

## REVIEW

## SUBJECT COLLECTION: CELL BIOLOGY AND DISEASE

# Extracellular vesicles – propagators of neuropathology and sources of potential biomarkers and therapeutics for neurodegenerative diseases

Natasha Vassileff, Lesley Cheng\* and Andrew F. Hill\*

## ABSTRACT

Neurodegenerative diseases are characterised by the irreversible degeneration of neurons in the central or peripheral nervous systems. These include amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD) and prion diseases. Small extracellular vesicles (sEVs), a type of EV involved in cellular communication, have been well documented as propagating neurodegenerative diseases. These sEVs carry cargo, such as proteins and RNA, to recipient cells but are also capable of promoting protein misfolding, thus actively contributing to the progression of these diseases. sEV secretion is also a compensatory process for lysosomal dysfunction in the affected cells, despite inadvertently propagating disease to recipient cells. Despite this, sEV miRNAs have biomarker potential for the early diagnosis of these diseases, while stem cell-derived sEVs and those generated through exogenous assistance demonstrate the greatest therapeutic potential. This Review will highlight novel advancements in the involvement of sEVs as propagators of neuropathology, biomarkers and potential therapeutics in neurodegenerative diseases.

**KEY WORDS:** Neurodegenerative disease, Amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Prion disease, Extracellular vesicles, Exosomes, miRNA biomarkers, Lysosomal dysfunction, Autophagy

## Introduction

Neurodegenerative diseases are characterised by the accumulation of insoluble protein aggregates that affect neurons, resulting in their progressive degeneration. Neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), prion diseases and amyotrophic lateral sclerosis (ALS). ALS is the most common adult-onset motor neuron disease defined by either a spinal or bulbar onset, both of which lead to motor neuron degeneration (Byrne et al., 2011; Wijesekera and Leigh, 2009). AD is the most common form of dementia, and is characterised by a progressive and irreversible decline in cognition (Lane et al., 2018; Mayeux and Stern, 2012). PD is the second-most common neurodegenerative disease and results from the death of dopaminergic neurons in the substantia nigra, which leads to the infamous clinical features of the disease – tremor at rest, muscular rigidity, akinesia and postural and gait instability (Kalia and Lang, 2015; Sveinbjornsdottir, 2016). Prion diseases in humans are infrequent and can be divided into acquired, inherited and sporadic, with sporadic Creutzfeldt–Jakob

disease (CJD) accounting for 85–90% of cases (Gambetti et al., 2003; Takada et al., 2017) (see Box 1 for more details). A common feature of these diseases is the aggregation of proteins, which disrupts normal cellular function. Additionally, the susceptibility of the neurons to cellular dysfunction is increased by their dependence on oxidative phosphorylation, decreased antioxidant production and differentiated post-mitotic state (Hall et al., 2012; Miana-Mena et al., 2011). Despite this knowledge, neurodegenerative diseases are currently incurable, and treatments predominantly focus on the alleviation of symptoms. The biggest risk factor in developing a neurodegenerative disease is age. With an increase in the aging population due to increased life expectancy, a better understanding of these diseases is of utmost importance.

In recent years, small extracellular vesicles (sEVs) have been determined to play a vital role in these neurodegenerative diseases. Here, we discuss recent findings showing that the selectively packaged cargo of sEVs, when altered by events including disrupted clearance pathways, promotes progression and dissemination of the diseases whilst simultaneously acting as a potential source of biomarkers for their diagnosis. Finally, we will highlight how the ability of sEVs to depict the state of their parental cells enables them to be used for the treatment of neurodegenerative diseases, depending on their origin.

## sEVs in neurodegenerative diseases

EVs are a class of heterogenous membranous vesicles created and secreted by all cells (Lötvall et al., 2014). EVs can be divided into large EVs, such as apoptotic bodies, which are 800–5000 nm in diameter that are released during cell death, and sEVs, including microvesicles, which are 50–1000 nm in diameter and produced from plasma membrane budding, and exosomes, which are 40–200 nm in size and originate from the endosomal trafficking system (Cocucci and Meldolesi, 2015; Crescitelli et al., 2013; Kalra et al., 2012; Pan et al., 1985). sEVs were initially believed to discard unwanted material; however, recently, they have been implicated in intercellular communication. The ability of sEVs to cross the blood–brain barrier (BBB) through transcytosis, enables them to facilitate this communication between the central nervous system (CNS) and distal organs (Chen et al., 2016; Haqqani et al., 2013; Matsumoto et al., 2017).

The unique characteristics of sEVs, created through their generation process, can distinguish them from other EVs; however, some features do overlap. Exosome biogenesis begins when endocytosed vesicles form early endosomes, where the vesicular material is either sent back to the plasma membrane for recycling via the endocytic recycling compartment, or the early endosomes mature into late endosomes (Kowal et al., 2014; Stoorvogel et al., 1991) (Fig. 1). The sorting process is controlled by Rab GTPases, including Rab11 (which has two forms in

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### Box 1. Neurodegenerative diseases

ALS is a genetically heterogeneous disease with large hexanucleotide expansions in chromosome 9 open reading frame 72 (C9ORF72) and mutations in superoxide dismutase 1 (SOD-1), TAR DNA-binding protein 43 (TDP-43, encoded by *TARDBP*) and fused in sarcoma protein (FUS) being well documented (Arai et al., 2006; DeJesus-Hernandez et al., 2011; Neumann et al., 2006; Rosen et al., 1993; Vance et al., 2009). TDP-43 aggregates, Lewy body-like hyaline, skein-like inclusions and Bunina bodies are commonly observed in ALS (Miki et al., 2018; Mizusawa, 1993; Okamoto et al., 2008).

AD is a multifactorial disease and can be divided into early-onset, attributed to mutations in the amyloid precursor protein (APP), presenilin 1 or 2 (*PS1* or *PS2*, also known as *PSEN1* and *PSEN2* genes, or late-onset, where apolipoprotein E-ε4 allele (APOEε4) is the greatest genetic risk factor (Cacace et al., 2016; Giri et al., 2016). AD is characterised by the aggregation of hyperphosphorylated Tau proteins in neurofibrillary tangles and amyloid-β (Aβ) 40 and 42 (Aβ40 and Aβ42) in extraneuronal amyloid plaques (Grundke-Iqbali et al., 1986; Masters et al., 1985).

PD can be divided into two subtypes, tremor-dominant and non-tremor dominant, the latter of which has a more-severe phenotype (Jankovic et al., 1990; Kalia and Lang, 2015). Penetrance of the disease is increased by mutations in α-synuclein, which characteristically aggregates in Lewy bodies, in addition to leucine-rich repeat kinase 2 (LRRK2), parkin (PRKN), PTEN-induced kinase 1 (PINK1) and protein deglycase (DJ-1), with genome-wide association studies enabling the identification of more risk loci (Beilina and Cookson, 2016; Lill, 2016; Spillantini et al., 1997).

Prion diseases attributed to mutations in the prion protein gene (*PRNP*) only account for 10–15% of cases, with the methionine/methionine polymorphism at codon 129 exhibiting greatest prevalence in sporadic and acquired CJD cases (Kobayashi et al., 2015; Parchi et al., 1996). Prion diseases are clinically characterised by progressive dementia with ataxia developing into akinetic mutism (Geschwind, 2015; Krasiński et al., 2016). These diseases are characterised by the aggregation of disease-associated scrapie isoform of the prion protein (PrP<sup>Sc</sup>), spongiform changes, neuron loss, astrogliosis and microglial activation (Iwasaki, 2017; Prusiner, 1982).

