

## REVIEW

# Extracellular Vesicles in neural cell interaction and CNS homeostasis

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## Abstract

Central nervous system (CNS) homeostasis critically depends on the interaction between neurons and glia cells. Extracellular vesicles (EVs) recently emerged as versatile messengers in CNS cell communication. EVs are released by neurons and glia in activity-dependent manner and address multiple target cells within and outside the nervous system. Here, we summarize the recent advances in understanding the physiological roles of EVs in the nervous system and their ability to deliver signals across the CNS barriers. In addition to the disposal of cellular components via EVs and clearance by phagocytic cells, EVs are involved in plasticity-associated processes, mediate trophic support and neuroprotection, promote axonal maintenance, and modulate neuroinflammation. While individual functional components of the EV cargo are becoming progressively identified, the role of neural EVs as compound multimodal signaling entities remains to be elucidated. Novel transgenic models and imaging technologies allow EV tracking in vivo and provide further insight into EV targeting and their mode of action. Overall, EVs represent key players in the maintenance of CNS homeostasis essential for the lifelong performance of neural networks and thus provide a wide spectrum of biomedical applications.

## KEYWORDS

axonal maintenance, blood–brain barrier, exosomes, in vivo imaging, microvesicles, neuroinflammation, neuron–glia interaction, synaptic plasticity

## 1 | INTRODUCTION: GENERAL ASPECTS OF NEURAL CELL COMMUNICATION AND SIGNIFICANCE OF EV SIGNALING

The nervous system in animal kingdom has evolved to integrate and process signals from all parts of the body and to execute behavior. Its core function, therefore, is founded on the communication of cells within the tissue but also across its borders. The cellular architecture of the nervous system is highly complex consisting of neurons, which

form electrical signaling networks, and different types of glial cells arranged within the network.<sup>1–3</sup> Neurons are postmitotic cells of sophisticated morphology: The heavily ramified dendrites receive and integrate signals through chemical synapses while their axons, which can be up to 1 m long in humans, are specialized for fast signal transmission. Glial cells including myelinating oligodendrocytes, astrocytes, and microglia continuously interact with neurons and actively shape and sustain the network, for example, through regulating synaptogenesis and modulating synaptic function, providing metabolic support, and

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promoting CNS homeostasis.<sup>4</sup> The CNS has only a limited capacity to regenerate, hence a progressive loss of neurons, for example due to homeostatic imbalance, leads to neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease (PD), or Amyotrophic lateral sclerosis (ALS). Glial dysfunction contributes to most neural diseases and can even drive neurodegenerative processes, as occurring in myelin disease. Overall, neural function and long-term homeostasis depends on fine-tuned cell communication, while maladaptive cellular interactions eventually lead to neural disease and neurodegeneration.

Intercellular communication in the brain is multimodal and has been systematically categorized into wiring transmission (one-to-one transmission) and volume transmission (one-to-many transmission)<sup>5</sup>: Wired signaling includes neurotransmission via electrical and chemical synapses, transfer of ions and small molecules through gap junctions as well as tunneling nanotubes, which allow the passage of cytoplasmic content including organelles through membrane extensions that function as conduits.<sup>6</sup> Volume transmission is characterized by paracrine transmission of signals through the interstitial space or the cerebrospinal fluid (e.g., neuromodulators acting via extrasynaptic receptors or growth factor/cytokine signaling).

In the recent years, EVs have entered the scene and broadened the view on neural cell communication. EVs including microvesicles shedding from the plasma membrane (also termed ectosomes), and exosomes secreted from multivesicular bodies (MVBs) into the extracellular space, are secreted from all types of neural cells.<sup>7-9</sup> EVs can convey signals through triggering surface receptors activating second messenger signaling cascades or by delivering their cargo, which comprises proteins, nucleic acids, and small molecules.<sup>10</sup> Thus, EVs have the capability to elicit profound effects and a phenotypic transformation in recipient cells. Intriguingly, EV release from neural cells appears generally controlled by neurotransmitter signaling, linking EV signaling to the degree of electrical activity within the cellular network (circuitry) of the releasing cells. Together, these features imply that extracellular vesicles may support local adaptive processes such as neural plasticity or maintenance of tissue homeostasis. Furthermore, EVs may carry their membrane protected cargo to distant sites and pass the brain barriers in both directions, allowing EVs either to enter the brain from the periphery or to exit via the circulation, the cerebrospinal fluid or the glymphatic system. Since neural EVs also transmit proteins involved in the pathogenesis of neurodegenerative diseases, they provide a window to the brain and represent attractive diagnostic and therapeutic targets.

Regarding their characteristics, in particular the ability to horizontally transfer complex biomolecules including nucleic acids, EVs represent exciting signaling modalities

