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REVIEW ARTICLE

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Most recent advances and applications of extracellular vesicles in tackling neurological challenges

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Abstract

Over the past few decades, there has been a notable increase in the global burden of central nervous system (CNS) diseases. Despite advances in technology and therapeutic options, neurological and neurodegenerative disorders persist as significant challenges in treatment and cure. Recently, there has been a remarkable surge of interest in extracellular vesicles (EVs) as pivotal mediators of intercellular communication. As carriers of molecular cargo, EVs demonstrate the ability to traverse the blood–brain barrier, enabling bidirectional communication. As a result, they have garnered attention as potential biomarkers and therapeutic agents, whether in their natural form or after being engineered for use in the CNS. This

Abbreviations: 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BBB, blood–brain barrier; CM, conditioned media; CNS, central nervous system; CSF, cerebrospinal fluid; DA, dopaminergic; DMSO, dimethyl sulfoxide; ECs, endothelial cells; ESCRT, endosomal sorting complexes required for transport; EVs, extracellular vesicles; FUS, fused in sarcoma; hADSCs-exo, human adipose-derived stromal cell exosomes; hASCs, human adipose stem cells; hBM-EPCs, human bone marrow endothelial progenitor cells; HD, Huntington's disease; IFNγ, interferon-γ; ILVs, intraluminal vesicles; IN, intranasal; ISEV, International Society for Extracellular Vesicles; IV, intravenous; KI, knock-in; mASCs-exo, murine adipose stromal cells; mBEC, mouse brain endothelial cell; MCAO, middle cerebral artery occlusion; mHtt, mutant huntingtin; MNs, motor neurons; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; MSCs, mesenchymal stem cells; MVBs, multivesicular bodies; NSCs, neural stem cells; PBS, phosphate-buffered saline; PD, Parkinson's disease; PEG, polyethylene glycol; REST, RE-1-silencing transcription factor; RRMS, relapsing-remitting MS; SC, stem cell; SHEDs, stem cells from human exfoliated deciduous teeth; SNpc, substantia nigra pars compacta; SOD1, superoxide dismutase one.

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review article aims to provide a comprehensive introduction to EVs, encompassing various aspects such as their diverse isolation methods, characterization, handling, storage, and different routes for EV administration. Additionally, it underscores the recent advances in their potential applications in neurodegenerative disorder therapeutics. By exploring their unique capabilities, this study sheds light on the promising future of EVs in clinical research. It considers the inherent challenges and limitations of these emerging applications while incorporating the most recent updates in the field.

KEYWORDS
biomarkers, central nervous system, extracellular vesicles, neurological disorders, therapeutics

1 | INTRODUCTION

The burden of neurological disorders on global health is significant and represents one of the leading causes of death.[1] Around 100 million people in the United States were affected by at least one of the neurological disorders in 2011[2] and nearly 10 million deaths were reported in 2019 due to neurological disorders.[3] The burden of neurological disorders is increasing and showing variations in the trends across the United States.[4] Currently, one in six people have been diagnosed with a neurological disorder worldwide.[5] Various neurological disorders such as amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), multiple sclerosis (MS), Parkinson's disease (PD), stroke, and Alzheimer's disease (AD) affect a large percentage of the aging population with no effective treatment options constitute a significant challenge for the clinical research. Despite the extensive research, improved diagnostic and therapeutic efficacy for central nervous system (CNS) diseases is urgently needed to design novel therapeutic strategies. Extracellular vesicles (EVs) are small, cargo-bearing, bilayer lipid membrane-enclosed structures involved in EV-mediated signaling.[6] EVs are gaining recognition in all significant nervous system cells, including neurons, astrocytes, oligodendrocytes, and microglia.[7] In nervous system pathologies, the dual role of EVs—either promoting physiology or causing pathology—makes them interesting to study and to overcome clinical challenges. This dual role suggests that EVs are essential regulators of neuronal health.[8] EVs' composition, quantity, and size show significant changes in the pathological state, which can support the development of disease biomarkers for specific diagnosis.[9] EVs are highly heterogeneous and currently classified based on the mechanism of biogenesis and size released by donor cells in the extracellular environment. The main EV subtypes are—exosomes, microvesicles, and apoptotic bodies. Once released, the EVs can be internalized via endocytosis or membrane fusion to release the contents into recipient cells.[10] As EVs are small "cell-free" products, pathological transformation tumors or thrombosis generation is limited. Due to the lack of immunoreactivity, EVs are considered biocompatible and critical mediators of neuroimmune crosstalk.[11] This positions EVs as highly promising therapeutic entities for addressing neurological disorders. The realm of research exploring the physiological roles of EVs in CNS diseases is vast and continuously expanding. Despite the acknowledged functional integrity of EVs, their significance in the CNS requires further elucidation through extensive research to surmount the existing

treatment challenges. This review concisely delineates translational approaches and the utilization of EVs in addressing evolving neurological challenges.

2 | EV

2.1 | Background

EVs are the small spherical and heterogeneous population of bilayer lipid-binding vesicles released into the extracellular environment, which function in intercellular communication.[6,10,12] They bear many biological functions and are implicated in several pathologies. EVs also have tremendous potential as biomarkers, therapeutic agents, and vehicles for therapeutic molecules.[13] Initially, EVs were considered debris secreted from cells. They are now recognized as more than just waste carriers because of their involvement in complex cell-to-cell communication mechanisms. EVs are important messengers of intercellular communication via the transfer of proteins, nucleic acids, lipids, and microRNAs (miRNAs). They can be isolated from diverse circulating body fluids or biofluids, including cerebrospinal fluid (CSF), blood plasma, saliva, urine, breast milk, semen, and bile.[14-18]

2.2 | General biology of EVs

EVs are nanoscale membrane particles derived from the biological system and released by almost all cells.[19] The combined term 'EV' was coined to describe these membranous particles, which were subsequently assigned different names reflecting their mode of biogenesis, functional characteristics, or morphology.[8] EVs are divided into three widely known subgroups based on the mode of biogenesis—(a) exosomes (30–150 nm); (b) microvesicles known as shedding vesicles or ectosomes (MVs, 100–1000 nm); and (c) apoptotic bodies or apoptosome (1–5 μm).[10,20] Table 1 provides a summary of the properties of EVs. The two most widely studied and characterized EVs are exosomes and microvesicles because these are released from the living cells involved in functions like proliferation and differentiation.[21] In the literature, the nomenclature is inconsistent, and the term 'EV' is often used as an umbrella term to encompass exosomes, microvesicles, and apoptotic bodies.[22]

2.2.1 | Exosomes

Exosomes are generated in a process from the endosomal system and are formed as intraluminal vesicles (ILVs) in the multivesicular bodies (MVBs) (Figure 1). This involves double invagination of the plasma membrane.[23,24] Endosomes are divided into early, late, and recycling endosomes.[25] The first invagination of the plasma membrane leads to the formation of early endosomes, which either fuse with endocytic vesicles for recycling/secretion/degradation or transform into late endosomes. The late endosomes accumulate ILVs formed by the inward budding of endosomal membranes containing proteins, nucleic acids, and lipids. Late endosomes containing multiple ILVs are called MVBs. MVBs are formed by inward invagination of the endosomal limiting membrane, the second invagination. This process results in MVBs containing several ILVs, the future exosomes. These MVBs can either fuse with lysosomes for degradation or fuse with the plasma/cellular membrane to release exosomes (ILVs).[23] The formation of ILVs involves two processes: (1) reorganization of the endosomal membrane to enrich tetraspanins[26] and (2) recruitment of endosomal sorting complexes required for transport (ESCRTs).[27] The ESCRT-dependent pathway is considered a key player for exosome biogenesis, but an ESCRT-independent pathway, namely the syndecan-syntenin-ALIX (ALG-2 interacting protein X) pathway, also exists for exosome biogenesis.[28]

TABLE 1 Key characteristics of EVs.

EVs	Diameter	Density	Morphology	Cellular origin	Origin	Content	Acronyms
Exosomes	30–150 nm	1.13–1.19 g/cm^3	Homogenous and cup shaped	MVBs	Endocytic pathway	Proteins, lipids, metabolites, and nucleic acids	Prostasomes
Microvesicles	100 nm–1 μm	Not well defined	Heterogenous and irregular	Plasma membrane	Budding	Proteins, lipids, metabolites, and nucleic acids	Ectosomes
Apoptotic bodies	1–5 μm	1.16–1.28 g/cm^3	Heterogenous and irregular	Plasma membrane	Blebbing	Nuclear fractions and cell organelles	Apoptotsomes

Abbreviations: EVs, extracellular vesicles; MVBs, multivesicular bodies.

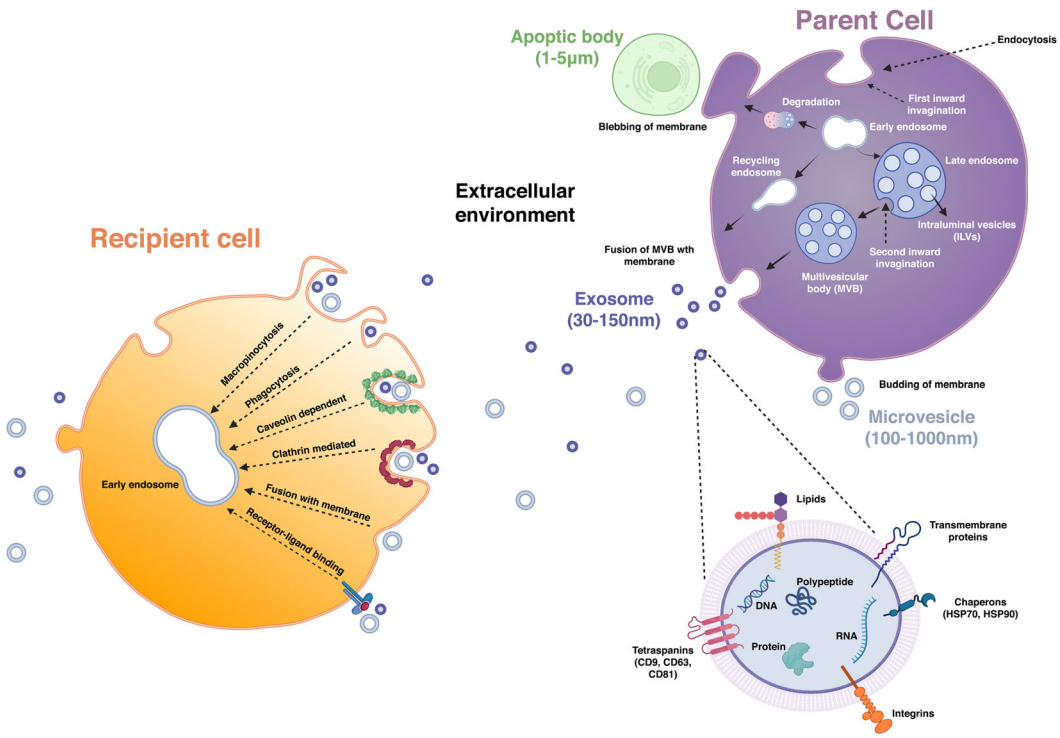

FIGURE 1 Biogenesis and modes of release of EVs into the extracellular environment. [Color figure can be viewed at wileyonlinelibrary.com]

2.2.2 | Microvesicles

Microvesicle biogenesis occurs via the plasma membrane's direct outward blebbing and fission (Figure 1).[29] The generation of microvesicles begins with the recruitment of cytoplasmic proteins and nucleic acids by ESCRT-dependent and independent pathways. Membrane blebbing is accompanied by factors such as vertical redistribution of phospholipids and contraction of actin-myosin machinery modulating membrane curvature and rigidity.[30,31] The microvesicles signify a unique mechanism of formation compared to the exosomes and apoptotic bodies because of the regulated release of microvesicles containing specifically enriched molecular cargoes.[32] Microvesicles represent a more heterogeneous population showing more sensitivity to external stimulation than exosomes.

2.2.3 | Apoptotic bodies

Both normal and diseased cells undergo a common mechanism of programmed cell death. Apoptosis initiates with condensation of the nuclear chromatin, followed by membrane blebbing and disintegration of the cellular content into distinct membrane-enclosed vesicles termed apoptotic bodies or apoptosomes (Figure 1).[33] These bodies are generally large and characterized by organelles. However, the apoptotic bodies are also considered important drivers in tissue regeneration and disease prevention.[34]

2.2.4 | EV release

The parent cell releases EVs into the extracellular environment. EVs navigate around the cells, intercellular junctions, nearby tissues, and remote organs for intercellular communication through blood, CSF, or lymph.[35] The functional or biological property depends upon the proteins and lipids present on the EV surface of the parent and recipient cells. The parent cells interact with recipient cells via direct fusion and internalization via endocytic pathways (phagocytosis, clathrin-mediated and caveolin-dependent endocytosis, macropinocytosis) (Figure 1).[36] After internalization, the recipient EVs can fuse with the endosome membrane and become ready to release cargo or degrade.[37] For proper vesicle docking and uptake by recipient cells, tetraspanins and integrins play an important role.[38]

2.2.5 | EV cargo

EVs are naturally loaded with proteins, lipids, and nucleic acids called EV cargo or EV content, depending on the type of parent cell (Figure 2). These contents differ depending on the mode of biogenesis, size, physiological/pathological state, and cell type. Due to extensive research in the EV cargo field, various data sets from different EV studies are available on online databases—Vesiclepedia, GTEx, ExoRBase, Exocarta, EV-TRACK, and exRNA Atlas.[39–44] This serves as a reference for future research.

