HHS Public Access

Author manuscript

Neurobiol Dis. Author manuscript; available in PMC 2020 October 01.

Published in final edited form as:

Neurobiol Dis. 2019 October ; 130: 104512. doi:10.1016/j.nbd.2019.104512.

Emerging roles of extracellular vesicles in neurodegenerative disorders

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Abstract

Extracellular vesicles (EVs) are heterogeneous cell-derived membranous vesicles which carry a large diversity of molecules such as proteins and RNA species. They are now considered to be a general mode of intercellular communication by direct transfer of biomolecules. Emerging evidence demonstrates that EVs are involved in multiple pathological processes of brain diseases including neurodegenerative disorders. In this review, we investigate the current knowledge about EV biology. We also provide an overview of the roles of EVs in related brain diseases, particularly in neurodegenerative disorders. Finally, we discuss their potential applications as novel biomarkers as well as the developments of EV-based therapies.

Keywords

Alzheimer's disease; biomarkers; exosomes; extracellular vesicles; interleukins; microtubule-associated protein tau; neurodegenerative disorders

Introduction

Extracellular vesicles (EVs) are small membranous vesicles bounded by a lipid bilayer and carrying diverse intraluminal cargos of proteins, lipids, and nucleic acids which are secreted into the extracellular milieu [1]. The secretion of EVs was initially described as a consequence of eliminating unneeded compounds from the cell [2]. However, EVs are now known to play vital roles in the intercellular communication that underlies various physiological processes and pathological functions of both recipient and parent cells [3]. The creation of EVs is conserved throughout evolution from bacteria to humans [4]. To our knowledge, EVs are most commonly grouped into three broad types according to their biogenesis: exosomes, microvesicles (MVs) and apoptotic bodies (Table 1) [3]. Most studies have focused on exosomes and microvesicles [5]. They are released from almost all cell types, including the cells of central nervous system (CNS) [6], and their functions in CNS

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are currently under active investigation [7–9]. Here, we examine the knowledge of EVs biology, focus on their roles in neurodegenerative disorders, and discuss their involvement in pathogenesis as well as in biomarkers for these diseases.

EVs subtypes

Exosomes

Exosomes, which were first termed in the 1980s [2], are small extracellular nano-size vesicles with typically 30 – 150nm in diameter [10]. Early endosomes undergo inward budding to form multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs) [11]. By fusion of MVBs with the plasma membrane, ILVs are released into the extracellular environment as exosomes [12]. Alternatively, MVBs can be fused with the lysosomal membrane, resulting in the degradation of the ILVs and their contents [13, 14]. As a consequence of their origin, exosomes contain enriched endosome-associated components, such as Annexins and flotillins [15], the ESCRT (endosomal sorting complex required for transport) component tumour susceptibility gene 101 protein (TSG101), and ALG-2-interacting protein X (ALIX) [16]. Otherwise, membrane proteins that play important roles in biogenesis of endosome or MVBs are also abundant on exosomes. These include tetraspanins such as CD9, CD63, CD81, which are considered as specific markers for exosomes [16, 17].

Exosomes were initially reported in sheep reticulocytes as a mechanism of removing unnecessary proteins and other contents during the maturation of reticulocytes to erythrocytes [2, 18, 19]. In the past decade, secreted exosomes were found not only in cells but the endogenous biofluids (blood, urine, cerebrospinal fluid, etc.) [20–22]. More recently, exosomes have been emerging as long-range messengers, capable of regulating growth and development [23], facilitating inter-cellular communication [24], modulating antigen presentation and inflammation [25, 26], and promoting various stages of tumorigenesis [27].

Microvesicles

Microvesicles, also known as microparticles or ectosomes, are larger than exosomes ranging in size typically from 100 – 1000nm in diameter [11, 23]. Beyond size, microvesicles are also distinguished from exosomes by the fact that they are generated directly by the outward budding of the plasma membrane and are subsequently released into the extracellular space [28]. A recent study identified annexin A1 as a specific marker for microvesicles [29]. However, the components of microvesicle membranes overlap considerably with that of exosomes. For example, although tetraspanins are considered as specific markers for exosomes, these proteins have also recently been observed in microvesicles and other vesicles [30, 31]. Additional experimental data and characterization methods are required to determine whether particular proteins are enriched on microvesicles relative to other specific EV-subgroups.

Apoptotic bodies

Apoptotic bodies are a subpopulation of EVs that are shed by the plasma membrane of apoptotic cells [1]. They are large EVs that range from 100 – 2000 nm in diameter, and

contain fragmented subcellular organelles for degradation [26, 32, 33]. The fate of apoptotic bodies is to be taken up by phagocytic cells for digestion, therefore they are not involved in inter-cellular communication as with exosomes and microvesicles [10].

A cautionary note

Although briefly categorized into three subtypes based on the size range, EVs are also found heterogeneous in their origin and molecular constituents, with considerable overlapped in phenotype [34]. Recent studies have applied different EV-isolated techniques to identify diverse subpopulations of EVs [17, 29, 35, 36]. Joanna K *et al* proposed four subcategories of exosomes (also termed small EVs) based on the expression pattern of tetraspanins, the classic exosome markers, from human dendritic cells by using differential centrifuge and iodixanol gradient floatation: $CD63^+$ $CD81^+$ $CD9^+$ EVs, $CD63^-$ $CD81^-$ $CD9^+$ EVs, $CD63^-$ $CD81^-$ $CD9^-$ EVs, and EVs enriched in other factors [17]. Moreover, a current study has identified two discernible exosome subpopulations (named Exo-S and Exo-L) and a distinct nanoparticle (the Exomere) which differ in size and contents from mostly reported particles by employing asymmetric flow field-flow fractionation, again highlighting the diversity of EVs and particles secreted by cells [36]. Thus, it's difficult to make a distinction between the vesicle types simply dependent on protein markers or size alone. To better interpret and replicate the experiment results among EV studies, combined EV extraction methods as well as improved techniques for accurate purification and characterization are recommended. Additionally, a crowdsourcing knowledgebase is now reliable for researchers in EV field to track latest EV biology and methodology [37].

EV Cargos: Nucleic acids and proteins

EVs were initially regarded as "body dust" and a consequence of loading unneeded compounds from the cell [2, 38]. A major breakthrough was the observation of nucleic acids (both mRNA and miRNA) in EVs and their transfer between cells mediated through EVs [39, 40]. Recently, various species of RNA have been detected within EVs. In addition to mRNA and miRNA, a large number of noncoding RNA, circular RNA, ribosomal RNA, transfer RNA fragments, and small interfering RNAs are also contained in EVs [41–45]. RNA profiling showed many enriched RNAs within EVs relative to the originating cells [45–47] and these EVs-derived RNA can be protected by RNaseA treatment [47], indicating the necessity and importance of RNA molecules loaded in EVs. Otherwise, DNA, including mitochondrial DNA and double-stranded DNA, has been found in EVs [48, 49], although there is a debate from a current study that small vesicles are not involved in active DNA release [29]. Nonetheless, increasing evidence indicates nucleic acids within EVs can be delivered and accepted by recipient cells, thus affecting gene expression [50, 51], regulating cell metabolism [52, 53], and facilitating disease progression [54–56].