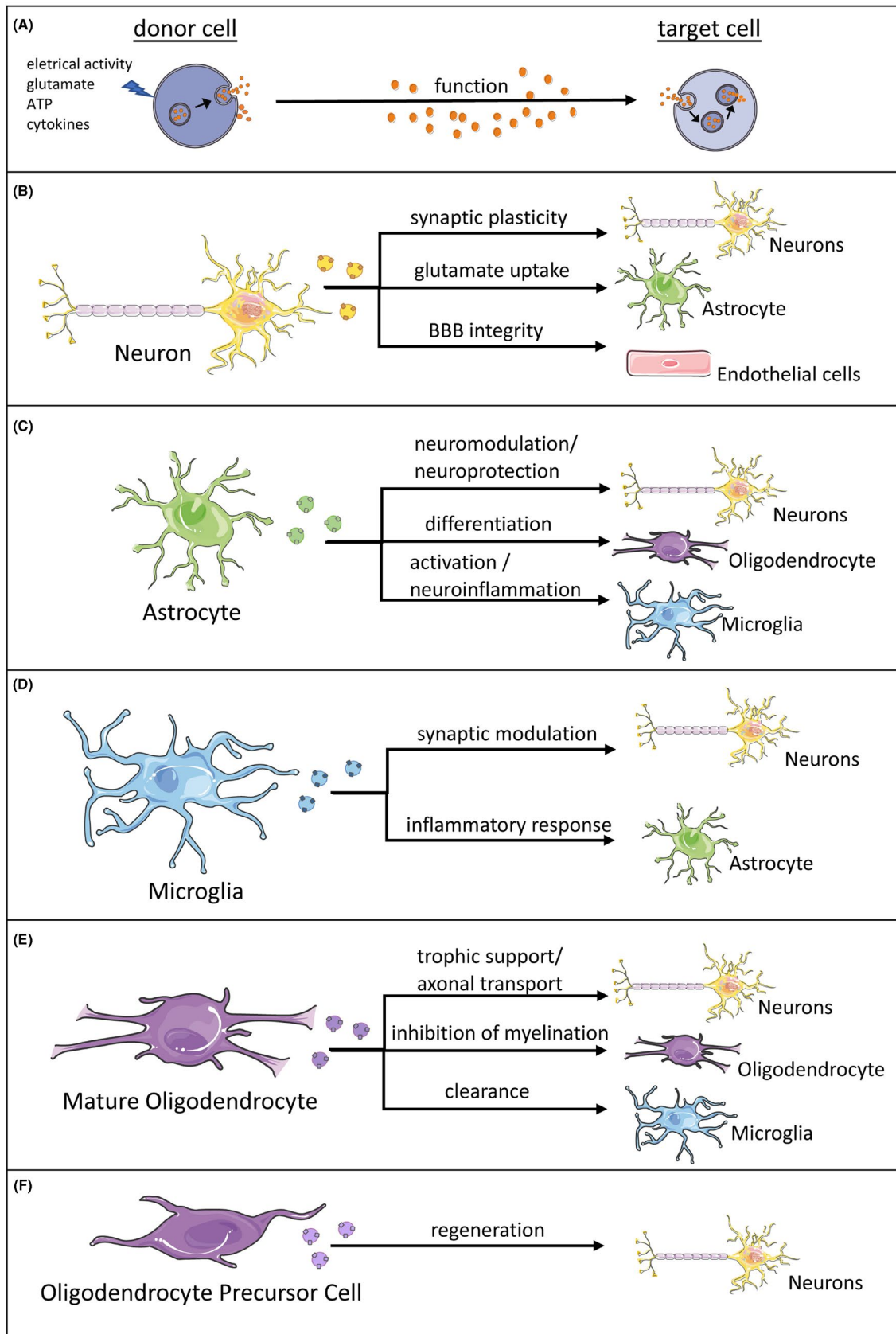
adding a new dimension to the interaction between neurons and glial cells. Here, we will provide a concise overview of EVs in neural cell communication and their functional implications. We will focus on physiological aspects and only briefly indicate the relevance for brain pathology (readers interested in neurodegeneration are referred to Hill, 2019 and the references therein<sup>11</sup>). After delineating the cellular travel routes and the fundamental modes of action, the current strategies for tracking and revealing the function of EVs *in vivo* will be discussed. Finally, we will summarize the current view on the exchange of EVs between the brain and the periphery.

## 2 | CELLULAR ROUTES OF NEURAL EVS

EVs are secreted by all CNS cell types. During early development, neural stem cells (NSCs) release EVs that affect neurogenesis and appear to regulate the switch between the neurogenic and gliogenic fate by delivering micro-RNAs. The influence on NSC fate, however, may differ depending on the regional origin or the specific developmental state of the NSCs.<sup>12,13</sup> In addition to their impact on neurogenesis, NSC-EVs exhibit immunomodulatory activity turning them to promising candidates for application in regenerative therapies of brain disease or injuries.<sup>14,15</sup>

In the mature brain, neurons as well as glia cells can interact homo- or heterotypically with their neighboring cells via the exchange of EVs (Figure 1). In general, this give-and-take between glia and neurons appears to promote neuronal survival and homeostasis, immune responses, and synaptic plasticity.<sup>7,8</sup> Strikingly, liberation of EVs occurs in response to neurotransmitter signaling and thus EV levels and the degree of EV action in the brain are controlled by neuronal electrical activity (Figure 1A). Secretion of the excitatory neurotransmitter glutamate from electrically active neurons stimulates EV release by neurons and oligodendrocytes.<sup>16,17</sup> Moreover, EV shedding from astrocytes and microglial cells is triggered by ATP, which is released at synapses as a co-transmitter and activates glial purinergic receptors.<sup>18,19</sup> Coupling of EV release to neurotransmitter signaling drives EV action preferentially in regions of high neural activity and likely reflects their enhanced demand in these areas.

Balancing of brain activity and its adaptation during learning is achieved through a range of plastic processes at synapses modulating the efficacy of neurotransmission and neuronal excitability (termed "synaptic plasticity"). For example, the number of neurotransmitter receptors in the postsynaptic membrane correlates with synaptic strength. Depolarized neurons release small EVs from MVBs mainly located in the somatodendritic compartment to dispose



**FIGURE 1** CNS EVs and their target cells. (A) Neurons and the different types of glial cells stimulated by neurotransmitter signaling or cytokines release EVs, which are committed to deliver their cargo to other cells of the CNS. Neuronal (B) and glial EVs (C–F) mediate a range of functions that regulate local adaptive processes and CNS homeostasis

neurotransmitter receptors as well as intra-neuronal miRNAs with proposed synaptic functions.<sup>16,20,21</sup> Plasticity at the level of synapses is also achieved by the selective elimination of unprofitable synapses through synaptic pruning. To this end, activity-mediated transfer of the Wnt-inhibitor PRR7 in neuronal small EVs eliminates excitatory synapses in neighboring neurons.<sup>22</sup> Wnt signaling mediated by EVs is also known to modulate synaptic signaling at the drosophila neuromuscular junction.<sup>23,24</sup> Two independent exciting studies demonstrated that the plasticity-associated protein Arc-1 self assembles with its own mRNA into capsid-like structures, which bud into EVs. Together with these EVs, Arc-mRNA can be transferred across synapses and undergo activity-dependent translation at postsynaptic sites.<sup>25,26</sup> Arc plays a crucial role during long-lasting information storage and mediates various forms of synaptic plasticity.<sup>27</sup> Modulation of synaptic signaling is also achieved by microglia-derived microvesicles, which appear to increase the probability of neurotransmitter release at presynaptic terminals and affect the efficacy of inhibitory synapses by transfer of endocannabinoids overall enhancing excitatory neurotransmission.<sup>19,28</sup> In summary, EVs play versatile roles in regulating synaptic signaling and plasticity (Figure 1 B–D).

