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Expert Opinion on Drug Delivery

ISSN: 1742-5247 (Print) 1744-7593 (Online) Journal homepage: www.tandfonline.com/journals/iedd20

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To cite this article: Naznin Bhom, Khonzisizwe Somandi, Poornima Ramburrun & Yahya E. Choonara (2025) Extracellular nanovesicles as neurotherapeutics for central nervous system disorders, Expert Opinion on Drug Delivery, 22:1, 69-84, DOI: 10.1080/17425247.2024.2440099

To link to this article: https://doi.org/10.1080/17425247.2024.2440099

Published online: 10 Dec 2024.

EXPERT OPINION ON DRUG DELIVERY
2025, VOL. 22, NO. 1, 69–84
https://doi.org/10.1080/17425247.2024.2440099

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REVIEW

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Extracellular nanovesicles as neurotherapeutics for central nervous system disorders

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ABSTRACT

Introduction: The blood-brain barrier (BBB) is a highly selective structure that protects the central nervous system (CNS) while hindering the delivery of many therapeutic agents. This presents a major challenge in treating neurological disorders, such as multiple sclerosis, where effective drug delivery to the brain is crucial for improving patient outcomes. Innovative strategies are urgently needed to address this limitation.

Areas covered: This review explores the potential of extracellular vesicles (EVs) as innovative drug delivery systems capable of crossing the BBB. EVs are membrane-bound vesicles derived from cells, tissues, or plant materials, offering natural biocompatibility and therapeutic potential. Recent studies investigating the permeability of EVs and their mechanisms for crossing the BBB, such as transcytosis, are summarized. Special emphasis is placed on plant-derived EVs (PDEVs) due to their unique advantages in drug delivery. Challenges related to the large-scale production and therapeutic consistency of EVs are also discussed.

Expert opinion: EVs, particularly PDEVs, hold significant promise as scalable and noninvasive systems for CNS drug delivery. However, critical barriers such as improving standardization techniques, manufacturing processes and addressing scalability must be overcome to facilitate clinical translation. Collaborative efforts in research and innovation will be pivotal in realizing the therapeutic potential of EVs for neurological conditions.

ARTICLE HISTORY
Received 17 September 2024
Accepted 5 December 2024

KEYWORDS
Blood brain barrier; central nervous system; extracellular vesicles; nanovesicles; plant-derived extracellular vesicles; transcytosis

1. Introduction: the blood brain barrier

The treatment of central nervous system (CNS) disorders remains a major global health challenge. CNS disorders comprise a group of disorders that affect the brain and spinal cord tissues [1]. Depending on where they manifest in the CNS, these disorders impair cognitive-motor function, disrupt the blood-brain barrier (BBB), and damage the neurons [2]. The Global burden of Disease (GBD) Report 2016 emphasized the importance of 18 major neurological disorders including stroke, migraine, Alzheimer's, epilepsy, and dementia, amongst others, contributing to GBD, as listed in Table 1 [2,3]. CNS disorders cause millions of deaths worldwide due to the existing gaps in drug treatment not directly entering deeper structures of the brain tissue as a result of the BBB [4]. Other factors include invasive methods of drug delivery, patient noncompliance due to the route of administration, or imprecise drug-to-receptor targeting.

Treating CNS disorders poses significant challenges because of the presence of the BBB, consisting of brain endothelial cells (BECs), pericytes, and astrocytes, as shown in Figure 1. This barrier serves to maintain the supply of oxygen and nutrients to the brain while preventing the entry of potentially harmful substances through tight junctions [4,6,7]. An array of specific transporters, receptors and enzymes controls the molecular traffic via the transcellular route to permit passage of certain nutrients and allow removal of waste products [5]. The integrity of the BBB is disrupted in several CNS disorders such as ischemic stroke, Alzheimer's disease, multiple sclerosis, and Parkinson's disease [8]. For example, in multiple sclerosis, the BBB integrity is disrupted by environmental or genetic factors which leads to invasion of inflammatory cells causing the protective sheath around the neurons to wear away and cause lesions in the brain. Thus, the integrity of the BBB is important to protect the brain. The disruption of the BBB leads to changes in tight junction and increased vesicular traffic which results in BBB leakage. BBB leakage results into increased inflammation and spread of the disease (Figure 1) [5].

The BBB interface allows small, lipid-soluble drugs (<400 Da) to permeate into the brain via lipid-mediated diffusion, whilst maintaining cellular communication with vascular tissues through paracellular diffusion or transcytosis [9]. A major problem with many drugs is the high molecular weight which prevents the drug from permeating through the BBB, for example: monoclonal antibodies do not cross the BBB due to its large molecular weight (Table 1). With several other requirements, another example consists of molecules with positive charges and low hydrogen bonding [10].

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Article highlights

- Extracellular vesicles (EVs) are a heterogeneous group of membrane-bound vesicles which are an emerging form of nanocarrier.
- EVs can be derived from different mammalian and plant sources such as cells, biological specimens, vegetables, fruits, plants or milk.
- EVs are researched for permeating the blood brain barrier (BBB) and have successfully shown to permeate the BBB due to their lipophilic membrane.
- Extraction of mammalian-derived EVs raises prolonged debate of the ethical implications of using tissues and cells. Other disadvantages include cell cultivation and cell expansion and associated costs and availability.
- Plant-derived EVs may potentially offer a unique strategy to cross the BBB however, it is a niche area of study due to lack of standardization methods of extraction and quantification.

To overcome the BBB, and improve the bioavailability of drugs, several strategies have been researched including noninvasive and invasive methods [11].

Noninvasive techniques include modifying the therapeutic agent, incorporating the drug into nanoparticles/liposomes, nose-to-brain drug (NtB) delivery systems and ultrasound techniques used for opening the BBB for drug delivery [12–14]. On the contrary, invasive techniques include brain implants and intraosseous drug administration into the skull [15,16]. These techniques have their own advantages such as sustained therapeutic release of the drug from nanoparticles, improved drug distribution in the brain through nose-to-brain drug delivery, and precise identification of injury location through brain implants/patches [17–19]. However, both the noninvasive and invasive techniques have clinical limitations such as toxicity of nanoparticles to cells; smaller absorption area in the nasal cavity for NtB drug delivery; higher risk of infections due to invasive surgical procedures; and hemorrhagic damage and transient blood-supply shortage due to the focused-ultrasound treatment for BBB permeation [20–24]. These techniques still need further preclinical research, such as *in vivo* and toxicology studies, to establish safety and efficacy before clinical implementation.

Other strategies include modification of drugs to overcome receptors such as adenosine triphosphate (ATP)-binding cassette transporter (ABCs) and influx mediators such as solute carrier family transporters (SLCs) [25]. Solute carrier proteins grant access to essential molecules consisting of amino acids and nucleic acids [10]. Hence this allows bimolecular phenolic compounds to cross the BBB. Nanomaterials, such as extracellular vesicles (EVs), can be derived from these phenolic compounds that interact with the SLCs to gain access to the BBB.

Extracellular vesicles (EVs) are a heterogenous group of membrane-bound vesicles with size ranges of 30–150 nm [26]. They originate from intracellular multivesicular bodies (MVBs) and are released by fusion of the MVBs with the cell membranes. EVs consist of different proteins, nucleic acids, lipids, and metabolites enclosed by a lipid membrane [27,28]. EVs are known to produce therapeutic effect through communication with other proteins, transporters, or receptors [29]. EVs also have the potential to function as therapeutic nanocarriers for drugs that work via receptor mediated endocytosis on the BBB interface [30,31].

Table 1. Summary of central nervous system disorders.

