Extracellular vesicles in neurodegenerative disease – pathogenesis to biomarkers

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Abstract

Reliable markers of diagnosis, disease activity and progression in neurodegenerative diseases are a research priority in the improvement of diagnosis and the development of effective disease-modifying therapies. The fact that neurodegenerative pathology is primarily associated with distinct subsets of cells in discrete areas of the CNS makes the identification of relevant biomarker molecules a challenge. The trafficking of macromolecules from the CNS to the cerebrospinal fluid and blood mediated by extracellular vesicles (EVs) presents a promising source of CNS-specific biomarkers. EVs are released by almost all cell types and carry a cargo of protein and nucleic acid that varies according to the cell of origin. EV output changes according to cell status and reflects intracellular events, so surface marker expression can be used to identify the cell type from which EVs originate. EVs could, therefore, provide an enriched pool of information about core neuropathogenic, cell-specific processes. This Review examines the current knowledge of the biology and function of EVs, discusses the evidence for their involvement in the pathogenesis of neurodegenerative diseases, and considers their potential as biomarkers of disease.

Key Points

1. Extracellular vesicles are secreted by cells of the CNS
2. Extracellular vesicles can be exosomes, microvesicles or apoptotic bodies, and their cargo reflects their cellular origin
3. Extracellular vesicles are implicated in neurodegenerative disease according to the prion-like hypothesis of propagation
4. RNA and protein that is associated with extracellular vesicles from the cerebrospinal fluid and serum have shown promise as early biomarkers for some neurodegenerative diseases but require validation
5. Robust identification markers for the most relevant, but relatively low abudance EVs present in CSF and blood is a major ongoing challenge
6. Focus is needed on the development of high yield extraction methods that are ultimately applicable to routine clinical use

The development of disease-modifying treatments for neurodegenerative diseases, such as Alzheimer disease (AD), Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS), is hampered by a lack of sensitive and specific biomarkers of disease activity and rate of progression[1]. These diseases follow a progressive course that is typically associated with accumulation of protein aggregates that vary in composition according to the disease [2-4]. The major pathological burden in neurodegenerative diseases is confined to the CNS, though aggregates of α-synuclein have been observed in the enteric nervous system of patients with PD[5]. Aggregate-associated proteins are detectable in the cerebrospinal fluid (CSF) and blood and form the basis of several biomarkers for neurodegenerative diseases. However, these proteins are present at extremely low concentrations and are estimated to account for less than one millionth of total CSF protein and one ten billionth of total blood protein[6]. Nucleic acid biomarkers, such as microRNAs (miRNAs), can be amplified, but are also present at low concentrations, and signal-to-noise ratio may similarly impede their use as biomarkers. Methods that eliminate abundant molecules without depleting disease-relevant molecules are, therefore, essential to develop meaningful neurodegeneration-specific biomarkers.

One strategy for isolating effective biomarkers is to focus on constituents of biofluid other than soluble proteins, such as extracellular vesicles (EVs). EVs, which are released by almost all cell types and vary in diameter (50–2,000 nm) , carry a cargo of protein, lipid and nucleic acid that varies according to the cell of origin[7]. In cell culture experiments, EV output changes according to the status of a cell; for example, the composition of protein and RNA cargo is different in the contexts of hypoxia and oxidative stress[8,9]. The composition of EV cargo also changes in diseases, such as sepsis and malignancy[10,11]. Little is known about the effects of neurodegenerative diseases on EV cargo, but EVs that derive from the CNS are found in the cerebrospinal fluid (CSF)

and the serum, they protect their cargo from degradation[12-14] and their cellular origin can be identified according to surface marker expression profiles[15]. Consequently, EVs have the potential to provide insight into core cellular and pathogenic processes in specific CNS cell populations. In this Review, we examine the current knowledge about the functions of EVs, provide an overview of the evidence that EVs are involved in neurodegenerative disease, and consider their potential as biomarkers of neurodegenerative disease.

[H1] Biogenesis, features and cargo of EVs

Research into the biology of EVs has increased considerably over the past decade, though the precise mechanisms that govern their formation, cargo loading, trafficking and release remain incompletely understood. A detailed account of these aspects is available elsewhere[16]; for the purposes of this Review, we will briefly outline the types of EV discussed herein.

All types of EV have important characteristics in common: they all have a fluid-filled lumen bounded by a lipid bilayer, carry an intraluminal cargo of protein and nucleic acid, and are secreted into the extracellular milieu. They can be classified according to their cell of origin (for example, prostasomes, ectosomes and cardiosomes from prostatic fluid, neutrophils or monocytes and cardiomyocytes, respectively), but are most commonly divided into three broad types according to their biogenesis: exosomes, microvesicles and apoptotic bodies [7] (Table 1). Most studies focus on exosomes and microvesicles (Table 1)[17].

[H2] Exosomes

Exosomes are generated by invagination of the endosomal limiting membrane to form multivesicular bodies (MVBs) that contain intraluminal vesicles. The tightly regulated mechanisms of intraluminal vesicle formation and loading relies on the endosomal sorting complex required for transport (ESCRT) proteins and tetraspanins, as well as ESCRT-independent mechanisms that involve, for example, ceramide (Figure 1)[16,18-20]. MVBs are trafficked to the plasma membrane, whereupon fusion of plasma and endosomal membranes leads to the release of exosomes into the extracellular environment [21].