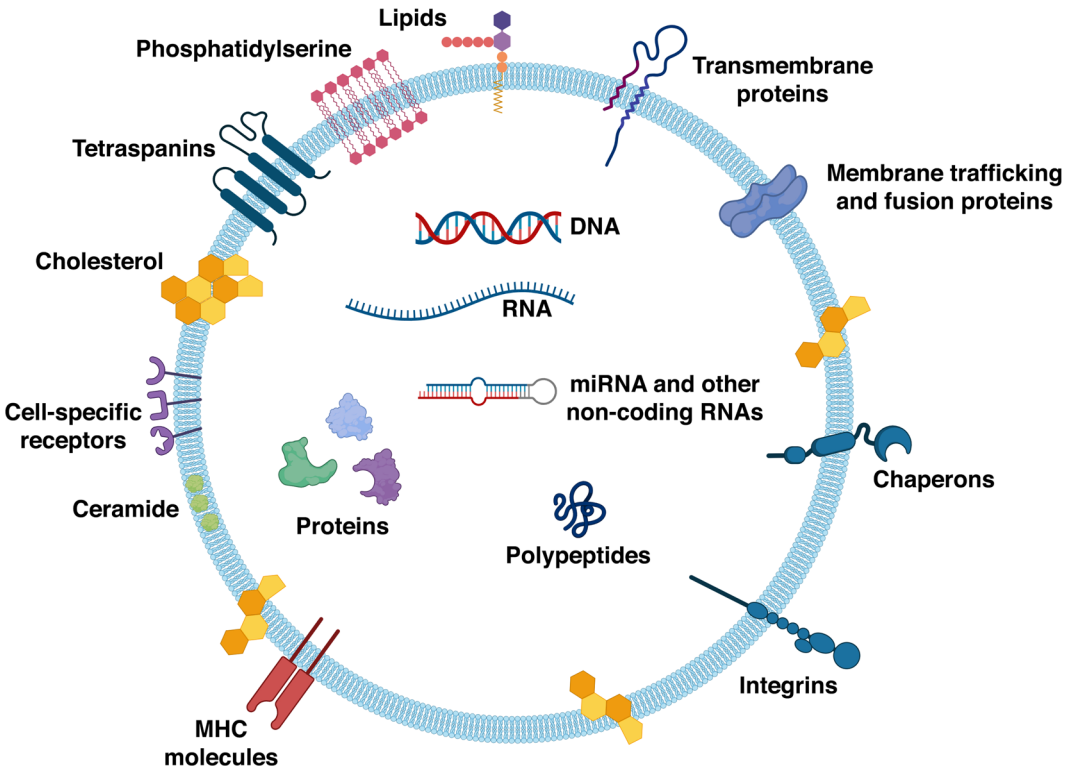

FIGURE 2 Schematic representation of EVs content. MHC, major histocompatibility complex; miRNA, microRNA. [Color figure can be viewed at wileyonlinelibrary.com]

EVs are enriched with a wide range of protein content, including transmembrane, membrane-associated, and luminally loaded soluble proteins, which are associated with mechanisms responsible for biogenesis.[45] The various tetraspanins (neither enzyme-linked nor catalytically active receptors) are CD9, CD37, CD63, and CD81.[46] Also, EVs carry ESCRT-related proteins: ALIX, TSG101, and syntenin[47]; EVs formation and release proteins: RAB27A, RAB11B, and ADP ribosylation factor 6; scaffolding transmembrane proteins: flotillin-1, flotillin-2, interleukin (IL)-6R, T-cell receptor, chimeric antigen receptor, notch receptors; molecular chaperons such as Hsp20, Hsp70, and Hsp90[48]; and other proteins like epidermal growth factor receptor, major histocompatibility complex (MHC)-I, MHC-II, and LAMP-1.[49-53] Cytosolic protein, actin, and tubulin are also sorted into EVs.[54] The lipid content of MVBs-derived EVs is enriched with sphingomyelin, cholesterol, phosphatidylserine, desaturated lipids, ganglioside GM3, and ceramide.[55] MVBs contain more phosphatidylserine than cellular plasma membranes. Therefore, lipid composition differences can reflect EVs' biogenesis, originating from either MVBs or the plasma membrane.[56,57] The most diverse content found in EVs is nucleic acids. EVs contain both coding and noncoding RNA, messenger RNA (mRNA), transfer RNAs, miRNAs, small nuclear RNAs, small nucleolar RNAs, mitochondrial RNAs, piwi-interacting RNAs, Y RNAs, circular RNAs, ribosomal RNAs, and long noncoding RNAs.[45] RNAs are packaged within a lipid bilayer, which protects them from RNase digestion. EV-associated DNA includes genomic double-stranded DNA, single-stranded DNA, mitochondrial DNA, and viral DNA.[58-61] The potential importance of EV-associated DNA is often underestimated and needs to be fully characterized. While it is widely accepted that EVs carry DNA cargo, the research focus on EVs' DNA has shifted. EVs' nucleic acids are potential biomarkers for the diagnosis of many diseases.

2.3 | Biological samples of EVs: Collection, handling, and storage

Before analyzing EVs, the pre-analysis phase, including collection, handling, and storage, is thought to affect the segregation, content, and functions of EVs.[62] EVs are present in different body fluids, as such, their origin, concentration, and composition are dependent on condition-whether physiological or pathological.[63-66] Collection and storage can, therefore, impact the EVs characteristics and the downstream characterization studies.

After collection, samples need to be handled gently. For example, centrifugation conditions can interfere with the properties of EVs by generating EV aggregates, a combination of EVs with diverse phenotypes and morphologies. It has been seen that with centrifugation, erythrocyte EVs were found as single EVs, whereas platelet EVs partly form aggregates.[67]

As per recommendations of the International Society for Extracellular Vesicles (ISEV), the EVs resuspended in phosphate-buffered saline (PBS) should be stored at −80°C in the siliconized vessels.[68] Biofluid samples are sometimes stored for short or long periods before EVs are isolated, depending upon the various conditions. Samples may also first be stored at 4°C and then frozen at −80°C,[69] which may depend upon the type of biofluid sample. For example, semen samples can be stored at −80°C without any impact on EVs for 2 years[70]; bronchoalveolar lavage fluid can be stored at −80°C for 4 days showing changes in protein composition and increased size.[71] Different studies have demonstrated that cryoprotective agents, trehalose[72,73] or dimethyl sulfoxide (DMSO),[74] are used for the prevention of cryodamage. In a systemic study by Gelibter et al.,[75] EVs were recovered from plasma using different storage conditions/times (at −80°C without any preservatives and with preservatives including trehalose, DMSO, glycerol, lyophilization, with sodium azide at 4°C) and techniques. They demonstrated that storage at −80°C results in a time-dependent decrease in EVs concentration and a significant reduction in EVs after 6 months of storage with marked implications in EVs functionality.[75] Few studies also mention no storage-dependent decrease in EVs load.[76,77] EVs stored at −80°C limit potential clinical applicability. Ideally, EVs should be isolated from fresh samples to eliminate the effects of cooling conditions. Some studies have suggested that EVs resuspended in PBS are unstable at 4°C showing low surface marker expression and changes in cargo expression, size, or number.[71,77,78]

Lyophilization is preferred for original particle size and concentration without losing cargo. Trenkenschuh et al.[79] showed that lyophilized samples are suitable at 2–8°C for 1 month. Colloidal stability can be maintained for at least 6 months using sucrose or potassium sodium buffer instead of PBS.[79] Therefore, storing EVs at −80°C or lower and avoiding repeated freeze–thaw cycles by storing samples as small aliquots is recommended.[80] Different biofluids and transportation pose challenges because of the impact on the integrity and function of EVs; alternatives like lyophilization or additives should be prioritized to improve the long-term storage stability without any implications for therapeutics.

2.4 | Different methods of EV isolation and characterization

The major limitation of the translational approach is the need for more specific markers because of the overlapping properties of exosomes and ectosomes. Therefore, there is an urgent need to develop techniques for isolation and more appropriate characterization to appraise EVs in diagnostics and therapeutics. One major challenge in the EV field is to improve and standardize methods of EV isolation and characterization.[81] Each method has its advantages and disadvantages. Clinical importance necessitates collecting the maximum amount of EVs, and to achieve the diagnostic goal, EVs should be isolated using techniques that provide maximum yield to confirm that the analyzed results are not misleading.[82]

2.4.1 | Isolation

The various commonly used EV isolation methods and their working principles with advantages and disadvantages are given (Table 2):

Ultracentrifugation

The most widely used and accepted methods are based on ultracentrifugation due to their reliability.[83] In differential centrifugation, EVs are isolated based on size and density through centrifugal force to separate the impurities from samples (cell culture media or biofluids).[84] The main advantage is an easy separation of EVs at low processing costs without extra chemicals.[85] Still, the generation of EV and non-EV aggregates, EV damage, and the need for efficient rotor types add to its disadvantages.[81,86,87] The rotors used for isolation are either swinging buckets or fixed angle. The swinging bucket swings horizontally, which is less efficient for pelleting the EVs. In fixed-angle rotors, EVs sediment against the tube wall, which first forces EVs on the walls and then to the bottom, which can lead to aggregation. Although the increased time of ultracentrifugation can result in a higher yield of EVs, it may result in the coaggregation of proteins in the pellet field.[88]

Regarding purity and yield, density gradient centrifugation isolates EVs into different layers based on the buoyant density of the solutions used in this method. The specific solutions are sucrose, iodinated gradient media, iohexol, or iodixanol.[89,90] With this method, no viral contamination is present because of pure preparation,[85] but it is laborious and time-consuming.[91] Combining density gradient centrifugation with size-exclusion chromatography to ensure improved yield and purity can reduce contamination to get highly pure EVs without interference with the ultrastructure.[92]

Size-exclusion chromatography

Size-exclusion chromatography is based on a single isolation column. The column contains porous beads with radii smaller than EVs.[93] Although EV aggregates are absent due to better separation of EVs, fast method, and high purity with preservation of integrity, the contamination with identical size particles and the need for specific equipment are limitations in its use.[94]

TABLE 2 Comparison of different isolation methods of EVs.

Isolation method	Principle	Advantages	Disadvantages
Density gradient centrifugation	Isolation of EVs into different layers based on the buoyant density of the solutions used (sucrose, iodinated gradient media, iohexol)	Pure preparation[84,85]	Laborious; time-consuming[86,87,88]
Size-exclusion chromatography	Isolation based upon single isolation column containing porous beads with radii smaller than EVs	Better separation; fast; high purity; preserve integrity[93]	Contamination with same size particles; specific equipment required[94]
Ultrafiltration	Isolation uses a porous filter of size-exclusion limits, which removes smaller molecules (non-EV) by flow-through	Multiple samples processed at the same time; no aggregates; less rupturing of EVs[95,97]	Filter plugging[98,99]
Immunoaffinity	Isolation using specific EV markers, which are bound onto a plate, bead, or chip	Purity and integrity of EV samples[100,101]	Nonspecific binding; high selectivity[102,103]
Precipitation	Isolation induces clumping of EVs in the form of pellet by decreasing the solubility	Easy protocol; less time; no additional equipment required[82]	Cost; impurities; differentiation[85]
Tangential Flow Filtration	Samples are passed through a filter membrane, tangentially at positive pressure relative to the permeate side, and EVs are collected as filtrate	Novel method; high yield; reproducible purity[112]	Contamination of proteins/lipid droplets[100]
Asymmetrical flow field fractionation	Isolation is based upon particle density and hydrodynamic properties by two perpendicular flows—forward laminar channel flow and variable crossflow	Gentle method; reproducible[113]	Low yield; time-consuming[114]
Microfluidics	Composition of two or more devices assembled into parallel operation	Fast; high throughput; single-cell sensitivity; EV morphology preservation[117,118]	Low yield
Nanoscale deterministic lateral displacement array	Size-based isolation that uses silicon processes to produce nanoscale DLD (nano-DLD) arrays of uniform gap sizes	Fast; reproducible[119,120]	Low yield; time-consuming

Ultrafiltration

The ultrafiltration or microfiltration technique uses a porous filter of some definite size with exclusion limits. It helps to eliminate the smaller molecules (non-EV) by flow-through.[95] This simple method allows multiple samples to be processed at the same time. No aggregates are formed, and fewer EVs rupture.[96,97] However, filter plugging leads to sample loss, and contamination of molecules of the same size is expected. Therefore, it is combined with another method.[98,99]

Immunoaffinity

The immunoaffinity or immunocapture of EVs relies on specific EV markers, typically monoclonal antibodies, affixed to a plate, bead, or chip.[100] This method is used after a centrifugation method.[101] It ensures the purity and integrity of the EV samples. The disadvantages are nonspecific binding, high selectivity, and difficulties with detachment and analysis.[102,103]

Precipitation

This approach is based upon commercially available kits, which induce the clumping of EVs in pellets by decreasing their solubility. It is accompanied by co-precipitation using albumin and apolipoprotein E.[104] Polyethylene glycol (PEG), lectins, and water, excluding polymers, are the commonly used agents.[105,106] Other options are the protein organic solvent precipitation method and commercially available kits such as Total Exosome Isolation Reagent (ThermoFisher Scientific), exoEasy (Qiagen), and miRCURY Exosome Isolation Kit (Exiqon).[107–110] Kits are easy to follow and are done in less time without additional equipment[82]; however, the precipitation methods have certain limitations. Commercial kits are usually costly. This method may not be suitable for large samples because of the high concentration of impurities. Moreover, the kits' components cannot differentiate between different types of EVs, making the analysis challenging.[85]

