, cell-to-cell transfer, huntingtin, prion-like, α-synuclein, tau.

INTRODUCTION

The pathogenesis of most neurodegenerative diseases is linked to the deposition of protein aggregates in the affected brains. In each disorder, aggregates result from the misfolding of specific proteins, for example, Aβ (amyloid-β) and tau in AD (Alzheimer's disease), α-syn (α-synuclein) in PD (Parkinson's disease), Htt (huntingtin) in HD (Huntington's disease) and the PrP^{C} (normal conformer prion protein) in prion disorders. These disease-associated proteins are very diverse in their primary sequences; however, when aggregated, they all share a similar tertiary structure (known as cross-β spine or amyloid), consisting of an ordered arrangement of β-sheets [1,2].

Although these different disorders seem to be unlinked, they all appear to arise from a general mechanism that involves the abnormal folding and aggregation of various proteins and therefore have been classed in a group of more than 20 disease-related amyloids called PMDs (protein misfolding disorders). Among PMDs, the best characterized are TSEs (transmissible spongiform encephalopathies), or prion diseases, in which the causative role for the accumulation of misfolded protein aggregates is well established. However, differently from all other diseases of this type, prion diseases are 'infectious'. The infectious agent, termed prion, is mainly composed of PrP^{Sc} (abnormal disease-specific conformation of prion protein) and is transmitted by the autocatalytic conversion of the natively folded prion protein (PrP^{C}) induced by the misfolded version of the protein (PrP^{Sc}) (seeding process). In this manner prions spread from the periphery of the body to the CNS (central nervous system) similarly to an infectious agent and in the same way can also be transmitted between individuals of the same or different species, as in the case of BSE [bovine spongiform encephalopathy; which in humans is caused by a variant of CJD (Creutzfeldt–Jakob disease)] [3,4]

Until recently, the spreading and transmission of disease by propagation of protein misfolding was thought to be peculiar to the prion protein. However, a series of recent and exciting studies has shown experimental evidence that 'prion-like' mechanisms underlie the pathological spreading of misfolded proteins that is observed in various neurodegenerative diseases [5,6]. The implications of these findings are extensive. On the one hand they question the use of transplants as a therapeutical approach, and on the other they indicate that it may be possible to bring prion as well as AD and PD to an abrupt and early halt by preventing cell-to-cell transmission.

In the present review, we focus on the evidence indicating the intercellular transfer of 'non-prion' protein aggregates, focusing on three major neurodegenerative diseases: AD, PD and HD. We highlight the mechanisms of cell-to-cell transfer based on current knowledge and discuss the possible implications in disease progression.

Abbreviations used: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; BiFC, bimolecular fluorescence complementation; CJD, Creutzfeldt–Jakob disease; CNS, central nervous system; COPII, coat protein complex II; CSF, cerebrospinal fluid; CSPα, cysteine-string protein-α; EE, early endosome; EEA1, EE antigen 1; ER, endoplasmic reticulum; ERC, recycling endosome; ESCRT, endosomal sorting complex required for transport; GFP, green fluorescent protein; HD, Huntington's disease; HEK, human embryonic kidney; Hsp, heat-shock protein; Htt, huntingtin; ILV, intraluminal vesicle; Lamp-1, lysosomal-associated membrane protein 1; LDCV, large dense-core vesicle; MVB, multivesicular body; NFT, neurofibrillary tangle; PD, Parkinson's disease; PK, proteinase K; PMD, protein misfolding disorder; polyQ, polyglutamine; PrP^{C}, normal conformer prion protein; PrP^{Sc}, abnormal disease-specific conformation of prion protein; α-syn, α-synuclein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; TNT, tunnelling nanotube; TSE, transmissible spongiform encephalopathy; VPS4A, vacuolar protein sorting-associated protein 4A.

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PRIONS, THE PRION PHENOMENON AND NON-PRION AMYLOIDS

TSEs, also known as prion diseases, are fatal neurodegenerative disorders present both in human and animals [7]. Like most neurodegenerative diseases, prion disorders are mainly sporadic with a small percentage being inherited [8,9]. Different from other neurodegenerative disorders, prion diseases are infectious because transmission of the pathology occurs between individuals and across species through exposure in the natural environment (e.g. variant CJD is transmitted from cows to humans through the ingestion of contaminated food) [3]. It is now widely accepted that the infectious agent consists of proteinaceous aggregates, called 'prions', that derive from the conformational change of the native PrP^{C} into its pathological counterpart, PrP^{Sc} (scrapie prion protein) [7]. The molecular mechanism underlying prion infectivity is the ability of prions to self-propagate via PrP^{Sc}-templated conversion of endogenous PrP^{C} molecules, a process known as prion replication [7]. Furthermore, PrP^{Sc} aggregates are able to transfer from one cell to another and once in the receiving cell they further propagate by acting as new conversion units or 'seeds', thus resulting in the transfer and spreading of the infection [10,11].

With the exception of AA amyloidosis (a form of systemic amyloidosis, a long-term complication of several chronic inflammatory disorders, and shown to be transmitted through the faeces among cheetahs) [12], the interorganism spread of non-prion misfolding diseases has not been observed [13]. Thus, although protein misfolding and aggregation are common features of neurodegenerative diseases, the concept of spreading and infectivity of aggregates in the CNS was confined to prion diseases [6,14]. However, evidence has suggested the unexpected possibility that intercellular 'prion-like' spreading mechanisms may be responsible for the propagation of protein misfolding in non-prion neurodegenerative disorders, involving both secreted proteins such as Aβ and cytosolic proteins such as tau, Htt and α-syn [6,13–18]. Conformational diversity and template conformation have been encountered in AD, PD and HD where they precede the formation of different Aβ, tau, α-syn and Htt conformers [19–22] (for a review see [23]). Like in prion diseases, misfolded forms of these disease-associated proteins can be transmitted experimentally in cellular and in animal models [24], where they can act as seeds to recruit the endogenous protein into aggregates [13–15,25–27]. Therefore, even if most neurodegenerative diseases are not transmitted from one individual to another like 'true prions', they might propagate in an analogous way within a single organism.

Of specific interest in the present review is the fact that many of these protein misfolding diseases initiate in a circumscribed area of the brain that is characteristic for each disease, and pathology progresses in a topographically predictable manner following anatomical connections [28] (Figure 1, upper panel). The classical explanation for these findings is that some brain areas are more resilient than others and resist the disease longer (cell-autonomous disease) [29–31] (Figure 1). However, evidence indicates that both in the case of α-syn in PD and of Aβ and tau in AD, the misfolded aggregates can be transmitted from neuron to neuron in a prion-like manner perhaps along neuronal paths, suggesting a non-cell-autonomous mechanism of disease progression [6,16,29,32] (Figure 1).

PATTERNS OF NEUROPATHOLOGY SPREAD

In the early stages of all major neurodegenerative diseases, the pathology, including protein aggregation and neurological dysfunction, is localized to a confined region of the nervous system, whereas in the later stages it affects other more distant areas of the brain. The pathological changes seem to progress in a systematic manner following predictable anatomical pathways that are specific for each disorder, suggesting a progressive spread of the disease throughout the nervous system (Figure 1, upper panel).

The characteristic progression of disease in brain tissue was first shown in the case of AD and PD by the work of Braak and colleagues on post-mortem tissues from affected individuals and control subjects [28,33,34]. Interestingly, in the case of AD the distribution of neuritic plaques does not seem to follow anatomical patterns and varies from one individual to another (see also below), whereas neurofibrillary tau tangles have a characteristic and progressive distribution pattern permitting the differentiation of six stages in the disease [28]. In the case of idiopathic PD, Braak and co-workers proposed that during disease progression aggregation of α-syn in the characteristic Lewy body structures [35] spreads in a systemic manner from the peripheral (or enteric) nervous system, or alternatively from the olfactory bulb to specific brain regions and then follows a defined pattern in the brain [33,34] (Figure 1, upper panel). Similarly, HD brain imaging studies have shown that cortical degeneration follows a topologically predictable pattern [36] and precedes degeneration in the striatum [6,37]. Furthermore, striatal projecting neurons are among the first to be affected [38] and within the striatum degeneration progresses following defined anatomical directions [37] (Figure 1, upper panel). Of interest, inclusions containing aggregated Htt are present in the regions of the brain that degenerate; however, the presence of visible aggregate does not always correlate with cell death [39] (see also below).

In prion diseases the initial site of propagation may occur outside the CNS [3]. However, as mentioned above, prions can disseminate beyond the tissue where they are produced and spread through the entire organism in a peripheral and systemic transmission via cells of the immune system, peripheral nerves and bloodstream [40]. Remarkably, most of the disease-associated misfolded proteins are found circulating in the CSF (cerebrospinal fluid) and plasma, which could facilitate their spread through the body, as has been reported for Aβ [41] (reviewed in [15]), α-syn [42–44], Htt [45] and AA amyloid [46]. Taken together, the above clinical findings suggest the possibility that 'the pathological triggers' underlying different neurodegenerative diseases could transfer from one neuron to another, thus contributing to the anatomical spreading of the pathology in the healthy tissue in a prion-like and non-cell-autonomous manner (Figure 1, lower panel). Recent experimental findings and clinical observations have supported this hypothesis by showing that in non-transmissible neurodegenerative disorders misfolded proteins can transfer between cells [6,16,29,32].