CNS Disease	Mechanism of disease	Diagnosis	Treatment	Why treatment needs modification?	Reference
Alzheimer's Disease	Accumulation of amyloid-ß plaques and intracellular neurofibrillary tangles leading to neuronal cell death.	Medical history, physical examination, cognitive assessments, and imaging tests.	Cholinesterase inhibitors, Memantine, Selective serotonin reuptake inhibitors (SSRIs) treat cognitive symptoms. Mono clonal antibodies slow disease progression.	Limited efficacy of the drug to the target site, provides symptomatic treatment instead of targeting disease, monoclonal antibodies are too large to cross BBB.	[48]
Parkinson's Disease	Progressive neuronal loss in the substantial nigra resulting in continual decrease in dopaminergic function.	Imaging tests, medical history and symptom review, full blood count, autoimmune screening, genetic testing.	Dopamine agonists, Monoamine oxidase (MAO) & Catecholamine 0-methly transferase (COMT) inhibitors.	Produces neurological adverse effects due to increased dosage of orally administered drug.	[32]
Multiple Sclerosis	Autoimmune demyelinating disease caused by an overactive immune system of the CNS.	Imaging tests, evoked potentials of the CNS circuits, CSF abnormalities	Skeletal muscle relaxant, benzodiazepines and anticonvulsants, disease-modifying therapies.	Treatment administered orally causes peripheral side effects and has limited benefits.	[36]
Epilepsy	Chronic brain disorder in which groups of nerve cells send the wrong signals and cause seizures.	Medical history, imaging and monitoring epilepsy, blood tests, and developmental, neurological and behavioral tests.	Antiseizure medicines	Severe side effects due to orally administered drug, and drug resistance	[28]
Glioblastoma	Malignant tumor cause by an abnormal cell growth	Signs and symptoms including size and location of tumor.	Surgical resection followed by radiotherapy and concomitant and adjuvant chemotherapy.	Immunosuppression caused by radiotherapy targeting normal healthy cells.	[48]
Traumatic Brain Injury	Damage to brain tissue caused by external mechanical force.	Symptoms, neurological exam, imaging tests	Individualized treatment from therapy to hospital admission.	Complexity of disease pathophysiology, BBB challenge, and lack of targeted therapeutic options.	[14]

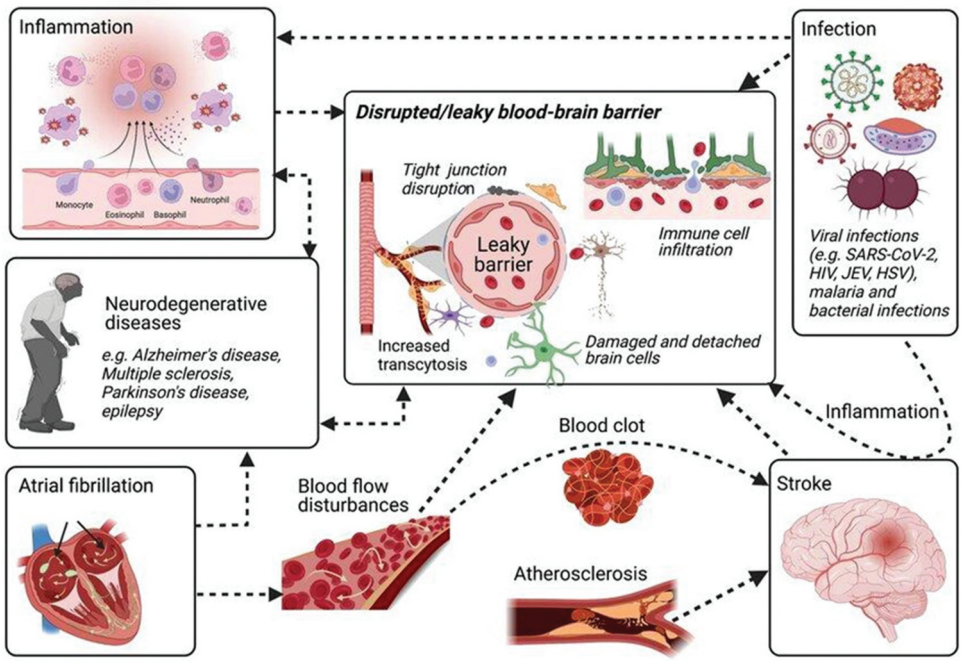

Figure 1. Structure of the blood-brain barrier (BBB) outlining the effects of central nervous system (CNS) diseases on the BBB structure (adapted and reproduced with permission from © Creative Commons CC by 4.0. Copyright 2023, Portland Press) [5].

This article provides a concise overview of the strategies investigated to enhance the permeation of neuroactive drugs across the BBB with emphasis on extracellular nanovesicles (EVs) as a trend for drug transport across the BBB. In addition, plant and mammalian derived EVs as co-therapeutics/carriers to treat CNS disorders are also discussed to guide further research development in this niche area.

2. Current neurotherapeutic strategies for drug transport across the BBB

2.1. Brain implant or patch

Brain implants have increasingly been used for neurological disorders and diseases. Malignant brain tumors are difficult to treat as the tumor cells can survive surgical reduction and radiation therapy resulting in tumor recurrence. Lee *et al.* investigated a biodegradable electronic patch (BEP) administered intracranially via a surgical procedure on mice, as shown in Figure 2(1), to release anti-cancer drugs directly in the brain. The BEP is made of flexible, adhesive, and biodegradable materials which locally deliver controlled release of anti-cancer agents. An advantage of biodegradable materials is that they dissolve within the human body, thus eliminating the need for invasive removal [32]. The drug release mechanism operates through introducing change in temperature of the patch, forcing it to open or close. This proposed system, however, still needs to be further investigated through longer term studies to observe the effect of hydrolyzed materials on the patch [33].

Other examples include silk microneedle (SMN) patches to deliver multiple therapeutic drugs for glioblastoma treatment and nitric oxide probes for permeation of the BBB [34,35]. These delivery methods are effective, but disadvantages of these techniques may include migration of the probe or implant leading to compromised efficacy, glial scarring, and increased risk of infection [36]. A common concern of brain implants is the introduction of a foreign object in the body which induces a foreign body reaction (FBR) – an often-ignored side effect that necessitates strategies to avoid FBR [37]. FBR has been known to surround the implant resulting in a highly mechanically stressed tissue environment which reduces patients' quality of life and non-adherence to treatment [38]. Other limitations include the lack of deep tissue penetration of such therapeutics, leakage of the drug into the cerebrospinal fluid (CSF) resulting in adverse neurological complications or mechanical mismatch of the patch with brain tissue [39].