Exosomes typically have diameters of 40–150 nm. Initially, transmission electron microscopy indicated that exosomes had a cup-shaped morphology, but cryoelectron microscopy of unfixed exosomes indicates that they are spherical, suggesting that the cup-shaped morphology is an artefact of fixation for conventional transmission electron microscopy[22]. The exosomal membrane contains components of the endosomal machinery, including tetraspanins, ALG-2-interacting protein X (ALIX), the ESCRT component tumour susceptibility gene 101 protein (TSG101) and flotillin-1[23].

[H2] Microvesicles

Microvesicles (also known as microparticles) form by outward budding of the plasma membrane, and are 50–2000 nm in diameter. The mechanisms of microvesicle formation and cargo loading are not as completely characterized as for exosomes. Formation and release of some microvesicles requires an interaction between TSG101 and arrestin domain-containing protein 1 (ARRDC1), and microvesicle shedding is enhanced by the VPS4 ATPase and the GTP-binding protein ARF6[24,25]. The release of microvesicles also depends on membrane lipid content: release is inhibited by cholesterol depletion, and the outer leaflet of the microvesicle membrane is enriched with phosphatidylserine, which is necessary for vesicle fusion and may enable microvesicle shedding through alterations in plasma membrane shape. The protein content of microvesicle membranes overlaps considerably with that of exosomes, preventing reliable distinction between the vesicle types by protein markers alone.

[H2] Apoptotic bodies

Apoptotic bodies are a heterogeneous population of cell fragments that form during apoptosis. They measure 50–5000 nm in diameter, have variable morphology, and contain the degradation products of subcellular organelles, including DNA, histones, organelle fragments and cytoplasmic components [7,26].

[H2] Cargo

[H3] Nucleic acids

Numerous attempts have been made to determine the relative amounts of different nucleic acids contained in EVs, but precise characterization seems to depend on the cell type studied and the protocol used. Various species of RNA have been detected within EVs; these species include messenger RNA, hairpin RNA and non-hairpin RNA, transfer RNA, non-coding RNA, micro RNA (miRNA), ribosomal RNA fragments, small nucleolar RNA, small nuclear RNA and small cytoplasmic RNA [27,28]. Some specific mRNAs are enriched in EVs relative to cells[29], and some evidence indicates disease-specific patterns of vesicular miRNA[29]. DNA — including double-stranded and single-stranded DNA, retrotransposons and mitochondrial DNA — has been detected in a subset of EVs, although predominantly in tumour-derived EVs[30-32].

[H3] Proteins

Over 40,000 proteins — nearly one quarter of the known human proteome — have been detected within EVs[33], although some data indicate that ~400 proteins account for 75% of the overall EV-associated protein mass[34]. EVs transfer information to target cells via receptor–ligand interactions at the surface membrane or internalisation of intraluminal contents via endocytosis or direct fusion with the plasma membrane[35].

[H1] EVs and the CNS

EVs have multiple functions in the CNS, including intercellular communication, maintenance of myelination, synaptic plasticity, antigen presentation and trophic support of neurons[36-38]. Additional roles of EVs that have been observed in other systems cannot be discounted in the CNS; for example, they can be involved in the disposal of unwanted protein, a role that might be particularly pertinent to

neurodegeneration [39]. The many functions of EVs in the CNS have been reviewed elsewhere,[40] and we will focus on the aspects of EVs that are relevant to neurodegenerative diseases and biomarker development.

Evidence indicates that EVs are secreted by neurons and glia in the CNS. Proteomic analysis indicates that EVs that are extracted from the CSF of healthy subjects contain neuron-specific markers, such as vesicle-associated membrane protein 2, a component of the presynaptic exocytotic machinery, and enolase 2 Similarly, microglia-specific proteins, such as integrin α-M and the oligodendrocyte marker transmembrane protein 132D have been detected in proteomic analysis of human EVs from CSF[12].

[H1] Movement of EVs to and from the CNS

Whether or not EVs move from the systemic circulation to the brain is unclear, and the evidence is limited. Proteomic analysis of EVs from the CSF of healthy humans detected the presence of several proteins that do not derive from the CNS, including proteins involved in the complement cascade, such as the complement component C8 β chain, and coagulation, such as the fibrinogen α chain and coagulation factor V[12]. Findings of the Cancer Genome Anatomy Project (cgap.nci.nih.gov) indicate that these proteins are expressed predominantly in the liver and found abundantly in blood. Combined, these findings could imply translocation of EVs from the blood to the CNS, but could alternatively indicate contamination of CSF samples with abundant plasma proteins during CSF extraction.

In several experiments, EVs have been used as a vehicle to bypass the blood–brain barrier. For example, intranasal administration of EVs has been used to deliver anti-inflammatory drugs to the brains of mice, thereby demonstrating that delivery of EVs to the CNS — principally the olfactory bulb in this study — is possible[41]. In this context,

EVs are thought to bypass the blood–brain barrier through olfactory neurons, although this mechanism is unlikely to be a major route for the passage of systemic EVs into the brain. Similarly, systemic administration of EVs has been used to deliver small interfering RNA to the brains of mice[42,43] although the non-physiological targeting method used makes the precise means of entry — whether via retrograde axonal transport or direct transendothelial movement across the blood–brain barrier — unclear. Nevertheless, blood–brain barrier dysfunction is thought to be a feature of AD, so transfer of EVs from the systemic circulation to the brain is a possibility in neurodegenerative disease[44].

