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Review

The interplay between autophagy and unconventional secretion in neurodegeneration

Maurizio Renna,[1,2,*] Raffaella Bonavita,[2] Grace Dixon,[1] Luigi Vittorio Verdicchio,[2] and Angeleen Fleming[1,3,4,*]
[1]Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK
[2]Department of Molecular Medicine and Medical Biotechnologies, University of Naples "Federico II", 80131 Naples, Italy
[3]Department of Medical Genetics, University of Cambridge, Cambridge Institute for Medical Research, The Keith Peters Building, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0XY, UK
[4]UK Dementia Research Institute, University of Cambridge, Cambridge Institute for Medical Research, The Keith Peters Building, Cambridge Biomedical Campus, Hills Road, Cambridge CB2 0XY, UK
*Correspondence: maurizio.renna@unina.it (M.R.), af425@cam.ac.uk (A.F.)
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SUMMARY

Within neurons, the misfolding and aggregation of certain proteins has been identified as a common feature of many late-onset neurodegenerative diseases (NDs). These aggregate-prone proteins include tau (in both primary tauopathies and in Alzheimer's disease) and alpha-synuclein in Parkinson's disease. There is strong experimental evidence that the upregulation of intracellular clearance pathways (autophagy and ubiquitin-proteasome pathways) can clear aggregate-prone proteins in experimental models. When the flux through these pathways is increased, the levels of aggregate-prone proteins are reduced, resulting in improved cell survival in both cell-based and animal models of ND. More recently, a third strategy for clearing proteins from cells has been identified, via the unconventional secretion of proteins out of the cell. However, secretion may also facilitate the spreading and propagation of disease through a prion-like process. This review explains how the autophagy and unconventional secretion pathways interact and how these impact ND.

INTRODUCTION

Autophagy is a cellular process that involves the degradation of cellular components, including proteins, via lysosomes. It is a crucial pathway for eliminating damaged or misfolded proteins, including those that are prone to aggregation.[1] This process is essential for numerous physiological functions, including adaptation to cellular stress, immune regulation, and metabolic homeostasis.[2] Autophagy plays a significant role in clearing both soluble and aggregated forms of aggregation-prone proteins, which are a hallmark feature of neurodegenerative diseases (NDs). Examples include alpha-synuclein (aSyn) in Parkinson's disease (PD), amyloid-beta (Aβ) and tau in Alzheimer's disease (AD), and mutant huntingtin (HTT) in Huntington's disease (HD). However, when autophagy is impaired, or its components are dysfunctional, aggregation-prone proteins can accumulate, leading to their misfolding and aggregation, potentially causing cellular damage and disease.[1]

Although there are currently no effective strategies that slow or prevent these NDs in humans, there is strong experimental evidence that the upregulation of intracellular clearance pathways (the autophagy-lysosome and ubiquitin-proteasome pathways) can clear aggregate-prone proteins in experimental models. When the flux through these pathways is increased, the levels of aggregate-prone proteins can be reduced and this results in improved cell survival in both cell-based and animal models of NDs.[3–6] More recently, a third strategy for clearing proteins from cells has been identified, which occurs via the unconventional secretion of proteins out of the cell. Protein secretion can occur via the canonical endoplasmic reticulum (ER)-to-Golgi secretory pathway or by means of non-classical pathways. The canonical pathway involves signal peptide-containing proteins that are directed to the ER, undergo post-translational modifications in the Golgi, and are transported via coat protein complexes and Rab protein-mediated anterograde vesicular transport.[7–9] In contrast, unconventional protein secretion (UPS) bypasses the Golgi and includes proteins lacking signal peptides or transmembrane proteins. UPS consists of four classes: direct translocation through plasma membrane pores, ABC transporter-mediated secretion, autophagosome/endosome-based secretion, and Golgi-independent secretion of membrane proteins. UPS is often triggered by cellular stress, activating alternative secretion mechanisms.[10,11] One route that has become a particular focus for ND research is the generation of extracellular vesicles (EVs). Interestingly, recent work has shown that these EVs can be taken up by neighboring cells.[12] Hence, the contents/cargoes of these EVs can be transferred into recipient cells, thereby allowing cell-to-cell communication. Within the context of NDs, this spreading of cargoes could have two opposing consequences: on one hand, this could be a route for clearing misfolded proteins from neurons, and the uptake of EVs by astrocytes or microglia may aid the removal of such aggregate-prone proteins. Alternatively, the spreading of misfolded and/or aggregate-prone proteins to surrounding neurons

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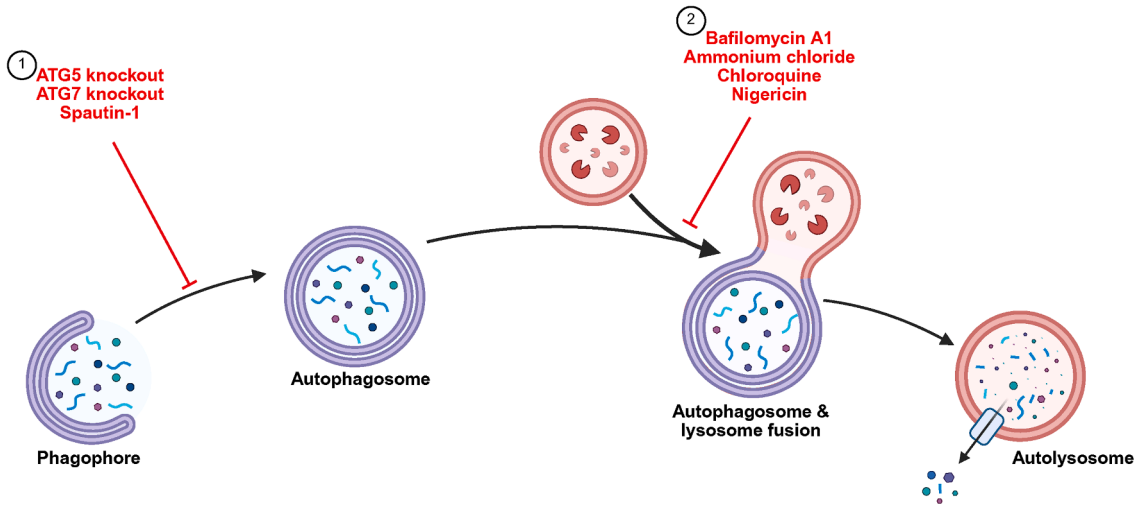


Figure 1. The autophagic pathway

During autophagosome biogenesis, a phagophore (or isolation membrane) forms within the cytoplasm. The phagophore expands, eventually enclosing cargo in a double-membraned autophagosome. Autophagosome biogenesis occurs at steady state but can be induced in response to a variety of cellular stress conditions such as nutrient deprivation. The mature autophagosome fuses with a lysosome, forming an autolysosome. The fusion event allows the lysosomal hydrolases to access the autophagosome contents. These hydrolases then break down the inner autophagosome membrane and degrade the sequestered contents (proteins, damaged organelles, and other cytoplasmic contents). The products of this digestion (amino acids, monosaccharides, and fatty acids) are subsequently released back into the cytoplasm. Initiation of autophagy can be inhibited (1) by knockdown/knockout of core autophagy genes or by the use of pharmacological inhibitors (examples used in the main text are shown). This prevents autophagosome formation. Autophagosome fusion with the lysosome can be inhibited (2) by lysosomotropic agents, such as Bafilomycin A1. Typically, these agents increase the lysosomal pH and result in a blockage of autophagy and a build-up of autophagosomes. Created in BioRender. Fleming, A. (2026) https://BioRender.com/nhe6smk.

may lead to the spreading and seeding of aggregates. Hence, UPS may complement classical degradation pathways, but also play a significant role in the pathophysiology of neurodegenerative diseases.

In this review, we provide an overview of the processes of autophagy and unconventional secretion and discuss the experimental evidence demonstrating how these two pathways interact. We focus on the recent advances in understanding the mechanisms regulating UPS, and how the secretion of ND-associated aggregate-prone proteins affects disease pathology.

AUTOPHAGY AND UNCONVENTIONAL SECRETION

Autophagy: mechanisms and regulation

Autophagy (from the Greek words auto "self" and phagein "eating") is a tightly regulated catabolic process by which cells degrade and recycle aggregate-prone proteins and damaged organelles to maintain cellular homeostasis. Autophagy is a "self-eating" mechanism fundamental for cell survival and development, and its dysregulation has been implicated in various diseases, including cancer and neurodegeneration.[1,13] There are three major types of autophagy: (macro)autophagy, (micro)autophagy, and chaperone-mediated autophagy (CMA). The most studied is (macro)autophagy, which is often referred to simply as autophagy and is the major focus of this review. Autophagy involves the formation of double-membrane vesicles called autophagosomes. In the first step of autophagosome formation, cytoplasmic constituents, including organelles, are sequestered by a unique membrane called the phagophore or isolation membrane. Elongation of the ends of the phagophore edges and their eventual fusion result in the formation of the autophagosome, a double-membrane structure, in which engulfed cytoplasmic material and cellular components are trapped. The autophagosomes traffic to and fuse with lysosomes, forming autolysosomes. The exposure of engulfed cargoes to lysosomal enzymes results in their degradation (Figure 1). Although autophagy was initially observed in the 1960s, significant progress in understanding its regulation began in the 1990s with the identification of the autophagy-related genes (*ATG* genes).[14] Screens performed in yeast identified a set of genes essential for survival in low nutrient conditions (the *Apg* genes), with their mammalian homologues, the *ATG* genes[14] identified soon after.