2.2. Nano-enabled systems and nose-to-brain drug delivery

Nano-enabled systems include liposomes, micelles, and polymeric nanoparticles which are made of materials that possess dimensions ranging from 10 to 100 nm in size with a distinct 3D functional compartment, as shown in Figure 2.3 [40–43]. Nanoparticles (NPs) provide a multipurpose system for loading a wide range of drugs whilst increasing the bioavailability of the drug, as shown in Figure 2(d) [11]. Multiple ways have been investigated for nanoparticles to cross the BBB such as through paracellular or transcellular pathways [48]. *In vitro* studies have shown that curcumin loaded polymeric nanoparticles results in reduced oxidative stress and inflammation in Alzheimer's disease. A study conducted by Muresan *et al.* investigated a patch containing a microneedle array and a layer loaded with polymeric NPs for the local delivery of chemotherapeutics [49]. In this study, the NPs were effective

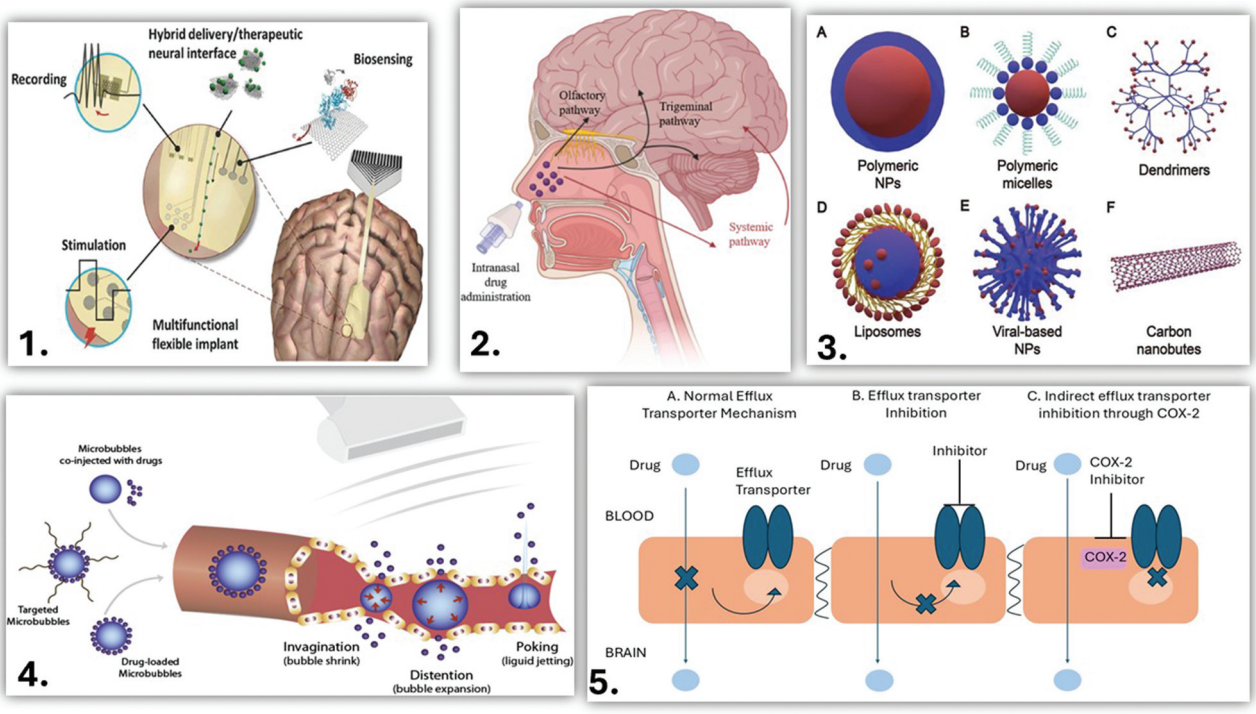

Figure 2. 1. Bioelectronic patch for brain drug delivery (reprinted and adapted with permission from © creative commons CC by 4.0. Copyright 2019, Wiley), 2. Nose-to-brain drug delivery (reprinted and adapted with permission from © RightsLink. Copyright 2023, Elsevier B.V. Ltd), 3. Types of nanocarriers for drug delivery (reprinted and adapted with permission from © Creative Commons CC by 4.0. Copyright 2021, MDPI), 4. Combination of focused ultrasound and microbubbles in the opening of the blood brain barrier (reprinted and adapted with permission from © RightsLink. Copyright 2020, Elsevier B.V. Ltd)), 5. Role of efflux transporters and inhibitors in BBB permeation [44–47].

in delivering cannabidiol (CBD) and olaparib, however, a burst release of these drugs was detected in the brain via *ex vivo* technique. NPs have also been investigated in combination with other strategies to cross the BBB.

Nose-to-brain drug delivery provides an alternative route to enhance drug delivery to the brain by bypassing the BBB, as shown in Figure 2.2. In NtB drug delivery, the drug is absorbed via two routes: 1) through respiratory epithelial cells into the blood circulation which needs to first cross the BBB to reach the target site; and 2) via the olfactory epithelium which directly enters the CNS through the trigeminal nerve pathway. Compared to other routes of administration, NtB drug delivery has been reported to increase the effective dose to the brain, while being noninvasive and bypassing first pass metabolism [50,51]. A recent study incorporated rivastigmine loaded lipid-polymer hybrid NPs for nose-to-brain drug delivery via a nasal gel to treat Alzheimer's disease [52]. The NP loaded gel was a success in terms of drug release showing sustained drug release for up to 3 days and increased bioavailability which was proven through an improvement in maximum concentration (C_{max}), half-life and mean residence time (MRT) [52].

Due to the unique physicochemical properties of the NPs, it is important to assess toxicology of nanoparticles as they may induce immunological response of cells to nanoparticles. The toxicological reports vary; however, it has been established that synthetic NPs exert an immunological response in the body [21]. In another study conducted by Semyachkina-Glushkovskaya and coworkers, a combination of the use of liposomes and photodynamic therapy (PDT) was tested as therapy for glioblastoma [53]. PDT is a mini-invasive procedure that allowed greater accumulation of the liposomes in the target site through the opening of the BBB – suggesting a 'least-toxic tool' compared to nanoparticles that are administered peripherally and cause greater adverse effects.

Liposomes made of polyethylene glycol (PEG), a type of nanoparticle as shown in Figure 2.3, were investigated for their delivery to the brain through the intranasal route using the chemical exchange saturation transfer (CEST) approach to enable image-guided NtB drug delivery in a noninvasive manner [51]. This study concluded that the mucus penetrating property of the PEG liposomes could facilitate the uptake of drug from the nasal mucosa to the brain bypassing the BBB. Vincristine nano emulsions in combination with a brain guider, borneol (BOR), were administered intranasally for nose to brain drug delivery to enhance drug distribution in the cortex for Alzheimer's disease [54]. The incorporation of BOR in the nano emulsion led to a significant drug distribution in the brain, – a method that overcomes the challenge of poor drug distribution when administered via the intranasal route. The NtB method is promising when used in conjunction with other drug delivery methods. An example of a promising drug delivery method in combination with NtB is a mucoadhesive powder formulation with a dedicated nasal device, tested in monkeys, which showed a higher distribution and uptake of the drug in the brain [55].

As much as nose-to-brain drug delivery bypasses the BBB exposing the brain to the treatment, it is necessary to address several important limitations such as low targeting efficiency

directly to the brain, drug distribution across different brain regions, the physiological condition of the nasal cavity and several others [56].

2.3. Inhibition of efflux transporters in the BBB

Drug delivery to the brain is hindered by the efflux transporters that exist on the BBB, as shown in Figure 2(c). Tournier *et al*. investigated the strategies to inhibit the ABCB1 (ATP binding cassette subfamily B member 1) and ABCG2 (ATP binding cassette subfamily G member 2) efflux transporters for the treatment of brain metastasis cancer with tyrosine kinase inhibitors (TKI) [57]. ABCB1 and ABCG2 are two adenosine triphosphate-binding cassette (ABC) transporters which prevent drug distribution to the brain [58]. The concomitant administration of the ABC inhibitors and TKIs resulted in improved drug delivery to the brain *in vivo*. Another study was done on the ABCB1 and ABCG2 efflux transporters for the penetration of olaparib both *in vitro* and *in vivo*. It was found that concomitant treatment with temozolomide (ABC inhibitor) and chemotherapeutic agent olaparib resulted in increased brain distribution of the drug [59]. A recent study used a derivative of probenecid to inhibit efflux proteins and increase cellular accumulation of vinblastine which is known to treat different types of cancer. The probenecid derivative selectively promoted apoptosis of cancer cells. However, inhibition of efflux proteins is insufficient to resolve multidrug resistant issues and may involve inhibiting other SLCs [60]. However, long term effects of ABC inhibitors have not been investigated which might result in brain penetration of potentially harmful agents and pharmaceuticals [61]. ABC transporters are the crucial gatekeepers of the brain which prevents CNS toxic drugs to pass through the brain. Another notable effect that needs to be considered is the reversibility of the process (inhibition of the efflux transporters) and the long-term impact on brain toxicity.