Synapses are engulfed by a subset of astrocytes, which intermingle with the pre- and postsynaptic membrane (termed “tripartite synapse”). Together, they build a cellular network regulating and maintaining synaptic homeostasis and efficacy dependent on neuronal synaptic activity.<sup>29</sup> Transfer of miR124-3p containing EVs from neurons to astrocytes leads to inactivation of two other miRNAs (miR-132 and miR-218) lifting a brake on expression of the astrocytic GLT1 transporter.<sup>30,31</sup> Higher levels of GLT1 allow increased glutamate uptake from the synaptic cleft and thus contributes to neurotransmitter homeostasis. In the opposite direction, astrocyte-derived small EVs deliver miR-26a-5p to hippocampal neurons regulating a number of neuronal proteins relevant for neuronal morphology and decreasing dendritic complexity relevant for receiving synaptic input.<sup>32</sup>

In addition to their synaptic functions, astrocytes are regionally heterogeneous cells that can adopt varying phenotypes and are involved in homeostatic processes including the inflammatory response. Release of EVs from astrocytes is promoted by pro-inflammatory cytokines such as IL1- $\beta$  and TNF- $\alpha$  indicating that these EVs modulate neuroinflammation during brain injury.<sup>33,34</sup> Strikingly, IL1- $\beta$ -induced EVs rapidly entered into the peripheral circulation and promoted the recruitment of peripheral leukocytes.<sup>34</sup> Morphine treatment of astrocytes induced the release of miR-138 in EVs promoting microglia activation via TLR7 signaling implicated in neuroinflammation observed in opioid abusers.<sup>35</sup> Furthermore, astrocyte-derived small EVs appear to facilitate neuroprotection against cell insult (reviewed in Ref. [36]), for example, by transferring neuroglobin to neurons, which

exhibits antioxidant, anti-apoptotic, and anti-inflammatory effects.<sup>37</sup> Together, astrocyte EVs appear to cover a broad spectrum of synaptic, homeostatic, and neuroprotective functions, reflecting their multifunctionality in the CNS (Figure 1C).

Microglia are macrophage-like cells that survey the brain, remove cellular debris by phagocytosis, and are key modulators of neuroinflammation linking the brain and the immune system. They react to brain injury or neurodegeneration by adopting reactive phenotypes that can dynamically vary between pro-inflammatory and pro-regenerative. EVs and their cargo released by microglia reflect the phenotype of the parent cells and may either promote inflammation (depending on cytokine cargo, e.g., IL1- $\beta$ , TNF- $\alpha$ ), contribute to neurodegeneration (e.g., by spreading of misfolded protein aggregates), or exhibit pro-regenerative functions, for example, by promoting remyelination in myelin lesions through stimulating oligodendrocyte precursor cell (OPC) migration and differentiation.<sup>38-41</sup> Thus, microglia-derived EVs appear to regulate and eventually propagate neuroinflammation but, moreover, also ameliorate pathology and promote recovery from CNS injury (Figure 1D).

Due to their ability to phagocytose, microglia clear EVs from the brain parenchyma.<sup>42</sup> Whether this clearance occurs in an immunologically silent manner or results in microglia activation most likely depends on the EV cargo and the state of the donor cell. Oligodendrocyte-derived EVs internalized by cultured microglia via macropinocytosis do not result in microglia activation, most likely reflecting a process of homeostatic turnover.<sup>43</sup> Moreover, EVs released from neurons challenged by a high load of pathology-associated misfolded proteins trigger a pro-inflammatory microglial response,<sup>44,45</sup> while glioma-derived EVs appear to drive microglia toward a tumor-supportive and immuno-tolerant state.<sup>46,47</sup> Thus, clearance of EVs by microglia regulates microglial activity and modulates their homeostatic functions.

To facilitate fast saltatory nerve conduction in the CNS, neurons are electrically isolated by the myelin membrane generated by oligodendroglial cells. Myelination is dynamic and activity-dependent myelin plasticity is associated with learning and memory.<sup>48</sup> EV-mediated autocrine signaling has been shown to inhibit the formation of myelin-like membrane sheets *in vitro* which may reflect a negative feedback loop balancing myelination<sup>49</sup> (Figure 1E). Myelin integrity and plasticity appears decreased in the aged brain. Interestingly, EVs derived from early passages of astrocytes provide homeostatic support facilitating differentiation of cultured OPCs to mature oligodendrocytes, whereas EVs collected from aged astrocytes that had adopted a senescence-like phenotype have lost this supportive effect. Thus, a senescence-associated secretory phenotype involving astrocyte-EVs may contribute to reduced myelin plasticity in the aged CNS.<sup>50</sup> During

pathological conditions such as brain lesion, NG2-positive OPCs invade the lesion and appear to promote neurite growth and regeneration by delivering retinoic acid to neurons via EVs<sup>51,52</sup> (Figure 1F).

Mature myelinating oligodendrocytes release EVs from MVBs upon stimulation with the neurotransmitter glutamate. Neurons that internalize these EVs are more robust toward various stress conditions and are able to sustain a high level of metabolic activity as well as axonal transport<sup>17,53</sup> (Figure 1E). Intriguingly, mice with secondary axonal degeneration due to the lack of the glial proteins PLP and CNP exhibit impaired oligodendroglial EV release and have lost the ability to promote axonal transport.<sup>53</sup> EV transfer from oligodendrocytes to neurons may thus provide a means of support, essential for long-term neuronal survival and axonal maintenance. Indeed, Ferritin heavy chain is a cargo of these EVs and provides antioxidant defense to neurons protecting from iron-mediated cytotoxicity. Interference with EV release by conditional deletion of Rab35 in oligodendrocytes results in neuronal death in the cortex and an enhanced susceptibility of neurons to oxidative damage.<sup>54</sup> In addition to neuronal transfer, oligodendroglial EVs may address astrocytes and microglia for clearance and immune surveillance of the microenvironment surrounding the axon-myelin unit.<sup>55</sup> Intriguingly, recent work suggests that viruses such as JC polyomavirus and Herpes simplex virus particles are contained within oligodendrocyte-derived EVs potentially increasing their infectivity and targeting spectrum.<sup>56,57</sup>

Conclusively, the close interaction of the different neural cell types via EVs appears crucial for maintaining overall brain homeostasis. Since EV release is regulated by neurotransmitter signaling, the action of EVs is directed to areas of high neural activity. In these areas, tissue homeostasis is challenged due to firing activity (metabolic and oxidative stress, high energy demand) and furthermore, local remodeling processes occur (synaptic plasticity). The homeostatic balance is lost during neurodegenerative disease, such as AD, PD, ALS, and Prion Disease, where proteostasis is perturbed and pathogenic or misfolded proteins (A $\beta$ , Tau,  $\alpha$ -synuclein, SOD-1, and prion protein) are disposed from cells via EVs. These EVs exhibit detrimental as well as beneficial functions during neurodegeneration (for reviews see<sup>11,42,58,59</sup>).

