

## Review

# Stem cell-derived extracellular vesicles in the therapeutic intervention of Alzheimer's Disease, Parkinson's Disease, and stroke

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## Abstract

With the increase in the aging population, the occurrence of neurological disorders is rising. Recently, stem cell therapy has garnered attention due to its convenient sourcing, minimal invasiveness, and capacity for directed differentiation. However, there are some disadvantages, such as poor quality control, safety assessments, and ethical and logistical issues. Consequently, scientists have started to shift their attention from stem cells to extracellular vesicles due to their similar structures and properties. Beyond these parallels, extracellular vesicles can enhance biocompatibility, facilitate easy traversal of barriers, and minimize side effects. Furthermore, stem cell-derived extracellular vesicles can be engineered to load drugs and modify surfaces to enhance treatment outcomes. In this review, we summarize the functions of native stem cell-derived extracellular vesicles, subsequently review the strategies for the engineering of stem cell-derived extracellular vesicles and their applications in Alzheimer's disease, Parkinson's disease, and stroke, and discuss the challenges and solutions associated with the clinical translation of stem cell-derived extracellular vesicles.

Keywords: stem cell; extracellular vesicle; neurological disease; drug delivery; clinical translation

## 1. Introduction

As the aging population has steadily expanded in recent decades, the incidence of Alzheimer's Disease (AD), Parkinson's Disease (PD), and stroke has continued to rise annually [1]. These conditions impact either the central or peripheral nervous systems, leading to impairments in brain function, spinal cord function, neuromuscular function, and peripheral nerves. These diseases are characterized by neuroinflammation, oxidative stress, mitochondrial dysfunction, impaired neuronal cells, disrupted blood-brain barrier (BBB) function, and the accumulation of abnormal proteins, resulting in lasting neurological deficits [2]. Despite remarkable strides and breakthroughs in pharmacological treatments, surgical techniques, and rehabilitation

modalities over the last fifty years, there is still no permanent solution; treatments only offer relief from symptoms and delay disease progression [3].

Stem cells (SCs), a category of undifferentiated cells with the potential for diverse specialization, self-replication, and self-renewal, represent a promising strategy. Their advantageous features, including a convenient source, minimal invasiveness, portability, and the ability to undergo directed differentiation in various inductive environments, underscore their potential in the realm of neurological therapeutics [4]. Various studies over the past few decades have shown that stem cell transplantation can promote neurogenesis and facilitate nerve healing post-injury [5]. However, stem cell application

encounters significant limitations due to the quality control, immune incompatibility, safety evaluation, ethical considerations and logical considerations, and tumorigenicity [6].

Extracellular vesicles (EVs) are small bilayer lipid structures discharged by most eukaryotic cells and tissue types [7-9]. Possessing structures and properties akin to cells, EVs present a distinctive advantage. SC-EVs differ from stem cells in that they neither replicate nor undergo uncontrolled division, which helps avoid issues related to the use of stem cells, such as the risk of tumor formation and the challenges of successful engraftment [10]. Importantly, SC-EVs possess the ability to cross the BBB to generate therapeutic impacts within the brain [11]. In addition, SC-EVs enhance the longevity and availability of therapeutic cargo in EV-based nanocarriers compared to EVs derived from other cells, thanks to the immuno-regulating characteristics acquired from the parent cells [12]. It has been also indicated that SC-EVs contribute to favorable outcomes, including extending therapeutic effects, stimulating the immune system, enhancing quality control, and maintaining long-term storage at  $-80^{\circ}\text{C}$  [13]. Furthermore, biomedical engineering technology can additionally optimize both the exterior and interior of SC-EVs, enabling them to target particular cells, enhance efficient BBB crossing, and attain specific therapeutic results [14, 15]. Inspiringly, the treatment efficacy of SC-EVs has been extensively explored in diverse neurological disorder models.

In this review, we summarize the methods of obtaining SC-EVs, including the isolation and differentiation of stem cells, and isolation and purification of extracellular vesicles. Then, we review the functions of native SC-EVs, including neuroprotection, angiogenesis and preservation BBB integrity, alleviation of neuroinflammation, and other functions. However, there are drawbacks including poor targeting efficiency, inconsistent therapeutic outcomes, and limited output efficiency, which can be solved by precondition, loading drug, and modified surface. Consequently, we summarize the strategies for the engineering of SC-EVs and their applications in AD, PD, and stroke. Ultimately, we outline the challenges linked to these extracellular vesicles in clinical translation and offer potential solutions.

## 2. From stem cell therapy to stem cell-derived extracellular vesicle therapy

The identification of SCs in the second half of the 19th century, recognizing them as an inherent mechanism for the body's development and self-repair, marked a revolutionary turning point in medical practice. This pivotal discovery substantially

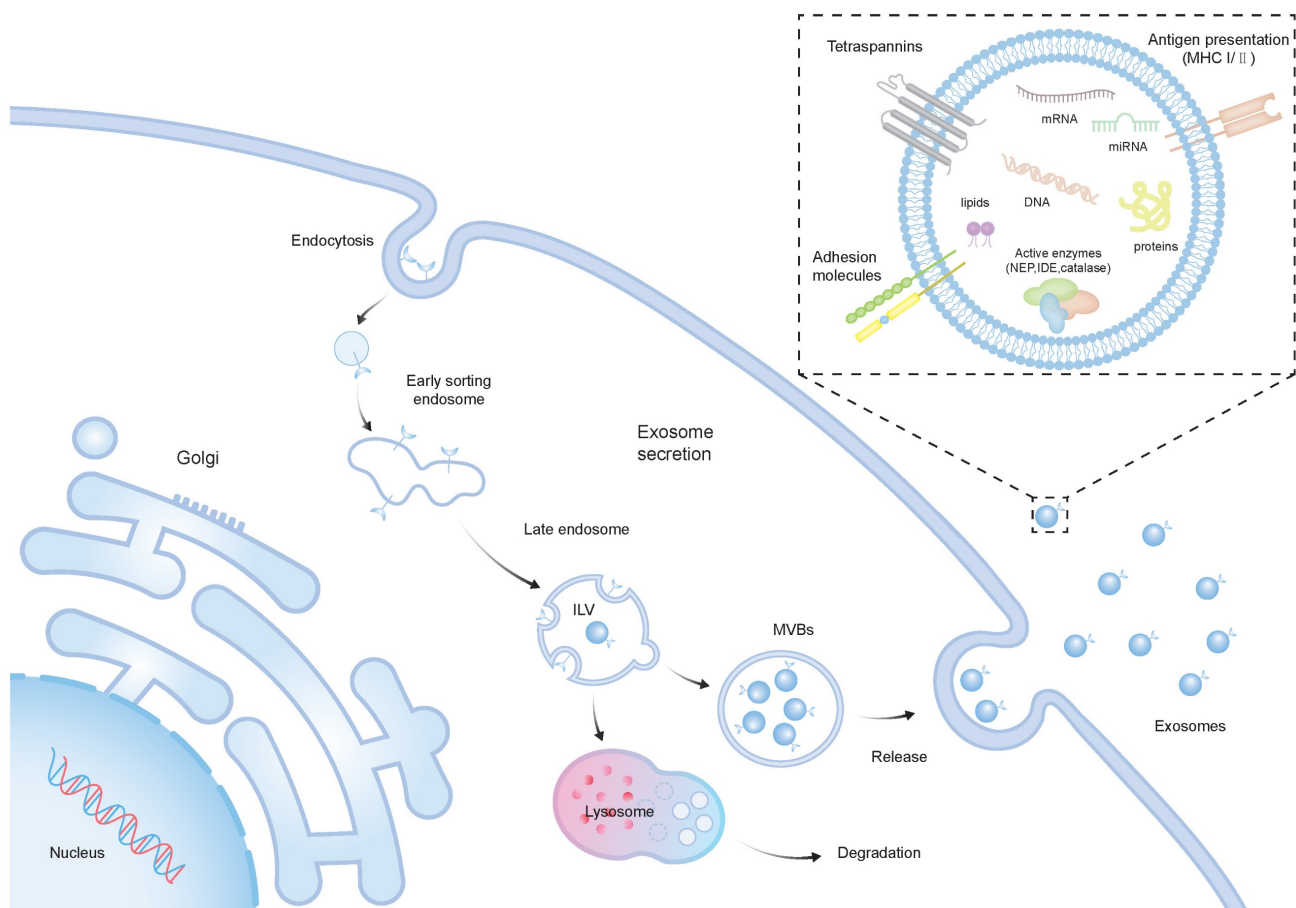
heightened interest in researching stem cells for the treatment of AD, PD, and stroke [16]. SCs are undifferentiated cells distinguished by remarkable attributes such as elevated proliferation, differentiation, and self-renewal capacities, setting them apart from specialized cells [17]. At present, SCs are commonly derived from four primary origins. The primary origins encompass (1) embryonic tissue, (2) fetal tissues (including the umbilical cord, amniotic fluid, and placenta), (3) particular sites within the mature organism, such as adipose, blood, bone marrow, skin, or skeletal muscle, and (4) differentiated somatic cells following genetic reprogramming, specifically induced pluripotent stem cells (iPSCs) [18]. SCs have various therapeutic potentials in addressing central nervous system (CNS) disorders, and they can contribute to neural injury repair through several mechanisms. Firstly, SCs can migrate to damaged neural sites, replacing dead or damaged nerve cells through a cell replacement mechanism, thereby repairing the compromised neural network. Then, SCs have the capability to secrete a significant quantity of active and nutrient factors, thereby stimulating neural cells, facilitating the regeneration and formation of new cells, and releasing angiogenic factors to encourage vascular generation at the site of injury. Moreover, SCs can modulate the immune response by adjusting the number of immune cells reaching the pathological site and secreting different levels of cytokines that mutually influence each other, providing a protective anti-inflammatory effect [19]. Nevertheless, the application of SCs faces notable limitations attributed to challenges in quality control, high cost, potential complications, one-off effects, as well as storage and transportation [20].

Recent investigations suggest that in models of neurological conditions, the treatment impact of SCs is associated with the paracrine mechanism, particularly involving EVs [21]. EVs, lipid membrane-enclosed extracellular structures, are typically released by various cell types. Moreover, they are frequently found in a range of bodily fluids, including breast milk, blood, and urine [22]. EVs encapsulate a rich cargo inherited from their parent cells, comprising cytosolic proteins, lipids, DNAs, and RNAs [23, 24]. They consist of exosomes (30–100 nm in diameter), microvesicles (200–1000 nm), and apoptotic bodies (500–5000 nm) [25]. The intricate process of EV biogenesis, with a primary focus on exosomes, mainly involves the internalization of the membrane to form early endosomes, and the maturation of early endosomes into late endosomes, resulting in the development of intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs) [7].

The majority of ILVs are expelled into the extracellular environment through a process where they merge with the cell's plasma membrane, resulting in the formation and release of exosomes [26] (Figure 1). As of now, over 9700 proteins, 3400 mRNAs, and 2800 miRNAs have been recognized within EVs. There is a diverse array of proteins that serve various functions in EVs from endosomes, peripheral membrane, and the cytosol, including receptor tyrosine kinases, and cytosolic signaling proteins [27]. The surface of EVs is adorned with numerous membrane proteins, among them annexins, flotillins, and tetraspanins, including CD81, CD9, CD63 [28].

In the context of regenerative medicine, SC-EVs have several advantages compared to cell-based therapies. An important benefit lies in the potential for SC-EVs to exhibit lower immunogenicity compared to their parent cells, contingent upon their origin. This could be attributed to the diminished presence of transmembrane proteins like MHC complexes on their surfaces [29]. In contrast to traditional nanocarriers such as liposomes, SC-EVs demonstrate superior biocompatibility and reduced

immunogenicity. These properties enable them to evade recognition and clearance by the host immune system following administration [30, 31]. Additionally, SC-EVs do not undergo replication following injection, reducing the risk of neoplastic growth and the latent transmission of viral pathogens [10]. Notably, SC-EVs demonstrate inherent capabilities to traverse tissue and cellular barriers, enabling their penetration through the BBB [11]. Furthermore, SC-EVs may exhibit intrinsic targeting properties, demonstrating an affinity for specific cells or tissues. This characteristic can be utilized to precisely transport drugs to their intended targets, thus minimizing off-target effects [32]. Moreover, in contrast to live cells, SC-EVs boast an extended shelf life, facilitating prolonged transport and storage capabilities. Interestingly, the surface and contents of SC-EVs can be finely tuned through advanced engineering and editing technologies to improve the therapeutic effects [33]. Numerous further instances highlight the successful preclinical applications of SC-EVs, with some summarized in subsequent sections of this review.



**Figure 1. The biogenesis and structure of exosomes.** The biogenesis of exosomes initiates with the inward budding of the plasma membrane, resulting in the creation of early sorting endosomes (ESEs), which may subsequently mature into LEs and MVBs. MVBs can either fuse with lysosomes for degradation or convey ILVs to the plasma membrane, releasing them into the extracellular space, thus forming exosomes. Exosomes are lipid membrane-enclosed extracellular structures, which include a variety of cellular proteins, lipids, DNAs, and RNAs with diverse biological functions.

