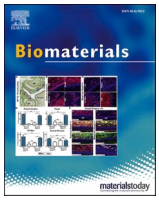
Biomaterials 293 (2023) 121949

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Exosomes based strategies for brain drug delivery

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ARTICLE INFO

Keywords:
Exosomes
Drug delivery
Neurodegenerative diseases
Glioma
Blood-brain barrier

ABSTRACT

Exosome application has emerged as a promising nanotechnology discipline for various diseases therapeutics and diagnoses. Owing to the natural properties of efficient drug delivery, higher biocompatibility, facile traversing of physiological barriers, and subtle side effects, exosomes shorten their way to clinical translation. Exosomes are nanoscale membrane-bound vesicles primarily involved in intercellular communication and exhibit natural blood-brain barrier (BBB) traversing ability, which enables their application as drug delivery vehicles for brain diseases treatment. Herein, we highlight recent exosome-based drug delivery endeavors for neurodegenerative diseases and brain cancer therapy, summarize the obstacles and future directions in clinical translation.

1. Introduction

Extracellular vesicles (EVs) are membrane-bound nano-microscale bodies secreted by almost all types of prokaryotic and eukaryotic cells [1,2]. They were first recognized in parasitic cells as debris and "cell dust" in human platelets and assumed without any significant role in biology [3]. Later their role was found by having biologically active substances as cargo [4]. Earlier, intercellular communication was thought to be mediated through soluble substances including interleukins, cytokines and soluble factors that were exchanged intracellularly. It is appreciated that EVs act as major conduits of long-distance communication among various cell types [5]. EVs are further classified as ectosomes and exosomes. Ectosomes originate from the outward budding of the cell membrane (microvesicles) or apoptotic bodies with a size range from ~100 nm to several μm, whereas exosomes originate from multivesicular bodies (MVBs) by double invagination of the plasma membrane, forming intraluminal bodies [6]. The plasma membrane is primarily invaginated to form a saucer-shaped structure termed an early sorting endosome containing engulfed proteins and genetic materials from the cytoplasm and cell membrane proteins resulting from membrane invagination. Early sorting endosome formation is also orchestrated by the endoplasmic reticulum and Golgi complex, as evidenced by finding their constituents in the early sorting endosome milieu. Early sorting endosomes then mature into late sorting endosomes that ultimately end up in MVBs by secondary fusing of the endosomal limiting membrane. The resulting MVBs comprise several intraluminal bodies, i.e., exosomes, either degraded by autophagosomes or lysosomes or released from the cell after fusion with the plasma membrane [7,8].

EVs have been reported to contain RNA, DNA, proteins, enzymes, metabolites, viruses, and even cellular organelles, i.e., mitochondria and cytoskeleton contents in relatively larger EVs [9–12]. They exist in many biological fluids, including blood, urine, saliva, cerebrospinal fluid, milk, amniotic fluid, cell culture media and even fruit tissue. Among EVs, exosomes have attracted considerable attention from biomedical researchers due to their suitable nanoscale size for biomedicines (~40–200 nm range) with an average of ~100 nm and a flotation density of 1.10–1.18 g mL^{-1} [6]. They are also abundantly present in body fluids at an average of approximately 3×10^6 exosomes per μL [13].

The exosome potential has been exploited in diagnostics and therapeutics of various neoplastic and non-neoplastic diseases [14,15].

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https://doi.org/10.1016/j.biomaterials.2022.121949
Received 13 April 2022; Received in revised form 12 November 2022; Accepted 5 December 2022
Available online 8 December 2022

