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REVIEW ARTICLE

Extracellular vesicles and intercellular communication in the central nervous system

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Neurons and glial cells of the central nervous system (CNS) release extracellular vesicles (EVs) to the interstitial fluid of the brain and spinal cord parenchyma. EVs contain proteins, nucleic acids and lipids that can be taken up by, and modulate the behaviour of, neighbouring recipient cells. The functions of EVs have been extensively studied in the context of neurodegenerative diseases. However, mechanisms involved in EV-mediated neuron–glial communication under physiological conditions or healthy ageing remain unclear. A better understanding of the myriad roles of EVs in CNS homeostasis is essential for the development of novel therapeutics to alleviate and reverse neurological disturbances of ageing. Proteomic studies are beginning to reveal cell type-specific EV cargo signatures that may one day allow us to target specific neuronal or glial cell populations in the treatment of debilitating neurological disorders. This review aims to synthesise the current literature regarding EV-mediated cell–cell communication in the brain, predominantly under physiological conditions.

Keywords: astrocytes; exosomes; extracellular vesicles; lipids; microglia; neural stem cells; neurons; oligodendrocytes; proteomics; RNA

Abbreviations

5HT, serotonin; ACR, acute cytokine response; ADEV-ATP, ATP-stimulated ADEVs; ADEVs, astrocyte-derived extracellular vesicles; AEA, *N*-arachidonoylethanolamine; ALIX, ALG-2-interacting protein X; ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ApoD, apolipoprotein D; ARRDC1, arrestin domain-containing protein 1; Asrgl1, asparaginase-like protein 1; BBB, blood–brain barrier; CB1, cannabinoid receptor type 1; CCL2, chemokine (C-C motif) ligand 2; CNP, 2′,3′-cyclic-nucleotide-phosphodiesterase; CNS, central nervous system; COX-2, cyclooxygenase-2; CREB, cAMP-responsive element-binding protein; CSF, cerebrospinal fluid; DPYSL2, dihydropyrimidinase like 2; DUBs, deubiquitinating enzymes; EE, environmental enrichment; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complexes required for transport machinery; EVs, extracellular vesicles; FLOTs, flotillins; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter 1; GM1, monosialotetrahexosylganglioside; GPI, glycosylphosphatidylinositol; GSK, glycogen synthase kinase; ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon gamma; ILVs, intraluminal vesicles; JNK, c-Jun N-terminal kinases; KCl, potassium chloride; L1CAM, L1 cell adhesion molecule; LAMP1, lysosomal-associated membrane protein 1; LOX, lysyl oxidase; LPS, lipopolysaccharide; Mac-1, macrophage 1 antigen; MAP1B, microtubule-associated protein 1B; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MDEVs, microglia-derived EVs; MFG-E8, milk fat globule-EGF factor 8 protein; MHC, major histocompatibility complex; miRNA, microRNAs; MMPs, matrix metalloproteinases; MOG, oligodendrocyte glycoprotein; mRNA, messenger RNA; MVBs, multivesicular bodies; MVEs, multivesicular endosomes; NCAM, neural cell adhesion molecule; NCM, neuronal conditioned medium; ncRNA, noncoding RNAs; NETO1, neuropilin and tolloid-like protein 1; NF-κB, nuclear factor κB; NLRP3, NLR family pyrin domain-containing 3; NMDA, *N*-methyl-d-aspartic acid; NSCs, derived from neuronal stem cells; ODEV, oligodendrocyte-derived extracellular vesicles; OPCs, oligodendrocyte precursor cells; P2X7, P2X purinoceptor 7; PDCD6IP, programmed cell death 6 interacting protein; PLP, proteolipid protein; PQ, paraquat; PTMA, prothymosin alpha; Rab31, Ras-related protein RAB31; ROS, reactive oxygen species; SPARC, secreted protein acidic and cysteine rich; STAT1, signal transducer and activator of transcription 1; Syn I, synapsin I; Syt4, synaptotagmin 4; TBC1D2B, TBC1 domain family member 2B; TEMs, tetraspanin-enriched microdomains; TLR4, Toll-like receptor 4; TSG101, tumour susceptibility gene 101 protein; VAMP7, vesicle-associated membrane proteins 7; VPS4, vacuolar protein sorting-associated protein 4; Vps4-Vta1, vacuolar protein sorting-associated protein 4-vesicle trafficking 1; Wnt, wingless-related integration site; WT, wild-type.

Rapid and targeted communication between different cell types of the central nervous system (CNS) is essential for optimal functioning of the brain. Glial cell populations (approximately 33–66% of total brain cell mass), including astrocytes, microglia and oligodendrocytes, serve many supportive roles for neurons, such as insulation and nourishment, removal of waste products, supply of neurotransmitter precursor molecules, protection from trauma, and act as migratory guidance cues for neural precursor cells [1,2]. Glial cells are also important in neural circuit maturation and the remodelling and pruning of synapses, both during development and adulthood [3,4]. Both glial and neuronal cell populations release extracellular vesicles (EVs) that contain cargos such as proteins, nucleic acids and lipid signalling molecules (Fig. 1). DNA, messenger RNA (mRNA) transcripts, microRNAs (miRNA) and noncoding RNAs (ncRNA) have been found in EVs, which can modulate gene expression in target cells [5–7]. Moreover, proteomic studies have found transcriptional regulatory proteins packaged into EVs that can trigger downstream signalling pathways in recipient cells [8]. Therefore, EV-mediated communication between neurons and glial cells likely results in both fast and long-term changes in the mRNA transcripts and proteome of target cells and serves as another important method of information transfer between neighbouring cells within functional neural ensembles.

Extracellular vesicles were once thought to be unwanted material released by cells. However, we now know that EVs are involved in both physiological and pathological intercellular communication processes and can regulate homeostatic signalling or trigger cytotoxic responses in target cells [5]. EVs travel from the cell of origin through the extracellular space by diffusion via interstitial fluid. Brain-derived EVs can also travel further through the body via the cerebrospinal fluid (CSF) [9,10] and blood [11,12]. Extraction of EVs from CSF and blood is a reliable source of molecular biomarkers that could be valuable in the clinical diagnosis of degenerative diseases [13,14]. From a biomedical research viewpoint, the study of EVs may facilitate the noninvasive interrogation of the physiology and pathophysiology of organ systems and tissues. It has been shown that all CNS cell populations generate and release EVs [15]. Although circulating EVs are capable of crossing the blood–brain barrier (BBB) in both directions, from the brain to the bloodstream and *vice versa*, the specific molecular mechanisms involved have not been fully elucidated [16]. A variety of methods for EV uptake by recipient cells have also been described. The different routes include clathrin-dependent endocytosis, which can be mediated by G protein-coupled receptors, and clathrin-independent pathways, including macropinocytosis, nonspecific lipid raft entry and receptor-mediated transcytosis

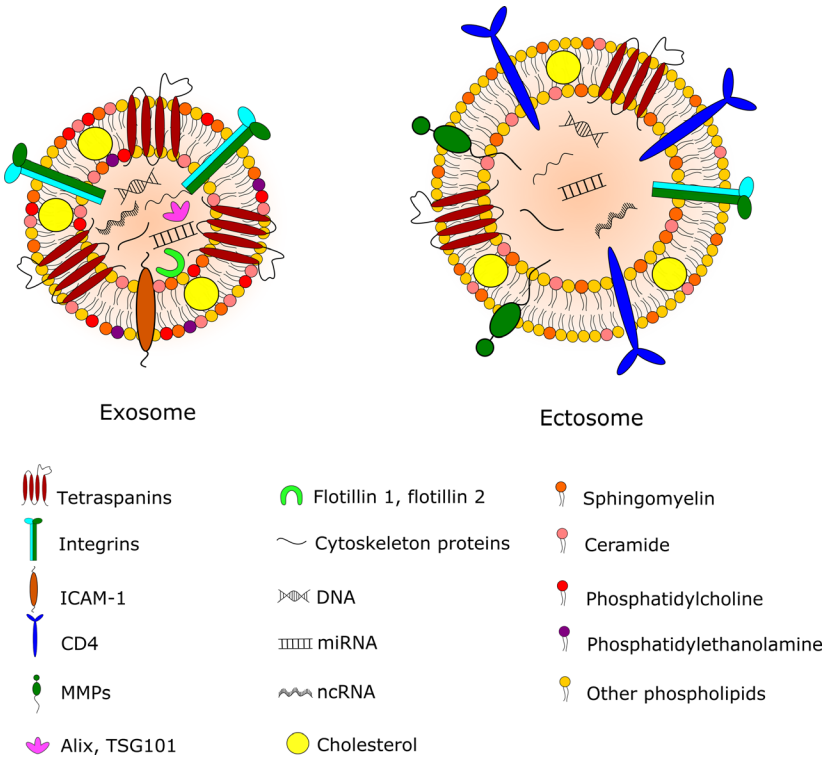

Fig. 1. Structure of exosomes and ectosomes. The membrane-spanning proteins, tetraspanins and integrins, are present in both types of EV. Cholesterol, sphingomyelin and ceramide are segregated during the formation of ectosomes and ILV budding. Phosphatidylcholine and phosphatidylethanolamine are common in exosomes, but they are not abundant in the plasma membrane domains, which is reflected in the ectosome structure. DNA, miRNA, cytoskeletal proteins and ncRNA are all present in both exosomes and ectosomes. Some proteins are exclusive for exosomes, like ICAM-1. ALIX and TSG101 are involved in the sorting of cargo into exosomes, whereas MMPs and CD4 are exclusive for ectosomes.