### 3. Methods of obtaining SC-EVs

#### 3.1 Isolation and differentiation of SCs

Recent technical advances enable the acquisition of SCs through three distinct methods: (1) direct isolation from primary tissues, such as fetal tissues and specific sites within the adult organism; (2) differentiation from pluripotent stem cells, including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs); (3) trans-differentiation from somatic cells, which could be subsequently genetically reprogrammed [18].

Isolation of SCs from various tissues can be carried out using a range of methods that rely on varied physical, chemical, or surface molecule-based attributes of cells. These methods may involve considerations such as cell size and density, the presence of distinct cell surface markers, or cell adhesion to synthetic substrates.

Principal physical separation techniques, including gradient centrifugation method, cell adherence method and membrane filtration method, are influenced by the physical properties of cells such as size, density and adherence. These methods are rapid, simple and cost-effective. Nevertheless, these techniques are primarily utilized for enriching SCs from the tissue or for initial separation, which is then followed by more precise separation techniques such as fluorescence-activated cell sorting (FACS) and magnet-activated cell sorting (MACS) [34, 35]. Immunoaffinity separation techniques are highly specific methods that leverage the unique expression of surface molecules on cells. These techniques utilize monoclonal antibodies that are tailored to bind with precision to these molecules. The antibodies are conjugated to either fluorescent markers or magnetic particles, enabling the targeted cells to be selectively identified and separated. While these methods offer high precision and specificity, they also face challenges related to cost-effectiveness and meeting specific market demands [36].

Besides, SCs can be differentiated from ESCs and iPSCs. For example, the application of synthetic coatings has demonstrated efficacy in facilitating the derivation of induced mesenchymal stem cells (iMSCs). Employing a synthetic polymer coating, poly 2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide, during the derivation process from iPSCs resulted in a notable high differentiation efficiency [37]. Likewise, Chen *et al.* illustrated that the treatment of iPSCs with SB431542, an inhibitor of the transforming growth factor beta pathway, in a two-dimensional (2D) culture system led to the generation of epithelial monolayer-like cells.

Subsequent induction of epithelial-mesenchymal transition under these conditions resulted in rapid and reliable differentiation into iMSCs [38]. Regarding neural differentiation, pluripotent SCs can be broadly categorized into two primary pathways: embryoid body (EB) formation and monolayer culture [39]. Morizane *et al.* demonstrated that inhibitors can downregulate bone morphogenetic protein and TGF $\beta$ /activin/nodal signaling pathways, thereby promoting neural lineage specification [40]. Surmac *et al.* also indicated that the Notch-related transmembrane protein Dlk1 can facilitate the neural progenitor differentiation through Notch signaling [41].

Lastly, SCs can be trans-differentiated from somatic cells. Song *et al.* proposed a comprehensive protocol for generating neural-like progenitors from MSCs derived from bone marrow and umbilical cord blood [42].

#### 3.2 Isolation and purification of SC-EVs

The isolation of SC-EVs holds paramount importance in unraveling their biological activity mechanisms and unlocking their potential applications in treatment. Various isolation methods are selected for different purposes and applications, including ultracentrifugation, size-based isolation techniques, polymer precipitation, and immuno-affinity capture techniques, which are among the commonly employed options [43]. Methods for isolating and purifying SC-EVs from culture medium or biological fluids are summarized in Table 1.

At present, ultracentrifugation (UC) is widely regarded as the isolation technique and is considered the gold standard for extracting and separating SC-EVs, including differential and gradient ultracentrifugation. UC primarily isolates the necessary components by leveraging the disparities in size and density among the various constituents in the starting mixture, rendering it well-suited for separating components with substantial differences in sedimentation coefficient [51]. Differential ultracentrifugation effectively isolates SC-EVs and can yield high-purity samples. Nonetheless, the process of subjecting samples to repeated rounds of centrifugation, especially when high centrifugal forces are used, may result in damage to the vesicles that is not readily reversible [52]. As for gradient ultracentrifugation, SC-EVs migrate to specific density layers according to their size and density, allowing for further purification and separation of EV subpopulations. However, the high viscosity of the sucrose solution reduces the rate of sedimentation for SC-EVs, leading to longer processing times [53].

**Table 1.** The methods used for SC-EVs isolation and purification

Methods	Principle	Advantages	Disadvantages	References
Differential Ultracentrifugation	Differential Centrifugation of particles based on their size and density	High purity; Most commonly used method	Time-consuming; Damage or aggregation of EV	[44]
Density Gradient Ultracentrifugation	Specific density layers based on their size and density	High purity; Avoiding exosomal damage	Time-consuming; Complex operation; Limited sample volume	[45]
Size-Exclusion Chromatography	Based on hydrodynamic Radius	High yield; Preserves EV integrity; Fast; low-cost	Contamination with other particles of similar size	[46]
Ultrafiltration	Based on their size using membranes with specific pore sizes	High yield; High purity; Simple operation; Not require specialized equipment	Contamination; Low specificity	[47]
Polymer Precipitation	Precipitation by PEG	Fast; Simple; Not require specialized equipment	Relatively impure product; Low sample recovery	[48]
Immunoaffinity chromatography	Antibodies or aptamers that specifically bind to surface markers on EVs	High specificity; high sensitivity	Limited by availability of specific antibodies; High cost	[49]
Microfluidic Isolation	Based on various physical and chemical properties	Precise control and automation; Fast	Expensive equipment; Limited throughput for large samples	[50]

A novel method for isolating SC-EVs from large samples involves utilizing size-exclusion chromatography (SEC). The separation mechanism behind SEC is based on the principle that larger macromolecules are unable to penetrate the gel pores and are instead eluted alongside the mobile phase through the gaps between the porous gels, while smaller molecules are trapped within the gel pores until they are eluted by the mobile phase [44]. SEC offers a rapid, straightforward, and cost-effective means of isolating SC-EVs. This method ensures that the SC-EVs retain their intact structure, consistent size, and experience minimal changes to their inherent biological properties. However, there is a possibility of contamination with particles of comparable size, leading to a potential decrease in purity [43].

Ultrafiltration serves as an alternative size-based separation technique that operates on the principle of membrane filtration, where SC-EVs are separated based on their size through membranes with different pore diameters. Ultrafiltration is simpler and faster than ultracentrifugation, does not require specialized equipment, and can readily be scaled up for clinical applications involving extracellular vesicles. However, ultrafiltration may sometimes result in EV damage due to shear forces applied. This can be minimized by carefully adjusting the pressure applied to the membrane [54].

Moreover, the method of polymer precipitation has been reported to produce SC-EVs comparable to the gold standard of the differential centrifugation. The method of polymer precipitation commonly utilizes polyethylene glycol (PEG) to facilitate the collection of SC-EVs. This process involves the reduction of the SC-EVs' solubility, followed by their isolation through centrifugation. The polymer precipitation method offers ease of operation with a quick analysis time and is well-suited for handling large sample volumes. Nevertheless, it is associated with relatively lower purity and recovery rates, potential false positives, and the challenge of removing the polymer residue, which may hinder

subsequent functional experimental analysis [55].

Furthermore, immunoaffinity chromatography, a method for separation and purification, relies on the precise interaction between antibodies and ligands to extract desired substances from heterogeneous mixtures. This technique ensures robust specificity, remarkable sensitivity, exceptional purity, and a high yield. However, its practical application is hindered by the high cost and the availability of specific antibodies [56].

In general, while every approach has its advantages and disadvantages, their drawbacks can be mitigated by integrating multiple purification techniques to improve both the quality and quantity.

## 4. The functions of native SC-EVs

Various studies have illustrated that administering SC-EVs can lead to the favorable outcomes in animals, including neuroprotection, angiogenesis and preservation of BBB integrity, alleviation of neuroinflammation, and other functions. The focus is particularly on MSC-EVs and NSC-EVs (Table 2).

### 4.1 Neuroprotection

Neuroprotection refers to strategies and interventions aimed at preserving the stability of the CNS microenvironment, as well as preserving and safeguarding the health and function of the nervous system, particularly the brain. According to the research, alterations in neurotrophic factor expression or their receptors are believed to contribute to neuronal deterioration and are involved in the pathogenesis of neurological diseases [86]. Inspiringly, EVs offer a promising therapeutic avenue for neurological disorders, as they are capable of transporting essential neurotrophic factors to the brain and stimulating neuroprotective mechanisms [87]. These neurotrophic factors (NTFs) are a class of endogenous biomolecules that include brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and glial cell-derived neurotrophic factor

(GDNF). They are pivotal in driving cell proliferation and differentiation within the nervous system, contributing to the maintenance and repair of neural tissues [88]. Moreover, molecular analysis has convincingly demonstrated the presence of NTFs in EVs derived from MSCs [89, 90]. It has been demonstrated that EVs derived from MSC therapy work as paracrine effectors responsible for promoting functional recovery through proteomics analysis of the EVs that identified many proteins that could be implicated in brain repair function [91]. For example, Liu *et al.* exhibited that administration of BMSC-EVs

via the lateral ventricle enhanced AD-like behavioral performance in mice injected with streptozotocin (STZ). The enhancements are likely due to the influence on glial cell activation, which leads to a decrease in neuroinflammatory responses, as well as the positive impact of BDNF related neuropathological changes within the hippocampal region [58]. Several studies have shown that NSC-EVs exhibit a therapeutic effect similar to MSC-EVs by transporting neurotrophic factors, including BDNF, NGF, and GDNF [92].

**Table 2.** Functions of native SC-EVs

Disease	Source of EVs	Animal Model	Administration Route	Therapeutic Effects	Mechanism of Action	Reference
AD	hUCMSCs	APP/PS1 mice	tail vein	↑cognitive impairments ↓hippocampal Aβ aggregation ↓neuronal loss	↓Nrf2	[57]
	mBMSCs	STZ mice	lateral ventricle tail vein	↑behaviors function ↓hyperactivation of microglia and astrocytes ↓inflammation	↑synapse-related proteins ↑BDNF	[58]
	hiMSCs	STZ mice	intracisternal	↓neuroinflammation ↓amyloid deposition ↓neuronal apoptosis	containing miR-223-3p ↓NLRP3/GSDMD	[59]
	rBMSCs	Aβ <sub>1-42</sub> -injected rats	lateral ventricle	↓cognitive dysfunction ↑cognitive function ↓Aβ plaques, Aβ deposition areas and levels of Aβ <sub>1-42</sub> -injected ↑NEP and IDE ↓inflammatory cytokine	containing miR-29c-3p ↓BACE1 ↑Wnt/β-catenin	[60]
	hBMSCs	5XFAD mice	intranasally	↑cognitive function ↓Aβ plaque	NA	[61]
	mNSCs	APP/PS1 mice	lateral ventricle	↑cognitive behavior ↑mitochondrial biogenesis ↓astrocyte activation	↑SIRT1 ↑PGC1c ↑NRF1 ↑COXIV	[62]
	PD	TMSCs	MPTP mice	intraperitoneally	↓the loss of DA neurons ↑nigro-striatal system function ↑motor function ↓oxidative stress	containing miR-100-5p ↓NOX4 ↓ROS ↑Nrf2
hUCMSCs		6-OHDA rats	tail vein lateral ventricle	↑motor function ↑dopamine content ↓neuronal damage ↓microglial activation	contain MiR-7, miR-125-5p, miR-122-5p, miR-126-3p, miR-199-3p	[64]
hBMECs		MPTP mice	intraperitoneally	↑angiogenesis	↑ICAM1-SMAD3/P38MAPK	[65]
hNSCs		6-OHDA mice	intracerebral	↑neuroprotection ↓dopaminergic neuronal loss ↓pro-inflammatory cytokines	containing hsa-mir-182-5p, hsa-mir-183-5p, hsa-mir-9, hsa-let-7 ↓ROS	[66]
stroke		rADMSCs	MCAO rats	lateral ventricle	↓brain injury ↑neuron viability ↓apoptosis	↓associated apoptotic pathways containing miR-22-3p ↓KDM6B ↓BMP2/BMF axis
	mBMSCs	MCAO mice	tail vein	↓infarct area ↓neuronal injury ↓apoptosis	containing KLF4 ↑lncRNA-ZFAS1 ↓Drp1 m6A modification by targeting FTO	[68]
	mNPCs mADMSCs	MCAO mice	femoral vein retroorbital	↑neurological recovery ↑neuroprotection ↑cell proliferation ↓pro-inflammatory	NA	[69]
	rBMSCs	MCAO mice	tail vein	↓infarct volume ↑neurological functions ↑microvessel density	↑miR-21-5p ↑VEGF, VEGFR2, Ang-1, and Tie-2	[70]
	BMSCs	MCAO rats	tail vein	↓cerebral infarction ↓BBB leakage ↓neurological function deficits	↑ZO-1 ↑Occludin ↓MMP activity ↓Caveolin-1	[71]