The autophagy process is mediated by autophagy proteins (encoded by *ATG* genes) and can be divided into five sequential steps: initiation; double-membrane nucleation and formation of a pre-autophagosome or phagophore; phagophore elongation and sequestration of cytoplasmic cargo; fusion of the autophagosome to a lysosome; degradation of sequestered cargo within the lysosomal compartment (Figure 1). Initiation of autophagy requires the ULK1 kinase complex, which is regulated by AMPK and mTOR. AMPK activates ULK1 through direct phosphorylation. The ULK1 complex, consisting of ULK1, FIP200, ATG13 and ATG101, stimulates the class III phosphatidylinositol 3-kinase (PIK3C3) complex, which is composed of VPS34, VPS15, Beclin-1, ATG14L/BARKOR, and p150. Once the phagophore is formed, nucleation occurs, enabling the recruitment of essential proteins and lipids. VPS34 generates phosphatidylinositol-3-phosphate (PI3P) at the phagophore, which is instrumental for the recruitment of WIPI proteins and the ATG5-ATG12-ATG16L1 ternary complex, as well as the delivery from different membranes of the ATG9-positive precursors.

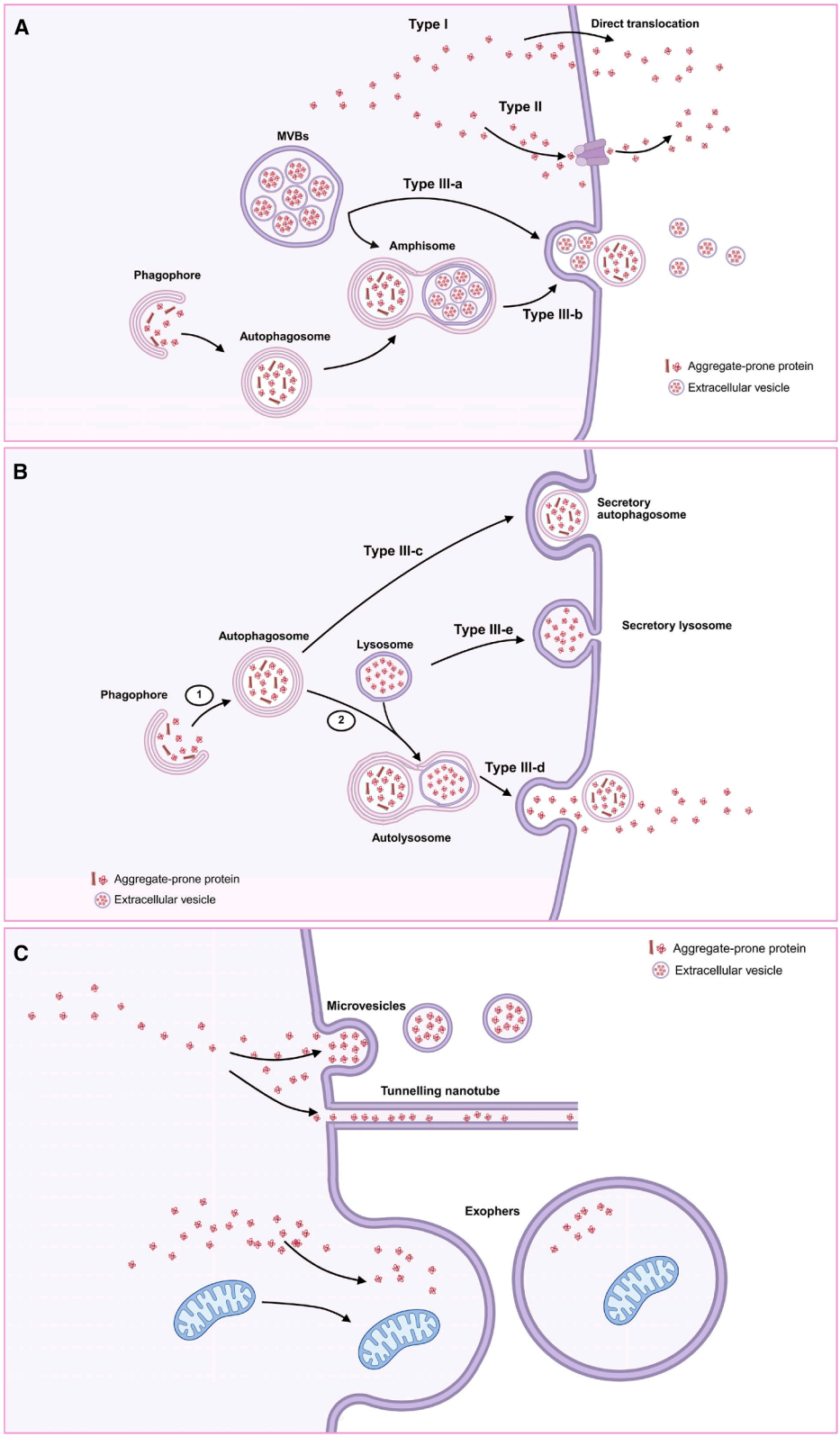


Figure 2. Unconventional protein secretion routes and intersections with the autophagic pathway

(A) In addition to the canonical ER-to-Golgi secretory pathway to the plasma membrane, proteins can follow unconventional secretion pathways. Leaderless proteins can be secreted by translocating across the plasma membrane (Type I) or through a pore on the plasma membrane (Type II). Type III unconventional

(legend continued on next page)

Beclin-1 plays a crucial role in this complex, acting as a regulatory hub whose interactions with various binding partners can either promote or suppress autophagy.[15] Elongation and closure of the autophagosome membrane requires the coordinated activity of two conjugation systems; the ATG12-ATG5-ATG16L1 ternary complex and the LC3 lipidation machinery. In the latter, ATG4 protease cleaves LC3 to generate cytosolic LC3-I, which is further recognized by ATG7, ATG3, and ATG12-ATG5-ATG16L1 ternary complex, leading to its conjugation to phosphatidylethanolamine on the phagophore membrane, forming LC3-II. LC3-II is widely used as a marker for autophagosome formation. LC3-II binds to LC-3 interacting region (LIR)-containing autophagy receptors, such as p62/SQSTM1, bound to cargo targeted for degradation. Fusion of mature autophagosomes with lysosomes is mainly mediated by the assistance of Rab GTPases, SNARE proteins, and the homotypic fusion and protein sorting (HOPS) complex. Upon fusion, lysosomal hydrolases degrade the inner membrane and enclosed cargo, and the degradation products can be recycled by the cell, whereas LC3-II bound to the outer membrane is cleaved by ATG4 to be reused.[16]

Autophagy regulation is tightly controlled by nutrient availability, energy levels, and stress signals. The most typical trigger of autophagy is nutrient starvation, particularly the deprivation of key nutrients such as amino acids, glucose, and lipids. Multiple key pathways and molecules play a role in regulating autophagy, with the mTOR signaling pathway being one of the primary and most effective regulators in dictating the activity of the pathway. In particular, the rapamycin-sensitive mTORC1 complex phosphorylates and inhibits the ULK1 complex, suppressing autophagy. Under stress or nutrient deprivation, mTORC1 is inhibited, which removes the suppression of ULK1, leading to autophagy activation.[15]

The inhibitory action of mTORC1 can be effectively counterbalanced by AMP-activated protein kinase (AMPK), which is an energy sensor that activates autophagy in response to low cellular ATP levels and glucose deprivation.[17] AMPK directly phosphorylates ULK1, promoting autophagy initiation. Under nutrient starvation, several transcription factors, such as TFEB and FOXO3A, can regulate the expression of autophagy-related genes. Under conditions of oxidative stress or growth factor deprivation, FOXO transcription factors can induce the up-regulation of autophagy-related genes.[18] Upon activation, TFEB translocates from the lysosomal membrane (where it is confined at steady-state by physical association to the mTORC1-Rag Regulator complex) to the nucleus, up-regulating genes associated with both autophagosome formation and lysosome biogenesis.[19–21]

Unconventional protein secretion and the interplay with autophagy

Conventional secretion follows a pathway that begins in the ER, where proteins destined for secretion are synthesized and initially folded while undergoing quality control. Once properly folded, signal peptide-containing proteins are transported to the Golgi apparatus for further processing, including modifications such as glycosylation. From the trans-Golgi network, vesicles carrying the processed proteins bud off and are targeted either to the plasma membrane or to endosomal compartments.[22] Proteins that do not contain signal peptides (“leaderless” proteins), are secreted by non-canonical mechanisms, known as unconventional protein secretion (UPS), that bypass the Golgi.[10,11] This process has been observed in a variety of organisms and is associated with multiple cellular functions, including, among others, immune regulation, intercellular communication, and tissue remodeling. Leaderless proteins can be secreted along three pathways, which are mediated by different mechanisms: pore-mediated translocation across the plasma membrane (Type I); proteins with a signal peptide and/or a transmembrane domain that enter the ER but bypass the Golgi apparatus and are secreted by ABC transporter (Type II); and autophagosome/endosome-based secretion (Type III)[11] (Figure 2). A fourth route, the Type IV pathway, does not lead to secretion *per se*, but describes the process by which some transmembrane proteins are routed to the plasma membrane without passing through the Golgi apparatus (also known as Golgi-bypass). These pathways can be broadly categorized as involving the direct secretion of proteins which lack a signal sequence across the plasma membrane (Types I and II) and routes that involve the secretion of cytosolic contents (not limited to proteins) via membrane-bound structures (Type III). Since there is little evidence of any interplay between autophagy and Type II and Type IV secretion, they are not covered further here.