2.4. Focused ultrasound and microbubbles

Ultrasound is a noninvasive imaging technique which is mainly used to make a diagnosis or to perform therapeutic procedures. Research has shown that ultrasound has beneficial effects on the neurons and microglial physiology without damaging the tissues involved (Figure 2.4) [62]. A study conducted on rat brain endothelial cells (RBE4) depicted that the BBB was stimulated by ultrasound and was shown to induce a reversible increase in intercellular spaces which can allow drugs to pass through the brain while under the effects of ultrasound. However, this method is not yet well known and further research is required on the long-term impact of ultrasound [14]. Another study concluded that transcranial focused ultrasound resulted in transient BBB opening with microbubbles administered systemically in patients diagnosed with Alzheimer's disease. Microbubbles are gas-liquid emulsions with a gaseous core surrounded by a biocompatible shell made of phospholipids, proteins and polymers as shown in Figure 2.4. This needs to be further investigated on the effect of ultrasound on the compartment changes in the brain and needs preclinical and clinical studies for delivering neurotherapeutic agents that may be used to promote brain health [62]. Thus, the combination of microbubbles and focused ultrasound seem promising. Other reported research focused on encapsulating doxorubicin in microbubbles and using focused ultrasound for drug delivery. This method opened the BBB, and doxorubicin was released directly into the brain tumor – maximizing the therapeutic efficacy for glioblastoma treatment [7]. These studies highlight the substantial potential for clinical translation when focused ultrasound is used in combination with other methods of drug delivery through the BBB.

3. An overview of extracellular nanovesicles and it's potential in neurotherapeutics

Nanocarriers are currently extensively studied due to their small size and the capability of carrying bioactive compounds. Extracellular vesicles are a group of membrane-bound vesicles with size ranges of 30–150 nm (Figure 3) [26]. They are naturally found in cells, tissues, biological specimens, or botanical therapeutics [64–67]. EVs go back several decades to the 1980s when they were referred to as 'micro-vesicles' or 'exosomes' [68,69]. They originate from intracellular MVBs and are released by fusion of the MVBs with the cell membranes. EVs consist of different proteins, nucleic acids, lipids, and metabolites enclosed by a lipid membrane (Figure 3) [27,28]. The inward budding of the exosomal membrane results in the presence of membrane proteins [70]. They are secreted through exocytosis of a cell, as shown in Figure 3, and they have gained popularity in the research industry due to the lipophilic membrane that encapsulates RNA, lipids, vacuoles, and proteins. EVs can produce a therapeutic effect as was demonstrated by Mas-Barques *et al*. who used EVs from human dental pulp stem cells (hDPSCs) to assess the antioxidant activity of the hDPSC EVs. The study shows that EVs can increase cell proliferation, migration, survival rate and inhibition of apoptosis, thus promoting wound healing and tissue repair [71]. Another study was done to confirm the neuroprotective effect of clonal mesenchymal stem cell (cMSC) EVs in traumatic brain injury (TBI). The study confirmed that the EVs entered the primary neurons and provided protective effects by improving amelioration of tauopathy [72].

Interest in EVs have increased over the years as it is progressively researched as potential biomarkers [31,73]. EVs are also known to produce a therapeutic effect through communication with proteins, transporters, or receptors on the BBB interface [29]. Some conditions that have been investigated for therapeutic EVs are pre-eclampsia, musculoskeletal disorders, colorectal cancer, and several others [29,74,75]. A first-in-human trial done on platelet-derived EVs (pEVs) as a potential therapeutic showed that pEVs administered to healthy volunteers in phase I clinical trials healed wounds rapidly, however further exploration is required in future clinical trials [76].

The lipid bilayer structure offers EVs the ability to encapsulate drugs [77]. This approach was used by Guo and coworkers who loaded glioblastoma cell-derived small EVs (GBM-sEVs) with DOX to suppress glioblastoma and the results revealed that GBM-sEVs-DOX could significantly inhibit the growth of solid tumors in nude mice after continuous intravenous injection for 7 days. GBM-sEVs-DOX enhanced suppression in GBM cell growth compared to DOX [78]. Another method involving

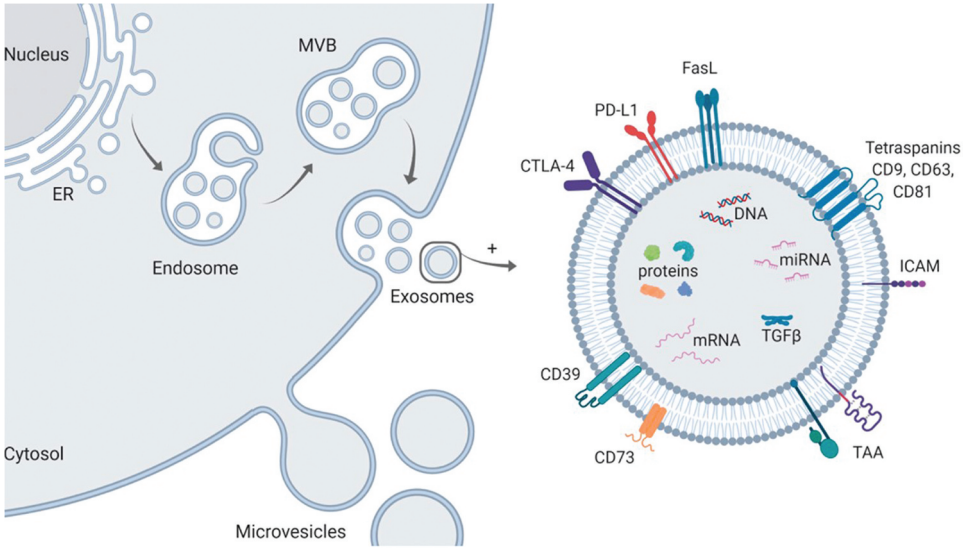

Figure 3. Schematic representation of EV biogenesis and molecular cargo. EVs are formed through inward budding of the endosomal membrane resulting in the formation of multivesicular bodies (MVB). Upon fusion of MVBs with the plasma membrane, exosomes are released in the extracellular space. In contrast, microvesicles are formed by simple budding of the plasma membrane. The molecular cargo of EVs consists of proteins, miRNA, mRNA, DNA, and lipids. On their surface, they carry the tetraspanins CD9, CD63, and CD81, commonly referred to as 'EV markers,' adhesion molecules (e.g. intercellular adhesion molecule ICAM) and – in case of tex – tumor-associated antigens (TAA), which are specific to the cell of origin. Further, the presence of immune suppressive proteins such as CTLA-4, PD-L1, fas-l, CD39, CD73, and TGFβ in EVs have been reported. (reprinted and adapted with permission from (adapted and reproduced with permission from © Creative Commons CC by 4.0. Copyright 2020, MDPI) [63].

EVs as nanocarriers is the use of plants to derive EVs. A large study was conducted using citrus fruits namely lemon, orange, tangerines, and grapefruit, which were used to derive EVs and were loaded with doxorubicin functionalized with heparin. An *in vitro* BBB model showed that the EVs could bypass the BBB and target the glioma tissues. It also exhibited excellent anti-tumor immunity by triggering secondary necrosis [79].