Disease	Source of EVs	Animal Model	Administration Route	Therapeutic Effects	Mechanism of Action	Reference
	hBMSCs	MCAO rats	tail vein	↓infarct volum ↓motor-coordination deficits ↓macrophage infiltrates ↑angiogenesis ↑neurogenesis	↓CD147 ↓VEGFR2 ↓VEGFA NA	[72]
	BMSCs	pMCAO rats	tail vein	↓cerebral infarction volume ↓BBB permeability ↑neurological function	↓Cav-1 ↑ZO-1 ↑Claudin-5	[73]
	hADMSC	MCAO rats	intranasally	↓infarct volume ↑long-term motor ↑behavioral impairment	NA	[74]
	hUCMSCs	MCAO mice	tail vein	↓TPA-induced disruption of BBB integrity ↓hemorrhage ↓astrocyte activation and inflammation	miR-125b-5p targete TLR4 ↓NF-KB signaling in astrocytes	[75]
	mNPCs	MCAO mice	femoral vein	↑poststroke BBB integrity ↓inflammatory cell recruitment	↓ABCB1 and MMP-9 regulation ↓NF-κB pathway	[76]
	mBMSCs	tMCAO P9 mice	ventricle intranasally	↓injury volume ↓cytokine/ chemokine accumulation	NA	[77]
	hBMSCs	MCAO rats	tail vein	↓infarct volume ↑neurological function ↓neuronal deat	↑IL-33 ↓ST2	[78]
	hBMSCs	MCAO young mice MCAO aged mice	intravenously	↓neurological deficits ↓infarct volume ↓brain edema ↓neuronal injury ↑anti-inflammation ↓leukocyte infiltrate ↓monocytes and activated T cells	NA	[79]
	rBMSCs	MCAO rats	tail vein	↓brain infarct area ↓brain water content ↑neurological function ↑M1-polarized microglia shifting toward M2 phenotype	↓NLRP3 inflammasome-related proteins ↓pyroptosis-related proteins	[80]
	hUCMSCs	MCAO mice	tail vein	↓infarct volume ↓behavioral deficits ↓microglia activation ↓neuroinflammation	containing miR-146a-5p ↓IRAK1/TRAF6 pathway	[81]
	hiNSCs	MCAO mice	lateral ventricle	↓inflammatory response ↓oxidative stress ↑NSCs differentiation ↓cerebral infarction ↓neuronal death ↓glial scarring ↑recovery of motor function	containing hsa-miR-30a-5p containing hsa-miR-7-5p	[82]
	rNSCs	MCAO/R Rat	tail vein	↓the infarction of brain tissues ↓neuronal pyroptosis	carrying YBX1 ↑stability of m6A-modified GPR30 by interacting with IGF2BP1 ↑GPR30 ↓activation of NLRP3 inflammasome through promoting NLRP3 ubiquitination by SPOP	[83]
	BMSCs of young monkey	cortical injury aged monkeys	intravenous	↑functional recovery ↓damaged oligodendrocytes	↑myelin-related genes ↑actively myelinating oligodendrocytes in sublesional white matter	[84]
	mNSCs	MCAO mice	left stratum	↑myelin maintenance ↓infarct volume ↓neurological function ↑OPC differentiation	containing miR-128-3p ↑myelin basic protein expression ↓BMP signaling	[85]

AD, Alzheimer's Disease; PD, Parkinson's Disease; h, human; r, rat; m, mouse; BMSCs, bone marrow-derived mesenchymal stem cells; UCMSCs, Umbilical cord mesenchymal stem cells; ADMSCs, adipose-derived stem cells; iMSCs, induced pluripotent stem cell-derived mesenchymal stem cells; NSCs, neural stem cells; T-MSCs, trophoblast stage-derived mesenchymal stem cells; NPCs, neural progenitor cells; iNSCs, induced pluripotent stem cell-derived neural stem cells; 5XFAD mice, 5 familial Alzheimer's disease mutations; MCAO, middle cerebral artery occlusion; pMCAO, permanent middle cerebral artery occlusion; 6-OHDA, 6-hydroxy-dopamine; Nrf2, nuclear factor E2-related factor 2; STZ, streptozotocin; BDNF, brain-derived neurotrophic factor; Aβ, amyloid β; BACE1, β-site amyloid precursor protein cleaving enzyme 1; SIRT1, sirtuin 1; PGC1c, peroxisome proliferator-activated receptor-γ coactivator-1α; NRF1, nuclear respiratory factor 1; COXIV, cytochrome C oxidase IV; ROS, reactive oxygen species; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; BMP2, Bone morphogenetic protein 2; BMF, Bcl-2 modifying factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; MMP, matrix metalloproteinase; Cav-1, Caveolin-1; BBB, blood-brain barrier; ABCB1, ATP-binding cassette transporter B1; MMP-9, matrix metalloproteinase 9; NF-κB, nuclear factor-kappa B; IL, interleukin; ST2, suppression of tumorigenicity 2 receptor; NLRP3, NACHT, LRR and PYD domain-containing protein 3; IRAK1, interleukin-1 receptor-associated kinase 1; TRAF6, TNF receptor-associated factor 6; YBX1, Y box binding protein; GPR30, G protein-coupled receptor 30; SPOP, speckle-type POZ protein; BMP, bone morphogenetic protein; OPCs, oligodendrocyte progenitor cells; MAPK, mitogen-activated kinase; KLF4, Kruupel-like factor 4; ZFAS1, zinc finger antisense 1; FTO, targeting obe- sity-associated protein; NA, not available.

Some researches revealed that EVs administered are effectively assimilated by dopaminergic neurons within the affected hemisphere of the brain. EVs demonstrated significant efficacy in ameliorating damage to the nigral-striatal dopamine system while concurrently mitigating microglial activation [64]. Wang *et al.* revealed that MSC-EVs effectively ameliorated cognitive impairments and neuronal loss. The involvement of the Nrf2 signaling pathway was observed in the mechanisms underlying the actions of MSC-EVs in APP/PS1 mice (Figure 2B) [57]. Besides, He *et al.* conducted a study assessing the impact of T-MSC-EVs, which are enriched with miR-100-5p, on dopamine neuron damage and oxidative stress in both MPTP-induced PD mouse models and MPTP-induced MN9D cells. The T-MSC-EVs were found to directly target the 3' untranslated region of the NOX4 gene. This specific targeting mechanism was shown to provide neuroprotection against the degeneration of dopamine neurons, maintain the integrity and functionality of the nigrostriatal pathway, reduce motor impairments, and decrease oxidative stress via the Nox4-ROS-Nrf2 signaling pathway in PD models [63]. Accumulating evidences have shown that treatment with MSC-derived EVs markedly ameliorated in vivo ischemia/reperfusion brain injury, enhanced neuron viability in vitro, and reduced apoptosis. Liu *et al.* demonstrated that MSC-EVs attenuated neuronal injury caused by oxygen and glucose deprivation (OGD) through the IL-33/ST2 signaling pathway in astrocytes, thereby reducing ischemia-induced brain injury in a mouse model [78]. Interestingly, MSC-EVs exhibited upregulation of miR-22-3p, and inhibition of EV-miR-22-3p led to heightened apoptosis and reduced neuronal survival. Mechanistically, miR-22-3p derived from MSC-EVs mitigated ischemic brain injury by suppressing KDM6B-mediated actions on the BMP2/BMF axis [67]. Exosomes enriched with KLF4 have shown considerable promise in mitigating the impact of MCAO in mouse models, effectively decreasing infarct size, neuronal injury, and apoptosis. This therapeutic effect is realized through the influence on the lncRNA-ZFAS1/FTO/Drp1 signaling axis. Importantly, KLF4 originating from BMSCs promotes the elevation of long non-coding RNA ZFAS1 levels. This increase in ZFAS1 expression subsequently leads to the inhibition of Drp1's m6A methylation, a regulatory event carried out by the RNA demethylase FTO [68]. Besides, MSC-EVs facilitated the restoration of functional neurological capacity and brain tissue restructuring in aged rats following a stroke. They effectively mitigated motor-coordination deficits in both young and aged rats at both doses administered. Notably, in aged rats,

EVs significantly decreased brain macrophage infiltration. Additionally, EV treatment promoted neurogenesis in the subventricular zone [69].

## 4.2 Angiogenesis and preservation BBB integrity

SC-EVs additionally contribute to the therapeutic effect through improving angiogenesis. Angiogenesis, the process of forming new blood vessels, serves as a vital physiological defense mechanism critical for replenishing oxygen and nutrient supply to injured brain tissue after ischemic events. By promoting blood vessel growth, angiogenesis holds promise in stabilizing brain perfusion and boosting neuronal survival, neurological recovery, and brain plasticity [93]. Xue *et al.* demonstrated that MSC-derived EVs maintain the transcriptional activity of human brain microvascular endothelial cells (HBMECs), which represent a predominant component of the brain's microvasculature. Additionally, MSC-derived EVs were observed to facilitate the recovery in a PD mouse model by promoting angiogenesis related to ICAM1 [65]. Hu *et al.* found that BMSC-EVs significantly improved neurological function, decreased the size of cerebral infarcts, and boosted the density of microvessels along with the expression of miR-21-5p in the context of cerebral ischemia. Additionally, in vitro studies revealed that BMSC-EVs also enhanced the functionality of human umbilical vein endothelial cells (HUVECs), as evidenced by increased proliferation, migration, and tube formation capabilities [70].

The BBB is a highly selective and protective barrier which controls the blood circulation to the brain and the CNS, preventing potentially harmful exogenous molecules from entering the brain and maintaining homeostasis. Thus, it assumes a pivotal role in both the initiation and management of AD, PD, and stroke [94]. Interestingly, MSC-EVs effectively traversed the BBB and exhibited selective accumulation within injured brain regions. Subsequently, these accumulated MSC-EVs mitigated tPA-induced BBB disruption and reduced hemorrhage by suppressing astrocyte activation and inflammation in murine ischemic stroke models. Mechanistically, miR-125b-5p, delivered by MSC-EVs, played a pivotal role in preserving BBB integrity through its targeting of TLR4 and inhibition of NF- $\kappa$ B signaling in astrocytes [75]. Some evidence showed that a single dose of EVs administered intranasally 24 hours post-focal permanent ischemic stroke in rats led to significant improvements. These improvements included preservation of BBB integrity, and enhanced vascular stability within the salvageable peri-infarct



region. Moreover, there was a noteworthy decrease in the volume of the infarct (Figure 2C) [74]. Li *et al.* demonstrated that administrations of both brain endothelial cell (BEC) and BMSC-derived EVs improved cerebral infarction, BBB integrity, and neurological deficits in MCAO rats. Furthermore, BMSC-EVs showed comparable inhibitory impacts to BEC-EVs on the Cav-1-mediated endocytosis of ZO-1 and Claudin-5. Notably, BMSC-EVs were found to suppress Cav-1 expression and improve neurological function [73]. Additionally, the elevated levels of CD147, Caveolin-1, vascular endothelial growth factor A (VEGFA), and vascular endothelial growth factor receptor 2 (VEGFR2) in brain microvessels were decreased following EVs administration. Moreover, metalloproteinase (MMP) activity inhibition was observed. [71]. Similarly, several studies have demonstrated that NSC-EVs successfully preserve BBB integrity in various disease models. For example, Zhang *et al.* discovered that EVs enhance poststroke BBB integrity by decreasing the expression of ATP-binding cassette transporter B1 (ABCB1) and downstream matrix MMP-9 activity in stroke mice [76].

### 4.3 Alleviation of neuroinflammation

Neuroinflammation is a key contributor to neurological diseases and injuries, and excessive inflammation can lead to neuronal damage. In patients suffering from neurological diseases, it is common to observe aberrant activation of astrocytes and microglia, along with elevated levels of pro-inflammatory molecules [95]. Emerging evidence has proven that SC-EVs play an important role in anti-inflammatory. The accumulation of MSC-EVs in the hemisphere ipsilateral to the occlusion leads to a reduction in injury volume, providing protection during the sub-acute injury phase. By 72 h, MSC-sEVs in the IN group were primarily localized within Iba1+ cells with retracted processes, as well as within GLUT1+ blood vessels in regions subjected to ischemic-reperfusion [77]. Treatment with MSC-EVs markedly decreased infarct volume, alleviated behavioral deficits, and mitigated microglial activation three days after transient brain ischemia. Zhang *et al.* demonstrated that miR-146a-5p contained within hUMSC-EVs diminishes microglia-mediated neuroinflammatory responses by targeting the IRAK1/TRAF6 pathway [81]. Lee *et al.* found that the intracerebral injection of NSCs-EVs into the substantia nigra (SN) region of PD model mice led to a noteworthy reduction in neuroinflammation in that area. This reduction was associated with a significant decrease in the presence of activated microglia, reactive astrocytes, and pro-inflammatory factors

within both the striatum and SN region, which is crucial for PD treatment (Figure 2D) [66].

The NLRP3 inflammasome, a complex assembly of proteins, is integral to the innate immune system's response to invading pathogens and signals of cellular distress by initiating the release of pro-inflammatory cytokines [96]. Some studies have demonstrated that SC-EVs can mitigate neuroinflammation by inhibiting NLRP3. For example, the intracisternal administration of iPSC-MSC-EVs effectively mitigated neuroinflammation and ameliorated cognitive deficits in an AD mouse model. This beneficial effect is attributed to the inhibition of NLRP3/GSDMD-driven neuroinflammation by miR-223-3p [59]. Additionally, NSC-EVs, enriched with Y box binding protein (YBX1), have been demonstrated to augment the stability of the m6A-modified G protein-coupled receptor 30 (GPR30) via interaction with IGF2BP1. This stabilization leads to an upregulation of GPR30, which in turn suppresses NLRP3 inflammasome activation by promoting the ubiquitination of NLRP3 by the speckle-type POZ protein (SPOP). Consequently, this mechanism significantly contributes to the suppression of neuronal pyroptosis in the context of ischemic stroke [83].