Evidence that EVs can cross the blood–brain barrier comes from a study in which examination of interactions between the systemic immune system and the CNS[45] suggested that EVs mediate transfer of mRNA from haematopoietic cells to cerebellar Purkinje cells. In this study, transgenic mice expressed Cre-recombinase under the control of a haematopoietic-cell-specific promoter on a background of a Cre reporter. The mice exhibited recombination in cerebellar Purkinje cells in the absence of marker-positive leukocytes in the cerebellum, indicating delivery of the recombinase across the blood–brain barrier. EVs from haemopoietic cells contained Cre-recombinase mRNA and induced recombination in Purkinje cells when directly injected into the cerebellum. The findings suggest that EVs are transferred across the blood–brain barrier. Recombination was not induced by systemic intravenous injection of EVs, but this might have been due to the infusion of insufficient quantities.

Understanding the transfer of EVs from the CNS to the periphery is crucial for the development of biomarkers of primary CNS disorders. EVs could move from the CNS to the systemic circulation by direct translocation into capillaries or draining venules, or by passing through interstitial fluid into the CSF and (via the arachnoid granulations and perinasal lymphatics) into the venous system[46,47]. Limited research suggests that

particles of a comparable size to that of EVs can migrate passively across arachnoid granulations from the CSF to the systemic circulation, indicating that EVs could migrate from the CSF to the blood in this way[48]. The discovery of lymphatic vessels in mammalian meninges that communicate with the cervical lymph nodes has revealed a way in which EVs could be involved in immune surveillance and antigen presentation in the CNS, as elsewhere in the body[49].

Endothelial cells that contribute to the blood–brain barrier also secrete EVs, carrying a protein cargo that is implicated in intercellular signalling[50]. These EVs could be a source of biomarkers of neurodegenerative disease, particularly diseases that involve blood–brain barrier disruption. Proteomic analysis of EVs in serum samples has revealed the presence of CNS-specific proteins — suggesting the presence of neuronal EVs in the serum — in healthy controls and in patients with PD or diabetes[51-53]. However, results between studies were inconsistent: different receptor subtypes were identified in different studies, and none were identified in one study. Similarly, glioblastoma-specific markers, such as EGFRvIII, have been found in serum, but these results might not indicate that EVs enter the serum from the brain in physiological conditions, as disruption of the blood–brain barrier is a feature of intracerebral neoplasms[54].

[H1] EVs in neurodegenerative diseases

In addition to their crucial physiological roles in the CNS, EVs are thought to contribute to the pathogenesis of neurodegenerative diseases. The mechanisms contributing to neurodegeneration are varied, but protein aggregates are widely considered to have a central role, in which case, the role of EVs in protein disposal would be especially pertinent. Toxicity of these aggregates might involve multiple mechanisms, including loss of function of the aggregated proteins, mitochondrial

dysfunction, interference with axonal transport, proteasomal inhibition, synaptic toxicity, and endoplasmic reticulum stress[55-60]. Alternatively, the accumulation of proteins could be a response to these dysfunctions or other upstream defects. There is evidence that EVs may have additional roles such as the sequestration of toxic oligomers[61], or contribute to the liberation of toxic oligomers from protein aggregates [62,63].

Prion diseases, such as Kuru and Creutzfeldt–Jakob disease, are transmissible neurodegenerative diseases that are associated with the accumulation of misfolded major prion protein (PRP^{C} for the native form, PRP^{Sc} for the misfolded form). Prion diseases seem to spread when PRP^{Sc} acts as a 'seed' that leads to misfolding of PRP^{C} [64]. This templating mechanism was initially thought to be unique to prion diseases, but evidence is mounting that similar templating of native proteins is involved in the pathogenesis of other neurodegenerative diseases, including AD, PD and ALS, and some inherited polyglutamine disorders, such as Huntington disease[65,64]. The concept of prion-like propagation in non-prion neurodegenerative disorders has led to speculation that misfolded proteins can spread between cells. Several mechanisms for this intercellular spread have been proposed, including the movement of protein within EVs (Figure 2)[66-68].

Several studies have examined the roles of EVs in various neurodegenerative diseases (Table 2). Below, we outline the evidence for the involvement of EVs in several conditions. Overall, evidence is mounting that EVs have a significant role in the propagation of templated protein aggregation, although the evidence is far from conclusive in conditions other than the classic prion diseases, and additional mechanisms cannot be excluded. In keeping with the complex role of EVs elsewhere in the body, it is likely that EVs have antagonistic roles according to their cellular or

subcellular origin, and propagate protein aggregations in some contexts and sequester or dispose of toxic protein in others.

[H2] Prion diseases

PRP^{C} misfolding can be transmitted in cell culture media in the absence of cells, indicating that the mechanisms do not require direct or close contact of cells (such as tunnelling nanotubes or direct surface contact)[69]. Instead, growing evidence indicates that prion disease is predominantly transmitted between cells via exosomes that contain PrP^{Sc}.

