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CONCISE REVIEW

STEM CELLS

Intranasal delivery of mesenchymal stem cells-derived extracellular vesicles for the treatment of neurological diseases

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Abstract

Neurological disorders are diseases of the central nervous system (CNS), characterized by a progressive degeneration of cells and deficiencies in neural functions. Mesenchymal stem cells (MSCs) are a promising therapy for diseases and disorders of the CNS. Increasing evidence suggests that their beneficial abilities can be attributed to their paracrine secretion of extracellular vesicles (EVs). Administration of EVs that contain a mixture of proteins, lipids, and nucleic acids, resembling the secretome of MSCs, has been shown to mimic most of the effects of the parental cells. Moreover, the small size and safety profile of EVs provide a number of advantages over cell transplantation. Intranasal (IN) administration of EVs has been established as an effective and reliable way to bypass the blood-brain barrier (BBB) and deliver drugs to the CNS. In addition to pharmacological drugs, EVs can be loaded with a diverse range of cargo designed to modulate gene expression and protein functions in recipient cells, and lead to immunomodulation, neurogenesis, neuroprotection, and degradation of protein aggregates. In this review, we will explore the proposed physiological pathways by which EVs migrate through the nasal route to the CNS where they can actively target a region of injury or inflammation and exert their therapeutic effects. We will summarize the functional outcomes observed in animal models of neurological diseases following IN treatment with MSC-derived EVs. We will also examine key mechanisms that have been suggested to mediate the beneficial effects of EV-based therapy.

KEYWORDS
experimental models, gene therapy, in vivo tracking, mesenchymal stem cells, nervous system, neuroimmune, transplantation

Significance statement

Treating neurological disorders is a challenging obstacle due to multifactorial and complex etiologies. The therapeutic benefits of intranasal administration of extracellular vesicles isolated from mesenchymal stem cells have gained much attention in recent years. Extracellular vesicles retain their parental cells advantages. Intranasal administration of extracellular vesical provides a non-invasive method to bypass the blood-brain barrier and target specific pathological regions. In the present study, the authors summarize the current research of mesenchymal stem cells-derived extracellular vesicles administrated by nasal pathways and their potential as a promising therapeutic approach.

Shay Herman and Idan Fishel contributed equally to this work.

1 | INTRODUCTION

There have been multiple reports over the last decade of improvements in various rodent models of neurodegenerative diseases or acute brain insults, following the transplantation of mesenchymal stromal cells (MSCs).[1-3] MSCs are multipotent adult stromal cells (https://www.sciencedirect.com/topics/immunology-and-microbiology/adult-stem-cell), which can be isolated from bone marrow (BM), umbilical cord (UC) (https://www.sciencedirect.com/topics/immunology-and-microbiology/umbilical-cord), placental, deciduous teeth, or adipose tissue (AT) (https://www.sciencedirect.com/topics/immunology-and-microbiology/adipose-tissue). They have the potential to differentiate into osteoblasts, chondrocytes (https://www.sciencedirect.com/topics/immunology-and-microbiology/chondrocyte), and adipocytes, as well as other cell types.[4] The understanding of mechanisms by which MSC exert their clinical effects is evolving and involves both paracrine and direct phagocyte activation.[5]

Cumulative data from recent research have indicated that relatively few MSCs engraft at sites of injury when administered intravenously (IV), because they are trapped in the capillaries of the lungs and rapidly cleared from the body. Of these, a minor fraction acquire certain phenotypic characteristics of neurons, although they do not fully differentiate to generate functional neurons within a brain lesion.[6-8] While MSCs are rapidly cleared from the body, their therapeutic benefits are typically preserved far longer, indicating that the effects are mediated by the cellular secretome, and specifically by small extracellular vesicles (EVs).[9,10] These small EVs are nanovesicles (30-150 nm) formed within multivesicular bodies in the endosomal system, or by shedding from the plasma membrane. They are released by a variety of cells, can pass the blood-brain barrier (BBB),[11] and are known mediators of intercellular communication. Accumulated evidence from recent years indicates that MSC-derived EVs may serve as a promising therapy for neurological disorders as a result of their ability to regulate neuroprotection, induce neurogenesis, modulate inflammation, and degrade misfolded protein aggregates.[12-14] They owe these extensive capabilities to their diverse cargo that may comprise a wide variety of nucleic acids, proteins, and lipids. These constituents reflect the characteristics of the cells from which the EVs are derived, and can modulate gene expression in recipient cells upon fusion with the plasma membrane.[15-17]

2 | THE ADVANTAGES OF EVs OVER MSCs

While MSCs have demonstrated significant efficacy in preclinical research, cell-based therapy has some notable disadvantages.[13] Exogenously administered MSCs may activate innate and adaptive immune responses that could lead to graft rejection,[14,15] trigger embolic phenomena,[16-18] or on rare occasions promote tumor cell growth in vitro,[19] or develop genetic mutations after prolonged culture.[20] In contrast, EVs are more stable, and can be readily isolated in high amounts from immortalized MSCs to ensure high reproducibility[21,22] and their therapeutic efficiency can be improved by preconditioning the parental cells.[4,23,24] Furthermore, MSCs EVs may be considered hypoimmunogenic,[25] comprehensive proteomic analysis of MSC EVs has not detected major histocompatibility complex (MHC) I or MHC II complex[26] and no immunogenicity has been reported in cross species studies.[27] However, while EVs lack the potential to form tumors themselves following administration, the possibilities that they may stimulate neoplasia[28-30] or induce procoagulant activity[16,31] require further analysis to avoid adverse effects.