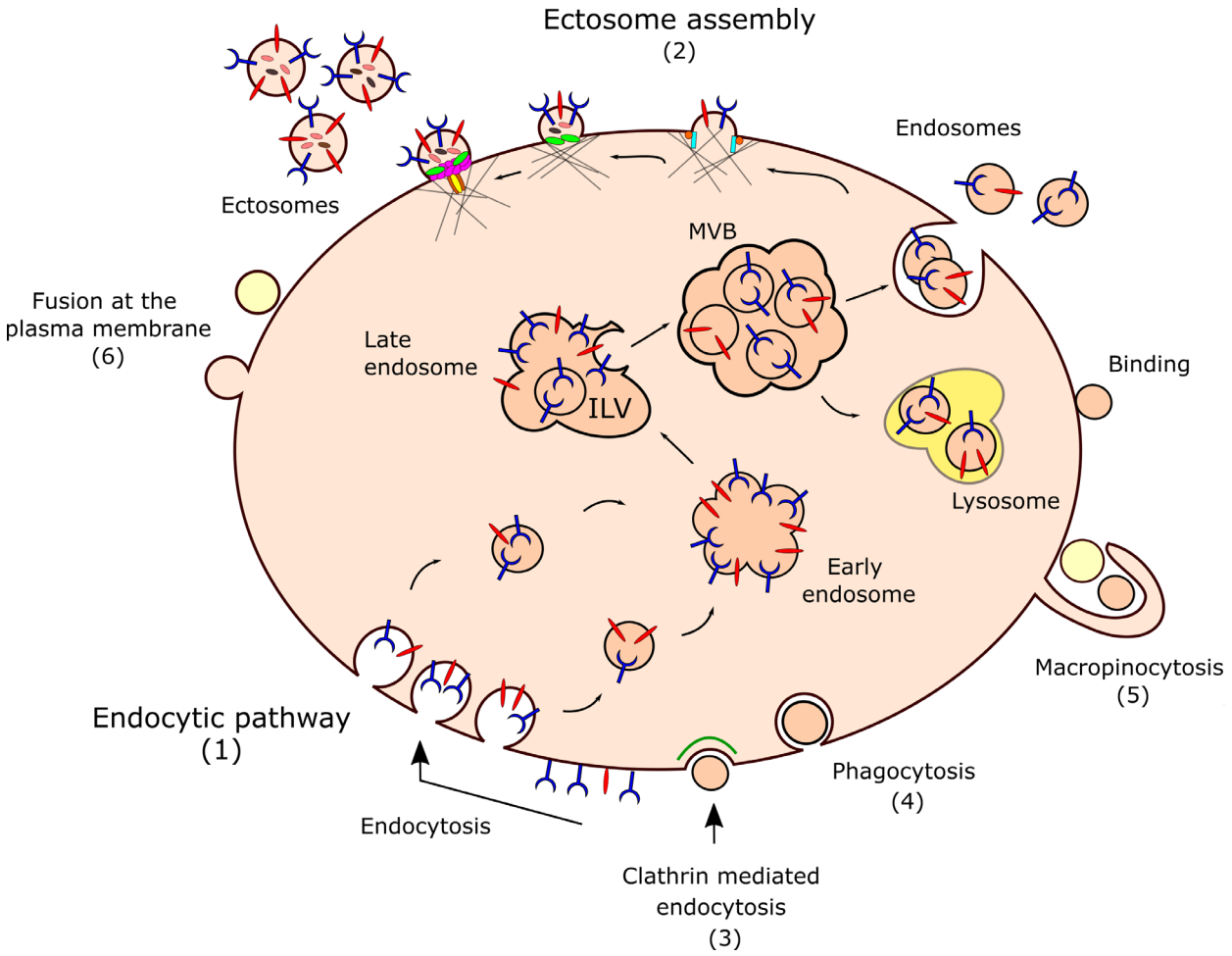

Fig. 2. Exosome and ectosome biogenesis and mechanisms of EV uptake. (1) Exosome biogenesis is performed via the endocytic pathway. Transmembrane proteins (red and blue) are endocytosed and trafficked to early endosomes followed by sorting to late endosomes. Budding of ILVs is carried out in late endosomes, leading to the formation of the MVB, which can release ILVs to the extracellular space. MVBs can also follow a degradation pathway by fusing with lysosomes. (2) Ectosome assembly is carried out through nucleation at the plasma membrane. Initially, transmembrane proteins (red and blue) are clustered in membrane domains leading to outward membrane budding. Proteins accumulate in the lumen by lipidic anchors of proteins, thus enhancing membrane curvature (orange circle). Flexibility in the cytoskeleton (grey lines) facilitates sorting of cytosolic proteins (pink) and RNA molecules (dark grey). ESCRT-III is mobilised to the plasma membrane (green), promoting the formation of a spiral-like structure (magenta). Finally, disassembly of the spiral is mediated by ATPase vacuolar protein sorting-associated protein 4 (orange-yellow). Different routes of EV uptake by recipient cells are shown including, (3) clathrin-mediated endocytosis, (4) phagocytosis, (5) macropinocytosis and (6) fusion at the plasma membrane.

(Fig. 2) [16,17]. Therefore, EVs may traverse endothelial cells of the BBB vasculature via several distinct mechanisms.

Extracellular vesicles can be grouped into three different categories based on their size and origin. Exosomes, the most studied type of EV, are small particles (50–100 nm) that originate in the cytosol by budding of intraluminal vesicles (ILVs) and are released by exocytosis in the form of multivesicular bodies (MVBs; Fig. 1, Table 1) [15,18]. Exosome cargo consists of proteins, RNAs and lipids, and neighbouring cells use exosomes as a method of paracrine transfer of molecular signals between cell populations. Exocytosis can be triggered in response to an exogenous stimulus, usually elevating cytosolic calcium concentration (i.e. regulated secretion), thereby inducing the formation of the SNARE protein complex, which enables both spatial and temporal control of exocytosis [19–21]. Exocytosis also occurs without stimulation, in a Ca^{2+}-independent manner (i.e. constitutive secretion) [22]. Ectosomes, also known as microvesicles or microparticles (50–1000 nm), are generated by outward budding from the

Table 1. Major features of exosome and ectosome biogenesis.

Features	Exosomes	Ectosomes
Membrane origin	Endosome, MVBs [15,18]	Plasma membrane [15,18]
Diameter	50–100 nm [15]	50–1000 nm [15]
Assembly	ESCRT [18] ESCRT-independent pathways (ceramide, RAB31 and tetraspanin routes) [39,42–44]	ESCRT [18], tetraspanin route [43,44]
Pinch off	ESCRT-III/Vps4 [18]	ESCRT-III/Vps4 [18]
Release mechanism	MVB exocytosis [18]	Shedding of cell membrane [18]
Markers	Tetraspanins (CD63, CD9, CD81), Alix, TSG101, LAMP1, syntenin 1, FLOT 1, FLOT 2, MFG-E8 [44,47–49]	Tetraspanins, TyA, C1q [18,47]
Segregated lipids	cholesterol, sphingomyelin and ceramide, phosphatidylcholine and phosphatidylethanolamine [18]	cholesterol, sphingomyelin and ceramide [18]
Membrane proteins	Integrins, L1CAM, tetraspanins, FLOTs [44,45,47–49]	Integrins, tetraspanins, MMPs, GP1b, GPIIb/IIa, P-selectin and the integrin [18,37,47]

plasma membrane and released into the extracellular space in response to various stimuli (Fig. 1, Table 1) [15,18]. Ectosomes contain cytoskeletal elements and can be loaded with proteins and genetic material from the cell of origin. Another type of EV is the apoptotic bodies (500–2000 nm), which are released during cell death [15]. Despite differences in the size and membrane of origin of exosomes and ectosomes, their assembly and release, as well as their interactions with target cells in the extracellular spaces, follow similar mechanisms. Details underlying the biogenesis and release of EVs into the extracellular space (see Box 1 and 2) are presented below (Fig. 2).