In the next two sections of the present review we will present and discuss the specific evidence supporting the transfer of different conformers of Aβ, tau, α-syn and Htt at cellular and molecular levels.

THE CELL: EVIDENCE OF INTERCELLULAR TRANSFER OF AMYLOID AGGREGATES

Misfolded protein aggregates can be considered as 'infectious' if they fulfil two conditions: (i) they can propagate from one cell to another and (ii) once in the recipient cell, they act as a seed to initiate aggregate formation by recruiting unfolded or oligomeric species of the same protein in a prion-like mechanism. Below we analyse the evidence supporting the intercellular transfer of α-syn in PD, of Aβ and tau in AD and of Htt in HD.

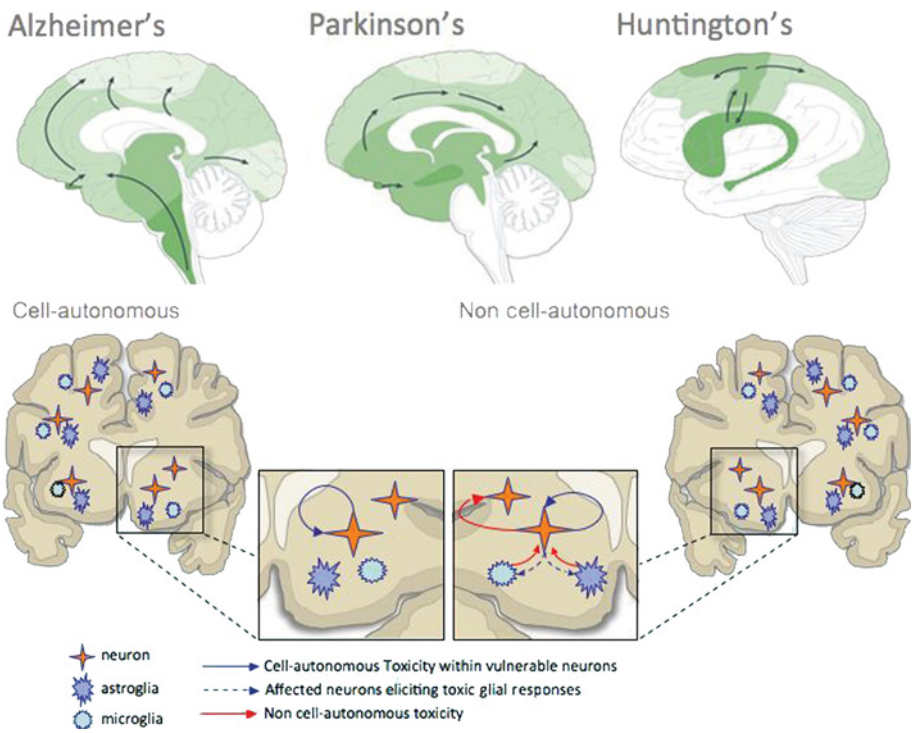

Figure 1 Progression of pathological changes in neurodegenerative diseases

Upper panel, schematic representation of the spatiotemporal progression of neuropathological changes during AD, PD and HD. This schema has been modified from previous publications [6,14]. A mid-sagittal view is shown for AD and PD and a lateral view for HD. Darker shading represents areas of the brain in which neuropathology develops earlier and the spreading patterns are indicated by arrows. In PD Lewy bodies and Lewy neurites appear first in the brainstem and the anterior olfactory structures. In AD neurofibrillary tangles appear first in the hippocampus, the basal nucleus of Meynert and the brainstem. In HD cortical degeneration seems to precede degeneration in the striatum. In each disorder, from the initial affected areas neuropathology progresses following predictable anatomical pathways. Lower panel, graphic representation of the concept of cell-autonomous and non-cell-autonomous degeneration with no anatomical reference to specific brain areas. Neurodegenerative diseases are traditionally viewed as diseases mainly affecting the most vulnerable neurons thus defining the characteristic feature of a given disorder. Selective degeneration and death occurs in neurons that are selectively susceptible to the disease-related alterations (indicated by the solid blue arrows, left-hand inset) and alone suffices to produce disease. Some brain areas are more resilient than others and resist the disease longer (cell-autonomous diseases, left-hand panel). Increasing evidence indicates that the convergence of damage occurring in multiple cell types, including neighbouring neuronal and non-neuronal cells (e.g. astroglia microglia) (red arrows and broken blue arrows, right-hand inset) is crucial to determine the specific clinical picture of various neurodegenerative diseases (non-cell-autonomous diseases, right-hand panel). This synergistic form of cellular disfunction may possibly account for selective neuronal loss in neurodegenerative diseases and in this frame prion-like transmission of protein aggregates might contribute to the gradual spreading of the pathology.

Cell-to-cell spreading of α-syn in PD

The first striking evidence for the intercellular spreading of amyloidogenic aggregates came from the post-mortem analysis of PD patients who had received a transplant of healthy embryonic neurons about 10 years prior to death, revealing that the grafted neurons were positive for Lewy bodies and Lewy neurites, which are mainly formed by misfolded α-syn [47–50]. The most likely explanation for these findings was that misfolded α-syn was transferred from the host to the graft cells (reviewed in [51,52]).

To further investigate this possibility, several *in vitro* and *in vivo* studies (in different cell types and PD mouse models) have been performed. In a pioneering work in 2009, Desplats et al. [53] injected GFP (green fluorescent protein)-labelled mouse stem cells into transgenic mice overexpressing human α-syn. After 1 week intracellular human α-syn immunoreactivity and occasionally inclusion bodies could be detected within the GFP-labelled cells, suggesting that host–graft transfer of α-syn had occurred [53]. In the same study, *in vitro* experiments showed that exogenous fluorescently tagged human α-syn could be internalized in cultured mouse cortical neuronal stem cells and that intracellular human α-syn was transferred from neuronal cells to GFP-labelled mouse stem cells under co-culture conditions [53]. In line with these findings, α-syn was shown to be transferred from the host (rodents overexpressing human α-syn) striatal tissue to transplanted embryonic mesencephalic neurons from wild-type animals [54,55]. Interestingly, α-syn was also shown to spread from neurons to glial cells, where it induced an inflammatory response, both *in vivo* (in humanized α-syn transgenic mice where expression of α-syn was restricted to neurons), and *in vitro* (in co-culture experiments between neuronal cells and astrocytes) [56]. These findings suggest a possible involvement of the glial tissue and of the pathogenic inflammatory astroglial response in the spread of the pathology (see Figure 1).

Seeding properties of α-syn

Exogenous recombinant α-syn delivered by different methods to recipient cells in culture has been shown to induce aggregation of cytoplasmic α-syn, supporting the existence of a 'prion-like' seeding mechanism *in vitro* [57–59]. Consistently seeding of misfolded α-syn has also been reported following intercellular transfer *in vitro* [54,60–62]. Even more impressively *in vivo* experiments support a prion-like propagation mechanism of the disease [54,55,60–62]. Specifically, different groups reported acceleration of α-syn aggregation in the brain of young pre-symptomatic transgenic mice [bearing the familiar PD-related A53T mutation (TgM83)] and an earlier onset of neurological symptoms following intracerebral inoculation of brain tissue from old transgenic diseased mice [63,64]. Furthermore, injection of recombinant α-syn fibrils into the brains of young pre-deposit

transgenic mice led to the same effects [63]. Importantly, the same group reported the transmission and spreading of α-syn pathology in wild-type mice following a single intrastriatal inoculation of synthetic α-syn fibrils. Lewy body-like inclusions appeared progressively in anatomically connected regions and recapitulated the neurodegenerative cascade typical of PD [65].

These results are particularly exciting because they indicate that α-syn pathology is sufficient to induce the pathological features of PD and that this requires aggregate spreading through neuronal interconnectivity, consistent with a 'prion-like' propagation mechanism.

Cell-to-cell spreading of tau and Aβ in AD

AD is characterized by the deposition of extracellular amyloid plaques predominantly composed of misfolded and aggregated forms of Aβ, as well as by the accumulation of intracellular hyperphosphorylated tau aggregates in the form of NFTs (neurofibrillary tangles). Tau is a microtubule-associated protein and was first identified from isolated brain microtubules [66] (reviewed in [67]). Besides AD, NFTs are present in many neurological disorders named tauopathies among which FTP (frontotemporal dementia) and PSP (progressive supernuclear palsy) are the most common (for a review see [68]).

Tau dissemination

Different evidence supports the intercellular transmission of tau aggregates in AD. Injection of brain extract from mutant P301S human tau-expressing mice into the brain of mice expressing wild-type human tau induced assembly of wild-type human tau into filaments [69]. Consistent with the stereotypical progression of neurofibrillary tau tangles in AD patients [13], aggregate formation increased over time and spread to anatomically connected regions [69] (reviewed in [6,23]). More recently, two independent studies provided evidence for a trans-synaptic spreading of tau pathology in transgenic mouse models expressing human mutant tau (P301L mutation) selectively in the entorhinal cortex [70,71]. Interestingly, over time human tau immunoreactivity was detected along synaptically connected neuronal circuits [70,71] and was found to co-aggregate with endogenous mouse tau [71]. These findings have been supported by *in vitro* experiments showing that externally applied tau aggregates are internalized in neuronal cell cultures and can induce the seeded polymerization of intracellular tau [60,72,73]. Furthermore, intracellular tau aggregates can transfer from cell to cell in co-culture experiments in C17.2 neuronal cells [72].