In cell culture models of scrapie, immunogold-labelled transmission electron microscopy (TEM) of released exosomes has detected normal and pathogenic forms of sheep prion proteins in exosomal membranes. Pellets generated by ultracentrifugation of the media from these cultures confer prion infectivity *in vitro* and in mice that express ovine PrP; after further fractionation by density gradient centrifugation, the exosome fraction is the most infective, suggesting that exosomes are the most likely vectors of intercellular PrP^{Sc} transmission. Some studies have even identified mechanisms by which PRP^{Sc} is loaded into the exosomal membrane[68,70-73]. Further evidence for the involvement of exosomes comes from studies that show potentiation or inhibition of exosomal release *in vitro* to alter prion infectivity[74], and that PRP^{C} is also enriched in exosome fractions from the CSF of healthy sheep[75].

[H2] Alzheimer disease: amyloid-β protein

The typical neuropathology of AD includes extracellular deposits of polymerized amyloid-β (Aβ) protein, and intracellular filamentous inclusions of

hyperphosphorylated tau protein, known as neurofibrillary tangles[76,77]. The relationship between these two aggregates and their exact role in the neurodegenerative cascade is not fully understood. The major components of Aβ plaques (polymers of soluble monomers $A\beta_{1-40}$ and $A\beta_{1-42}$) undergo nucleated polymerization upon reaching a threshold concentration *in vitro*, and this polymerization is facilitated by the presence of a polymer 'seed', suggesting that plaque components are susceptible to templated aggregation[78]. Amyloid pathology can also be seeded in primate and murine models by injection of aggregate-containing brain homogenate into wild-type animals[79,80].

Overall, the role of EVs in the biology of AD seems to be complex and nuanced. Several lines of evidence indicate that EVs have a role in amyloid pathology in AD. For example, some cleavage of amyloid precursor protein to Aβ peptides occurs in the endosome, and analysis of exosome fractions by Western blotting and immunogold-labelled TEM has shown that some of the Aβ peptides are released from cells in association with exosomal membranes[81]. Furthermore, the propensity of soluble amyloid peptides to form fibrils is increased by the presence of EVs[82]. In addition, EV-associated proteins are enriched in amyloid plaques, a finding that is in keeping with the hypothesis that EVs are involved in plaque biology, though the nature of this relationship is not clear.

Emerging evidence indicates that EVs have a role in both formation and degradation of amyloid plaques[81]. Neuronal EVs can bind to soluble amyloid peptides, thereby mitigating plaque formation and enhancing degradation of amyloid in microglia [82]. In addition, purified neuronal EVs ameliorate amyloid-mediated disruption of synaptic plasticity in the rat hippocampus by protecting against the neurotoxic effects of Aβ[83]. Purified neuronal EVs also ameliorated amyloid-mediated disruption of synaptic plasticity in the rat hippocampus[83]. Furthermore, though inhibition of exosome

secretion results in reduced amyloid aggregation, infusion of neuroblastoma-derived EVs decreased amyloid deposition in mouse models of AD[84]. Microglial EVs promote disaggregation of Aβ into amyloid oligomers, but contrary to the effects of neuronal EVs, this process leads to increased toxicity[62,63,82,85]. The seemingly contradictory roles of EVs in amyloid biology appear to relate to the cell of origin, in keeping with accumulating evidence that microglial dysfunction contributes to Alzheimer disease pathogenesis.

[H2] Tauopathies

Intracellular neurofilamentous inclusions of hyperphosphorylated tau are characteristic of several rare neurodegenerative diseases in addition to AD, including progressive supranuclear palsy, corticobasal degeneration and some forms of frontotemporal dementia[86]. In AD, characteristic patterns of this tangle pathology in the brain correspond with disease severity[3].

Injection of brain homogenates from mice that express mutant tau into the cerebral cortex of wild-type mice leads to aggregation in the wild-type animals. The aggregation is delayed, suggesting that initial seeding of protein aggregation becomes self-propagating, and is remote from the injection site, an observation that implicates intercellular transfer that could be mediated by EVs[87]. In keeping with this hypothesis, tau isoforms have been detected in EV ultracentrifugation pellets from cell culture models of tauopathies, though the proportion of extracellular tau that is within EVs is unknown[88].

In one study, transgenic mice that express mutant human tau under the control of a neuron-specific promotor have been used to study EV-associated tau propagation in more detail[89]. Tau-containing exosomes isolated from these mice by sucrose density gradient separation, and therefore likely to be of high purity, were sufficient to transfer

tau to cultured neurons. Furthermore uptake of tau by neurons *in vivo* was greater after injection of tau-containing exosomes than injection of tau alone. Further evidence for the involvement of EVs in tauopathies comes from studies of CSF isolated from patients with AD: disease-associated tau phosphoforms, such as that phosphorylated at Thr-181, are enriched in the EV fraction[88].

[H2] Parkinson disease

[H3] α-*Synuclein*

α-synuclein is one of the most strongly implicated proteins in the aetiopathogenesis of PD. The physiological function of α-synuclein remains unclear, but propagation of its misfolding and aggregation is a prominent feature of both genetic and sporadic forms of PD. Inclusions of α-synuclein are also features of several other neurodegenerative diseases, collectively known as synucleinopathies, including PD with dementia, dementia with Lewy bodies, and multiple system atrophy[90-92].

A large body of evidence from studies in cell culture and animal models indicates that α-synuclein oligomers or aggregates have a role in the pathogenesis of PD; seeded propagation is a leading hypothesis of the means of interneuronal transmission of pathology. In humans, the observation of Lewy bodies within mesencephalic stem cell transplants in recipients supports this hypothesis, implying host-to-graft transfer of α-synuclein pathology[96].