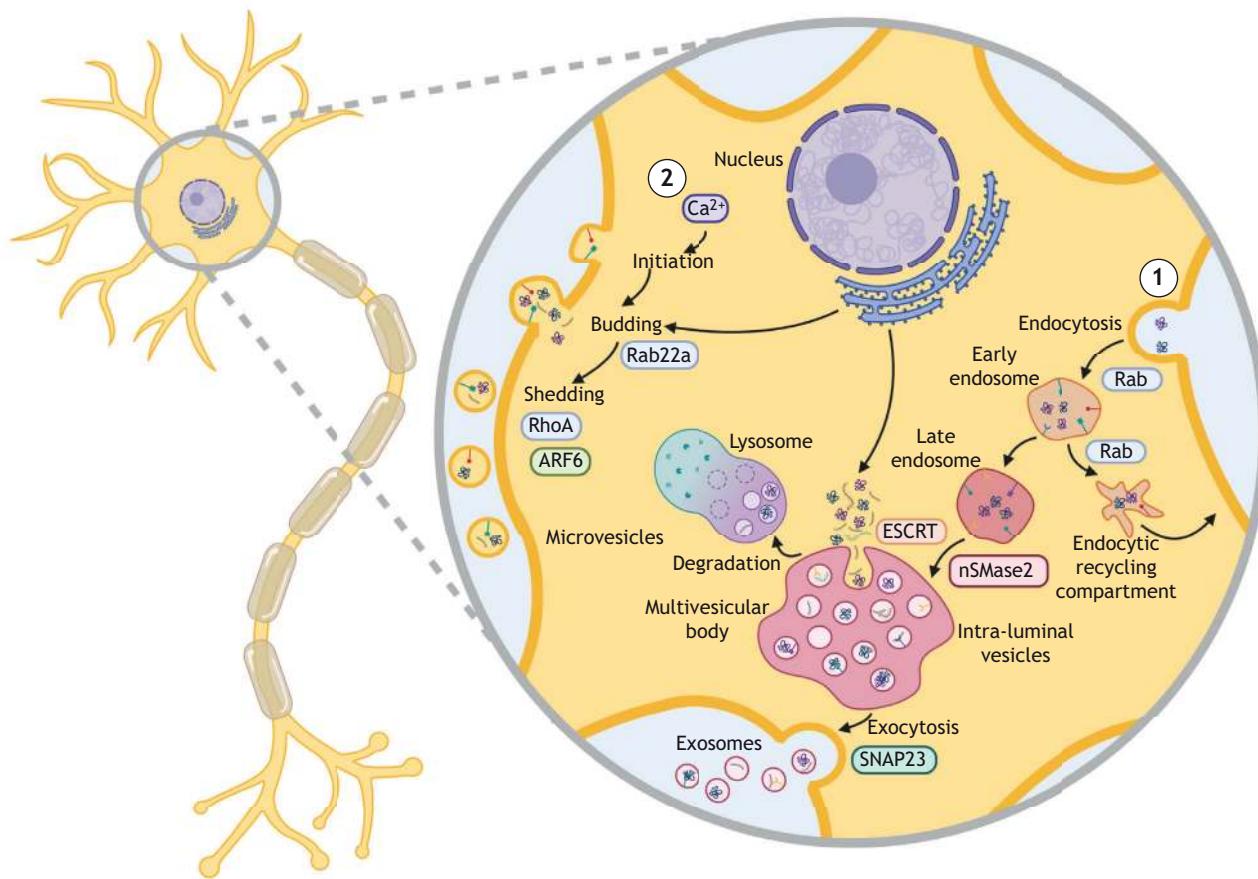
mammals, Rab11a and Rab11b), which has been well documented in neuronal EV generation (Stenmark, 2009). At the late endosomes, exosome cargo, which can include proteins, RNA such as microRNA (miRNA), DNA and lipids, is transferred to the limiting membrane of the multivesicular body (MVB), causing it to invaginate and form intra-luminal vesicles (ILVs) (Kahlert et al., 2014; Pan et al., 1985; Skotland et al., 2017; Valadi et al., 2007). The sorting and formation of ILVs are controlled by endosomal sorting complexes required for transport (ESCRT) proteins, such as vacuolar protein sorting-associated protein (VPS) 4 (VPS4; VPS4A and VPS4B in mammals), tumour susceptibility gene 101 (tsg101) and their accessory proteins, or by ESCRT-independent mechanisms, involving neutral sphingomyelinase 2 (nSMase2; also known as SMPD3), which is vital for neuronal EV biogenesis, in addition to lipids, heat-shock proteins (HSPs) and tetraspanins (Falguières et al., 2008; Nazarenko et al., 2010; Trajkovic et al., 2008). ILVs are either degraded through MVB fusion with lysosomes, or released into the extracellular fluid upon MVB fusion with the plasma membrane as exosomes; this fusion event has been reported to be assisted by synaptosomal-associated protein 23 (SNAP23) in neuronal cells (Buschow et al., 2009; Verweij et al., 2018). In contrast, microvesicle biogenesis begins with an initiating event where cytosolic Ca<sup>2+</sup> activates cytoskeletal remodelling proteins and induces changes in the lipid and protein constituents of the plasma membrane (Taylor and Bebawy, 2019; Tricarico et al., 2017). This results in the recruitment of contractile proteins, changes to hydrostatic

pressure and alterations to the curvature and rigidity of the membrane causing it to bud outwards (Muralidharan-Chari et al., 2010; Taylor and Bebawy, 2019; Tricarico et al., 2017). Vertical and lateral redistributions of cargo then occur, leading to the selective packaging of microvesicles, believed to be assisted by Ras-related protein Rab22a (D'Souza-Schorey and Clancy, 2012; Wang et al., 2014). Finally, a fission event occurs where the opposing membranes are drawn together allowing for scission of the microvesicle into the extracellular space. This shedding step is speculated to involve ADP-ribosylation factor 6 (ARF6) and RhoA (Muralidharan-Chari et al., 2009, 2010; Sedgwick et al., 2015) (Fig. 1). Upon release into the extracellular fluid, the sEVs travel to their target cells where they are taken up through energy-dependent processes (Escrevente et al., 2011). Once inside, the sEVs can alter the biological function of the cell, which in neurodegenerative diseases can lead to further aggregation of misfolded proteins (Emmanouilidou et al., 2010; Gomes et al., 2007).

### Protein content of sEVs

The spread of neurodegenerative disease-associated proteins is enabled by their prion-like properties and is facilitated by sEVs. In prion diseases, sEVs facilitate the spread of cellular prion protein (PrP<sup>C</sup>) and its scrapie isoform (PrP<sup>Sc</sup>) (Fevrier et al., 2004). Stereotaxic injection of sEVs containing PrP<sup>Sc</sup> into mice lead to prion disease symptoms, including motor impairment (Cervenakova et al., 2016; Vella et al., 2007). Furthermore, PrP<sup>C</sup> was found to bind, deliver and co-secrete with amyloid beta (Aβ) in AD sEVs (Qin et al., 2019). In ALS, the prion-like properties of TDP-43 (also known as TARDBP) and misfolded SOD-1, and enrichment of both full-length TDP-43 and its C-terminal fragments (CTFs) in sEVs point to their ability to seed aggregation and propagate their acquired conformation in recipient cells (Ding et al., 2015; Grad et al., 2014; Nonaka et al., 2013). Furthermore, sEVs containing phosphorylated TDP-43 (p-TDP-43), oligomeric TDP-43 and dimeric TDP-43 were found to elicit toxicity in recipient neurons (Feiler et al., 2015; Sproviero et al., 2018). Similarly, AD brain-derived EVs elevated in Aβ oligomers have been implicated in propagating spread as blockage of the formation, secretion and uptake of EVs abolished Aβ oligomer transfer of its acquired conformation (Sardar Sinha et al., 2018). Recently, sEV lipids have been found to promote these conformational transitions, specifically monosialotetrahexosylganglioside (GM1), which binds to and promotes fibrilization of Aβ peptides in AD, and ganglioside lipids, which accelerate conformational changes of α-synuclein and its subsequent aggregation in PD (Dai et al., 2020; Gaspar et al., 2019). Finally, a range of upregulated proteins unique to PD, and FUS in ALS, have been detected in sEVs (Jiang et al., 2019a; Kamelgarn et al., 2016), demonstrating that EVs facilitate propagation of disease-associated proteins.