Aβ dissemination

Although the distribution of tau tangles correlates better with cognitive decline than the distribution of Aβ plaques [28], both cellular and animal models indicate that Aβ accumulation (intracellular soluble oligomeric form) drives the disease [74,75] and precedes tau-related neurotoxicity [76,77]. Previous studies have shown that intracerebral injection of brain extracts from AD patients in transgenic mice [78–82] and rats [83] expressing human APP (amyloid precursor protein) resulted in deposition of cerebral extracellular Aβ plaques, suggesting that Aβ has 'infectious' properties and is capable of seeding AD pathology in rodents.

However, these studies have been unable to differentiate between direct neuron-to-neuron transmission of intracellular Aβ and the secondary induction of endogenous Aβ production [78–80]. Interestingly, intraperitoneal administration of Aβ-rich brain extracts induced cerebral amyloidosis. This required a prolonged incubation time and a larger Aβ inoculum compared with direct intracerebral injection, suggesting that this might be a less efficient route [81]. Nevertheless, these observations are reminiscent of the replication of peripherally applied prions (e.g. following ingestion of contaminated food) and of their propagation within the CNS. They also infer the ability of Aβ seeds (like infectious prions) to travel between different cell types (see below).

Of interest, Wang et al. [84] recently showed that intracellular Aβ fusion proteins were able to quickly spread from cell-to-cell in cultured primary rat astrocytes and neurons. In addition, extracellular Aβ was taken up by astrocytes and transferred to primary neurons under co-culture conditions [84], suggesting that, like α-syn, Aβ can also transfer between different neuronal cell types. Furthermore, by performing microinjection into electrophysiologically defined primary hippocampal rat neurons, Nath et al. [85] recently demonstrated a direct neuron-to-neuron transfer of soluble oligomeric Aβ. These findings were supported by *in vitro* experiments in a human donor–acceptor culture cell model showing that Aβ transfer depends on direct cellular connections [85]. Overall, the combined *in vitro* and *in vivo* evidence suggest that Aβ (especially in the form of oligomers, see below) can drive disease progression, even if the extracellular plaque load is poorly correlated with the degree of cognitive decline.

Cell-to-cell spreading of Htt in HD

The hallmark of HD is the presence, in the cytosol and/or in the nucleus, of aggregates of mutant Htt containing polyQ (polyglutamine) repeats above a threshold of 35–40 repeats [86,87]. *In vitro* experiments have shown that synthetic polyQ peptides or recombinant fragments of mutant Htt are readily taken up by cells in culture [88,89] and can seed the polymerization of a soluble Htt reporter protein [89]. These aggregates persisted for over 80 generations in prolonged cell culture despite their dilution in dividing cells, suggesting a self-sustaining seeding process similar to prion replication [89]. However, the natural cell-to-cell transmission of Htt, measured indirectly from the seeded polymerization of the Htt reporter, was rather inefficient in co-culture of HEK (human embryonic kidney)-293 cells, but was drastically increased upon selective lysis of the donor cells [89]. This suggested a transfer mechanism linked to the death of the aggregate-producing cells. Low rates of movement of the mutant Htt (103QHtt-V1 and 103QHtt-V2 plasmids) were also reported recently by BiFC (bimolecular fluorescence complementation) [90] (see below).

Although suggestive, none of these studies has provided direct evidence for Htt aggregate transfer. We have recently addressed this issue in co-culture of neuronal cells and primary neurons transiently expressing polyQ aggregates of a mutant Htt fragment (GFP–480-68Q) ([91,92], and M. Costanzo and C. Zurzolo, unpublished work). Using both flow cytometry and fluorescence microscopy, we found that mutant Htt aggregates formed within donor cells are actively transferred to receiving cells in co-culture, independently of cell death (see also below) (M. Costanzo and C. Zurzolo, unpublished work).

However, it should be noted that the relevance of these observations in HD pathogenesis is still unclear. Indeed, upon autopsy, fetal grafts of striatal tissue from HD patient brains have been shown to be susceptible to disease-like neurodegeneration, but abnormal Htt aggregation was not observed within a decade of the transplant [93], in contrast with what has been found in grafted cells from PD patients [47–50].

THE MOLECULES: MECHANISMS OF TRANSFER OF PROTEIN AGGREGATES

The evidence reported above for the different disease-associated protein aggregates supports the existence of an underlying 'prion-like' process in the propagation of these neurodegenerative disorders. However, as in the case of prions, the mechanism of intercellular transfer is not clear (reviewed in [5]). In the next section we review the current knowledge and speculate on possible mechanisms.

Cell-to-cell transfer of protein aggregates requires two consecutive steps: (i) exit from a 'donor' cell that produces the aggregates and (ii) uptake by a healthy 'acceptor' cell. By taking into account the different biochemical properties of the various disease-associated proteins, different mechanisms can be invoked. Exit could either result from the passive release of aggregates from dying cells or might require an active mechanism involving either conventional or unconventional secretion (see Box 1). On the other hand, entry into the recipient cells could be mediated by (i) direct penetration trough the plasma membrane, (ii) fluid phase endocytosis or (iii) receptor-mediated endocytosis (Box 2). All of these different mechanisms of release and uptake imply the passage of aggregates through the extracellular milieu. Alternatively, intracellular aggregates could bypass this step and be transferred directly between the cytosol of neighbouring cells using TNTs (tunnelling nanotubes; Box 3).

Molecular mechanism of α-syn secretion

Monomeric and aggregated α-syn forms are secreted into the extracellular space via an unconventional secretion pathway (Box 1) [94,95], as demonstrated by the finding that in neuronal cells extracellular α-syn levels are not affected by brefeldin A treatment, a classical inhibitor of the ER (endoplasmic reticulum)–Golgi trafficking involved in conventional secretion [96,97] (Figure 2, top left-hand panel, and Table 1). Concurrent evidence suggest that release of α-syn in healthy cells occurs through a vesicular mechanism (Figure 2, top left-hand panel). Consistently, a small part of the protein is found in the membrane vesicle fraction in tissue and cell homogenates, and its secretion is blocked by low temperature, which inhibits membrane fusion [96,97]. Topologically, vesicle-associated α-syn has a luminal localization and does not appear to be bound to the cytosolic surface of vesicles [96,97]. How cytosolic α-syn enters these vesicles is not clear. It has been shown that α-syn interacts with membranes mainly through association with anionic head groups of phospholipids [98–100]. Therefore free cytosolic α-syn might establish a dynamic interaction with the cytosolic surface of vesicles, which could contribute to its entry in the vesicular lumen either by interacting with membrane proteins or by penetrating the membrane directly [101,102]. It was indeed reported that α-syn can permeabilize model membranes by forming stable pore-like oligomers [103,104].

In healthy cells only a small fraction of α-syn is secreted, whereas the majority of the protein is retained in the cytoplasm [96,97]. However, in neuronal cells subjected to various stress conditions including oxidative stress, proteolytic or autophagic stress, both translocation into vesicles and release of α-syn in its monomeric and aggregated state increases [96,97]. Consistently, the truncated, misfolded and oxidatively modified α-syn forms appear to be enriched in the vesicular fraction and to be preferentially secreted [97]. This suggests that vesicle translocation and consequent exocytosis are conformation-sensitive processes [97] and can be seen as an attempt by the cells to get rid of damaged/misfolded α-syn proteins. This hypothesis is supported by the finding that vesicular α-syn appears to be more prone to aggregation compared with the cytosolic protein [96]. Furthermore, inhibition of protein synthesis does not reduce the levels of secreted α-syn, indicating that the increased release of the protein upon stress conditions derives from a damaged pre-existing pool of α-syn within the cells [97].

The exact identity of α-syn-containing vesicles is still unknown. Early electron microscopy and density-gradient experiments suggested that they had properties similar to the LDCVs (large dense-core vesicles) [96,105]. More recently, it has been reported that α-syn is associated with vesicles whose identity is compatible with exosomes in neuronal cells and can be secreted in association with these structures [106–108] in a Ca^{2+}-dependent manner (Figure 2, top left-hand panel, and Table 1). Furthermore, the use of Ca^{2+} ionophores, which raise cytosolic Ca^{2+} concentrations, resulted in increased α-syn secretion. Conversely, the use of a membrane-permeant Ca^{2+} chelator {BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetra-kis(acetoxymethyl ester)]} reduced the levels of secreted α-syn [106]. In contrast with these observations, Hasegawa et al. [109] did not recover α-syn in the exosomal fraction of neuronal culture medium. They showed that impaired biogenesis of MVBs (multivesicular bodies) in cells expressing a VPS4A (vacuolar protein sorting-associated protein 4A) dominant-negative mutant (E228Q) (Box 2) inhibits lysosomal targeting of α-syn and facilitates its secretion (Table 1). Dominant-negative Rab11 restored partially α-syn secretion levels confirming the role of Rab11 in the secretion of α-syn through the recycling endosomal

Box 1 Conventional and unconventional protein secretion

Most secretory proteins are transported to the extracellular space or to the plasma membrane following the conventional ER – Golgi secretory pathway. The presence of N-terminal or internal signal peptides allows entry of the nascent proteins into the ER lumen from which they exit at specialized membrane domains called ER exit sites, in association with cargo-containing COPII (coat protein complex II)-coated vesicles [207]. Subsequently, the newly synthesized proteins reach the Golgi apparatus where they are modified, processed and sorted towards their final destination.