Tangential flow filtration (TFF)

TFF is a rapid and efficient technique that concentrates and filters the EVs simultaneously.[111] This method employs a sequential filtration technique whereby EV samples are tangentially passed through a filter membrane under positive pressure about the permeate side. This process allows for effective collection of EVs as filtrate.[112] Being a closed system, it gives a high yield of EVs (10–100× more) with more reproducible purity. However, TFF-isolated EVs may contain proteins and lipid droplets.[100]

Asymmetrical flow field fractionation

Asymmetrical flow field fractionation is another novel and gentle method in which isolation of EVs is based upon particle density and hydrodynamic properties by two perpendicular flows; the first is forward laminar channel flow, and the second is variable crossflow.[113] This highly reproducible technique ensures efficient separation, but the yield is low and time-consuming.[114]

Microfluidics

Microfluidic technologies are trending because these systems are composed of two or more devices assembled into parallel operations.[115] These systems have high specificity and accuracy. The methods are classified into three categories based on microfluidics: size, immunoaffinity, or dynamic categories.[116] Microfluidic-based methods are fast, have high throughput and single-cell sensitivity, preserve EV morphology, and are label-free but low-yield.[117,118]

Nanoscale deterministic lateral displacement array

Nanoscale deterministic lateral displacement array is a size-based EV isolation technology that uses silicon processes to produce nanoscale DLD (nano-DLD) arrays of uniform gap sizes ranging from 25 to 235 nm on a single chip capable of parallel processing of sample fluids. This is a promising alternative technology for fast and reproducible EV isolation.[119,120]

2.4.2 | Characterization

While methods for isolating EVs have significantly advanced, the characterization or analysis of EVs still lacks sufficient development. There is an urgent need for more consistent and reproducible EV characterization methods. EVs' integrity and efficacy are imperative because of their potential to be used in the development of drug delivery.

As per the guidelines for the minimal information required for studies of EVs (MISEV2023), it is crucial to describe the source of EVs and the EVs preparation process using the following parameters: (A) Total Protein Concentration: The concentration of total proteins in the EVs sample should be reported. This information helps assess the protein content and potential cargo within the EVs. (B) Total Particle Number: The total number of

particles in the EVs sample should also be provided. This quantification allows for a better understanding of the EVs yield and can assist in comparing results across different studies. By including the total protein concentration and particle number in the description of EVs source and preparation, researchers can provide essential quantitative data that contributes to the standardization and comparability of EVs studies. The identification EVs should include the following parameters: (1) The presence of at least one transmembrane or glycophosphatidylinositol (GPI)-anchored protein (tissue or nontissue specific); one cytoplasmic protein; purity controls include proteins found in most common major non-EV co-isolated structures; and if studying EV subtypes, proteins from proteins from intracellular compartment-associated proteins, and secreted proteins. (2) Images of single EVs are required at high resolution using electron microscopy (scanning and transmission) and related techniques, including atomic force microscopy (AFM), or cryo-electron microscopy (cryo-EM). (3) Single-particle analysis techniques for biophysical features such as resistive pulse sensing (electric field displacement) or light scattering properties—nanoparticle tracking analysis (NTA), high-resolution flow cytometry, multi-angle light scattering, or dynamic light scattering (DLS). Raman spectroscopy is valid for chemical composition measurement per ISEV guidelines (Table 3).[121,122]

TABLE 3 Established standards and characterization methods specific to EVs as per guidelines for the minimal information for studies of EVs (MISEV2023).

Characterization	Parameters	Methods	Measurement type
Total protein concentration	At least one transmembrane or GPI-anchored protein One cytosolic protein Purity controls, including major non-EV co-isolated structures EV subtypes—proteins from intracellular compartment-associated proteins and secreted proteins	SDS-PAGE, mass spectrometry, NGS	Bulk
Morphology	Single EV images at high resolution for morphological characterization	Electron microscopy (scanning and transmission), cryo-EM, AFM	Individual
Total particle number	Biophysical features—size and concentration	Flow cytometry, NTA, TRPS, multi-angle light scattering and DLS	Individual, bulk
Total cargo	Total RNA quantification, lipids, nucleic acids (miRNA, mRNA), specific RNA profiles, lipid compositions, and proteomes	Colorimetric assays, fluorescence of membrane intercalating dyes, total reflection FTIR or chromatography, Agilent Bioanalyzer, Quant-iT RiboGreen RNA Assay, Qubit miRNA Assay kit, RNA sequencing, lipidomics, proteomics	Individual, bulk
Nonprotein markers of EVs	Colocalization with protein markers	Lipid mass spectrometry, Raman spectroscopy, fluorescent probes such as membrane labels or intraluminal dyes	Individual, bulk

Abbreviations: AFM, atomic force microscopy; cryo-EM, cryo-electron microscopy; DLS, dynamic light scattering; EV, extracellular vesicle; FTIR, Fourier-transform infrared spectroscopy; mRNA, messenger RNA; miRNA, microRNA; NGS, next-generation sequencing; NTA, nanoparticle tracking analysis; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; TRPS, tunable resistive pulse sensing.

(A) Protein amount

The primary methods to quantify the total protein content of EVs can be determined by Bradford assay or bicinchoninic acid assay and fluorometric assays, but these techniques need extra purification to remove other protein contaminants.[121] Results may vary depending on whether detergent is used to disrupt EVs. Western blot analysis is a convenient method compared to other methods. The general EV markers are the tetraspanins family of proteins (CD9, CD63, and CD81).[123] Only about 30% of EVs coexpress two of these three tetraspanins. Other surface markers include ESCRT machinery proteins (Alix, TSG101, VSP40); Syntenin-1; heat shock proteins (HSP70, HSP90, HSP60); flotillin-1; and TSG101. However, not all markers must be expressed by all EVs. The search for EV markers is always ongoing and extensive.

(B) Particle number

NTA. NTA is considered one of the methods to characterize EVs. It tracks the EVs based on the Brownian motion of individual particles to measure concentration and particle size distribution.[124] The Stokes–Einstein equation is used to measure the mean velocity and calculate the size of particles. NTA is considered necessary in analyzing subpopulations of EVs, because it can track the EVs from 30 to 1 μm size.[125] The only issue with NTA is that it measures the other particles showing the same motion as EVs without any distinction.[82]

DLS. The other method that measures size distribution and zeta potential (relative stability of particles in a solution) of EVs is the DLS.[126] To determine the size and distribution, intensity changes are measured by illuminating a laser through particles with light scattering at specific angles with particles ranging from 1 nm to 6 μm.[127] It is reliable only for monodispersed suspensions and cannot distinguish between EVs and particles of the same size.

Flow cytometry. Flow cytometry is a more powerful and robust technique for the qualitative and quantitative characterization of EVs. A standard flow cytometer detects particles above 200 nm; therefore, a more sensitive flow cytometer is required to characterize smaller particles. A flow of cells is hydro-dynamically focused in a flow chamber to enable single-cell illumination by several lasers.[128]

Tunable resistive pulse sensing (TRPS). TRPS is another reliable EV size and concentration distribution method. In this method, a nano-size pore separates two fluid chambers (EV sample from an EV-free electrolyte solution. As the particles move through the nano-sized pore, the altered flow of ions is measured via a resistive pulse.[129]

Fluorescence correlation spectroscopy (FCS). FCS is based on analyzing the fluctuation of fluorescent signals resulting from the diffusion of labeled molecules moving through a confocal detection volume.[130] These fluctuations can determine particle number, size, and binding of specific antibodies to vesicles.[131]

Atomic force microscopy (AFM). AFM is another imaging technique to assess EVs' surface features and morphology in a three-dimensional space with a nanometer resolution.[132] AFM also helps to get the unique information—stiffness and elasticity of the vesicles.[133]

Microscopy. The traditional method for measuring particle size and distinguishing between EVs is scanning electron microscopy.[134] In contrast, transmission electron microscopy details the size and structure using immunogold particles specific to the EVs.[135] These methods are considered semiquantitative, and sample dehydration or vacuum procedures can affect the characteristics of EVs.[88]

2.5 | Different routes of EVs administration for therapeutics

Alongside investigating the beneficial effects of EVs originating from different cellular sources, many studies have explored strategies for the neurotherapeutic administration of EVs.[136-141] These studies have used two approaches to deliver EVs into the CNS to treat neurological disorders: local or peripheral nanovesicle administration (Figure 3).

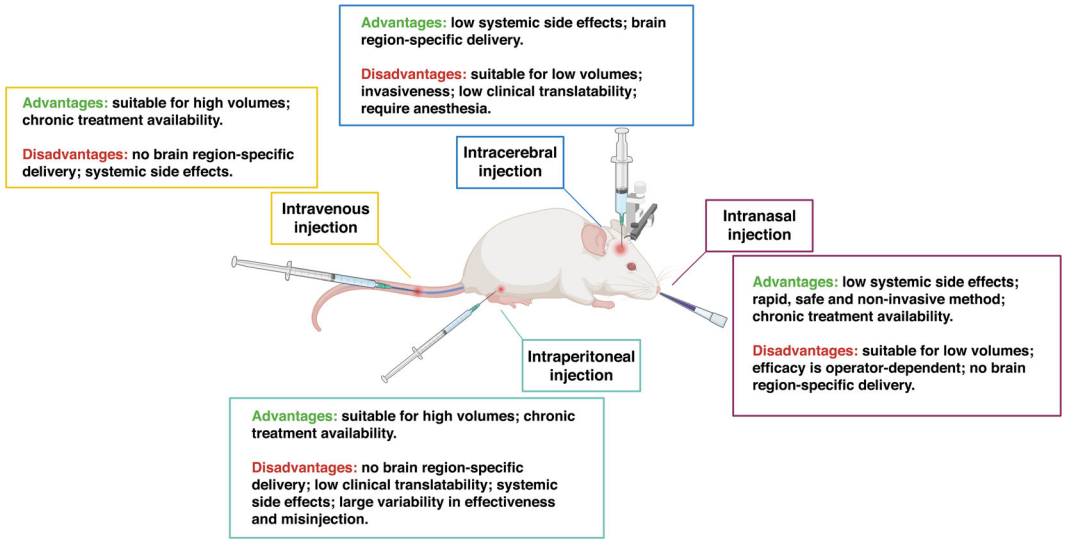

FIGURE 3 Different approaches for the delivery of EVs into the CNS. Intracerebral injection of EVs through stereotaxic apparatus represents the main local dispensation route. Peripheral routes of administration include intranasal delivery and intravenous or intraperitoneal injection. The advantages and disadvantages of each route of administration are described. [Color figure can be viewed at wileyonlinelibrary.com]

2.5.1 | Local route

Stereotaxic intracerebral administration

Stereotaxic intracerebral injection of EVs has been performed in different murine brain sites, including the hippocampus, neocortex, and lateral ventricles. Administration of mesenchymal stem cell (MSC)-derived EVs in dentate gyrus or CA1 area in mouse models of AD counteracted cognitive impairment by stimulating adult neurogenesis in the subventricular zone and inhibiting beta-amyloid deposition, respectively.[136,137] Injection of MSCs-derived EVs into the neocortex also ameliorated AD phenotype and reduced dystrophic neurites in both the neocortex and hippocampus, suggesting the potential capability of these vesicles to spread their immunoregulatory potential inside the brain.[142]

Intracerebral administration

Intracerebral administration of EVs represents an efficacious but also invasive approach with limited translational potential, especially for chronic diseases that may require multiple treatments. Some studies tried central vascular approaches as alternative routes of EV administration. Seras-Franzoso et al.[138] demonstrated that EVs derived from cells overexpressing lysosomal enzymes were able to efficiently reach different brain areas and deliver their protein cargo when intra-arterially administrated through the cannulation of the external carotid artery. In particular, the biodistribution assay showed enrichment of EVs in the hemisphere where arterial cannulation was performed.