### 3 | CARGO OF NEURAL EVS: HOW DO THEY ACT?

Although some of the EVs can be broken down by phagocytic cells such as microglia, the active participation of EVs in signal transmission in the normal and diseased brain

is undisputed. However, their mode of action and specific functional components are not completely uncovered so far. EVs may signal through the activation of signal transduction pathways or the functional transfer and retrieval of the EV cargo. Possibly, EVs operate as a complex molecular entity and elicit a compound response in target cells. With regard to neural EVs, proteins, miRNAs, mRNAs, and lipids were identified as active components. To the best of our knowledge, DNA has not yet been identified in neural EVs as a functional player so far, which, however, may well be the case for EVs originating from transformed cells such as glioma cells.<sup>60</sup> In addition to the delivery of cargo to target cells, EVs may operate as independent metabolic units in the microenvironment. NSC-EVs contain asparaginase activity which converts metabolites and has the potential to influence the metabolic milieu of the microenvironment.<sup>61</sup>

Selective cargo loading into neural EVs can occur in ESCRT-dependent as well as ESCRT-independent fashion.<sup>10,62</sup> The specific mechanisms of EV cargo selection appear to differ between cell types and are not well described for neural cells. In general, cargo loading or enrichment is achieved by posttranslational modifications of proteins including ubiquitination, phosphorylation, sumoylation, isoprenylation, palmitoylation, ISGylation, deimination, and glycosylation, which is recognized by a sorting machinery such as the ESCRT complex (reviewed in Ref. [63]). For example, hyperphosphorylation of Tau-protein (involved in AD pathology and serving as a biomarker), leads to its increased secretion via astrocyte-derived EVs.<sup>64</sup> With regard to miRNA sorting, RNA-binding proteins (e.g., hnRNP A2/B1, YBox protein 1, and Ago) appear to recognize defined miRNA motifs and inclusion into EVs is mediated by posttranslational modification of the RNA-binding protein or other interacting proteins.<sup>65-67</sup> While these sorting mechanisms were defined in nonneural tumor cells, it is presently unclear whether the same principles of miRNA sorting to EVs apply to primary neural cells.

Intriguingly, in neurons Arc-mRNA is recruited to EVs by self-assembly of the Arc protein into virus-like capsids, a process that may be evolutionary related to retroviral budding and is conserved between vertebrates and invertebrates.<sup>25,26</sup> Upon EV-mediated transfer to neighboring neurons, Arc-mRNA can undergo activity-dependent translation and is required for synaptic plasticity. Remarkably, these two elegant studies provide a complete exploration of the EV life cycle, from cargo sorting in the donor to cargo function in the target cell. It remains to be determined whether other mRNAs are delivered in similar fashion between neural cells.

MiRNAs are by far the most heavily studied cargo in CNS EVs, largely examined in the context of disease, and their qualification as biomarkers (reviewed in Ref. [68]). However,

the molar ratio of miRNAs contained within EVs and the biological significance in the physiological context are under debate and remains difficult to assess.<sup>69-71</sup> Proteins with enzymatic activities such as Cre or luciferase are utilized as reporters providing the proof of principle for their functional transfer (see below). However, the biological activity of natural protein cargo has been assessed only in a few cases and requires sophisticated transgenic experimental setups, as exemplified in the case of Ferritin heavy chain transfer to neurons via oligodendrocyte-derived EVs<sup>54</sup> or the delivery of NADPH-oxidase-2 from macrophages to injured neurons facilitating neuronal regeneration after lesion through ROS generation.<sup>72</sup> Intriguingly, during nervous system regeneration after injury or under inflammatory conditions, even larger organelles such as ribosomes or mitochondria may be transferred via EVs.<sup>73,74</sup>

Isolation of EVs from CNS cells is achieved from primary cultured neurons or glia cells as well as neural cell lines, which are cultured in chemically defined media in the absence of serum. A recent study raised awareness that commercial supplements such as B27 commonly used for neural cell culture are contaminated with miRNAs (e.g., miR451 and miR-122) that co-purify with EVs and may be easily misinterpreted as highly enriched EV-associated miRNAs in RNA-seq or qPCR experiments.<sup>75</sup> The contaminating miRNAs were introduced by only a single component identified as catalase, which actually also co-isolated with EVs. Therefore, RNA and protein cargo of EVs identified by means of RNA-seq and proteomics, require careful assessment and validation. Moreover, recent studies investigating the heterogeneity of EVs and other particles released by cells indicated that RNA-binding proteins expected to bind miRNAs rather fractionate with non-vesicular particles independent of classic EVs.<sup>76</sup> Further studies are required to reveal the heterogeneity of bulk isolated EVs and the implications of non-vesicular particles in the neural domain.

## 4 | IN VIVO: HOW TO STUDY EVS IN THE BRAIN?

So far, the vast majority of EV research has been performed from *in vitro* or *ex vivo* collection of cell culture supernatant. To study the incidence and functional effects of EVs in their natural environment in the healthy and diseased brain *in vivo*, mainly three strategies can be adopted: (a) Isolation and characterization of EVs directly from the tissue or accessible body fluids, (b) Imaging and tracking of labeled EVs and subsequent identification of their target cells in the tissue, and (c) Inactivation of EV release by genetic means to study the phenotypes that develop following EV loss of function.

## 4.1 | Biofluids and tissue isolation

Brain-derived EVs appearing in the cerebrospinal fluid (CSF) and blood provide a promising source for early biomarkers of neurodegenerative disease and brain tumors through liquid biopsy. The brain is an immune-privileged organ that is shielded by the blood-brain barrier (BBB), a vascular wall being impermeable to most cells, drugs, metabolites, and proteins. Whole-blood liquid biopsies to assess the status of CNS health are based on the assumption that brain-derived EVs carrying markers reflecting their diseased parent cells might pass the BBB into the peripheral circulation.<sup>77,78</sup> CSF circulates within the brain and spinal cord and is in direct contact with the interstitial space, hence CSF collection requires an invasive lumbar puncture and thus thorough ethical considerations.

In fact, the largest proportion of neural EVs will be cleared from the interstitial space by surrounding cells and never reach these body fluids. Recent efforts aimed at recovering neural EVs from the brain parenchyma starting with frozen human, macaque, or mouse brain tissue.<sup>79-83</sup> The basic idea is to dissociate the tissue under mild conditions with as few as possible damage to the cells and collect EVs present in the interstitial fluid. The challenge is to minimize co-isolation of non-EV contaminants with the same physiochemical properties such as intracellular vesicles, intraluminal vesicles, or membranous particles released from broken cells during tissue harvest, processing, or storing.<sup>79,84</sup> Following gentle tissue disruption (mechanical procedures, enzymatic digestion), EV separation can be performed by ultracentrifugation,<sup>85</sup> density gradient ultracentrifugation,<sup>79-81,83</sup> size-exclusion chromatography,<sup>82,83</sup> or precipitation.<sup>86</sup> However, it is unavoidable that during brain dissociation artificial membrane fragments are generated from thin axons, dendrites (spines), synapses, and myelin, which form small vesicles co-purifying with interstitial EVs. The purification methodology of myelin and in particular of synaptosomes is remarkably similar. In addition, the pre-analytical conditions (species, death-to-processing period, and storage conditions) greatly impact the yield, purity, and stability of the recovered EV fractions and require thorough reporting and standardization as recommended by the MISEV and EV-TRACK initiatives, which impart essential guidelines for increasing transparency and harmonizing EV research.<sup>84,87</sup> A stringent characterization including screening for potential contaminants following EV enrichment from brain tissue is mandatory to meet the standards and requirements for the definition of EVs and subtypes.<sup>79,82,88</sup> Until now, an absolute separation of EVs from cellular compartments and a resolution of the existing EV subtypes is unreachd. However, a direct comparison of different brain states, for example, healthy versus diseased brain tissue, may provide an important indication

for the involvement of EVs in a specific pathology or brain disease that may be pursued by follow-up studies.