In the case of proteins secreted into the extracellular medium the secretion pathway can be constitutive (for proteins that are secreted as fast as they are synthesized, e.g. extracellular matrix proteins such as collagen and fibronectin) or regulated (in response to a specific signal as in the case of synaptic secretion or in response to hormones, e.g. insulin). Transport of cargos within the secretory pathway involves a series of membrane fusion events catalysed by SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptors) that are localized on both opposing membranes v-SNAREs (vesicle SNAREs) from vesicles and t-SNAREs (target SNAREs) from target membranes [208].

The term 'unconventional protein secretion' is used to describe trafficking pathways that target proteins to the cell surface or the extracellular space and do not follow the 'conventional' ER–Golgi pathway (reviewed in [94,95]). They include signal-peptide-containing proteins that have been shown to exit from the ER in a COPII vesicle-independent manner or to bypass the Golgi apparatus, as well as cytoplasmic and nuclear proteins that lack an ER signal peptide. In this particular case four different secretion mechanisms have been proposed including: (i) direct translocation from the cytoplasm across the plasma membrane, (ii) release via secretory lysosomes, (iii) release via exosomes following fusion of MVBs to the plasma membrane (for exosomes see Box 2) and (iv) microvesicle shedding (for reviews see [94,95]). In the last three scenarios, the released protein is surrounded by a membrane, whereas the translocation across the plasma membrane requires interaction either directly with membrane lipids as shown in the case of FGF2 (fibroblast growth factor 2) [209] or through membrane channel or transporter proteins (for a review see [95]). More recently, an autophagy based unconventional secretion pathway, termed 'autosecretion', has emerged that involves non-degradative autophagosome intermediates (for a review see [210]).

pathway [110] (Figure 2, top left-hand panel, and Table 1). Overall these data suggest that exosomes are not involved in α-syn secretion. More recently, Danzer et al. [108] reported that α-syn oligomers can be found in two different extracellular fractions: one associated with exosomes and one membrane free. One possible explanation for this finding is that α-syn oligomers can be secreted via different secretory pathways (exosome-dependent or -independent) [108] (Figure 2, top left-hand panel).

Of interest, α-syn is predominantly enriched at the presynaptic terminal where it is loosely associated with the distal pool of synaptic vesicles (for a review see [111]). Earlier reports had indicated that α-syn may act as a co-chaperone in co-operation with the chaperone CSPα (cysteine-string protein-α), a synaptic vesicle protein containing a DNA-J domain typical for Hsp (heat-shock protein) 40-type co-chaperones (reviewed in [112]). The synaptic vesicle localization and co-chaperone activity of CSPα suggest that it may function in preventing the accumulation of non-native potentially toxic molecules during the continuous operation of a nerve terminal. Interestingly, in fruitflies CSPα may regulate Ca^{2+} influx [113,114] or mediate Ca^{2+}-triggered exocytosis [115] at nerve terminals, as well as in transfected cells where CSPα has multiple, sometimes opposing, effects on exocytosis [116–119]. Thus it is possible that neuron-to-neuron transfer of α-syn could take place across the synaptic cleft. However, this possibility has not been directly explored.

Although quite interesting, all of these observations remain preliminary and more experiments are required to identify the different pathways and the molecular factors possibly regulating synuclein release. In addition, the specific mechanism of α-syn release might depend on the conformational state of the protein, as seems to be the case for internalization (see below).

Molecular mechanism of α-syn internalization

Owing to the fact that under stress conditions and misfolding, there is an increase of α-syn secretion that becomes very relevant for the spreading of aggregates into diseased brain, the fate of extracellular α-syn has been the subject of many studies [120]. Extracellular α-syn can be cleared by microglia [121] or by extracellular proteases [122] or can be internalized into cells (Figure 2, top left-hand panel). In particular Sung et al. [123] have shown that in neuronal hippocampal cells and primary cortical neurons recombinant α-syn was internalized in a Rab5A-dependent endocytic process (see Box 2) (Table 1). Internalized α-syn was mainly detected in the intracellular soluble fraction (rather than in the particulate membrane fraction) and resulted in the formation of granular cytoplasmic aggregates positive for ubiquitin, synaptophysin and tau proteins, all markers of Lewy body pathology in PD. Exposure to extracellular α-syn resulted in neuronal cell death that appeared to be directly correlated with α-syn–Rab5A-specific endocytosis. Indeed, expression of a GTPase-deficient Rab5A (mRab5A/S34N) resulted both in incomplete endocytosis of α-syn and in a significant decrease in cytotoxicity in cell culture [123]. Rab5 is known to modulate transport between the plasma membrane and early endosomes, where it selectively localizes [124–126]. Thus these data strongly support the involvement of a vesicular endocytic pathway in α-syn internalization (Figure 2, top left-hand panel).

Box 2 Endocytic mechanisms

Endocytosis occurs by various mechanisms, which are either clathrin dependent or independent. Clathrin-dependent endocytosis requires recognition and packaging of cargo proteins into clathrin-coated vesicles. It is well characterized and is the most common mechanism for receptor-mediated endocytosis [211]. By contrast, clathrin-independent endocytosis is less well studied and can come in different forms [211,212]. The most commonly reported non-clathrin-mediated endocytosis involves the caveolin coat. Three mammalian caveolin proteins have been identified, with caveolin-1 likely to be necessary for caveolae biogenesis. Caveolin-1 binds to the glycosphingolipid GM1 that is associated with specialized domains of the plasma membrane known as lipid rafts [150,151]. Rafts are membrane microdomains enriched in glycosphingolipids and cholesterol that have the property to cluster different lipids and proteins and thereby mediate precise cellular functions. Several proteins and receptors have been shown to be internalized by raft-mediated endocytosis and the most common markers are GPI-APs (glycosylphosphatidylinositol anchor proteins) [213]. Both clathrin-dependent and -independent endocytosis requires the use of different molecules downstream, which are involved in different steps of the internalization process (e.g. clustering of the receptor, formation of the bud, constriction and pinching off). For example, dynamin is involved in the abscission of clathrin-coated pits and has been additionally implicated in non-clathrin mediated endocytosis. Overall, the non-clathrin-mediated pathway, different from the clathrin-dependent one, is defined by its sensitivity to cholesterol depletion [211].

Finally, molecules can enter the cell through fluid phase endocytosis that mediates the non-selective uptake of solute molecules, nutrients and antigens. It is initiated from the formation of surface membrane ruffles that give rise to large endocytic vacuoles resulting in the uptake of the entrapped extracellular components [211].

Independently of the endocytic mechanism, cargo proteins are targeted to the EEs that constitute an intracellular sorting platform for internalized proteins. The small GTPase Rab5 and its effector EEA1 are required for the generation and the maintenance of EEs. From the EE, cargos can move either to ERCs (recycling endosomes) or to late endosomes where they are targeted for degradation by fusing with lysosomes. From the ERCs, proteins can recycle to the plasma membrane in a process that requires the small GTPase Rab11. In the EEs some cargoes are selected to enter the MVBs. From the limiting membrane of MVBs proteins can be incorporated into ILVs (intraluminal vesicles) by invagination of the MVB membrane. MVBs can either fuse with lysosomes and deliver ILVs to the lysosomal lumen where they are degraded or, alternatively, can fuse with the plasma membrane, thus releasing ILVs into the extracellular space as exosomes (for reviews on exosomes see [152,153]). Proteins are sorted and packaged into exosomes in a regulated manner, involving the different components of the ESCRT (endosomal sorting complex required for transport) machinery. After protein sorting into ILVs has been completed, a multimeric AAA-type ATPase, Vps4, binds to the ESCRT machinery and disassembles it. The Vps4-dependent dissociation process is therefore the final distinguishable step of cargo sorting and is a prerequisite for ILV formation [214]. Indeed, the use of a dominant-negative mutant of Vps4 impairs protein sorting to ILVs and exosome formation.

Box 3 TNTs

TNTs were discovered only a few years ago as a novel form of cell-to-cell communication [185]. They were first recognized in cultured PC12 neuronal cells as long thin actin-containing bridges that do not contact the substratum and extend up to 100 μm in length with diameters ranging from 50 to 200 nm [185]. Since then, TNTs have been found in many cell types, from neuronal cells and primary cells to immune and epithelial cells in culture, acting as conduits for the exchange of cytosolic and membrane-bound molecules and organelles, as well as for the spreading of pathogens. In particular, they have been shown to transfer both viruses and bacteria as well as proteinaceous aggregates linked to several neurodegenerative diseases [186,187]. In neuronal cells F-actin (filamentous actin) is the major component, whereas in other cells TNTs can contain only actin or both actin and microtubules (as in cells of the immune system) or be composed of cytokeratin filaments (in the case of epithelial cells) [187]. Cargoes can be uni- or bi-directionally transported inside TNTs. Unilateral transfer occurs when a donor cell (usually the cell that initiated TNT formation) transfers material to an acceptor cell, whereas bilateral transfer happens when both cells mutually exchange materials (found mainly in non-neuronal cells). The reasons for these different transport mechanisms are the TNT structural components (actin only compared with both actin and microtubules) and the specific molecular motors involved. Unidirectional transfer is found when only actin is present, whereas transfer appears to be bidirectional in the presence of both actin and microtubules. Both inside transit of vesicles and organelles (endosomes, lysosomes, mitochondria, Golgi and ER), as well as surfing of cargoes on the extracellular TNT membrane or in the intracellular leaflet have been reported [187]. Interestingly, in neuronal cells TNTs have been reported to mediate bidirectional spread of electrical signals leading to the activation of low-voltage-gated Ca^{2+} channels in the connected cells. These TNTs are characterized by the presence of the gap junction protein connexin43 at one side of the nanotube at the level of the junction with the connected cell [186,215].