In the Type I pathway, the formation of plasma membrane pores allows cytoplasmic cargos known as cytoplasmic leaderless proteins to translocate across the plasma membrane. FGF2 and HIV TAT are constitutively secreted, and their unconventional secretion is not stimulated by stress. These proteins form a self-made lipidic pore that allows them to directly translocate across the plasma membrane to reach the extracellular space (Figure 2A, Type I). FGF2 and TAT interact with PI(4,5)P2, self-oligomerize, and their oligomerization drives membrane insertion and pore

protein secretion requires a membrane-bound organelle carrier, such as exosomes generated in MVBs (III-a), which are released by fusion at the plasma membrane, or amphisome-mediated secretion (III-b) or via secretory autophagy routes shown in (B).
(B) Once the phagophore (1) has formed, the maturation, trafficking, and intracellular transport of autophagosomes is dictated by molecular determinants associated with the outer membrane (see main text for details). In normal conditions, the autophagosome will be transported to and fuse with the lysosomal compartment to generate the autolysosome (2), which allows the degradation of autophagosome content. During UPS, secretory autophagosomes can fuse either with MVBs to form a hybrid structure known as an amphisome (shown in A, Type III-b) or directly with the plasma membrane (Type III-c). In addition, autolysosomes can fuse with the plasma membrane (Type III-d). Finally, lysosomes can fuse with the plasma membrane, releasing their contents via secretory lysosome-mediated plasma membrane fusion (Type III-e).
(C) Cytosolic components can also be secreted in microvesicles (0.1–1.0 μm diameter) formed from blebbing of the plasma membrane, which subsequently become pinched off. Larger extrusions of the plasma membrane generate larger vesicles termed exophers (1–50 μm diameter). These can contain organelles and large protein complexes, as well as aggregated and soluble proteins, and other cytoplasmic components. Tunneling nanotubes (TNTs) are also formed by cytoskeletal protrusions from the plasma membrane, but form as thin tubules rather than vesicles. TNTs can extend between two cells, allowing transfer of cytosolic contents, including ions, free proteins, nucleic acids, vesicles, and organelles. Diagrams not drawn to scale. Created in BioRender. Fleming, A. (2026) https://BioRender.com/v05i4vn.

Table 1. Examples of cytosolic components and cargo proteins undergoing autophagy-dependent and -independent unconventional secretion

Protein	Secretion/Transfer mechanism
IL-1β	EVs (including exosomes)[28,29] ATG/LYSO[30] micro-vesicle shedding[31]
FGF2	direct transfer[24]
Galectins	direct transfer[32,33] EVs (including exosomes)[34]
Annexins	direct transfer[35] EVs (including exosomes)[36]
HMGB1	ATG/LYSO[30,37]
Acb1	ATG/LYSO[38]
PGK1	direct transfer[39]
HIV-TAT	direct transfer[23,40] EVs (including exosomes)[41]
FABP4	EVs (including exosomes)[42] ATG/LYSO[27]
HSPBs	EVs (including exosomes)[43–45] ATG/LYSO[46]
ATG8/LC3	ATG/LYSO[47]
Transferrin receptor	ATG/LYSO[48]
Mitochondria	EVs (including exosomes)[49,50]
Histones	EVs (including exosomes)[51]

formation.[23,24] Translocation across the plasma membrane is also triggered by inflammation. Secretory autophagy is implicated in the release of pro-inflammatory cytokines, particularly IL-1β and IL-18, both of which play crucial roles in immune responses. This function has been observed in macrophages through the hyper-permeabilization of the macrophage plasma membrane, which precedes cell lysis.[25] When inflammation is the trigger, IL-1β secretion utilizes the Type I pathway. However, when nutrient deprivation (i.e., starvation) is the trigger, IL-1β secretion occurs through the Type III pathway via secretory autophagosomes[25] (Figure 2B, Type III, route c).

Secretion through the Type III pathway is dependent on the formation and release of vesicular structures (Figures 2A and 2B). In the Type III pathway, upon stress induction, membrane-bound organelles are diverted from their normal function and become secretory. These vesicular structures are proposed to be derived from endosomes (Figure 2A, Type III routes a & b), autophagosomes and/or lysosomes (Figure 2B, Type III, routes c-e).[26] The signal peptide-lacking protein FABP4 is secreted by adipocytes after nutrient deprivation. Its secretion is independent of the conventional ER-Golgi secretory pathway and is mediated by both endosomes and secretory lysosomes.[27] Further examples of cytosolic components are provided in Table 1.

There are three additional routes by which cytosolic components are secreted (Figure 2C), and which share a common mechanism of being driven by cytoskeletal rearrangements. Cytosolic components can become trapped in microvesicles (0.1–1.0 μm diameter). These form from blebs which initially occur on the outer surface of the plasma membrane and then become pinched off. Cytosolic contents can also be transferred between cells over distances of up to 100 μm via tunneling nanotubes (TNTs). These are formed by cytoskeletal protrusions from the plasma membrane, which can either have closed or open ends, the latter allowing cell-to-cell transfer of cytosolic contents, including ions, free proteins, nucleic acids, vesicles, and organelles.[52] Recently, an additional population of vesicles has been identified, termed exophers. Whilst these structures share similarities with microvesicles, in that they arise from extrusions of the plasma membrane, exophers are considerably larger (2–15 μm diameter) and hence can contain organelles, large protein complexes as well as aggregated and soluble proteins, and other cytoplasmic components. Exophers were first observed in *C. elegans* and were shown to be extruded from neurons of adult nemotodes, and their production was enhanced in response to proteasome and autophagy inhibition, suggesting a compensatory role for removing proteins and damaged organelles when intracellular clearance mechanisms are perturbed.[53]

Therefore, although autophagy and secretion were once considered largely independent processes, with autophagy focused on degradation and secretion dedicated to biosynthesis and transport, the discovery that UPS as part of normal cellular physiology has led to a growing body of research demonstrating shared regulators and molecular crosstalk between these pathways. This is particularly evident under stress conditions, highlighting an intricate balance between degradation and export in cellular homeostasis.

Mechanisms regulating secretory autophagy

A specialized form of autophagy, defined "secretory autophagy," can contribute to the regulated transport and release of cytosolic proteins into the extracellular environment. Like conventional autophagy, secretory autophagy begins with the formation of autophagosomes. However, these bypass the terminal fusion with the lysosomal compartment and are rather diverted either directly to the plasma membrane or to multivesicular bodies or secretory autolysosomes, to ultimately allow the release of their content into the extracellular space.[54] Soluble NSF Attachment Protein Receptors (SNAREs) and Rab GTPases are essential for mediating vesicular trafficking in both autophagy and unconventional secretion. For instance, the SNARE protein syntaxin-17, which is involved in the fusion of autophagosomes with lysosomes, also plays a role in regulating exocytosis. Similarly, Rab GTPases, particularly Rab8 and Rab11, function in both autophagosome transport and secretory pathways, highlighting their dual roles in intracellular trafficking.

Cargo proteins targeted for secretory autophagy typically bear distinct structural motifs or post-translational modifications that signal their entry into this pathway. Notable examples include IL-1β[55] and galectins,[56,57] which are recognized to be secreted via this mechanism. In secretory autophagy, cargo selection is often regulated by specific adaptor proteins or receptor-ligand interactions that recognize proteins destined for secretion. Selective recruitment to autophagosomes by specific mechanisms involves chaperones, lysosomal membrane proteins, or components of the ESCRT machinery. Equally, cytosolic proteins that are ubiquitinated or phosphorylated can be recognized by selective autophagy receptors.[58] Autophagy receptors such as p62/SQSTM1, NBR1, TOLLIP, and Optineurin are well known for mediating autophagic degradation. However, some of these receptors can also identify and bind specific cargoes destined for

secretion, directing them into autophagosomes for export rather than degradation.[54] A common feature of these autophagy receptor proteins is the presence of a protein domain, termed the LC3-interacting region (LIR), which binds to LC3 or other ATG8 proteins allowing the selective recruitment and packaging of cargoes into autophagosomes.[59–62] Following cargo loading, autophagosomes designated for secretion avoid lysosomal degradation and instead fuse with the plasma membrane, releasing a single-membraned vesicle into the extracellular environment.[54]