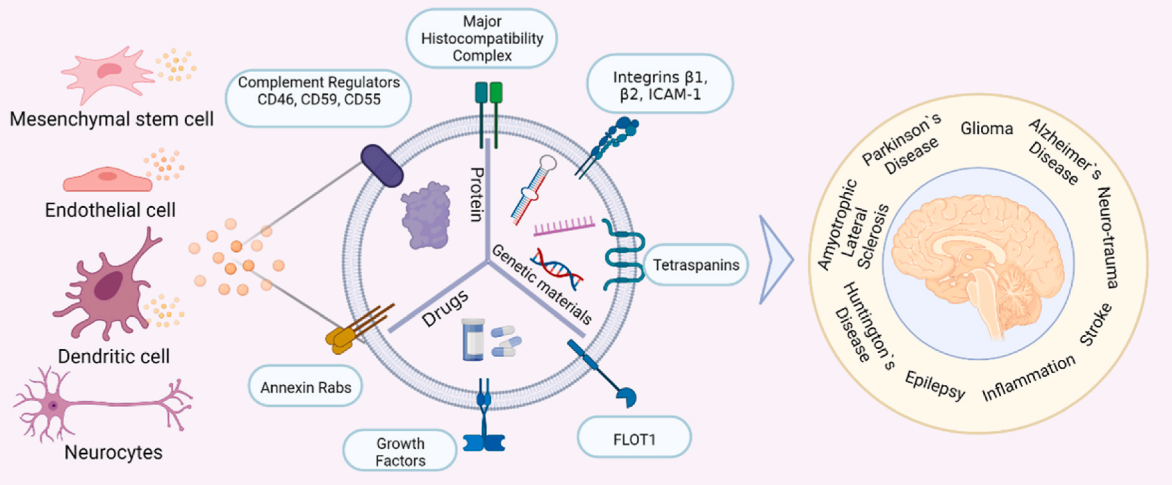

Fig. 1. Illustration of exosome cell origin, structure, and cargo types employed in brain disease therapy and theranostics.

Interestingly, exosomes isolated from agricultural products show particular promise in clinical applications. In particular, exosomes isolated from fruits and milk are potentially more suitable for gastrointestinal (GI) ailments and survive in the gastrointestinal microenvironment with enhanced absorption. Indeed, several foodstuff-derived exosomes have successfully qualified for clinical trials. Exosomes also mirror the surface protein expression of the parent cell and cellular cytoplasmic contents, i.e., 'cargos' loaded during

Table 1
Exosome associated clinical trials registered in NIH by November 2022.

Title	NIH identifier	Type	Conditions	Notes or Interventions
The Pilot Experimental Study of the Neuroprotective Effects of Exosomes in Extremely Low Birth Weight Infants	NCT05490173	Phase: Not Applicable Enrollment 10	•Premature Birth •Extreme Prematurity •Preterm Intraventricular Hemorrhage •Hypoxia-Ischemia, Cerebral •Neurodevelopmental Disorders	Other: Exosomes derived from mesenchymal stromal cells (MSCs)
Application of Circulating Exosomes in Early Diagnosis and Prognosis Evaluation After Intracerebral Hemorrhage	NCT05035134	Observational Enrollment 300	•Intracerebral Hemorrhage •Circulating Exosomes	N/A
Cohort Study of Blood Biomarkers for TES	NCT04928534	Observational Enrollment 120	•Chronic Traumatic Encephalopathy •Traumatic Encephalopathy, Chronic •Traumatic; Encephalopathy, Postcontusional	•Diagnostic Test: Blood tests (including exosomes), Cognitive function tests, head MRI (plain scan and DTI sequence) examination and head PET (FDG-PET, Tau-PET and Amyloid-PET) examination
Allogenic Mesenchymal Stem Cell Derived Exosome in Patients With Acute Ischemic Stroke	NCT03384433	-Phase: •Phase 1 •Phase 2 Enrollment: 5	•Cerebrovascular Disorders	•Biological: exosome
Focused Ultrasound and Exosomes to Treat Depression, Anxiety, and Dementias	NCT04202770 Pending due to Covid-19	Phase: Not Applicable Enrollment: 300	•Refractory Depression •Anxiety Disorders •Neurodegenerative Diseases	•Other: Exosomes
Extracellular Vesicles as Stroke Biomarkers	NCT05370105	Observational Enrollment: 100	•Stroke •Rehabilitation	•Other: blood withdrawal
The Role of Acupuncture-induced Exosome in Treating Post-stroke Dementia	NCT05326724	Phase: Not Applicable Enrollment: 30	•Exosome •Post-stroke Dementia •Acupuncture	•Device: Acupuncture
LRRK2 and Other Novel Exosome Proteins in Parkinson's Disease	NCT01860118	Observational Enrollment: 601	•Parkinson's Disease	Inhibited NF-kB and MAPK to reduce neuroinflammation mediated by microglial cells
the Safety and the Efficacy Evaluation of Allogenic Adipose MSC-Exos in Patients With Alzheimer's Disease	NCT04388982	Phase: •Phase 1 •Phase 2 Enrollment: 9	•Alzheimer Disease	Biological: low dosage MSCs-Exos administrated for nasal drip •Biological: mild dosage MSCs-Exos administrated for nasal drip •Biological: high dosage MSCs-Exos administrated for nasal drip

Box 1
Highlighted merits of exosomes for brain diseases.

Compared with other nanodelivery systems.

- Exosome is non-toxic and has immunomodulatory role.
- Some has natural navigating capacity for the lesion site and BBB penetrating ability.
- Along with uploaded cargo, inherited content may also be therapeutic.

Compared with cell transplantation.