EVs show a great potential for targeting brain disease and neurological conditions by overcoming the BBB. In addition, it carries therapeutic properties imparted by the origin of the EVs: mammalian cells or plants. The composition of the EVs depends on the source of the cell from which it was isolated. They function by mediating intercellular communication through mRNAs and miRNAs [26]. Some examples of EVs extracted from biological tissues or cells include cardiac-progenitor-cell-derived EVs, brain endothelial cell-derived EVs, and urinary-derived EVs [30,80,81].

EVs that function as therapeutic nanocarriers for drug delivery work via receptor mediated endocytosis [30]. Other roles of EVs includes angiogenesis (formation of new blood vessels), cell death, receptor-ligand signaling, regulation of gene transcription and translation, and tumorigenesis (transformation of normal cells into cancer cells) [66,82]. Compared to other nanomaterial, EVs are naturally occurring, have higher biocompatibility, prolonged half-life in blood circulation, low to no immunogenicity, lower toxicity and faster penetration into tissues [27,30,82]. EVs can penetrate biological membranes without inducing an immunological response due to their natural compatibility which makes them different to liposomes with immunogenic synthetic components and coatings [83]. The immunogenicity of EVs can range from non-immunogenic to specifically immunogenic against diseased cells [84]. Current research is focused on the standardization of terminology, extraction, and isolation methods to improve experimental reproducibility of EVs [85].

EVs are known to inherit the properties of the cells they originate from. A study comparing EVs derived from two different cells namely, human neonatal fibroblast cell line (HDFn) and SH-SY5Y cells assessed the impact of cell origin on cellular uptake. It was proved that the presence of like proteins between EVs and target cells increased the cellular uptake of EVs by those specific target cells. An improved understanding of the molecular mechanisms that influence cell targeting must be established to develop more sophisticated and biomimetic drug delivery tools [86].

Mammalian EVs are extracted from cells, bacteria, fungi, biological specimens, or tissues (Figure 4(a)) [80,87–90]. The production process of cell based EVs occurs in two phases where cells are firstly cultured and thereafter placed in a medium for EV production. There are several limitations associated with mammalian extracellular vesicles, such as generating a sufficient number of cells *in vitro* or through biological fluids [91]. EVs derived from cells are extensively researched in the fields of cancer therapy, brain injury and gene therapy, to name a few.

One study reported a cell-derived nanocarrier of empty breast tumor cell-derived EVs loaded with TPP/P53 to induce cell death through P53-mediated endogenous apoptosis for the treatment of breast cancer [92]. Mammalian EVs are not only used for cancer but other disorders as well, such as traumatic brain injury; neuropathic pain; gene therapy; desiccation-induced dry eye, and ovarian cancer [72,93–96].

Research using mammalian EVs is drastically growing due to their intrinsic therapeutic potential, but is it ethically feasible and cost-effective? Many questions arise regarding EVs derived from cells. Flamant *et al.* described the challenges

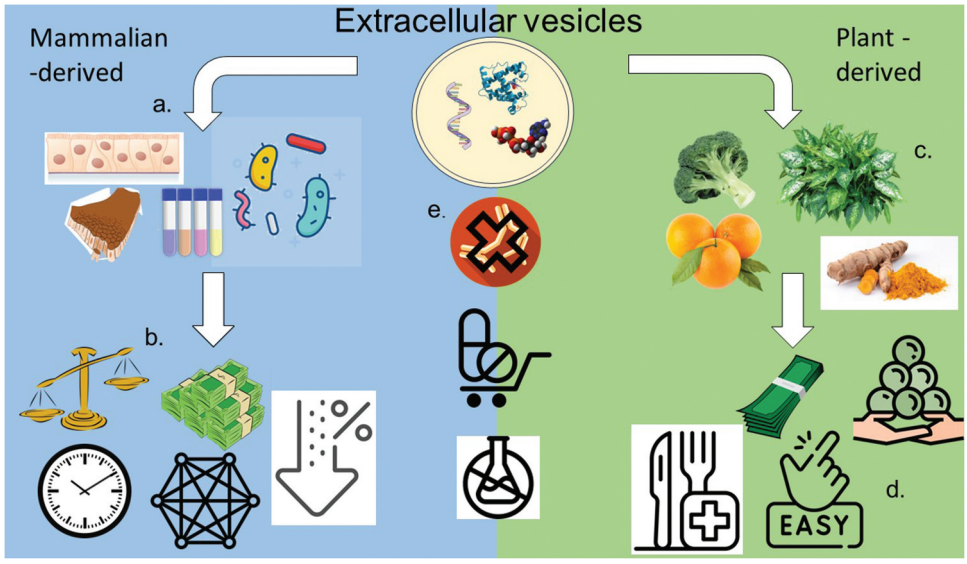

Figure 4. Comparison of mammalian-derived EVs and plant-derived EVs. (a) Mammalian-derived EVs can be extracted from cells, bacteria, biological specimens, or tissues. (b) The use of mammalian EVs raises matters such as ethical aspects, high costs but low yield, complex and time-consuming methods. (c) Plant-derived EVs include extraction from medicinal plants, herbal plants, and food sources such as fruits or vegetables. (d) Plant-derived EVs are easier to produce, turn food into medicine, resulting in a higher yield with low costs. (e) Both plant and mammalian-derived EVs produce low immunogenicity, low toxicity, and can be used in drug delivery.

associated with MSC-derived EVs in terms of the large amount of cell production and the high costs associated with production. Another challenge is the bone marrow collection from where the MSCs are harvested which involves donors, an invasive medical procedure and elevated hospital costs [97]. There is no doubt that mammalian EVs have a broad clinical application, however, there are several disadvantages associated with its application and clinical translation.

In contrast, plant-derived EVs (PDEVs) are structurally similar to mammalian EVs. Plant-derived EVs include isolating EVs from roots, vegetables, fruits, flowers, seedlings etc. Plenty of research has been done on plant-derived EVs with different approaches as a therapeutic and as a nanocarrier. Plant-derived EVs are nontoxic, safe for humans and can modulate cellular processes and cell communication [98,99]. Plant-derived EVs are preferable to those obtained from human or plant cell cultures because the large-scale synthesis of EVs via cell culturing is expensive and time-consuming [100]. Plant-derived EVs may be a promising candidate for use as nanocarriers because of their natural biomimetic composition, non-immunogenic properties, and potentially cost-effective production with higher yields, as summarized in Figure 4 [101].

The use of plant raw materials is an ideal strategy in the pharmaceutical field as they naturally possess useful physicochemical, biological, and pharmacological properties for potential therapeutic applications. An example of PDEVs include cannabis-derived EVs (C.EVs) for the treatment of hepatocellular carcinoma (HCC), where the C.EVs were exposed to two HCC cell lines and was revealed to decrease the viability of the two liver cancer cell lines with no effect on normal cells [102]. Similar effects could be investigated for CNS diseases using botanical and medicinal plants. A feature that may be different in plant-derived EVs in comparison to mammalian-derived EVs is the inner secondary metabolite content and other phytocompounds that the plant carries which could function as potential therapeutic agents [102]. In comparison to mammalian-derived EVs, plant-derived EVs do not have ligands for cell targeting [103].