The proteins carried by sEVs are also capable of promoting inflammation. Increased levels of interleukin (IL)-6 in sEVs from sporadic ALS patient plasma and mutant SOD-1G93A in *in vitro*-derived sEVs are believed to trigger inflammation (Chen et al., 2019; Gomes et al., 2007; Massenzio et al., 2018). sEVs isolated from PD patient serum, containing elevated levels of α-synuclein, IL-1β and tumour necrosis factor (TNF), increased α-synuclein levels, neuronal death, microglial activation and impaired motor coordination, when stereotactically injected into mice (Han et al., 2019). Given the resemblance of α-synuclein to Toll-like receptors, these results suggest that sEV-carried α-synuclein promotes neuroinflammation in recipient cells, including microglia (Han et al., 2019).



**Fig. 1. Overview of sEV biogenesis.** (1) In exosome biogenesis, endocytosed material forms early endosomes, which can either be sent back to the plasma membrane for recycling via the endocytic recycling compartment or mature into late endosomes. This sorting process is controlled by Rab GTPases. At the late endosomes, exosome cargoes, including proteins, RNA, DNA and lipids, are transferred to the limiting membrane of the now multivesicular body (MVB), causing it to invaginate and form intra-luminal vesicles (ILVs). The sorting and formation of ILVs is either controlled by ESCRT proteins or proceeds by ESCRT-independent mechanisms such as facilitation by nSMase2 in neurons. The MVB can then either fuse with lysosomes, resulting in ILV degradation, or with the plasma membrane with the assistance of SNAP23 in neurons, resulting in ILVs being released as exosomes. (2) In microvesicle biogenesis, an initiating event involving  $\text{Ca}^{2+}$  occurs; this leads to the activation of proteins involved in cytoskeleton remodelling and changes in the lipid and protein constituents of the plasma membrane, causing outward budding. Cargo is then vertically and laterally redistributed, leading to the selective packaging of microvesicles thought to involve Rab22a. The opposing membranes are then drawn together, allowing the microvesicle to pinch off into the extracellular space; this shedding step is assisted by ARF6 and RhoA. Both types of sEVs can then travel to and be taken up by recipient cells. Image created with BioRender.com.

The ability of sEVs to facilitate neuronal cell–glial cell communication demonstrates the non-autonomous nature of neurodegenerative diseases (Box 2). This is observed in ALS where dipeptide repeats (DPRs), translated from intronic repeats in C9ORF72, in sEVs are taken up by astrocytes, microglia and mature oligodendrocytes (Westergard et al., 2016). Furthermore, CNS-derived sEVs, containing SOD-1 and its aggregates, predominantly originate from neurons and astrocytes (Silverman et al., 2019). sEVs from PD patients, when injected into mice, activate microglia, resulting in elevated sEV production. These secreted sEVs are then transferred to recipient neurons where the  $\alpha$ -synuclein seeds oligomerisation (Xia et al., 2019). Rat brain slices experiencing microglial activation also exhibit dopaminergic neuronal death and upregulated microglial sEV release, and these effects abolished by inhibition of EV generation (Tsutsumi et al., 2019). In AD, murine brain-derived EVs enriched in high-molecular-mass amyloid precursor protein (APP) C-terminal fragments (APP-CTFs) and oligomers, are believed to propagate disease following uptake by microglial cells (Lauritzen et al., 2019). These findings implicate microglial sEVs in neurodegeneration. APP and hyperphosphorylated (p)Tau levels in AD patient plasma EVs also

correlate with decreasing cognition as the disease progresses from mild to severe (Perrotte et al., 2019). Neuronal cell-derived EVs containing mutated presenilin-1 were stereotactically injected and p-Tau and Tau aggregates were found in the hippocampus of the mice (Aulston et al., 2019). Alternatively, stereotaxic injection of oligomeric A $\beta$ , into the same mouse model, following injection of hippocampal neuronal stem cell-derived sEVs preserved memory function and decreased oligomeric A $\beta$  (Micci et al., 2019). Therefore, cell-specific uptake in addition to EV origin dictates whether the EVs protect or propagate neurodegeneration.

sEV-facilitated propagation of neurodegenerative diseases is assisted by altered intracellular protein transportation and trafficking. In PD, an increased association of mutant  $\alpha$ -synuclein with EVs compared to wild-type  $\alpha$ -synuclein suggests mutated  $\alpha$ -synuclein, owing to its altered physiological function, may favour vesicular secretion (Gustafsson et al., 2018). In prion diseases, induction of endosomal-derived sEV release with the ionophore monensin demonstrated an increase in PrP $^{\text{Sc}}$  transmission to recipient cells, enhanced PrP $^{\text{C}}$  to PrP $^{\text{Sc}}$  conversion and inhibited PrP $^{\text{C}}$  Golgi-dependent trafficking (Guo et al., 2016). Additionally, treatment with

## Box 2. CNS cells and their EVs

### CNS cells

The CNS is primarily comprised of neurons, specialised cells that conduct electrical impulses in the nervous system and supporting glial cells. Microglia are phagocytic cells involved in immune responses and can polarize into M1 (pro-inflammatory) or M2 (anti-inflammatory) activation states, the former is associated with neurodegenerative diseases (Sarlus and Heneka, 2017). Astrocytes are specialized glial cells that are essential for many CNS functions and can become reactive in response to CNS injury, a characteristic of neurodegenerative diseases (Sofroniew and Vinters, 2010). The CNS also includes oligodendrocytes, which insulate axons with myelin sheath and whose dysfunction is associated with neurodegeneration, ependymal cells, which line the ventricular system and are involved in CSF production, and neural stem cells, which generate progenitor cells for neurons and glia (Mot et al., 2018; Ottoboni et al., 2020).

### EV mechanisms in CNS cells

Although classic EV biogenesis is generally conserved across cell types, internalisation of EVs is cell type specific. In the CNS, clathrin-dependent endocytosis is the preferred method by neurons, micropinocytosis by microglia, and receptor-mediated endocytosis by dendritic cells (Fitzner et al., 2011; Montecalvo et al., 2012; Mulcahy et al., 2014).

### CNS cell-derived EV biomarkers

With advances in immunoprecipitation techniques, it is now possible to isolate cell-specific EVs from heterogenous EV solutions such as biofluids. Specifically, in the context of neurodegenerative diseases this has been achieved with the isolation of neuronal cell-derived EVs and astrocyte-derived EVs (Goetzl et al., 2015; Mustapic et al., 2019; Winston et al., 2019).

### Intercellular communication between CNS cells

Although, within the CNS, EVs are endocytosed by many different cell types, some cells exhibit preferences for specific EVs. sEVs from primary neurons are preferentially endocytosed by other neurons (Chivet et al., 2014). By contrast, in neurodegenerative diseases, neuronal EVs are readily internalised by astrocytes and microglia (Fernandes et al., 2018; Morel et al., 2013). Additionally, sEVs released by oligodendrocytes and circulatory EVs entering the CNS are preferentially endocytosed by microglia (Fitzner et al., 2011; Li et al., 2018). Finally, microglial EVs are known to target neurons for functions including modulation of synaptic activity (Antonucci et al., 2012).