Other advantages are that EVs administered by the intranasal (IN) or intravenous routes are able to penetrate the brain parenchyma and migrate to cells in the brain while their parental BM-MSCs do not.[32] Indeed, the therapeutic effects of EVs have been reported to be comparable or significantly greater than those of the parental MSCs.[33,34] Therefore, although accurate comparisons of functional outcomes are difficult due to the large differences in quantity, size, and biodistribution, as well as dosage regimen and the possible added value of the MSC secretome, substituting EVs for their parental MSCs could be a promising therapeutic direction for neurological disorders.

3 | TREATMENT OF CNS DISORDERS BY NASAL ADMINISTRATION

Over the past decade, there has been increasing interest in the exploitation of an alternate direct route from the nose to the brain via the olfactory region. Such IN delivery has the potential to bypass the BBB in a noninvasive manner and deliver therapeutic materials, such as viral vectors,[35] proteins,[36,37] peptides,[38] stem cells,[39-41] and EVs[34,42,43] to the brain.

IN administration of EVs provides an effective strategy to treat a variety of CNS pathologies, including neurodegenerative diseases such as Parkinson's disease (PD),[44] MS,[34] and Alzheimer's disease (AD),[45-47] psychiatric disorders including autism[32,48] and schizophrenia,[49] and neurotrauma such as that caused by perinatal brain injury,[50] status epilepticus (SE),[42,51] and traumatic brain damage (TBI).[52]

Practically, IN administration offers a number of benefits for patients, as it represents a painless, noninvasive, simple drug delivery system, which is manageable and easily repeatable. Typically, there is also a rapid onset of action and a favorable tolerability profile.[53] Moreover, IN administration increases the bioavailability of drugs in the brain since it prevents their absorption through the gastrointestinal tract and hepatic presystemic metabolism.[54,55]

IN administration also has some limitations that should be considered before use. The anatomy and physiology of the nasal cavity impose restrictions on the volume of drug administered.[56] In addition, as part of the defense system from xenobiotics, the nasal cavity contains degrading enzymes such as cytochrome P450, peptidases, and proteases that can metabolize drugs and reduce their impact.[53,57] Other defense mechanisms, including mucociliary clearance and efflux transporters (P-glycoprotein), may also limit the absorption of drugs.[58-60] In addition, physical and pathological conditions such as allergy, polyps, and the common cold may affect nasal absorption,[61] which also depends on physicochemical properties of the drugs and their formulations, such as drug molecular weight and size, or the solubility and stability.[56,62-64] More research

needs to be done to determine precisely how these parameters affect the nasal administration of EVs and their stability.

3.1 | Anatomy of nasal cavity

The nasal cavity is divided longitudinally into two halves by the nasal septum. Each of the two halves contains three regions: the nasal vestibule, the respiratory region, and the olfactory region.

The nasal vestibule is the entrance to the nose, the area inside the nostrils, while the respiratory region constitutes the major portion of the nasal surface area. The respiratory region contains three structures termed the superior, middle, and inferior turbinates, which are designed to generate a turbulent airflow to humidify and warm the incoming air and increase the contact between the inhaled air and the mucosal surface. The third, olfactory region, is located in the roof of the nasal cavity and is covered with pseudostratified columnar epithelium, termed olfactory epithelium. The olfactory region enables direct access to the CNS through the olfactory nerve, which bypasses the BBB.[65]

3.2 | Nose-to-brain drug transport mechanism

The respiratory region has the largest nasal surface area, and is innervated by the maxillary branch of the trigeminal nerve, which enters the CNS along the pons,[56] and serves as an entry point to both the caudal and rostral brain areas.[66,67] Therefore, the respiratory region has the potential to be a significant destination for the delivery of EVs to the CNS.

The olfactory epithelium pathway is believed to employ both intracellular and extracellular forms of transport,[68] where intracellular transport includes endocytosis into the olfactory sensory neurons (OSN), followed by intraneuronal transport along the axon and through the cribriform plate into the olfactory bulb[69] (Figure 1).

Alternatively, the observation that molecules have also been shown to enter the CNS through the systemic route, suggests another possible route for EVs to enter the CNS. Once the drug crosses the nasal epithelium, it may enter the systemic circulation due to the rich supply of nasal blood vessels. Once in a blood vessel, it can cross the BBB or blood-CSF barriers, and enter the brain through receptor-mediated transcellular transport or endocytosis depending on the drug properties[70] (Figure 1).

In a mice model of Alzheimer's disease, it was shown that the majority of PKH-labeled MSC-derived EVs delivered IN, could be seen to accumulate in the layer of immature olfactory sensory neurons.[49] EVs were observed to pass through the cribriform plate, concentrate in the glomerulus, and then progress to the next grade nerve cells, the mitral cells, before being transported further to other brain areas.[47] The definitive pathway by which EVs reach the CNS will require more research and further analysis is crucial to determine the exact pathways by which EVs reach the brain after IN administration.