Although the intercellular transfer of exosome cargos into the target cell occurs through fusion or endocytosis, little is known about the molecular mechanisms that control EV recognition by target cells. Interestingly, it has been suggested that the recipient cell may stimulate the secretion of EVs from neighbouring cells, although the mechanisms behind this type of 'on demand' EV release by neurons and glia is not fully understood [23,24,25]. Despite significant advances in our understanding of EV-mediated intercellular communication in the brain, it is important to note that our current knowledge is mostly limited to *in vitro* cell culture models. Therefore, mechanisms underlying EV-mediated intercellular communication in the intact CNS are yet to be uncovered. There are a comprehensive literature and an increasing number of studies on the role of EVs in the pathogenesis of neurodegenerative diseases [5,26], including Alzheimer disease [27,28,29], Parkinson disease [30] and frontotemporal dementia [31]. However, the role of EVs in neuron–glial communication under normal physiological conditions remains to be elucidated. Therefore, we review here the current body of knowledge on EVs as mediators of cell–cell communication between neurons, astrocytes, microglia, oligodendrocytes and neural stem cells (NSCs) under physiological conditions.

Extracellular vesicle-mediated intercellular communication in the CNS

Characterisation of EVs that originate in the CNS is challenging because neurons generally release low amounts of EVs. However, several studies have found EV proteomic signatures that can help to identify the most likely cell of origin in the CNS. This promises to increase our understanding of intercellular communication between neurons and glial cells [15]. To date, only a few markers that are specific for CNS-derived EVs have been identified. Neural cell adhesion molecule (NCAM) and L1 cell adhesion molecule (L1CAM) are widely used to detect and isolate CNS-derived EVs. However, it is important to note that these markers are not exclusive to CNS-derived EVs since they are also expressed in the peripheral nervous system. Additionally, NCAM is expressed in the heart, adrenal gland and peptic cells, while L1CAM is present in the distal renal tubules [45]. In the case of astrocyte EVs, the glutamate aspartate transporter (GLAST), and the Na^+/K^+ ATPase are present in both exosomes and ectosomes, whereas the cannabinoid receptor type 1 (CB1) is exclusive for ectosomes [46]. ALG-2-interacting protein X (ALIX; also known as programmed cell death 6 interacting protein, PDCD6IP), syntenin 1, heat-shock proteins and the tetraspanins CD81, CD63, CD9 are the most common 'general' exosome markers. Other typical exosome markers include lysosomal-associated membrane protein 1 (LAMP1), Flotillins 1, 2

Box 1. Extracellular vesicle biogenesis – ESCRT-dependent pathways

Assembly of small exosomes and larger ectosomes relies on endosomal sorting complexes required for transport machinery (ESCRT) [18]. Exosomes can form via the endocytic pathway (Fig. 2). Vesicles generated at the plasma membrane by endocytosis, or those that originate at the Golgi complex, can fuse with the membranes of early endosomes [32]. ESCRT-0 initiates vesicle budding in early endosomes by recognising and engaging ubiquitinated proteins, while ESCRT-I and ESCRT-II complexes cluster proteins and form an ESCRT cargo-enriched zone [33]. Vesicle maturation occurs via inward budding of the membrane into the lumen of endosomes, forming intraluminal vesicles (ILVs). EV cargo is sorted and packaged into maturing vesicles with the help of ALG-2-interacting protein X (ALIX) and tumour susceptibility gene 101 protein (TSG101) [34]. ESCRT-III assembly results in vesicle budding and the sequestration of proteins. ESCRT-III is then disassembled by vacuolar protein sorting-associated protein 4-vesicle trafficking 1 complex (Vps4-Vta1 complex), resulting in ILV sorting [33]. Fusion of multivesicular bodies (MVBs) with the plasma membrane, which is mediated by a SNARE complex involving the vesicle-associated membrane protein 7 (VAMP7) and v-SNARE [20,18], results in exosome release.

In contrast, ectosomes are assembled by regulated outward budding of plasma membrane microdomains or lipid rafts, which are enriched in cholesterol and glycosphingolipids (Fig. 2) [18,35]. The assembly of ectosome luminal cargo is carried out through myristoylation and palmitoylation, resulting in the binding of cytoplasmic proteins to the plasma membrane. Cytoplasmic proteins accumulate in the lumen and generate membrane curvature [18]. As cytoskeletal tension loosens, RNA molecules and cytosolic proteins are sorted into ectosomes. The ESCRT-I subunit, TSG101, relocates to the plasma membrane and interacts with ALIX and with arrestin domain-containing protein 1 (ARRDC1) [36]. ESCRT-III is also crucial for the pinching-off and release of ectosomes.

Membrane proteins, like tetraspanins and integrins, are present in both exosomes and ectosomes. However, others are exclusive to certain ectosomes, such as matrix metalloproteinases (MMPs), the membrane glycoproteins GP1b and GPIIb/IIa, P- selectin, and the macrophage-1 antigen (Mac-1) [37,18]. Intercellular adhesion molecule 1 (ICAM-1) appears to be present exclusively in exosomes [25].

Box 2. Extracellular vesicle biogenesis – ESCRT-independent pathways

Alternative exosome biogenic mechanisms operate in parallel to ESCRT, or when ESCRTs are inactivated (Table 1) [38]. For example, ceramide triggers the budding of ILVs into multivesicular endosomes (MVEs) by generating raft-based microdomains, thus causing negative curvature of the membrane. The outer leaflet of the cell membrane contains high concentrations of sphingolipids from which ceramides are formed, via SMase activity [39,40]. Ceramide mixes poorly with other lipid raft components, showing self-assembling capability, which can induce the coalescence of small microdomains into larger ceramide domains, thus promoting domain-induced budding [40,41]. In addition, the cone-shaped structure of ceramide seems to enhance spontaneous negative membrane curvature by creating a large distinct area between membrane leaflets [39,40].

Another ESCRT-independent pathway involves active Ras-related protein, Rab31, and the formation of ILVs and exosomes while MVE degradation is avoided [42]. When Rab31 concentration is high in late endosomes, it can encounter active epidermal growth factor receptor (EGFR) which, in turn, activates Rab31 via tyrosine phosphorylation. Active Rab31 then engages flotillins (FLOTs) in lipid rafts to enhance EGFR entry into MVEs to form ILVs. To avoid MVE degradation, Rab31 recruits TBC1 domain family member 2B (TBC1D2B) to inactivate Ras-related protein, Rab7a. This supresses the fusion of MVEs with lysosomes, thus allowing the secretion of exosomes [42].

In addition, tetraspanins are important for the sorting of proteins, mRNAs and microRNAs into EVs [43,44]. Although little is known with regard to how specific proteins or nucleic acids are routed toward EV sorting, tetraspanin-enriched microdomains (TEMs) may play a role in defining the protein content. TEMs are ubiquitous specialized membrane platforms composed of tetraspanins in close associations with transmembrane proteins and lipids, including integrins, metalloproteinases and immunoglobulin-superfamily receptors. Evidence suggests that insertion of CD81 into TEMs may be necessary for protein inclusion into the exosome structure [43]. TEMs act as a platform that supports the compartmentalization of receptors and signalling proteins in the plasma membrane and facilitates the selection of receptors and intracellular components to be exported into exosomes [43].

(FLOTs 1, 2), tumour susceptibility gene 101 protein (TSG101) and milk fat globule-EGF factor 8 protein (MFG-E8) [44,47–49]. Using high-resolution mass spectrometry-based proteomics, Chiasserini *et al.* [50] generated a dataset of proteins present in EVs isolated from human CSF and identified proteins and exosome-associated biomarkers of neurodegenerative diseases. Indeed, to date, most research has focused on identifying EV cargos associated with neurodegenerative diseases and their potential use as diagnostic [51–53] and therapeutic tools [54–58].