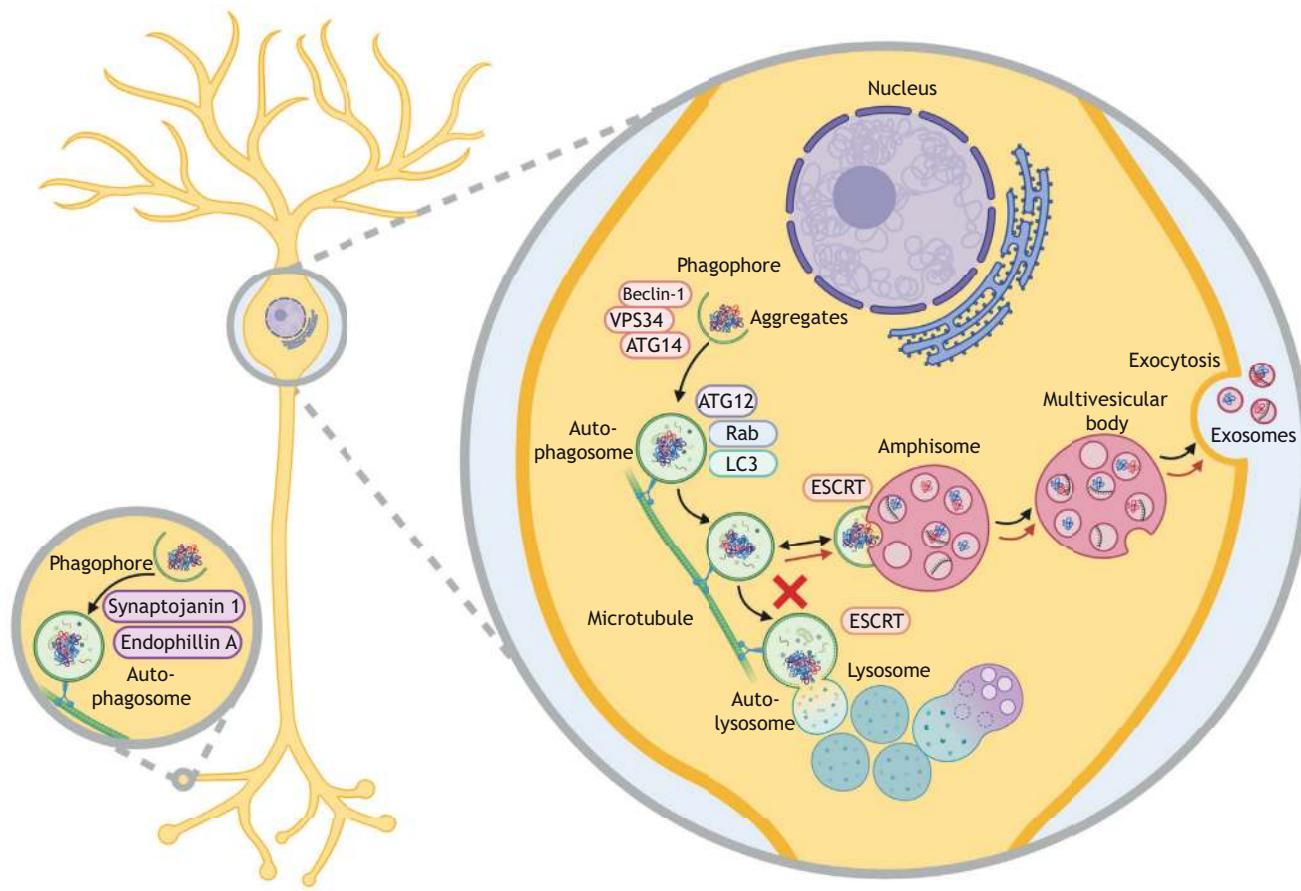
GW4869, which targets nSMase2, decreased sEV secretion and subsequent PrP<sup>Sc</sup> transmission (Guo et al., 2015, 2016). This effect was not observed in infected cells where nSMase2 was inhibited with shRNA, further eluding to alterations in PrP trafficking (Guo et al., 2015). Furthermore, proteasomal inhibition and inhibition of cyclophilin B, an endoplasmic reticulum foldase, increased PrP aggregation and sEV secretion, including PrP, by bypassing Golgi-dependent trafficking (Pan et al., 2018). These studies allude to the upregulation of Golgi-independent trafficking pathways in small-EV-mediated propagation of prion diseases and, in general, neurodegenerative disease-associated alterations in intracellular protein trafficking. The ability of sEVs to be selectively packaged with cargo not only enables them to facilitate further aggregation or neuroinflammation in neurodegenerative diseases, but also enables them to depict the state of their parental cells whom may be experiencing dysregulated physiological processes such as disrupted clearance pathways.

### Lysosomal dysfunction in neurodegenerative diseases and their impact on sEVs

Cells initially attempt to refold misfolded proteins with the help of chaperones such as HSPs (Penke et al., 2018). However, when these proteins cannot be refolded due to their accumulation, the

aggregates they form, as seen in neurodegenerative diseases, are cleared through macroautophagy (hereafter referred to as autophagy) (Metcalf et al., 2012). Autophagy is the process by which cytosolic components and damaged organelles are packaged for lysosomal degradation (Fig. 2) (Parzych and Klionsky, 2014). The process begins with VPS34 (also known as PIK3C3), beclin-1 and autophagy-related protein (ATG) 14 (ATG14) initiating nucleation and formation of a phagophore (reviewed in Guo et al., 2018; Mehrpour et al., 2010; Saha et al., 2018). The phagophore surrounds the intended material and is induced by ATG12, Rab and the microtubule-associated protein light chain 3 family proteins MAP1LC3A, MAP1LC3B and MAP1LC3C (collectively denoted LC3) to elongate and encapsulate the material forming an autophagosome (Mehrpour et al., 2010). The autophagosome travels down microtubules, where it can fuse and exchange material with MVBs, forming amphisomes, before reaching the lysosomes, with whom it fuses creating autolysosomes ready for material degradation (Fader and Colombo, 2009; Xu et al., 2018a). Furthermore, in neuronal cells, synaptic activity, endophilin A, an endocytic adaptor, and synaptotagmin 1, a lipid phosphatase, are known to induce or assist in autophagosome biogenesis at the pre-synaptic terminals (Soukup et al., 2016; Vanhauwaert et al., 2017; Wang et al., 2015). Both the formation of autolysosomes and amphisomes are assisted by ESCRT proteins, such as VPS4 and tsg101, which are vital for exosome biogenesis. Thus, fusion with MVBs and use of ESCRT and Rab proteins demonstrates the close relationship between autophagy and sEV biogenesis. In addition, this alludes to sEV secretion as a compensatory mechanism for lysosomal dysfunction that is caused by the accumulation of misfolded proteins (Eitan et al., 2016; Mizushima, 2007; Murrow et al., 2015). Furthermore, an upregulation of MVB fusion with the plasma membrane during times of oxidative and metabolic stress has been demonstrated to relieve toxic protein accumulation through EV secretion, lessening the burden inflicted on the lysosomal and proteasomal recycling compartments (Eitan et al., 2016).

The close relationship between sEV release and autophagy has been reported to play a vital role in neurodegenerative diseases. The ALS-associated proteins ubiquilin-2, valosin-containing protein, optineurin, and p62 (also known as SQSTM1) assist in recruiting proteins to the autophagosome for degradation (Mandrioli et al., 2019). Furthermore, HSP  $\beta$ 8, which is induced by proteasome inhibition, promotes clearance of mutant SOD-1, TDP-43 and DPRs of C9ORF72 by autophagy (Mandrioli et al., 2019). Additionally, the transcription profile of mutant-SOD-1-expressing mouse spinal cords demonstrated differential expression of exosomal and lysosomal genes, indicating that these processes are affected together in ALS (Henriques et al., 2018). In AD, neurons exposed to A $\beta$ 1-40 and A $\beta$ 1-42 were found to contain enlarged MVBs, increased endolysosomal permeability and A $\beta$  fibril-like structures in MVBs and lysosomes (Willén et al., 2017). Furthermore, ESCRT proteins, such as VPS4 and tsg101, accumulate with A $\beta$ 42 in enlarged vesicles, with mutated VPS4 increasing A $\beta$  aggregation by blocking MVB fusion with lysosomes, an effect abolished by exogenous autophagy induction (Willén et al., 2017). Similarly, neuronal cell-derived blood EVs from AD patients show elevated amounts of lysosomal markers, ubiquitylated proteins and increased levels of the endosomal/lysosomal enzyme cathepsin D, which is responsible for neuronal lysosomal dysfunction (Goetzl et al., 2015). sEVs isolated from PD patient plasma showed enrichment in  $\alpha$ -synuclein and its oligomers when compared to healthy controls, as well as significantly decreased levels of GCase (GBA), a lysosomal enzyme and



**Fig. 2. Autophagy and sEV biogenesis.** Autophagy begins when VPS34, beclin-1 and ATG14 form a complex and initiate phagophore nucleation and formation. The phagophore surrounds the intended cargo before elongating and encapsulating it, resulting in the formation of an autophagosome. This step is assisted by ATG12, Rab and LC3 proteins. In neurons, autophagosome biogenesis involves synaptjanin 1 and can be induced by synaptic activity and endophilin A. The autophagosome travels along microtubules to the microtubule-organising centre where it fuses with lysosomes creating an autolysosome, which allows lysosomal enzymes to degrade the material. As the autophagosome travels along microtubules, it can fuse and exchange material with MVBs, forming amphisomes. Both the formation of the autolysosome and the amphisome is controlled by ESCRT proteins. The similarities between autophagy and sEV biogenesis, including fusion with MVBs and use of ESCRT and Rab proteins, demonstrates a potential relationship between the two processes and the compensatory role sEV secretion may play when autophagy is deregulated as indicated by the red arrows. Image created with BioRender.com.

important PD risk factor (Cerri et al., 2018; Xia et al., 2019). Furthermore, dopaminergic neurons from PD patients exhibited enlarged lysosomes and autophagosomes, increased expression of the autophagy markers LC3 and beclin-1, and increased secretion of  $\alpha$ -synuclein not associated with sEVs, which may be due to the indirect measurement of sEV-associated  $\alpha$ -synuclein used in the study (Fernandes et al., 2016). These results demonstrate a connection between autophagy and sEV packaging.