Emerging evidence suggests that EVs have a role in the prion-like propagation of aggregated α-synuclein, predominantly the intercellular spread of aggregates[97,98]. α-synuclein is packaged into EVs for extracellular transport [94], and released into culture medium from neuroblastoma cells that express wild-type α-synuclein. α-Synuclein is detectable in the EV ultracentrifugation fraction of this media, and intraluminal α-synuclein can be detected within vesicles with immunogold-labelled

TEM; these observations imply that, though much α-synuclein might be released independently of EVs, some is released within EVs[94]. Other evidence supports this hypothesis. α-Synuclein release is calcium-dependent and increased by disruption of lysosomal acidification and by insensitive inhibition of classical protein secretion, implying the involvement of a nonclassical secretion method, such as release within EVs[94,99]. Lysosomal dysfunction — a PD-relevant stress condition — increases the release of α-synuclein-containing vesicles[100]. Furthermore, the propensity of α-synuclein to aggregate is increased by the presence of EVs, and EV-associated α-synuclein is more likely to be taken up by cells in culture than are free α-synuclein oligomers[97,98], suggesting a role for EVs in intercellular transfer of α-synuclein.

[H3] Leucine-rich repeat kinase 2

Leucine-rich repeat serine/threonine kinase 2 (LRRK2) is another key PD-related protein that strong evidence suggests has a role in regulation of and transport by the EV trafficking pathway. As for α-synuclein, the precise function of LRRK2 remains to be discovered, but evidence that it is pathogenic in PD is overwhelming: the gly2019ser mutation causes the most common form of late-onset autosomal dominant PD, and dysfunction of LRRK2 is thought to contribute to pathology in sporadic PD [101]. LRRK2 is known to co-localize with MVBs, and is released from cells in association with exosomes[102].

[H2] Amyotropic lateral sclerosis

The most characteristic neuropathological feature of ALS is cytoplasmic inclusions that are positive for the 43 kDa nuclear DNA binding protein TAR DNA-binding protein 43 (TDP-43), which is encoded by *TARDBP* [103,104]. The inclusions contain hyperphosphorylated, ubiquitylated aggregates that are predominantly C-terminal fragments of TDP-43. Mutations in *TARDBP*, albeit <1% of cases of ALS, are found

almost exclusively within the C-terminus[105]. ALS-associated mutations in this region are associated with an increased propensity of TDP-43 to aggregate in cell culture models, although aggregation might depend on the presence of the RNA-binding motif RRM2[106,107].

Similar TDP-43-positive inclusions are seen in ~50% of patients with FTD and nearly all patients with ALS, including familial ALS and FTD linked to *C9orf72* hexanucleotide repeat expansions, the most common single cause of both conditions[108,109]. In the remaining <3% of patients with ALS, the aggregates consist of the protein produced by the mutant gene, commonly superoxide dismutase 1 (SOD1) or, more rarely, fused in sarcoma (FUS) [110,111].

When human neuroblastoma cells that express TDP-43 are exposed to insoluble TDP-43 from the brains of patients with ALS or FTD, they develop TDP-43-positive inclusions, indicating that templated aggregation occurs[112]. Templated propagation of SOD1 aggregates has also been observed. Spontaneous aggregation of SOD1 *in vitro* is accelerated in the presence of preformed seeds and in the presence of spinal cord homogenate from mice that express mutant SOD1, but not in the presence of homogenate from mice that overexpress wild-type SOD1[113]. SOD1 aggregates are secreted and subsequently taken up by neighbouring cells to form seeds for further aggregation of intracellular SOD1[114].

The involvement of EVs in the propagation of aggregates in ALS is unclear, but some evidence indicates that they have a role. TDP-43 is enriched in the EV fraction of conditioned media in which TDP-43-expressing neuroblastoma cells have been cultured, and has been detected in EVs extracted from the CSF of patients with ALS and FTD; both findings indicate that at least some TDP-43 is released in EVs, supporting the hypothesis that this mechanism is a means of propagation[112,115]. In a

study published in 2015, use of a luciferase fragment–TDP-43 fusion peptide demonstrated that oligomeric TDP-43 is packaged into exosomes and that exosomal TDP-43 is taken up preferentially over extracellular TDP-43, thereby leading to greater toxicity[116]. The authors employed the same technique to demonstrate the transfer of oligomeric TDP-43 between neurons and axonal transport of TDP-43 after uptake from culture medium. The means by which aggregated SOD1 propagates is controversial, but there is evidence of intercellular transfer via direct secretion of aggregates and secretion in EVs[114,117,118].

[H1] EVs and biomarkers of neurodegeneration

Several properties of EVs make them excellent candidates for harbouring biomarkers. Their cargo of protein and nucleic acid is likely to reflect core pathogenic intracellular processes, in contrast to biofluids, which have a high probability of contamination and are likely to contain less relevant molecules. Secreted EVs can have remote as well as local effects , and those produced in the CNS can be transferred to the CSF, or to distal sites via the bloodstream. CSF and blood are amenable to sampling, and blood is particularly convenient for repeated sampling to monitor treatment responses.