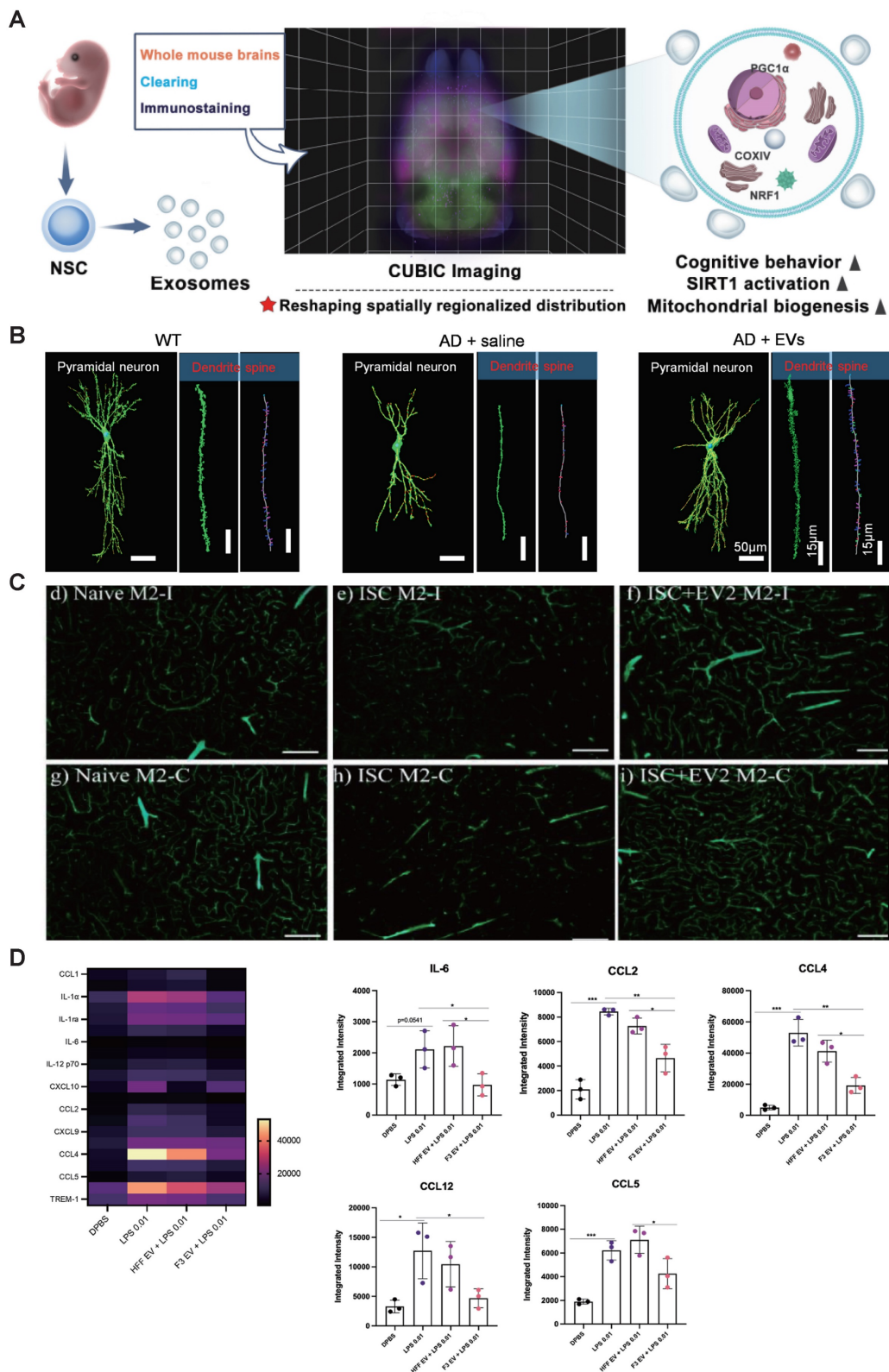
There is a wealth of research indicating that EVs alleviate the recruitment of inflammatory cells. EVs significantly reduced leukocyte infiltration, particularly polymorphonuclear neutrophils, monocytes, and macrophages, within the ischemic brain tissue of older mice. Additionally, MSC-EVs have caused a noteworthy reduction in the peripheral blood levels of monocytes and activated T lymphocytes [79]. However, the immunomodulatory effects of EVs are subject to dynamic changes and have a finite duration. Zheng *et al.* indicated that NSC-EVs had no impact on leukocytes, monocytes, B cells, or T cells in MCAO mice. Instead, NSC-EVs were discovered to counteract post-ischemic peripheral immunosuppression, resulting in elevated levels of T and B lymphocytes in the bloodstream [69].

### 4.4 Other functions

Pathological proteins, exemplified by amyloid- $\beta$  (A $\beta$ ) plaques and hyperphosphorylated tau, play a central role in AD pathology, which results in synaptic disruption and neuronal degeneration. The substantial benefits of removing these abnormal proteins are evident in the treatment of AD [97]. Recent research has revealed that MSC-EVs delivered miR-29c-3p to neurons, effectively inhibiting  $\beta$ -site amyloid precursor protein cleaving enzyme 1 (BACE1) expression while activating the Wnt/ $\beta$ -catenin pathway. This dual mechanism led to increased expression of A $\beta$  decomposition-related

factors (NEP and IDE), reduced formation of amyloid- $\beta$  ( $A\beta$ ) plaques and deposition areas, as well as lowered levels of  $A\beta$ 1-42 and inflammatory cytokine levels [60]. Similarly, Cone *et al.* demonstrated that a reduced  $A\beta$  plaque burden was

noted in the hippocampus of mice treated with EVs. Notably, diminished colocalization between glial fibrillary acidic protein (GFAP) and  $A\beta$  plaques was evident in the brains of EV-treated mice relative to those treated with saline [61].



**Figure 2. The functions of native SC-EVs.** (A) NSC-EVs remodel the abnormal distribution of mitochondrial biogenesis-related proteins throughout the brain and enhance mitochondrial function. Adapted with permission from [62], copyright 2023 Medknow. (B) The administration of MSC-EVs restores hippocampal neuronal morphology in mice.

Adapted with permission from [57], copyright 2021 Springer Nature. (C) The parameters of brain blood vessels were evaluated in the region of the supplementary motor cortex following the administration of extracellular vesicles on the 42nd day post-treatment. Adapted with permission from [74], copyright 2021 MDPI. (D) Anti-inflammatory effect of EVs on LPS-treated cells. Copyright 2022 Lee E. J. et al. Adapted with permission from [66], copyright 2022 BMC.

SC-EVs also exert a therapeutic effect enhancing the differentiation of oligodendrocyte progenitor cells (OPCs). Fibrinogen deposition hindered remyelination post-MCAO by impeding oligodendrocyte progenitor cells (OPC) differentiation through the activation of ACVR1, the type I receptor of bone morphogenetic protein (BMP) signaling. Hou *et al.* discovered that exosomal miR-128-3p derived from NSCs significantly elevated myelin basic protein expression in OPCs while suppressing BMP signaling. Moreover, NSC-derived exosomal miR-128-3p conferred protection against fibrinogen-induced demyelination linked to BMP signaling, resulting in a decrease in infarct volume and improvement in neurological function following MCAO [85]. In primate models, EVs treatment led to a reduction in damaged oligodendrocyte density and improved myelin maintenance. The study's findings were confirmed by observing an elevation in the expression of genes associated with myelin production and a rise in the number of oligodendrocytes actively involved in myelination within the white matter areas surrounding lesions. The enhancements in myelination were positively linked to the pace of motor function restoration, suggesting that improved maintenance of myelin contributes to the recuperation process following brain injury in older primates [84].

Interestingly, SC-EVs can improve mitochondrial biogenesis. Li *et al.* demonstrate that EVs derived from neural stem cells markedly enhance mitochondrial biogenesis by activating the sirtuin 1 (SIRT1) - peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC1 $\alpha$ ) signaling pathway and increasing the synthesis of nuclear respiratory factor 1 (NRF1) and cytochrome C oxidase IV (COXIV). Moreover, these EVs inhibit astrocyte activation, although they do not suppress amyloid- $\beta$  production (Figure 2A) [62].

In conclusion, these studies underscore the promising therapeutic applications of both MSC-EVs and NSC-EVs in the treatment of neurological disorders. Both types of EVs offer similar therapeutic benefits, albeit through different combinations of mechanisms that promote neural repair, including neuroprotection, angiogenesis and preservation BBB integrity, alleviation of neuroinflammation, and other functions. However, the choice of the most appropriate SC-EVs for particular neurological diseases remains uncertain. While some studies have shown that NSC-EVs may be more effective than MSC-EVs, for instance, in a comparative analysis of the therapeutic impacts of NSC-EVs and MSC-EVs, it

was determined that NSC-EVs were more effective. They demonstrated superior results in mitigating neural injury and bolstering the systemic immune system's response within a mouse model of thromboembolic stroke [98]. This finding is supported by another study showing that NSC-EVs outperformed MSC-derived EVs in addressing neuroinflammation [99]. Besides, in a head-to-head evaluation, the therapeutic efficacy of NPC-EVs was determined to be at least as effective as that of MSC-EVs. The benefits are clear, not just in terms of boosting cells' resistance to damage caused by a lack of oxygen in laboratory settings, but also in promoting the regeneration of brain tissue after a stroke and aiding in the recovery of neurological functions within a mouse model of stroke [69]. Nevertheless, these findings should not be construed as indicating that MSC-EVs are inherently less advantageous than NSC-EVs. Therefore, further evidence and a better understanding of the underlying mechanisms are needed to facilitate the translation of these findings into clinical applications.

## 5. Engineered SC-EVs for therapies

Although SC-EVs have considerable promise as a therapeutic strategy, there are a few drawbacks such as low targeting efficiency, inconsistent treatment outcomes, and limited production yield [100]. These challenges can be addressed through various engineering biotechnologies (Figure 3).

### 5.1 SC-EVs engineered techniques

#### 5.1.1 Preconditioned SC-EVs

The generation of SC-EVs is affected by the cell type and surrounding conditions, yet it can be enhanced through artificial means. There are two prevalent techniques employed for the preconditioning of SC-EVs: (1) improving the conditions within which cells are cultured, such as by employing three-dimensional (3D) culture techniques, and (2) applying external stimuli to the cells.

3D culture is a laboratory technique used to culture and study cells in an environment that more closely resembles the natural three-dimensional structure of tissues and organs within the human body. Some studies have demonstrated that MSCs within a hollow fiber bioreactor increased the total production of EVs by up to 19.4-fold in 3D culture [101], and when combined with tangential flow filtration (TFF), MSCs can enhance the yield of EVs by as much as 100-fold [102]. Besides, stem cells can

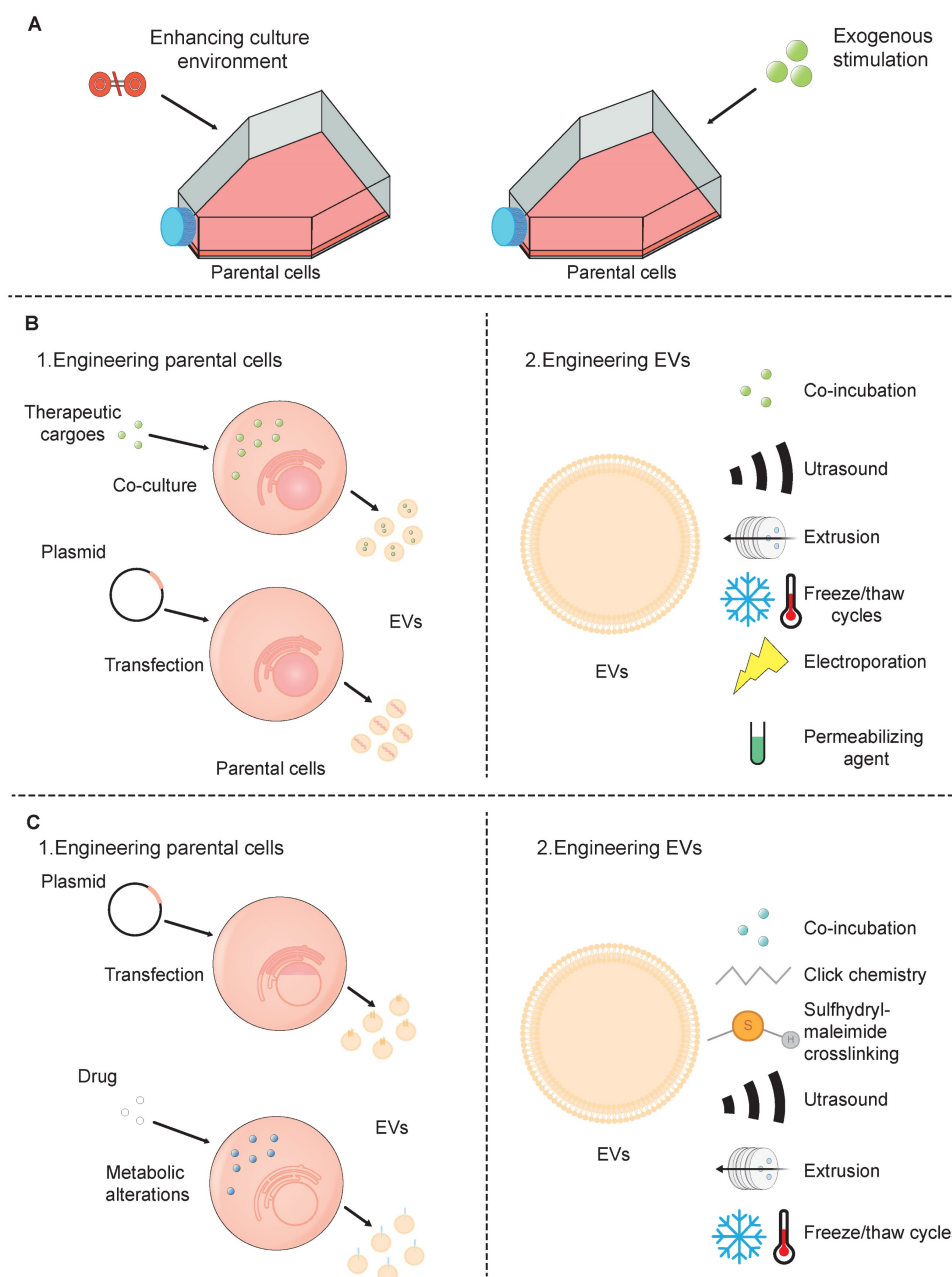
undergo preconditioning during cell culture to enhance the bioactivity of SC-EVs, such as hypoxia [27, 103, 104], proinflammatory cytokines [105, 106], Chemical stimuli [107], low electric currents [108], and collagen scaffolds [109]. However, it is crucial to examine the contents generated within SC-EVs that could potentially have adverse effects on therapeutic outcomes because preconditioning can impose stress on parental cells.