EV engineering methods can be employed to resolve the targeting specificity issue. Li *et al* engineered ginger-derived EVs (GDEVs) to feature a ligand arrow tail RNA for cell targeting in the assessment of tumor-suppression abilities of the GDEVs. It was proven to be a biocompatible delivery system when administered through the oral route [103]. Some of the routes of administration of PDEVs include the oral route, which is highly preferred, and it is expected for PDEVs to have a high gut tolerance due to its natural origin. Secondly, IV injections which are unavoidable in some cases, such as monoclonal antibodies for TBI (listed Table 1), have also been studied but is reported to cause higher toxicity. Another route of administration is intranasal administration which is encouraged due to its direct connection to the brain, and transdermal delivery which is a good candidate for skin infections or melanomas [104].

4. Extracellular vesicles as a novel strategy for permeating the BBB

Penetrating the blood-brain barrier (BBB) and delivering drugs to the brain at therapeutic levels is a major challenge in treating many CNS diseases. Finding a delivery system that is nontoxic and allows for maximum effectiveness is important in treating CNS disorders. Currently, the utilization of EVs in nanomedicine is gaining popularity because of their ability to potentially penetrate the blood-brain barrier (BBB). EVs consist of lipids that facilitate penetration through various biological membranes. A study by Wang et al. utilised turmeric-derived exosome-like nanoparticles enriched with lipids such as 51.2% digalactosyl diacylglycerol, 12.6% monogalactosyl diacylglycerol, 10.7% phosphatidylcholine, 11.1%

phosphatidylinositol and 12.7% phosphatidic acid for improved permeation across the BBB [104]. Thus, lipophilic EVs of nanoparticulate size showed promising penetration through the BBB compared to microvesicles. The amphipathic framework of EVs allow the loading of both hydrophilic and lipophilic drugs, mRNAs, and several other bioactives, however, the exact mechanism for drug loading into EVs is not fully understood [105]. Methods used to load the EVs with drugs include sonication, incubation, electroporation, transfection, freeze-thaw cycles, or a combination of either listed methods [64,106–110]. The targeting ability of EVs is dependent on the collection of proteins including both cytosolic and membrane proteins, as shown in Figure 3 [105]. Discussed below are examples of EVs derived for the treatment of brain disorders or investigated for permeating the BBB.

Table 2 provides a summary of the examples of EVs for BBB permeation. Research is not only limited to these few papers, but there is plenty of ongoing research on the use of EVs to treat brain disorders due to their ability to permeate the BBB. EVs do not only offer their lipophilic properties for a potential drug delivery system, but low immunogenicity, biocompatibility and low cytotoxicity as well [80,111].

4.1. Mammalian-derived EVs used in CNS disorders and BBB permeation

As listed in Table 2, several mammalian-derived EVs have been investigated for the ability to cross the BBB interface. An example of mammalian EVs for the treatment of CNS disorders include EVs derived from clonal mesenchymal stem cells (cMSC) [72]. MSCs can cross the BBB which makes it a potential delivery strategy for CNS diseases [119]. In one study, clonal MSCs were therefore used to derive EVs loaded with antibodies to treat traumatic brain injury. The CMSC-EVS were confirmed to provide protective effects in neurons that were exposed to nutrition deprivation stress [72]. The study also stated that the EV biodistribution in the brain is influenced by their cell source, route of administration, and the dose. Several studies have been conducted on MSC EVs; however, the feasibility of EV separation and up-scale of production is questionable.

EVs extracted from biological samples such as urine have also been investigated to cross the BBB. Bai *et al*. extracted EVs from urine (U-EVs) collected from healthy people and used it to assess the BBB permeation [112]. The U-EVs were loaded with platinum and resveratrol nanoparticles (Res-Pt @EVS) with some nanoparticles functionalized on the surface of the EVs. The Res-Pt@EVs were engineered with a rabies virus glycoprotein (RVG) targeted peptide. Using an *in vitro* BBB model, the study showed that engineered EVs with photothermal conversion and targeting peptides on the surface can better cross the BBB. Furthermore, the Res-Pt@EVs-RVG system demonstrated enhancements in cognitive function and reduction in glial cell activation. However, U-EVs did not exhibit therapeutic effects; instead, they served only as a nanocarrier by encapsulating the Res-Pt nanoparticles within. The multi-targeted system showed great promise of treatment of early Alzheimer's disease [112]. The need for significant engineering of the U-EVs is the limiting factor which may make it difficult to achieve complexity in the production of such a system, thus presenting a challenge for the reproducibility of the drug delivery system.

A study investigated EVs derived from neural stem cells (NSCs), medial ganglionic eminence (MGE) cells, and interneurons, each loaded with GABA for seizure treatment, demonstrating that these vesicles could deliver GABA across the blood-brain barrier (BBB) to effectively control seizures. Whilst promising, this approach has notable limitations. The extended culture periods required – 45 days for interneurons and 25 days for MGE cells, highlight the constraints of this method for scalable clinical applications, as these long timelines hinder timely EV production. Furthermore, the study relies on iPSC-derived cells, which limit them to specific cell lineages and introduces challenges in consistency and quality control [113]. Mammalian EVs are not only restricted to cells and biological specimens but also include milk fluid samples. A study done by Zhou *et al*. used bovine milk derived EVs (BEVs) to assess the BBB permeability of the EVs. The study showed that orally ingested BEVs tested on pups crossed the BBB and accumulated primarily in the brain. BEVs were also shown to alter gene expression and promote neuronal growth in the brain [114]. The study seemed to promote the notion of using EVs derived from human milk in infant feeding formulas to promote neurological function, however this raises the ethical aspects of using human milk to derive EVs.

EVs derived from human plasma were also investigated for permeating the BBB using blood samples collected from anonymous healthy volunteer [115]. The results from the study conducted by Jakubec *et al*. showed that the plasma-derived EVs had a BBB permeability of 1.4% and retention of 88.4% of EVs which was comparable to liposomes. A BBB model was set up using porcine brain endothelial cells and rat astrocytes. The factor that contributed to the BBB permeation was the high phospholipid content of the plasma-derived EVs as proven by mass spectrometry analysis. The human plasma derived EVs showed BBB permeability similar to liposomes but unlike liposomes, it accumulated in the brain endothelial cells. This research provides a strategy which could be further investigated in drug delivery systems for exploiting the high accumulation potential of EVs in brain endothelial and other neural cells [115].

Engineering EVs with surface decoration of BBB-targeting ligands have been studied for the treatment of glioblastoma. The study introduced the combination of brain endothelial cell derived EVs (bEnd.3 – EVs) and photodynamic therapy (PDT) by loading a photosensitizer (TPP-Ce6) in the EVs resulting in mitochondrial damage of the cancer cells [30]. The results showed that TPP-Ce6 accumulated in the mitochondria for effect and the bEnd.3-EVs significantly boosted the aqueous stability of TPP-Ce6 and was able to effectively target the brain tumor after efficient BBB permeation. This study offers a new system of using a combination of cell derived EVs with PDT to target glioblastoma, however more insights are needed to implement it clinically [30].

Table 2. Extracellular vesicles investigated for CNS disorders.