Apart from nucleic acids, EVs are highly enriched in protein contents. These include endosome associated proteins (e.g., Annexins, TSG101, ALIX and Rab GTPase) and transmembrane proteins or lipids (e.g., tetraspanins, cholesterol, sphingomyelin), which are involved in the biogenesis of EVs [15, 16, 57–59]. They were identified from a variety of cells by proteomic analysis and SDS-PAGE followed by immunoblotting [60]. Recently, the

manually curated web-based database Vesiclepedia (http://www.microvesicles.org/) catalogs proteins, RNAs, and lipids identified in different classes of EVs from 41 species which are easily accessed for EV study [61]. In addition, the sub-database ExoCarta (http://www.exocarta.org) lists the corresponding data of both exosomes and microvesicles from independent human studies [62]. According to the current version, 41,860 proteins, >7540 RNA and 1116 lipid molecules have been detected within EVs from human samples (Table 2). It has to be noted that the purification methods of EVs in these reports are not ideal and likely contain contaminated molecules. A further validation is necessary with the EV samples isolated from biospecimens according to the recent recommendations as published in MISEV2018 [63]. Thus, the fact that EVs are loaded with enriched biomolecules which can be targeted to the recipient cells within the nervous system opens an entirely new perspective on cell-cell conversation in the brain.

Biogenesis of EVs

Because exosomes and microvesicles differ from their derivation, the cellular machineries involved in their formation and release are likely different in spite of the overlapped mechanistic components (Figure 1). The ESCRT-dependent mechanism was initially interpreted as the biogenesis of ILVs and MVBs, thereafter giving rise to the speculation on its potential role in exosome formation [64, 65]. It's known that the ESCRT machinery mediates the formation of ILVs in a stepwise manner: ESCRT-0, and ESCRT-I complexes are responsible for recognition and subsequently location of ubiquitinated membrane proteins at the limiting membrane of MVBs, while the ESCRT-II and ESCRT-III as well as their accessories (e.g., TSG101, ALIX and VPS4) perform membrane budding and scission of ILVs [66, 67]. Aside from the ESCRT-dependent mechanism, exosomes can also be generated in an ESCRT-independent manner (Figure 1). Trajkovic *et al.* first showed biogenesis of proteolipid protein-containing exosomes derived from the oligodendrocyte cell line is dependent on neutral sphingomyelinase 2 (nSMaseII), disruption of which reduced the secretion of exosomes [59, 68]. Sphingomyelinase can hydrolyse the membrane lipid sphingomyelin to ceramide, and ceramide may then induce the aggregation of microdomains, which promotes domain-induced inward budding of ILVs that are secreted as one class of exosomes [59, 69]. Studies in melanocytes also indicate a luminal domain-dependent pathway and CD63 dependent pathway for MVBs formation which are ESCRTs independent [70, 71]. In addition, tetraspanins, the four transmembrane proteins, are directly involved in the formation of ILVs and exosomes [70, 72–75]. The cone-shaped structure of tetraspanins could induce microdomain formation in membrane and then promote the inward budding of exosomes [76].

Although still largely undefined, the biogenesis of microvesicles requires the involvement of several molecular machineries within the plasma membrane. ESCRT proteins, including ESCRT-I and III, and the generation of ceramide by sphingomyelinase also have an important role in microvesicle biogenesis which is partially common to exosomes [67, 77]. Otherwise, lipids and Ca^{2+}-dependent enzymatic machineries, including aminophospholipid translocases, scramblases and calpain, within the plasma membrane are reported to drive membrane budding and formation of microvesicles [67, 78, 79]. Small GTPases such as

RHO family and ARF6, which regulate cytoskeletal rearrangements, have also been implicated in microvesicles formation [44, 80, 81].

As mentioned above, cytosolic proteins and nucleic acids are enriched in EVs and additional mechanisms may be involved in their sorting. The chaperone heat shock proteins such as HSP70 and HSP90, and heat shock cognate protein (HSC70), which are found coimmunoprecipitated with MHC II together with tetraspanins and enriched in exosomes from most cell types, are suggested to be contributing to sorting soluble proteins to ILVs and exosomes [82–84]. Otherwise, some evidence demonstrates that cytosolic proteins can be incorporated into ILVs and exosomes in an ubiquitylation or farnesylation dependent manner [85, 86]. For RNA species, the sorting mechanism is far from clear. Previous studies revealed ESCRT-II as an RNA binding complex or miRNA-induced silencing complex (miRISC) which mediates RNA silencing process and may function to sort RNAs into exosomes [87, 88]. Recent findings described a broad role of YBX1 in sorting not only miRNA but some small noncoding RNA species into exosomes [89, 90]. One study from cancer cells revealed that a zipcode-like 25 nucleotide (nt) sequence in the 3′-untranslated region (3'UTR) of mRNAs appears target mRNAs to microvesicles [91]. In addition, the neuronal gene encoded protein Arc is suggested to package RNA by self-assembling into virus-like capsids that are then loaded in extracellular vesicles [92].

Uptake of EVs by recipient cells

After entering into the extracellular space, EVs can target to recipient cells and deliver their cargos which mediate the physiological processes and pathological progress. EV uptake requires the interactions with surface receptors at the plasma membrane, followed by their fusion with target cells or endocytosis [93]. Several molecules including integrins, tetraspanins, lipids, lectins, proteoglycans, and extracellular matrix (ECM) components, are known to mediate these interactions [67]. For example, previous work from Nazarenko group suggested that exosomal tetraspanin-integrin complexes are involved in target cell binding [94]. In their experiment, they confirmed that Tspan8 can form complexes with integrin alpha4 as well as CD54 to mediate exosome-uptake [95]. Integrins are also shown to interact with cell adhesion molecules such as ICAMs [96] and ECM proteins such as fibronectin [97] at the cell surface, which drive the endocytosis of EVs. In addition, lipids on the surface of EVs can aid in vesicle docking to the cell membrane. Blockade of phosphatidylserine can prevent exosome uptake in microglia [98], and suppression of its binding protein annexin-V is found to disrupt the cellular uptake of tumor-derived microvesicles [99]. Of note, the mode of interactions between EVs and the recipient cells is likely determined by the specificity of proteins at the surface of EVs and cell membrane, which therefore might account for the target cell specificity [67]. In the nervous system, exosomes released upon synaptic activation selectively bind to neurons but not glial cells [100]. CD63-enriched exosomes can bind to both neurons and glia cells while exosomes lacking CD63 specifically bind to neurons [101]. Additionally, microglial vesicles display different dynamics of interaction with the surface of astrocytes compared with microglia [102]. These findings provide us new insights into the intercellular communication in brain, however, the underlying molecular mechanisms that specify the target of EVs still remains elusive.