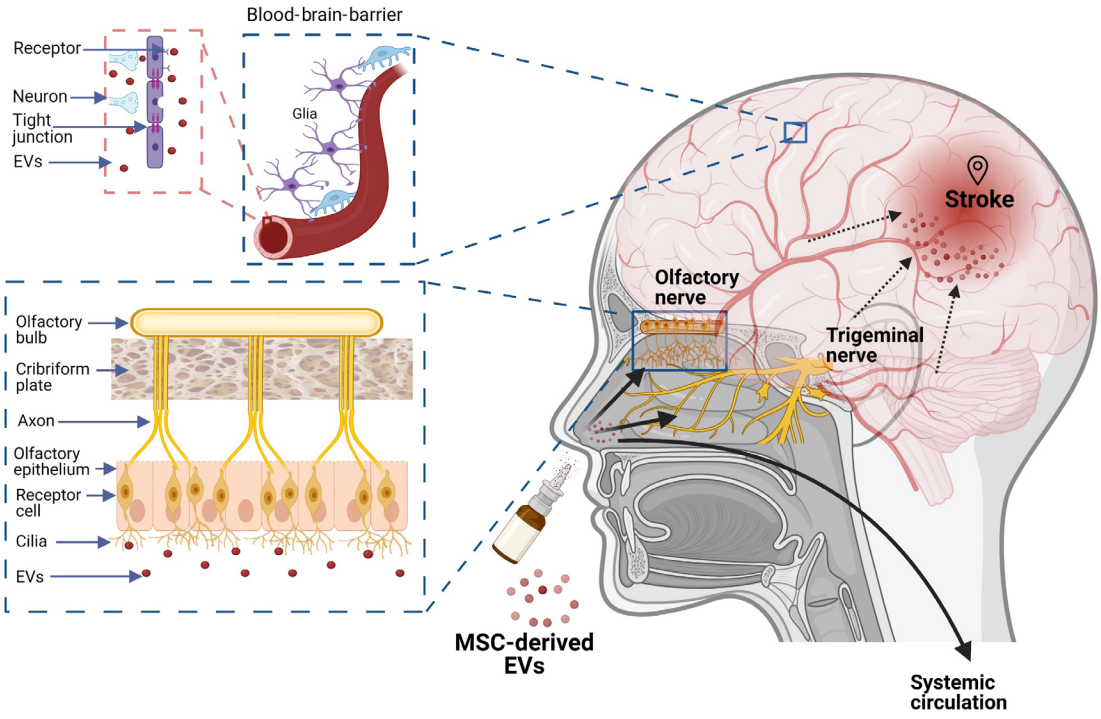

FIGURE 1 Schematic diagram of key aspects of EVs transport through the nose to the CNS. The olfactory and trigeminal nerves can serve as direct nose-to-brain routes that bypass the BBB. The top box depicts transport through the following route: EVs are absorbed by the cilia of olfactory receptor cells and pass through the axons to the olfactory bulb. Small amounts of EVs may enter through the indirect pathway by systemic circulation and need to pass the BBB through receptor-mediated transcellular transport or endocytosis (lower box). EVs will actively migrate to the area of injury or inflammation regardless of the mode of entry. BBB, blood-brain barrier; CNS, central nervous system; EVs, extracellular vesicles

3.3 | Translation from rodent studies to treating humans with IN drug administration

IN administration has been tested in a variety of animal models including mice, rats, rabbits, and monkeys. When translating the information obtained from laboratory rodents into clinical trials, it is important to consider the anatomical and physiological differences. For instance, as rats are obligate nasal breathers who rely heavily on their sense of smell, their nasal passages are more complex than those of primates, who are oronasal breathers. Rats have a large olfactory area that covers approximately 50% of the nasal cavity (the nasal cavity is approximately 10 cm^2), while humans have a small area of olfactory epithelium in the roof of the nasal cavity (the nasal cavity is approximately 180 cm^2), which constitutes only 3% of the nasal cavity.[70,71] Due to this difference, olfactory transport is likely to be more significant in rats than in humans, for the same compounds. In addition, there is a difference in the rate of CSF renewal: rats have only 150 μL CSF that is replaced every 1 hour (24 times a day), while adult humans have 160 mL that is replaced every 5 hours.[64] These differences can affect the diffusion rate of drugs from the CSF into the brain parenchyma after IN administration. It is therefore crucial to adapt the treatment dose of EVs to each animal model, by taking these anatomical and absorptions parameters into consideration.