- Avoiding teratoma formation due to exosomes lack of nucleus and cannot replicate *in vivo*.
- Better quality control, exosomes can be easily sterilized via filtration and can store at −80 °C for long term.
- BBB penetration is readily achievable when exosomes are systemically injected.
- Exosomes have a higher surface-to-volume ratio with the potential for modulating ligand-gated signaling pathways.
- Exosomes can achieve better tissue penetrating through transcytosis.
- Drug loading in exosome is more feasible than loaded into cells.
- Microenvironment or niche significantly impact the behavior of transplanted cells *in vivo* and may defect the supposed aim.

biogenesis. The nature of these proteins and cargos largely controls the biological activity mediated by the exosomes, ranging from immunomodulation and induction of apoptosis to the enhancement of proliferation. Exosomal surface proteins and contents have been comprehensively reviewed in Ref. [6].

Specific cell types generate exosomes with characteristic properties; for example, exosomes derived from the mesenchymal stem or stromal cells (MSC) are bestowed with immunosuppressing and tumor homing ability. Likewise, nanotechnology-based interventions can tune exosomes for specific applications. The incorporation of desired cargos (RNA interference; RNAi, gene editing materials, vaccine content, proteins, nanomaterials, therapeutic agents, diagnostic probes, AAV virus) dictates their intended biomedical application [16–18]. Interestingly, naturally occurring exosomes have the upper hand over synthetic counterparts (i.e., nanomaterials; especially liposomes) for biomedical applications due to their biogenic origin, and they can readily cross physiological barriers, e.g., blood-brain barrier (BBB), placental barrier, etc. and have enhanced bioavailability in GI tract. The size of exosomes is relatively more suitable for cellular applications as most cellular organelles, DNA, proteins, and ligand receptors have nanoscale architecture, therefore, constituting excellent scaffolds for exosome interplay. The exosome origin, surface moieties and modifications, loaded cargoes as well as the potential applications in brain diseases were presented in (Fig. 1).

In brain biology, the exosomes' role is pivotal. The neuronal exosomes communicate with other supporting cells present in the brain milieu, including axon myelination and integrity cells, oligodendrocytes, and microglia [19]. The neurotransmitters and glutamate trigger the release of exosomes to deliver a payload to adjacent and remote neurons. The oligodendroglia exosomes have the natural ability to protect the axons from oxidative stress by delivering catalase and superoxide dismutase-1 [20,21]. Considering the natural biological role and abundance of naturally occurring cell receptor amenable proteins on their surface that otherwise should be decorated on the synthetic nanomedicine surface, the exosome application is ideal for delivering desired cargo to the brain.

The BBB is highly permeable to exosomes isolated from specific cells such as mesenchymal stem cells (MSC), neurons, and inflammatory cells; they lend themselves to brain disease applications. However, these applications still remain exploratory as bench research and further spatial-temporal efforts will be needed for their commissioning to bedside applications. Considering that initial results in models of brain diseases are encouraging, herein, we review the latest design developments and experimental findings in neurodegenerative diseases and brain cancer.

Exosomes have an excellent drug-carrying capacity and can accommodate hydrophobic and hydrophilic drugs. The benefits of tumor homing ability, extended blood circulation half-life, excellent BBB traversal, lower toxicity, hypo-immunogenicity, and reflection of the 'inheritance' from the parent cell and cellular affinity make exosomes excellent drug delivery vehicle for various diseases, including brain ailments [22]. Exosomes' significant functions include intercellular communication, removal of unwanted cellular metabolites or constituents, and/or providing a potential scaffold for cellular proliferation. Noteworthy, in tumors, exosomes can promote angiogenesis, favorable modulating tumor microenvironment, and immunosuppression that paves the way for tumor growth and metastasis [23,24]. *In vivo*, exosome tendency for accumulation in specific organs, prolonged blood plasma half-life, and evading the reticuloendothelial system depend on the 'inherited' factors mentioned above. For example, exosomes expressing cluster of differentiation 43 (CD43), a T-cell marker, use this as a shield to protect it against immune cells [25].

Exosome-based applications for brain diseases therapeutics development has a history of around one decade, and several exosome associated clinical trials was initiated and registered at NIH, highlighted in Table 1. Contrary to the synthetic drug delivery system, exosomes have the natural ability to cross various physiological barriers. In brain diseases theranostics, BBB penetration is one of the essential milestones that can easily be achieved via exosomes. Interestingly, exosomes from stem cells, neural origin, and macrophages can achieve higher BBB penetration. Upon neuro-insult, bone marrow and adipose tissue stem cells are mobilized and localized in the brain after traversing the BBB. They initiate tissue repair by secretion cytokines, certain growth factors, and exosomes [26]. Experimentally, exosomes isolated from bone marrow stromal cells (BMSC) can promote neurite growth and branching, leading to functional recovery in neurodegenerative conditions [27]. Likewise, BBB endothelial cells are highly responsive to exosomes isolated from mesenchymal stem cells (MSC), endothelial cells, macrophages, normal neural cells, and healthy or cancer cells compared to exosomes isolated from other types of somatic cells (Box 1 highlights the merits of exosomes for brain diseases employment).