Putative neuron-derived exosomes are detectable through the presence of L1CAM [5,59,60]. L1CAM is a member of the immunoglobulin superfamily cell adhesion molecules that regulate cell–cell adhesion. L1CAM is recognised as an important component of the ligand–receptor network involved in axonal growth and guidance [60–62]. However, cell subtype-specific EV markers released from distinct neuronal and glial cell populations have not yet been defined [15]. Therefore, specific functions for EVs produced by astrocytes, oligodendrocytes and microglia are largely unknown. Interestingly, only a small percentage of exosomes carry miRNAs, suggesting that only certain exosome subtypes contain a sufficient miRNA load to exert gene silencing in target cells [63]. These small ncRNA molecules seem to be particularly important for modulating gene expression and regulating CNS functions [64]. Approximately 70% of all known miRNAs are expressed in the human brain. Although there are several studies describing miRNAs as biomarkers of neurodegenerative diseases [13], more research into miRNAs that are secreted by the brain under physiological conditions is required (Table 3).

Neuron-derived EVs modulate synaptic plasticity

Active neurons secrete exosomes containing lipids, proteins and RNA transcripts that can modify protein expression, neurotransmission and neurogenesis in neighbouring glial cells, neurons and stem cells (Fig. 3) [65]. Moreover, exosome-mediated communication may facilitate the transfer of information, both anterogradely and retrogradely, across synapses. The constitutive release of exosomes from N2a neuroblastoma cells has been studied under *in vitro* cell culture conditions and they were found to bind indiscriminately to rat primary hippocampal neurons and glial cells, although they were endocytosed preferentially by the astrocytes and oligodendrocytes. In contrast, exosomes released by primary cortical glutamatergic neurons into culture medium tended to preferentially bind to hippocampal neurons at their presynaptic terminals where they could then be endocytosed. Therefore, *in vivo* there may be selective recognition receptors on different cell types that facilitate targeted internalisation of exosomes at neuronal synapses [66]. Internalised exosomes likely modify post-transcriptional mRNA trafficking and translation and induce local changes in synaptic plasticity [67,68]. Goldie *et al.* [68] studied the subcellular distribution of miRNA in both resting and depolarised human neuroblastoma (SH-SY5Y) cells. Decreased expression of miRNA was detected in the neurites of potassium-depolarised cells, whereas the exosomes produced by these cells were enriched with miRNAs and microtubule-associated protein 1B (MAP1B). Four of these miRNAs (miR-638, -149*, -4281 and let-7e) were found to be negatively regulated by repeated neuronal depolarisation. Interestingly, these miRNAs regulate the expression of mRNAs involved in synaptic plasticity [68]. MAP1B is known to have essential roles in axon guidance, neuronal regeneration and neurite branching [69]. MAP1B also regulates the morphology of postsynaptic spines on dendrites of glutamatergic neurons [70–72]. Therefore, anterograde signalling by EVs released from active presynaptic compartments may be involved in regulating learning and memory formation. Presynaptic release of EVs can also modulate retrograde signalling by the postsynaptic cell, which may be important during brain development, axon guidance or synaptic plasticity [73]. For instance, synaptotagmin 4 (Syt4)-containing EVs are released from muscle cells and communicate with presynaptic terminals of motor neurons at the Drosophila neuromuscular junction. Syt4 is a membrane trafficking protein and its expression is regulated by neuronal activity [74]. Syt4 localises to brain-derived neurotrophic factor-containing vesicles in hippocampal neurons [75] and regulates synaptic plasticity and memory formation. Moreover, postsynaptic release of Syt4-containing exosomes may regulate the presynaptic quantal release of neurotransmitters, thus facilitating synaptic tuning [73].

Glutamatergic neurons also form α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-containing exosomes after bursts of synaptic activity or ionomycin-triggered elevations in cytosolic calcium levels [62]. The formation of AMPA receptor-containing exosomes may be a mechanism used by neurons to decrease AMPA receptor numbers locally at synapses that undergo plastic changes, similar to the well-documented phenomenon of AMPA receptor internalisation and recycling [76]. This loss of AMPA receptors may, therefore, be a homoeostatic mechanism to adjust the strength of synapses [62]. Exosomes