4 | IN ROUTE TO DELIVER EVs TO THE BRAIN

PKH lipophilic dyes are commonly used to observe and track the distribution of EV distribution in the brain.[72] These dyes are highly fluorescent and stain membranes by intercalating an aliphatic region into the exposed lipid bilayer (https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/lipid-bilayer) of the vesicles. It is important to remember that while the technique is useful, the dyes may form nanoparticles that could lead to false-positive signals,[73] or alter the size of EVs, thereby affecting the cellular uptake and biodistribution.[74]

PKH26-labeled EVs rapidly reached the brain, as quickly as 30 minutes after IN administration, and the concentration peaked 1 hour post administration. Although they were detected in a variety of brain areas, their primary location was in the olfactory bulb. Immunostaining revealed that the EVs were predominantly distributed in neurons but were also found in microglia and astrocytes. Importantly, treatment with EVs did not induce any obvious morphological changes in other tissues, indicating that the IN administration of EVs is safe.[42,47,75]

The ability of EVs to cross the blood-brain barrier and be taken up by cells in the brain is critical for their therapeutic effect. In murine models of disease, MSC-derived EVs specifically targeted and accumulated in regions associated with stroke, autism, PD, AD, and SE up to 96 hours post IN administration, while in healthy controls the EVs displayed a diffuse pattern of migration and were cleared by 24 hours. The accumulation of EVs was highly correlated with a neuroinflammatory signal in the damaged brains, suggesting that the homing mechanism is inflammation-driven.[42,43,76] MSC-derived EVs treated with proteinase K to remove membrane proteins,[32] or pertussis toxin to block chemokine receptors,[43] were not able to migrate and localize to the site of injury or inflammation with the same efficiency, and lost their therapeutic advantage, indicating a crucial role for membrane proteins and receptors in the neuroinflammation-mediated chemotaxis and homing abilities. These findings suggested that EVs have the ability to target and accumulate in different cell types and in damaged regions of the brain (Figure 2).

5 | THERAPEUTIC ASSESSMENT OF EVs AFTER INTRANASAL ADMINISTRATION

Accumulating evidence indicates that IN administration of MSCs, components of their secretome, or derived EVs, produce therapeutic effects that are similar to conventional routes, that is, intraperitoneal (IP) or intravenous (IV) injections.[77-79] MSC-derived EVs administered IN or IV were able to penetrate the brain parenchyma and migrate to sites in the brain in a similar fashion.[32] While IN administration holds great promise as a direct and quick route to the brain, further investigation is needed to examine the differences between IN and other administration routes with respect to the minimum effective dose (MED), systematic biodistribution[80,81] (taking into account presystemic metabolism), and speed of localization in brain damaged tissues. Taken together, the noninvasive and easily repeatable nature of IN administration holds great promise for CNS therapy.

6 | CHALLENGES TO STANDARDIZATION OF ISOLATION AND CHARACTERIZATION OF EVs

While treatment with MSC-derived EVs appears promising, the large differences in isolation methods, reported concentrations, and characterization techniques make it a challenge to compare the functional outcome of EVs in different preclinical murine models of neurological diseases. Recent comprehensive guidelines "minimal information for studies of extracellular vesicles 2018 (MISEV2018)"[82] have been published in an attempt to unify EVs research and consolidate characterization protocols.

There are currently no specific markers to identify EVs subtypes and subcellular origin (with the exception of live imaging of vesicle release). While most reports still utilize the term "exosomes", which implies an endosome-origin, the biogenesis pathway of the vesicles remains unclear and EVs can therefore not be differentiated from plasma membrane-derived vesicles, which share membrane protein composition and size (50-200 nm).[82,83] For this reason, Théry et al recommend the generic term of small extracellular vesicles (EVs).[82]

A variety of methods are used to isolate EVs from other non-EVs components of the matrix. The most common are ultracentrifugation-based methods, based on density, size, and shape. These include both