Following uptake by recipient cells via different mechanisms, extracellular vesicles may undergo various subcellular fates [67]. Current studies combined live-imaging and super-resolution methods to track EVs and found, in most cases, the internalized vesicles are shuttled within endocytic pathway which are ultimately directed to the lysosome for degradation [103– 105]. Otherwise, it is believed that a small portion of internalized EVs may release their contents into the cytoplasm of the recipient cells, instead of being digested, by fusion with plasma membrane and the limiting membrane of the multivesicular bodies [67, 104]. This process is still poorly understood but it is a mechanism of importance for extracellular vesicles to exert regulatory effects on the recipient cells.

EVs in the CNS and neurodegenerative disorders

Both neurons and glia in the nervous system are known to release EVs [106, 107]. These EVs can move from the central nervous system to the systemic circulation by direct transfer into capillaries or through interstitial fluid into the CSF [1, 108]. In several experiments, EVs isolated from body fluids such as blood and cerebrospinal fluid (CSF) are shown to contain neuron- or glia-specific markers. For example, Goetzl *et al.* found some neuron-associated proteins, such as synaptotagmin and synaptophysin, were present in L1CAM (a neural adhesion protein) immunoprecipitated plasma-derived exosomes [109]. The proteome data from healthy CSF EVs also suggests a high proportion of brain-derived proteins including neuron-specific markers, such as vesicle-associated membrane protein 2 and enolase 2, microglia-specific protein integrin α- M, and oligodendrocyte protein transmembrane protein 132D [110].

Within the nervous system, EVs could be secreted from one cell type and targeted to the other (Figure 2) [107, 111, 112], and are suggested to involve multiple functions. EVs transfer not only membrane components but also nucleic acids between different cells, emphasizing their role in intercellular communication [112]. Microglia-derived EVs have been proposed to contain the cytokine interleukin-1β (IL-1β) and regulate inflammatory response [113]. There is also evidence that microvesicles from microglia can stimulate synaptic activity [114]. Similar to microglia, astrocytes release Nef in EVs to mediate neurotoxicity and synapsin 1 to promote neurite outgrowth [115–117]. Oligodendrocyte-derived exosome-like vesicles are reported to participate in myelin formation and maintenance [118], as well as in trophic support of neurons [8]. Taken together, these findings described above indicate that EVs have crucial physiological roles in the CNS. In addition, EVs are considered to contribute to the pathogenesis of many primary CNS disorders such as neurodegenerative diseases. Pathogenic protein aggregates are widely considered to be contributing factors to neurodegeneration. These aggregates would cause toxicity in cells and involve multiple mechanisms including neuronal death [119], synaptic dysfunction [120], and immune activation [121]. Several pathology-associated proteins including prions [122], amyloid peptide [123], Tau [10], and α-synuclein [124] are found present within EVs. These molecules released from EVs are thought to spread between neural cells contributing to disseminating pathogenesis, as well as enter into circulatory system which can be detected by non-invasive tools (Figure 2) [107]. Below, we will outline the roles of EVs in brain diseases particularly the aspects that are relevant to neurodegenerative diseases, as well as their potential biomarker development (Table 3).

Alzheimer's Disease

Alzheimer's Disease (AD) is a degenerative brain disease and the most common cause of dementia which affects appropriately 30 million patients worldwide [6]. AD is characterized by deposits of amyloid− β (Aβ) protein called Aβ plaques, and abnormally folded hyper-phosphorylated tau protein known as neurofibrillary tangles (NFTs) in patients' brains [125, 126]. Either the Aβ plaques or tau tangles are thought to spread in AD in the manner of a prion disease, where oligomeric proteins act as 'seeds' to undergo nucleated polymerization to form eventual pathogenic aggregates [127]. Although the neuropathology of AD has been clearly studied in the past 30 years, the mechanism of how these pathogenic proteins act in the neurodegenerative cascade remains elusive.

The role of EVs in the pathology of AD begins to uncover this cascade since β-cleavage of the amyloid precursor protein (APP) was found in early endosomes followed by delivery of Aβ to multivesicular bodies [123]. Some exosomal proteins such as Alix and Flotilin-1 were also enriched in the Aβ plaques in AD patient brains, suggesting that exosomes may contribute to Aβ deposits and the pathogenesis of AD. Furthermore, direct evidence of the role of extracellular vesicles in AD comes from isolating neuron-derived exosomes (NDEs) or astrocyte-derived exosomes (ADEs) from plasma in AD patients using neuron-specific antibodies against L1 cell adhesion molecule (L1CAM) or neural cell adhesion molecule 1 (NCAM1), and astrocyte-specific antibodies against glial fibrillary acidic protein (GFAP). Fiandaca *et al.* found levels of amyloid β 1–42 ($A\beta_{1-42}$) in NDEs from AD were significantly higher than those from case-controls 1 to 10 years before diagnosis, which might be developed as a prediction of AD [128]. In addition, levels of cellular survival factors (e.g. low¯density lipoprotein receptor¯related protein 6, heat¯shock factor¯1, and repressor element 1¯silencing transcription factor) in NDEs were significantly lower in Alzheimer's disease patients than controls, possibly explaining the decreased neuronal resistance to neurotoxic proteins in AD [129]. Recently, Goetzl and their colleagues, isolating ADEs from AD patients, noticed significantly raised levels of complement including C1q, C4b, C3d, and pro-inflammatory factors including IL-1, TNF- α , IL-1 β compared to matched controls [130]. Further analysis revealed levels of complement proteins and some complement regulatory proteins in ADEs were associated with the staging of disease, for example, higher complement proteins and lower regulatory proteins in patients at AD2 stage than that at AD1 preclinical stage.