Further evidence for the compensatory role that sEV packaging may play in times of autophagy dysregulation has come from studies utilizing autophagy inhibitors. In ALS, cells treated with autophagy or proteasome inhibitors exhibited increased TDP-43 levels in their sEVs, indicating clearance of TDP-43 aggregates from the cells (Iguchi et al., 2016). Treatment of cells with sEVs containing TDP-43-CTFs increased autophagy and expression of the active lapidated form of LC3 (LC3-II), beclin-1 and the oncogene p53 (also known as p53) in the recipient cells, suggesting that TDP-43-CTFs may induce autophagy through a p53-dependent pathway (Ding et al., 2015). Similarly, in AD, inhibition of VPS34 leads to increased release of sEVs and the enrichment of APP-CTFs therein (Miranda et al., 2018). Furthermore, the detection of lysosomal substrates, ubiquitin and p62 in these sEVs indicate that the material is destined for disposal and

becomes packaged into the EVs owing to the autophagic defects caused by VPS34 inhibition (Miranda et al., 2018). Inhibition of metalloproteases involved in A $\beta$  degradation increased their presence along with A $\beta$  in sEVs, indicating that the MVB is a site of A $\beta$  degradation (Pacheco-Quinto et al., 2018). Inhibition or dysfunction of the autophagy-lysosomal pathway, which regulates  $\alpha$ -synuclein clearance in PD, increased  $\alpha$ -synuclein shuttling to MVBs and their fusion with autophagosomes, as well as increased  $\alpha$ -synuclein,  $\alpha$ -synuclein aggregates and lysosomal marker levels in EVs (Minakai et al., 2018). The EV-enriched  $\alpha$ -synuclein and its aggregates were also more transmissible and neurotoxic compared to those from EVs isolated from control neuronal cells (Alvarez-Erviti et al., 2011; Fussi et al., 2018). Likewise, treatment of cells with an autophagy inhibitor increased endosomal-derived sEV release and also increased total PrP $^C$  and PrP $^{Sc}$  levels within the sEVs in addition to their infectivity abilities; this effect was reversible upon treatment with an autophagy activator (Abdulrahman et al., 2018). These results suggest that impairment of or low levels of physiological autophagy are capable of increasing packaging of both PrP $^C$  and PrP $^{Sc}$  into EVs, thus facilitating spread and potentially explaining the high susceptibility of some cells, such as catecholaminergic neuronal cells, to prion infection depending on their basal autophagy levels (Abdulrahman et al., 2018).

Interestingly, neurodegenerative disease-associated dysregulation of autophagy has been observed in non-neuronal cell types, including microglia. In ALS, autophagic impairment caused by SOD-1 accumulation in microglial cells increases their toxicity to neurons (Massenzio et al., 2018). Although the microglia released less mutant SOD-1 in sEVs, they internally accumulated more mutant SOD-1 in their lysosomes, expressed less LC3, released more pro-inflammatory cytokines and expressed more markers indicative of autophagy impairment (Massenzio et al., 2018). In PD, stereotaxic injection of sEVs containing  $\alpha$ -synuclein into mice resulted in their uptake by microglia, where they accumulated in lysosomes, induced autophagic inhibition and activated the microglia (Xia et al., 2019). Furthermore, autophagic dysregulation in microglia can be induced by sEVs enriched in the neuronal miRNA miR-19a-3p (Zhou et al., 2019). Thus, the protein content of sEVs is not solely responsible for propagating pathology, as small RNAs also play a significant part in disseminating neurodegenerative diseases as further discussed below.

### The role of sEV miRNA in neurodegenerative diseases

In addition to proteins, the dysregulation of miRNA has been implicated in neurodegenerative diseases. miRNAs are ~22-nucleotide-long non-coding RNA species, and are transcribed in all cells, where they function in post-transcriptional regulation of gene expression (Box 3) (Lee et al., 1993; Wightman et al., 1993). These miRNAs can be packaged into sEVs, and once released into the extracellular fluid, can mediate cell-to-cell communication and facilitate pathogenesis in neurodegenerative diseases (Valadi et al., 2007).

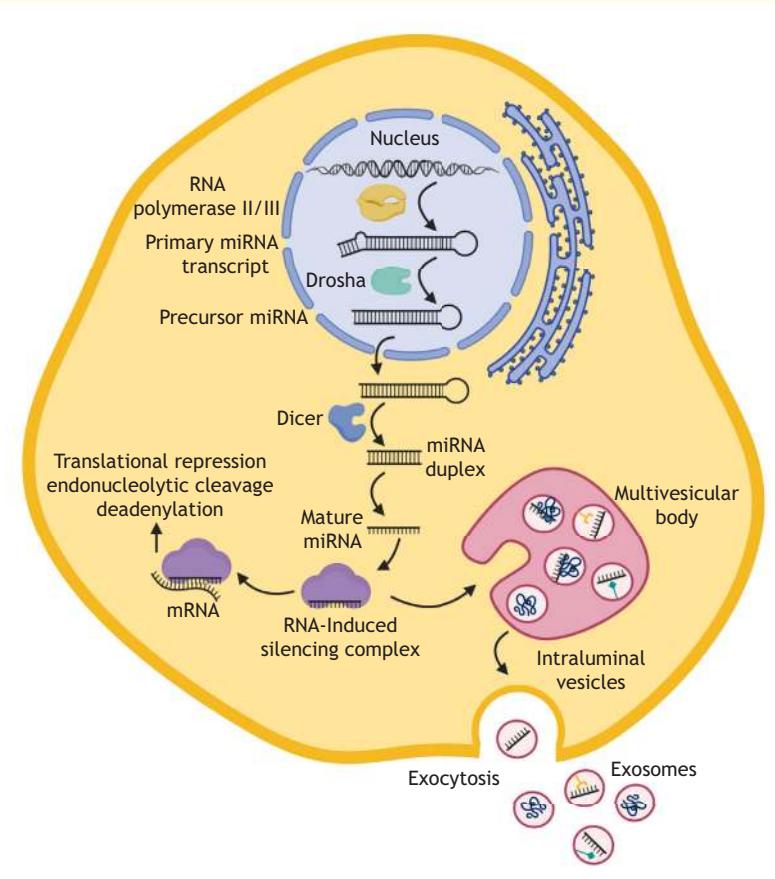
In contrast to what is found for ALS, AD and PD, only a small panel of differentially expressed miRNA have been identified in sEVs isolated from prion-infected cells (Table S1) (Bellingham et al., 2012).

### sEV miRNA in ALS

sEVs in ALS have been found to carry miRNAs believed to contribute to disease progression (Table S1). sEVs isolated from

### Box 3. miRNA biogenesis

miRNA biogenesis begins with the transcription of independent genes and introns into primary miRNA transcripts (pri-miRNA) by RNA polymerase II (see figure showing an overview over miRNA biogenesis). The RNA hairpins are then cleaved by Drosha and DiGeorge critical region 8 (DGCR8), an endoribonuclease microprocessor complex (Gregory et al., 2004; Han et al., 2006; Lee et al., 2003, 2004). The generated 70 bp precursor miRNA (pre-miRNA) is exported into the cytoplasm by exportin 5, where it is cleaved by the endoribonuclease Dicer into a 20-bp miRNA duplex (Lee et al., 2002). The mature miRNA strand of this duplex associates with the RNA-induced silencing complex (RISC) and guides it to target mRNAs to whose complementary 3' untranslated end it binds (Gregory et al., 2005; Lim et al., 2005). This binding induces translational repression and accelerated mRNA degradation through targeted endonucleolytic cleavage or deadenylation of the mRNA, all of which downregulate gene expression (Wu and Belasco, 2008). Alternatively, these miRNAs can be packaged into sEVs where, once taken up by recipient cells, they can downregulate gene expression and facilitate pathogenesis of neurodegenerative diseases (Valadi et al., 2007) (see figure).