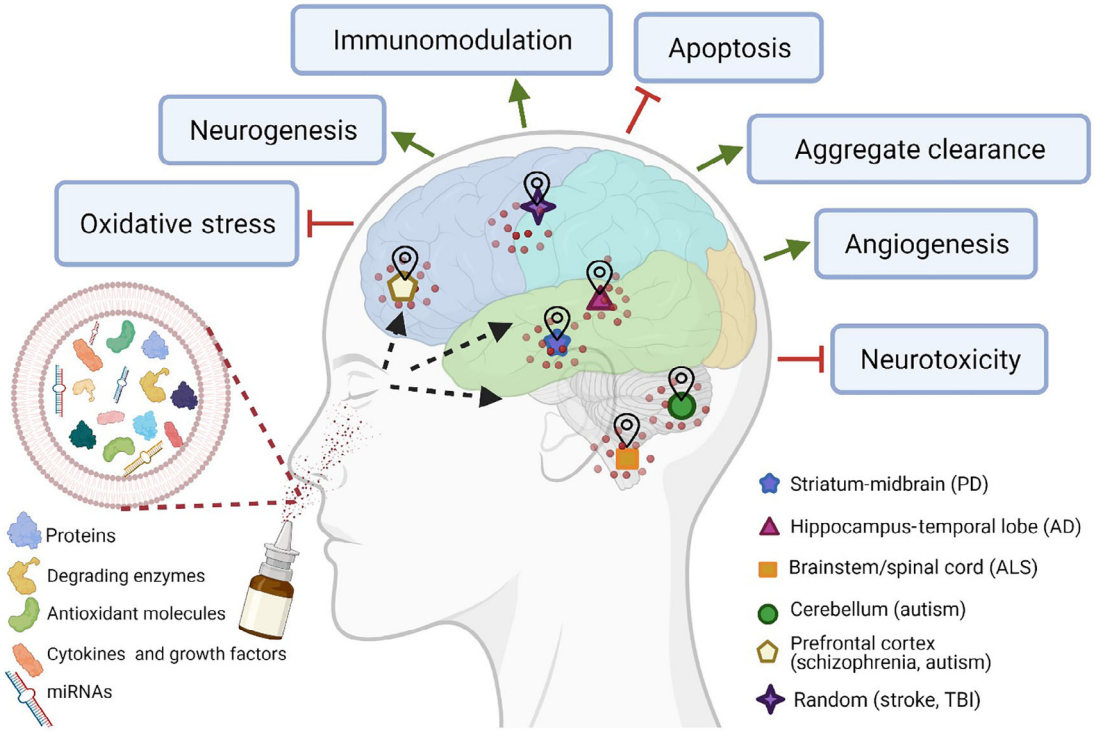

FIGURE 2 Schematic diagram illustrating the major brain regions affected in neurodegenerative diseases, to which EVs actively migrate following IN administration. The figure presents the key mechanisms and cargo used by MSC-derived EVs to mitigate these pathologies and induce regeneration. EVs, extracellular vesicles; IN, intranasal; MSC, mesenchymal stem cells

differential ultracentrifugation and density gradients. Although, differential centrifugation is the gold-standard method for isolating EVs, it requires large starting volumes and may induce mechanical damage due to the high speed of the ultracentrifugation.[84] Alternative methods developed to deal with these challenges include size based techniques, precipitation, filtration, microfluidics, and immunoaffinity.[82,83] While most labs use differential centrifugation to isolate EVs, some use ion-exchange chromatography[42,51] or a commercial isolation kit[34,79] that could lead to variations in the concentration, purity, size, and therapeutic efficacy of the isolated vesicles.[85,86]

Physical and biochemical analyses can further characterize and assess the purity of an EVs preparation, where physical analysis provides information about the morphology, size, and concentration of the vesicles. Size and particle concentration are generally measured by light scattering technologies such as nanoparticle tracking analysis (NTA) or dynamic light scattering (DLS). Visual imaging techniques such as transmission electron microscopy (TEM), are often used to examine EV morphology, with biochemical analysis such as immunodetection (western blotting or flow cytometry), mass spectrometry, or microfluidic assays, to detect established EVs markers. A negative control of proteins not expected to be in EVs, is routinely used to assess purity. The observation that applying different isolation methods to the same cell line generates EVs with different proteomic profiles, further complicates the comparison of preclinical therapeutic effects.[87]

Quantification of EVs is a major obstacle when comparing functional outcomes. The most commonly used methods are total protein (measured by various colorimetric assays) or total particle number (measured by light scattering technologies). While each method is acceptable, protein quantification may depend on the assay protocol and may be confounded by co-isolated protein contaminants. The finding that it is not always possible to deduce protein concentration from the number of particles and vice versa has prompted the recommendation to use more than one quantification method and report a ratio of proteins: particles, in order to compare published results.[82]

Furthermore, additional challenges to an accurate comparison of the therapeutic effect of EVs relate to the different source or preconditioning treatments of the parental MSCs. Taken together, the inadequate quality control and lack of standardization of protocols across study groups makes comparisons difficult. Reproducibility and consistency of preparation will be required for EVs to be successfully implemented as a clinical treatment.

7 | SUGGESTED MECHANISMS OF ACTION OF MSC-DERIVED EVs

The varied actions of EVs are generally attributed to their vast and diverse cargo, which may include cytokines and growth factors, signaling lipids, mRNAs, and regulatory miRNAs.[88,89] These factors influence key processes and pathways such as immunomodulation, angiogenesis, and neurogenesis, which are all altered in CNS disorders. Indeed, multifactorial neurological diseases might be expected to

gain particular benefit from simultaneous effects on multiple pathways.[90] Specifically, EVs derived from MSCs have been shown to contain immunoregulatory cytokines and growth factors, including TGFβ1, interleukin-6 (IL-6), IL-10, and hepatocyte growth factor (HGF).[91] In addition, they may also contain miRNAs that can contribute to neurogenesis and neural plasticity. These include miR-133b that can promote neural remodeling,[92] miR-219 that promotes CNS myelination,[93] miR-1000 that has a role in regulating neurotransmission release, and members of the miR-17-92 cluster, which have known roles in inhibiting phosphatase and tensin homolog (PTEN), a major intrinsic impediment to axonal growth.[94] Other miRNA species found in MSC-derived EVs, such as miR-21[95] or miR-223,[96] have antiapoptotic effects. Transfer of functional miRNAs to neural cells can promote neurite remodeling and plasticity, or inhibit apoptosis, which subsequently promotes functional recovery.[97] In addition, VEGF, extracellular matrix metalloproteinase inducer (EMMPRIN), wnt3a, pentraxin 3 (PTX3), and MMP-9 have all been detected in MSC-derived EVs. These proteins play a vital role in stimulating angiogenesis and neurogenesis, which are fundamental for tissue repair.[98-101] Enzymes such as neprilysin (NEP), found in EVs, have the ability to degrade aggregates of misfolded protein,[45] and a proteomic analysis also identified antioxidant molecules such as Cu/Zn superoxide dismutase 1 (SOD1), antioxidant proteins thioredoxin (TXN) and peroxiredoxin-6 (PRDX6), and heat shock protein 70 (HSP70), which are able to lower oxidative stress and neurotoxicity.[102] It is interesting to speculate that the multiple properties of MSC-derived EVs are exquisitely suited to synergistically target the complex and manifold aspects of CNS diseases. However, understanding the particular molecular mechanisms by which EVs exert their function would facilitate the generation of advanced EV-based therapies for neurological disorders.