Extracellular vesicles	Source	CNS disease	Mechanism of treatment	Outcome	BBB Permeation	References
Mammalian derived extracellular vesicles	Urine	Alzheimer's disease	EVs were engineered and loaded with rabies virus glycoprotein, resveratrol, and platinum nanoparticles to target the brain and increase BBB permeation	Little effect on the proliferation of neural cells.	Yes	[112]
	Human neural stem cells (NSCs), medial ganglionic eminence (MGE) cells, and GABAergic interneurons (INs)	Seizures	EVs were loaded with GABA to assess BBB permeability and decrease in seizure intensity.	Decrease in seizure *in vivo* with improvements in cognitive behaviour	Yes	[113]
	Clonal mesenchymal stem cells (Cmsc)	Traumatic brain injury	The Cmsc-EVs has neuroprotective effects *in vitro*. It was administered intravenously and there was an improvement in the cognitive function.	Yes, the neuronal cells showed no difference after 96 hours of exposure.	No data available	[72]
	Milk	BBB permeation	The study suggests that milk-derived small EVs cross the BBB and deliver messages that alters gene expression and promote neuronal growth in the brain.	No data available	Yes	[114]
	Human plasma	BBB permeation	EVs were isolated from human plasma and were able to permeate the BBB however, they accumulate in the endothelial cells which provides a higher contact time resulting in greater therapeutic outcome.	No data available	Yes	[115]
	Bend.3 (Brain endothelial cells	Glioblastoma	Bend.3 EVs were used in combination with PDT to treat GBM.	92% cells retained viability.	Yes	[30]
Plant derived extracellular vesicles	Grapefruit	Brain tumor	Antiproliferation, bypasses the BBB and enhances glioma-targeting ability of DNs	Yes	Yes	[116]
	Onion	Alzheimer's disease	Inhibits inflammation by regulating the NF-kB signaling pathway	90%	No data available	[100]
	Momordica charantia	Glioblastoma	Prevents proliferation of glioma cells and inhibits glioma growth and metastasis by penetrating the BBB	Yes, no proliferation detected on neuronal cells	Yes	[117]
	Ginseng	Glioma	Increased apoptosis rate of C6 glioma cells compared to a negative control group.	Proliferation of C6 cells did not decrease after treatment.	Yes	[118]

4.2. Plant-derived EVs for CNS disorders and BBB permeation

PDEVs are considered potential alternatives to synthetic liposomes or nanoparticles. In recent years, studies have begun to investigate the therapeutic potential of EVs derived from plants, as mentioned earlier – plants possess CNS therapeutic effects which can be an added benefit to the ability of crossing the BBB. Discussed below are a few examples of PDEVs research studies that have shown a CNS effect or has crossed the BBB, as outlined in Table 2.

Grapefruit-derived EVs were studied to treat cancer and to assess the permeability of the EVs [116]. The grapefruit-derived EVS were surrounded by doxorubicin loaded nanoparticles (DN) which provided a high drug loading capacity per microgram of EVs and efficient glioma-targeting ability. The integrated system crossed the BBB and showed maximal brain tumor uptake via receptor-mediated transcytosis and membrane fusion, therefore providing anti-glioma efficacy *in vivo* [116]. Functionalizing the DN on the surface of grapefruit-derived EVs provided a novel approach to the design of EV-based drug delivery systems. The study proved that grapefruit-derived EVs efficiently bypassed the BBB due to its natural cellular uptake ability. Interestingly, the study showed that isolated pristine grapefruit-derived EVs permeated the BBB to a greater extent compared to grapefruit-derived EVs functionalized with DNs due to the EVs unique intracellular uptake ability. The grapefruit-derived EVs had better αvβ3 receptor-mediated transcytosis effect than DNs which contributed to their ability in crossing the BBB [116].

Onion-derived extracellular vesicles (Onex) were investigated for anti-inflammatory effects on RAW 264.7 macrophage cells. Inflammation is a prevailing element in many brain-related illnesses, with the study focusing specifically on Alzheimer's disease as one of these conditions. Onex vesicles inhibited inflammation by restraining the pro-inflammatory cytokines and disrupting the signaling pathway [100]. The study also showed the effect of long-term preservation of Onex vesicles; there was no significant size difference between the fresh and preserved Onex vesicles, however the concentration of Onex vesicles significantly decreased which depicts that the Onex vesicles deteriorated with time. A long-term preservation method of extracellular vesicles would include the use of preservatives such as metal oxides, or surface modifications through coupling reactions of peptides, proteins or other molecules [100,120,121].

Momordica charantia (MC) is an herbal plant that is used as traditional folk medicine for stroke treatment, and it is known to have neuroprotective effects. MC-derived extracellular vesicles-like nanovesicles (MCELNs) were investigated to enhance the therapeutic efficiency of MC as it has poor affinity for brain tumor tissues due to the BBB. The MCELNs were dyed and injected intraperitoneally into a mouse xenograft model to assess the BBB permeability; it was shown via system imaging that the MCELNs cross the BBB and accumulate at the tumor tissue. MCELNs prevented proliferation of the cancer cells resulting in inhibited glioma growth and metastasis [117].

5. Different pathways for delivery of EVs through the BBB interface

It is known that EVs can cross the BBB due to its lipophilic properties. However, the mechanism of EV permeation through the BBB is not fully understood and requires further studies. It is proposed that endocytosis may be a mechanism used for EV permeation. Endocytosis may include different types of pathways including receptor-mediated endocytosis, lipid raft-mediated endocytosis, or micropinocytosis as shown in Figure 5. However, this requires extensive investigations to fully understand the mechanisms. In recent research that used brain endothelial cell-derived EVs (bEnd.3 EVs) loaded with

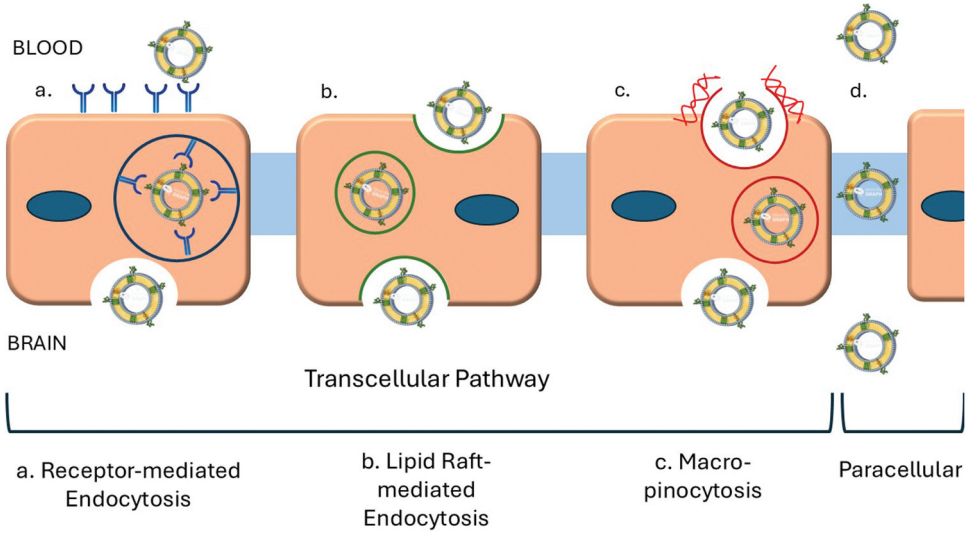

Figure 5. Potential mechanisms of extracellular vesicles crossing the BBB. a. Receptor-mediated endocytosis – is an active targeting method, in which ligands bind to a specific type of receptor found on the endothelial cells. b. Lipid raft-mediated endocytosis – is the use of lipid rafts to move molecules/proteins from the cell surface to the intracellular space. c. Macropinocytosis – is a form of fluid-phase endocytosis in which cells use ruffles of the membrane to take up the molecules into the endocytic vesicles. d. Paracellular diffusion – is a passive process that occurs when substances pass through the intercellular spaces in-between the endothelial cells.