Despite the challenges of extracting EVs from biofluids at the required purity (Box 1), EVs have already shown promise as a pool of enriched biomarkers in a variety of diseases, particularly several types of cancer. For example, in melanoma, increasing serum EV levels are associated with progression to disseminated disease[119]. Evidence indicates that the levels of EVs expressing tumour-associated surface proteins, such as epithelial cell adhesion molecule, in EVs has diagnostic and prognostic value in colorectal cancer [120], and that expression levels of epithelial growth factor receptor subtypes predict treatment responses in glioblastoma multiforme[54]. In 2016, an RNA-based assay to detect the product of the fusion gene *EML4-ALK* in the plasma of

patients with lung cancer became the first EV-based test to receive FDA approval for clinical use to predict responses to certain chemotherapies[121].

[H2]EV Protein

To date, just one study has taken a proteomic approach to the discovery of neurodegenerative disease protein biomarkers in EVs. Principal component analysis was used to compare protein expression in EVs from the serum of PD patients with that from disease controls with ALS and healthy controls. Differential expression of specific proteins — including syntenin 1, a protein involved in the formation and trafficking of exosomes — distinguished between patients and controls[52]. Exposure of cortical neurons to EVs from patients with PD during a stress paradigm improved survival compared with exposure to EVs from controls or to protein-free liposomes, a finding that could indicate a systemic protective response to neurodegenerative disease.

Target-driven approaches, involving immunological assays, have also been used to quantify EV-associated candidate protein biomarkers of disease. Evidence from such an approach indicates that low levels of α−synuclein in the EVs from CSF might help to distinguish patients with Lewy body dementia from those with other conditions, including PD[122]. The same study revealed lower levels of α-synuclein in EVs from the CSF in patients with PD than in healthy controls, but levels did not differ between patients with PD and those with other conditions, such as progressive supranuclear palsy, preventing its use as a specific biomarker of PD. Similarly, a small study of LRRK2 in urinary EVs revealed no difference between LLRK2 levels in patients with PD and those in controls[102].

EVs that express L1CAM, consequently presumed to be CNS-derived, have been analysed to determine whether they could provide protein biomarkers of disease.

Elevated levels of α-synuclein have been observed in these EVs from patients with PD; although the assay did not achieve appropriate sensitivity or specificity for use in diagnostics, it does show potential for further development[13] particularly because α-synuclein was not detected in EVs from the CSF of healthy individuals[12]. A similar approach has revealed altered levels of proteins that are associated with AD (though notably not $A\beta_{1-42}$) and lysosomal proteins in L1CAM-positive EVs prior to the clinical onset of dementia[123,124]. These results require validation, but suggest the potential for a test that predicts development of AD.

Other findings hold promise for the development of a marker for early or presymptomatic AD pathology. An analysis of phosphorylated tau isoforms associated with EVs in the CSF of patients with pathologically-classified early (Braak stage 3) or late (Braak stage 5–6) AD and healthy controls revealed elevated levels of disease-associated isoforms in early AD that fall in later stages[88]. Although promising, this finding requires validation in appropriate cohorts.

EV—based protein biomarker research in ALS is extremely limited, with a single study in which EVs were extracted from the CSF of patients with ALS, FTD and healthy controls identified no difference in the levels of exosomal TDP-43[115], although the sensitivity of current assays for TDP-43 in biofluids is notoriously variable[125,126].

[H2] EV miRNA

Dysregulation of several miRNAs in brain tissue and biofluids has been reported for several neurodegenerative diseases, but the precise roles of these miRNAs in disease pathogenesis remains to be defined. Circulating, extracellular miRNAs are remarkably stable in biofluids such as blood and CSF, and are protected by membranes in EVs[127].

This stability, combined with the fact that the genes that are mutated in several neurodegenerative diseases encode proteins that regulate miRNA biogenesis, makes miRNAs an attractive pool of potential biomarkers of neurodegenrative disease[128].

Several studies have investigated the potential of miRNAs as biomarkers of AD. Early miRNA profiling studies reported upregulation of several miRNAs in the blood and CSF of patients with AD[129,130]; miRNA-9, miRNA-29a and b, and miRNA-146a are just some of the circulating miRNAs that have subsequently been consistently identified as being dysregulated in AD. The targets of these miRNAs are implicated in the formation of toxic proteins and the inflammatory processes associated with the pathogenesis of AD[131-134].

Several lines of evidence indicate that miRNA dysregulation is involved in the pathogenesis of PD[135-137], and the mutated form of LRRK2 interacts with Ago1 and Ago2, two components of the miRNA biogenesis machinery[128]. Only a few studies have assessed the expression of miRNAs in the blood and CSF in PD[138-140], and these studies have identified non-overlapping sets of potentially dysregulated miRNAs. A target-driven study has also revealed that expression of the miRNAs 103a-3p, 30b-5p and 29a-3p is elevated in peripheral blood, and this elevation might contribute to a PD-specific miRNA signature[141]. Putative target genes of these dysregulated miRNAs include *PARK7*, *GPR37*, *CDC42* and *BCL2*, all of which are thought to be involved in the pathogenesis of PD.

Dysregulation of miRNAs in ALS might be associated with dysfunction of TDP-43 and FUS, as these proteins are involved in the biogenesis of miRNAs through interactions with the RNA processing Drosha complex [142]. In keeping with this hypothesis, patterns of miRNA dysregulation overlap between sporadic ALS, familial ALS and presymptomatic carriers of known predisposing genetic mutations[133,143].

Downregulation of miR-1825 and miR-1234-3p has been seen in serum from patients with ALS, and evidence strongly suggests that their target genes *NXPH3* and *NLE1* are involved in the pathogenesis of ALS [144].