## 4.2 | Tracking of brain EVs

### 4.2.1 | Imaging of fluorescently labeled EVs

In order to study EV fate, the use of fluorescent labels is a reasonable approach to image EVs in a tissue. Lipophilic dyes incorporated into the EV membrane are relatively easy to handle but come with side effects such as staining of non-EV structures, flipping between membranes, and influence on EV membrane functionality, which may create artificial results. Lipid dyes have largely been used in biodistribution studies demonstrating the entry of EVs from the periphery into the brain.<sup>89,90</sup> It is apparent from these studies that the EV-labeling dye in target cells is uniformly distributed likely independent of the subcellular compartments involved in EV uptake, indicating that the label has dispersed away from EVs at least within the target cell. Keeping in mind these obstacles, EV studies utilizing lipid dyes require careful controls.<sup>84</sup>

In contrast, selective tagging by genetic engineering of EV-associated proteins with fluorescent or enzymatic reporters enables tracking of EVs via live imaging (with the caution that large tags may influence trafficking). The tetraspanin CD63 is widely used for tagging, since it is considered as a reliable marker for EVs and exosomes. Adverse effects of CD63-GFP overexpression indeed were observed in transgenic rats ubiquitously expressing human-CD63-GFP.<sup>91</sup> These can be circumvented by promoter-directed expression of the transgene in specific cell types of interest. Expression of CD63-GFP under control of the Sox2 promoter, driving expression in NSCs, was used to track NSC-derived EVs in the neurogenic niche during development.<sup>92</sup> As expected CD63-GFP was expressed in NSCs and proliferating astrocytes but not in differentiated neurons and glial cells when analyzed in a neurosphere differentiation assay in vitro. In the embryonic brain, CD63-GFP signals were detected in NSCs located in the subventricular zone and also at sites distant to the EV-secreting NSCs. However, targeting of labeled EVs to NSCs and astrocytes was further studied in vitro using neurospheres and primary astrocytes. In vivo imaging of CD63-GFP-EVs may be complicated by the fact that epifluorescence of CD63-GFP expressing cells creates a strong background covering relatively faint signals produced by only few labeled molecules associated with EVs, which are quickly turned over in target cells. To avoid background fluorescence, the pH-sensitive pHluorin-CD63 can be employed as it acquires fluorescence upon release from acidic late endosomal MVBs to the pH-neutral extracellular space.<sup>93-95</sup> While pHluorin was successfully used for live-tracking of EVs involved in inter-organ communication in zebrafish,<sup>96</sup> it

will be tempting to apply this technique for imaging of EVs in the mammalian brain. In the mouse brain, live imaging of fluorescent EVs released from implanted glioblastoma cells was performed by intravital 2-photon imaging, confirming glioma EV uptake by microglia and monocytes in the tumor microenvironment.<sup>97</sup>

Transgenic models employing EV reporters were further refined by utilizing Cre-mediated excision of floxed stop sequences to allow for cell type-specific and temporally controlled activation of CD9-GFP or CD63-GFP reporters upon crossing with specific inducible Cre drivers.<sup>31,98,99</sup> In so-called TIGER-mice (*transgenic-inducible GFP EV reporter*), CD9-GFP expression was induced by crossing to CAG-CreER<sup>T2</sup> or Nestin-CreER<sup>T2</sup> mice and subsequent application of an estrogen analog to achieve astrocyte expression of CD9-GFP.<sup>98</sup> Combining different lines of arguments, the study implied that EVs released by astrocytes during development act on microglia and exhibit immunomodulatory properties. However, it was unclear from this study, why ubiquitous CreER<sup>T2</sup> expression (controlled by the CAG or the Nestin promoter active in NSCs) did not result in reporter activation in a broader spectrum of CNS cells. Furthermore, EV tracking was not performed on the basis of CD9-GFP labeling but surprisingly by intraventricular injection of DiI-labeled EVs isolated from cultured astrocytes.

To label CD63-positive EVs in inducible and cell type-dependent fashion, stop-flox-CD63-emGFP mice were generated and crossed to Cdh5-CreER<sup>T2</sup> mice, labeling EVs derived from vascular endothelial cells including brain endothelium.<sup>99</sup> While GFP-positive EVs were found in the circulation, further tracking of EVs and their potential target cells in the brain (or other tissues) was not performed. It seems apparent from the above-mentioned studies that fluorescent labeling of EVs by genetic means has its limitations at least for EV tracking in the brain due to a donor cell-related background fluorescence as well as a lack of sensitivity and resolution of imaging techniques.

A recent study by Men et al. avoided background fluorescence by local activation of the CD63-GFP reporter using AAV-mediated Cre delivery.<sup>31</sup> Injection of AAV8-CaMKII-Cre into the hippocampus or sciatic nerve of stop-flox-CD63-GFP mice resulted in a locally restricted neuronal expression of CD63-GFP. CD63-GFP-labeled EVs released from infected neurons were detected at distal sites, where AAVs had not penetrated and neurons remained unlabeled. In these areas, astrocytes were identified as target cells internalizing neuron-derived CD63-GFP-labeled EVs. Furthermore, co-injection of Cy5-miR-124-3p allowed visualization of miRNA delivery in association with neuronal EVs to these astrocytes. In addition to fluorescent imaging, comprehensive immuno-EM characterization of brain tissue was performed providing further evidence that the release of CD63-positive EVs occurred in

activity-dependent fashion from the somato-dendritic but not the axonal compartment of neurons, which is consistent with previous findings.<sup>16,31</sup>

Overall, the availability of these inducible transgenic reporter lines represents a huge advance for the field, providing a powerful means to resolve persistent questions referred to their origin, fate and function in different tissues including the brain. However, tracking of fluorescently labeled EVs provides insight into general EV dynamics but is not necessarily conclusive for functional cargo delivery or retrieval.