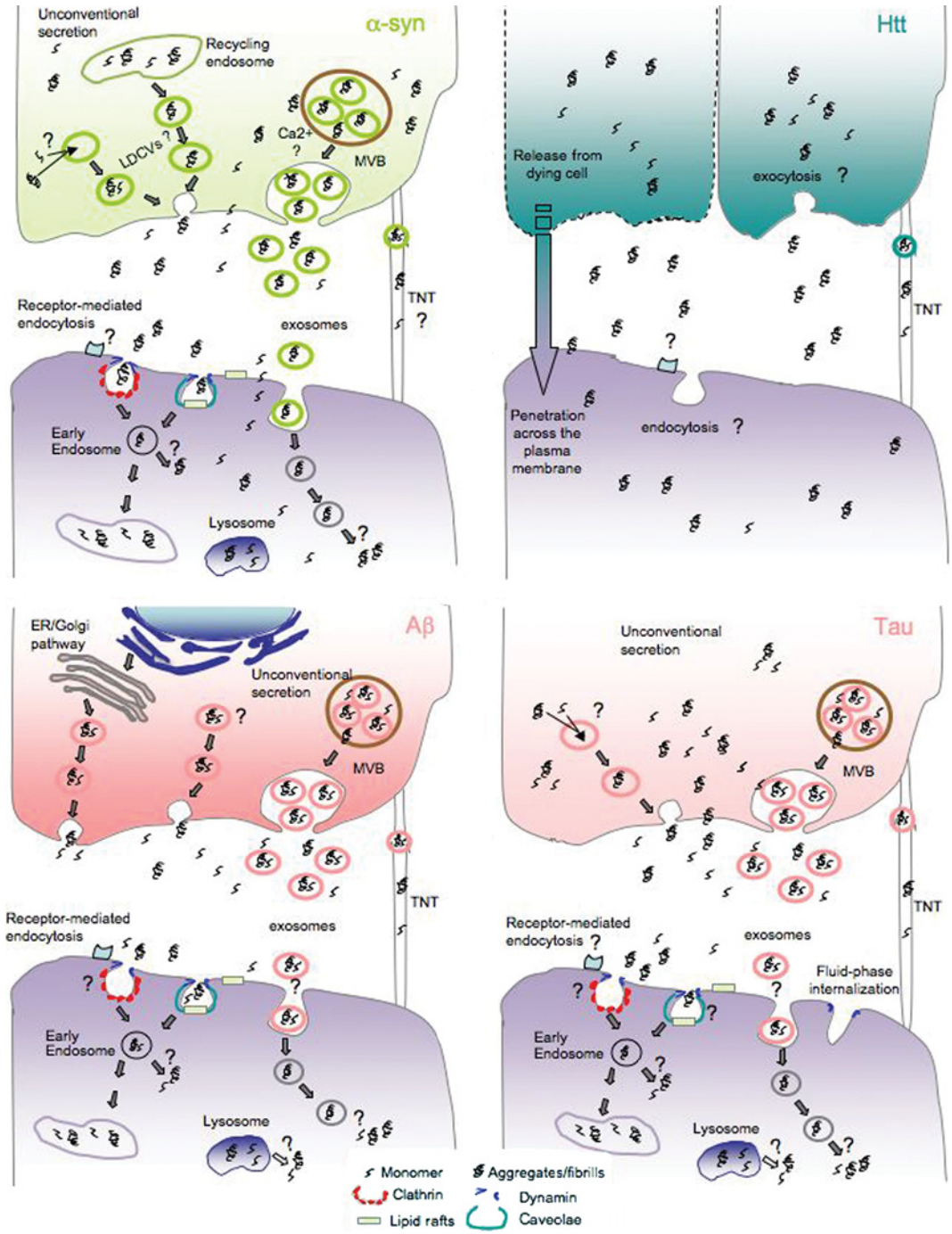

Figure 2 Possible routes for cell-to-cell transfer of cytosolic (α-syn, Htt and tau) and secreted (Aβ) proteins

In each panel donor (upper cell) and acceptor cells (lower cell) are depicted. Despite being a cytosolic protein α-syn associates with vesicles via mechanisms that are still unknown and is secreted in the extracellular space via ER–Golgi independent exocytosis. The identity of α-syn containing vesicles is unknown. The involvement of the recycling endosome pathway, LDCVs and exosome-mediated release (possibly triggered by intracellular Ca^{2+}) have been proposed (top left-hand panel). α-Syn can be internalized via exosome-mediated transport or following endocytosis (top left-hand panel). How α-syn-containing exosomes are taken up and whether this requires a fusion step with the plasma membrane are unknown. The endocytic process requires dynamin and occurs both through clathrin-dependent and -independent pathways (involving caveolin-1 and lipid rafts). A protein receptor on recipient cells could facilitate α-syn internalization, but there are still no experimental evidence for specific candidates. Monomeric α-syn internalization was also reported to occur though binding to the ganglioside GM1 in lipid rafts (independently of clathrin, caveolae and dynamin). Once internalized α-syn localizes along the endocytic pathway from which it must escape and translocate in the cytosol using mechanisms that are still unknown. Different from aggregated α-syn forms, monomers seem to passively diffuse across the plasma membrane. TNTs remain an unexplored intercellular transfer mechanism for α-syn (top left-hand panel). Htt aggregates have been proposed to be passively released from dying cells and to directly access the cytosolic compartment of recipient cells by translocating across the plasma membrane as they do not appear to be associated with membrane vesicles. We have recently proposed that aggregated Htt transfers from healthy donor cells to acceptor cells via TNTs. There is currently no experimental evidence for exocytic/endocytic transfer mechanisms (top right-hand panel). Aβ is a secretory protein generated and processed along the ER–Golgi secretory pathway, but it has also been shown to be released by cells in association with exosomes. Other unconventional secretory pathways may also be involved. Aβ peptides have been shown to be internalized in a lipid raft/caveolae-mediated endocytic pathway dependent on dynamin. GM1-dependent endocytosis in lipid rafts has also been reported. Moreover, several receptors have been proposed to facilitate Aβ internalization (see the text and Table 1 for details). Recently, TNTs have been shown to mediate intercellular transfer of Aβ particles, whereas cell-to-cell transfer of Aβ containing exosomes has not

Table 1 Mechanisms and molecules involved in the intercellular trafficking of α-syn, tau, Aβ and Htt

APOE, apolipoprotein E; LRP1, low density lipoprotein receptor-related protein 1; NMDA, *N*-methyl-D-aspartate.

	Exit		Entry		
Protein	Mechanism	Molecules	Mechanism	Molecules	Reference(s)
α-Syn	Unconventional vesicular secretion	Rab11			[96,97,109,110]
	Exosomes	VPS4A	Exosomes		[106–108]
			Endocytosis	Rab5A, dynamin 1, clathrin, caveolin-1, GM1	[54–56,59–62,123,129,130,149]
			Uptake at nerve terminals		[61]
	Non-vesicular translocation across the plasma membrane		Non-vesicular translocation across the plasma membrane		[102,129,149]
Tau	Unconventional non-vesicular secretion				[163]
	Exosomes				[160,161]
			Endocytosis	Dynamin	[72,73,165]
	TNTs		TNTs		S. Abounit and C. Zurzolo, unpublished work
Aβ	ER–Golgi secretion				For a review see [154]
	Synaptic stimulation				[155–157]
	Exosomes				[158,159]
			Endocytosis	Dynamin, caveolin-1, GM1	[166] [167]
			Receptor-mediated endocytosis	α_7-acetylcholine receptor, NMDA, LRP1/APOE, sortilin	[174–181]
	TNTs		TNTs		[84]
Htt	Non-vesicular translocation across the plasma membrane				[89]
	TNTs		TNTs		M. Costanzo and C. Zurzolo, unpublished work

However, together with its different adaptors, Rab5 can mediate the delivery to EEs of various cargoes internalized by different (clathrin-dependent or -independent) endocytic mechanisms [126–128] (Box 2). Of interest, different studies pointed to a role for dynamins as well as clathrin-mediated endocytosis in α-syn internalization [56,62,129,130] (Figure 2, top left-hand panel, and Table 1). Dynamins (Box 2) are GTPases involved in vesicle fission in both clathrin-dependent and -independent endocytic pathways [131,132]. Three dynamin isoforms have been identified, with dynamin 1 and dynamin 3 being highly expressed in neurons and mainly involved in synaptic vesicle and clathrin-mediated endocytosis [133,134]. Expression of a dominant-negative mutant of dynamin 1 (K44A dynamin 1) [135] reduced α-syn (fibrils and syn secreted from overexpressing SH-SY5Y cells) uptake in neuronal cells and primary astrocytes [56,129]. Furthermore, genetic suppression and pharmacological inhibition of the dynamin GTPases significantly decreased the internalization of α-syn oligomers in neuronal and oligodendroglial cells and also inhibited transfer of α-syn in neuronal as well as neuronal/oligodendroglial co-cultures [62]. These data were supported by *in vivo* experiments in rats where, following intracortical injection of recombinant α-syn, neuronal uptake was attenuated by co-injection of dynasore, a non-competitive inhibitor of the GTPase activity of dynamin [54,136]. Although dynamins are involved in both clathrin-dependent and -independent endocytosis [131,132], the involvement of clathrin-mediated endocytosis in the internalization of α-syn in microglia was supported by a proteomic analysis that identified clathrin as a requirement for microglia activation following internalization of α-syn aggregates [130].