To date, literature relating to EV-specific miRNAs as biomarkers of neurodegenerative disease is limited. Low levels of EV-associated miRNA-193b in the serum and CSF have been observed in patients with mild cognitive impairment and established AD, suggesting that this measure has diagnostic potential[145]. A study published in 2015 identified seven miRNAs that were underexpressed in patients with AD; these miRNAs included miR-342-3p, which is overexpressed in brain tissue, but did not include miR-193b[146]. Another study that used a commercial kit for extracting EV-associated miRNAs from serum identified a pattern of miRNA expression that could distinguish patients with AD from healthy controls with a sensitivity of 87% and a specificity of 77%, showing the potential of this method[147]. Once again, however, similarities in the relative abundance of miRNA species with other studies was limited, miR342-3p was the only species in common with a previous study. Similarly, a further study that compared CSF exosomal miRNA in patients with AD, patients with PD and healthy controls identified a specific pattern of miRNA dysregulation for each disease, but did not corroborate the findings of other studies[148].

[H1] Concluding remarks

Extracellular vesicles are strong candidates as vehicles of connectome-based propagation of neurodegenerative disease. Notwithstanding the controversies around this proposed mechanism of propagation, the cell-specific cargoes that EVs carry give them the potential to harbour disease-specific molecular signatures. This property, combined with the evidence that EVs are released from disease-relevant cells into accessible biofluids, makes them an apparently ideal source of biomarkers. Whether

EV-associated biomarkers are most suitable for diagnosis or predicting prognosis, however, remains to be determined.

Though EVs provide an attractive means of accessing detailed information about a cell population of interest (a 'liquid biopsy'), biomarker studies in neurodegeneration have produced little overlap with preclinical and histopathological work. Aside from α-synuclein in EVs from the serum, protein biomarkers from EVs that have been identified so far have not included the proteins that make up the hallmark inclusions. This disappointment could be due to a lack of conformation-specific assays. EV miRNA biomarkers show promise, but are in need of validation, and reproducibility across studies is poor.

The difficulty in harnessing the properties of EVs to yield useful biomarkers is principally due to the small numbers of EVs that are secreted by disease-relevant cells and are detectable in the CSF or bloodThese difficulties are exacerbated by the complexity and technical limitations of current EV extraction methods in terms of EV yield and purity.

Future research must focus on the development of techniques enabling high-yield capture of relevant EV population more likely contain relevant candidate markers. In the few promising biomarkers of neurodegenerative diseases to date, validation in appropriate cohorts is needed. The recent introduction of a clinically approved EV-based assay shows promise for the wider development of routine assays, though this clinical translation mandates the development of robust, reproducible extraction protocols applicable to individual patients.

Figure 1. Extracellular vesicle biogenesis and intercellular interactions. Microvesicles form by outward budding of the plasma membrane (1), regulated by proteins including the endosomal sorting complex required for transport (ESCRT) components, ADP ribosylation factor 6 (ARF6) and dependent upon membrane lipid content[24]. Exosomes are initially produced by invagination of the endosomal membrane, forming the multivesicular body (MVB). Exosomal cargo is determined by several mechanisms, including sorting by ESCRT proteins (2) or ceramide-mediated transport (3)[20]. Exosomes are released from the cell after trafficking of the MVB to the plasma membrane (4), a process that involves the Rab family of small GTPases and the soluble NSF attachment protein receptor (SNARE) complex[72,149,150]. Extracellular vesicles (EVs) are released into the extracellular space and can enter the systemic circulation. EVs interact with acceptor cells at the cell surface via receptor-ligand interactions (5), or by mixing of EV cargo with cellular contents after fusion (6) or endocytosis (7), thereby delivering protein, mRNA and non-coding RNAs to acceptor cells[151,152].

Figure 2 | Proposed mechanisms of prion-like propagation of aggregates in neurodegenerative disease. Extracellular vesicles (EVs) have been proposed as vehicles by which aggregated protein is transferred between cells, assecretion of neurodegeneration-associated proteins as intraluminal or surface cargo of EVs has been observed (1). These vesicles can be taken up by other CNS cells, leading to seeded aggregation of the native protein that leads to toxicity[70,81,88,94,112] (2). Components of the EV biogenesis and trafficking machinery, including the Rab GTPase family, SNARE complex and syntenin-1, have also been implicated in the pathogenesis of neurodegenerative diseases [52,153-155]. Other proposed mechanisms of aggregate propagation are direct secretion and subsequent uptake by nearby cells (3) and direct movement of aggregates from cell to cell via tunelling nanotubes[67,156].