2.5.2 | Peripheral route

Intravenous (IV) administration

Regarding the peripheral administration of EVs, the most common approach is the IV injection (e.g., caudal vein, jugular vein, retro-orbital vein).[139] The reticuloendothelial system rapidly traps most IV-injected EVs and localizes

inside the spleen and liver with a minimal fraction reaching the brain.[140,143] However, depending on the cellular generation source, some EVs have a natural brain barrier-crossing capacity, presumably transported via receptor-mediated endocytosis. IV-administered, microglia-derived EVs showed hippocampus targeting capability and colocalization with neuronal, microglial, and astrocytic markers in a mouse model of an injured brain.[144] The difference in brain biodistribution has also been reported for brain cancer cells injected into the cardinal vein in the zebrafish. EVs released by ECs derived from mouse brain endothelioma showed higher brain targeting capacity than vesicles obtained from glioblastoma-astrocytoma, neuroectodermal, or glioblastoma cell lines.[145] Fluorescently labeled EVs originating from dendritic cells were also detected in the brain 24 h after IV administration.[140]

Moreover, both intraperitoneal or IV injections of macrophage-derived EVs were able to induce neuroprotective effects in experimental models of neurodegenerative disorders, probably due to the longer circulation time of these vesicles because of their capacity to avoid blocking by mononuclear phagocytes.[146,147] The most studied EVs for their efficacy in neurological disorders originated from stem cells (SCs) due to their secretome neuroprotective and anti-inflammatory properties.[148] IV administration of EVs produced by interferon γ-activated MSCs reduced neuroinflammation in an experimental autoimmune encephalomyelitis mouse model.[141] MSC-derived EVs have also been shown to reduce the trauma brain injury-related lesion size and neurobehavioral alteration after injection via the retro-orbital route. Still, no data about their biodistribution were reported.[149]

Intranasal (IN) administration

The less invasive method to deliver EVs inside the brain is the IN route. The connection among nasal mucosa, microcirculation, and olfactory and trigeminal nerves allows rapid absorption and a diffuse brain delivery, reducing systemic side effects.[150] EVs secreted by white blood cells have been demonstrated to efficiently accumulate in the brain following IN administration.[151] Neural SC (NSC)-derived EVs have been reported to reach the hippocampus via the IN route and interact with the neurogenic niche.[152] Recent studies provided quantitative neuroimaging visualization of in vivo biodistribution of IN-administrated MSC-derived EVs by novel techniques.[153,154] Betzer et al.[155] reported that gold nanoparticle-labeled EVs showed higher brain accumulation after IN administration than IV injection. The cellular source of EVs may influence biodistribution. Indeed, other studies described equal brain targeting for EVs after different routes of administration (i.e., IV, IN, or stereotaxic injection).[156]

Several studies have developed novel engineering approaches to modify the surface of EVs and enhance their brain-targeting capacity.[157] In mouse models of PD and AD, the IV administration of EVs transfected with a neuron-specific rabies viral glycoprotein (RVG) peptide fused to the lysosomal-associated membrane protein 2B demonstrated enhanced delivery of small interfering RNA (siRNA) targeting α-synuclein or amyloid precursor protein cleaving enzyme 1.[158,159] These studies indicated that multiple routes of EV administration may target different brain regions and show therapeutic potential against neurological disorders. However, several variables, such as EV characteristics (e.g., isolation techniques, size, molecular cargo, surface molecules), transport mechanisms, and pathologic conditions, can influence the EV brain delivery efficiency. More in-depth studies about EV biodistribution, pharmacokinetics, and novel strategies to improve brain targeting via engineered EVs will strengthen their potential in clinical applications.

2.6 | Noninvasive imaging of EVs

The noninvasive imaging of EVs may help elucidate their in vivo properties for successful translation as cell-free therapeutic vehicles. Despite EVs' advancements and known benefits, detailed knowledge of EVs biodistribution, bioavailability, pharmacokinetics, various mechanisms, or toxicity is still lacking for the effective translation of EVs-based therapy. Therefore, noninvasive imaging techniques are crucial to facilitate translational aspects of EVs in vivo.

The EV labeling methods are direct or indirect (Figure 4).[160] Direct labeling refers to direct contact between EVs and labeled molecules (contrast agents, dyes, radionuclides, and so on). Indirect labeling refers to labeling

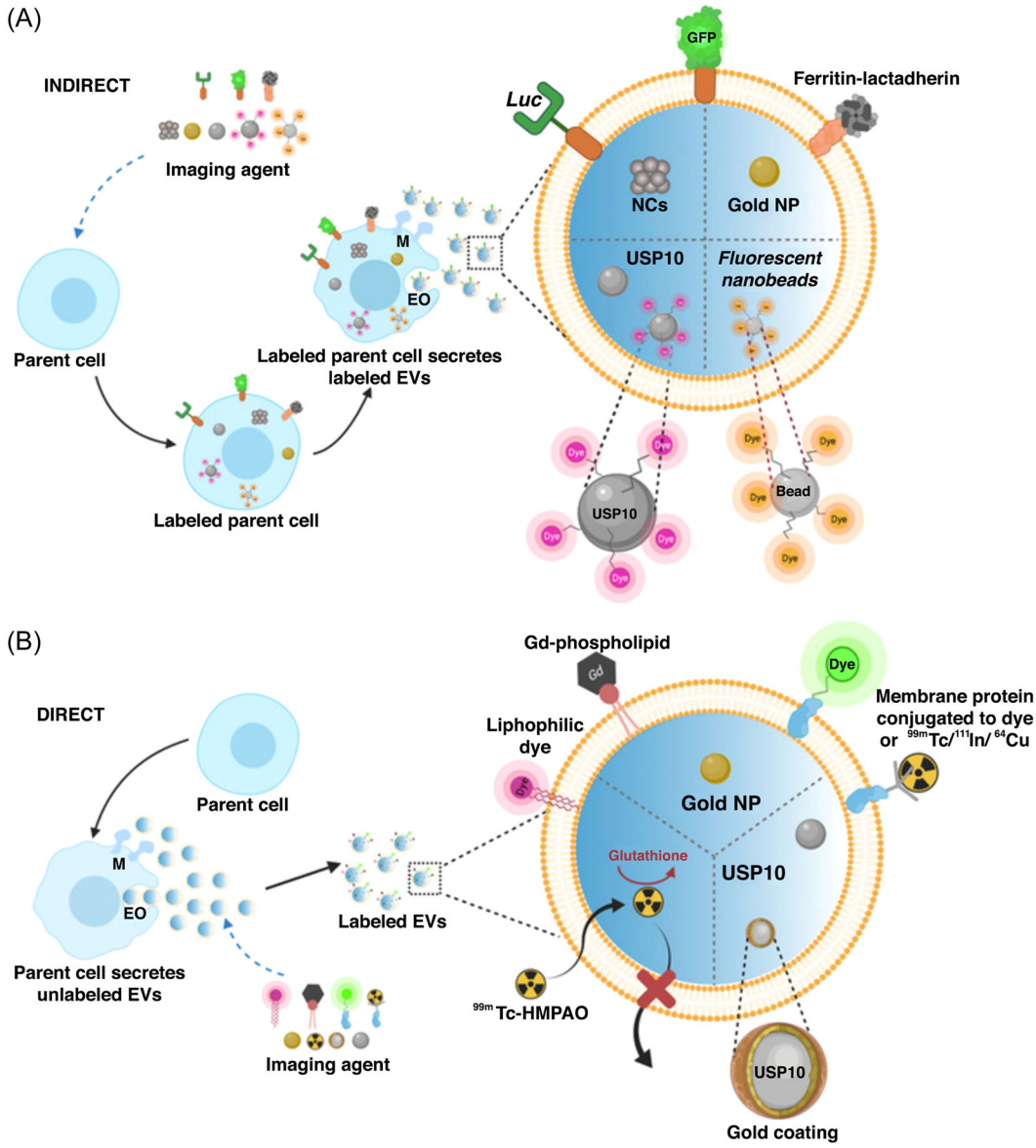

FIGURE 4 Schematic depiction of the various methods to label EVs using indirect (A) or direct labeling (B). Figure adapted, with permission, from *J Extracell Vesicles*. 2022 Jul;11(7):e12241. doi: 10.1002/jev2.12241). GFP, green fluorescent protein. [Color figure can be viewed at wileyonlinelibrary.com]

parent cells or genetic engineering (a viral vector carrying a reporter gene), which releases labeled EVs.[161] The strategies to label EVs directly can be either physical or chemical. In one of the strategies, EVs can be labeled with near-infrared wavelength lipophilic dyes (DiR, Cy7, PKH, DiD) and membrane permeant dyes (Calcein AM) to detect intact vesicles.[162] Labeled fluorescent dyes can be seen in tissues even after the degradation or internalization of EVs.

Moreover, lipophilic dyes may accelerate the aggregation of EVs after entering the blood circulation and extravasation in tissues.[163] The other physical strategy is electroporation, in which temporary micropores are

generated in the phospholipid membrane. An electric field increases the permeability of labeling agents and superparamagnetic iron oxide nanoparticles. This decreases membrane integrity and aggregation of EVs in the off-target organs.[164] In sonication, a mechanical shear force generates ruptures in EV membranes for the loading agent. The main problem is the time the membrane takes to reverse the ruptures, leading to insufficient loading.[165] In the extrusion procedure, EVs and cargo pass through an extruder for membrane recombination, but the loss of intrinsic cargo is common.[164] The chemical strategy, which is considered efficient, tends to increase the size of the EV and, therefore, alters pharmacokinetics and biofunctionality, which is surface modification. This is conducted via covalent binding involving crosslinking reactions (azide-alkyne cycloaddition), forming a stable triazole bond.[164]

The current imaging modalities, depending on the labeling agent, optical imaging (fluorescence and bioluminescence), and bioluminescence resonance energy transfer, are widely used techniques for biodistribution of EVs in in vivo and ex vivo animal models.[166-169] ^{1}H magnetic resonance imaging (MRI) is used for high spatial and soft tissue resolutions but requires a high concentration of MRI agents for attachment to EVs.[170-172] Computed tomography (CT) imaging uses ultra-small gold nanoparticles as a tracer because of their biocompatible nature.[153,173] Nuclear imaging, including single-photon emission CT (SPECT) and positron emission tomography (PET), is a comparatively expensive technique.[174,175] Magnetic particle imaging is a relatively new imaging modality that detects magnetic nanoparticles without tissue depth.[164,176] MRI, CT, SPECT, and PET can be used for whole-body imaging, whereas other imaging techniques can be used for small animal-body imaging.[177]

3 | THERAPEUTIC APPLICATION OF EVS IN DIFFERENT NEURODEGENERATIVE DISORDERS

3.1 | ALS

ALS is a progressive neurologic disease that involves degeneration of the upper and lower motor neurons (MNs) in the cortex, brain stem, and spinal cord. The cause of ALS is unknown. In 90%–95% of ALS disorders the disease is sporadic with no genetic link, and in 5%–10% it is familiar with several gene mutations involved in the adult onset, including Cu/Zn superoxide dismutase one (*SOD1*), chromosome 9 open reading frame 72 (*C9orf72*), TAR DNA-binding protein (*TARDBP*), and fused in sarcoma (*FUS*). SOD1 is one of the most mutated genes associated with ALS, and G93A mice containing multiple copies of a human mutant form of SOD1 are the most widely used model of ALS disease. The diversity of ALS pathogenesis makes it ineffective for pharmaceutical treatment. In recent years, SCs or EVs derived from SC have immense potential in treating different neurodegenerative diseases, thus providing a new method as a potential therapy for ALS.

Among various SCs isolated from different sources, the advantage of MSCs was shown both in experimental models of ALS and in clinical studies.[178-182] Recently, the mechanism of MSC action has been modified. The effect of transplanted MSCs is now predominantly associated with their paracrine properties. It is known that transplanted MSCs modify the functions of the host microenvironment through the release of different secreted factors and EVs.[183-185] Recently, the novel therapeutic strategies proposed in ALS concern using EVs derived from MSCs. EVs can maintain effective cargo of parent cells. After transplantation, EVs cross the blood–brain barrier (BBB) to reach the sites of CNS injury and transmit different proteins, lipids, and genetic materials to the neural cells of the host. Current research indicates that EVs have immense potential in neuroprotective activity and alleviate the pathology of different neurodegenerative diseases in vitro and in vivo.

Several in vitro studies have found that MSC-EVs can protect and repair nerve injury in ALS. The Mariotti group conducted a study to examine the impact of exosomes derived from murine adipose stromal cells (mASCs-exo) on a motor neuron (MN)-like cell line called NSC-34.[186] These NSC-34 cells were transfected with various human mutant SOD1 transgenes and were engineered to overexpress specific human SOD1 mutants, particularly the G93A mutation. This cell line serves as an in vitro model of ALS, a neurodegenerative disease affecting MNs. The

study aimed to investigate how mASCs-exo influenced the NSC-34 G93A cells in the context of ALS. Adding mASCs-exo into the NSC-34 G93A ALS model mouse cell line previously exposed to the oxidative (H_2O_2) insult rescued the cells from death with a median value of 60% viability compared to control cells cultured without exosomes.[187] The authors suggested that the beneficial effect of mASCs-exo documented in previous studies could be due to the secretion of miRNAs that play a protective role via the apoptosis-inhibiting pathway, for example, miRNA21, miRNA222, and miRNAlet7a, whose presence and neuroprotective function have been previously identified in ASCs-exosomes. Further studies from this group demonstrated that mACSs-exo treatment of NSC-34 (G93A) cells once exposed to H_2O_2, which determined a high cell death, rescued the cells from apoptosis. The expression of apoptotic markers accompanied the increase in NSC-34 (G93A) cell viability, the reduction of proapoptotic proteins caspase-3 and Bax protein, and the rise of antiapoptotic Bcl-2α protein.[188]

The neuroprotective effect of exosomes isolated from mice adipose stromal cells was also presented by studies of the influence of mASCs-exo on the energy metabolism of NSC-34 (G93A) cells, and particularly the mitochondrial respiratory capacity, which was reduced due to the presence of mutated SOD1 protein. Calabria et al.[189] demonstrated that mACSs-exosomes added to NSC-34 (G93A) cells that show a reduced mitochondrial oxidative capacity were able to restore mitochondrial respiratory capacity. Through the utilization of high-resolution respirometry and cytofluorimetry analysis, it was discovered that exosomes derived from mASCs-exosomes were able to rescue the reduced complex I-linked phosphorylated activity observed in SOD1 (G93A) mice tissue, as well as in NSC-34 (G93A) cells induced by mutant SOD1 protein. Furthermore, treatment with mASCs-exosomes effectively restored the decreased mitochondrial membrane potential of NSC-34 (G93A) cells to values similar to those observed in control cells.[189] Lee et al.[190] presented related results using human adipose-derived stromal cell exosomes (hADSCs-exo) cocultured with primary neuronal SCs isolated from G93A mice. The presence of hADSCs-exo normalized the decreased levels of mitochondrial proteins such as pCREB and PGC-1α in G93A cells. Concomitantly, hADSCs were shown to ameliorate the SOD1 protein level and slow the progression of aggregate formation in the cytoplasm of differentiated G93A neuronal cells in vitro.