Consistent studies revealed a prominent role of EVs in the AD pathology, however, the relationship is not clear. Numerous evidence indicates that EVs play a neurotoxic role in AD. Higher levels of myeloid MVs were found in the CSF of AD and MCI patients than controls, suggesting the role of EVs in propagation of disease [131]. Besides, the increased myeloid EVs was thought to be released by activated microglia and contain neurotoxic Aβ forms which could drive AD degeneration [132]. Moreover, AD patients' brains derived exosomes contain elevated toxic Aβ oligomers that can promote the neuron-to-neuron transfer of oligomers which cause toxicity in culture [133]. Therefore, EVs might be a potential therapeutic target for AD treatment. For example, inhibition of nSMase2, a key regulatory enzyme involved in ESCRT-independent exosome biogenesis with a small molecule GW4869, can reduce Aβ deposits both in vitro and in vivo [134]. Additionally,

genetic depletion of nSMase2 can ameliorate AD pathology and improve cognitive deficits in 5×FAD mouse model of Alzheimer's disease [135]. There are also some studies suggesting EVs may play a neuroprotective role in AD such as aiding in the degradation of amyloid plaques. Neuron-derived exosomes assist in conformational changes of A β to nontoxic amyloid fibrils and promote their uptake by microglia [98]. These Aβ were further transported to lysosomes for degradation. Additionally, exosomes derived from N2a cells or human CSF may ameliorate the synaptic-plasticity-disrupting activity of both synthetic and AD brain-derived Aβ in vivo [136]. These controversial studies demonstrate EVs would act as a 'doubled-edged sword' in the amyloid accumulations. In the future, more details of EVs, for example cell of origin, in vivo and vitro, and stage of disease, are needed for functional analysis.

Hyper-phosphorylated tau-based NFTs have also been proposed to contribute to the diffusion of neurodegeneration including AD, and this tangle pathology proceeds in a characteristic and predictable spatiotemporal manner [137, 138]. What is the underlying mechanism accounting for the sequential dissemination of tau? Apart from direct cell to cell contact for tau propagation, accumulating evidence recently supports that EVs are engaged in tau spread particularly as a form of long distance communication. Sudad *et al.* identified exosome-associated tau which is phosphorylated at Thr-181 (AT270), an established phosphotau biomarker for Alzheimer disease (AD), in human CSF samples from early AD [139]. Phosphorylated tau was also present in exosomes derived from plasma in patients with AD or frontotemporal dementia (FTD) [128]. The exosomal levels of P-T181-tau and P-S396-tau in AD were significantly higher than that in healthy controls even 1 to 10 years before AD diagnosis. Currently, induced pluripotent stem cells (iPSCs) have become ideal tools for the postnatal generation of specific diseased cell types from patients [140]. Researchers differentiated AD patient-derived iPSCs into functional neurons and detected more aggregation-competent Tau from neuron-derived EVs compared to healthy controls [141], which provides important insights of EV in the pathology of AD. Furthermore, in our previous study, more details of the EVs-associated tau propagation were provided by developing a mouse model with rapid tau spread. We demonstrated that microglia can spread tau via exosome secretion, and inhibiting exosome synthesis with GW4869 significantly reduced tau propagation in vitro and in vivo [7].

For the application of EVs to the potential biomarker in disease, It was mentioned that levels of $A\beta_{1–42}$ and cellular survival factors from NDEs [128, 129], complements from ADEs [130], and phosphorylated tau isoforms from exosomes in CSF and plasma [128, 139] are associated with development of AD. In addition, several studies have identified some dysregulated miRNAs as potential biomarker for AD. A MicroRNA profiling study reported an opposite pattern of the microRNA expression in the exosome-enriched CSF AD samples and control samples; miR-9–5p and miR-598 are present in most of control CSF samples but not in AD samples [142]. Deep sequencing of plasma exosomal miRNAs from control and AD patients screened a panel of seven miRNAs to predict AD status with 83–89% accuracy [143]. Of these miRNAs, miR-342–3p is the most significant and brain-enriched in tissue expression. Notably, it's common with two previous studies which reported circulating mir-342–3p is down-regulated in AD [144, 145]. Though promising, the literature of EV-

associated miRNAs as biomarkers in AD is still limited and these findings require validation in different cohorts.

Amyotrophic lateral sclerosis (ALS)

The major pathological proteins of ALS include TAR DNA-binding protein 43 (TDP- 43), Cu/Zn superoxide dismutase 1 (SOD1) and fused in sarcoma (FUS), abnormal accumulations of which would result in motor neuron death in spinal cord, brain stem, and cortex [146–148]. These aggregates are also thought to propagate in a manner similar to prion proteins by which misfolded proteins seed at a focal site before spreading to contiguous neuroanatomical regions [149, 150]. Familial ALS (FALS) accounts for 5–10% of all cases, while the others are sporadic (SALS). No evidence shows difference in clinical characterization between FALS and SALS patients, suggesting that they may share common pathogenic mechanisms [151].

EVs, as new-discovered intercellular messengers, are raising more interests in the study of neurodegenerative diseases including ALS. The propagation of ALS has been proposed to be mediated through release and uptake of protein aggregates (SOD1, TDP-43, FUS) in EV-dependent pathways [150, 152, 153]. It was observed that wild type and mutant SOD1 were respectively present in exosomes within the supernatant medium from wild type and mutant SOD1 stably expressed NSC-34, a motor neuron-like cell line [154]. Moreover, astrocyte-derived exosomes were found to efficiently transport mutant SOD1 to spinal neurons and induce motor neuron death in primary astrocyte cultures overexpressing mutant SOD1 [155]. The authors believed that these exosomes might help limit the formation of intracellular aggregates and prevent from toxicity. But on the other side, exosome release is also considered to be a pathway for spreading disease [155].

Similar to SOD1 protein, TDP-43 was detected in secreted exosomes from Neuro2a cells and primary neurons, as well as from CSF of ALS patients [156, 157]. Application of exosomes isolated from brains of ALS patients, but not from healthy controls, to Neuro2a cells causes pathological TDP-43 aggregates, suggesting that secreted exosomes might contribute to propagation of TDP-43 proteinopathy [156]. This was supported also by another study that oligomeric TDP- 43 is packaged into exosomes, and is preferentially taken up by recipient cells, thereby leading to greater toxicity than free TDP-43 [158]. More recently, the researchers observed increased size of MVs and exosomes isolated from plasma of sporadic ALS patients compared to healthy controls [150].

Plasma-MVs and-exosomes of ALS patients are enriched in ALS associated proteins including SOD1, TDP-43, phospho-TDP-43, and FUS, suggesting that EVs can be a reliable biomarker [150]. In addition, Xu Q *et al.* reported the down-regulation of serum-derived exosomal miR-27a-3p which targets the genes promoting osteoblast mineralization in ALS patients compared to healthy subjects [159]. The author suggested it may be involved in the development of ALS, and therefore could be a candidate indicator for the diagnosis of ALS in the clinic. Several studies demonstrated exosomes from adipose-derived stem cells exert a neuroprotective effect in an in vitro model of ALS including increasing motoneuron survival

and restoring mitochondrial protein function, demonstrating that EVs could be potential as a novel therapeutic target to halt the course of ALS [160–162].

Huntington's Disease

The neuropathological feature of Huntington's disease (HD) is intracellular inclusions consisting of mutant HTT protein, which is encoded by mutant *HTT* gene with a trinucleotide repeat CAG in the first coding exon [163]. The mutant HTT protein contains the expansion of polyglutamine (polyQ) repeat which can form protein aggregates and fibrils and induce neurotoxicity in brain [164].