SOD-1G93A motor neurons contain elevated miR-124 and are preferentially taken up by microglia (Pinto et al., 2017). In response, the recipient microglia are activated, show upregulated inflammatory markers and experience decreased phagocytic activity, indicative of impaired protective abilities induced by the sEVs (Pinto et al., 2017). Microglia transduced with SOD-1G93A were also found to upregulate pro-inflammatory markers and packaging of SOD-1 into sEVs (Vaz et al., 2019). Upon stress induction, the microglial sEVs downregulated miR-155 and miR-21, and upregulated miR-146a (a negative regulator of pro-inflammatory miR-155) and miR-125b, in contrast to their parental cells (Vaz et al., 2019). The response of astrocytes to ALS neuronal cell-derived sEV exposure has also been investigated. Neuronal sEVs that contain miR-124a were taken up by astrocytes, leading to increased levels of miR-124a and glutamate transporter-1 (GLT-1; also known as SLC1A2) (Morel et al., 2013). This is a crucial finding, given a significant decrease in GLT-1 is observed in end-stage ALS patients (Rothstein et al., 1995), suggesting that astrocytes may have a neuroprotective role. Conversely, EVs isolated from C9ORF72-mutated astrocytes were found to negatively impact survival, neurite length and node number when applied to motor neurons (Varcianna et al., 2019). Furthermore, these EVs contained decreased levels of miR-494-3p, a negative regulator of axonal guidance and maintenance that is downregulated in ALS post mortem spinal cord tissue (Varcianna et al., 2019). Finally, EVs isolated from ALS patient serum show downregulated levels of miR-27a-3p, which was suggested to encourage osteoblast mineralisation by facilitating muscle–bone communication (Xu et al., 2018b). Taken together, these studies demonstrate the ability of deregulated miRNAs in EVs to activate microglia and promote neuroinflammation.

#### sEV miRNA in AD and PD

Similar to what occurs in ALS, miRNAs in AD sEVs can activate microglia (Table S1). A panel of miRNAs, including several implicated in ALS neuroinflammation, were enriched in AD neuronal sEVs that were taken up by microglia, where they colocalised with lysosomes and induced upregulation of pro-inflammatory markers (Fernandes et al., 2018). sEVs isolated from treated microglia contained higher levels of miR-21, pointing to microglial dysregulation caused by AD neuronal sEVs (Fernandes et al., 2018). In PD, miRNAs in sEVs derived from  $\alpha$ -synuclein-transfected neuronal cells suppressed autophagy when applied to microglia (Zhou et al., 2019). Moreover, neurons treated with manganese, an inducer of Parkinsonian features, upregulated their secretion of sEVs, which were enriched in miRNA involved in protein aggregation, autophagy and inflammation (Harischandra et al., 2018). sEV miRNAs from PD cerebrospinal fluid (CSF) were also found to be involved in ubiquitin-mediated proteolysis, axon guidance and neuronal synapses (Gui et al., 2015). Panels of differentially expressed miRNAs in sEVs from AD CSF compared to control CSF have also been reported. Several of these sEV miRNAs were differentially expressed compared to the CSF, demonstrating their selective packaging into sEVs (McKeever et al., 2018; Riancho et al., 2017). Furthermore, miR-193b was significantly decreased in AD murine CSF and serum EVs when compared to CSF and EVs from wild-type mice (Liu et al., 2014). Given that miR-193b is a negative regulator of APP expression, its decreased expression may assist in AD propagation by preventing APP inhibition in target cells (Liu et al., 2014). Likewise, miRNAs that are decreased in AD plasma EVs, compared to that of healthy controls and patients with dementia with Lewy bodies, affect APP

regulation, in addition to neuronal differentiation and morphology (Gamez-Valero et al., 2019). Interestingly, AD serum EVs contain differentially expressed miRNA compared to that in the total serum, suggesting heterogeneous miRNA secretion (Barbagallo et al., 2019). Serum PD EVs contain increased levels of miR-137, and injection of PD mice with EVs containing inhibitors (antagonomirs) against miR-137 gradually reduced PD symptoms, indicating that EV-derived miR-137 might induce oxidative stress by downregulating oxidation resistance protein 1 (OXR1), a sensor of oxidative stress, in recipient cells (Jiang et al., 2019b). Furthermore, unique miRNA expression patterns were observed in sEVs isolated from PD serum and plasma compared to control samples (Cao et al., 2017; Yao et al., 2018). sEVs from neuronal stem cells containing miR-322 and miR-485, were determined to significantly reduce the binding of  $\text{A}\beta$  oligomers to dendritic processes in hippocampal synaptosomes (Micci et al., 2019), confirming the importance of EV origins. miR-34a, a regulator of stress-response elements, is also increased in sEVs isolated from AD primary neurons (Sarkar et al., 2016). Finally, given the prevalence of AD and PD, comparison of sEV miRNA between these two major neurodegenerative diseases has been conducted. This comparison revealed unique panels of differentially packaged sEV miRNAs associated with each disease (Table S1).

Taken together, the differences in miRNA composition of EVs further reflects the complexity of neurodegenerative diseases. Despite all of them leading to neuronal loss, each disease has unique pathological characteristics, including their miRNA content, which enables their EVs to serve as biomarkers for the differential diagnosis of neurodegenerative diseases, as discussed next.

#### EVs in the diagnosis of neurodegenerative diseases

##### EV biomarkers in AD

Currently, AD can be diagnosed as pre-clinical and progress to mild cognitive impairment (MCI) followed by Alzheimer's dementia (Lane et al., 2018). However, these diagnostic criteria can be difficult to apply and additional biomarkers are needed. sEVs isolated from CSF show the greatest promise, as their panels of miRNA predict early-onset AD with high area under the curve scores (AUCs), a measure of the diagnostic accuracy of a given test; scores 0.8–0.9 are considered excellent and scores 0.9–1.0 are outstanding (McKeever et al., 2018) (Table 1). Similarly, miRNA from plasma EVs predict AD with high AUCs (Cha et al., 2019; Lugli et al., 2015). Alternatively, a panel of 15 differentially expressed miRNA identified in serum sEVs that correlate with APOE $\epsilon$ 4 status (see Box 1) was able to predict AD with a sensitivity of 87% (Cheng et al., 2015). Furthermore, three unique differentially expressed serum EV miRNA exhibited a combined AUC of 0.995 for diagnosing MCI (Yang et al., 2018). Increased levels of  $\text{A}\beta$ 42, total-tau (t-Tau), and p-Tau in neuronal cell-derived serum EVs isolated from AD patients, compared to those isolated from MCI patients and healthy controls, successfully distinguished between AD, MCI and healthy controls (Jia et al., 2019). Moreover, decreased levels of SNAP25 in neuronal cell-derived serum EVs predicted AD with a high AUC and positively correlated with Mini-Mental State Examination scores, an evaluation of cognitive function, demonstrating their potential as a biomarker for synaptic integrity (Agliardi et al., 2019). Elevated levels of complement component 4B, fragment Bb and the complement C5b–C9 membrane attack complex in astrocyte-derived EVs predicted MCI patients who would progress to AD with 100% sensitivity (Winston et al., 2019). In addition to miRNA, piRNAs (piRNAs) also exhibit diagnostic biomarker potential. sEVs isolated from AD