8 | THERAPEUTIC EFFECT OF EVs IN NEUROLOGICAL DISORDERS

8.1 | Neurodegenerative diseases

Neurodegenerative diseases are a diverse group of disorders that are characterized by the progressive degeneration of structure and function in the central or peripheral nervous system. This is thought to be mediated by reactive oxygen species released by mitochondrial dysfunction, aggregated misfolded proteins, and inflammation.[103-105] In an APP/PS1 AD mouse model, 2 weeks of IN treatment with AT MSC-derived EVs reduced beta-amyloid deposition and microglia activation thereby reversing the neuron shrinkage and loss, and rescued memory deficits.[47] Similarly, BM MSC-derived EVs loaded with NEP administered intranasally for 14 days, led to improvement in spatial learning and memory deficits in a virally induced rat model of AD, and a decrease in the number of beta-amyloid plaques in the hippocampus compared with control animals. The treatment decreased the neuronal damage and inhibited inflammation and apoptosis by immunomodulation and suppression of oxidative stress.[45] EVs isolated from BM MSCs pretreated with proinflammatory cytokines exerted an anti-inflammatory effect in a triple-transgenic AD mice model that led to decreased microglial activation. Hyperactivated microglia may contribute to synapse loss, therefore modulation of this activation could explain the observed increase in dendritic spine density in treated mice compared with controls.[46] IN treatment with EVs prepared from stem cells from exfoliated deciduous teeth caused a notable increase in the density of tyrosine hydroxylase (TH) positive cells in both the striatum and the substantia nigra pars compacta of 6-OHDA treated rats that led to improvement in all gait parameters. Interestingly, although the effect was maintained up to 10 days posttreatment, there was a deterioration by day 19, indicating that the therapeutic benefits of EVs could be time dependent. A proteomics analysis of the protein expression profile in EVs indicates that their therapeutic effect on neurodegenerative diseases models is in part obtained through protein-mediated pathways by immunomodulation and suppression of oxidative stress and neurotoxicity.[47,102] Information from recent studies on MSC-derived EVs given IN in neurodegenerative rodents models is reviewed in Table 1.

8.2 | Neurodevelopmental disorders

The etiologies of major neurodevelopmental disorders such as schizophrenia and autism are unclear, with evidence supporting a combination of genetic factors and environmental insults, including viral infection during pregnancy. They commonly involve disruption of synapse formation and other deficiencies during critical periods in embryonic development and up to early childhood. The currently approved pharmacological treatments are antipsychotics that target the comorbid behaviors and do not modify the pathophysiology of the disease. Since EVs can modulate gene expression and promote regeneration of cells, they may represent a promising therapy for neurodevelopmental disorders. In a murine model of autism spectrum disorder (ASD) involving BTBR T+tf/J (BTBR) and genetic Shank3B knock-out mice, repeated IN administration of BM MSC-derived EVs significantly improved several core symptoms related to social interactions and maternal behavior, and led to a reduction in repetitive behaviors and cognitive rigidity, and an increase in vocalization. These beneficial effects were maintained for up to 6 months after treatment. It is thought that attenuation of GABA-mediated pathways in the prefrontal cortex as a result of EVs treatment may contribute to the improvement in behavioral deficits observed in ASD mice.[32,48] Normalization of the number of parvalbumin-positive GABAergic interneurons as well as reduced glutamate levels in the CSF were also seen in a murine phencyclidine (PCP) induced model of schizophrenia after IN treatment with BM MSC-derived EVs. In addition, there was also an improvement in schizophrenic behaviors such as social interactions and disruption in prepulse inhibition. EVs were able to migrate and localize in the prefrontal cortex of treated mice, which is a crucial region involved in the pathogenicity of schizophrenia.[49] These results indicate that treatment with EVs can prevent or ameliorate symptoms of neurodevelopmental disorders in animal models, with the therapeutic potential to activate molecular and protein changes in the neurons. This induces regenerative mechanisms, leads to a restoration of the

TABLE 1 Intranasal treatment with MSC-derived EVs in odels of neurodegenerative and neurodevelopmental disorders