The ER and Golgi apparatus act as a key interaction hub in coordinating autophagy and secretion through inter-organelle crosstalk. Notably, the ER-to-Golgi Intermediate Compartment (ERGIC) serves as a membrane source for autophagosome biogenesis.[63,64] Additionally, TMED10 has been identified as a translocator of leaderless cytosolic proteins to the ERGIC, from which these proteins are subsequently incorporated into autophagosomes.[65,66] Furthermore, secretory vesicles derived from the Golgi can often be redirected to the autophagic pathway under stress conditions, highlighting a dynamic interchange.[67] Sec22b, a protein involved in trafficking between the ER and the Golgi, is also involved in unconventional secretion, regulating the cargo loading in autophagosomes and promoting their secretion at the plasma membrane.[68] During ER stress induced by ER-to-Golgi transport blockade, ER-phagy receptors ATL3 and RTN3L induce the formation of ER tubulovesicular structures (ER-TBs).[69] Misfolded CFTR protein is retained in the ER, and ΔF508-CFTR cannot reach the cell surface via the conventional ER-to-Golgi secretory pathway; however, the ER form of core-glycosylated ΔF508-CFTR can be delivered to the cell surface via the activation of a Golgi-independent UPS pathway.[70] ER-TBs are involved in the stress-induced UPS of ΔF508-CFTR.[69] These structures also facilitate the Golgi-independent transport of the SARS-CoV-2 spike protein, since the SARS-CoV-2 S protein does not appear to travel solely via the Golgi-dependent route.[69,71]

Multiple signaling pathways regulate secretory autophagy, including mTOR, AMPK, and ULK1. While mTOR is a central regulator of canonical autophagy in response to nutrient availability, it can also influence the secretory pathway, particularly in controlling the biogenesis of secretory vesicles and their exocytosis.[72] Secretory autophagy can also be triggered via mTOR-independent pathways, such as the JNK pathway, especially during stress-induced secretion.[73] AMPK, a metabolic sensor that activates autophagy during energy stress, also modulates protein trafficking and secretion.[74] In response to nutrient availability, mTORC1 phosphorylates GRASP55, which keeps it localized to the Golgi apparatus. However, during nutrient starvation and mTORC1 inhibition, GRASP55 becomes dephosphorylated and can move from the Golgi to other locations, such as autophagosomes and MVBs, and facilitates their secretion.[75] The TOR-autophagy spatial coupling compartment (TASCC) is a specialized cellular compartment facilitating the biosynthesis of secretory proteins such as interleukin-6/8. Pro-inflammatory signals such as TNF-α and IL-1β further enhance secretory autophagy, often requiring autophagy-related proteins ATG5 and ATG7.[73,76]

Disruption of the autophagic degradation pathway is closely linked to the modulation of exosome secretion. Secretory autophagy during lysosome inhibition (SALI) is a novel secretory autophagy pathway that employs EVs and nanoparticles and acts as a mechanism to preserve protein and cellular homeostasis when autophagic or lysosomal degradation is impaired.[77] Upon endo-lysosomal dysfunction or impaired autophagosome maturation of autophagic cargo receptors, these components can be loaded into EVs and released to prevent their intracellular accumulation. As such, this secretion route is highly regulated and relies on multiple ATGs required for autophagosome formation, as well as the small GTPase RAB27A.[78] For example, chloroquine induces the accumulation of Atg8 orthologs and autophagy receptors inside small EVs in a lipidation-dependent manner, suggesting that EV composition varies in response to lysosomal inhibition.[47] Rubicon, a negative regulator of autophagy, recruits WIPI2d to endosomes to promote exosome biogenesis. Notably, Rubicon and WIPI2d are responsible for the age-dependent variation in exosomal composition from both the quantitative and qualitative standpoint.[79] In addition, the analysis of the interstitial fluid content of EVs in a murine cancer model found elevated levels of EV-associated proteins specifically involved in mitophagy and secretory autophagy.[49] These observations reinforce the connection between autophagy, unconventional secretion, and EVs. Secretory autophagy not only regulates EVs secretion but, in some cases, components of the autophagic pathway can themselves be secreted through the same mechanism.

In addition, secretion via the formation and release of exophers is also tightly linked with autophagy regulation. Neuronally restricted knockdown of early acting autophagy genes in *C. elegans* leads to increased exopher formation and is associated with increased lifespan.[80] Importantly, this association was only observed with knockdown of early-acting and not late-acting autophagy genes (i.e., genes involved in autophagy initiation and autophagosome formation rather than those involved in autophagosome-lysosome fusion and cargo degradation). Although numerous studies have shown that autophagy upregulation is associated with increased lifespan (reviewed in[81]), these apparently contradictory findings may be explained by the additional non-canonical roles of proteins involved in autophagosome biogenesis such as in phagocytosis and secretion.[82] Although initially characterized in *C. elegans*, recent studies have observed exophers containing damaged mitochondria released from mammalian cardiomyocytes and taken up by surrounding macrophages.[83] Similarly, exopher-like structures have been found in vertebrate optic nerve axons involved in the secretion of dysfunctional mitochondria, enabling their uptake and degradation by astrocytes.[84]

Extracellular vesicle biogenesis

EVs are small, membrane-bound particles secreted by cells into the extracellular environment. These vesicles play a crucial role in intercellular communication and have emerged as key mediators in various physiological and pathological processes.[85,86] EVs are classified into different subtypes, including exosomes (30–150 nm in diameter), microvesicles (100-1,000 nm), secreted autophagosomes (1–2 μm), exophers (2–15 μm), and apoptotic bodies, based on their size, biogenesis, and molecular composition. They carry a diverse cargo of bioactive molecules such as proteins, lipids, RNA, and DNA, reflecting the cell of origin and its state.

The ESCRT machinery is involved in MVB biogenesis and is responsible for the sorting of ubiquitinated proteins into intraluminal vesicles. ESCRT-0 recognizes and retains ubiquitinated proteins in the late endosomal membrane, then ESCRT-I/II triggers the initial involution of the limiting membrane into the MVB lumen and forms a spiral-shaped structure that constricts the budding neck, where the ATPase VPS4 drives membrane scission.[87,88] The interaction between syndecan and ESCRT mediates exosome formation. The small cytosolic adapter protein syntenin connects syndecan to the ESCRT-III-associated protein ALIX. Syndecan clustering stimulates syntenin-ALIX-ESCRT-mediated sorting and exosome production.[89–91]

EVs have garnered significant attention for their roles in modulating immune responses, promoting tissue repair, and influencing cancer progression. They also serve as potential biomarkers for disease diagnosis and prognosis, owing to their presence in various biological fluids such as blood, urine, saliva, and cerebrospinal fluid (CSF).[92,93] In addition, the most recent advancements in EV research have highlighted their therapeutic potential, particularly in drug delivery systems, regenerative medicine, and as targets for therapeutic interventions. As our understanding of EVs deepens, they offer exciting opportunities to unravel complex biological pathways and develop innovative clinical applications. For this reason, it is important to characterize the molecular regulators of biogenesis and the delivery of these vesicles. By doing so, it will be possible to identify novel druggable targets.

UNCONVENTIONAL SECRETION OF NEURODEGENERATIVE DISEASE-ASSOCIATED AGGREGATE-PRONE PROTEINS

Within neurons, the misfolding and aggregation of certain proteins have been identified as a common feature of many late-onset NDs.[3] Upregulation of autophagy has been demonstrated to be effective for ND-associated aggregate-prone proteins in both cell and animal models and is the focus of a variety of novel therapeutic approaches.[3,94] However, our growing understanding of the interplay between autophagy and other protein clearance routes (e.g., the ubiquitin-proteasome system (reviewed in[94,95]) and with UPS, highlights the need for an holistic approach to assess how changes in one pathway might affect others. Autophagic flux declines with age, and disease-associated mutations in aggregate-prone proteins are also known to compromise steps in the autophagy-lysosome clearance pathway.[1] In addition, many of the proteins identified by Genome-Wide Association Studies (GWASs) that are risk variants for different NDs play functional roles in autophagosome trafficking and clearance.[1] The evidence for the different routes of unconventional secretion of the major ND-associated aggregate-prone proteins is summarized in Table 2. Many of these studies rely on the over-expression of the aggregate-prone protein, and therefore need to be interpreted with caution, since over-expression in itself may alter unconventional secretion routes. This is a very important consideration, since the secretion of these proteins via various alternative routes has been demonstrated to be able to induce seeding and spreading in experimental models. Here, we focus on the experimental evidence for the unconventional secretion of endogenous aSyn and tau and how this is affected by autophagy.

aSyn and tau are two of the most extensively studied aggregate-prone proteins and are associated with the most prevalent NDs, namely Parkinson's disease (PD) and Alzheimer's disease (AD). aSyn is widely expressed in neurons, and its misfolding and aggregation is observed in PD and other NDs collectively known as Lewy Body Diseases.[118] The intracellular accumulation and aggregation of tau is the major hallmark of NDs termed tauopathies. Primary tauopathies, where accumulation and aggregation of tau is the primary disease driver, include Pick's disease, progressive supranuclear palsy, corticobasal degeneration, and argyrophilic grain disease. In secondary tauopathies, such as AD, tau accumulation is an important driver of neurodegeneration but its accumulation is thought to be secondary to an upstream cause.[119]

Unconventional secretion of non-vesicular ("free") alpha-synuclein

aSyn is highly expressed in presynaptic terminals, where it binds to synaptic vesicle membranes and plays a functional role in regulating neurotransmitter release.[120] aSyn interacts with SNARE proteins and synapsins and plays a role both in the assembly of the SNARE complex and in the docking of vesicles with the plasma membrane.[121] During neurotransmitter release, some aSyn is released into the synapse and can be detected extracellularly. Over-expression of aSyn, which is often used in experimental models to study both UPS routes and to model disease pathology, has been shown to result in the elevated secretion of both vesicular and free aSyn.[122] Here, however, we have focused on experimental studies that have examined the secretion of endogenous aSyn, as this allows a more direct interpretation of how UPS may affect the pathology of neurodegenerative diseases.