Table 1 Characteristics of EV subtypes

	Origin	Size	Morphology	Markers	Cargo	CNS functions
Exosomes	Invagination of endosomal limiting membrane to form multivesicular bodies (MVB). Released on fusion of MVB with plasma membrane	40–150 nm	Typically appear cup-shaped with TEM, but spherical with cryo-EM	ESCRT components: ALIX, TSG101, flotilin, tetraspanins (CD9, CD63)	Surface and intraluminal proteins including MHC class II, cytokines, adhesion molecules and neurotransmitter receptors mRNA, non-coding RNA, DNA Enriched in phosphatidylserine, sphingomyelin, cholesterol	Neuronal exosomes upregu astrocytic glutamate transp Possible role in synaptic pla disposal of neurotransmitte Oligodendrocyte exosomes neuronal survival and chan rate Cytokines released in astroc exosomes Microglial exosomes contai class II and have a putative role
Microvesicles	Outward budding of plasma membrane	50–2000 nm	Variablyspherical, discoid and cylindrical	Considerable overlap with exosomes Glycoprotein Ib, externalized phosphatidylserine	Surface and intraluminal proteins including cytokines, adhesion molecules; mRNA, non-coding RNA Enriched in phosphatidylserine, sphingomyelin and cholesterol	Secretion of cytokines in mi microvesicles Alteration of synaptic plasti modifying transmitter relea neuronal excitability by mic microvesicles
Apoptotic bodies	Fragmentation of cells undergoing apoptosis	50–5000 nm	Heterogeneous	Phosphatidylserine	DNA, histones, organelle fragments	Uptake by meningothelial c anti-inflammatory effects, t limited CNS-specific study

Table 2

Evidence for the involvement of EVs in neurodegenerative diseases, and possible EV-associated biomarkers

Pathology	*In Vitro* evidence	*In Vivo* evidence	Biomarker*
Prion diseases	PrP^{c} and PrP^{Sc} found in exosomal membranes[68,70] Exosome fractions confer prion infectivity[71,73] Altering EV output affects prion infectivity[73]	PrP^{c} enriched in exosomal fraction in sheep[75]	None
AD: amyloid	Amyloid-β released from cells in EVs [81] Amyloid fibril formation enhanced in the presence of EVs[82]	Neuronal EVs capture amyloid-β and decrease amyloid plaque burden when administered intracerebrally[63,85] EVs ameliorate amyloid-β-induced synaptic disruption[83] Inhibition of EV secretion reduces plaque load in overexpression models[84] EV proteins enriched in amyloid plaques[81]	Elevated plasma levels of LAMP1 and Cathepsin D in plasma EVs of AD patients and presymptomatic individuals[123] Differential miRNA patterns in AD, presymptomatic and healthy control CSF and blood[145-148]
Tauopathies	Tau isoforms detected in culture media ultracentrifugation pellet[88]	Disease-associated phosphoforms found in EV fraction of patients with AD[88]	Disease-associated phosphoforms found in EV fraction of patients with early AD[88] Elevated EV total tau and tau phosphoforms in AD and presymptomatic individuals[124]
Synucleinopathies	α-synuclein detected in EV extraction pellet[94] α-synuclein associated with EVs that are preferentially endocytosed[97] Serum EVs from patients with Parkinson disease are neuroprotective[52]	α-synuclein is detectable in human CNS-derived serum EVs isolated by immunoaffinity capture[13]	α-synuclein is elevated in serum EVs from patients with Parkinson disease[13] CSF EV α-synuclein in dementia with Lewy bodies is decreased compared with PD[122]

			Differential serum EV protein expression in patients with Parkinson disease[52] CSF EV miRNA profile altered in PD[148]
ALS	TDP-43 is enriched in the ultracentrifugation pellet of conditioned media[112] SOD-1 is found in EVs[117]	TDP-43 is enriched in EVs extracted from humans[115]	None

*The biomarkers specified are measures that have been shown to discriminate between disease states and healthy controls of disease controls, or provide prognistic information. All studies lack validation owing to the nascence of the field. AD, Alzheimer disease; CSF, cerebrospinal fluid; EV, extracellular vesicle; PrP^{c}, native prion protein; PrP^{Sc}, misfolded prion protein.

Box 1 Extravesicular extraction

Traditional extracellular vesicle (EV) extraction methods are based on differential centrifugation to separate EVs from other fluid constituents according to density[119]. Exosome extraction requires centrifugal forces in excess of 100,000g. Typically, the vesicle pellet is washed in buffer prior to a subsequent ultracentrifugation step, though contamination with proteins that are not associated with EVs, and possibly miRNA in complex with AGO2, remains an issue[157]. This contamination is not necessarily detrimental to biomarker discovery, but can be overcome by the use of density gradient centrifugation, albeit at the expense of additional vesicle loss and prolonged protocols[158].

Size exclusion chromatography is a newer technique that has been used successfully to extract EVs from cell culture medium and plasma, with yields and exclusion of contaminants that are comparable to those achieved with density gradient centrifugation[35,159]. However, extraction on the basis of size cannot distinguish between EVs and other similar sized particles, such as lipoproteins in blood, and contaminating protein and miRNA[160].

Other developments have included immunocapture methods, in which antibodies against surface antigens are used to capture vesicles. Contamination is still an issue with this method, but it does enable selection for a specific subpopulation of EVs on the basis of surface protein expression[158]. This approach has been exploited to specifically extract EVs that originate from the CNS, based on their expression of the neuronal lineage marker L1CAM[14,125].

Commercial kits are also available for the extraction of EVs. These kits are based on precipitation methods, and typically use polyethylene glycol. Coprecipitation of

contaminating proteins is common with these methods, limiting their use in protein marker discovery, but the same techniques can provide a high yield of RNA [159].

Overall, selection of a method for EV extraction depends on several factors, including the abundance of biofluid contaminants, the molecule of interest (typically protein or RNA) and the abundance of these particles in the fluid. Despite efforts to standardize extraction procedures, no clear consensus has been reached about which extraction method to employ in different circumstances.

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