**Table 1. Potential EV miRNA biomarkers for neurodegenerative diseases**

Cargo with increased expression in disease-isolated EVs compared to control EVs	Cargo with decreased expression in disease-isolated EVs compared to control EVs	AUC, specificity or sensitivity	Bio-specimen type	References
<b>Alzheimer's disease</b>				
Early onset: miR-605-5p Late onset: miR-605-5p	Early onset: miR-451a, miR-16-5p and miR-125b-5p Late onset: miR-451a and miR-125b-5p	Increased miRNA AUC: 0.73 and 0.77 Decreased miRNA AUC: 0.95, 0.76, 0.71, 0.85 and 0.78 Young onset combined AUC: 0.976 Late onset combined AUC: 0.847	Cerebro-spinal fluid	McKeever et al., 2018
miR-361-5p, miR-30e-5p, miR-93-5p, miR-15a-5p, miR-143-3p, miR-335-5p, miR-106b-5p, miR-101-3p, miR-424-5p, miR-106a-5p, miR-18b-5p, miR-20a-5p and miR-582-5p	miR-1306-5p, miR-342-3p and miR-15b-3p	Combined sensitivity: 87%	Serum	Cheng et al., 2015
miR-135a and miR-384	miR-193b  miR-342-3p, miR-141-3p, miR-342-5p, miR-23b-3p, miR-24-3p, miR-125b-5p and miR-152-3p	Increased miRNA sensitivity and specificity: 94.4% and 94%, 97.2% and 99% Decreased miRNA sensitivity and specificity: 92.5% and 83% Combined AUC: 0.995 Combined AUC: 0.915	Serum	Yang et al., 2018
A $\beta$ 42, t-tau and P-T181-tau	miR-132 and miR-212  SNAP-25	AUC: 0.77 and 0.84 AUC: 0.826	Plasma	Lugli et al., 2015
		AD vs Control combined AUC: 0.98 AD vs MCI combined AUC: 0.88 MCI vs Control combined AUC: 0.85	Plasma	Jia et al., 2019
C4b, fragment Bb and C5b-C9		Combined sensitivity: 100%	Plasma	Winston et al., 2019
miR-27a-3p, miR-30a-5p, miR-34c, piR_019949 and piR_020364	piR_020364	miRNA and piRNA combined AUC: 0.83 piRNAs alone combined AUC: 0.86	Cerebro-spinal fluid	Jain et al., 2019
<b>Parkinson's disease</b>				
Ser(P)-1292, LRRK2		AUC: 0.73, sensitivity: 60%, specificity: 89%	Urine	Fraser et al., 2016b
Ser(P)-1292, LRRK2		PD and G2019S mutation vs without AUC: 1 Ser(P)-1292 LRRK2 in G2019S mutation carriers with PD vs without AUC: 0.844, sensitivity: 100%, specificity: 63%	Urine	Fraser et al., 2016a
Ser(P)-LRRK			Urine	Wang et al., 2017
DJ-1 and $\alpha$ -synuclein		AUC: 0.703 and 0.607	Plasma	Zhao et al., 2019
Tau		AUC: 0.607	Plasma	Shi et al., 2016
$\alpha$ -synuclein		AUC: 0.657, sensitivity: 71.2%, specificity: 50.0%	Plasma	Shi et al., 2014
	$\alpha$ -synuclein	AUC: 0.675, sensitivity: 66.7%, specificity: 71.1%	Serum	Si et al., 2019
$\alpha$ -synuclein			Plasma	Cerri et al., 2018
$\alpha$ -synuclein		AUC: 0.941, sensitivity: 92%, specificity 86%	Saliva	Cao et al., 2019
let-7f-5p, miR-27a-3p, miR-125a-5p, miR-151a-3p and miR-423-5p, miR-10b-5p, miR-22-3p and miR-151-3p combined with $\alpha$ -synuclein		Combined AUC: 0.82, sensitivity: 90%, specificity: 80% Combined AUC: 0.96, sensitivity: 97%, specificity: 90%	Cerebro-spinal fluid	Dos Santos et al., 2018
miR-153, miR-409-3p, miR-10a-5p, and let-7g-3p	miR-1 and miR-19b-3p	Increased miRNAs: AUC: 0.93, 0.90, 0.95 and 0.95 Decreased miRNAs: combined AUC: 0.94	Cerebro-spinal fluid	Gui et al., 2015
miR-24 and miR-195	miR-19b	Combined AUC: 0.946, specificity: 90.0%, sensitivity: 85.3%	Serum	Cao et al., 2017
miR-331-5p		AUC: 0.849	Plasma	Yao et al., 2018

CSF contained a panel of miRNAs and piRNAs that were capable of predicting AD with high accuracies; these accuracies were increased further when combined with p-Tau and A $\beta$ 42:A $\beta$ 40 ratios and when

piRNAs were used to predict MCI to AD development (Jain et al., 2019). These findings demonstrate the promising potential of EV small RNA species in diagnosing neurodegenerative diseases.

### EV biomarkers in ALS

ALS is diagnosed based on the revised El Escorial criteria and the Awaji algorithm (Brooks, 1994; Brooks et al., 2000; Carvalho and Swash, 2009). However, there is currently no definitive diagnostic test, nor is there an early detection test (Scarfino et al., 2018). Many studies have examined differentially expressed genes and proteins, including cytokines, in the blood and CSF of ALS patients with differing success (Moreno-Martinez et al., 2019; Vu and Bowser, 2017). Neurofilament heavy and light chain levels showed promising positive correlations with ALS progression, but lacked specificity for ALS, as did the CSF levels of TDP-43 (Gaiani et al., 2017; Gendron et al., 2017; Kasai et al., 2019; Majumder et al., 2018; Verde et al., 2019). Despite this, sEVs isolated from ALS CSF were found to contain a panel of promising differentially expressed mRNAs (Table 1) (Otake et al., 2019). Additionally, miR-27a-3p was significantly decreased in EVs isolated from ALS serum, thus showing promise as a biomarker (Xu et al., 2018b). Given sEV biomarker discovery is a novel field for ALS studies, comparisons to other neurodegenerative diseases, such as AD and PD, for which sEV biomarker discovery is well established, may be of use.

### EV biomarkers in PD

PD is currently diagnosed based on the appearance of motor parkinsonism with a rest tremor or rigidity; however, there is a need for biomarkers for easier diagnosis (Postuma et al., 2015). Urine is the easiest biofluid to collect, and urine sEVs from PD patients exhibit elevated levels of Ser(p)-1292 LRRK2, a kinase well associated with PD (Fraser et al., 2016a,b; Wang et al., 2017) (Table 1). Neuronal cell-derived plasma EVs are more difficult to collect, but they were found to contain increased levels of DJ-1 (also known as PARK7) and  $\alpha$ -synuclein, which inversely correlated with GCase activity, as well as increased Tau, and could predict PD with moderate AUCs (Cerri et al., 2018; Shi et al., 2016, 2014; Zhao et al., 2019). However, a recent study detected decreased levels of  $\alpha$ -synuclein in neuronal cell-derived serum EVs that were able to predict and differentiate between different onsets of PD (Si et al., 2019). These findings suggest that caution is required when utilizing different blood components for EV biomarker isolation. Interestingly, EVs isolated from the saliva of PD patients containing elevated  $\alpha$ -synuclein levels predicted PD accurately (Cao et al., 2019). Furthermore, several panels of both elevated and decreased miRNAs in CSF EVs predicted PD with high AUCs, which increased when combined with CSF  $\alpha$ -synuclein (Dos Santos et al., 2018; Gui et al., 2015). In addition to CSF EV, miRNAs in serum and plasma EVs accurately predicted PD (Cao et al., 2017; Yao et al., 2018). Thus, EV miRNAs appear more promising as biomarkers for the diagnosis of PD than EV proteins.