Several studies found that administering MSC-EVs in neurological diseases attenuated inflammatory processes through the modulation of immune cell activity.[191–193] In ALS pathogenesis, SOD1 (G93A) activates NLRP3 inflammasome in microglia by promoting IL-1β secretion, leading to neuroinflammation and neurotoxicity.[194] Recently, Giunti et al.[195] demonstrated that MSCs-derived EVs downregulated neuroinflammation in vitro in a model of ALS. The authors showed that the exposure of the primary microglia, acutely isolated from late symptomatic SOD1 (G93A) mice to MSCs-EVs modulated pro-inflammatory phenotype of SOD1 (G93A) microglia by increasing Cxcr1 and Nr4a2 expression. Their previous studies suggested that the immunomodulatory effect of MSC-EVs was exerted by the anti-inflammatory effect of miR-467f and miR-466q, in which EVs are enriched.

The Mariotti group tested the neuroprotective in vivo effect of MSCs-derived EVs in ALS. The authors transplanted mASCs-exo into SOD1 (G93A) mice IV or IN.[186] The results demonstrated that repeated infusion of mASCs-exosomes improved the impaired motor function in the host. The stereological analysis of lumbar motoneurons in graft recipients revealed that IV or IN exosome administration increased the number of surviving MNs, indicating that mASCs-exo can protect MN loss in SOD1 (G93A) mice. Moreover, SOD1 (G93A) mice injected with mASCs-exo showed a higher innervated neuromuscular junction number and attenuated the degeneration of skeletal muscle fibers compared to control untreated mice. The authors found a decreased astrocyte activation observed in SOD1 (G93A) mice during the disease progression without mASCs-exo administration. Interestingly, exosomes infused with IN have been observed in the brain of SOD1 (G93A) mice, indicating the ability of mASCs-exo to home the lesioned sites in ALS.

Recently, capillary alterations within CNS have been identified in ALS disorders. The structural and functional changes of ECs lead to vascular leaking, allowing detrimental factors to penetrate CNS and profound MN degeneration in patients and animal models of ALS disease.[196,197] Previous research by Garbuzova-Davis et al.[198] showed that systemic transplantation of human bone marrow endothelial progenitor cells (hBM-EPCs) into SOD1 (G93A) mice restored capillary structures and enhanced MN survival in the brain cortex and spinal cord and

improved behavior test performance compared to control mutant mice. Based on these findings, Garbuzova-Davis et al.[199] suggested that EVs derived from endothelial progenitors may alleviate CNS vasculature damage in mouse models of ALS. The in vitro studies revealed that EVs isolated from hBM-EPCs cocultured with mouse brain EC (mBEC) line previously exposed to ALS mouse plasma collected from symptomatic SOD1 (G93A) mutants improved the morphology and viability of impaired mBEC affected by cell exposure to plasma derived from ALS mice.[199] These results suggest that EV treatment may restore the damaged ECs and decrease blood capillary permeability to avoid the entry of detrimental peripheral factors contributing to MN damage in ALS.

EVs are considered the intriguing prognostic and diagnostic markers for ALS, which can be isolated from blood, urine, saliva, and CSF.[200] These EVs are not only investigated based on EV cargo but also on the EV's size. The leukocyte-derived large EVs from the CSF of ALS patients were found to be slightly correlated with disease progression. The misfolded SOD1 has yet to be found in the CSF-derived EVs.[201] It has been seen that miR-124-3p level is associated with ALS severity, and the authors implied that this might serve as a prognostic biomarker.[202]

Also, the decreased mean size of EVs was investigated in the serum of ALS patients.[203] Large EVs isolated from the plasma of ALS patients were found to be enriched with SOD1. miR-27a-3p, miR-24-3p, miR-1268a, miR-3911, and miR-4646-5p can also be potential markers from the serum of ALS. The authors also described miR-15a-5p and miR-193-5p, with either diagnostic potential or associated with ALS progression.[204] Studies have shown that blood might be appropriate for identifying disease and progression. Still, technical challenges must first be addressed toconsider EVs as biomarkers for untreatable diseases.

Overall, these studies demonstrated the beneficial effect of EVs in MN protection in ALS. Experimental results with different disease models support the potential use of EVs in clinical therapy of ALS disorders and their use in the prognosis and diagnosis of the disease. EVs offer a promising cell-free treatment approach in contrast to traditional SC-based therapies for ALS patients. In ALS patients, EV infusion demonstrated the ability to counteract the progressive degeneration of MNs, reduce pro-inflammatory markers, and extend the survival of the recipients. This highlights the potential of EVs as a novel therapeutic tool that could provide significant benefits in treating ALS compared to conventional SC therapies.

3.2 | HD

HD is a hereditary neurodegenerative disorder caused by the aggregation of mutant huntingtin (mHtt). This mutation leads to the generation of polyglutamine (polyQ) protein, which has neuronal toxicity. Accumulation of mHtt results in cognitive deficits and compulsory movement, correlated with a selective loss of striatal neurons and cortical atrophy.[205] HD seems to be the most amenable of CNS diseases to early intervention due to its genetic predictability. However, the failure of drugs designed to treat HD requires searching for new therapeutic options, among them SCs and derivate transplantation.

Lee et al.[206] investigated the therapeutic role of EVs derived from human adipose SCs (hASCs) using an in vitro model of HD. The results revealed that co-incubation of hASC-EVs with NSCs isolated from R6/2 transgenic HD mice increased cell survival and ameliorated mitochondrial dysfunction of NSCs. Additionally, hASC-EVs upregulated PGC-1 and phosphorylated cAMP-response element binding protein (CREB) and improved abnormal apoptotic p53, Bax, and cleaved caspase-3 levels, reducing cell apoptosis. Immunocytochemical analysis showed that hASC-EVs decreased mHii aggregates in R6/2 mice-derived NSCs.

The protective effect of EVs in HD mice was observed by Hong et al.[207] Transplantation of exosomes derived from astrocytes isolated from the brains of wild type (WT) murine pups into the striatum of full-length Htt 140Q knock-in (KI) adult mice diminished aggregation of mHtt in the neural cells of the exosome-infused brain area.[207] Seven days after the astrocytic exosome transplantation, a significant decrease in mHtt aggregate density was found in the ipsilateral striatum of KI graft recipients.

Experimental studies conducted in HD models have shown that different modifications of EVs may achieve better efficacy. Recently, Joshi et al.[208] evaluated the potential of EVs isolated from C17.2 NSCs overexpressing DNAJB6, a member of the DNAJ chaperone family, which was reported to inhibit polyQ aggregation in vitro efficiently. The results revealed that DNAJB6-enriched EVs abrogate polyQ aggregation in vitro and in vivo. Coculture of EVs enriched in DNAJB6 with EGFP-Htt (Q74) HEK293T suppressed polyQ aggregation in the cells compared with untreated cells. Similarly, intrathecal injection of DNAJB6-EVs into R6/2 HD transgenic mice reduced the formation of HTT aggregates in graft recipients' brain and spinal cord compared to control mice.[208]

Other studies have shown that genetic modification of EVs by loading them with noncoding miRNA or siRNA expanded their therapeutic potential. It is known that among miRNAs, miRNA-124, which is a brain-specific miRNA, regulates the proliferation of neural progenitor cells, controls neuronal survival, and regulates neuronal differentiation.[209–211] Previous studies have explored the alteration of miRNA-124 in HD. Johnson et al.[212] observed that the expression of miRNA-124 in the brain of HD patients and transgenic mouse models was decreased and led to the dysregulation of RE-1-silencing transcription factor (REST), resulting in neuronal damage. In a study conducted by Lee et al.,[213] it was discovered that exosomes derived from HEK-293 cells enriched with miRNA-124, a miRNA with high expression levels, exhibited therapeutic efficacy in R6/2 transgenic mice with HD. The findings from this study provide evidence that exosomes carrying miRNA-124 hold potential as a therapeutic intervention for HD.[213] Transplantation of miRNA-124 exosomes into the striatum of R6/2 mice reduced the expression of REST protein in the brain of the recipients compared to the control (nontreated) counterparts. Unfortunately, no significant improvement was found in the behavioral symptoms 1 week after miRNA-124 exosome delivery; however, longer time intervals have yet to be studied.

siRNA is known to guide mRNA cleavage through RNA-induced silencing complex, which can provide effective long-term gene silencing. Delivery of siRNA to target HTT mRNA silencing in neurons of HD disorders remains an important challenge in therapy. Wu et al.[214] tested the therapeutic potential of siRNA loaded in exosomes delivered systemically into BACHD and N171-82Q transgenic mice models of HD. The infusion of modified exosomes expressing the neuron-specific RVG loaded with siRNA targeting human Htt transcript significantly reduced Htt expression in the brain of graft recipients. Moreover, N171-82Q mice receiving Htt-siRNA RVG exosomes showed behavioral improvement in rotarod performance. In other studies, with human siRNA targeting Htt RNA, lipid-conjugated siRNA was used to load EVs. In in vitro studies, Didiot et al. observed that hydrophobically modified siRNA-loaded exosomes derived from the human U87 glioblastoma cell line were internalized into mouse primary cortical neurons and promoted silencing of Htt mRNA and Htt protein in these cells.[215] Unilateral infusion of such siRNA-loaded exosomes into the striatum of WT mice resulted in a statistically significant reduction of Htt mRNA in striatal and cortical regions on both sides of the mouse brain. Similarly, in vitro studies by Biscans et al.[216] showed that exosomes isolated from Wharton's jelly-derived MSCs loaded with cholesterol-conjugated siRNA induced silencing of Htt mRNA in primary mouse neurons.

The research is still going on to analyze the composition of EVs, looking for EVs as HA biomarkers. No differences were found in the number of EVs and correlation with disease state using platelets and platelet-derived EVs for search them as potential biomarkers as mHtt was absent.[217] The miRNAs studied concerning disease pathophysiology have yet to be explored. Significantly increased miRNAs were found in the CSF samples in the prodromal HD gene polyQ expansion carriers (miR-135b-3p, miR-520f-3p, miR-4317, miR-3928-5p, miR-140-5p, and miR-8082).[218] In the blood plasma of symptomatic HD patients, another 13 miRNAs (miR-877-5p, miR-223-3p, miR-223-5p, miR-30d-5p, miR-128, miR-22-5p, miR-222-3p, miR-338-3p, miR-130b-3p, miR-425-5p, miR- 628-3p, miR-361-5p, miR-942) were found to be upregulated in the plasma samples of symptomatic patients.[219] These studies suggest that administering exogenous EVs into transgenic animals of HD models may reduce neuronal dysfunction in disease disorders and prove that EVs could hold promise as a treatment for HD patients.

3.3 | MS

MS is a chronic autoimmune disease of the CNS. Although the exact etiology of MS is currently unknown, it is generally believed that environmental and genetic factors evoke an immune response causing pathological features, including inflammation, cell infiltration, demyelination, axonal degeneration, oligodendrocyte apoptosis, and dysfunction of the BBB.[220,221] Complex interactions between several types of immune cells, such as T and B cells, dendritic cells, macrophages, and NK cells, cause immune-mediated damage in the CNS. Along with the immune cells, glial cells function as nonclassical immune cells in the pathogenesis of MS. Despite the increasing number of MS patients, no effective therapy still exists. Medications currently in use only focus on delaying the adverse effects but are ineffective in a complete cure. There is an immense need to find therapies that will be effective therapeutically and minimize drug side effects. The use of EVs to target MS has evoked much interest among researchers and many studies reported the therapeutic benefits in MS.[222,223]

Proteomic analysis of oligodendrocyte-secreted EVs revealed the presence of many myelin-forming proteins such as myelin basic protein, myelin oligodendrocyte glycoprotein (MOG), proteolipid protein, and 2′3′-cyclic-nucleotide-phosphodiesterase.[224-226] Additionally, Casella et al.[224] found that IV injections of oligodendrocyte-derived EVs decreased disease pathophysiology in a myelin antigen-dependent manner (Figure 5). In the experimental autoimmune encephalomyelitis mice model, immune tolerance was restored via the induction of immunosuppressive monocytes and apoptosis of autoreactive CD4+ T cells.[224] Studies have also reported that mesenchymal stromal cell-derived EVs can have direct or indirect effects on oligodendrocyte precursor cells and microglia to significantly improve neurological outcomes by inducing myelin regeneration and alleviating neuroinflammation in mouse models.[227-229] Systemic injection of EVs isolated from interferon-γ (IFN-γ) activated MSC resulted in a clinical recovery with improved motor skills and reduced neuroinflammation and demyelination in mice. RNA sequencing and proteomic analysis revealed that these EVs contained anti-inflammatory and/or neuroprotective RNAs and proteins.[141]

EVs derived from dendritic cells, when stimulated with low levels of IFN-γ, were found to carry miRNAs such as miRNA-219, which plays a role in oligodendrocyte differentiation and anti-inflammatory pathways. These EVs exhibit a remarkable ability to induce oxidative stress tolerance and enhance remyelination in hippocampal brain slices. Increased CNS myelination was further confirmed in vivo by administering EVs isolated from IFN-γ-stimulated dendritic cells.[230] These results indicate that EVs have a significant role to play in promoting myelin regeneration and treating MS. As EVs are composed of different biologically active compounds, they could significantly contribute to an immune response in MS, such as the delivery of self-antigens, cytokines, and peptide–MHC complexes, and that could account for initiation and continuation of the autoimmune response by triggering autoreactive T and B cells.[231-233] Nevertheless, the biological cargo, including proteins, lipids, and nucleic acids, makes EVs particularly attractive as a diagnostic tool to detect specific EVs' subpopulations and biomarkers to determine disease stage, severity, and progression.