#### 4.2.2 | Enzymatic reporters

Instead of GFP reporters, enzymatic reporters such as Cre recombinase or luciferase delivered together with EVs provide a highly sensitive readout for the successful transmission of EV cargo to the target cells.<sup>100-102</sup> Luciferase may be applied for the quantitative assessment of cargo delivery or real-time imaging but is incompetent to achieve a permanent labeling of recipient cells. An interesting *in vitro* attempt was recently introduced by deJong et al. using the CRISPR-Cas9 system to track functional RNA transfer.<sup>103</sup> Though it might be a promising tool, translation to model organisms has not yet been reported. Notably, implementation of Cre shares the advantage of mediating a permanent genetic recombination of target cells (usually documented by activation of a fluorescent reporter gene) with an extraordinary sensitivity. This means that only a few molecules of Cre conveyed via EVs within a small window of development can result in a permanent labeling of target cells and their offspring that persists throughout lifespan. Thus, the readout of target cells is independent of the EV dose the cells received and the timing of the analysis. Furthermore, transgenic Cre driver as well as Cre reporter mouse lines are already available and can be applied for EV research. Using a hematopoietic Cre driver crossed to stop-flox YFP reporter line, it was shown that Cre-containing EVs originating from peripheral immune cells enter the brain and recombine neurons in particular under inflammatory conditions and following increased neuronal activity.<sup>100,104</sup> Careful controls such as transplantation of the hematopoietic Cre driver bone marrow into reporter mice or injection of isolated Cre-carrying EVs were performed to provide the proof that recombination is indeed due to the transfer of EVs and not resulting from reporter leakiness. Despite tracking of glioma-derived EVs targeting astrocytes, neurons and leukocytes in the tumor microenvironment after transplantation of Cre-engineered tumor cells,<sup>105,106</sup> the Cre system has not yet been successfully used to track the target cells of neuron- or gliaderived EVs within an unprocessed healthy tissue. We are presently making use of CreER<sup>T2</sup>-drivers to track the target cells of oligodendrocyte-derived EVs in the brain (Schnatz,

Müller and Krämer-Albers, unpublished). CreER<sup>T2</sup> expression in EV-producing cells has the advantage that, in absence of tamoxifen, CreER<sup>T2</sup>-protein is present in the cytoplasm. Due to its interaction with HSP90, CreER<sup>T2</sup> is included in the EV cargo, resulting in efficient delivery to target cells and their permanent recombination upon tamoxifen treatment. To demonstrate that target cell recombination indeed is mediated by EVs, the CreER<sup>T2</sup> reporter mice can be further crossed to knockout models interfering with EV release, such as Alix<sup>fl/fl</sup>, Rab27<sup>fl/fl</sup>, or Rab35<sup>fl/fl</sup> mice,<sup>54,107,108</sup> which should wipe out the observed target cell recombination. Alix is an ESCRT-associated protein involved in the biogenesis of EVs both at MVBs<sup>109</sup> and at the plasma membrane, while Rab27 and Rab35 were shown to control EV secretion largely from MVBs in different cell types.<sup>110,111</sup> Although mapping of EV target cells using this sophisticated transgenic methodology is laborious and time-consuming, it has a huge potential to provide spatio-temporal maps of EV transfer in the healthy and the diseased brain (as well as other tissues). Since the brain is of complex morphology composed of distinct regions and characterized by pronounced cellular heterogeneity, EV transfer is likely to occur region-specific and altered with different brain states.

#### 4.3 | Inactivation of EV release

To study the functional role of EVs in the brain, conditional deletion models that affect or specifically inactivate EV release, such as the above mentioned conditional Alix<sup>fl/fl</sup>, Rab27<sup>fl/fl</sup>, or Rab35<sup>fl/fl</sup> mice, are essential. Of note, phenotypic analysis of these mice can be complicated and may require refined strategies, for instance a distinct behavioral test battery. Conditional knockout of Rab35 (CNP<sup>Cre/+</sup>/Rab35<sup>fl/fl</sup> mice) has been recently used to interfere with EV release from oligodendrocytes demonstrating that cortical neurons degenerate due to oxidative damage when they do not receive oligodendroglial EVs.<sup>54</sup> Furthermore, null mutants of oligodendroglial PLP or CNP (representing EV cargo) are characterized by impaired EV release and suffer from axonal degeneration providing genetic evidence that oligodendroglial EVs provide essential support to axons.<sup>53</sup> Additional studies looking at the functional impact of impeded EV release and transfer between brain cells are required to uncover the likely broad range of EV functions in the brain.

### 5 | BRAIN-PERIPHERY INTERACTION

The brain parenchyma is surrounded by protecting barriers: the BBB, the blood-cerebrospinal fluid barrier (BCB), and the arachnoid barrier. These barriers ensure a selective transport of nutrients and other molecules between the CNS



and the periphery which is crucial for brain homeostasis.<sup>112</sup> Additionally, they provide a line of protection against CNS infiltration by immune cells, bacteria, viruses, and other harmful impact from the periphery. Under various pathological conditions, the CNS barriers display a reduced integrity which leads to permeability of substances that have adverse effects on the CNS functions. Regulation of brain vascular integrity is highly complex but however seems to be partly controlled by EV-mediated signaling between neurons and brain vascular endothelial cells through influencing expression of the junctional protein VE-cadherin.<sup>113</sup> Evidence accumulates that EV signaling to and across the BBB or BCB participates in brain–periphery communication in health and disease (Figure 2A–C).