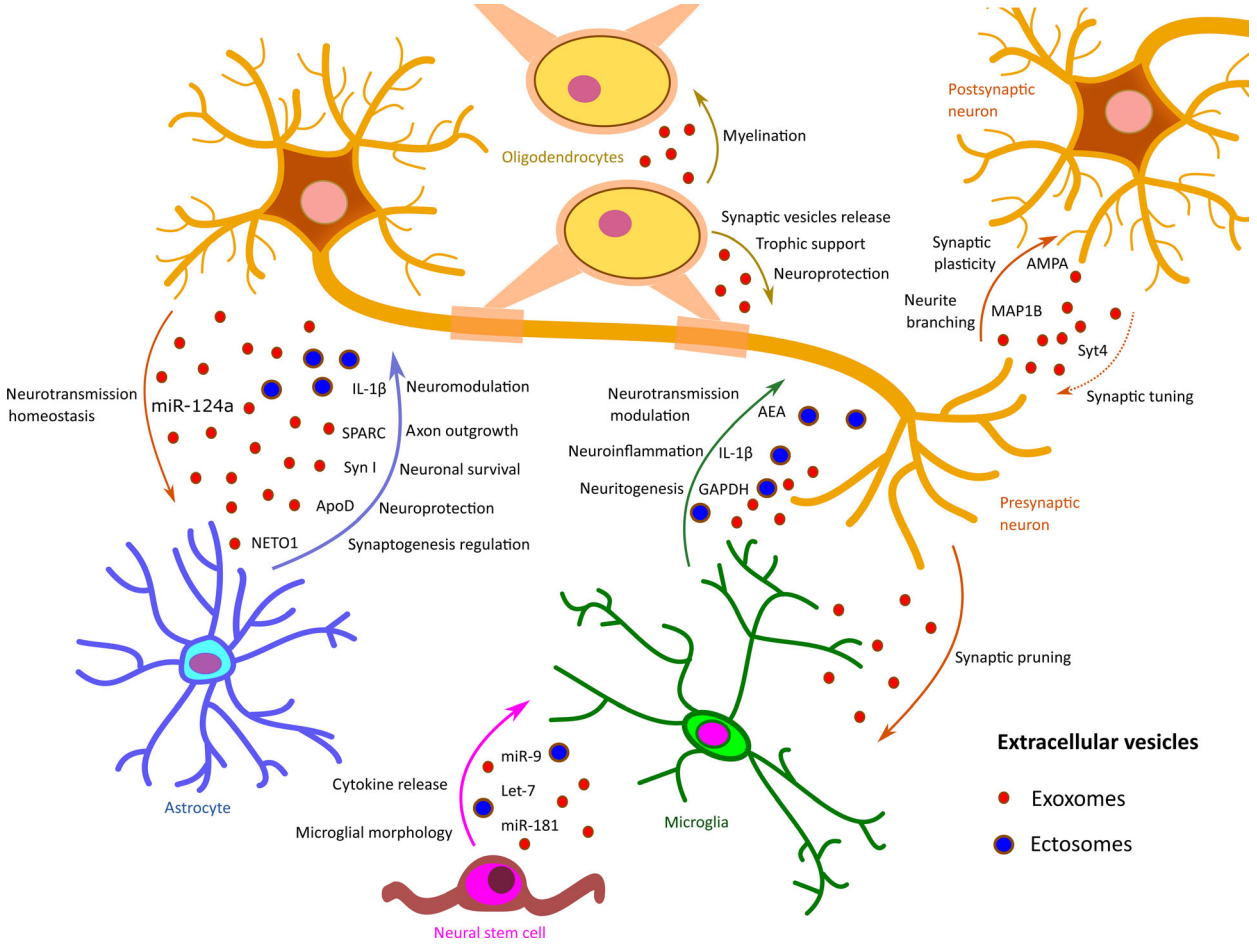

Fig. 3. EV-mediated intercellular communication in the CNS. Neuron-derived exosomes can modulate synaptic plasticity via AMPA receptor transfer to postsynaptic terminals. Synaptic tuning may be facilitated by the retrograde transport of exosomes composed of Syt4 protein to the presynaptic boutons. Moreover, MAP1B-loaded exosomes may influence neurite branching during brain development. Neurons also produce exosomes loaded with miR-124a that are internalised by astrocytes, which promotes the homeostatic maintenance of neurotransmission. Astrocytes can secrete neuromodulatory IL-1β-containing ectosomes that are internalised by neighbouring neurons. Astrocytes also produce exosomes loaded with various proteins, including SPARC, Syn I, ApoD and NETO1 that can be internalised by neurons and promote axon outgrowth, neuronal survival, neuroprotection and synaptogenesis, respectively. Microglia are known to produce both exosomes and ectosomes loaded with GAPDH which is involved in neuritogenesis. Microglial ectosomes loaded with IL-1β and AEA are internalised by neurons and are involved in neuroinflammation and the modulation of neurotransmission. Internalisation of neuronal exosomes by microglia can trigger synaptic pruning. Exosomes produced by oligodendrocytes have been shown to provide trophic support to neurons, enhance neuroprotection and are involved in the regulation of synaptic vesicle release. Oligodendrocyte-derived exosomes can also be internalised by neighbouring oligodendrocytes to modulate myelination. NSCs produce EVs loaded with miRNAs, including miR-9, Let-7 and miR-181 that trigger cytokine release from microglia and promote morphological transitions in microglial cells towards less complex nonstellate shapes. These versatile functions of neuronal and glial-derived EVs enhance the complexity of cellular communication in the CNS.

are capable of fusing with the cell membrane of postsynaptic neurons, and therefore, the addition of functional AMPA receptors to the postsynaptic bouton will further modulate synaptic strength [66]. The presence of glutamate receptor subunits within exosomes of neuronal origin suggests that other ion channels may also travel from neuron-to-neuron and influence their intrinsic properties [61]. Consistent with Lachenal *et al.* [62], the presence of AMPA receptors in neuron-derived EVs was detected by Fauré *et al.* [61]. They isolated and characterised exosomes of neuron- and astrocyte-cell origins from rat primary cortical cell cultures. Nineteen proteins were found, of which two are mainly expressed in astrocytes, that is GLAST1 and ceruloplasmin, the latter being a glycosylphosphatidylinositol (GPI)-anchored ferroxidase [62]. L1CAM and

AMPA receptor subunits (GluR2/3) were also found in the exosome fractions. The presence of AMPA receptors in neuron-derived EVs strongly suggests a role in the modulation of synaptic plasticity.

Neuron-derived EV signalling to glial cells

Neurons communicate with astrocytes through a variety of mechanisms, including exosome-mediated transfer of miRNAs. This can regulate protein expression in perisynaptic astrocytes which, in turn, can modulate synaptic function and neurotransmission. For instance, exosomes carrying small RNAs and miR-124a have been isolated from neuron-conditioned medium and shown to be internalised by primary astrocytes. This causes an increase in both miR-124a and glutamate transporter 1 (GLT-1) protein expression levels in the target cells [77]. GLT-1 (also known as excitatory amino acid transporter 2) is crucial for the homeostatic maintenance of synaptic glutamate levels and for preventing neuronal excitotoxicity [78]. Moreover, severe reductions in GLT-1 protein expression can lead to excitotoxicity and degeneration of motor neurons in patients with amyotrophic lateral sclerosis (ALS) and in rodent models of the disease [77]. Interestingly, an increase in GLT-1 protein expression was detected after stereotaxic injection of miR-124a into the ventral grey matter of the spinal cord of SOD1 G93A mice, a mouse model of ALS. Therefore, neuronal exosome cargoes may contain complementary combinations of proteins and miRNAs that help astrocytes to maintain homeostasis of neurotransmission in the CNS [79]. Interestingly, neuron-derived exosomes contain a distinct subset of miRNA compared to the miRNA profile of the parent neuron. Men *et al.* [79] detected 168 miRNAs only found in neurons and a subset of 95 miRNAs present in their secreted exosomes. For example, miR-669, miR-466, miR-297a-5p, and miR-3082-5p were enriched in the exosomes. The authors suggest that these miRNAs, along with the neuron-specific miR-124-3p, are potentially internalised into astrocytes and that miRNA-124-3p can upregulate GLT1 by suppressing GLT1-inhibiting miRNAs [79].

Neuron–microglial communication also occurs via neuron-derived exosome secretion, which can stimulate synaptic pruning by causing the upregulation of complement factors in microglia [80]. In an attempt to identify the underlying mechanisms of this process, Bahrini *et al.* [80] stimulated neuronal differentiation and synapse formation in PC12 cells, followed by the induction of neuronal degeneration. A mouse microglial cell line (MG6) was then co-cultured with the PC12 cells, resulting in the engulfment and phagocytosis of PC12 neurites. The MG6 cells were also pre-incubated with exosomes secreted by differentiated PC12 neurons following depolarisation. This caused an increase in the removal of degenerating neurites. Increased expression of complement component 3 in MG6 cells was thought to be responsible for the accelerated removal of neurites from degenerating PC12 cells. The *Cfb* and *C3* genes, which encode for complement factor B and complement component 3, respectively, were found to be highly upregulated (56-fold and 25-fold, respectively) in exosome-engulfed MG6 cells. Since C3 mRNA was not detected in exosomes, but only in exosome-engulfed MG6 cells, these findings suggest that neuron-derived exosomes induced (in an undetermined way) the C3 mRNA expression in MG6 cells, rather than the direct transfer of C3 mRNA from PC12 cells [80].

Astrocyte-derived extracellular vesicles

Astrocytes are crucial in the regulation of CNS homeostasis, synaptogenesis and cognitive function [81] by supplying trophic factors to neurons, actively clearing excess neurotransmitter from synapses, and helping to maintain the structure of the BBB [32]. Astrocytes also communicate with neurons and other glial cells by releasing gliotransmitters and neuromodulators that regulate synaptic plasticity. Moreover, astrocytes can release EVs containing a variety of cargos including proteins and RNAs that can modify protein expression in target cells. Cortical astrocytes produce ectosomes loaded with the proinflammatory cytokine, IL-1β, which acts as a neuromodulator in the healthy CNS [82]. Bianco *et al.* [46] found that ectosome shedding in rat primary astrocytes is triggered by acid sphingomyelinase following activation of the ATP receptor, P2X purinoceptor 7 (P2X7). When P2X7 is activated, acid sphingomyelinase moves to the plasma membrane resulting in the shedding and release of IL-1β-loaded ectosomes (referred to as 'microparticles' in their study). Interestingly, p38 mitogen-activated protein kinase inhibitors reduce acid sphingomyelinase activation [46].

Astrocyte-derived EVs can also stimulate neurite outgrowth, enhance neuronal survival and maturation and increase neuronal excitability (Table 2). For instance, You *et al.* [83] stimulated human primary astrocytes using ATP and evaluated the effects of astrocyte-derived EV (ADEV) internalisation on neurite outgrowth and firing frequency of neurons. Label-free quantitative proteomic studies showed the

Table 2. Protein cargo identified in EVs isolated from neurons, astrocytes, microglia and oligodendrocytes.

EV type	Stimulus	EV-producing cell	Protein/cargo	Function	Recipient/target cell	Outcome in target cell/ microenvironment	References
Exosome	Glutamatergic synapse	N2a neuroblastoma cells	–	–	Bound to hippocampal neurons Endocytosed by hippocampal astrocytes and oligodendrocytes	–	[66]
		Cortical neurons	–	–	Hippocampal neurons	–	[66]
Exosome	Potassium-induced depolarisation	Human neuroblasts	MAP 1b	Plasticity	–	–	[68]
Exosome	Spaced stimulation	Cell line S2 (Drosophila melanogaster)	Syt4	Quantal neurotransmitter release	Motor neurons (presynaptic terminals)	Stimulation of synaptic plasticity	[73]
Exosome	Synaptic activity and cytosolic calcium (ionomycin)	Glutamatergic neurons	AMPA receptor	Fast excitatory synaptic transmission	Postsynaptic neurons[a]	–	[62]
Exosome	Potassium-induced depolarisation	Rat cortical primary neurons	L1 GluR2/3	Neuronal adhesion protein Subunit of AMPA-type glutamate	–	–	[61]
		Rat cortical primary astrocytes	GLAST1 Ceruloplasmin	GLT GPI-anchored ferroxidase	–	–	
Exosome	Neuronal degeneration	PC12 neuronal cells	–	–	Microglial cell line (MG6)	Removal of PC12 cell dendrites by MG6 ↑ C3 mRNA in MG6	[80]
Exosome	Activation of ATP receptor P2X7	Rat primary astrocytes	IL-1β	Proinflammatory cytokine and neuromodulator of healthy CNS	–	–	[46]
Exosome	ATP	Human primary astrocytes	Proteins with GTPase, MHC receptor and cell adhesion activities	Cell growth, maintenance, transport and cell communication	CD1-mouse primary neurons	↑ Neuronal maturation ↑ Spike activity	[83]
Exosome	PQ (ROS)	Human astrocytic cell line	ApoD	Survival and functional integrity of neurons	Neuroblastoma cells	Neuroprotection	[48]
Exosome	KCl	Mouse primary astrocytes	Syn I		Hippocampal mouse neurons	↑ Neurite outgrowth ↑ Neuronal survival	[88]
Exosome	ATP-stimulated	Rat primary astrocytes	RPLT10 NETO1 DPYSL2 SPARC	Neurite outgrowth Synaptogenesis regulation Actin reorganisation Axon outgrowth, AMPA receptor regulation	Neurons[a]	Neuronal survival, excitability and neurite outgrowth[a]	[49]
	IL-10		Proteins involved in Gap junction signalling and regulation, neuronal CREB signalling				