Neuronal activity is a major driver of endogenous aSyn release. In cultured neurons, blocking neuronal activity with tetrodotoxin resulted in a reduction of aSyn in the extracellular medium, whereas increasing excitatory transmission with glutamate led to elevated extracellular aSyn levels.[123] These findings were confirmed *in vivo* by manipulating neuronal activity using micro-dialysis to locally apply picrotoxin (a $GABA_A$ receptor antagonist) or tetrodotoxin and simultaneously measuring endogenous aSyn in the interstitial fluid from the hippocampus and striatum of mice.

Since autophagosomes are known to form in the presynaptic compartment and then undergo retrograde transport along the axon to the cell body for fusion with lysosomes, it is important to consider how autophagy affects presynaptic neurotransmission, as this may impact the secretion of aSyn. In mice with conditional neuronal knockout of the core autophagy gene *Atg5*, the quantitative proteomic analysis of key synaptic proteins (including aSyn) showed that their turnover was unaltered in ATG5-deficient neurons. However, loss of neuronal autophagy resulted in the accumulation of tubular ER in axons. This was caused by the absence of a selective form of macroautophagy, termed ER-phagy, which prunes and removes damaged parts of the ER.[124] The accumulation of ER resulted in an increase in calcium release from ER stores, hence an increase in excitatory neurotransmission.[125] A similar increase in neurotransmitter release was observed in autophagy-deficient dopaminergic neurons in conditional *Atg7* knockout mice. When the effect of this conditional knockout was studied in transgenic mice expressing human

Table 2. Main neurodegenerative disease-associated proteins known to undergo unconventional secretion and intercellular spreading

Neurodegenerative disease	Proteins	Secretion/Transfer mechanism	Model and detection method
Huntington's disease (HD)	HTT	EVs[43]	Over-expressed wt and mHTT detected in conditioned media, and uptake observed in recipient cells (western blot). Endogenous HTT detected in the vesicular (CD63 and CD9-positive) fraction of conditioned media[#].
		ATG/LYSO[96,97]	Over-expressed wt and mHTT were detected predominantly in the free (non-vesicular) fraction of conditioned media. Intracellular HTT co-localized with lysosome markers and was reduced when lysosomal secretion was inhibited.[51] Over-expressed mHTT secreted (western blot; conditioned media) in GRASP55-dependent manner and effects abrogated in autophagy-null cells.[96]
		TNT[98]	Over-expressed mHTT visualized in TNTs between transfected and neighboring cells.
Alzheimer's disease (AD)	Aβ peptides	EVs and exosomes[99]	Over-expressed Aβ localized with MVBs. Electron microscopy of exosome enriched fractions showed co-localization of Aβ and exosome markers.
	tau	direct transfer[100]	Over-expressed tau is predominantly detected in the (non-vesicular) fraction of conditioned media. Intracellular tau clusters at the plasma membrane and secretion did not require ATP, and were affected when plasma membrane lipids were altered, and were facilitated by HSPGs.
		EVs and exosomes[101]	Density gradient fractionation of EVs from the brains of patients with AD. Cryo-ET is used to observe tau filaments tethered to the luminal side of the EV limiting membrane. Tau-containing EVs had a diameter of >300 nm.
		ATG/LYSO[102]	Over-expressed tau (and endogenous tau in primary neurons) was detected in conditioned media and increased by autophagy induction.
		TNT[103]	Extracellular tau (monomers and fibrils) stimulates TNT formation. Tau fibrils visualized moving between transfected and neighboring CAD cells within TNTs.
Parkinson's disease (PD)	aSyn	EVs and exosomes[104–106]	Over-expressed aSyn was detected in the vesicular fraction of conditioned media. Vesicular fraction positive for Alix and contained 50–140 nm vesicles (EM analysis).[104] Vesicular fraction analyzed by Nanosight (100-300 nm) vesicles (EM analysis).[105] Secreted, vesicular EVs taken up by non-transfected cells.[106]
		ATG/LYSO[107]	Pathogenic aSyn species were identified in lysosomes isolated from the brains of Tg-aSynA53T mice. aSyn secreted from primary neurons was non-vesicular, and release was reduced by dominant-negative

(*Continued on next page*)

Table 2. ***Continued***

Neurodegenerative disease	Proteins	Secretion/Transfer mechanism	Model and detection method
			VAMP7 (and correlated with reduced Cathepsin-L secretion).
		TNT[108–110]	Recombinant human aSyn fibrils taken up by CAD cells and visualized moving from donor to acceptor cells via TNTs.[110] Similar observations in ESC-derived astrocytes[108] and from SH-SY5Y cells and human PD-patient derived pericytes.[109]
Amyotrophic lateral sclerosis (ALS) and Frontotemporal dementia (FTD)	SOD1	ATG/LYSO[111]	Over-expressed SOD1 was detected in CM. Levels were reduced by autophagy inhibition and by blocking autophagosome biogenesis. Knockdown of SNARES required for ATG-LYS fusion blocked SOD1 secretion, as did knockdown of SNAP23 (known to be required for LYSO exocytosis.
		EVs and exosomes[112]	Over-expressed SOD1 was detected in the vesicular fraction of conditioned media and on the exterior surface of exosomes by immuno-EM.
	TDP43	ATG/LYSO[113]	Blocking lysosome acidification increases the secretion of vesicular TDP43, confirmed by immuno-EM, and causes TDP43-containing autophagosomes to localize to the plasma membrane. Secretion is reduced by blocking autophagosome biogenesis.
		TNT[114]	Intercellular transmission of TDP43 aggregates, induced by exposure to ALS-FTD-patient CSF, visualized within TNT-like structures.
		EVs and exosomes[114]	Over-expressed TDP43 was detected in the vesicular fraction of conditioned media and in vesicles (50–100 nm) by immuno-EM.
	FUS	EVs[115]	Proteomics analysis identified exosome proteins as FUS interacting partners, confirmed in the vesicular fraction of CM (positive for exosome markers).
Prion disease	PrPSc	TNT[116]	Transfer of PrPSc observed between infected and naive cells via TNTs.
		EVs and exosomes[117]	PrPSc in the vesicular fraction of conditioned media and confirmed on membranes of 50-90 nm vesicles by immuno-EM.

Summary of experimental evidence supporting a role for different unconventional secretion routes for the main neurodegenerative disease-causing, aggregate-prone proteins. Most studies are based on the over-expression of the disease-causing protein. Detection methods used for analyzing vesicular sections are listed. #indicates information reported in Supplementary data of the cited article (Figure S9).

wildtype aSyn, Lewy body-like aggregates and neuronal loss were significantly increased but PD-associated movement and behavioral deficits were significantly reduced.[126]

Secretion of alpha-synuclein via secretory autophagy or lysosomal exocytosis

Although not directly addressed in these studies, the secretion of aSyn was assumed to be mediated via increased synaptic vesicle release, i.e., an increase in the "normal" or physiological route of aSyn secretion. However, other studies have demonstrated that alternative secretory routes exist and are increased by the stimulation of the autophagy-lysosome pathway. Recent work by Nakamura et al. studying the release of endogenous aSyn from mouse primary cortical neurons confirmed that enhanced neuronal activation, triggered by either exogenous glutamate or via KCl-induced depolarization, resulted in an increase in aSyn secretion. This neuronal activation resulted in increased autophagic flux and, importantly, upregulation of autophagy alone (i.e., in unstimulated neurons) was sufficient to increase aSyn secretion. Fractionation of the secreted media

from activated neurons demonstrated elevated aSyn secretion in both vesicular and free forms. Since treatment with chloroquine, a blocker of autophagosome-lysosome fusion, increased aSyn secretion in neurons, and knockdown of ATG5 reduced aSyn secretion in both basal and glutamate-stimulated conditions, this suggests that its secretion is mediated via the secretion of autophagosomes.[127]

Secretion of alpha-synuclein in extracellular vesicles

The most extensively studied form of aSyn secretion is in the form of vesicular secretion (reviewed in[128]). EVs containing monomeric and oligomeric forms of aSyn were first identified by Lee et al. from conditioned media of cultured neurons over-expressing wildtype aSyn.[129] Proteasome inhibition and disruption of mitochondrial function, two cellular functions associated with PD pathology, increased the secretion of aSyn-containing EVs.[129] Treatment with Bafilomycin A1 or ammonium chloride, which results in an increase in lysosomal pH and therefore inhibits autophagic flux, also increased the release of aSyn-containing EVs from cultured neurons over-expressing aSyn.[130] However, other studies have found that aSyn secreted from over-expressing HEK293 or SH-SY5Y cells is mainly within the supernatant (i.e., free) rather than in EVs in conditioned media.[131] This may reflect differences in detection methods and purification of vesicles, the expression levels of transfected aSyn, alternative routes for the sorting and secretion of different conformational forms of aSyn, but nonetheless, demonstrates the importance of measuring both free and EV-contained aSyn when analyzing secretion. In addition, it is important to note that the vesicular fraction of conditioned media analyzed in these studies is likely to contain vesicles ranging from 30 nm to 10 μm hence may comprise MVB-derived exosomes, large extracellular vesicles, ectosomes, as well as secreted autophagosomes. While the majority of these studies have used over-expression of aSyn in either cell culture or mouse models, the presence and pathological relevance of EV-mediated secretion of endogenous aSyn is undeniable. Brain-derived EVs containing aSyn have been isolated from CSF and plasma and used extensively to study both intercellular transmission (reviewed in[128]) and for the identification of potential biomarkers (see future directions).