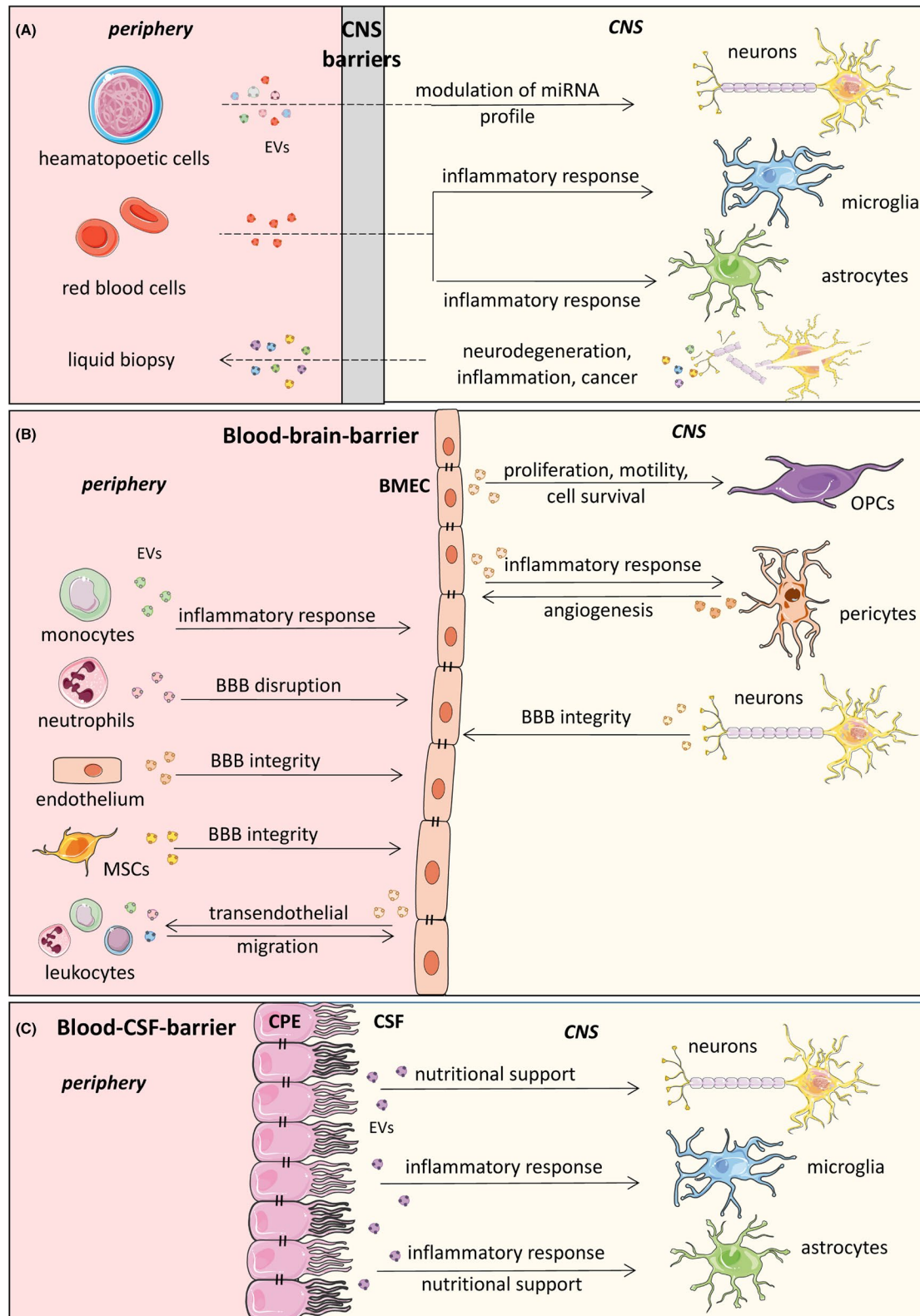
The detection of brain cell-derived EVs in the circulation has gained importance for the noninvasive diagnostics of different CNS pathologies (Figure 2A). Evaluation of the cargo of brain-derived EVs separated from plasma is used for the development of diagnostic strategies in AD,<sup>114–116</sup> PD<sup>117</sup> as well as gliomas and brain metastasis.<sup>118,119</sup> Further progress in multiparametric single EV detection technologies, for example, microfluidics or imaging flow cytometry, may help to implement circulating brain EVs as biomarkers for the real-time monitoring of disease and therapeutic effects.<sup>120,121</sup> Following CNS inflammation, astrocytes and brain endothelial cells were observed to release EVs into the circulation, which contribute to the subsequent immune response.<sup>34,122</sup> Destruction of the BBB is considered the major cause for EV release into the circulation, though the exact EV signaling routes and functional mechanisms are unknown. Possibly EVs, play a critical role in initiating the acute phase response to CNS injuries modulating systemic inflammatory events, which influence the secondary phase of injury and recovery.<sup>123</sup>

Circulating EVs are considered to be able to naturally overcome the CNS barriers and transfer information to brain cells, which seems to be promoted under inflammatory conditions (Figure 2A). EVs derived from different cell lines with the characteristics of macrophages, fibroblasts, T-cells, keratinocytes, and various cancer cell types were shown to target distinct brain regions with differing uptake kinetics also in response to inflammatory conditioning.<sup>124</sup> These observations indicate that entry to the brain might be a universal characteristic of EVs while the triggers and mechanisms of EV uptake seem to differ between EV subtypes. EVs released by cells of the hematopoietic lineage target the brain parenchyma and were shown to transfer cargo including functional Cre reporter mRNA to neurons, which was accompanied by an altered mRNA profile in the recipient cells. This signaling occurred rarely under physiological conditions but was highly elevated in response to different inflammatory stimuli or neuronal activity.<sup>100,104</sup> Likewise, inflammatory conditioning triggered human red blood cell (RBC)-EVs, which

carry  $\alpha$ -synuclein, to enter the mouse brain and target microglia where they induced a pro-inflammatory phenotype. RBC-EVs derived from PD patients further elevated the pro-inflammatory effect on microglia.<sup>125</sup> In a PD mouse model, RBC-EVs transporting  $\alpha$ -synuclein oligomers were taken up by the processes of astrocytes, where glutamate uptake was negatively affected, indicating that RBC-EV-mediated accumulation of  $\alpha$ -synuclein may contribute to the perturbation of glutamate homeostasis in PD.<sup>126</sup> While these observations provide profound evidence that EVs are indeed able to mediate periphery-to-brain signaling, the underlying molecular mechanisms of EV brain entry remain to be investigated.

The BBB is built by the brain microvascular endothelial cells (BMEC), which form a layer of cells lining the brain microvessels and are steadily connected by tight junction proteins limiting paracellular transport (Figure 2B). Transport of macromolecules across the endothelial barrier is facilitated by receptor-mediated transcytosis, which is also discussed as primary mechanism for EV trafficking over the BBB. BMECs are layered with pericytes, which cover the abluminal side of the endothelial cell barrier, and astrocytes, whose endfeet reach the endothelial surface. Together, these cells form the neurovascular unit and their intercellular exchange is mandatory for BBB integrity and function.<sup>112</sup> Studies directly addressing the mechanism of EV crossing over the endothelial cell layer of the BBB suggested that the EV-transport mechanism is energy-dependent and can follow dynamin-, clathrin-, and caveolin-mediated as well as macropinocytotic transcellular routes rather than paracellular diffusion.<sup>126–129</sup> Additionally, it was shown that uptake of blood-derived EVs by BMECs is, at least in part, mediated by transferrin receptor-mediated endocytosis.<sup>128</sup> More detailed studies examining the efficiency of EV transfer across the BBB in *ex vivo* models or *in vivo* upon genetic manipulation of candidate molecules regulating barrier functionality are required to unravel the different mechanisms of EV trafficking over the CNS barriers under homeostatic and inflammatory conditions.