Ebrahimkhani et al.[234] found that the serum EVs miRNA profiles reflected physiological alterations linked to MS initiation and progression. When relapsing-remitting MS (RRMS) patients were compared to the progressive disease and healthy controls, a cluster of nine miRNAs (miR-15b-5p, miR-23a-3p, miR-223-3p, miR-374a-5p, miR-30b-5p, miR-433-3p, miR-485-3p, miR-342-3p, miR-432-5p) were found to be differentially expressed[234] and serum-derived EV miRNA hsa-miR-122-5p, hsa-miR-196b-5p, hsa-miR-301a-3p, and hsa-miR-532-5p were deregulated during relapse in RRMS patients.[235] Microarray of EVs from the serum of MS patients showed upregulation of let-7i, miR-19b, miR-25, and miR-29a compared to healthy controls.[236] Although the relationship between EV proteins and lipid cargo in MS patients remains unknown, Galazka et al.[226] described a strong correlation between the concentration of EV-derived MOG in serum and CSF of MS patients. Elevated levels of EV-derived MOG were found in serum of RRMS during relapse and in secondary progressive MS. In the CSF, MOG levels were higher in all MS groups compared to controls.[226] Bhargava et al.[237] reported altered levels of Toll-like receptors 3 and 4 in serum EVs of RRMS patients and lipidome analysis showed a relevant increase in sulfatides in MS patient plasma EVs compared to healthy controls.[238] Increased lipid classes in EVs and cholesterols, phospholipids, and sphingolipids may play a significant role in remyelination,.[239]

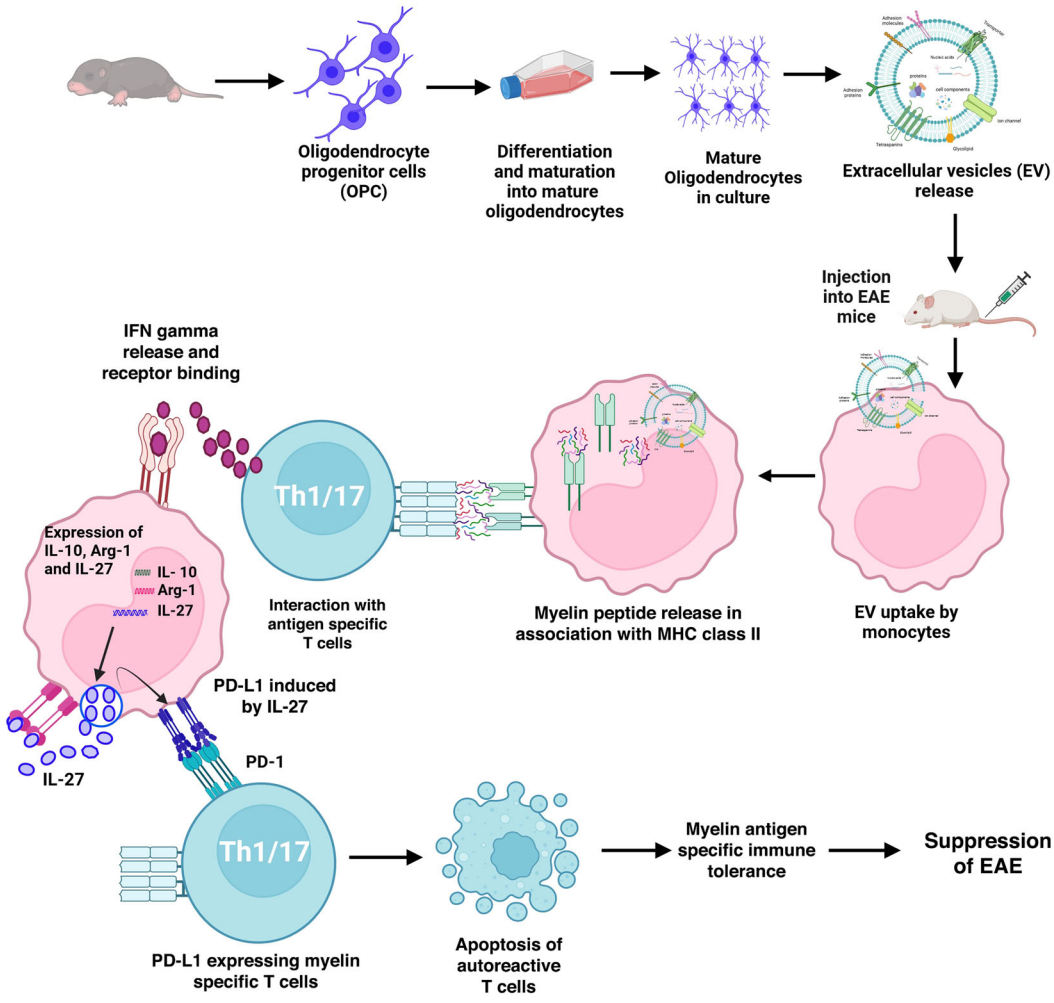

FIGURE 5 Mechanism of EV-induced myelin antigen-specific immune tolerance in experimental autoimmune encephalomyelitis (EAE) mice model. IL-10, interleukin-10; IL-27, interleukin-27; MHC, major histocompatibility complex. [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | PD

PD is the second most prevalent neurodegenerative disease and the most common movement disorder, affecting nearly one million people in the United States.[240,241] Over the course of the disease, PD patients develop both nonmotor and motor deficits, the latter of which primarily results from the progressive loss of dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc) and their terminals in the striatum. It is now widely accepted that the degeneration of the DA nigrostriatal system is likely triggered by the accumulation of oligomers of the protein α-synuclein found in Lewy bodies, the neuropathological hallmark of the disease.[240,241]

Remarkably, nearly 80% of the dopamine-producing neurons in the SNpc are lost before most PD patients develop their first motor symptoms (i.e., tremors, postural imbalance, bradykinesia, and so on). However, before these midbrain-associated changes, Lewy bodies have been observed in the vagus olfactory bulb and dorsal motor nucleus, causing anosmia, sleep disturbance, constipation, and other nonmotor symptoms.[240,241] As this early

prodromal syndrome often occurs years before SNpc involvement, there is a window of opportunity during which therapies can be initiated to protect and preserve "at risk" midbrain dopamine neurons.

Various potential therapeutics capable of enhanced neuroprotection/neurorestoration in PD models, including the products of different stem and brain cell types, have been identified.[241–243] However, their therapeutic promise has often been short-lived due to the BBB, which can limit or prevent their transport into the brain, reducing their clinical efficacy. Using EVs to deliver therapeutics to the brain is a much-needed game changer.

We now know that cells, such as MSCs, NSCs, astrocytes, and microglia, naturally shed EVs containing potentially restorative growth factors, cytokines, mRNAs, and miRNAs. Unlike their parent cells, EVs readily cross the BBB or nasal mucosa to transport their contents to the brain, and because EVs are membrane-bound, their cargo remains stable during delivery. Moreover, EVs produce little to no immunogenicity in host tissue, especially those derived from autologous cells (Figure 6).[242]

Of further significance, the contents of EVs can be modified in the laboratory. Thus, EVs can be exogenously loaded with preferred products and/or drugs using standard laboratory methodologies (i.e., permeabilization, electroporation, sonication, and so on).[151,242] Moreover, "designer EVs" can be generated in cells engineered to overexpress potentially therapeutic molecules, or conversely, deleterious proteins, mRNAs, and miRNAs in parent cells can be reduced using knockdown technology. EVs, therefore, represent a promising new delivery platform for treating neurological disorders such as PD.[243]

In PD, as in other neurodegenerative diseases, SCs and other cell types play an important neuroprotective role in rescuing vulnerable dopamine neurons in the SNpc.[243,244] These beneficial effects have been posited to arise partly from the EVs released by these cells onto target brain cells. Consistent with this notion, conditioned media (CM) from these cells rich in EVs often replicates many therapeutic effects in culture models of PD.[243]

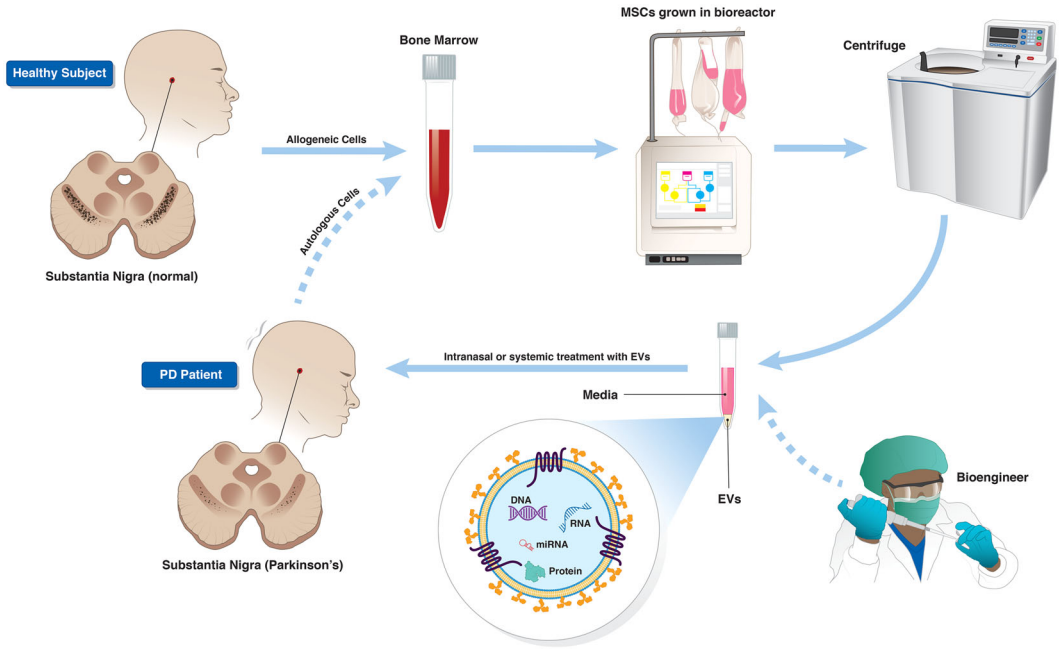

FIGURE 6 Schematic depicts that allogeneic or autologous cells from various sources can be harvested and expanded in culture, and EVs are isolated by centrifugation and/or filtration. EVs then can be systemically or intranasally delivered to the patient with or without prior engineering in the lab. [Color figure can be viewed at wileyonlinelibrary.com]

Consequently, CM has been the primary source of EVs for isolation, molecular analysis, and experimental manipulation.[242,245–247]

Jarmalavičiūtė et al.[248] found that EVs isolated from the CM of MSCs derived from human exfoliated deciduous teeth (SHEDs) effectively protected human dopamine neurons from 6-hydroxydopamine (6-OHDA)-induced toxicity in a culture model of PD. The authors later showed that SHED-derived EVs administered IN could rescue dopamine neurons of the SNpc and their terminals in the striatum, reversing motor deficits in a 6-OHDA treated rat model of PD in vivo.[249] Moreover, CM from SHEDS later replicated this EV effect in a rat rotenone model of PD.[250]

As with SHED-derived MSCs, EVs derived from cultured human umbilical cord MSCs were able to ameliorate 6-OHDA toxicity in dopamine neurons differentiated from the human SH-SY5Y cell line, and this neuroprotective effect was partially blocked by co-treatment with an inhibitor of EV release.[251] Similarly, EVs from umbilical cord SCs, administered IV, significantly reduced the degeneration of dopamine neurons in the SNpc of 6-OHDA rats, confirming that systemic EVs can effectively cross the BBB into the brain to affect target cells.[251]

In other studies, cells were first engineered to increase or decrease specific components in their EV cargo to further enhance their ability to rescue dopamine neurons in PD. Qu et al.[252] used EVs derived from blood to safely deliver prepackaged dopamine across the BBB, achieving more widespread distribution in the brain and greater therapeutic efficacy than that observed with free dopamine. In 2015, Haney et al.[152] loaded the potent antioxidant catalase into monocyte/macrophage EVs. They showed attenuated oxidative stress/inflammation and augmented neuroprotection in both in vivo and in vitro PD paradigms. Building on this concept, Kojima et al.[253] developed a system of genetically encoded steps in MSCs to increase the number of microvesicles containing concentrated catalase mRNA delivered to target brain cells, thereby mitigating inflammatory signals and enhancing rescue of DA terminals in the striatum in PD.