### 5.1.2 Drug-loaded SC-EVs

As cell engineering progresses, researchers now possess the capability to genetically program cells in a way that enables them to incorporate specific cargo

into SC-EVs. This cargo includes a range of therapeutic agents such as peptides, proteins, chemical compounds, and nucleic acids. The current strategies for engineering SC-EVs can be broadly categorized into two directions: (1) pretreating the donor cells, followed by the isolation of drug-loaded SC-EVs from stem cells; and (2) directly loading drugs in isolated SC-EVs.

Strategies for the pretreatment of donor cells are typically classified into two primary types: (1) direct co-culture of donor cells with drugs; and (2) genetic engineering of donor cells. In the initial approach, drugs are cultivated in conjunction with the donor cells, leading to their inherent presence within the



**Figure 3.** Strategies for the engineering of SC-EVs. (A) preconditioning of parental cells; (B) loading of therapeutic cargoes; and (C) surface modification.

SC-EVs that are subsequently released [110]. This technique is quite simple and straightforward, eliminating the need for further modifications of the SC-EVs. Nevertheless, the transfection efficiency obtained through this approach tends to be on the lower side, which limits its application to primarily chemical substances that have a low level of cytotoxicity [111]. In the second method, donor cells are transfected with plasmid encoding genes of interest to express or overexpress specific therapeutic molecules. When these cells release SC-EVs, they carry the desired drug cargo, which can enable a continuous collection of SC-EVs, mitigate adverse impacts on molecules, maintain a biocompatible composition, and safeguard the physicochemical parameters of SC-EVs. However, its time-consuming and complex nature for establishing a producing cell line and its limitation in being applied primarily to SC-EVs derived from stem cells rather than isolated SC-EVs or those obtained from body fluids [112].

Directly loading drugs in SC-EVs can be delivered using three main approaches: (1) incubating SC-EVs with a drug or dye solution, which facilitates their permeation through diffusion or endocytic uptake. During the incubation of cargoes, SC-EVs successfully encapsulated various low molecular weight compounds [113]. While this method is cost-effective and easy to execute, its efficiency is compromised due to the limited capacity within SC-EVs, the small size of their pores, and the hydrophobic properties of their lipid membranes, which collectively restrict drug penetration. (2) loading drugs into SC-EVs using physical strategies, including ultrasound, extrusion, freeze/thaw cycles, and electroporation. After being mixed with therapeutic drugs, ultrasound exposure can lead to either partial disruption of EV membrane integrity, allowing drug diffusion inward, or complete membrane disruption, leading to the release of EV contents. Subsequently, the membranes may self-assemble to encapsulate the drug solution used during sonication [114]. EV-like nanovesicles, constructed from the plasma membrane of parent cells, are primarily generated by extrusion through a series of polycarbonate membrane filters with decreasing pore sizes [115]. These EV-like nanovesicles address the constraints linked to low yield, thus providing a method for the extensive production of extracellular vesicles. The freeze-thaw technique for loading SC-EVs with drugs begins with combining the pharmaceutical agents with the SC-EVs at room temperature. This mixture is then subjected to rapid freezing, either at  $-80^{\circ}\text{C}$  or by immersion in liquid nitrogen. After the freezing process, the sample is allowed to thaw back to room

temperature. This technique leverages the temporary formation of ice crystals, resulting in the disruption of plasma membranes and significant structural and functional changes within the cell membranes. These changes include lateral phase separation, and membrane fusion [116]. Consequently, this method can cause aggregation of EVs, leading to a heterogeneous distribution of EV sizes. Moving on to electroporation, this is a distinct technique that employs brief electrical pulses. These pulses create transient openings, or pores, in the plasma membrane [117]. Through these pores, drugs or nucleotides can efficiently diffuse into SC-EVs. Electroporation is frequently utilized for the encapsulation of larger biomolecules, such as siRNA or miRNA, into SC-EVs. Nonetheless, certain research has suggested that this process may lead to the aggregation of EVs. Significantly, Johnsen *et al.* demonstrated that the inclusion of trehalose in the electroporation buffer could effectively maintain the structural integrity of SC-EVs and avert their aggregation [118]. (3) loading drugs into SC-EVs using permeabilizing agents. For example, saponin, a type of surfactant molecule, has the ability to interact with cholesterol present in cell membranes. This interaction results in the formation of pores that facilitate an increase in the permeability of the membrane [119]. By increasing permeability, saponin allows for the passage of various proteins into the SC-EVs, including the model enzyme catalase. Significantly, this method not only enhances the transport of proteins but also retains the structural integrity and enzymatic functionality of complexes like catalase [120].

The techniques used to incorporate molecules and pharmaceuticals into Small Extracellular Vesicles (SC-EVs) differ in their effectiveness. As previously noted, methods such as sonication and extrusion have been found to be the most efficient in terms of loading capacity within SC-EVs, outperforming other strategies like freeze-thaw cycles and passive incubation [116]. However, it is important to consider the characteristics of the drugs and to find the most optimized techniques.

### 5.1.3 Surface-modified SC-EVs

Surface modification enables the introduction of distinct functionalities into natural SC-EVs by targeting peptides or sites for chemical alteration through genetic manipulation [121]. Much like the process of loading cargo, the process of surface functionalization of SC-EVs can be broadly categorized into two distinct approaches: (1) alteration of the donor cells, which is then followed by the extraction of the SC-EVs with the desired modifications from the stem cells; (2) alteration of the

SC-EVs' surface, which is succeeded by the removal of any residual modifying agents to obtain a purified SC-EV preparation.

The genetic modification of donor cells is typically performed using plasmid vectors that carry genes for targeting ligands. These ligands are engineered to be fused with various types of transmembrane proteins [122]. The genetic engineering of donor cells offers a final EV product free from chemical contaminants and with high specificity in modification. However, this approach comes with drawbacks. One significant challenge is the considerable effort involved in developing a genetically modified cell line that is capable of effectively generating the targeted molecules. Additionally, there is the possibility that the fusion of peptides with the targeting segment could interfere with the normal functions of the proteins present on the EV membrane. Moreover, donor cells can undergo metabolic alterations through endogenous synthesis or specific cleavage processes. For instance, SC-EVs with an elevated expression of mannose were produced by growing cells in an environment containing kifunensine, an inhibitor of mannosidase enzymes. This treatment leads to an increased accumulation of mannose residues on the glycoproteins present on the cell surface [123]. However, the metabolic modification approach for obtaining modified SC-EVs has limitations, including a restricted range of applications and the inability to extend this method to isolated SC-EVs.

In contrast to the modification of donor cells, the methods of modifying the EV surface, including co-incubation, chemical, and physical strategies, are easier. SC-EVs can be directly modified by co-incubating them with specific peptides, which interact with the SC-EVs through non-covalent binding to their surface [124]. The drawbacks of using peptides as targeting tools stem from their inherent instability, rendering them vulnerable to degradation or hydrolysis. To address the challenge of protease susceptibility, D-amino acid isomers can be incorporated into peptides that are more resistant to proteolytic degradation [125]. Alternatively, the stability of peptides can be enhanced by introducing glycosylation sites within their sequence [126]. When it comes to the chemical modification of EV surfaces, a common approach involves the covalent attachment of targeting agents to the amino groups present on the surface proteins of SC-EVs. A widely used technique for this purpose is click chemistry, which includes two main types: copper-catalyzed azide-alkyne cycloadditions (CuAAC) and strain-promoted alkyne-azide cycloadditions (SPAAC) [127]. This method involves a two-step process: first, the EV

proteins are chemically modified to introduce alkyne groups; second, these modified proteins are conjugated with an azide-containing molecule. The propensity of alkyl and azide groups to react and form triazole rings is superior to that of conventional cross-linking agents. This enhanced reactivity affords greater precision and control during the conjugation process at the specific target site [128, 129]. Importantly, the conjugation process is designed to be non-disruptive to the fundamental properties of SC-EVs. It does not change the size or the cell-binding characteristics of the SC-EVs, ensuring that both their structural and functional integrity are maintained. Besides, sulfhydryl-maleimide crosslinking is also a promising approach. Sulfhydryl groups are ubiquitously found in the majority of proteins such as membrane proteins. As a result, SC-EVs can be equipped with a wide variety of functional molecules through the formation of a biocompatible bond between sulfhydryl groups and maleimide moieties [130]. Physical methods include ultrasound, extrusion, and freeze-thaw cycling, as the above.

## 5.2 The applications of engineered SC-EVs

Enhancements to the therapeutic efficacy of SC-EVs could be achieved by improving their target recognition, biodistribution, and their ability to efficiently cross the BBB through manipulation and modification of the surface or content using engineered approaches, ultimately leading to improved treatment outcomes. SC-EVs have utility in AD, PD, and stroke (Table 3).

### 5.2.1 The applications of engineered SC-EVs in AD

AD, a form of neurodegenerative disorder, is marked by a gradual decline in cognitive functions [151]. In AD, the brain exhibits the presence of amyloid beta ( $A\beta$ ) plaques, an overabundance of hyperphosphorylated Tau protein, and the formation of neurofibrillary tangles (NFTs). These aggregates interfere with the normal functioning of neurons, leading to cytotoxicity. The cytotoxicity arises from various disruptions, including the leakage of ions across cell membranes, irregularities in calcium levels, and the deterioration of the electrical potential of the membrane [152, 153]. These processes result in neuronal apoptosis, synaptic degeneration, and a spectrum of cognitive and functional impairments, encompassing learning, behavioral, and motor deficits [154].

In recent years, an increasing body of evidence has underscored the therapeutic potential of engineered SC-EVs in AD. Preconditioning has been shown to enable EVs to modulate function and improve production. EVs sourced from MSCs that

had been conditioned with the secretome of lipopolysaccharide (LPS)- or A $\beta$ -activated microglia showed heightened potency in curbing neuroinflammation, reducing demyelination, and

improving memory and anxiety-related behavioral impairments in murine models of neuroinflammatory conditions [131].