BMECs release EVs at both the apical and basal sides of the endothelial cell layer with a wide range of potential signaling functions (Figure 2B). Proteomic analysis of BMEC-EVs identified several proteins involved in intercellular signaling processes as well as receptor-mediated transcytosis of molecules across the BBB.<sup>130</sup> Under hypoxic conditions, as emerging after traumatic brain injury (TBI) or stroke, changes in protein and miRNA cargo of BMEC-EVs were observed indicating a possible contribution to vascular remodeling.<sup>131,132</sup> Following TNF treatment, acting as a pro-inflammatory stimulus known to increase BBB permeability, the proteomic signature of BMEC-EVs was altered and included proteins involved in inflammatory signaling.<sup>133</sup> Consistently, inflammatory conditioning increased the release of BMEC-derived EVs, which were taken up by cultured pericytes and stimulated



**FIGURE 2** Brain–periphery interaction mediated by EVs. (A) EVs derived from peripheral cells enter the brain by crossing its different barriers. EVs produced by degenerating neural cells reach the circulation, utilized for liquid biopsy. Dashed lines indicate that the mechanism of transfer across the barrier is unknown. (B) EVs interacting with BMECs at the blood–brain barrier and their functions. (C) Blood–CSF barrier and effects of CPE-derived EVs on neural cells. CNS, central nervous system; BMEC, brain microvascular endothelial cell; BBB, blood–brain barrier; OPC, oligodendroglial progenitor cell; MSC, mesenchymal stem cell; CPE, choroid plexus epithelial cells; CSF, cerebrospinal fluid

an inflammatory response in the cells, possibly transmitted by miRNAs.<sup>134</sup> Next to pericytes, OPCs are potential targets of BMEC-derived EVs. Brain as well as non-brain endothelial cell-derived EVs had a beneficial effect on proliferation, motility, and cell survival of OPCs.<sup>135</sup> These observations provide a first hint of the contribution of BBB-derived EVs to maintaining brain homeostasis.

Furthermore, BMECs are targeted by EVs from the circulation acting as peripheral triggers. EVs derived from platelets, monocytes, and neutrophils can be internalized by BMECs mediating an inflammatory response (expression of cytokines and adhesion molecules) or disrupting barrier integrity.<sup>136-138</sup> Additionally, EVs seem to directly affect transendothelial migration of leukocytes in an inflammatory state into the brain. Both, BMEC-derived EVs and T-cell blast-derived EVs were observed to facilitate transmigration of leukocytes.<sup>139,140</sup> In contrast to these rather harmful pro-inflammatory effects, peripheral EVs can also contribute to the alleviation of adverse impact on the brain. Following TBI, EVs derived from non-brain endothelial cells as well as mesenchymal stem cells improve BBB integrity and lead to decreased brain swelling.<sup>141,142</sup> Similarly, bone marrow endothelial progenitor-derived EVs diminished the damage on brain endothelial cells introduced by ALS mouse plasma, modeling BBB disintegration during ALS.<sup>143</sup> These observations underline the potential of cells of the BBB to actively contribute to brain homeostasis via EV-mediated signaling, rather than only forming a limiting barrier of the CNS.

Little is known about signaling functions of pericyte-derived EVs. Brain pericytes are characterized by expression of the PDGF $\beta$ -receptor, which upon stimulation with PDGF-BB triggers the release of large EVs containing a range of pro-regenerative growth factors (BDNF, bFGF, bNGF, VEGF, and PLGF), while inflammatory stimulation resulted in higher concentrations of EV-associated cytokines.<sup>144</sup> These observations indicate that pericyte-derived EVs are involved in the regulation of CNS inflammation and neuroprotection, although the cellular targets of these EVs remain to be investigated. Moreover, pericyte EVs may play a critical role during hypoxic conditions. In response to HIF pathway activation, EV signaling stimulates the wound healing activity of endothelial cells, likely by promoting angiogenesis.<sup>145</sup> In addition, pericyte EVs could be involved in the pathological processes leading to the development of hypertension. EVs released by brain pericytes of spontaneous hypertensive rats exhibit an altered miRNA profile compared to those of normotensive rats, including enhanced abundance of miRNAs critical for developing hypertension.<sup>146</sup> These findings indicate that pericyte EVs may considerably contribute to CNS vascular maintenance and regeneration.

EVs also contribute to the cellular signaling from the periphery to the CNS via the BCB (Figure 2C). In a model of nutrient transport over the BCB, it was shown that folate

can be transported from blood to brain via internalization by receptor-mediated endocytosis in choroid plexus epithelial cells (CPE) followed by package into intraluminal vesicles, which are released into the CSF as exosomes.<sup>147</sup> These exosomes are then transporting folate from CSF across the ependymal cell layer to the interstitial fluid where they are taken up by astrocytes and neurons. In response to systemic inflammation, the formation and release of EVs from CPE are increased.<sup>148</sup> These EVs contain pro-inflammatory miRNAs, which deliver a pro-inflammatory stimulus to microglia and astrocytes. Furthermore, CPE EVs seem to be involved in the manifestation of disease states. CPE infected with JC polyomavirus release EVs carrying virions which target and infect astrocytes.<sup>149</sup> Intriguingly, CPE can be preconditioned by EVs derived from acute lymphoblastic leukemia cells to allow lymphoblast entry into the brain without altering the barrier function.<sup>150</sup> These findings underscore the importance of EV-mediated periphery-to-brain signaling at the BCB in addition to the BBB.

Conclusively, EVs emerge as versatile elements mediating cross talk between brain barrier cells, peripheral cells, and brain cells. Selective CNS import and export of EVs appears to be modulated by inflammatory conditions or injury. However, the molecular mechanism utilized by EVs to cross these barriers and the factors regulating the transfer remain to be determined.

## 6 | BIOMEDICAL IMPLICATIONS AND CONCLUSION

Progress in the understanding of EVs in nervous system physiology identifies EVs as local modulators of various homeostatic processes in the CNS. Neural EVs preferentially act in areas of high neuronal electrical activity where homeostasis is challenged. They affect neurotransmission, regulate synaptic plasticity, promote axonal maintenance, or modulate immune and inflammatory responses. Defining the functional cargo of neural EVs will be a major task in the future. Since EVs appear to deliver their homeostatic signals across the CNS barriers, EVs are eligible for CNS regenerative therapies and could serve as vehicles for drug delivery to the brain.<sup>151</sup> Stem cell therapies involving mesenchymal stem cell-derived EVs or NSC-EVs, which address neural cells directly in the CNS domain or can even act indirectly in the periphery by modulating the inflammatory response, are being explored and already revealed promising results.<sup>152,153</sup> Further understanding of the basic concepts of EV target cell interaction and the molecular pathways employed by EVs to favor CNS homeostasis will be key to the successful implementation and translation of these therapies. Recent technological advances in *in vivo* imaging and tracking of EVs as well as transgenic mouse models interfering with EV release are providing valuable tools to further resolve the impact of

EV-dependent cellular cross talk in the brain and across its borders.

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## CONFLICT OF INTEREST

The authors declared that no conflict of interest exists.

## AUTHOR CONTRIBUTIONS

EMKA drafted the manuscript. All authors were involved in writing and editing of the manuscript.

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