Similarly, the levels of miRNAs of known importance in PD pathogenesis (i.e., miR16-1, miR354b-c, miR-153) or PD neuroprotection (i.e., miR-133b, miR-143, miR21) have been manipulated in EV-producing cells to lessen α-synuclein aggregation and neuroinflammation, and to promote the rescue of dopamine neurons in PD.[243,254,255] Thus, an inhibitor of miR-21[256] or a synthetic antagonist of miR-155 was shown to effectively decrease the production of pro-inflammatory cytokines, reduce microglial activation, and promote neuroprotection in in vitro and in vivo models of PD. Likewise, EVs engineered to carry siRNA[159] or small hairpin RNAs[257] have been used to lower levels of α-synuclein RNA and reduce toxic aggregation of the protein in transgenic and nontransgenic models of PD. Alternatively, α-synuclein mRNA levels have been directly modified in target cells by miR-7, which binds the untranslated region of the *SNCA* gene and blocks its transcription.[258] Thus, a better understanding of miRNAs' role in PD pathogenesis and how to best modify their expression in EVs through genetic engineering or synthetic reagents (agomirs and antagomirs) will be critical for using therapeutic EVs effectively in PD.

EVs may also serve as a valuable source of potential disease biomarkers, as neurons, astrocytes, microglia, and ECs secrete them. Indeed, EVs isolated from CSF, serum, and brain lysates of PD patients have been shown to contain aggregated α-synuclein, which can be transferred to and produce PD pathology in healthy DA neurons in vitro[259-261] and in vivo.[259,262-265] EVs found in the saliva[266,267] and urine[268-270] of PD patients may also contain early biomarkers (i.e., RNAs, miRNAs), capable of distinguishing sporadic from certain inherited forms of PD (i.e., DJ-1, LRRK2 mutations).[269,270] Although it remains to be seen whether the levels of EV-derived biomarkers increase as PD progresses and disease severity worsens, their potential diagnostic value is indisputable.

Although the study of EVs is still in its infancy, remarkable progress has been achieved in the last decade, particularly for neurodegenerative diseases such as PD. We now know that EVs can participate in the spread of toxic α-synuclein oligomers from one cell to another in vitro and in the midbrain. Likewise, we know that PD-associated changes in growth factors, proteins, RNAs, and miRNAs are often mirrored in the composition of EVs, from brain cells into blood, urine, and CSF. As such, EVs may provide critical early biomarkers to diagnose PD and properly stage the disease. We can use these insights and the latest technological advances to manipulate the contents of EVs to help lower α-synuclein, oxidative stress, and neuroinflammation and better protect "at risk" midbrain dopamine neurons in PD models.

Learning how to dose and best administer therapeutic EVs to slow or halt disease progression will be critical for moving this exciting platform from the lab bench into PD patients in the future.

3.5 | Stroke

A stroke is caused by an insufficient supply of oxygen and nutrients to an area of the brain.[271] This event leads to neuronal death, gliosis, and activation of neuroinflammatory responses.[272] In the last decade, numerous studies reported the beneficial effects of EV administration on neuroinflammatory pathways and behavioral deficits in experimental stroke models by elucidating different molecular mechanisms.[273] IV administration of MSC-derived EVs in a middle cerebral artery occlusion (MCAO) rat model attenuated neuromotor deficits and increased neurogenesis and angiogenesis processes.[274] Among the reported molecular mechanisms, EVs decreased oxidative stress and inhibited the expression of pro-inflammatory cytokines.[192,275]

Moreover, Deng et al.[276] demonstrated that intracerebroventricular injection of MSC-derived EVs rescued synaptic plasticity and cognitive deficits in a mouse model of transient ischemia. EVs released by NSCs showed more effective therapeutic effects, including reduced neuroinflammation, motor deficits, and enhanced neuroprotection in a thromboembolic stroke mouse model compared to vesicles originating from MSCs.[277] Interestingly, intracerebral injection of EVs generated from interferon-stimulated NSCs improved therapeutic effects in an ischemic rat stroke model.[278]

Glia cell preservation and metabolism also appeared to play a vital role in maintaining ischemia-related molecular responses and brain function. In an MCAO mouse model, IV administration of NSC-derived EVs 2 h after artery occlusion ameliorated motor function after 1 day and reduced the infarct size 4 days after reperfusion, respectively, through preservation of astrocyte function.[279] IV treatment with EC-derived EVs has also been reported to counteract cognitive deficits in a transient cerebral ischemia mouse model by modulating astrocyte and BBB function.[280] Furthermore, Pei et al.[281] showed that IV administration of astrocyte-derived EVs reverted autophagy pathways and increased neuronal survival in an MCAO rat model.[282]

EVs are potential biomarkers for changes during stroke in the CSF, urine, and blood.[282] The common biomarkers are cell adhesion molecule L1 (L1CAM), GluR2/3, GPI-anchored prion protein, neural cell adhesion molecule (NCAM), CD81, tubulin-β, neuron-specific enolase, including miRNA-9, miRNA-124, miRNA-124-A, miRNA-128, miRNA-153, and miRNA-219.[283–285] Elevated procoagulant EVs in plasma for up to 7 days after intracerebral hemorrhage (ICH) in comparison to controls[286]; anti-inflammatory monocyte-derived EVs were found to be elevated in ICH patients[287] is suggestive of a preferential release of EVs that counteract post-ICH inflammation. As increased expression of EVs in CSF is also seen, it is considered a better predictor of 1-week mortality than plasma EVs.[288]

Several studies have reported results that seemingly conflict with the above-described beneficial effects of EVs in stroke experimental models. Otero-Ortega et al.[289] did not detect any reduction of the infarct size upon EV administration, and Nalamolu et al.[290] showed no efficacy on motor and sensory functions. The timing of the artery obstruction, reperfusion, EV administration, and molecular and clinical evaluations may explain these differences in neuropathological and functional outcomes. A clinical trial (Allogenic MSC-Derived Exosome in Patients with Acute Ischemic Stroke -NCT03384433) is evaluating both the safety and efficacy of MSC-derived EVs enriched with miR-124 in acute ischemic stroke patients.

3.6 | AD

AD is an irreversible neurodegenerative disorder characterized by the alteration of synaptic functions.[291,292] Although a common understanding of the pathogenesis is still debated, evidence from studies on both experimental models and patients highlights the role of neuroinflammation, oxidative stress, and alteration of protein homeostasis as key

processes triggering neurodegeneration in AD.[293,294] To date, no curative therapy has been developed, and the current pharmacological approaches only aim to slow down the death of neurons. The therapeutic application of EVs in AD may exert multiple effects, including the delivery, even in deep brain regions, of neurotrophic and anti-inflammatory factors stimulating neuroplasticity and fostering endogenous antiaging mechanisms inside the brain.[295] Many studies have reported the potential benefits of MSC- and NSC-derived EVs against synaptic dysfunction and cognitive deficits in AD experimental models.[296,297] Molecular hallmarks of brain insulin resistance and alteration of neurotrophin signaling have been demonstrated in AD experimental models.[298,299] IN administration of EVs secreted by NSCs has been reported to counteract the brain insulin resistance-related cognitive decline by rescuing the brain-derived neurotrophic factor/CREB-related pathway in the hippocampus.[300]

Moreover, intracerebral injection of these vesicles has been demonstrated to prevent synaptic plasticity and memory deficits by reducing the binding of Aβ oligomers to hippocampal synapses and attenuating their synaptotoxic activity.[301] Accordingly, stereotaxic injection of MSC-derived EVs into the neocortex of APPswe/PS1dE9 AD mice at the preclinical stages reduced the Aβ levels and number of dystrophic neurites in both the neocortex and hippocampus.[142] Nakano et al.[302] demonstrated that intracerebral injection of these vesicles in AD models transferred the miR-146a into astrocyte, promoting both astrocytic function and synaptogenesis and leading to the rescue of cognitive function. Even the systemic administration (i.e., via retro-orbital vein) of NSC-derived EVs attenuated alterations of both cognitive and anxiety-related behaviors in the 5 × FAD AD mouse model.[303]

EVs originating from human MSCs have also shown antioxidant and neuroprotective effects. In particular, they sustained the survival of hippocampal neurons in the presence of Aβ in vitro by inducing the expression of catalase enzymes and decreasing reactive oxygen species production.[304] SC-derived EVs have also been proposed to reduce amyloid-beta plaque accumulation and protect against synaptic loss and cognitive decline by modulating the levels of circulating pro-inflammatory cytokines and inhibiting microglia activation. Li et al.[305] reported that intracerebral injection of NSC-derived EVs in APP/PS1 mice ameliorated mitochondrial function and synaptic activity without reducing Aβ levels while decreasing inflammatory responses. Therefore, EVs released by MSCs were able to mitigate neuroinflammatory pathways both in vitro and in vivo. Studies have demonstrated that EVs originating from cytokine-treated MSCs could reduce the release of inflammatory interleukins IL-6 and IL-1β from microglia and the number of reactive cells in the hippocampus and neocortex.[306] Engineered EVs have also been used as a biotechnological approach in experimental models of AD. IV-infused MSC-derived EVs were targeted at the brain by conjugating the vesicles with the CNS-specific RVG peptide. RVG-tagged EVs rescued memory deficits in APP/PS1 mice by reducing tumor necrosis factor-α, IL-β, and IL-6; the activation of astrocytes; and the Aβ levels in the brain.[307]

According to Fiandaca et al.,[308] the authors found significantly higher levels of amyloid β 1–42 in neuron-derived exosomes (NDEs) from plasma of AD patients in comparison to case–controls 1–10 years before diagnosis, using L1CAM or NCAM1, and glial fibrillary acidic protein, which might be developed as a prediction of AD.[308] Also, higher complement and lower regulatory proteins have been seen in NDEs from the plasma of the patients at the AD2 stage than at AD1 preclinical stage.[309] Moreover, increased myeloid MVs were found in the CSF of AD patients compared to the controls.[310] More studies are needed for the involvement of EVs as biomarkers in AD.

One clinical trial exploring the effects of IN administration of MSC-derived EVs in AD patients with mild-moderate dementia has begun in Shanghai, China (ClinicalTrials.gov Identifier: NCT04388982).

Table 4 summarizes the numerous EV resources, administration methods, and their mechanistic effects on the neurological disorders discussed above.

4 | ADVANTAGES OF EVS OVER SYNTHETIC NANOPARTICLES

Synthetic nanoparticles have advanced clinical drug delivery methodologies since the 1990s.[311] Despite their widespread use, the intricate molecular targeting strategies associated with synthetic nanoparticles often encounter formidable challenges, particularly concerning their interactions within the biological milieu, which

TABLE 4 Comprehensive overview of EVs isolated from different parent cell types, routes of administration, diseases treated, and main outcomes.

Pathology	EVs source	Mechanistic effect/result	Administration route	Animal model
ALS	1. WJ-MSCs	Enhanced expression of inflammatory and chemotactic markers in plasma	Intrathecal	Spinal cord injury[178]
	2. mASCs-exo	Improved impaired motor function, increased the number of surviving MNs	IV; IN	SOD1 (G93A)[186]
	3. hUCB-MSCs	Ameliorated muscle atrophy and rate of neuromuscular degeneration in skeletal muscle, reduced intracellular ROS levels	Intramuscular	hSOD1 G93A[179]
HD	1. C17.2 NSCs	Reduced the formation of HTT aggregates	Intrathecal	R6/2[208]
	2. Astrocytes	Reduced density of mHtt aggregates and decreases the expression of αB-crystallin	Stereotaxic	HD140Q KI[207]
MS	1. Oligodendrocyte-derived MSCs	Immune tolerance was restored via induction of immunosuppressive monocytes and apoptosis of autoreactive CD4+ T cells	IV	EAE[224]
	2. IFNγ-activated MSCs	Improved motor skills, reduced neuro-inflammation, and demyelination in mice	Systemic	EAE[141]
	3. Dendritic cells	Directly impact oligodendrocytes to increase myelination	IN	Ref. 230
PD	1. CM of MSCs from human exfoliated deciduous teeth	Rescued dopamine neurons of the SNpc and their terminals in the striatum; reversed motor deficits	IN	6-OHDA[249]
		Increased tyrosine hydroxylase in the striatum and decreased α-synuclein in both the nigra and striatum	IV	Rotenone[250]
	2. Cultured human umbilical cord MSCs	Reduced the degeneration of dopamine neurons and can traverse BBB	IV	6-OHDA[251]
Stroke	1. MSCs	Improved functional recovery and enhanced neurite remodeling, neurogenesis, and angiogenesis	IV	MCAO[274]
		Restored impaired basal synaptic transmission and synaptic plasticity, with inhibiting ischemia-induced COX-2 expression	Intracerebro-ventricular	Ischemia[276]

TABLE 4 (Continued)

Pathology	EVs source	Mechanistic effect/result	Administration route	Animal model
	2. NSCs	IFN-γ-hNSC-Exo showed therapeutic effects in comparison to hNSCs-Exo	Intracerebral	Ischemic stroke[278]
		Improved cellular, tissue, and functional outcomes	IV	Thrombo-embolic stroke[277]
		Exhibited reduced infarct volumes and preserved astrocyte function	IV	MCAO[279]
	3. ECs	Counteract cognitive deficits in a transient cerebral ischemia mouse model by modulating astrocyte and BBB function	IV	tMCAO[280]
	4. Astrocytes	Enhanced neuronal viability; inhibited apoptosis and expressions of caspase-3 and Bax	IV	MCAO[281]
AD	1. MSCs	Reduced Aβ plaque burden and amount of dystrophic neurites in the cortex and hippocampus	Stereotaxic	APP/PS1[142]
		Increased level of miR-146a and a decreased level of NF-κB observed in astrocytes	Intracerebral	APP/PS1[302]
		Plaque deposition and Aβ levels decreased; reduced activation of astrocytes	IV	APP/PS1[304]
	2. NSCs	Improved CREB activity, TrkB signaling and memory	IN	High fat diet[300]
		Protected synapses from Aβo-binding and Aβo-induced LTP inhibition	Intracerebro-ventricular	Nestin-δ-HSV-TK[301]
		Protected against synaptic loss in the AD brain	IV	5xFAD[303]
		Significant improvement in cognitive performance and synaptic morphology	Intracerebral	APP/PS1[305]