EVs are recently considered as an important regulator in HD propagation. Both polyQ protein and the repeat RNA were found incorporation into EVs in a model of human 293T cells with the overexpression of mutant *HTT* gene [164]. A higher level of the repeat RNA was loaded into EVs than the level of the normal mRNA, indicating the potential role of EVs in transferring toxic repeat RNAs. This is supported by further experiment that these EVs can be taken up by striatal mouse neural cells though no apparent toxicity was observed probably due to a short incubation period [164]. Otherwise, a more recent study determined a neuroprotective role of cell-type specific EVs in HD [165]. The researchers observed the defective exosome release from astrocytes in a mutant huntingtin HD140Q knock-in (KI) mouse model. Further, injection of normal astrocytic exosomes into the striatum of HD140Q KI mice could reduce the density of pathogenic aggregates [165]. Exosomes from adipose-derived stem cells, which are known to secrete various neurotrophic factors and also studied in ALS, are shown to decrease protein aggregates and ameliorate mitochondrial function in an in vitro HD model [166]. All these findings define a progressive role of EVs in HD propagation but also reveal a potential therapeutic effect of EVs on disease.

Parkinson's Disease

Parkinson's disease (PD) is characterized by the pathologic accumulation and aggregation of α- synuclein (α-syn) in the neuronal soma (Lewy bodies) and neurites (Lewy neurites) [167, 168]. α-syn aggregates are formed in a step-wise manner that misfolded monomers lead to the formation of oligomers and protofibrils, which eventually deposit as fibril and insoluble aggregates [169]. In PD, Lewy bodies are initially resident in brain stem as well as the substantia nigra at early stages, which become more widespread to cerebral cortex and other brain regions as the disease progresses.

Recent evidence suggests that EVs may play a vital role in the hierarchical spreading of toxic α-syn within brain. α-syn was found elevated in serum-derived EVs from PD patients [170]. It has been proposed that exosomes can participate in the inter-neuronal transmission of α-syn by using neuronal-like cells [124]. Oligomeric α-syn has been detected half within the exosomes and half on the extracellular space by fusion of α-syn to humanized Ganussia Luciferase [171], indicating the presence of EVs-associated α-syn. Additionally, these exosomal α-syn oligomers are more efficient to be taken up by recipient cells and induce toxicity compared with free α-syn oligomers. Further evidence indicates that exosomes can trigger the aggregation of α-syn, probably due to the optimal catalytic environments for

nucleation they provide [172]. The authors also concluded that this reaction is more likely to be mediated by phospholipids which are enriched in vesicular membranes. Another study supports this hypothesis. The lipid peroxidation product 4-hydroxynonenal (HNE) was found to increase not only the aggregation of endogenous α-syn but the secretion of EVs containing toxic α-syn as a consequence of degeneration in primary neurons [173]. Moreover, stereotaxic injection of these EVs into the striatum of wild-type mice leads to the spread of synuclein pathology to anatomically connected brain regions including the cerebral cortex, substantia nigra, and hippocampus [173]. Notably, a current study demonstrated a new mechanism based on EV-associated α-syn for PD progression: α-syn-rich EVs produced by periphery erythrocytes could cross the blood-brain barrier and provoke microglial inflammatory responses to initiate CNS α-syn-related pathology [174]. All these findings support a role of EVs in the transcellular propagation of α-syn.

In addition to α-syn, other PD-related proteins such as PARK9 and LRRK2 may be involved in EVs trafficking pathway thereby regulating α-syn transport [169]. For example, overexpression of PARK9 results in increased release and loss of function mutations in PARK9 results in decreased secretion of α-syn into extracellular space via exosomes [175]. Similar functions have been implicated in LRRK2, too. LRRK2 is known to colocalize with MVBs, and the R1441C LRRK2 mutant has been reported to increase the number and size of MVBs; LRRK2 dysfunction would disrupt the dynamics of vesicular release [1, 176]. Another study demonstrated that LRRK2 interacting with Rab5b regulates endocytosis of synaptic proteins, suggesting LRRK2 might also have a role in EVs-associated uptake [177]. Moreover, LRRK2 is released in exosomes from cells [178].

Levels of autophosphorylated LRRK2 (P-S1292) were found to be elvated in urinary exosomes and are correlated with the severity of cognitive impairment and difficulty in accomplishing activities of daily living, which is a promising candidate biomarker for PD [179]. Also, EV-associated miRNAs are useful for diagnosis of PD though the studies are limited [180]. Gui *et al.* reported that miR-1 and miR-19b-3p are significantly reduced in PD CSF exosome, while miR-153, miR-409–3p, miR-10a-5p, and let-7g-3p are elevated [181]. Further pathway analysis demonstrated that these molecules are targeted to Neurotrophin signaling and Dopaminergic synapse. Another group collected serum-derived exosome-like microvesicles from PD patients and validated the downregulation of miR-19b, the upregulation of miR-195 and miR-24 in PD compared to healthy controls [182].

Prion Diseases

Prion diseases such as Creutzfeldt–Jakob disease are associated with the aggregates of misfolded prion protein (PrP^{C}) [183]. PrP^{C} misfolding results in the generation of pathological prion protein (PrP^{Sc}), a key factor in the pathophysiology of prion diseases [184]. Different mechanisms of how prion proteins move and progressively spread between cells have been proposed, including direct release and uptake by nearby cells, and intercellular transfer through tunneling nanotubes [24, 185].

Increasing evidence indicates that exosomes represent a novel and efficient way for prion transmission. Saá *et al.* first reported the presence of PrP^{Sc} in exosomes isolated from

plasma in a prion disease mouse model [186]. After, they injected these EVs to PrP^{C} transgenic mice and found the transmission of prion occurs, suggesting exosomes are the most likely carriers of intercellular PrP^{Sc} transmission [187]. Further evidence identified the mechanisms by which PrP^{C} or PrP^{Sc} is packaged into exosomes. Guo *et al.* proves that the neutral sphingomyelinase pathway regulates exosome biogenesis and packaging of PrP^{C} into these vesicles by inhibition of the pathway using GW4869 [188]. Moreover, packaging of PrP^{C} into exosomes is dependent on nSMase2 whereas PrP^{Sc} packaging occurs independently of nSMase2, providing new insights into prion transmission and identify a pathway which might be targeted to avoid the infection [189].

In addition, some miRNAs were found dysregulated in exosomes from prion infected samples. A small RNA deep sequencing of exosomes released by prion-infected neuronal cells demonstrated a distinct miRNA signature compared to non-infected exosomes (increased let-7b, let-7i, miR-128a, miR-21, miR-222, miR-29b, miR-342–3p, and miR-424 levels with decreased miR-146a levels) [43]. These results might not only be utilized for diagnostic biomarker but provide better understanding of the pathogenic mechanisms in prion disease.