Compelling evidence that the autophagy pathway plays a role in modulating the EV-mediated secretion of endogenous aSyn comes from two studies, which predominantly used over-expression models but validated their findings in primary neuronal cultures from wildtype animals[132] and human iPSC-derived dopaminergic neurons.[133] In the first study, autophagy inhibition by Bafilomycin A1 treatment resulted in a 2-fold increase in vesicular aSyn secreted from wildtype rat primary neurons and a 1.4-fold increase in EV-associated aSyn secreted from neurons from transgenic rats over-expressing human wildtype aSyn. Analysis of the protein profiles of these EVs demonstrated two different populations, namely, EVs which were positive for classical MVB-EV protein markers (e.g., FLOT1, CD81) and ones displaying a typical autophagosome-like protein profile (LC3-II and p62/SQSTM1). Ultrastructural characterization revealed distinct autophagosomes and MVBs under basal conditions and an accumulation of vesicular structures with intralumenal content, including proteins, non-processed cytoplasmic parts, as well as small intralumenal vesicles under Bafilomycin A1 treatment conditions.[132]

In a study investigating the regulation of autophagy by p62/SQSTM1, Oh et al. demonstrated that the S-nitrosylation of p62/SQSTM1 (SNO-p62) resulted in autophagic inhibition and an increase in the release and spread of aSyn. They observed SNO-p62 in A53T hiPSC-derived dopaminergic (hiPSC-DA) neurons increased compared with isogenic, gene-corrected controls. This corresponded with increased levels of p62/SQSTM1 and LC3-II in the EV fraction of conditioned media from A53T hiPSC-DA neurons compared with isogenic control and a ∼3-fold increase of extracellular aSyn in both the conditioned media supernatant and EV fractions in A53T hiPSC-DA neurons. This study provides a mechanism by which the inhibition of autophagic flux results in increased aSyn secretion. In addition, SNO-p62 was found to be elevated in human LBD patient brains, providing a link between altered autophagy-mediated aSyn secretion and disease.[133]

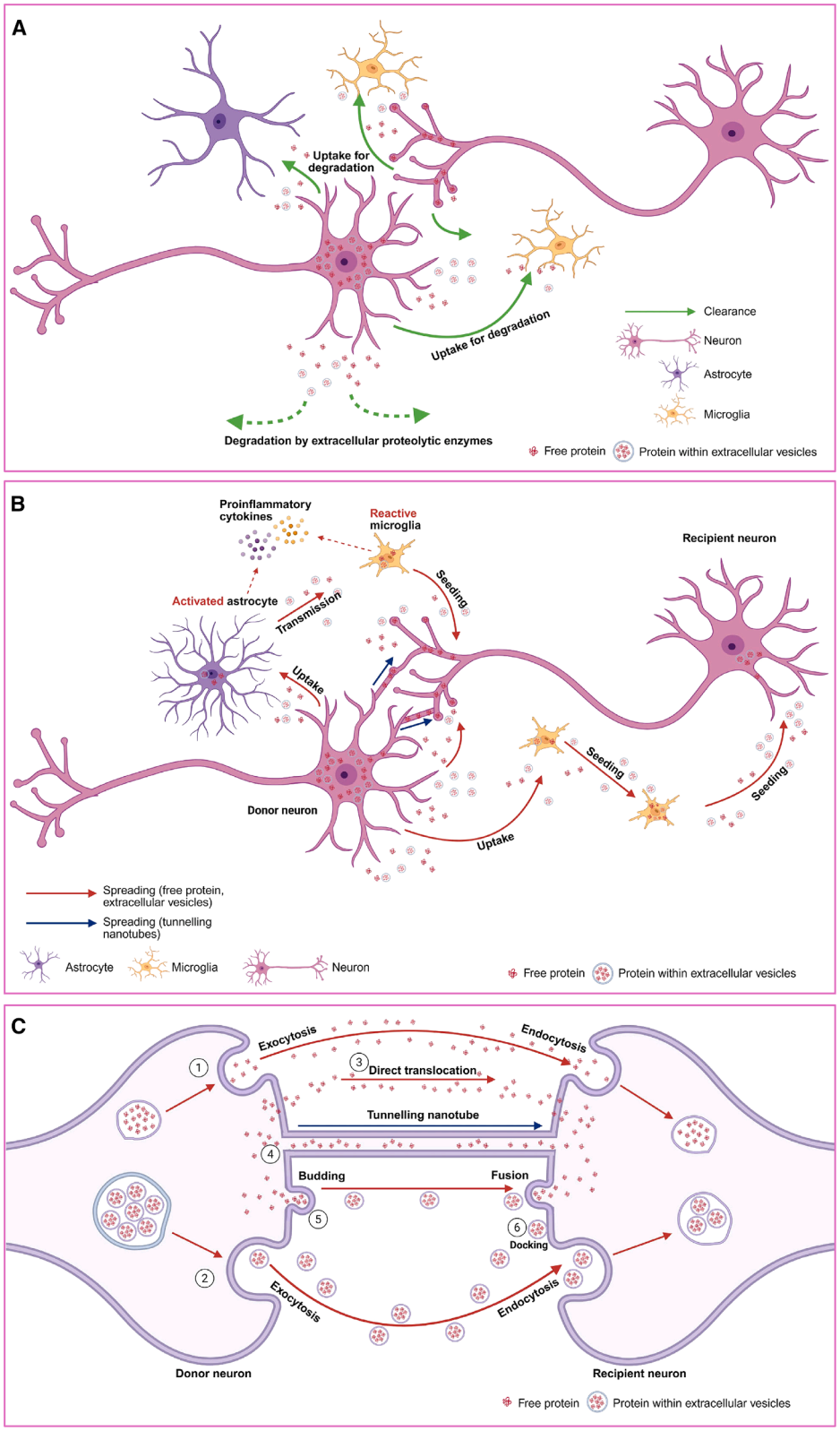
Unconventional secretion of non-vesicular ("free") tau

Tau is a microtubule-associated protein that is essential for regulating the structure and dynamics of microtubules.[119] By binding to microtubules and interacting with motor proteins, tau can modulate transport efficiency along axons. Phosphorylation of tau switches it from a microtubule-bound to an unbound state and, in pathological conditions, hyperphosphorylation prevents microtubule binding. This causes cytoplasmic tau levels to increase, leading to its aggregation into insoluble paired-helical filaments (PHFs) and neurofibrillary tangles (NFTs).[119]

The role of autophagy in mediating UPS of tau has been studied in SH-SY5Y cells over-expressing tau and confirmed for endogenous tau secretion in mouse primary cortical neurons. The conditioned media of these neurons was analyzed following treatment with nigericin, which raises lysosomal pH and therefore blocks autophagy, or by co-treatment with nigericin and spautin-1, which is an inhibitor of autophagy initiation. Tau secretion was enhanced by blocking autophagosome-lysosome fusion (nigericin treatment), and this effect was abrogated by co-treatment with spautin-1, although whether secreted tau was free or vesicular was not determined.[102] Studies using primary cortical neurons have demonstrated that, similar to aSyn, secretion of endogenous tau is stimulated by neuronal activity in a calcium-dependent manner and co-incident with the exocytosis of presynaptic vesicles,[134] with the majority of secreted tau being free rather than contained within vesicles. In addition, whilst investigating the secretion of aSyn by the secretory autophagy route, Nakamura et al. confirmed that glutamate-induced neuronal activation led to the elevated secretion of endogenous tau and SOD1, an effect that was blocked by ATG5 knockdown.[127]

Tau secretion in extracellular vesicles

Tau is present in EVs isolated from ND patient samples, and the levels of phosphorylated tau in EVs isolated from brain tissue, blood, and CSF of patients with AD are significantly increased in samples collected from individuals even prior to their clinical diagnosis of AD.[135,136] The clinical and pathological relevance of this vesicular pool of tau is underlined by the extensive studies on their uptake and the subsequent spreading and seeding of aggregates (reviewed in,[137] and see future directions). However, the majority of studies rely on models in which tau is



(legend on next page)

over-expressed, and analysis of conditioned media from primary cortical neurons has shown that this results in a 2– to 3-fold increase in the small EV markers Hsc70 and TSG101 in tau-P301S over-expressing compared to wildtype neurons.[138] An important study comparing endogenous tau in the conditioned media from 3 different neuronal models (hiPSC-derived cortical neurons, rat primary hippocampal neurons, and N2A cells), using sensitive ELISA methods to quantify protein levels, found that more than 1% of secreted tau was within the pellet of the conditioned media (i.e., within vesicular structures). In addition, this study found that the majority of secreted tau was a C-terminally truncated form, which lacks the microtubule-binding region considered to be necessary for self-aggregation.[139]

Spreading of alpha-synuclein and tau via extracellular vesicles

While not the focus of this review, it is important to highlight some of the studies that have shown that unconventional secretion can lead to the spreading of aggregate-prone ND-associated proteins but may also be a route for their clearance and removal (see Figure 3). One of the primary functions of microglia is to maintain homeostasis in the CNS by phagocytosing dying cells and cellular debris as well as misfolded or aggregated proteins, which are secreted by neurons (reviewed in[140]). Astrocytes are also able to clear aggregates and have been shown to compensate when the microglial population is ablated or when microglial phagocytosis is impaired.[141] EVs may also play a protective role in NDs by extruding pathological molecules from cells, which may be a route for their clearance (reviewed in[142]). In addition, the exciting new discovery that 20S proteasomes can be packaged, secreted by EVs, and delivered to recipient cells highlights a new mechanism for clearing tau.[143] Cultured primary astrocytes and microglia can take up aSyn-containing EVs derived from mouse brains and clear fibrillar aSyn via the endo-lysosomal route[144] (Figure 3A). However, the activation state of both astrocytes and microglia can be altered by the engulfment and accumulation of aggregate-prone proteins, leading to the release of inflammatory cytokines, which drive inflammation[145,146] (Figure 3B).