The fate of internalized α-syn

Following internalization through the endocytic pathway, a partial co-localization of recombinant α-syn with markers for EEs (Rab5A) [62,123] and lysosomes [Lamp-1 (lysosomal-associated membrane protein 1)] was reported [62] (Figure 2, top left-hand panel). However, the majority of internalized α-syn is located within the intracellular soluble fraction and is assembled into high-molecular-mass oligomers and in Lewy-body-like cytoplasmic inclusions [54,55,60–62]. This indicates that once internalized α-syn must escape the endocytic pathway in order to access the cytosol, which is reminiscent of the behaviour of bacterial AB toxins [137]. Translocation of α-syn into the cytosol will also impair trafficking to lysosomes and degradation [129,138].

One of the questions that has captured the attention of a number of researchers is how α-syn exits the endocytic compartment. Structurally, α-syn can be divided into three functional regions: an N-terminal region (1–60 aa), containing four imperfect apolipoprotein A1-like repeats that mediate lipid membrane binding; a centrally located hydrophobic amyloidogenic region (61–95 aa); and an acidic C-terminal domain (96–140 aa) with chaperone activity [139]. The N-terminal domain of α-syn has been shown

been yet reported (bottom left-hand panel). Tau is released in the extracellular space via unconventional secretion through a constitutive release (possibly occurring by direct translocation across the plasma membrane) or in association with exosomes. Internalization of tau exosomes in recipient cells has still to be demonstrated. Tau aggregates have been shown to be internalized via fluid-phase internalization. The endocytic process seems to be dynamin dependent and to involve small oligomeric species, but not long fibrils or monomers. Because of the lack of experimental evidence it is not possible to exclude a role for other endocytic pathways, e.g. receptor-mediated, clathrin-dependent and caveolae-mediated endocytosis in different cell types. Once internalized tau localizes along the endocytic pathway from which it must escape in order to gain access to the cytosol. Results (M. Costanzo and C. Zurzolo, unpublished work) indicate that tau aggregates can hijack TNTs for intercellular transfer (bottom right-hand panel).

to bind to membranes and promote curvature in a similar manner to endophillins (for a review see [140]). On the other hand the N-terminal region of α-syn has also been shown to be necessary for membrane translocation [102]. Finally, similar to many oligomeric species of amyloid proteins, α-syn is also able to permeabilize membranes either by forming transbilayer protein channels or by thinning of the hydrophobic core of the lipid bilayer owing to the incorporation of the oligomers between the tightly packed lipids [141,142]. Despite all of these studies, the specific mechanism of α-syn escape in the cytosol remains an unanswered question that appears to be crucial to understand the mechanism of α-syn transmission and seeding between neighbouring cells (see below).

Previous studies that specifically addressed the cell-to-cell transfer of α-syn by using different cultured cells of human and rodent origins as well as transgenic mice further supported the involvement of endocytosis in the uptake of α-syn from the extracellular space [53–55,60,61] (Figure 2, top left-hand panel, and Table 1). Of particular interest is the finding of Angot et al. [55] who reported for the first time that *in vivo*-transferred α-syn puncta co-localize with a marker for EEs, EEA1 (EE antigen 1) in the recipient cells (grafted dopaminergic neurons in transgenic rat brains) suggesting an endocytic localization in the brain. These data are particularly exciting because they suggest that synuclein oligomeric species produced by diseased cells are present in the endocytic compartments of a recipient cell. In addition the authors showed that some of the transferred α-syn is resistant to PK (proteinase K) digestion and does not react with anti-phospho-α-syn antibodies, thus suggesting that some of the α-syn forms that transfer to the transplanted dopaminergic neurons are not aggregated or phosphorylated [55]. However, which form(s) of α-syn are taken up (monomeric, oligomeric or aggregated forms) by the recipient cells and in what ratio is not yet clear (see also below). Moving along the endocytic pathway α-syn would be targeted to the lysosomal compartment where it could be finally degraded [129,138].

The finding that, following endocytosis, transferred α-syn is able to seed aggregation and therefore transmit protein misfolding in recipient cells [54,55,60–62] implies, as we discussed above, that it escapes the endocytic pathway and translocates in the cytosol where it can encounter the native endogenous protein. Alternatively, it is possible that the transfer of α-syn initiates a cell stress response leading to α-syn aggregation in the recipient cell and further transmission to neighbouring cells. Interestingly, in PD patients lysosomal function has been shown to be decreased (together with other biochemical abnormalities) [107,143]. This could exacerbate the propagation of a pathological form of α-syn which, once transferred to recipient cells, are no longer degraded but act in the cytosol to seed aggregate formation.

Conformational dependent α-syn endocytosis

One of the questions that is still not clear is whether the mechanism of internalization and the fate of transmitted α-syn depends on its oligomerization state. *In vivo*, both monomeric and oligomeric forms of α-syn have been detected in human blood plasma and CSFs [42–44,144]. *In vitro*, Lee et al. [129] have examined the internalization of various forms of extracellular recombinant α-syn including monomers, oligomers and fibrils in neuronal cells. Indeed, in both differentiated human neuroblastoma cells and primary rat cortical neurons, the mechanism of internalization is dependent on the assembly state of the protein: aggregated α-syn forms (both fibrils and oligomers) can enter into the cell via receptor-mediated endocytosis, whereas monomeric α-syn seems to passively diffuse across the plasma membrane (Box 2) (Figure 2, top left-hand panel, and Table 1) [102,129]. Consistently, uptake of exogenous monomeric α-syn was not inhibited by low PK treatment of the cells or by the expression of a dominant-negative dynamin 1 [129]. Moreover, after internalization monomeric α-syn was localized in the cytosol and rapidly removed from cells and released into the culture medium. The use of proteasomal and lysosomal inhibitors did not slow down the kinetics of release, suggesting that monomers are not degraded within the cells, but possibly translocate across the plasma membrane [129]. On the contrary, upon uptake, fibrillar and aggregate forms of α-syn probably enter the endocytic pathway because they were shown to be associated to the membrane fraction and to co-localize partially with marker proteins for caveolae-derived endosomes (caveolin-1), EEs (EEA1) and late endosomes (Lamp-1). Finally they end up in lysosomes where they undergo lysosomal degradation [129].

Different from recombinant monomeric α-syn, wild-type α-syn does not freely diffuse [129,145] and is retained within the cell. The reason for this discrepancy is not known. One possibility is that the native protein, differently from the recombinant one, establishes interactions with other cytoplasmic proteins that would retain it in the cytoplasm [129,145]. Alternatively, the native cytoplasmic protein might exist in a conformational state different from a disordered monomer and therefore be incompatible with the translocation mechanism. Indeed, recent studies reported that α-syn produced in *Escherichia coli* cells [146] or isolated from mammalian cells and tissue [147] exists predominantly as a tetramer enriched in α-helical structure. However, the biochemical state of intracellular native α-syn as well as of the recombinant purified protein is still controversial [62,148].

Interestingly, Park et al. [149] reported that the internalization of monomeric recombinant α-syn at low concentrations into BV-2 microglial cells was inhibited by low temperature and ATP deprivation. However, at higher concentrations monomeric α-syn uptake was not sensitive to temperature, indicating that, within the same cells, different mechanisms of internalization can operate depending on the levels of extracellular α-syn [149] (Table 1). They also reported that at low doses monomeric α-syn internalization occurred in a clathrin-, caveolae- and dynamin-independent manner, but was mediated by the binding to the ganglioside GM1 in lipid rafts. Lipid rafts are highly ordered specialized microdomains of the plasma membrane enriched in glycosphingolipids and cholesterol [150,151]. Binding of α-syn to GM1 was reported also in artificial small unilamellar vesicles and was accompanied by the formation of α-syn oligomers [100]. Therefore it is possible that, following binding to cell-surface GM1, monomeric α-syn oligomerizes and that this then triggers its internalization into microglia [149].

Mechanisms of α-syn transfer: exosomes or synaptic transmission?

How the different mechanisms of exo- and endo-cytosis described above participate to the transfer between cells is not clear. Exosomes released from SH-SY5Y cells overexpressing α-syn have been shown to transfer α-syn to naïve cells [107] (Figure 2, top left-hand panel, and Table 1). Moreover, Danzer et al. [108] have recently reported that exosome-associated α-syn oligomers are more prone to internalization than exosome-free α-syn oligomers. In addition, exosome-associated α-syn appeared to be more toxic when compared with free α-syn oligomers as measured by the level of caspase 3/7 activation. Topologically, exosome-associated α-syn is localized both at the cell surface and in the lumen of the vesicles [108].