Discussion

In the past decade, the major progress on EVs was finding their active roles in cell-to-cell communication, both under healthy and pathological conditions. The properties that EVs can be accessibly isolated from biofluids and carry cell-specific cargoes, including proteins and nucleotides, give them the potential to harbor disease-specific molecular signatures [1]. Therefore, vesicles have been proposed as useful candidate biomarkers as well as promising therapeutic targets in neurological diseases. For example, the levels of Aβ1–42, P-T181-tau, and P-S396-tau in plasmal exosomes predict the development of AD 1 to 10 years before clinical diagnosis [128]. High complement proteins including C1q, C4b, C3d were found in exosomes from AD patients [130]. For PD, levels of α-syn and autophosphorylated LRRK2 were elevated and found correlation with the severity of disease [170, 179]. In addition, a distinct miRNA signature in exosomes has also been revealed in other neurodegenerations [43].

Development of EV biomarkers show promise; however, reproducibility across studies is poor. One reason is that the pathology of neurodegenerative disorders is associated with distinct subsets of CNS cells, and the heterogeneity of EV populations and technical limitations of current EV extraction methods in terms of EV yield and purity make the identification of reliable biomarkers a challenge [1]. In order to solve these problems, researchers applied immunoaffinity enrichment to immunoprecipitate CNS-specific exosomes from AD samples via cell-specific antibodies, (e.g., neuron-specific L1CAM and astrocyte-specific EAAT1), and indeed obtained some promising results [128, 130, 190]. However, no study shows that these proteins are present in EVs isolated from the different cell types. Therefore, identification of cell-type specific EVs markers is necessary for comprehensive understanding of the sources of pathological EVs and the potential therapeutic targets for halting propagation of diseases. Future research will focus on enabling high-yield capture of EVs and shed light on the nature of the different extracellular

vesicle subpopulations that could be associated with distinct pathological stages in a given disease.

Aside from developing new biomarkers and therapeutic targets for neurodegenerative diseases, EVs themselves could be used as non-invasive vectors to deliver defined compounds for therapeutics. The best advantages is that EVs are biocompatible and self-derived, and therefore they are immunologically inert and less likely to trigger innate and adaptive immune responses [191]. Mesenchymal stem cell-derived EVs has been tested to treat acute kidney injury [192], stroke [193] and ischemic brain injury without immunogenicity [194]. A previous study successfully designed the engineered exosomes which were loaded with short interfering RNAs (siRNAs) to target the CNS cells and silence expression of BACE1, a therapeutic target in AD, in wide-type mice [195]. Genetically engineered microvesicles expressing suicide gene can effectively inhibit schwannoma tumor growth [196]. Moreover, application of EV-based drug delivery has been registered in serval clinical trials [197]. Undoubtedly, these aforementioned use of EVs have addressed the potential of EVs as novel therapeutics in various diseases.

Despite the enormous therapeutic potential, there are still many questions of EVs remaining to be answered [107]. It is unclear how EVs are sorted to disparate targets and how recipient cells discriminate among EVs. The mechanisms of EVs uptake by recipient cells also remains unknown, as well as the decision of EV cargoes' destination of lysosome for degradation or to be utilized by recipient cells. More new in vivo models combined with powerful imaging methods to track the biogenesis, secretion, uptake, and fates of EVs will help us further understand the basic functions of EVs and promote the translation of researches into the clinical applications.

Acknowledgements

We would like to thank Samuel Hersh for editing the manuscript. This work is funded in part by Nancy Lurie Marks Family Foundation (TI), Robert E. Landreth and Dona Landreth Family Foundation (TI), BrightFocus Foundation (A2016551S), Cure Alzheimer's Fund, NIH R01AG054672 (TI), RF1AG054199 (TI), R56AG057469 (TI).

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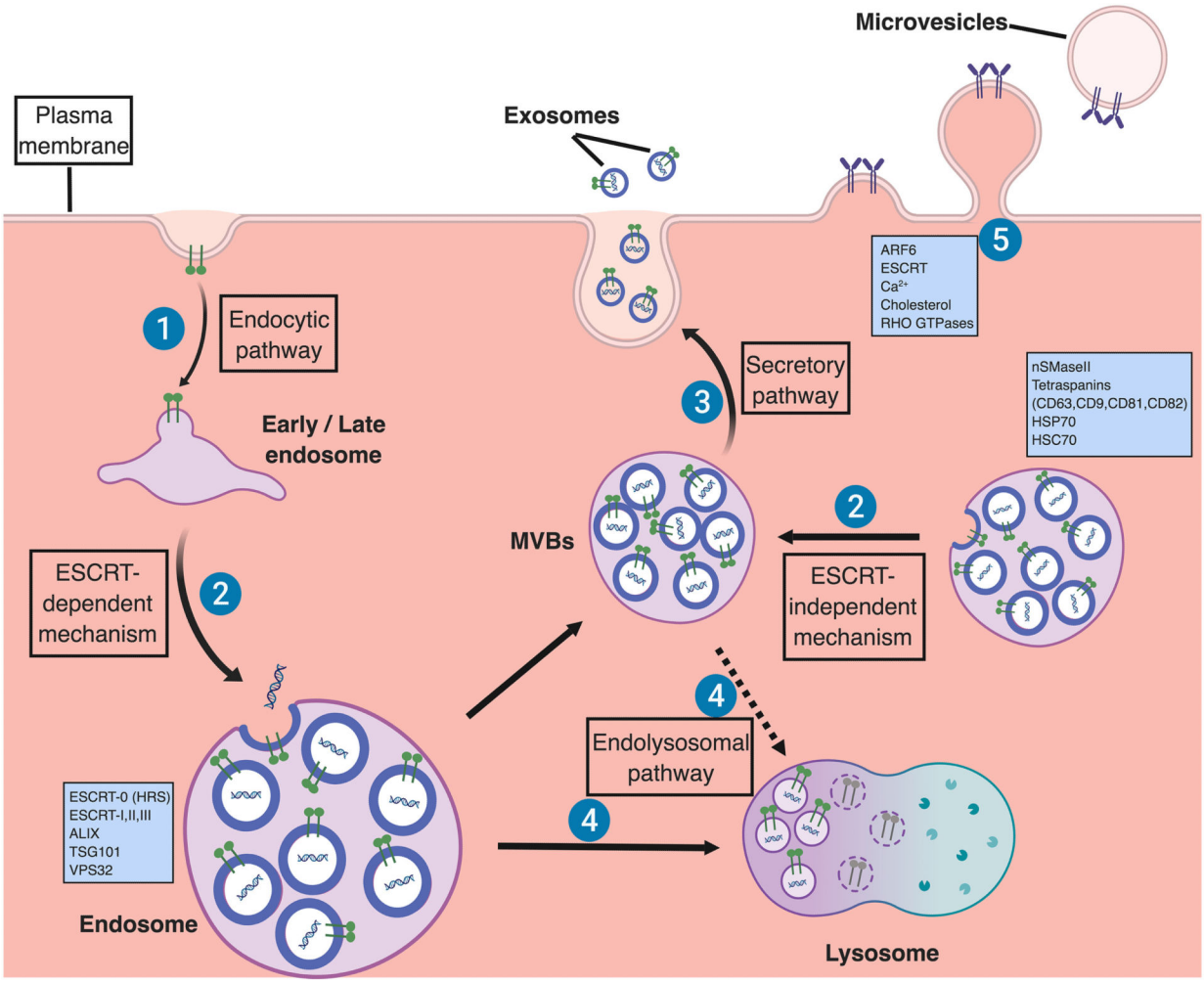