In recent years, a large body of work has focused on the role of EVs in the spreading of ND-associated proteins,[147] summarized in Figures 3B and 3C. Cultured neuroglioma cells and microglia can take up preformed aSyn fibrils (PFF) and release them within EVs, and these can then be taken up by neuronal cells.[148] When PFF-containing, neuroglioma-derived EVs were injected into the tail vein of mice, the PFFs could be detected in the brain 1 day later and resulted in the formation of aSyn aggregates.[148] In addition, direct injection of PFF-containing, microglia-derived EVs into the mouse striatum resulted in aSyn phosphorylation and aggregation, and induced neurodegeneration of dopaminergic neurons in the substantia nigra and striatum.[149] Similarly, EVs isolated from the brains of tau transgenic mice are capable of seeding tau aggregation when applied to fluorescent reporter cell lines.[150] In addition, EVs from primary neurons over-expressing tau or from brain extracts from patients with progressive supranuclear palsy or Pick disease can be taken up by astrocytes, resulting in the disruption of mitochondrial morphology and function.[151] Activated microglia release higher levels of EVs than inactivated microglia, and in AD mouse models, these activated microglia secrete EVs containing tau protein, which can propagate to neurons and promote the progression of pathology.[152]

Importantly, these findings have been validated using patient-derived samples. For instance, CSF-derived EVs from patients with PD increased aSyn aggregates when applied to reporter cell lines when compared to those from controls or patients with LBD.[153] In addition, EVs from the plasma of patients with PD were taken up with high affinity by microglia both in culture and when injected into the striatum of mice, and this human (EV-derived) aSyn was detected in cortical brain regions, suggesting microglial-neuronal transmission *in vivo*.[154] Biomarkers can be used to determine the cellular origin of EVs, and microglia/macrophage-derived EVs isolated from the CSF of patients with PD have been demonstrated to induce aSyn aggregate formation when applied to cultured neurons.[149] Similarly, tau-containing EVs isolated from the plasma or serum of patients with AD can cause tau accumulation when applied to lab-cultured neurons.[155,156] One important consideration in these studies is that the presence of EVs alone can affect aggregation since both empty and aSyn-containing EVs can accelerate the *in vitro* aggregation of aSyn to the same extent; in addition, the differential membrane phospholipid composition can also attenuate or accelerate this process.[157]

Since the secretion of aggregate-prone proteins can occur via multiple routes, and estimates from conditioned media and interstitial fluid from mouse brains suggest that less than 1% of secreted aSyn and tau is within vesicles, it is important to consider that spreading between neurons can also occur via non-vesicular routes (summarized in Figure 3C). The majority of studies in this area have used preformed fibrils (PFFs) or seeds, either directly applied in cell culture system on injected into the brain or intravenously in mouse models, to investigate their propensity to induce further aggregation and spread pathology (reviewed in[128,158]). However, the unconventional secretion of these proteins is not the only consideration, since monomeric and

Figure 3. Modes of secretion and uptake of aggregate-prone proteins in the nervous system

(A) Mechanisms of protein uptake and clearance. Secreted proteins can be released from neurons either as "free" protein or within membrane-embedded vesicular carriers. Free protein can be directly degraded by extracellular proteolytic enzymes, or both vesicular and free protein can be taken up by astrocytes and microglia for intracellular lysosomal degradation.

(B) Mechanisms of protein spreading and induction of pathology. Uptake of secreted protein triggers the activation of astrocytes and microglia, resulting in the release of proinflammatory cytokines that can induce neuroinflammation in surrounding tissue. Protein secreted by a donor neuron can be taken up by a recipient neuron (spreading). This may occur directly or involve uptake and transmission by astrocytes and microglia. Aggregated forms of the protein that have spread to the recipient neuron can induce aggregation (seeding).

(C) A simplified overview of protein transmission between neurons. Protein held in vesicular structures (e.g., endosomes, autophagosomes, lysosomes) (1) and multi-vesicular bodies (2) are released by exocytosis and taken up via endocytosis. Free protein is translocated directly (3), through tunneling nanotubes (4) or by the formation of micro-vesicles (5). In addition, vesicles can dock onto the plasma membrane of the recipient neuron but are not taken up (6). Created in BioRender. Fleming, A. (2026) https://BioRender.com/swu7ib1.

oligomeric/aggregated forms are likely to be released from dying cells both as vesicular and free forms.

FUTURE DIRECTIONS

New models to study unconventional secretion

These studies highlight the complexity of ND pathophysiology, with dysregulation extending to the metabolic and immune systems, as well as to the functionality of the blood-brain barrier. Such features often manifest in inflammation, oxidative stress and cell death, making it challenging to identify pathologies that result from aggregate formation from those that might induce it.[159] To disentangle the roles for unconventional secretion in the pathogenesis of neurodegenerative disease, there is a need for models specifically suited to studying secretion, uptake and pathological processes.[160] While direct visualization of aggregates benefits from the sub-cellular resolution provided by *in vitro* techniques,[161] these models may lack the complexity of an intact nervous system.

In this regard, microfluidic chips provide a useful tool to study cellular communication between different cell types (e.g., neurons and glia), allowing the analysis of the spreading of secreted proteins in the absence of cell contact.[162] Different cell types can be grown in separate compartments, hence with spatial separation and size exclusion by the incorporation of porous membranes between compartments. These devices have the potential to be used with high content imaging, enabling the analysis of the trafficking and uptake of secreted proteins and vesicular structures. Greater complexity can be achieved with the use of brain organoids, three-dimensional cultures of neurons and glia. Comparison of the EV population secreted from forebrain and hindbrain organoids has been used for proteomic and lipidomic analysis[163] and from cerebral organoids for microRNA analysis,[164] the latter identifying some EV-derived microRNAs that are associated with ALS, HD, and other neurodegenerative diseases. Perhaps one drawback of the organoid system is that it models the embryonic brain and hence may not recapitulate the features of late onset disease.

To fully understand the complexity of how autophagy and unconventional secretion affect disease pathology, the best approach would be to perform studies in an *in vivo* model. While mouse models are ideal for recapitulating the pathophysiology of neurodegenerative disease, their application for studying protein secretion remains limited by the need for *ex vivo* analysis required for cellular-level visualisation.[160] Recent progress in this area has come from the development of exosome reporter mice, which have provided valuable insights into exosome-mediated neuronal-astrocyte communication and provide a powerful tool for both visualizing the localization and for immuno-isolation analysis of EVs derived from distinct cell types.[165,166] Important progress in understanding the biogenesis of intraluminal vesicles has come from studies using a combination of cultured cortical neurons from knock-in mice with GFP-tagged human CD63 and *in vivo* analysis. As observed in previous studies,[167] Ca^{2+}-dependent glutamatergic stimulation increased neuronal EV release. Using cell-type specific expression of the reporter, the resultant increase in spreading between cortical neurons was confirmed *in vivo.*[168]

An alternative approach, which has the potential to address the requirement for *in vivo* visualization EVs and free secreted protein in normal and pathogenic states, is the use of zebrafish. Their high degree of tissue and genetic homology, a fully sequenced genome, coupled with ease of genetic and pharmacological manipulation, are features that make them powerful models for neurodegenerative disease research.[169] Crucially, the zebrafish larva is transparent and approximately 2 mm in length, facilitating the use of confocal microscopy for the non-invasive visualization of the CNS at cellular and subcellular resolution *in vivo.*[170] Hence, a variety of tools have been developed to enable the live-tracking of proteins and organelles at the cellular and subcellular level in the zebrafish model.[171–173]

The direct tracking of extracellular vesicles *in vivo* using live zebrafish models has been reported using two methods; in one study, human melanoma vesicles bound to the fluorescent probe, MemBright, were injected at the one-cell stage and tracked at larval stages,[174] and an alternative approach was the generation of transgenic zebrafish expressing EVs tagged with a fluorescent protein.[175] High-speed confocal imaging enabled live tracking of vesicle velocity, behavior, and fate, even depicting the direct visualization of uptake by macrophages and neutrophils.[174]