Exosomes can deliver proteins to recipient cells in different ways, including endocytosis, fusion with the plasma membrane, and also through ligand–receptor interaction at the cell surface (for reviews see [152,153]). The exact mechanism of α-syn-containing exosome uptake is unknown. However, exosome internalization and consequent delivery of α-syn oligomers into recipient cells requires membrane integrity because it could not be achieved upon sonication [108]. An important point is to address whether this process can be involved in the propagation of aggregated α-syn and therefore whether it would lead to seeding of endogenous α-syn misfolding. Moreover, additional studies are needed to verify the presence of exosome-associated α-syn in biological fluids from PD patients, as this will provide strong evidence for the *in vivo* involvement of the exosomal pathway in PD.

Recently Volpicelli-Daley et al. [61] demonstrated that aggregates of α-syn which are able to act as seeds in primary neurons can be taken up at the nerve terminals, suggesting that transfer might occur at the synaptic cleft (Table 1). Trans-synaptic transmission might explain the finding that in wild-type mice the propagation of α-syn pathology occurs in anatomically connected regions in a step-wise progressive manner [65]; however, further studies are required to specifically address this possibility.

To date the precise mechanism(s) of α-syn release and uptake by recipient cells remains elusive. Many of the differences that we have discussed can be related to the different experimental systems and forms of α-syn utilized. However, it is likely that different mechanisms may coexist within single cells and could be differently regulated depending on the cell type and on the α-syn form. The use of more physiological *in vitro* settings, e.g. endogenously produced α-syn in primary neurons combined with *in vivo* experiments is required to fully understand the molecular factors involved in α-syn transfer and their relevance for progression of the disease.

Molecular mechanism of Aβ and tau secretion

Aβ peptides ($A\beta^{40–42}$) are generated within neurons through ER–Golgi processing of the APP [154] (Figure 2, bottom left-hand panel, and Table 1). Aβ peptides have been shown to be secreted by neurons upon synaptic stimulation [155–157] to be released by cells in association with exosomes [158,159] (Figure 2, bottom left-hand panel, and Table 1). Remarkably, exosomal proteins such as Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] and flotillin-1 have been found to accumulate in amyloid plaques in the brains of PD patients suggesting that exosomes might play a role in the trafficking of Aβ peptides *in vivo* [158].

More recently, two independent studies have described the secretion of tau protein by exosomes [160,161] (Figure 2, bottom right-hand panel, and Table 1). However, exosome-associated tau is only observed in overexpressing systems [160,161], and endogenous tau was not detected in exosomes derived from primary embryonic neuronal cell cultures [162]. These findings suggest that exosomes may contribute to eliminate the excess of intracellular tau by releasing the protein into the extracellular space. Consistently, a constitutive release of tau into the extracellular space was proposed to occur through unconventional secretion as it could be inhibited by low temperature, but not by inhibitors of the conventional ER–Golgi pathway (Figure 2, bottom right-hand panel, and Table 1). Furthermore secreted tau was not found in the membrane pellet of the culture supernatant, indicating that constitutive tau release occurs via direct translocation of the protein through the membrane, proportionally to its intracellular concentration [163]. Consistently, tau protein can be detected in CSF notably at very high levels in AD patients. Of interest, tau was found associated with exosomes in the CSF of early AD patients [160], which may indicate that diseased cells can release tau in higher amounts by active exosomal secretion. Alternatively, tau aggregates may be released in the extracellular space from degenerating cells as suggested by the presence of 'ghost tangles' in the brain of AD patients.

Molecular mechanism of Aβ and tau internalization

After its release in the intercellular space, (misfolded/fibrillar) tau can be internalized by the neighbouring cells, where it can induce the fibrillization of the wild-type protein [20,60,69,71–73]. However, the mechanism of internalization and the mode of transmission are not clear [23,164]. Interestingly, tau aggregates showed partial co-localization with dextran, a bulk-endocytosis marker, indicating an uptake through fluid-phase internalization [72,165] (Box 2, Figure 2, bottom right-hand panel, and Table 1). As for α-syn, the internalization of tau seems to be dependent on its aggregation state [165]. In that study it was shown that extracellular low-molecular-mass tau aggregates and short fibrils were taken up by primary neurons in culture, whereas monomers and long fibrils (either synthetic or purified from brain extracts) were not, suggesting the existence of a relationship between the oligomerization state of extracellular tau and its endocytosis. Furthermore tau uptake was dynamin-dependent, but did not require clathrin and occurred both at the axonal terminal and at the cell soma (Figure 2, bottom right-hand panel, and Table 1). Once internalized, tau co-localized with markers of EEs and late endosomes and was reported to travel along the axons in a retrograde manner towards the soma in association with lysosomal vesicles [165] (Figure 2, bottom right-hand panel). This finding supports the possibility that a trans-synaptic passage might have a role in the spreading of tau pathology. More experiments are needed to understand the specific mechanism by which tau aggregates are incorporated into recipient cells, how they traffic inside the cells, and how and where they could eventually seed the misfolding of the wild-type cellular protein.

Similarly to tau and α-syn, exogenous $A\beta^{42}$ has also been shown to be internalized in mouse cortical neurons and human neuroblastoma cells (SH-SY5Y cells) and to be delivered to the late endosomes/lysosomal compartment [166] (Figure 2, bottom left-hand panel). This process was suggested to play a role in the intracellular formation of amyloidogenic Aβ aggregates (that could be further delivered to the extracellular space) both by concentrating the internalized peptides in the vesicular lumen and by exposing them to an acidic environment that would facilitate their misfolding [166]. At low lysosomal pH, $A\beta^{42}$ did form insoluble aggregates leading to lysosome disruption [167]. In that recent study, Omtri et al. [167] reported that the internalization of the amyloidogenic peptide $A\beta^{42}$ in differentiated PC12 cells occurs via a dynamin-dependent endocytic mechanism sensitive to the plasma membrane content of cholesterol (Box 2). Indeed mbCD (methyl β-cyclodextrin) treatment, which extracts cholesterol from cell membranes (thereby disrupting lipid rafts), significantly reduced $A\beta^{42}$ uptake. Moreover, in the presence of cholera toxin, which binds to the ganglioside GM1 (localized in the lipid rafts), internalization of $A\beta^{42}$ was also reduced. The same authors also observed that $A\beta^{42}$ co-localizes with caveolin-1, which is closely associated with lipid rafts and serves as a scaffold protein to co-ordinate membrane proteins and to regulate cell signalling and caveolae-mediated endocytosis. Altogether these observations suggest that $A\beta^{42}$ is internalized in neuronal cells

upon binding to the GM1 receptor in a raft/caveolae-mediated endocytic pathway [167] (Figure 2, bottom left-hand panel, and Table 1). Although these are interesting findings, their relevance in the brain is not clear because caveolin-1 is not normally expressed in mature neurons [168] and caveolin expression in neurons has been detected independently of any conclusive evidence for caveolae [169,170].

Following internalization Aβ^{42} has been found within late endosomes and lysosomes [166,167] (Figure 2, bottom left-hand panel). Of interest, the neuronal uptake of the less amyloidogenic peptide Aβ^{40} appears to be energy independent and non-endocytic [166,167,171–173]. Whether Aβ^{40} is just a less toxic version of Aβ^{42} and/or plays a protective role by counteracting the toxic effect of Aβ^{42} within neurons is not fully understood. However, the fact that they are internalized by distinct pathways suggests that Aβ^{40} and Aβ^{42} have differential effects within neurons. Other reports have proposed that endocytosis of Aβ^{42} may be facilitated by the presence on the neuronal cell surface of different receptors, such as α_7 nicotinic acetylcholine receptor [174–176], NMDA (*N*-methyl-D-aspartate) receptor [177], LRP1 (low density lipoprotein receptor-related protein 1)/APOE (apolipoprotein E) [178–180] and sortilin, a p75NTR (p75 neurotrophin receptor) co-receptor who shuttles other proteins within the cells [181] (Figure 2, bottom left-hand panel, and Table 1).

Involvement of TNTs in Aβ transfer

All the evidence presented above supports the hypothesis that, as for α-syn, Aβ and tau are also packaged into endocytic vesicles upon internalization in receiving cells, and require a mechanism that allows them to gain access to the cytosol in order to exert their seeding activity. As for α-syn, and for Aβ peptides, studies conducted on phospholipid bilayers and in vesicles made of total brain lipids demonstrated the ability of Aβ^{40} and Aβ^{42} to intercalate into the phospholipid bilayer of the plasma membrane mainly by establishing electrostatic interactions between charged peptide residues and lipid headgroups (for reviews see [182,183]). Whether this property enables Aβ proteins to passively diffuse across the plasma membrane and reach the cytoplasm needs to be carefully examined.