Figure 1. Biogenesis of extracellular vesicles.

Exosomes are secreted from the multivesicular body (MVB), which is formed by invagination of the endosomal membrane. Initially, extracellular cargoes are targeted at the plasma membrane to form early endosomes by endocytic pathway (1). Early endosomes undergo transition to late endosomes and inward budding in which exosomal cargos including specific proteins and nucleic acids are further loaded to form multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) [11]. Endosomal sorting complex required for transport (ESCRT)-dependent mechanism, which are regulated by ESCRT proteins (ESCRT-0, I, II and III) and their accessories (ALIX, TSG101, VPS32) [66, 67], and ESCRT-independent mechanism, which are regulated by neutral sphingomyelinase 2 (nSMaseII), tetraspanins, and the chaperone heat shock proteins (HSP70, HSC70), can develop ILVs (2) [59, 70, 73, 83]. Some of MVBs can be further fused with the lysosomal membrane, resulting in the degradation of the ILVs and their contents for recycling as an endolysosomal pathway (3) [13, 14]. Alternatively, ILVs are released into the extracellular space as exosomes via a secretory pathway (4) [12]. For microvesicles, they are formed by direct outward budding of the plasma membrane, a process which is regulated by the ESCRT

components and ADP ribosylation factor 6 (ARF6), some small GTPases, lipids, and Ca^{2+}-dependent enzymatic machineries [1]. (Figure was created with BioRender. com)

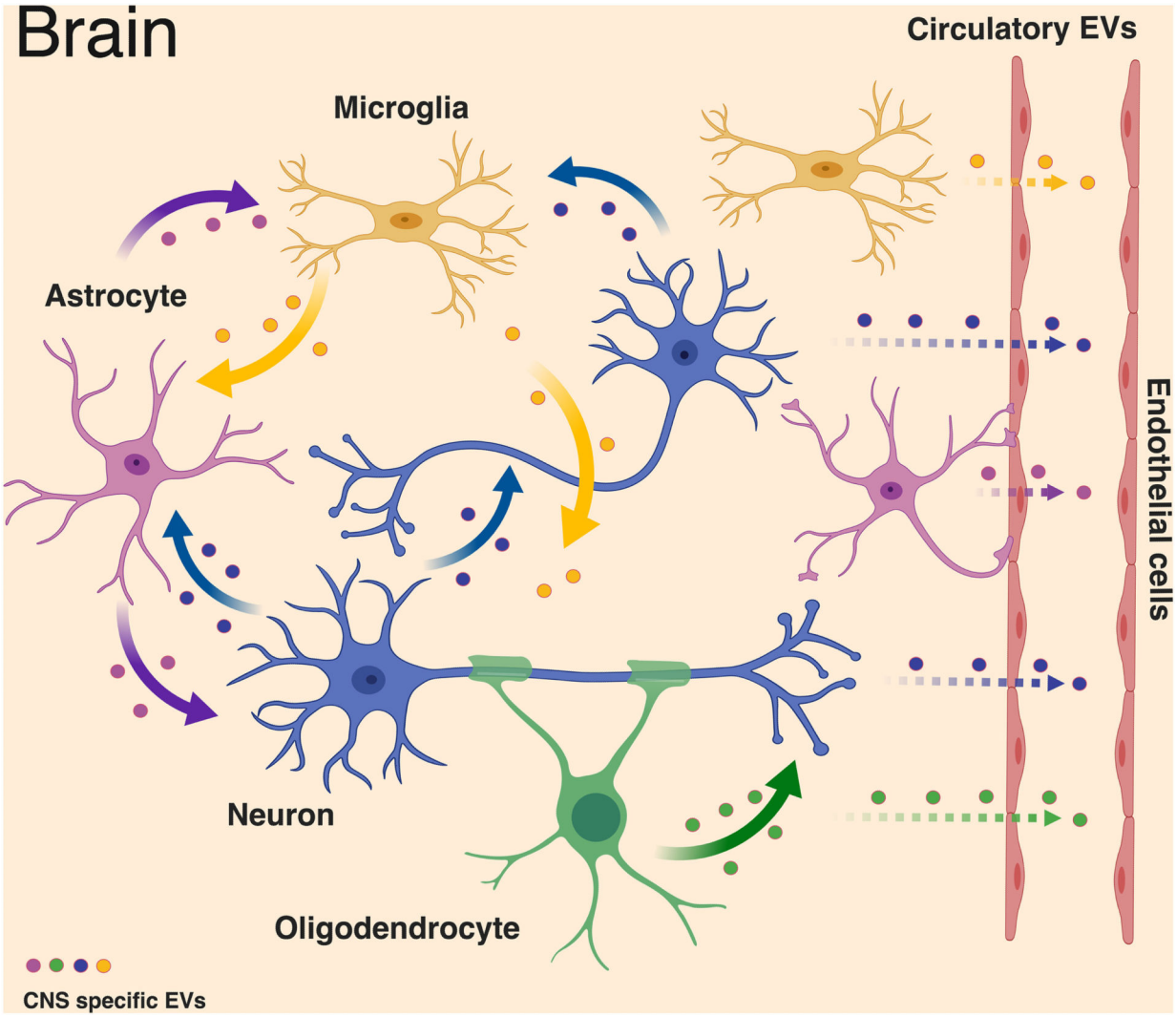

Figure 2. Intercellular communication of neural cells derived extracellular vesicles in brain.