Table 2. (Continued).

EV type	Stimulus	EV-producing cell	Protein/cargo	Function	Recipient/target cell	Outcome in target cell/ microenvironment	References
	IL-1β		C3, PTMA, LOX	Immune response			
Exosome	IL-1β	Mouse astrocytes (*in vivo*)	GM1 nSMase	Cell–cell recognition Hydrolase sphingomyelin	Liver cells	Transmigration of leukocytes into the brain	[93]
		Primary astrocytes	Ceramide	Neuronal apoptosis		Induction of 1L-1β, IL-6, TNFα and CCL2 in the liver	
Exosome	Ethanol	Mouse primary astrocytes	TLR4 NFκB-p65 IL-1R Caspase-1 NLRP3	Signalling in inflammation Signalling in inflammation Inflammation response Immunity/inflammation Immune response	Mouse cortical neurons	↑ Neuronal levels of COX-2 ↑ Neuronal levels of miR-146a	[95]
Exosome	Glutamate stimulation	Mouse primary oligodendrocytes and Oli-neu cells	–	–	Mouse primary microglia, astrocytes, neurons	–	[98]
			Hsc70	Protein folding and degradation	Mouse primary neurons	↑ Neuroprotection	
			–	–	Neurons (mouse brain)	–	
	Oxidative stress (H_2O_2)		–	–	Mouse primary neurons	↑ Cell survival	
	Nutrient deprivation (absence of B27)		–	–	Mouse primary neurons		
Exosome	Oxygen-glucose deprivation	Primary and cell line oligodendrocytes	Superoxide dismutase, catalase	Cell protection from free radicals	Primary mouse neurons	↑ Resilience to oxidative stress ↑ Phosphorylation of CREB, GSK-3a/b, GSK-3b and JNK ↑ Firing rate	[99]
Exosome	NCM	Rat primary oligodendrocytes	–	–	Rat primary oligodendrocytes	↓ Differentiation and myelin formation	[97]
Exosome	ionomycin	Mouse primary oligodendrocytes	PLP CNP MBP MOG Stress protective proteins	Myelination Cell signalling Stabilise myelin sheath Myelination Neuroprotection	Oligodendrocytes[a]	Regulation of myelin production and trophic support for axons[a]	[96]
EVs	–	Mouse and human NSC	Asrgl1	Asparaginase activity	–	Affects concentration of critical nutrients in the microenvironment.	[107]
EVs	Th1 and Th2 cytokine cocktails	Adult mouse NSC	IFN-γ	Immune activation	Fibroblast cells	Activation of Stat1, Neuroprotection	[109]
Ectosome	ATP	Microglia cell line	GAPDH	Glycolysis		Neuroinflammation and neuritogenesis in the brain[a]	[114]

Table 2. (Continued).

EV type	Stimulus	EV-producing cell	Protein/cargo	Function	Recipient/target cell	Outcome in target cell/ microenvironment	References
Exosome	5HT	Mouse primary microglia	–	–	–	–	[117]
Ectosome		Microglia cell line and rat primary microglia	Anandamide	Presynaptic transmission (AEA)	GABAergic neurons	Inhibition of presynaptic transmission through CB1 receptors	[118]
Exosome	Wnt-3	Rat primary microglia	WntA GAPDH Ubiquitin	Calcium regulation Glycolysis Proteosomal degradation	Primary rat neurons	Generation of morphogen gradients during CNS development[a]	[119]
	Wnt	Primary cortical neurons	Wnt-3	Calcium regulation	–		

[a]Recipient/target cell or outcome in target cell/microenvironment that have been suggested by the authors rather than proven experimentally.

presence of exosomal proteins in ADEVs, including endosomal sorting complexes required for transport machinery (ESCRT), tetraspanins such as CD9, CD81 and CD63, and PDCD6IP (ALIX). Astrocyte-specific proteins were also found, including glial fibrillary acidic protein, GLAST1 and glucose transporter member 1. Interestingly, the ADEVs were enriched with proteins that possess GTPase activity, major histocompatibility complex (MHC) receptor activity, and function as cell adhesion molecules, and therefore, exert a range of biological effects including neuronal cell growth and homeostatic communication. Mouse primary cultured neurons exposed to these ADEVs for three days displayed accelerated neuronal maturation and an increase in their spiking activity [83].

Apolipoprotein D (ApoD), which is predominantly expressed in the nervous system during normal development and ageing [84], is also transported from astrocytes to neurons via EVs. ApoD is expressed by astrocytes and myelinating cells to promote the survival of neurons by reducing free radical-generating lipid hydroperoxides [48,85–87]. Pascua-Maestro *et al.* [48] demonstrated that ApoD is transported from astrocytes to neurons via EVs (exosomes), where it is internalised. A reduction in neuroprotection was detected when conditioned media from ApoD knockout astrocytes was incubated with the SH-SY5Y neuroblastoma cell line. This media only provided partial protection against oxidative stress challenges, while EVs from an ApoD-positive astrocytic cell line (human astrocytoma 1321N1) exerted full neuroprotection [48]. ApoD was only internalised by SH-SY5Y neurons when it was loaded into exosomes, resulting in a protective effect from reactive oxygen species (ROS) generated by paraquat (PQ) treatment. Interestingly, no internalisation of the ApoD-free soluble form was detected in neurons [48].

Synapsin I (Syn I) can also be released from murine cortical astrocytes via exosomes upon stimulation with high concentrations of extracellular potassium chloride (KCl), oxygen/glucose deprivation (ischaemia) or treatment with hydrogen peroxide (oxidative stress). ADEVs containing Syn I promote neurite outgrowth and survival of mouse hippocampal neurons [88]. Several studies have shown that the cargo content of ADEVs is stimulus-dependent. ADEVs are known to be secreted constitutively and enhance neuronal survival and neurite outgrowth. However, Datta Chaudhuri *et al.* [49] studied the cargo composition of ADEVs using quantitative proteomic analysis after subjecting astrocytes to multiple stimuli. Rat primary astrocytes were exposed to a trophic stimulus, an inflammatory stimulus, and an anti-inflammatory

stimulus using ATP, IL-1β and IL-10, respectively. In ATP-stimulated ADEVs (ADEV-ATP), ribosomal protein L10 and neuropilin and tolloid-like protein 1 (NETO1) were detected, which induce neurite outgrowth [89] and regulate synaptogenesis [90], respectively. In addition, dihydropyrimidinase like 2 (DPYSL2) and secreted protein acidic and cysteine rich (SPARC) were found in ADEV-ATP. DPYSL2 is involved in actin reorganisation in growth cones and in dendritic patterning [91], while SPARC promotes axon outgrowth and regulates the level of postsynaptic AMPA receptors at maturing synapses [92]. The unique protein cargos identified in ADEVs stimulated by IL-10 were found to be involved with gap junctions and neuronal cAMP-responsive element-binding protein (CREB) signalling. On the other hand, IL-1β-stimulated ADEVs contained proteins that modulate peripheral immune responses including C3, prothymosin alpha (PTMA) and lysyl oxidase (LOX). These data indicate that modulation of neuronal excitability by astrocyte EVs is stimulus-dependent [49].