**Table 3.** Applications of engineered SC-EVs

	Methodology	Source of EVs	Animal Model	Administration Route	Therapeutic Effects	Mechanism of Action	Reference
Pre-condition	secretome of lipopolysaccharide or amyloidbeta activated microglia	hMSCs	5xFAD mice	intranasally	↓microglia and astrocyte activation  ↓amyloid deposition ↓demyelination ↓memory loss and motor and anxiety-like behavioral dysfunction	↑miRNAs target key genes on the TLR4 signaling pathway	[131]
	hypoxic	mBMSCs	MCAO mice	intravenously	↓infarct areas ↓behavioral deficits	↑miR-214-3p ↓PTEN/ Akt signaling	[132]
	hypoxic	hBMSCs	MCAO mice	tail vein	↑microvascular length and branching point density ↓neuronal degeneration  ↓brain atrophy	↑miR-126-3p, miR-140-5p, let-7c-5p ↓miR-186-5p, miR-370-3p, miR-409-3p 52 proteins differentially abundant	[133]
	cerebral infarct tissue extracts	hUCMSCs	MCAO rats	tail vein	↑neurological recovery ↑vascular remodeling	↑miRNAs and their target genes which is beneficial to vascular smooth muscle	[134]
	lithium	mBMSCs	MCAO mice	femoral vein retroorbital	↑neurological function ↑neurological recovery ↑neuroregeneration ↓inflammation	↑miR-1906 ↓TLR4 ↓NF- $\kappa$ B ↓proteasomal activity ↓inducible NO synthase ↓cyclooxygenase-2 expression	[135]
	BHD	rNSCs	MCAO rats	tail vein	↑neurological recovery  ↑NSCs proliferation and differentiation	↑miR-124-5p miR-9a-5p miR-137-5p miR-184	[136]
Drug-loading	SHP2	mBMSCs	A $\beta$ <sub>1-42</sub> -injected mice	intravenously	↓synaptic loss  ↓cognitive decline ↓neuronal cells apoptosis ↓neuroinflammation	↑mitochondrial damage-induced apoptosis ↓NLRP3 inflammasome	[137]
	CB2 receptor agonist AM1241	mBMSCs	APP/PS1 mice	tail vein	↑learning and memory	↓calcium-Erk signaling pathway	[138]
	FTO-targeted siRNAs	hUCMSCs	MPTP mice	intravenously	↓neuronal apoptosis ↑neuronal regeneration ↓neuronal death	↑TH	[139]
	BDNF	hiMSCs	MCAO mice	intranasally	↓ $\alpha$ -Syn ↑functional behavior ↑neural repair ↓infarct volume reduction ↑neurogenesis ↑angiogenesis ↑synaptic plasticity ↑fiber preservation ↓inflammatory-cytokine expression	↑neuroprotection-related genes ↓inflammation-related genes ↑BDNF/ TrkB signaling	[140]
	BDNF	hNSCs	MCAO rats	striatum of the ischemic hemisphere	↓glial response ↓infarct volume  ↑neurological function ↓activation of microglia ↑differentiation of endogenous NSCs into neurons	NA	[141]
	IncRNA KLF3-AS1	mBMSC	MCAO mice	intravenously	↓cerebral infarction ↑neurological function ↑cell viability ↓apoptosis ↓inflammatory injury	↑Sirt1 deubiquitinating ↓miR-206 ↑USP22	[142]

Methodology	Source of EVs	Animal Model	Administration Route	Therapeutic Effects	Mechanism of Action	Reference	
	miR-181a-2-3p	mADMSCs	6-OHDA mice	tail vein	↓ROS production ↓inflammatory factors ↓activation of microglia ↓apoptosis ↓oxidative stress ↓α-syn ↓4-HNE	↓EGR1 ↓NOX4/p38 MAPK	[143]
	miR-188-3p	mADMSCs	MPTP mice	intravenously	↓autophagy ↓pyroptosis ↑proliferation	↓CDK5 ↓NLRP3	[144]
	miR-17-92	rBMSCs	MCAO rats	intravenously	↑neuro-functional recovery ↑axonal extension and myelination ↑axon-myelin remodeling ↑electrophysiological recovery	↓PTEN ↑PI3K/Akt/mTOR	[145]
	miR-210 miR-126	mNPCs	MCAO mice	tail vein	↓infarct volume ↓neurological deficits ↓neural apoptosis ↓ROS production ↑spine density of dendrites	↓Nox2 ↑BDNF ↑p-TrkB/TrkB	[146]
<b>Surface-modification</b>	RGD-4C	hNPCs	MCAO mice	tail vein	↓inflammatory response	↓MAPK	[33]
<b>Other</b>	Hydrogel	hADMSCs	5×FAD mice	intranasally	↓memory deficits ↓neuronal damage ↑neurogenesis	NA	[147]
	HAD hydrogel	mNSCs	MCAO mice	ventricle	↑neurological functions ↑infarct volume and angiogenesis ↑cerebral angiogenesis and anti-inflammation	NA	[148]
	RVG BACE1 siRNA caspase-3 siRNA	mBMSCs	3xTg-AD mice	intranasally	↓cognitive deficits ↓Aβ plaques ↓apoptosis of neurons ↓reactive astrocytes ↑AD pathologies	↓BACE1 ↓caspase-3	[149]
	curcumin	mBMSCs	MPTP mice	intranasally	↑movement and coordination ability ↓α-synuclein aggregates ↑neuron function recovery ↓neuroinflammation	NA	[150]

h, human; r, rat; m, mouse; BMSCs, bone marrow-derived mesenchymal stem cells; MSCs, mesenchymal stem cells; UCMSCs, Umbilical cord mesenchymal stem cells; NSCs, neural stem cells; ADMSCs, adipose-derived stem cells; NPCs, neural progenitor cells; iNSCs, induced pluripotent stem cell-derived neural stem cells; 5XFAD mice, 5 familial Alzheimer's disease mutations; MCAO, middle cerebral artery occlusion; UCMSCs, umbilical cord mesenchymal stem cells; 6-OHDA, 6-hydroxydopamine; TLR4, toll-like receptor-4; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Akt, protein kinase B; BHD, buyang huanwu decoction; SHP2, tyrosine phosphatase-2; Aβ, amyloid β; EGR1, growth-response-1; CDK5, cell division protein kinase 5; FTO, m6A demethylase fat mass and obesity-related protein; TH, tyrosine hydroxylase; α-syn, α-synuclein; mTOR, mammalian target of rapamycin; BDNF, brain-derived neurotrophic factor; Nox2, NADPH oxidase 2; TrkB, BDNF receptor tyrosine kinase receptor B; ROS, reactive oxygen species; Sirt1, silent mating type information regulation 2 homolog 1; USP22, ubiquitin specific peptidase 22; RGD, arginine-glycine-aspartic acid; MAPK, mitogen-activated protein kinase; HAD, adhesive hyaluronic acid; RVG, rabies virus glycoprotein; BACE1, β-site amyloid precursor protein cleaving enzyme 1; siRNA, small interfering RNAs; SPIONs, superparamagnetic iron oxide nanoparticles; P, penetratin; NA, not available.

Besides, many EVs exert an effect by containing therapeutic drugs. For example, Zhu *et al.* employed biocompatible EVs derived from MSCs as carriers for delivering the CB2-targeted medication AM1241 (EVS-AM1241) to mitigate neurodegenerative progression. This intervention facilitated Aβ phagocytosis, fostered neurogenesis, and ultimately enhanced learning and memory in AD model mice. These effects were mediated through the calcium-Erk signaling pathway (Figure 4B) [138]. Additionally, Xu *et al.* utilized lentivirus to infect MSCs with the Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP2) gene, thus obtaining MSC-EVs enriched with a high level of SHP2 (MSC-EVs-SHP2) in the context of AD. Consequently, MSC-EVs-SHP2 significantly induce mitophagy, leading to the improvement of mitochondrial damage-induced

apoptosis and the inhibition of NLRP3 activation in neuronal cells [137]. Mitophagy not only reduces neuronal cell apoptosis and neuroinflammation but also leads to the restoration of synaptic loss and the amelioration of cognitive decline in a mouse model of AD. Additionally, ADMSCs were transfected with a miRNA-22 mimic to produce miRNA-22-loaded EVs, utilized for the treatment and neural repair of AD. EV-miRNA-22 demonstrated the ability to enhance motor function, promote nerve cell survival, and reduce the expression of inflammatory factors in APP/PS1 mice. Moreover, EVs loaded with miRNA-22 have been observed to reduce the secretion of inflammatory cytokines *in vitro* by suppressing the process of pyroptosis [155].

Drawing from the enzymatic activity of membrane proteins found in MSC-EVs, Huang *et al.*



have successfully engineered and created a hydrogel made up of self-assembling peptides, which are specifically designed to be degradable by enzymes present in cellular membranes. Upon intranasal delivery, this smart release hydrogel prolonged the retention of MSC-EVs at the site of administration, enabling a controlled release of MSC-EVs. This approach effectively mitigated neuronal damage, fostered neurogenesis, and ameliorated memory deficits in 5×FAD AD model mice [147]. To improve siRNA delivery to brain neurons via nasal administration, Li *et al.* developed lesion-targeting EVs. These MSC-derived EVs were modified with the RVG peptide, which binds to neuronal acetylcholine receptors. Following intranasal delivery, these EVs penetrated the nasal mucosa, reaching afflicted brain regions. Within the cytoplasm, EV cores facilitated controlled siRNA release in the high-ROS environment, leading to decreased BACE1 and caspase-3 levels, thus reducing A $\beta$  plaques and neuronal apoptosis. Additionally, MSC-derived EVs decreased reactive astrocyte numbers. Collectively, these strategies synergistically ameliorated AD pathologies and cognitive deficits [149].

These findings suggest a promising therapeutic approach that may help mitigate the symptoms of Alzheimer's disease. However, it is essential that additional clinical studies are conducted to fully assess the efficacy and safety of this strategy in a medical context.

### 5.2.2 The applications of engineered SC-EVs in PD

PD, ranking as the second most prevalent chronic neurodegenerative condition globally [156], is distinguished by several key neuropathological features. These include a significant loss of dopaminergic (DA) neurons within the substantia nigra (SN) region of the brain, a consequent decrease in dopamine levels within the striatum, and the formation of intracellular aggregates composed of the  $\alpha$ -synuclein protein [157]. The complex pathophysiology of PD stems from a convergence of multiple molecular pathways and cellular mechanisms. These include disruptions in the regulation of  $\alpha$ -synuclein protein homeostasis, impairments in mitochondrial function, increased oxidative stress, perturbations in calcium ion balance, deficits in axonal transport processes, and the detrimental effects of neuroinflammation [158]. PD's defining symptoms encompass motor dysfunctions, such as bradykinesia, rigidity, tremors, and gait disturbances, in addition to non-motor manifestations such as pain, fatigue, depression, and cognitive deficits [159]. Current therapeutic approaches for PD predominantly center on surgical interventions and a

limited array of pharmaceutical options, while potential future treatments explore avenues like deep brain stimulation, transplantation of dopaminergic neurons derived from stem cells, and gene therapy as potential curative strategies [160, 161].

Recent research has demonstrated that the administration of specially engineered SC-EVs has led to significant improvements in a 6-hydroxydopamine (6-OHDA) induced mouse model of Parkinson's disease. These improvements include the reduction of neuronal cell apoptosis, amelioration of neurobehavioral deficits, suppression of neuroinflammation, decrease in oxidative stress, and the dispersion of abnormal protein aggregates. Notably, the therapeutic impact of EVs is primarily dependent on their contents, which can be modified to activate or suppress specific processes in target cells. For example, EV-encapsulated miR-181a-2-3p derived from MSCs could potentially mitigate oxidative stress in PD by enhancing SH-SY5Y cell proliferation and boosting superoxide dismutase (SOD) levels. Additionally, it suppresses apoptosis and reduces levels of malondialdehyde (MDA) and reactive oxygen species (ROS) by modulating the expression of growth-response-1 (EGR1) through the inhibition of NOX4/p38 mitogen-activated protein kinase (MAPK) signaling. This process helps prevent the apoptosis of dopamine neurons [143]. Meanwhile, Li *et al.* also employed miR-188-3p-enriched EVs derived from ADMSCs to target cell division protein kinase 5 (CDK5) and NLRP3 in PD model, resulting in the restraint of autophagy and pyroptosis [144]. Using si-FTO to suppress FTO expression simultaneously decreased the overproduction of  $\alpha$ -synuclein and the underproduction of tyrosine hydroxylase, which helped to reduce neuronal death in PD models. Furthermore, EVs derived from MSCs effectively delivered si-FTO to the striatum of animal brains. This resulted in a significant reduction of  $\alpha$ -synuclein levels, protection of dopaminergic neurons, and a recovery of tyrosine hydroxylase expression in PD mice brains (Figure 4C) [139]. Interestingly, Peng *et al.* have developed a sophisticated self-targeting nanocarrier named PR-EXO/PP@Cur. This breakthrough technology merges the healing potential of extracellular vesicles derived from MSC-EVs with the powerful anti-inflammatory and therapeutic effects of curcumin. The PR-EXO/PP@Cur nanocarrier has shown remarkable capabilities, including the autonomous navigation through multiple cellular membranes and the targeted delivery of its cargo directly into the cytoplasm of specific cells. PR-EXO/PP@Cur effectively targets the complex pathologies of PD through a synergistic three-pronged strategy. By increasing the concentration of the therapeutic agent

at the site of action, it reduces the accumulation of  $\alpha$ -synuclein aggregates, facilitates the restoration of neuronal function, and alleviates neuroinflammation [150].

Although SC-EVs hold promise as a strategy against PD through multiple mechanisms, translating preclinical findings to clinical studies poses significant challenges because none of the currently used animal models fully replicate all the defining features of PD. Developing more robust disease models is essential to gain a deeper understanding of the pathophysiology.

### 5.2.3 The applications of engineered SC-EVs in stroke

Stroke remains a leading cause of both mortality and disability across the globe. Regrettably, to date, no universally effective treatment strategy has been established [162]. It disrupts the typical operation of specific brain areas, resulting in enduring brain damage and impairments in both motor and cognitive functions [163]. Ischemic stroke (IS), the most common form of stroke, is marked by an initial ischemic episode that leads to a lack of blood flow and oxygen supply to the brain tissue [164]. Frequently, these obstructions are triggered by blood clots, which are the primary cause of ischemic strokes [165].