### EV biomarkers in prion diseases

Prion diseases are currently diagnosed based on several medical-imaging techniques and analyses, with neuropathology required for a definitive diagnosis (Manix et al., 2015; Zanusso et al., 2016). CSF real-time quaking-induced conversion (RT-QuIC), a technique in which CSF PrP<sup>Sc</sup> induces conversion of PrP<sup>C</sup> *in vitro*, is the current gold standard for diagnosis with a sensitivity of 92% and a specificity of 100% (Green, 2019). In addition, biomarker levels in CSF are examined for the presence of 14-3-3 proteins and elevation of  $\alpha$ -synuclein, Tau and neurofilament light chain, with the latter two showing promise as blood biomarkers (Kanata et al., 2019; Lattanzio et al., 2017; Llorens et al., 2018; Thompson and Mead, 2019; van Eijk et al., 2010). Given the invasiveness of CSF sampling, there is a need for a blood-based

test, which could involve sEVs, as their uniquely packaged cargo, which is protected from enzymatic degradation, may enable for the early detection and differentiation between different forms of prion diseases.

### EVs as therapeutics for neurodegenerative diseases

The unique properties of sEVs can also be exploited for therapeutic development, and below, we discuss recent efforts in this area.

### EV therapeutics in AD

Research into EV therapeutics for AD has been extensive. sEVs isolated from microglia initially exposed to Tau and lipopolysaccharide (LPS) followed by incubation with GW4869 or siRNA against nSMase2, contained decreased levels of Tau and propagated less Tau compared to sEVs isolated from control microglia not incubated with GW4869 or siRNA (Asai et al., 2015). Similarly, intraperitoneal injection of GW4869 into AD mice decreased levels of sEVs in the brain and serum and A $\beta$ 1-42 plaque load, area and number in the cortical region of the brain (Dinkins et al., 2014). Thus, inhibition of nSMase2 appears to have therapeutic value. Furthermore, intraperitoneal injection of curcumin-containing sEVs into AD mice reduced Tau phosphorylation and neuronal death, as well as improving memory and spatial learning, and hence showing promise in recovering neuronal function (Wang et al., 2019). These same beneficial effects, in addition to rescued cognition and reduced plaque deposits, were also observed following systemic administration of EVs isolated from hypoxic-preconditioned mesenchymal stromal cells into AD mice (Cui et al., 2017). These EVs further reduced neuronal A $\beta$  levels, astrocyte and microglial activation, and levels of the proinflammatory cytokines TNF and IL-1 $\beta$  in the microglia of AD mice, while upregulating the number of dendritic spines and anti-inflammatory cytokines in the microglia, pointing to increased A $\beta$  degradation and containment of the inflammatory response (Cui et al., 2017). Recently, several insulin receptor substrates present in plasma-derived neuronal EVs helped determine the efficiency of certain treatments targeting insulin in AD patients; given insulin resistance in the brain is implicated in AD and affects A $\beta$  and Tau processing, this was a crucial finding (Athauda et al., 2019; Mustapic et al., 2019). Thus, EV biomarkers can be used in clinical trials to help identify off-target effects and to target pathways through a blood-based test, enabling for more in-depth monitoring during clinical trials. Finally, cells upregulated in  $\alpha$ -secretase and downregulated in  $\beta$ -secretase, cultured on a 3D graphene scaffold, produced EVs that reduced A $\beta$  production, accumulation and deposition and increased its clearance, when administered to AD mice (Yang et al., 2019). These EVs further rescued memory and cognitive behaviour, and attenuated microglial activated neuroinflammation in the AD mice (Yang et al., 2019), demonstrating the therapeutic efficiency of EVs.

### EV therapeutics in ALS

The therapeutic potential of EVs in ALS is a novel field. Adipose-derived stem cells (ADSCs) have evoked the most interest, owing to their secretion of beneficial factors such as cytokines and neurotrophic factors through routes including EVs (Shukla et al., 2020). Indeed, administration of ADSC-derived EVs to SOD-1G93A neurons significantly reduced aggregation of mutant SOD-1 protein, demonstrating promise as therapeutics for ALS (Bonafede et al., 2016; Lee et al., 2016). In addition, the application of glycoursoodeoxycholic acid (GUDCA) and

dipeptidyl vinyl sulfone (VS) to SOD-1G93A microglia that experience inflammation upregulated the packaging of miR-155 and miR-21, a protective agent against oxidative stress in microglia, into EVs (Vaz et al., 2019). Therefore, GUDCA and VS appear to mediate inflammatory neurotoxicity in microglia, preventing the spread of neuroinflammation through sEVs. Finally, small bioactive molecules have been designed to inhibit translation of DPRs from C9ORF72 RNA and subsequent formation of poly(GP) inclusions and RNA foci in neurons (Su et al., 2014). Packaging of these small molecules into EVs, in order to protect them from degradation in the body and to enable their safe distribution throughout the CNS, may benefit certain ALS patients.

### EV therapeutics in PD

The therapeutic potential of EVs has been well investigated in PD. The creation of the exosomal transfer into cells (EXOtic) device increased production of sEVs, their RNA packaging and cytosolic delivery capabilities, and enabled them to specifically target the brain (Kojima et al., 2018). Subcutaneous implantation of this device loaded with catalase mRNA, an enzyme known to protect neurons from oxidative stress-induced damage, into mice challenged with LPS successfully attenuated CNS neuroinflammation (Kojima et al., 2018). Macrophage sEVs loaded with catalase by a different approach elicited an anti-inflammatory effect when administered into mice (Haney et al., 2015). This was indicated by decreased astrogliosis and microglial activation, and decreased neuron death, evident by increased dopaminergic neuron survival and preserved motor function that is believed to be caused by attenuation of oxidative stress mediated by the catalase (Haney et al., 2015, 2013). Alternatively, murine blood sEVs loaded with dopamine and intravenously administered into mice lead to slow dopamine release in the brain, attenuating the dopaminergic neuronal death characteristic of PD pathology (Qu et al., 2018). Additionally, sEVs isolated from PD patient blood and applied to stressed neurons activated less caspase-3, as determined through immunohistochemical analysis, compared to those isolated from healthy patients, and improved metabolic activity in the neurons, indicating a neuroprotective effect (Tomlinson et al., 2015). Targeting of  $\alpha$ -synuclein has also shown therapeutic promise. EVs loaded with the DNA aptamer F5R2, which recognizes  $\alpha$ -synuclein, were applied to neurons; the aptamer then reduced  $\alpha$ -synuclein phosphorylation and aggregation by inhibiting fibril recruitment of endogenous  $\alpha$ -synuclein and preserved synapsin II and SNAP-25 protein levels, thus rescuing synaptic protein loss caused by  $\alpha$ -synuclein fibrils (Ren et al., 2019). These EVs also improved motor functions when intraperitoneally administered into PD mice (Ren et al., 2019). Finally, attenuation of endosomal-derived sEV biogenesis, through nSMase2 targeting, decreased accumulation and aggregation of high-molecular-mass  $\alpha$ -synuclein, and transfer of oligomeric aggregates between cells (Sackmann et al., 2019). These studies demonstrate that inhibition of sEV production in diseased cells, or exploitation of sEVs with beneficial properties may enable them to become a future therapeutic avenue for neurodegenerative diseases.

### Conclusions

Neurodegenerative diseases are characterised by the progressive loss of neurons. These diseases are currently incurable and lack early and sometimes definitive diagnostic tests, thus requiring immediate attention. EVs have provided insights into the complex nature of neurodegenerative diseases. EVs play a vital role in

propagating neurodegenerative diseases; they are released from diseased cells and, as their cargo represents the state of their parental cells, can promote protein misfolding in receiving cells. Furthermore, the contents of EVs can be altered by dysregulation of autophagy due to its overlap with sEV biogenesis, which can result in further dissemination of misfolded and aggregated proteins. miRNAs in EVs activate microglia and promote loading of inflammatory molecules into their sEVs; however, they also show the biggest promise as biomarkers for early diagnosis. Nevertheless, there are discrepancies in the differentially packaged cargo among specific EVs that may be attributed to different sample collection methods, sampling at different stages of the disease, and inherent differences between *in vivo* and *in vitro* models. Finally, EVs may be exploited therapeutically, and here, stem cell-derived EVs, EVs produced with assistance from exogenous devices and specifically-packaged EVs demonstrate the greatest promise. It is important to note that EVs can have both propagating and protective roles in neurodegenerative diseases, depending on their origin. Therefore, the source, unique characteristics and properties of EVs can be exploited to achieve a better understanding of neurodegenerative diseases, and ultimately for diagnostic and therapeutic purpose.

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### Competing interests

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