The stimulating effects of IL-1β on the formation of EVs by astrocytes were demonstrated in a study of brain injury in mice [93]. Intrastriatal administration of IL-1β was found to promote EV shedding from astrocytes, which rapidly entered into the peripheral circulation, resulting in the induction of peripheral acute cytokine responses (ACR). These EVs were found to promote transmigration of leukocytes into the brain through suppression of peroxisome proliferator-activated receptor α in hepatocytes which increased nuclear factor κB (NF-κB) activity in the liver. NF-κB activation induces peripheral ACR, which primes leukocytes to transmigrate to the injury site. Hence, in this case, ACR resulted in the induction of the inflammatory cytokines IL-1β, IL-6, TNFα and the chemoattractant chemokine (C-C motif) ligand 2 (CCL2) in the liver followed by the transmigration of leukocytes to the brain injury site. In addition, IL-1β can induce a rapid release of EVs from cultured primary astrocytes and promotes the formation of membrane microdomains enriched with monosialotetrahexosylganglioside (GM1), nSMase2 and ceramide. These data support previous studies in which the production of ceramide by nSMase is involved in plasma membrane fusion events [39,94].

Similarly, Ibáñez *et al.* [95] demonstrated a modulatory role for ADEVs in the immune response. Primary wild-type (WT) and Toll-like receptor 4 (TLR4)-knock out ($TLR4^{-/-}$) astrocytes were treated with ethanol to determine whether ADEV content was modified and if this affects neuroinflammation. After ethanol treatment, there was an increase in the number of EVs produced by WT astrocytes and an increase in inflammatory protein cargo, such as TLR4, NFκB-p65, IL-1R, caspase 1, NLR family pyrin domain-containing 3 (NLRP3) and miRNAs including miR-146a, miR-182 and miR-200b (Table 3). On the other hand, there were no changes in the number of EVs produced by $TLR4^{-/-}$ astrocytes, nor did their content change when treated with ethanol. These results suggest that secretion of EVs by astrocytes is dependent on the TLR4 response. Additionally, ethanol-treated WT astrocyte-derived EVs were found to be internalised by naïve mouse cortical neurons resulting in an increase in neuronal levels of the inflammatory protein cyclooxygenase-2 (COX-2) and miRNAs (e.g. miR-146a), as well as mRNA levels of IL-1β. This increase in inflammatory-related proteins resulted in enhanced neuronal cell death via apoptosis [95].

Oligodendrocyte-derived EVs (ODEVs)

Myelin formation by oligodendrocytes is controlled by a range of extracellular factors, including signals from the extracellular matrix and from axons. Close physical contacts between oligodendrocytes and neurons can trigger mechanotransduction signals that promote proper myelination, and this can enhance the long-term survival of certain axons. Oligodendrocytes also release EVs (ODEVs) that provide trophic support to neurons and their secretion can be stimulated by neural activity and neurotransmitter signals [96,97]. Oligodendrocyte-derived exosomes contain myelin proteins, such as proteolipid protein (PLP), which can be used as a marker to identify these particular EVs [98]. Frühbeis *et al.* [98] showed that ODEV secretion is promoted by activity-dependent release of glutamate from neurons and mediated by Ca^{2+} influx through N-methyl-D-aspartic acid and AMPA receptors on oligodendrocytes [98]. Oligodendrocyte-derived exosomes were also found to be internalised by target neurons *in vitro* and *in vivo*. Interestingly, co-culture of mouse primary oligodendrocytes with mouse cortical neurons and other glial cells showed that ODEVs were internalised by 21% of the mouse cortical neurons, 93% of the mouse primary microglia, 3% of the mouse primary astrocytes and 2% of the oligodendrocytes. *In vivo*, neurons can internalise ODEVs by endocytosis. Moreover, when cultured *in vitro* under stressful conditions, including oxidative stress (with H_2O_2) or nutrient deprivation (absence of B-27 supplement), neurons survived better when co-cultured with oligodendrocytes or oligodendroglial exosomes [98]. Oligodendrocyte-derived exosomes also promote the survival of neurons under oxygen–glucose deprivation

Table 3. miRNA identified in EVs isolated from neurons, astrocytes, microglia and oligodendrocytes.

EV type	Stimulus	EV-producing cell	RNA	Recipient cell/organ	Outcome	References
Exosome	Potassium-induced depolarisation	Human neuroblasts	miR-638, -1915, -3196, -1908, -1228, -4281	–	–	[68]
Exosome	Conditioned medium	Neuron	miR-124a	Primary culture astrocytes	↑ miR-124a and GLT1 protein expression	[77]
Exosome	–	Murine primary neurons	miRNA-669, -466, -297a-5p, -3082-5p, -124-3p	Astrocytes*	–	[79]
Exosome	Ethanol	Primary astrocytes	miRNA-164a, -182, 200b	Cortical neurons	↑ Neuronal levels of COX-2 ↑ Neuronal levels of miR-146a	[95]
Exosome	EE	–	miR-219	OPCs NSCs Ageing rat brain	↑ Differentiation and myelin production, remyelination ↑ Proliferation ↑ Myelination in ageing rat brain	[102]
EVs	–	human NSC (hNSC) line	(hsa)-miR-1246, -4488, -4508, -4492, and -4516	–	–	[108]
EVs	–	Mouse primary neonatal subventricular zone NSCs	miR-9, Let-7, miR-181	Mouse primary microglia	Cytokine release, Negative feedback loop affecting NSC proliferation	[111]

conditions (a model of cerebral ischaemia) [99]. Primary mouse neurons grown under these ischaemic conditions displayed increased metabolic activity compared to control neurons. This resilience to ischaemia may be due to the transfer of superoxide dismutase and catalase via ODEVs. ODEV treatment also promoted protein kinase B activation and an increase in the phosphorylation of CREB, glycogen synthase kinase (GSK)-3a/b, GSK-3b and c-Jun N-terminal kinases (JNK) in recipient neurons. Electrophysiological studies revealed an increase in action potential firing rate in primary neurons when exposed to ODEVs. The exosomes were isolated from both primary mouse oligodendrocytes and the oligodendrocyte precursor cell (OPCs) line, Oli-neu cells. Authors stated that the increase in the firing rate is consistent with the notion that oligodendrocyte-derived exosomes support the metabolic activity of neurons [99]. For instance, ODEVs increase the release of synaptic vesicles (at the presynaptic site of neurons) through the induction of sphingolipid metabolism and thus increase action potentials in postsynaptic neurons. This mechanism has been previously observed in microglia-derived ectosomes that increase spontaneous and evoked excitatory transmission in hippocampal neurons [100].

Exosomes produced by rat primary oligodendrocytes can also signal to neighbouring OPCs to inhibit their differentiation and thus reduce myelin formation [97]. To study whether neurons played a role in oligodendrocyte-derived exosome secretion, rat primary oligodendrocytes were treated with neuronal conditioned medium (NCM). Interestingly, the number of differentiated oligodendrocytes increased, as well as their size and complexity, after treatment with NCM. However, addition of ODEVs to oligodendrocytes that were previously treated with NCM caused a decrease in NCM-mediated cell surface area extension [97]. This inhibitory effect was mediated by the Fyn-RhoA-ROCK signalling pathway, in which Baer *et al.* [101] have shown to be involved in myelin-mediated inhibition of OPC differentiation. These data show that neurons likely control ODEV secretion to fine-tune myelination [97]. Krämer-Albers *et al.* [96] demonstrated that exosomes secreted by mouse primary oligodendrocytes were enriched in PLP and 2′,3′-cyclic-nucleotide-phosphodiesterase (CNP). After treatment of cell cultures with ionomycin, there was a significant production of PLP-containing exosomes suggesting that elevations in cytosolic calcium levels may regulate their secretion. Proteomic analysis of the ODEVs revealed the presence of myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and stress-protective proteins such as glutathione S-transferase P and peroxiredoxin 1 [96]. This suggests that ODEVs are likely to

be involved in regulating myelin formation, although the mechanisms underlying how internalisation of ODEVs by oligodendrocytes can influence myelin production are yet to be fully elucidated.