Interestingly, a recent study has shown the importance of direct cell–cell contact in the transmission of Aβ soluble oligomeric species in an *in vitro* co-culture of primary rat hippocampal neurons and human neuroblastoma cells, which resulted in progressive cytotoxicity of the acceptor cells after 3 days of co-culture with the cell population producing Aβ [184]. The same study suggested the involvement of the endolysosomal pathway as most of the Aβ oligomers co-localized with lysosomes in the acceptor cells and were also observed in EEs in the neurites connecting donor and acceptor cells. Furthermore, Wang et al. [84] have shown that extracellular Aβ, once internalized in primary astrocytes, is found in TNTs established with co-cultured neurons (Figure 2, bottom left-hand panel, and Table 1). TNTs are thin actin-rich membrane bridges connecting the cytoplasm of distant cells [185], thereby allowing intercellular exchange of cell components, but also of pathogens (Box 3) [186,187]. Their involvement in the intercellular spreading of prions has already been shown [188]. TNTs have also been shown to allow the spreading of death signals between immune cells [189]. Therefore it is possible that TNTs act as transport conduits for other prion-like protein aggregates and their toxic signals. Interestingly, Wang et al. [84] reported that when TNTs were induced by H_2O_2 treatment in co-cultures between astrocytes expressing Aβ and healthy neurons, there was an increase in neuronal cell death compared with control or non-stressed co-cultures. They suggested that under stress conditions the increased number of TNT connections resulted in a higher transfer of Aβ to the neurons and that this led to cell death. However, we cannot exclude the fact that under H_2O_2 conditions neuronal cells are more sensitive to noxious stimuli, and therefore that the increased cell death is not linked to higher transfer of Aβ. Quantitative experiments measuring the amount of cell transfer via TNTs are needed.

How Aβ aggregates are transported along TNTs is not clear. Following uptake, endocytic vesicles containing Aβ might shuttle within TNT structures. Alternatively, Aβ oligomers could surf along TNTs upon insertion in the lipid bilayer. Further study is required to specifically address this question. Remarkably, we have been able to detect the presence of tau short fibrils within TNTs between neuronal cells (S. Abounit and C. Zurzolo, unpublished work), pointing to a more general role for TNTs in the spreading of protein aggregates (see also below).

Molecular mechanism of Htt cell-to-cell transfer

By contrast with the oligomeric species of α-syn, tau and Aβ described above, in the case of mutant Htt, fibrillar aggregates do not appear to be associated with membrane vesicles upon internalization after challenging HEK-293 cells [89] (Figure 2, top right-hand panel). Deep-etch transmission electron microscopy revealed that aggregates of synthetic polyQ peptides or recombinant fragments of the protein localize directly to the cytosolic compartment where they appear to be attached to the actin cortex in the absence of any surrounding endomembranous structure [89]. Consistently, a strong co-localization was observed by immunofluorescence with ubiquitin, proteasome subunits and Hsp70, all cytosolic 'quality control' components, but not with markers of endosomes, lysosomes or autophagosomes [89]. These data indicate that polyQ aggregates in the state of fibrils do not accumulate in an endosomal compartment, but that they are able to directly penetrate biological membranes thereby gaining access to the cytosol [89] (Figure 2, top right-hand panel, and Table 1).

In HD, deposition of protein aggregates is an early event in the pathogenic cascade and precedes neurodegeneration. A more recent study from Kopito and colleagues has shown that internalization of polyQ fibrils depends on both trypsin-sensitive and -insensitive saturable sites on the cell surface, thus implicating the involvement of as yet unidentified cell surface proteins in this process [190]. Binding to the cell surface and cytoplasmic entry into recipient cells did not appear to be dependent on electrostatic interactions at the level of the plasma membrane [190]. Fibrillar aggregates enriched in β-sheets seem to bind preferentially to the cell surface compared with amorphous aggregates enriched in α-helices, suggesting an important role for β-sheet-rich assemblies in the interaction and the consequent internalization of polyQ species [190].

So far, it has not been explored whether and by which mechanism small oligomeric assemblies or even monomeric species of mutant Htt might transfer between cells and which fate they might follow in the recipient cells. Monomers and oligomers diffuse much more easily than large fibrils and therefore they are better candidates for efficient transfer between neighbouring cells. One way to address this question *in vitro* could be the use of BiFC constructs, a fluorescence microscopy technique based on the use of two halves of a fluorescent protein fused to interacting proteins of interest [191]. Such an approach has

been recently taken by Herrera et al. [90] who fused an exon 1 mutant Htt fragment to the non-fluorescent half of the Venus protein to assess transmission of Htt between cells. Because Venus becomes functionally reconstituted and emits fluorescence only when Htt dimerizes inside the cells, they could show that the Htt mutant fragment had passed between co-cultured cells. Although this study did not address specifically the cell-to-cell transfer of mutant Htt either in terms of protein species (i.e. monomers compared with oligomers) or intercellular trafficking pathways involved, it showed that this model can be a powerful tool to study the dynamics of mutant Htt transfer between cells [90].

Recently we have begun to address this question in neuronal co-cultures and found that transfer of mutant exon 1 Htt aggregates (GFP–480-68Q) occurs quite efficiently between intact, viable neurons (M. Costanzo and C. Zurzolo, unpublished work). This suggests an active transfer mechanism that could contribute to the early stage of HD pathogenesis and to the progression of the disease in the brain. However, in later stages spreading of the aggregates upon their passive release from dead or dying cells, as suggested [6,89], can also be envisaged and might contribute further to the progression of the disease.

We addressed specifically the transfer mechanisms and found that in our experimental setting intercellular transfer between co-cultured neuronal cells and in primary neurons does not occur through the supernatant, but requires cell-to-cell contact, thus excluding an exo/endo-cytic mechanism. We also showed that Htt aggregates formed within one cell access the cytoplasm of neighbouring uninfected cells via TNTs (M. Costanzo and C. Zurzolo, unpublished work) (Figure 2, top right-hand panel, and Table 1), similarly to what was previously shown for PrP^{Sc} [188], Aβ particles [84] and tau (S. Abounit and C. Zurzolo, unpublished work). Because polyQ aggregates are cytosolic or nuclear, and do not appear to be associated with membrane vesicles upon internalization [89], a passage in the form of aggregate through TNTs can be envisaged. In addition, Htt can interact with acidic phospholipids enriched on the cytoplasmic leaflet of the plasma membrane [192,193], therefore a surfing process along the TNT membrane could also be possible [187]. Further research is clearly necessary to understand the mechanisms by which these proteinaceous assemblies hijack TNTs.

FROM PROTEIN AGGREGATION TO NEUROTOXICITY: AN IMPERFECT FIT

The existence of a direct relationship between propagation of protein aggregates and neurotoxicity remains elusive. In most neurodegenerative diseases protein aggregate deposition does not appear to be associated with neuronal disfunction and degeneration. In PD patients there is no evidence of neuronal loss in transplanted tissues despite the presence of Lewy body aggregates [47,49,194], whereas in a mouse model of PD host-to-graft transfer of α-syn resulted in caspase 3 activation [53]. In the case of HD, autopsy of transplanted patients revealed that fetal grafts of striatal tissue were susceptible to neurodegeneration, displaying increased caspase 3 activation, vacuolization and decreased structural integrity in the absence of abnormal Htt aggregation [93]. Tau and Aβ aggregate spreading in mouse models was not associated with neuronal loss [69,79].

One approach to determine the relationship between aggregate propagation and neurodegeneration would be to identify the molecular species that are responsible for propagation of disease-linked protein misfolding and for neurodegeneration. Indeed, several studies suggest that the formation of large protein aggregates is beneficial for cell survival. Evidence from several independent studies of different proteins indicates that oligomers might be the most toxic species in pathogenesis [195–197]. Soluble oligomers are small assemblies of misfolded proteins that are present in the soluble fraction of tissue extracts and usually include structures ranging in size from dimers to 24-mers [196]. Interestingly, both synthetic and natural oligomers have been shown to induce apoptosis in cell cultures at very low concentrations [198–200], as well as to block long term potentiation in brain slice cultures [201] and to impair synaptic plasticity and memory in animals [202,203]. Fibrils can also elicit toxicity in cultured cells, albeit at much higher concentrations than oligomers and protofibrils [195]. Specifically in the case of prion diseases the infectious agent has been clearly distinguished from the toxic one [204–206]. Exploring whether and by which mechanism small soluble oligomeric assemblies or even monomeric species of the disease-associated proteins transfer misfolding and trigger toxic cascade events in recipient cells is fundamental for the understanding of the molecular basis of disease progression and for the development of more effective therapies.

CONCLUDING REMARKS AND PERSPECTIVES

In the present review we have shown that a large body of data suggest that both cell-autonomous and non-cell-autonomous processes might have a role in the pathogenic cascades of different neurodegenerative diseases. Progressive accumulation of protein misfolding can be the result of events occurring separately in single cells and/or prion-like transmission mechanisms that alter cell-to-cell communication between cells. More studies *in vivo* are required to understand how, to what extent and at which stage of the disease the cell-autonomous and non-cell-autonomous mechanisms contribute to disease progression. Crucial questions that need to be addressed are which species are toxic *in vivo* and whether it is possible to link propagation of protein misfolding (in a prion-like manner) and neurodegeneration.

In addition to *in vivo* studies it is essential to develop better model systems to study the transfer of amyloidogenic proteins in cell culture. This will allow a clear understanding of the mechanisms of protein aggregate spreading at the cellular and molecular levels, thus contributing to the understanding of the disease and to the development of novel therapies. Because infectious prions [188], Aβ [84] and polyQ aggregates (M. Costanzo and C. Zurzolo, unpublished work) transfer from cell-to-cell through TNTs, it is tempting to speculate that they might constitute a general mechanism for the spreading of different β-sheet-enriched proteinaceous aggregates [187]. Exploring the mechanisms by which cells form TNTs and how the transfer of material is regulated within these structures is essential for a better understanding of the mechanisms of aggregate spreading. Conversely, the identification of specific TNT markers allowing the characterization of these structures in tissues and organs is critical to confirm their role in the progression of protein misfolding *in vivo* [187].

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