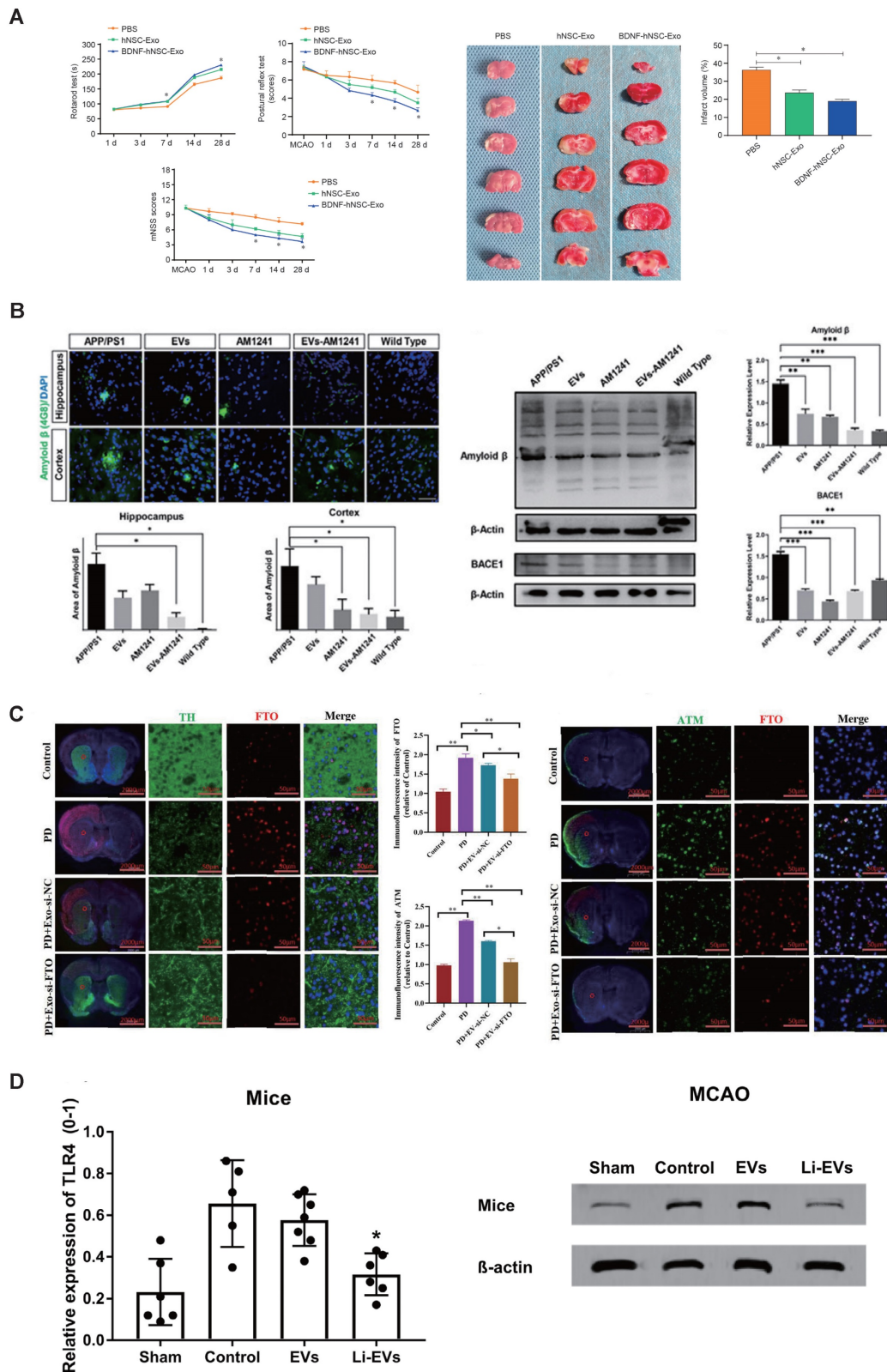
In contrast to the treatment strategies for neurodegenerative diseases, the primary therapeutic focus in stroke treatment is on mitigating neuroinflammation, enhancing neuroprotection, and fostering neuroregeneration. Literature suggests that the primary driver of neuronal damage in stroke cases is the inflammatory reaction initiated by glial cells [166]. Over recent years, an increasing amount of research has underscored the promising therapeutic effects of SC-EVs that have been treated under hypoxic conditions. For example, Wu *et al.* demonstrated that the administration of hypoxia-treated EVs (Hypo-EVs) can lead to significant improvements in behavioral deficits and a reduction in the size of infarcted areas in a mouse model of stroke. This therapeutic effect is believed to be mediated by a specific regulatory pathway involving miR-214-3p, which is enriched in Hypo-EVs. The miR-214-3p pathway works by downregulating the PTEN/Akt signaling cascade, a process that is instrumental in enhancing the recovery of neurological function after ischemic and reperfusion injuries [132]. EVs sourced from MSCs subjected to hypoxic conditions have shown a significant improvement in microvascular growth within ischemic tissue. This was evidenced by an increase in both microvascular length and branching point density, as observed using 3D light sheet microscopy over a period of up to 56 days. Furthermore, these EVs have been associated with a decrease in delayed neuronal degeneration and brain

atrophy, which collectively contribute to improve neurological recovery [133]. Besides, the findings revealed that infarct-preconditioned EVs, in comparison to normal EVs, significantly enhanced vascular remodeling and neurological function recovery post-stroke. The upregulation of specific miRNAs and their target genes associated with vascular smooth muscle cells underscored the crucial role of vascular remodeling in stroke recovery [134]. Notably, Haupt *et al.* discovered that intravenous administration of EVs preconditioned with lithium led to enhanced neurological recovery and neuroregeneration for up to 3 months compared to controls and EV-treated mice. Specifically, Li-EVs exhibited significantly elevated levels of miR-1906, identified as a novel regulator of TLR4 signaling. Li-EVs effectively decreased posthypoxic and postischemic TLR4 levels, leading to inhibition of the NF- $\kappa$ B signaling pathway, reduced proteasomal activity, and decreased expression of inducible NO synthase and cyclooxygenase-2. These collective effects resulted in diminished post-stroke cerebral inflammation (Figure 4D) [135].

Furthermore, the treatment of drug-loaded SC-EVs led to significant improvements functional levels in stroked models. Zhou *et al.* engineered MSCs to overexpress BDNF and purified the resulting EVs using anion exchange chromatography. In a mouse model of ischemic stroke, intranasally administered EVs were able to specifically target the region surrounding the infarct. The enrichment of EVs with BDNF substantially amplified their therapeutic potential, leading to marked improvements in behavioral functions and neural reparative processes. These enhancements included a decrease in the size of the infarct, stimulation of neurogenesis, promotion of angiogenesis, enhancement of synaptic plasticity, and the maintenance of nerve fibers. Additionally, this treatment reduced inflammatory cytokine expression and glial response. The intranasal delivery of both EVs and BDNF-loaded EVs upregulated genes associated with neuroprotection while downregulating inflammation-related genes. Additionally, BDNF-loaded EVs specifically activated the BDNF/TrkB signaling pathway in the ischemic brain [140]. Similarly, hNSC-EVs loaded with BDNF after 24 hours of co-incubation displayed the capacity to enhance cell survival and induce differentiation. In a rat ischemic stroke model, BDNF-hNSC-EVs efficiently suppressed microglial expression and stimulated the differentiation of endogenous NSCs into neurons, consequently leading to a reduction in infarct volume and an enhancement of neurological function (Figure 4A) [141]. Inspiringly, BMSC-derived EVs containing lncRNA KLF3-AS1 exhibited a

reduction in cerebral infarction and improvement in neurological function in MCAO mice by enhancing Sirt1 deubiquitinating. Mechanistically, KLF3-AS1

inhibited the ubiquitination of Sirt1 protein by inducing USP22 expression.



**Figure 4. The applications of engineered SC-EVs.** (A) BDNF-hNSC-Exo enhances neurological function and reduces brain damage in rats following cerebral ischemia. Adapted with permission from [141], copyright 2022 Medknow. (B) EVs-AM1241 alleviated the pathological neurodegenerative changes in AD mice [138], copyright 2023 ELSEVIER. (C) Synergistic loading of si-FTO into exosomes effectively protects against dopaminergic neuronal death in a Parkinson's disease model in vivo. Adapted with permission from [139], copyright 2023 BMC. (D) Li-EVs decrease the expression of TLR4 in mice. Adapted with permission from [135], copyright 2021 WILEY.

Furthermore, KLF3-AS1 acted as a sponge for miR-206, leading to the upregulation of USP22 expression [142]. Moreover, Xin *et al.* discovered that the improved neuro-functional recovery observed in stroke cases, as a result of miR-17-92 cluster-enriched MSC EVs, can be linked to an augmentation in axonal extension and myelination. The activation of the PI3K/Akt/mTOR pathway, which is thought to be a consequence of PTEN downregulation, is likely responsible for facilitating these beneficial effects on neural repair and function [145]. In MCAO mice, both NPC-EVs and endothelial progenitor cell-derived EVs (EPC-EVs) were found to decrease infarct volume and neurological deficits, reduce neural apoptosis and ROS production, and enhance dendritic spine density. These effects were found to be connected to the reduction in Nox2 expression and the upregulation of BDNF, along with the phosphorylation of its receptor, known as p-TrkB/TrkB. Notably, the simultaneous use of NPC-EVs and EPC-EVs demonstrated a synergistic effect, yielding superior results compared to the use of either type of EVs in isolation. Furthermore, the synergistic effects of EPC-EVs-miR-126 + NPC-EVs-miR-210 were superior to those of NPC-EVs + EPC-EVs [146].

In addition, engineered EVs can also improve the efficiency of therapy by surface modifications. Recently, Tian *et al.* have shown that through intravenous injection, RGD-C1C2-bound EVs can specifically target the affected area in ischemic brain injury. This protein has a high affinity for self-assembly onto the surface of the EVs, facilitating the precise homing of RGD-EVs to the lesion site. Consequently, they strongly suppressed the inflammatory response by inhibiting the MAPK pathway, which is an inflammation-related pathway [33].

Additionally, a hyaluronic acid (HA) hydrogel modified with catechol can encapsulate NSC-derived EVs while preserving their activity [167]. This hydrogel mimics the 3D microenvironment of tissues, promoting endogenous cell adhesion and proliferation in ischemic regions. The controlled release of NSC-derived EVs from the adhesive HA hydrogel shows promise in enhancing cerebral angiogenesis and improving neurological function post-ischemic stroke [148].

In conclusion, these findings suggest a strategy that could potentially reduce infarct volume and provide implications for alternative therapeutic approaches to stroke. However, future preclinical studies on the therapeutic potential of SC-EVs in stroke should focus more on gaining a comprehensive understanding of the mechanisms of action of EVs.

## 6. Route of administration

Discussion about the administration route of SC-EVs-based therapy is increasing in recent years, including intravenous injection, intracerebral injection, intraventricular injection, and intranasally.

Previous research has suggested that intravenous administrations of SC-EVs can ameliorate neurological impairments. In a comparative analysis between femoral vein and retroorbital injection as delivery routes, the study observed a consistent biodistribution pattern for NPC-EVs under both ischemic and non-ischemic conditions. NPC-EVs were found in peripheral organs like the liver and lungs, in addition to their presence in the brain. Nevertheless, the liver and lungs exhibited a higher concentration of these EVs compared to the brain [69]. As a result, to counteract the tendency of SC-EVs to accumulate in the liver, spleen, gastrointestinal tract, and lungs after intravenous injection, higher doses are required. Notably, administering EVs via the tail vein and lateral ventricle led to improved behavior in PD model rats, with no significant difference observed between the two injection techniques [64]. However, in the case of AD-like behavioral performance enhancement in mice injected with streptozotocin, the administration of BMSC-EVs via the lateral ventricle, rather than through the tail vein, has shown better outcomes [58]. Nonetheless, it's essential to acknowledge that intracerebral and intraventricular injections are highly invasive and have low compliance.

In recent years, the intranasal (IN) route has garnered increasing attention due to its distinctive transport pattern [168, 169], which presents a compelling method for the direct and non-invasive introduction of pharmaceuticals or cellular therapies into the brain. The nasal cavity is equipped with olfactory sensory neurons in the olfactory region and branches of the trigeminal nerve in the respiratory region, both of which have exposed nerve terminals under the mucosal layer. This arrangement allows drugs to penetrate the epithelial layer and be transported to the olfactory bulb or pons along the axons of these neurons. From there, the drugs can enter the perivascular space of the brain's blood vessels or spread through the cerebrospinal fluid, leading to a broad distribution across the central nervous system. This route of administration bypasses the digestive system and liver metabolism, which can improve drug absorption and decrease the amount needed to reach therapeutic levels [170]. Pharmacokinetic research has shown that although the nasal epithelium may reduce the bioavailability of drugs delivered intranasally, the concentration of the

drug in most central nervous system regions is roughly tenfold higher following intranasal administration compared to systemic injection when blood drug levels are the same [171]. Moreover, the non-invasive characteristic of intranasal delivery is a significant benefit. Devices for intranasal delivery, such as sprays, atomizers, nasal drops, or other similar tools, offer simple application methods and minimize the risk of harm to patients [172].