TPP-Ce6 for the treatment of glioblastoma, an *in vitro* study was done with bEnd.3 and U87MG cells labeled with a deep red-fluorescent dye which determined if the bEnd.3-TPP-Ce6 could enter cells via the endocytic pathways. The results showed that most of the bEnd.3 EVs were colocalized with the deep red-fluorescent dye indicating that they entered the cells via endocytosis. Most of the bEnd.3 EVs were localized in the mitochondria [30]. An *in vitro* BBB model was used by Shan *et al*. to detect the BBB transcytosis ability of the EVs. EVs isolated from macrophages loaded with panobinostat and siRNA effectively transversed the BBB *in vitro* through transcytosis [122].

In another recent study, the permeability of engineered EVs namely acid-cleavable transferrin decorated engineering exosome (ACTE) was used to study the transcytosis effect using an *in vitro* BBB permeability model where bEnd.3 cells were seeded with GL261 cells to mimic the BBB. It was proved that the cellular uptake of the engineered EVs were higher than normal EVs which suggests that EVs can effectively travel through transcytosis in the BBB [123]. You *et al*. extensively reviewed how proteins and lipids are involved in cellular communication in the brain to enhance the permeation of EVs deeper into the tumor tissue. Proteins such as albumin have been shown to bind to receptors on the BBB and the abundance of transferrin on the BBB has been proven to be a good source to extract EVs to enable BBB penetration. In addition, the presence of glucose transporters makes it a great candidate to mimic glucose-like EVs to permeate the BBB [124]. There are numerous studies done on mammalian EVs proving BBB permeability through transcytosis, however, the consideration for the presence of proteins and integrins essential for cellular communication are not thoroughly investigated.

Plant-derived EVs have also been proved to cross the BBB as shown in Table 2, however there are very few studies outlining the exact mechanisms of BBB permeation. One study investigated the mechanism of BBB permeation of ginseng-derived exosome-like nanoparticles (GEN) by using a series of inhibitors of different pathways. The inhibition of pinocytosis and phagocytosis resulted in ~ 60% reduction in cellular uptake. In contrast, the inhibition of endocytosis, phagocytosis and lipid-raft mediated endocytosis (Figure 5) had no effect or slightly disturbed the transcytosis of the GEN. The blockage of the transmembrane protein of tight junctions typically involved in paracellular transport resulted in 98.2% and 91.2% cellular uptake, respectively, compared to 100% in the control experiment [118]. Although this proves that EV permeation occurs through several mechanisms, such pathways need to be investigated *in vivo* in a BBB model as well.

There is very limited information of EVs crossing through the BBB *in vivo*. A few studies have highlighted the permeation of EVs in human, mouse and Zebrafish models [125].

6. Conclusion and future perspectives

To conclude, this literature review explored the current challenges in treating central nervous system (CNS) disorders, particularly in overcoming the restrictive nature of the blood-brain barrier (BBB). Traditional strategies for BBB permeation, while valuable, often fall short due to significant limitations, including potential toxicity, limited efficiency, and lack of specificity. These challenges underscore the need for innovative approaches to safely deliver therapeutic agents across the BBB.

EVs have emerged as a promising alternative, offering inherent biocompatibility, targeted delivery potential, and the ability to cross biological barriers, including the BBB. EVs can be of plant or mammalian origin. Many studies have focused on extracting EVs and using them as co-therapeutic agents or developing them as nanocarriers for neurotherapeutics. Studies have evaluated the entrapment of various drugs, nucleic acids and biologics. Most research is focused on the mammalian EVs due to their properties such as long circulation time, homologous targeting, and immune escape, however the ethical aspect of using mammalian tissues or cells have been a prolonged debate. Other disadvantages, such as cell cultivation and cell expansion associated with the extraction of mammalian EVs, are a challenge for upscaling drug delivery systems [77,126]. Due to their biomimetic framework, the human body does not produce an immunological response to all kinds of EVs. Plant EVs exhibit distinct advantages in terms of cost-effectiveness, ease of production, and reduced risk of immune response, making them an appealing option for future drug delivery applications.

The underlying mechanisms of BBB permeation, including receptor-mediated transcytosis and vesicular trafficking, provide insights into how EVs may effectively transport therapeutic molecules into the CNS. Plant-derived EVs, in particular, hold promise for addressing the existing limitations of conventional approaches and demonstrate potential for advancing CNS-targeted therapies. Further studies are warranted to optimize their use and fully harness their therapeutic potential, making them a compelling subject for future research in the field of CNS drug delivery and bypassing the BBB.

Some hurdles in PDEV research includes the lack of standardized PDEV extraction methods, storage conditions, and stability issues. According to De las Hazas, large-scale production of PDEVs is challenging to the isolation methods showing limited scalability potential, low levels of yield and purity [64]. Several isolation methods have been employed; however, this is classed on the basis of yield, morphology, density and size of the PDEVs. Isolation methods for scalability is required to implement PDEVs clinically. Another concern is the loading method – there is no standardization on loading drugs into PDEVs to ensure dose uniformity and reproducibility. Moreover, a deep understanding of how drug loading methods could change the physical and chemical structure of EVs should be encouraged [127]. Regulatory barriers arise due to several fundamental studies and clinical trial data being unavailable and leading to uncertainty in decision-making regarding the regulatory approval processes [128]. Other limitations include limited knowledge on the surface marker proteins and how they affect the mechanism of action of the PDEVs [80]. The presence of heterogeneity and establishing uniformity in the extraction of EVs makes it difficult to produce EVs on a larger commercial scale [129].

The composition of PDEVs varies depending on the material of origin, conditions of storage, and seasonal changes of the plant. PDEVs can potentially offer a unique strategy to cross the BBB and treat many CNS disorders. PDEVs is a great but niche area of research. Botanical and medicinal plants are currently used as raw materials to extract many active pharmaceutical ingredients hence, to take advantage of these properties to extract extracellular vesicles may prove to be a major change in drug delivery systems. Future perspectives include exploring PDEVs as drug delivery carriers for crossing the blood-brain barrier (BBB) due to their low immunogenicity, cost-effectiveness, higher yield, and potential to convert botanicals into neuroactive delivery vehicles. However, extensive research is still needed to standardize PDEVs.

7. Expert opinion

Extracellular vesicles (EVs) present a novel and promising approach for treating central nervous system (CNS) disorders that are currently limited by the permeation of the blood-brain barrier (BBB). The BBB restricts the entry of therapeutic agents, but EVs, owing to their nanoscale size, lipid membrane, and inherent therapeutic properties, are an ideal candidate that bypass the BBB, hence enabling targeted drug delivery and additional therapeutic effects. EVs consist of natural components, which subsequently contribute to minimal immunogenicity, resulting in low or negligible toxicity, a crucial advantage for clinical applications that seek to avoid synthetic materials and instead utilize more biocompatible nanovesicle structures.

Despite the promising potential, EV research is currently constrained by challenges in standardizing extraction, bioactive loading, and characterization methods. Overcoming these obstacles is essential for achieving reliable and reproducible results, which would not only enhance our understanding of EVs but also optimize their therapeutic applications. While mammalian-derived EVs are already advancing into clinical trials, issues related to ethical considerations, extraction time, and resource availability have hindered their scalability and widespread clinical success.

Plant-derived extracellular vesicles (PDEVs) are emerging as a viable alternative, offering advantages such as renewable availability and complex biocompatible structures, that have the capacity to carry bioactive molecules across the BBB. This offers a greener and more cost-effective approach yet requires extensive research to optimize and standardize extraction and bioactive loading protocols. Given the rapid evolution of the EV field, continued exploration is essential, not only for CNS disorders but also for improving treatments for diseases with low bioavailability concerns. The potential of EVs to transform therapeutic strategies is vast, making ongoing investigation critical to unlocking their full clinical application.

Funding

This research was funded by the National Research Foundation (NRF) of South Africa SARChI Grant UID 64814.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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