In the central nervous system, EVs could be secreted from one cell type and targeted to the others to engage multiple functions. Microglia have been proposed to secrete EVs containing the pro-inflammatory cytokine interleukin-1 β (IL-1 β), which mediates inflammasome response and the aminopeptidase CD13, which provides metabolic support [113, 198]. Similar to microglia, astrocyte-derived EVs play both neuroprotective and neurotoxic roles such as containing synapsin 1 to promote neurite outgrowth and Nef to mediate neurotoxicity [115–117]. Oligodendrocytes secrete myelin molecules and stress-protective proteins in EVs, which are reported to participate in myelin formation and maintenance [118] as well as trophic support of neurons [8]. Neurodegenerative disorder-associated proteins such as prions [122], amyloid-β peptide [123], Tau [10], and α-synuclein [124] can also be released from EVs of the neural cells, leading to the spread of protein aggregate seeds and disease progression. In addition, these EVs could be exported through blood-brain barrier as circulatory EVs, which can be used for disease-specific biomarkers [1]. (Figure was created with BioRender. com)

Table 1.

Summary of characteristics of extracellular vesicles subtypes

EVs subtype	Alias name	Size	Origin	Known markers	Biogenesis mechanisms	Cargos
Exosomes	• Dexosomes • Nanovesicles • Exosome-like vesicels	• 30–150 nm	• Inward budding of early endosome to form multi vesic-ular bodies (MV Bs) • Fusion of MVB s with the plasma memb rane	• Endosome-associated components: Annexins, flotillins • ESCRT components: TSG101, ALIX • Tetraspanins: CD9, CD63, CD81	• ESCRT-depedent mechanism: ESCRT-0, -I, -II and-III, TSG101, ALIX, VPS4 • ESCRT-indepedent mechanism: nSMase2, CD63	• Surface proteins including adhesion molecules, tetraspanins • Nucleotide acids: mRNA, non-coding RNAand DNA • Lipids including sphingomy elin and cholesterol
Microvesicles	• Microparticles • Ectosomes • Oncosomes	• 100 – 1000nm	• Outward budding of the plasma memb rane	• Overlapping considerably with exosomes • Glycoprotein 1b, external phosphatidyl serine • Annexin A1	• ESCRT-I, III • Aminophospholipid translocases, scramblases and calpain • RHO family and ARF6	• Surface proteins including adhesion molecules and tetraspanins • Nucleotide acids: mRNA and non-coding RNA • Lipids including phosphatidylserine and sphingolipids
Apoptotic bodies		• up to 5μm	• Shedding by the plasma memb rane of apoptotic cells	• Phosphatidyl serine		• DNA, histones, organelle fragments and cytoplasmic components

Table 2.

Overview of EVs cargos based on ExoCarta and Vesiclepedia database (updated on 4/30/2019)

	Studies	Protein entries	mRNA entries	miRNA entries	Lipid molecules	Species
ExoCarta	286	41,860	4,946	2,838	1,116	Human
Vesiclepedia	1,254	349,988	27,646	10,520	639	41

Table 3.

Current knowledge of EV involvement in neurodegenerative diseases

Disease	EVs subtype	Evidence	Biomarker development
Alzheimer's disease	• Exosome	• Exosomal proteins are enriched in the Aβ plaques [122] • Levels of Aβ1–42 and cellular survival factors in plasma NDEs, complement in plasma ADEs are dysregulated in AD patients [127] • AD patients' brains derived exosomes can spread toxic Aβ oligomers and cause toxicity in neurons [132] • Exosomes would assist in the degradation of amyloid fibrils through uptake by microglia [98] [135] • Different pathogenic phosphorylated tau isoforms are present in AD assoicated exosomes [127] [138] • microglia can spread tau via exosome secretion, and inhibiting exosome synthesis significantly reduces tau propagation in vitro and in vivo [7]	• Levels of Aβ1–42, P-T181-tau and P- S396-tau in plasmal NDEs is higher 1 to 10 years before diagnosis of AD [127] • Higher complement proteins and lower regulatory proteins are found in patients at AD2 stage than that at AD1 preclinical stage [129] • Some exosomal miRNAs including miR-9–5p, miR-598 and mir-342–3p are downregulated in AD [141] [142]
	• Microvesicle	• Higher number of myeloid MVs are found in the CSF of AD patients and MCI patients than controls [130]	
Amyotrophic lateral sclerosis	• Exosome	• Mutant SOD1 is present in exosomes from ALS cell model NSC-34 [153] • Astrocyte-derived exosomes can efficiently transport mutant SOD1 to spinal neurons and induce motor neuron death in primary astrocyte cultures overexpressing mutant $_{SOD1}$ [154] • TDP-43 is detected in exosomes from in vitro neuron cells and CSF from ALS patients [155] [156] • Exosomes from brains of ALS patients could cause pathological TDP-43 aggregates in Neuro2a cells and induce intracellular toxicity [155] [157]	• Serum-derived exosomal miR-27a- 3p is downregulated in ALS patients compared to healthy subjects [158]
	• Microvesicle	• Plasma-MVs of ALS patients are enriched in ALS pathological proteins such as phospho-TDP-43 [149]	
Hungtington's disease	• Undefined	• Both polyQ protein and its repeat RNA were found incorporation into EVs which	

Disease	EVs subtype	Evidence	Biomarker development
		can be taken up by neural cells [163]	
	• Exosome	• Astrocytic exosomes could reduce the density of pathogenic aggregates in the striatum of HD140Q KI mice [164] • Exosomes from adipose-derived stem cells decrease protein aggregates and ameliorate mitochondrial function in an in vitro HD model [165]	
Parkinson's disease	• Exosome	• Toxic α-synuclein has been detected within the exosomes and is more efficient to induce toxicity in cells compared with free α- synuclein oligomers [170] • Exosomes can trigger the aggregation of α-synuclein mediated by lipids enriched in vesicles membrane [171] [172] • Stereotaxic injection of α-synuclein related EVs into the striatum leads to spread of synuclein pathology to anatomically connected brain regions including cerebral cortex, substantia nigra and hippocampus [172] • α-synuclein -rich EVs produced by periphery erythrocytes could cross the blood-brain barrier and might initiate PD [173] • Other PD-related proteins such as PARK9 and LRRK2 are also associated with EVs and have a role in EVs trafficking [174–177]	• α-synuclein is elevated in serum- derived EVs from PD patients [169] • Levels of autophosphorylated LRRK2 (P-S1292) in urinary exosomes are elevated in PD and are correlated with the severity of cognitive impairment and difficulty in accomplishing activities of daily living [178] • Some miRNAs levels are altered in CSF- and serum-exosomes from PD patients (e.g. miR-1, miR-19b, miR-195, miR-24) [180] [181]
Prion disease	• Exosome	• PrP^{Sc} is present in exosomes isolated from plasma in a prion disease mouse model [185] • Injection of PrP^{Sc} contained EVs to PrP^{C} transgenic mice accelerates the transmission of prion [186] • Inhibition of nSMaseII by GW4869 reduces PrP^{C} or PrP^{Sc} packaging into exosomes [188]	• A distinct miRNA signature in exosomes from prion-infected neuronal cells [43]