In an ETH1 stroke model, the use of glucose-coated gold nanoparticles (GNPs) for labeling, in conjunction with CT imaging, revealed that the IN route resulted in better accumulation in the brain's injured area compared to the intravenous route [173]. Zhou *et al.* found that intense fluorescence was observed surrounding the periinfarct region of the brain after the intranasal administration of EVs, with minimal signal detected in other organs such as the heart, lung, liver, spleen, and kidney, suggesting a targeted delivery of EVs to the lesion area. Furthermore, the nasal mucosa structure resembled that of the PBS group, exhibiting no apparent abnormalities, thus indicating the absence of mucosal toxicity associated with the intranasal delivery of BDNF-EVs in mice [140]. Rohden *et al.* illustrated that intranasal injection of EVs 24 hours after ischemic stroke in rats led to enhancements in EV quantity, blood-brain barrier integrity, and vascular stabilization within the recoverable peri-infarct zone [74]. Losurdo *et al.* conducted a study where they utilized intranasal delivery of EVs sourced from cytokine-preconditioned MSCs to provide neuroprotective and immunomodulatory effects in 3xTg AD mice. This therapeutic strategy effectively reduced the activation of microglial cells, increased the density of dendritic spines, and regulated the inflammatory response in the treated mice [105].

Nevertheless, the primary concentration of ongoing preclinical studies is on substantiating the therapeutic efficacy of IN delivery, particularly in the context of EV-based treatments for CNS disorders. A majority of intranasal delivery research is performed on animal models, particularly rodents, whose anatomical structures and olfactory regions differ significantly from those of humans [174]. Future investigations may need to include experiments on large animals, considering the substantial differences between human and rodent nasal cavities. Experimentation on large animal models is crucial for gaining insights into how different formulations affect the pharmacokinetics of a drug. Such studies in larger animals provide data that are more closely aligned with the pharmacokinetic profiles observed in humans, offering valuable and relevant information for translational research. Additionally, efforts in

biotechnology are necessary to prolong the residence time of drugs within the nasal cavity, thereby increasing bioavailability [149, 150]. Integrating EVs into formulations such as hydrogels could extend mucus retention and enhance bioavailability. Moreover, the development of appropriate formulations can enhance the compatibility of EVs with IN delivery systems. This optimization not only streamlines the administration process but also enhances the overall efficiency of drug delivery [147]. Therefore, there is still considerable room for improvement in the IN delivery of EVs. Despite challenges, intranasal delivery offers significant promise and could become a pivotal element in the clinical application of therapies based on EVs.

## 7. The challenges and prospects of clinical translation

### 7.1 Clinical studies of SC-EVs

Over recent years, an expanding pool of research has underscored the promising therapeutic effects of EVs that originate from stem cells. This evidence has fostered a perception that EV-based therapies may offer superior safety and a wider range of applications compared to traditional cell therapy methods, even though clinical data have yet to be fully realized. A number of studies focused on the utilization of EVs or exosomes are registered on <https://beta.clinicaltrials.gov> (accessed on 18 October 2023), including NCT05490173, NCT05370105, NCT05326724, NCT05035134, NCT04928534, NCT04388982, NCT04202770, NCT03384433, NCT01860118. However, a large proportion of those are observational studies or not applicable. Notably, only two studies are in phase 2. They are the clinical trial NCT04388982 and NCT03384433. The registered clinical trial NCT04388982 investigates the safety and efficacy of EVs derived from allogenic adipose MSCs in the treatment of mild to moderate dementia caused by AD. While NCT03384433 evaluates improving patients with acute ischemic stroke who received miR-124-Loaded EVs derived MSCs.

Although the number of currently registered clinical studies is relatively limited, there is a noticeable increase in interest in these therapies. Looking ahead, it is anticipated that an increase in clinical studies will pave the way for the emergence of EV-based drugs on the market.

### 7.2 The challenges of SC-EVs therapeutic strategies

In recent decades, significant strides have been made in comprehending the biological characteristics and exploring potential therapeutic uses of SC-EVs in

the realm of neurological disorders. However, there are several challenges of clinical translation to overcome.

Initially, the heterogeneity of SC-EVs is shaped by numerous factors, including donors and tissue origins, cell compositions, culture environments, diverse batches, methods of isolation and purification, as well as storage conditions of EVs. Variances in donors' health status, genetic makeup, gender, and age contribute to these diversities [175]. Subsequently, tissues derived from different sources manifest distinct attributes, further contributing to this heterogeneity. Li *et al.* extensively examined the heterogeneous landscape of MSC-EVs, focusing on BMSC-EVs, UCMSC-EVs, and ADMSC-EVs, which are among the most intensively utilized. Their findings revealed that UCMSC-EVs exhibited superior drug loading and delivery capabilities, thereby offering promising prospects for future translational applications [176]. Similarly, the culturing environment significantly influences SC-EVs yield and function, including factors such as the use of artificial preconditioning, various culture methods, and specific culture conditions [134]. There is heterogeneity in surface markers, biophysical properties, and content among different batches of SC-EVs. Moreover, differences in EV isolation and purification methods, along with variations in EVs storage conditions, can alter the composition of SC-EVs. Simultaneously, the characterization methods and detection techniques for SC-EVs are not yet standardized. Therefore, it is crucial to establish standardized protocols for the cell source, production process, and content of SC-EVs. This standardization is essential for reducing the side effects associated with the variability of SC-EVs and for guaranteeing their consistency and reproducibility in therapeutic applications [177]. Recent studies have elucidated some established physicochemical properties of EVs. Morphological examination of EVs can be conducted using transmission electron microscopy (TEM), which provides detailed imaging. Meanwhile, the particle size and concentration can be precisely measured with the Flow NanoAnalyzer [178]. For protein content analysis, the BCA protein assay kit [47] offers a reliable quantitative method. Furthermore, the presence and quantity of cytosolic proteins derived from the parent cells, as well as specific EV markers like CD9, CD63, and CD81, can be assessed through flow cytometry. With the development of EV analysis techniques and the continuous deepening of research and understanding of EVs, it is necessary to establish quantitative and specific indicators for EVs from different stem cell sources, thus improving the quality standards of SC-EVs.

Then, the yield of SC-EVs is indeed a significant challenge in the clinical translation of EV-based therapies and diagnostics. Identifying methodologies that yield high quantities and are effective in producing and purifying SC-EVs is crucial. It is necessary to continually refine SC-EVs isolation techniques to optimize both yield and purity. The ultimate goal is to establish a standardized and uniform SC-EVs manufacturing process that adheres to current Good Manufacturing Practices (GMP). SCs are typically cultured in Petri dishes, or culture bottles, limiting the surface area for cell growth. The multi-layer cell factory cultivation process enables the medium-scale expansion and cultivation of SCs. However, this method requires a significant amount of manpower and space, and cells may be unevenly distributed across the layers. Therefore, it is indispensable to achieve highly repeatable cell cultivation and collection of cell supernatant by using a large-scale automated cell culture device. Bioreactors are essential tools for the large-scale production of extracellular vesicles due to their capability for dynamic monitoring. The hollow fiber bioreactor system, in particular, offers a dynamic environment that is conducive to cell cultivation. It also features a continuous medium collection system, which is vital for the efficient collection of the produced medium [179]. A stirred-tank bioreactor can be used for the suspension cultivation of stem cells or their attachment to microcarriers suspended in the stirred vessel. The choice of suitable microcarriers is crucial for ensuring both the quantity and quality of cells, and it is closely related to the quality of SC-EVs collection and purification. Hollow fiber bioreactors allow stem cells to adhere directly to the coated surface of hollow fibers. Nonetheless, stem cells are known to be mechanically responsive, undergoing changes in their phenotype as a reaction to shear stress, cell clustering, and variations in the substrate they encounter within the dynamic environment of a 3D bioreactor [176]. Thus, during the adherent cell culture process, real-time monitoring of cell proliferation status and quality is challenging. Son *et al.* discovered that culturing MSCs in a 3D spheroid within a micro-patterned well led to enhanced EV production. Compared to traditional 2D culture techniques, the EVs derived from the 3D culture platform, in terms of particle number, size, and purity, showed higher consistency not only across multiple batches from the same donor but also among different donors [180]. Similarly, Han *et al.* developed a gelatin methacryloyl (GelMA) hydrogel for the 3D culture of MSCs. Their work suggests that a 3D environment can mimic the natural biological niches of MSCs, which significantly boosts the stem-like

properties of these cells and, in turn, enhances the yield of MSC-derived exosomes [181].

Furthermore, maintaining the stability of SC-EVs during storage is crucial prior to clinical translation. The stability of stored extracellular SC-EVs is impacted by several factors, such as storage conditions, temperature, choice of storage solution, storage duration, and the occurrence of freeze-thaw cycles. Therefore, it is imperative to store SC-EVs in an environment not exceeding  $-80^{\circ}\text{C}$ , while also avoiding repeated freeze-thaw cycles or significant temperature fluctuations [182].

Finally, assessing the safety of EV-based therapies is crucial. Potential adverse effects, immunogenicity, and off-target effects must be thoroughly investigated in preclinical studies and during clinical trials.

## 8. Prospects and conclusions

SC-EVs continue to hold promise as versatile agents, given their capacity to encapsulate abundant cargo, traverse the BBB, and exhibit high biocompatibility and low immunogenicity, coupled with their ease of engineering for modifications. The progress of SC-EVs in the realm of neurogenesis undeniably heralds a new era in the treatment of neurological diseases, potentially opening the door to clinical applications for nerve damage repair in the future.

However, several avenues require further exploration, including the identification of the optimal source of SC-EVs derived from stem cells exhibiting neurotherapeutic behavior, elucidating the neuroprotective properties of proteins or miRNA cargo within SC-EVs, and investigating molecular mechanisms such as the cargo selection process and the methods through which cells uptake SC-EVs. As technology continues to advance, we can expect to deeper exploration of the biologically active mechanisms and key components of extracellular vesicles. High-throughput techniques, such as single-cell transcriptomics and proteomics, have the potential to intricately unravel the molecular composition and dynamic changes of extracellular vesicles. Moreover, dynamic simulation and big data machine learning will play pivotal roles in simulating the release, transmission, and interaction processes of extracellular vesicles across various microenvironments. This not only aids in predicting the interactions between extracellular vesicles and recipient cells but also provides targeted experimental design and more efficient data interpretation. Simultaneously, methods based on big data and machine learning can harness large-scale clinical data to gain a deeper understanding of the biomolecular

characteristics of extracellular vesicles, thereby fostering the development of personalized medicine that provides clinicians with more precise disease diagnoses and treatment plans. In the future, attention to surface modifications of extracellular vesicles aiming to enhance the targeted delivery of nucleic acids may be a focus. Moreover, considering the integration of CRISPR technology holds promise for achieving even greater precision in gene editing and therapeutic outcomes.

In this review, we provide a comprehensive overview of the methods employed to obtain SC-EVs, encompassing the isolation and differentiation of stem cells, and the isolation and purification of extracellular vesicles. Subsequently, we delve into an exploration of the inherent functions of native SC-EVs. Under physiological conditions, EVs play a pivotal role in neuroprotection, angiogenesis and preservation BBB integrity, alleviation of neuroinflammation, and other functions. Moving forward, we examine strategies for engineering SC-EVs to enhance their therapeutic effects and yield. This leads us to a focused discussion on their applications in AD, PD, and stroke. Finally, we describe the challenges associated with these extracellular vesicles in clinical translation and propose potential solutions.

## Abbreviations

A $\beta$ : amyloid  $\beta$ ;  
 $\alpha$ -syn:  $\alpha$ -synuclein;  
 AD: Alzheimer's disease;  
 Akt: protein kinase B;  
 BACE1:  $\beta$ -site amyloid precursor protein cleaving enzyme 1;  
 BBB: blood-brain barrier;  
 BDNF: brain-derived neurotrophic factor;  
 CNS: central nervous system;  
 EB: embryoid body;  
 EVs: extracellular vesicles;  
 FTO: targeting obesity-associated protein;  
 GDNF: glial cell-derived neurotrophic factor;  
 GMP: good manufacturing practices;  
 HBMECs: human brain microvascular endothelial cells;  
 HUVECs: human umbilical vein endothelial cells;  
 IEP: insulin-degrading enzyme;  
 IL: interleukin;  
 iMSCs: induced mesenchymal stem cells;  
 iNSCs: induced pluripotent stem cell-derived neural stem cells;  
 iPSCs: induced pluripotent stem cells;  
 IS: ischemic stroke;  
 MAPK: mitogen-activated kinase;

MCAO: middle cerebral artery occlusion;  
 MMP: matrix metalloproteinase;  
 mTOR: mammalian target of rapamycin;  
 NEP: neprilysin;  
 NF- $\kappa$ B: nuclear factor-kappa B;  
 NPCs: neural progenitor cells;  
 NTFs: neurotrophic factors;  
 OGD: oxygen and glucose deprivation;  
 OPCs: oligodendrocyte progenitor cells;  
 PD: Parkinson's disease;  
 PTEN: phosphatase and tensin homolog deleted

on chromosome ten;

RGD: arginine-glycine-aspartic acid;

ROS: reactive oxygen species;

RVG: rabies virus glycoprotein;

SCs: stem cells;

siRNA: small interfering RNAs;

Sirt: silent mating type information regulation 2  
 homolog;

SHP2: tyrosine phosphatase-2;

VEGF: vascular endothelial growth factor;

VEGFR: vascular endothelial growth factor  
 receptor.

## Competing Interests

The authors have declared that no competing interest exists.

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