			Dosage regimen							
Disorder	**MSC source**	**Isolation method**	**# doses**	**Total protein per dose**	**Total # of particles per dose**	**Characterization of EVs**	**Animal model**	**Clinical improvement**	**EVs suggested mechanism**	**Reference**
AD	Rat BM + Neprilysin	Differential centrifugation method	Multidose	50 μg	N/A	SEM, DLS, EVs markers-CD9, HSP70	Lentiviral-transduced mutant APP rats	Functional recovery and reduced inflammation and apoptosis	Immunomodulation and suppression of oxidative stress	45
AD	Cytokine-preconditioned human BM	Differential centrifugation method	2	15 μg	7.5×10^9	NTA, EVs markers-CD9, HSP70, CD63	3xTg mice	Reduced activation of microglia cells and increased dendritic spine density	Immunomodulation	46
AD	Human AT	Differential centrifugation method	Multidose	1 mg/kg	N/A	TEM, NTA, Coomassie blue staining EVs markers-Alix, TSG101	APP/PS1 mice	Ameliorate neuronal damage, promote neurogenesis, and rescue memory deficits	Evs proteins are neuroprotective and promote neurogenesis	47
ALS	Murine AT	Commercial isolation kit	Multidose	1 μg	N/A	TEM, TRPS, EVs markers-CD9, HSP70	SOD1 mice	Improved motor performance, protected lumbar motoneurons, reduced inflammation	miRNAs suppress the activation of neurotoxic astrocytes	79
Autism	Human BM	Differential centrifugation method	Multidose	N/A	2×10^9	NTA, EVs markers- CD63, CD9, negative marker-Calnexin	BTBR mice	Functional recovery	miRNAs affect immunomodulation and migration	32
Autism	Human BM	Differential centrifugation method	Multidose	N/A	2×10^8	TEM, NTA, EVs markers-CD81 CD63	Shank3B KO mice	Functional recovery, increase of GABARB1 in the prefrontal cortex	Molecular and protein changes in the neurons, increase in expression in GABA Ra1	48
MS	Murine AT	Commercial isolation kit	Multidose	10 μg	N/A	SEM, DLS, EVs marker-CD63	EAE mice	Reduced clinical score and spinal lesions, elevated immunomodulatory responses	Anti-inflammatory properties	34
PD	Human exfoliated deciduous teeth	Differential centrifugation method	Multidose	N/A	3×10^8	TEM, NTA	6-OHDA induced rats	Improved gait and preserved the expression of TH	Effects on GABAergic, glutamatergic, and cholinergic pathways	44

(Continues)

TABLE 1 (Continued)

Disorder	MSC source	Isolation method	Dosage regimen			Characterization of EVs	Animal model	Clinical improvement	EVs suggested mechanism	Reference
			# doses	Total protein per dose	Total # of particles per dose					
PD	Human exfoliated deciduous teeth	Differential centrifugation method	Multidose	N/A	2.85×10^8	TEM, NTA, EVs markers-Syntenin 1, HSP70, MFG-E8	6-OHDA induced rats	Improved motor functions and normalized TH expression	Neuroprotective and antioxidative actions, transport of annexins	102
Perinatal brain injury	Human Wharton's jelly	Differential centrifugation method	1	50 mg/kg	N/A	TEM, EVs antibody array—CD63, CD81, ALIX, FLOT1, ICAM1, EpCAM, ANXA5, TSG101. negative marker-GM130	Hypoxic–ischemic and an inflammatory insult in rats	Rescued myelination and cell count, improved learning ability	Oligodendroglia maturation, neuroprotective properties	106
Perinatal brain injury	Human Wharton's jelly	Differential centrifugation method	1	50 mg/kg	N/A	TEM, EVs Antibody Array-CD63, CD81, ALIX, FLOT1, ICAM1, EpCAM, ANXA5, TSG101 negative marker-GM130	Hypoxic–ischemic and an inflammatory insult in rats	Reduced microglia-mediated neuroinflammation	Suppression of transcription and secretion of proinflammatory cytokines	50
Schizophrenia	Human BM	Differential centrifugation method	Multidose	N/A	2.4×10^7	TEM, NTA EVs markers-CD81 CD63	PCP-treated mice	Improved behavior and biochemical properties, prevented reduction in GABAergic interneurons, decreased glutamate levels	Immunomodulatory, anti-inflammatory, and neuroprotective effects	49

TABLE 1 (Continued)

Disorder	MSC source	Isolation method	Dosage regimen			Characterization of EVs	Animal model	Clinical improvement	EVs suggested mechanism	Reference
			# doses	Total protein per dose	Total # of particles per dose					
SCI	Human BM loaded with PTEN siRNA	Differential centrifugation method	Multidose	N/A	1.72×10^8	NTA, TEM EVs markers-CD9 CD81 negative marker-calnexin	Rat laminectomy spinal cord injury	Functional recovery, reduced neuroinflammation and gliosis, increased axonal regeneration and angiogenesis, structural and electrophysiological improvements	Transfer of pro-angiogenic microRNAs, inhibits the activity of A1 neurotoxic reactive astrocytes	43
SE	Human BM	Ion-exchange chromatography	1	N/A	10×10^{10}	NTA EVs marker-CD63	Kainate-induced SE rat	Colocalization of EVs in neurons in areas of brain injury	Incorporation into microglia at site of injury	42
SE	Human BM	Ion-exchange chromatography	2	15 μg	7.5×10^9	NTA EVs markers-CD63 CD81 (CD9 negative)	Pilocarpine hydrochloride-induced SE mice	Reduced inflammation, preserved neurogenesis, and improved cognitive and memory function	Anti-inflammatory effects, neuroprotection	51
TBI	Human endometrial	Differential centrifugation method	1	500 μg/kg	N/A	TEM, DLS EVs marker-TSG101	Hippocampal penetrating brain injury mouse model	Reduced sensorimotor deterioration, improved neurological recovery, locomotion speed preservation	Proinflammatory and anti-inflammatory cytokines, vascular remodeling. VEGF, GM-CSF, and IL-6 factors in EVs-related to angiogenesis, neurogenesis, and neuroprotective effects	52
Ischemic stroke	Human AT	Differential centrifugation method	1	200 μg/kg	N/A	Photon correlation spectroscopy, TEM, EVs markers-CD63 CD81	Focal permanent ischemia rats	Decreased infarct volume, improved BBB integrity, improved motor, and behavioral performance	Angiogenesis	107