To complement the *in vivo* analysis of EVs, a recent study has also reported methods for the characterization of extracellular vesicle size, shape, abundance, and surface markers in zebrafish.[176] Nanoparticle tracking analysis and flow cytometry enabled the proportion of vesicles of each size within a sample to be calculated, as well as an estimate of the proportion of ''large'' vesicles that are actually clusters of smaller ones. The methods were tested in larvae; therefore, if run in parallel with *in vivo* imaging, could provide a sensitive characterization of the behavior and structural composition of vesicles in a disease phenotype – or stage – of interest. In addition, the examination of vesicle morphology using transmission electron microscopy analysis revealed what appeared to be heat-shock proteins associated with exosomes in the zebrafish.[176] This finding may be particularly pertinent to the study of unconventional secretion in ND, since heat-shock proteins have been implicated in both the spreading and clearance of aggregate-prone proteins and the regulation of unconventional secretion.[177,178]

Transgenic zebrafish have also successfully been used to directly visualize aggregate-prone protein clearance from the neurons *in vivo*. In transgenic zebrafish expressing human tau or aSyn fused to a photoconvertible fluorescent protein, direct quantification of tau accumulation and clearance can be analyzed within individual neurons over time and can be coupled with chemical and/or genetic manipulation of clearance pathways to assess their relative contributions.[179,180] Using the tau-expressing model, a novel therapeutic mechanism for tau clearance was recently identified via the induction of lysosomal secretion induced by carbonic anhydrase inhibition.[181] Interestingly, the transgenic line developed to study EVs used an exosomal protein tagged to pHluorin,[175] a pH-sensitive GFP variant designed to fluoresce when vesicles enter the non-acidic extracellular space. Similar probes have previously been used to track synaptic vesicle movement[182] and to measure autophagic flux.[172] Hence, deploying

Table 3. Mechanisms regulating the unconventional secretion of tau and aSyn

Protein/Mechanism		Secretion/Transfer mechanism
nSMase2 and ceramides	tau	EV biogenesis is triggered by ceramide enrichment at the plasma membrane from neutral sphingomyelinase2 (nSMase2)-mediated cleavage of sphingomyelin. PDDC, the first potent, selective, orally bioavailable, and brain-penetrable nSMase2 inhibitor.[200]
FGFR3	tau	Extracellular tau binds to the extracellular domain of FGFR3 and is internalized upon ligand (FGF2) binding.[201]
Gal3	tau	Gal3 is upregulated in microglia after exposure to neuron-derived hyperphosphorylated tau, resulting in microglial activation and an increase in EVs released. Gal3 binding to internalized tau enhances its fibrillation, and secreted EVs increase tau accumulation in recipient cells.[202]
cholesterol and sphingomyelin	tau	Tau clusters at the plasma membrane and their rate of secretion dependent on the cholesterol and sphingomyelin content. Increasing neuronal plasma membrane cholesterol or depleting sphingolipids increased tau secretion.[100]
Slingshot Homolog-1 (SSH1) and SQSTM1	tau	SSH1 dephosphorylates SQSTM1, enabling it to bind to ubiquitinated cargoes.[138]
HSPG	tau	Reducing cell surface HSPG decreased tau secretion.[100] Misfolded tau binds HSPGs and can be internalized through macro-pinocytosis into neurons, where it can be transported both anterogradely and retrogradely.[203,204]
TPPP/p25α	aSyn	p25alpha co-localizes with aSyn and facilitates its incorporation in autophagosomes. p25alpha reduces autophagosome motility and hinders autophagosome-lysosome fusion but increases alpha-synuclein autophagy-dependent secretion.[205]
SNAREs	aSyn	Neuronally generated pathogenic species of aSyn accumulate within neuronal lysosomes in mouse brains and primary neurons. Neurons release these pathogenic α-synuclein species via SNARE-dependent lysosomal exocytosis.[107]
DNAJC5	aSyn	The translocation of cytosolic aSyn to the endosomal membrane compartment is dependent on DNAJC5 and requires its palmitoylation.[206]
HSPB5/CRYAB	aSyn	CRYAB-containing autophagosomes are not able to fuse with lysosomes, and instead, these are diverted to the exocytic pathway. In aSyn expressing cells, over-expression of CRYAB leads to increased secretion.[46]

(*Continued on next page*)

Table 3. *Continued*

Protein/Mechanism		Secretion/Transfer mechanism
PARK9/ATP13A2	aSyn	ATP13A2 is essential for maintaining lysosomal pH. In loss-of-function mutations, associated with Kufor-Rakeb syndrome, a juvenile form of parkinsonism, defective lysosomal degradation causes aSyn accumulation.[207] Over-expression of ATP13A2 decreases cellular toxicity and promotes the exosome-mediated secretion of the mutant protein.[208]
PELI1	aSyn	aSyn preformed fibrils impaired autophagy flux via the upregulation of PELI1, which in turn, resulted in the degradation of LAMP2 in activated microglia.[149]
sumoylation	aSyn	SUMO protein modification acts as a Ubiquitin-independent ESCRT sorting signal for the extracellular vesicle pathway and regulates the extracellular vesicle release of aSyn.[209]
UBL3 and MGST3	aSyn	UBL3) acts as a post-translational modification factor that regulates protein sorting to exosomes/sEVs.[210] aSyn interacts with UBL3, and this interaction is downregulated after silencing microsomal glutathione S-transferase 3 (MGST3).[211]
SNAP29	aSyn	aSyn decreases the abundance of the v-SNARE protein SNAP29, an SNARE protein that mediates autophagolysosome fusion.[212]
TNFRSF10B	aSyn	TNFRSF10B downregulation in microglia inhibits the secretion of aSyn-containing exosomes and the release of pro-inflammatory factors.[213]
SCAMP5	aSyn	SCAMP5 inhibits autophagy flux by blocking the fusion of autophagosomes and lysosomes and stimulates the unconventional secretion of aSyn.[214]
LGAL3	aSyn	LGALS3 (galectin 3) mediates the release of SNCA following damage to endocytic vesicles and mediates recruitment to the secretory autophagy pathway.[215]

combinations of tagged proteins may enable secretion to be observed directly, *in vivo*, to understand the mechanisms of relevance to disease.

Advances in methods to characterize extracellular vesicles

One of the challenges of characterizing the role of EVs in normal physiology and disease is to assess and establish their cellular origin. While the identification of EVs can be performed by imaging using markers as described above is a powerful tool to look at *in vivo* dynamics, it has limited utility for the characterization of their contents (e.g., for approaches such as proteomics or identification of nucleic acid cargoes). Traditional techniques for EV characterization have relied on size selection methods (e.g., density gradient separation or size exclusion chromatography) followed by the recognition of particular proteins by western blot, ELISA, or mass spectrometry, and typically require large volumes of starting material.[183] Advances in microfluidic technologies have enabled the enrichment and the detection of EVs in small volumes and at high throughput. These approaches have been driven, in large part, by the potential for detecting EVs within biological fluids as biomarkers of disease.[184] Microfluidic devices can be used for EV extraction and separation using physical (size) and chemical properties[185] or immunological methods[186–188] and/or for biomolecular characterization through the sensitive and rapid detection of EV-associated proteins and RNA.[189]

In the context of neurodegeneration, brain-derived EVs secreted from neurons, oligodendrocytes, microglia and astrocytes can be isolated from human CSF and plasma and measurements of aSyn and tau from purified EVs are reportedly more reliable than from conventional patient-derived samples, such as CSF, blood and urine[93,190] and can be used to quantify the ratio of different tau isoforms that are predictive of

frontotemporal dementia.[191] Hence, recent work has focused on the identification of novel diagnostic biomarkers within purified EVs.[192,193] Whilst this diagnostic potential is not the focus of this review, the development of microfluidic-enrichment technologies is an important advance in EV analysis and is being employed in biological and pre-clinical research studies as well as for diagnostic purposes.[153,192,194–199]

Outstanding questions

It is likely that unconventional secretion from neurons is a highly dynamic process and reflects the physiological state of the brain, both during normal aging as well as in pathological conditions. In this context, the fate of secreted proteins, expressed at physiological levels, remains poorly understood. It is not clear how secretion pathways, rate, and the fate of proteins may vary under normal and pathological conditions *in vivo*. In this regard, EVs are of particular interest, since there may be specific signatures which determine whether an EV will be rapidly taken up by neighboring cells, interact with the extracellular matrix, or instead be simply diluted in the interstitial fluid. Over the past ten years, researchers have identified a variety of proteins regulating unconventional secretion of aSyn and tau (Table 3) with much effort focusing on the mechanisms and pathways by which these proteins act. One important challenge is to understand how the selective cargo loading of these EVs is regulated. Likewise, little is known about the mechanisms regulating the trafficking and the biological activity of the EVs internalized by recipient cells. These questions are important since the trafficking pattern of EVs, and especially that of seed-carrying EV subpopulations, is likely to affect disease progression. In addition, the identification of reliable markers is needed to identify neuronal and glial EVs and distinguish them from other EVs in biological fluids[216] for the purpose of biomarker studies, and the functional relationship between levels of EV biomarkers and disease progression is an area that has yet to be explored and is essential for the development of biomarkers for clinical trials.

Finally, since there is strong experimental evidence for crosstalk between autophagy and unconventional secretion, it is important to consider how therapeutic interventions targeting the autophagy pathway (e.g., inflammation, cancer, and ND[217–219]), might impact unconventional protein secretion and therefore disease progression.

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AUTHOR CONTRIBUTIONS

The concept and content of the review were devised by M.R. and A.F. with the contribution of R.B. All authors contributed to the writing of the article. Figures were prepared by G.D. and A.F., tables were prepared by L.V.V., M.R., and A.F. The article was edited by M.R. and A.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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