Abbreviations: 6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; BBB, blood–brain barrier; CM, conditioned media; EC, endothelial cell; HD, Huntington's disease; hUCB-MSCs, human umbilical cord blood-derived mesenchymal stem cells; IFN-γ, interferon-γ; IN, intranasal; IV, intravenous; KI, knock-in; mASCs-exo, murine adipose stromal cell; MCAO, middle cerebral artery occlusion; mHtt, mutant huntingtin; MN, motor neurons; MS, multiple sclerosis; MSCs, mesenchymal stem cells; NF-κB, nuclear factor-κB; NSCs, neural stem cells; PD, Parkinson's disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; SOD1, superoxide dismutase one; WJ-MSCs, Wharton's jelly-derived MSCs.

can substantially hinder their clinical efficacy.[312] In recent years, synthetic nanoparticles have also emerged as a promising approach to target and treat CNS disorders. Initially, scientists used poly(lactic-co-glycolic acid)-based nanoparticles for drug delivery,[313] but due to their limited capacity to carry drugs and their tendency to cause localized immune reactions, researchers shifted to lipid-based nanoparticles.[314] However, despite being safer and more effective, lipid nanoparticles still face issues like off-target accumulation and the risk of complement activation-related pseudo-allergy reactions, which has slowed their clinical adoption.[315] One of the notable setbacks in the synthetic nanoparticles domain is the documented failure of ligand-targeted nano-delivery approaches,[311] highlighting the pressing need for nuanced design considerations and advanced computational modeling techniques to decipher the complex interplay between nanoparticles and biological systems. While synthetic nanoparticles offer distinct spatial and temporal drug distribution advantages, their intricate designs may also significantly hinder cost-effective manufacturing processes. In contrast, EVs emerge as a promising alternative. With their naturally occurring complex bilayers and diverse cargo, EVs share structural similarities with liposomes and exhibit innate capabilities for efficiently transporting biomolecules to target cells. Moreover, EVs can be sourced from various origins, including cell cultures and biological fluids, rendering them a versatile and potentially superior platform for drug delivery.[24] The utilization of EV structures, honed through evolutionary selection, presents an intriguing avenue to circumvent numerous design and manufacturing challenges inherent in nanomedicine.[312] Specifically, the swift clearance of synthetic nanoparticles by macrophages in the liver presents a major challenge, as it hampers targeted delivery. To overcome this obstacle, numerous synthetic nanoparticles approved for clinical use have been modified with PEG. This modification efficiently decreases macrophage absorption, extending their circulation half-life from hours to several days.[311] However, using PEG is not without its drawbacks, prompting exploration into EV-based cargo delivery as an alternative strategy to evade immunological clearance, leveraging the intrinsic nature of the carrier. In addition to potentially surpassing synthetic carriers in biodistribution profiles, EV-based therapy offers the unique advantage of exploiting cellular processes for drug loading and surface modifications. Through genetic engineering, cells can be tailored to express and package protein- and RNA-based therapeutic agents and target ligands within the EVs field.[316] Leveraging cellular machinery for drug loading and EV surface modifications presents notable advantages, as it mitigates the risk of RNA and protein degradation or damage inherent in nanoparticle synthesis processes. Furthermore, EV endocytosis pathways or fusion events with recipient cell membranes hold promise for facilitating intracellular delivery, thereby targeting therapeutic agents to specific intracellular compartments or organelles. However, the realization of EV-based drug delivery faces its own challenges, including issues related to EV isolation, characterization, and concerns regarding rapid hepatic clearance, all of which necessitate further investigation and optimization.[317] Despite these hurdles, the immense promise of EV-based drug delivery is underscored by clinical trials reporting favorable safety profiles, indicating their potential as highly effective drug carriers with reduced adverse effects. Overcoming manufacturing challenges such as scalability and reproducibility will be paramount for the widespread adoption of EV-based drug delivery systems, heralding a new era in personalized and targeted therapeutic interventions.

5 | REGULATORY CONSIDERATIONS AND CLINICAL USE OF EV THERAPEUTICS

Emerging therapeutic modalities centered on EVs hold significant promise across diverse medical applications. While formal clinical approval for EV-based therapeutics is pending, early-phase clinical trials have explored the efficacy of autologous dendritic cell-derived EVs in cancer immunotherapy and allogeneic MSC-derived EVs for regenerative and anti-inflammatory purposes.[318] Noteworthy advancements include using tissue-specific EVs, such as lung spheroid cell-derived exosomes in idiopathic pulmonary fibrosis treatment.[319] The regulatory approval of

lipid nanoparticles, pioneered by Alnylam Pharmaceuticals for delivering siRNAs to manage hereditary transthyretin amyloidosis in 2018, represents a significant milestone in nanoparticle-based drug delivery systems. Lipid nanoparticles have played a prominent role in developing coronavirus disease 2019 vaccines by Moderna and Pfizer. However, existing lipid nanoparticles pose challenges due to their synthetic nature, necessitating extensive modifications to optimize their efficacy and safety. In contrast, being of natural origin, EVs offer inherent advantages for therapeutic use, possessing intrinsic biological properties that make them highly suitable for therapeutic interventions.[320]

EV-based therapies, categorized within the pharmaceutical realm as biologicals, adhere to established regulatory frameworks governing biological products in major regions like the US, Australia, Japan, and the European Union. Tailored criteria specific to EV-based therapies may be necessary to ensure their appropriate classification,[321] which is crucial for integrating EVs into clinical practice. Implementing regulatory measures concerning the manufacturing and application of novel therapies is critical, emphasizing safety considerations from multiple perspectives, including donor, recipient, product, production, clinical use, and biovigilance.[321] Despite established effectiveness, uncertainties persist regarding EV-based treatments' precise mechanisms of action. Challenges such as standardization of isolation protocols, purity of EV preparations, and potential side effects of chronic administration remain unresolved, making the reproducibility of experimental studies and clinical applications involving EVs challenging.

In addition, despite extensive research, the broader immunogenic or toxic effects of EVs are not well understood, particularly in large animal studies and clinical trials. Although efficacy is a primary focus in early preclinical development, it is equally important to identify potential toxicities and understand their underlying causes for experimental therapies. This knowledge gap is crucial, as therapeutic EVs, predominantly derived from human cell lines, will undergo testing in various small and large animal disease models during preclinical development before advancing to human trials. Similar to other biological therapeutics, such as cell-based therapies and monoclonal antibodies, which can induce immune responses, it is essential to assess the immunogenic potential of therapeutic EVs comprehensively. Previous studies have shown that the immunogenicity and toxicity of EVs depend on factors such as the animal models used for testing and the source and composition of the EVs.[322] Immortalized cell lines are commonly utilized for bulk production of EVs due to their ability to provide an infinite supply of cells for EV generation, rapid proliferation, and ease of genetic modification.[140,323] Among these cell lines, the HEK293T human embryonic kidney cell line is frequently employed for EV production in various studies, owing to its capacity to yield large quantities of EVs, ease of high transfection efficiency and growth.[322] However, despite these advancements, there is still limited knowledge regarding the immunogenicity of EVs in clinical trials,[324] highlighting the need for further research in this area.

In the commercial realm, numerous companies are dedicated to developing and applying EVs across various medical domains. However, intrinsic limitations of native EVs include challenges related to long-term maintenance of parental cell culture with minimal metabolic/phenotypic variation, as well as biodistribution and clearance rates.[139,325,326] Novel approaches such as synthetically tailored mimetic extracellular nanovesicles, EV synthetics (synEVs), and EV hybrids (hEVs) have emerged to address these limitations.[327] Unlike cell-derived EVs, synEVs lack targeting and recognition molecules, necessitating functionalization techniques such as bioconjugation and cargo loading to achieve use-specific synEVs. Additionally, fusion with EVs (native or mimetics) yields hEVs, a recent development comprising native EV components and synthetic liposomes.[328] These hybrids offer advantages such as loading versatility, targeting capabilities, and stability, potentially surpassing both EVs and liposomes. Thus, hybrid EVs represent a promising alternative with combined benefits applicable to theranostics.

As interest in EV-based therapies intensifies, enhancing design, manufacturing, and clinical administration processes is imperative. However, fundamental challenges such as low production yields, scalable and standardized EV generation, potency monitoring, regulatory compliance, and targeting capabilities must be addressed to pave the way for successful clinical translation.[329] Isolation techniques, such as ultracentrifugation, size-exclusion chromatography, and polymer-based precipitation methods, exhibit variable efficiencies and lack standardized

protocols, resulting in heterogeneous EV populations.[84] Scaling up production to meet clinical demands is further hindered by the absence of universally applicable, scalable isolation methodologies. Concentrating EVs from biological fluids presents another formidable challenge, given their low abundance and susceptibility to loss or contamination during enrichment processes.[68,327,330] Simultaneously, selecting the optimal route of administration is paramount, necessitating meticulous consideration of biodistribution dynamics, target tissue tropism, and systemic clearance rates.[140,327] Moreover, ensuring the stability and integrity of EVs during storage and transportation is crucial for maintaining their therapeutic efficacy. Addressing these multifaceted challenges will be pivotal in harnessing the full clinical potential of EVs as versatile mediators of intercellular communication and therapeutic delivery systems.

Delivering effective EV-based therapeutics also poses numerous challenges, particularly in determining appropriate dosing strategies to achieve therapeutic benefits while minimizing adverse effects. Critical considerations include selecting the right dose, assessing its efficacy, and determining optimal administration parameters such as route, frequency, and timing. To ensure the reliability of EV therapies, it is imperative to develop robust potency assays that can accurately measure therapeutic effects both in vitro and in vivo.[331–333] However, the lack of standardized potency assays limits their widespread acceptance and utilization. Before EV-based therapeutics can be approved for clinical use, thorough analytical assessments and rigorous testing for safety and efficacy are essential prerequisites.[334] These assessments encompass various aspects, including molecular fingerprinting, potency assays, and mechanistic studies, to comprehensively evaluate the therapeutic efficacy and safety profiles.[335,336] Despite advancements in analytical techniques, standardized methods for quantifying EV concentration and determining appropriate dosages are still lacking.

Early-stage development must monitor variables influencing the transition to clinical stages. Modifying current technologies for increased scalability can eventually meet clinical and commercial demands for EV-based medicinal production. Quality control remains a significant challenge. Moreover, data on the effects of EVs in treating neurological disorders often vary across different protocols and routes of administration, even for the same disease, hindering comparisons of efficacy among approaches. Intensified research efforts in this direction hold significant potential for establishing therapeutic protocols and elucidating the long-term effects of chronic administration, which is critical for clinical translation.

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

Neurological disorders represent a significant challenge in healthcare, necessitating early diagnosis and effective treatment strategies to halt or reverse the progression of these diseases. To address this urgent need, ongoing research efforts are focused on developing better therapeutic options to alleviate the burden of these disorders. In this context, EV-based therapeutics have emerged as a promising avenue for adjuvant treatments and biomarkers. The field of EV research in neurosciences is rapidly expanding, with increasing attention being directed toward understanding the potential of EVs in combating neurodegenerative disorders. EVs play a crucial role in intercellular communication by transferring various bioactive molecules, such as proteins, nucleic acids, and lipids, between cells. Their ability to traverse biological barriers and deliver cargo to specific target cells makes them an attractive option for therapeutic intervention. Despite the tremendous potential of EVs, several hurdles must be overcome before their application as an effective therapeutic agent. One significant challenge lies in understanding the biodistribution and pharmacokinetics of EVs in the context of neurodegenerative diseases. Comprehensive research is needed to elucidate how EVs interact with different cell types within the CNS, their distribution in various brain regions, and the factors influencing their clearance from the body. Such knowledge is essential for optimizing the therapeutic potential of EVs and ensuring their safe and efficient delivery to the target sites.

Furthermore, the development of effective therapeutic strategies utilizing EVs requires extensive investigation. Although initial studies have shown promising results, further research is needed to explore the specific

mechanisms by which EVs exert their therapeutic effects in different diseases. Understanding the cargo composition of EVs, including specific proteins, RNA molecules, or other bioactive molecules, and their functional roles in disease modulation is crucial for tailoring EV-based therapies to specific neurodegenerative disorders. In summary, EV-based therapeutics hold immense promise for addressing the challenges of debilitating neurodegenerative diseases. However, to fully harness the potential of EVs, it is imperative to conduct additional research focused on unraveling their biodistribution, pharmacokinetics, and the underlying mechanisms of their therapeutic effects. Such investigations will provide valuable insights that can drive the development of effective and targeted therapeutic strategies, ultimately improving the diagnosis, treatment, and overall management of neurodegenerative diseases.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article, because no new data were generated in this study.

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