Abbreviations: AD, Alzheimer's disease; ALS, Amyotrophic lateral sclerosis; BBB, blood-brain barrier; DLS, dynamic light scattering; EVs, extracellular vesicles; MS, Multiple sclerosis; MSC, mesenchymal stem cell; N/A, not available; NTA, nanoparticle tracking analysis; PCP, phencyclidine; PD, Parkinson's disease; SEM, scanning electron microscope; SCI, spinal cord injury; SE, status epilepticus; TBI, traumatic brain damage; TEM, transmission electron microscopy; TH, tyrosine hydroxylase.

expression of impaired neurotransmitters, and promotes axonal regrowth. Information from recent studies on MSC-derived EVs given IN in neurodevelopment models is described in Table 1.

8.3 | Neurotrauma

Neurotrauma has a major epidemiological and economic impact on society. SE, spinal cord injury (SCI), TBI, stroke, and neonatal encephalopathy all share a convergent pathophysiology. While a traumatic injury is an "event," it actually resembles a chronic disease process that involves progressive loss of neurons and myelin sheaths, and lifelong neurological defects. Currently available drugs provide only symptomatic treatment with a narrow therapeutic time window available for effective application. Cell-based therapy could provide a novel solution to repair and regenerate the injured central nervous system.[108-110] In this context, sensorimotor deterioration was reduced, and neurological recovery was improved in a mouse model of penetrating hippocampal injury, when the mice treated IN with a single dose of EVs 24 hours post injury.[52] EVs loaded with PTEN small interfering RNA (siRNA), induced dorsal root ganglia neuron outgrowth and successfully inhibited PTEN expression in the damaged tissue of rats with complete spinal cord injury for up to 8 weeks after treatment. They also promoted significant functional and sensory recovery, motor improvement, and faster restoration of the urinary reflex when administered 2 to 3 hours after injury. Functional recovery was accompanied by reduced neuroinflammation and gliosis, increased axonal regeneration and angiogenesis, and improvement in structural and electrophysiological parameters.[43] Similarly, MSC-derived EVs inhibited the production of proinflammatory molecules, prevented microgliosis, reduced myelination, and improved learning abilities in rats with perinatal brain injury.[50,106] EVs treatment after SE reduced neuron loss and inflammation, and enabled the maintenance of normal neurogenesis, and preservation of cognitive and memory function.[51] A single intranasal administration of MSC-derived EVs, 24 hours after a focal permanent ischemic stroke in rats, was enough to significantly reduce the infarct volume, improve the integrity of the blood-brain barrier, and restabilize vascularization. In addition, the treatment restored the long-term motor and behavioral performance that had been impaired by the stroke.[107] The mechanism is thought to be by preventing secretion of proinflammatory cytokines and inducing anti-inflammatory factors, inhibiting apoptosis of cells after injury, and promoting angiogenesis and cell regeneration. This effect was retained even when treatment was delayed by up to 24 hours post injury, indicating an advantage of using EVs over the short therapeutic window of current drugs. Information from recent studies on MSC-derived EVs given IN in Neurotrauma rodents models is summarized in Table 1.

9 | SUMMARY

IN administration of MSC-derived EVs has wide therapeutic potential for diverse neurological disorders. EVs can migrate to specific regions in the brain through neuroinflammation-mediated chemotaxis and homing abilities, which minimizes the systematic distribution. While the therapeutic capabilities of EVs are comparable to those of the parental MSC, they exhibit additional advantages due to their small size, low immunogenicity, and migrating abilities. Treatment by IN provides further advantages over cell transplantations or other routes. The therapeutic effect of EVs can be attributed to their ability to transport hundreds of proteins, growth factors, peptides, miRNAs, and other molecules to recipient cells and thereby mediate neuroprotection, angiogenesis, immunomodulation, cell regeneration, and aggregate clearance. Thus, EVs safety profile and broad spectrum of therapeutic effects, beneficial advantages in various neurological disorders and numerous preliminary clinical data indicate great promise. While there is still a need for further studies to determine optimal dosing and administration regimens, and to identify the pathways involved in their mechanism of action, MSC-derived EVs administered intranasally have immense potential and seem a novel therapeutic approach to neurological disorders. Moreover, EVs could be enhanced or loaded with approved drugs, molecules, and nucleic acids, opening a vast potential for directed and synergistic therapies. While more investigation is needed to effectively load EVs, recent research has indicated significant progress in the field.[43,111,112] Once limitation of reproducibility and consistency of preparation will be standardized in the clinic, MSCs EVs could change to way brain and CNS disorders are treated today.

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CONFLICT OF INTEREST

D.O. (with others) has submitted several patent applications related to EVs. All were assigned to "Ramot" at Tel Aviv University. The other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

S.H. and I.F.: writing—original draft preparation; D.O.: writing—review and editing. All authors have read and approved the published version of the article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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