Interestingly, parabiotic exposure of aged Wistar rats to a youthful systemic milieu, including serum exosomes, promotes the differentiation of OPCs, myelin production, and improves remyelination in hippocampal slice cultures after acute lysolecithin-induced demyelination [102]. Serum-derived exosomes from rats exposed to environmental enrichment (EE) exerts a similar promyelinating effect and promotes the proliferation of NSCs. Both, young and EE serum-derived exosomes were found to be enriched in miR-219, which is necessary to produce myelinating oligodendrocytes by reducing the expression of inhibitory regulators of differentiation [103]. In addition, nasal administration of exosomes from young rats enhances myelination in the ageing rat brain [102]. Therefore, serum-derived exosomes from young and healthy donors could, in theory, be one day used as neurotherapeutics to promote the repair and regeneration of the aged and damaged CNS.

Neural stem cell-derived EVs

Extracellular vesicles derived from NSCs are promising therapeutic tools for the treatment of neurodegenerative diseases. Studies that compare cellular uptake/internalisation of EV cargo from liposomes versus naturally packaged EV cargos will be important to guide drug development studies [104,105]. NSC-derived EVs have several important functions in both physiological and pathological conditions [106]. They express markers such as PDCD6IP (ALIX), TSG101, and the tetraspanins CD63 and CD9 and package the enzyme asparaginase-like protein 1 (Asrgl1). Therefore, NSC-derived EVs demonstrate intrinsic enzymatic activity, acting as independent metabolic units with asparaginase activity, and can thus modify the concentrations of nutrients in the extracellular environment and regulate cellular physiology [107]. NSC-derived EVs may also influence cell growth and regeneration in the CNS. For instance, Stevanato *et al*. [108] demonstrated that NSC-derived EVs released from an immortalised human NSC (hNSC) line contain 113 miRNAs, the most abundant being miR-1246, miR-4488, miR-4508, miR-4492, and miR-4516. The miR-1246 targets p53 pathway and is involved in cell growth and apoptosis [108]. Cossetti *et al*. [109] demonstrated that RNA and protein cargo contained in NSC-derived exosomes are modified by cytokine stimulation (Th1 and Th2 cytokine cocktails). The interferon gamma (IFN-γ) pathway was activated in NSCs when exposed to a proinflammatory cytokine cocktail, which was mirrored in the EV cargo released. The transcription factor, signal transducer and activator of transcription 1 (STAT1), is activated by interferons which trigger the expression of IFN-stimulated genes involved in antiviral defence, tumour-suppressive functions, and also provides resistance to DNA-damaging agents [110]. Hence, Cossetti *et al*. [109] showed that NSC-derived exosomes can stimulate neuroprotective mechanisms in target cells. Recently, Morton *et al*. [111] provided evidence that mouse neonatal subventricular zone NSCs release EVs that can regulate microglial morphology and physiology and express the miRNAs (miR-9, Let-7, miR-181). These EVs induced the transition of microglial cells towards less complex nonstellate morphologies that express CD11b and Iba1. Moreover, NSC-derived EVs modulated transcriptional programmes and cytokine release from microglia, thus inducing a negative feedback loop that decreased NSC proliferation [111]. Therefore, there are likely bidirectional interactions *in vivo* between adult-born NSCs and microglia via the release and uptake of EVs.

Microglia-derived EVs

Microglia, the resident macrophages of the CNS, regulate neuroinflammation and innate immunity in the brain and spinal cord. Microglia are the first line of defence against pathogens, toxins and malignant cells in the CNS and act to maintain tissue homeostasis [112]. Hence, microglia are surveyors and monitors of the brain parenchyma and react to danger signals. Microglia-derived EVs (MDEVs) are an important form of intercellular communication in the brain. Several studies have identified aminopeptidase CD13 and the monocarboxylate transporter 1 as specific markers for MDEVs [112,113]. Extracellular ATP, via the activation of P2X7 receptors, is an important stimulant of vesicle shedding in microglia. Takenouchi *et al*. [114] showed that ectosomes (microvesicles) form in microglial cells following activation of P2X7 receptors by ATP, resulting in the release of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into the extracellular space [114]. GAPDH is a key glycolytic enzyme expressed on the surface of macrophages where it functions as a transferrin receptor [108]. It is also involved in the regulation of neuritogenesis in neurons [115,116]. To investigate the mechanism of GAPDH release, lipopolysaccharide (LPS)-primed MG6 mouse microglial cells were exposed to ATP which resulted in the formation of EVs (exosomes and ectosomes) enriched in GAPDH. GAPDH was found to facilitate

LPS-induced phosphorylation of p38 MAP kinase in MG6 cells. Taken together, these findings indicate that GAPDH release might be involved in the regulation of neuroinflammation and/or neuritogenesis in the brain [114].

Astrocyte-derived ATP can induce vesicle shedding (ectosome formation) from nearby microglia, followed by IL-1β release. Cytokine-loaded EVs secreted by microglia are thought to coordinate inflammatory responses across multiple regions of the brain. In addition to extracellular ATP, serotonin (5HT) also stimulates EV secretion from microglia. Glebov *et al.* [117] demonstrated the expression of 5HT receptors in microglia, including 5-HT2A, 5-HT2B, and 5-HT4, and their role in exosome secretion. A significant increase in cytosolic Ca^{2+} and exosome release was detected upon 5-HT treatment of mouse primary microglia and the BV-2 microglial cell line. Moreover, co-culture of microglia with embryonic stem cell-derived serotonergic neurons stimulates exosome secretion from microglia through 5-HT receptor-mediated mechanisms [117].

Microglia-derived EVs can also modulate presynaptic neurotransmission via the endocannabinoid system. Endocannabinoids are secreted in EVs produced by microglial cells (both rat primary cells and the murine N9 cell line). The endocannabinoid anandamide, also known as *N*-arachidonoylethanolamine (AEA), is expressed on the surface of MDEVs and can stimulate CB1 receptors, thus inhibiting presynaptic neurotransmission in GABAergic neurons [118]. Hooper *et al.* [119] have shown the internalisation of microglia-derived exosomes by neurons after EV isolation from wingless-related integration site (Wnt)-3a-treated microglia. Wnt-3a is a signalling protein and proto-oncogene that displays critical roles in neurodevelopmental processes and neurological diseases [120]. Interestingly, Wnt-3 has also been shown to stimulate exosome secretion in microglia. Initially, carrier-free Wnt-3a was found to be internalised into primary cultured rat microglia followed by release of Wnt-3a-containing exosomes from rat primary microglia [119]. The microglia-derived exosome secretion was regulated by a GSK 3-independent mechanism. Similarly, Wnt-3a-containing exosomes were also secreted by primary cortical neurons upon Wnt-3a treatment. Proteomic analyses of the microglia-derived exosomes showed the presence of a total of 45 proteins involved in metabolism, cellular architecture, protein synthesis and protein degradation, for example, GAPDH, ubiquitin, and ribosomal subunits. The authors suggest that the Wnt3a-containing exosomes might serve to produce morphogen gradients during CNS development [119]. Despite the importance of microglia in the CNS, there is still much to learn regarding the multiple roles of MDEVs in regulating glial and neuronal function.

Concluding remarks

Extracellular vesicles function as a complex mode of communication between neurons and glial cells of the CNS, under both physiological and pathological states. EVs have been implicated in important processes including synaptic plasticity, myelination and neurogenesis, as well as modulation of neuroinflammation. However, most studies are based on results from cell culture of human or rodent primary cells or cell lines. Although *in vitro* models have been very useful in uncovering the mechanisms of EV binding and internalisation by target cells, they provide only a limited understanding of physiological roles of brain EVs in living animals. Moreover, purely *in vitro* studies may provide misleading information in terms of target cell specificity of EVs by confining the EVs to a particular and unnatural cell culture environment. Therefore, further *in vivo* studies are needed for a more reliable and complete understanding of the role of EVs in intercellular communication in the CNS in health and disease. There is also a paucity of research on intercellular communication via EVs in the brain under physiological conditions and healthy ageing. Deciphering the role of EVs under physiological conditions is crucial for developing therapies to cure or alleviate neurological diseases. For instance, CSF-derived EVs extracted from young healthy humans could be administrated to older patients suffering from neurological diseases to promote a healthier brain physiology. However, more information on the specific mechanisms of cell recognition and internalisation of EVs by recipient cells is required to develop targeted treatments. Additionally, how cargoes are unloaded and processed by the recipient cells remains to be unravelled. Another outstanding aspect that remains to be revealed is how EVs cross the BBB. Mechanistic understanding of these complex processes warrants further investigation.

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Author contributions

GKS and LRL conceived the review topic. LRL prepared the figures and wrote the first draft of the

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