2. The mechanism of exosome-based BBB penetration

BBB is a physiological barrier primarily comprised of brain capillary endothelial cells preventing the brain environment from potentially harmful substances. The BBB is characterized by non-fenestrated endothelial cells, forming tight junctions and directly amenable to small-

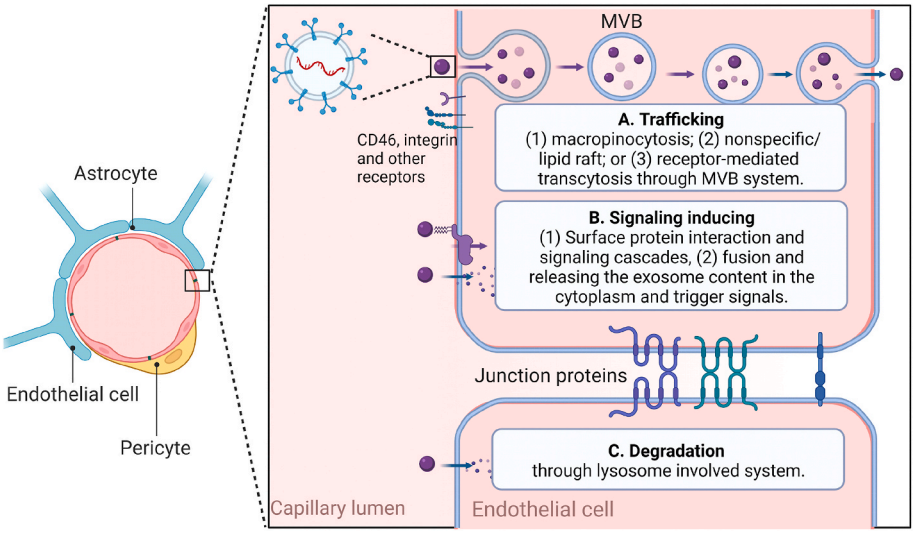

Fig. 2. A mechanistic illustration of exosomes' blood-brain barrier trafficking, signaling inducing and degradation. There are several routes for exosomes interact with BBB, including A) trafficking through 1) macropinocytosis, 2) lipid raft or nonspecific exosome-endothelium interaction, and 3) receptor-mediated transcytosis, exosomes can trafficking from the multivesicular bodies to the plasma membrane as *de novo* intraluminal vesicles in the receiving cells. The anticipated fate of exosomes also involved in B) degradation by lysosomes associated pathway, C) signaling induction through backfusion event in the multivesicular bodies to release its contents in the cytoplasm or interact with G protein receptor on the cell surface to induce cell signaling cascade or interacted with the cell surface, or fusion to the membrane and release the payload and initiate molecular events in the endothelial cells.

sized molecules (less than 400 Da or less than nine hydrogen bonding) and gaseous exchange (CO_2 and O_2) [28]. In contrast, large molecules are selectively transcytosed by a specific transport system [29]. Such selective behavior of BBB prevents the penetration of most chemotherapeutic agents or biotech drugs and limits their therapeutic efficacy in brain ailments. Therefore, despite the availability of a wide variety of disease ameliorating agents, only 2% of drugs can traverse the BBB, thus limiting their ameliorating effect in brain diseases [30].

The BBB ensures the express entry of various hormones, small molecules, ions, and nutrients into the brain milieu [31]. Small molecules can only achieve a cell-capillary distance of <20 μm in half a second, thus making it the most feasible route for brain ailments therapeutics [32,33]. Receptor-mediated transcytosis is a potent route for exosomes to achieve BBB penetration. BBB expresses a significantly higher number of cell receptors, including transferrin, low-density lipoproteins receptors family, intracellular adhesion molecules (ICAM 1), insulin, and glucose receptors (GLUTs) as compared to other somatic cells [34,35]. Therefore, targeting these receptors via exosome-based nanomedicine could enhance drug delivery to the brain.

The BBB penetrating capacity of exosomes was intensively studied. A recent study revealed that CD46 is one of the major receptors responsible for internalizing cancer cells' isolated exosomes to the brain via BBB and promoting tumor metastasis [36]. Likewise, the neural stem cells (C17.2) derived exosomes have been reported to interact with endothelial cells via the heparan sulfate proteoglycans (HSPGs) receptor for endocytosis to deliver cargo across BBB [37]. Similarly, glutamate receptors functionalization of exosomes has been reported with efficient BBB crossing ability and delivery of neurogenic/neurotrophic factors including *Ascl1, Brn2,* and *Myt1l* to the brain for electrophysiological modulation and drive pro-neurogenic programming [38]. Recently Chen et al. investigated the detailed mechanisms of exosome BBB traversing. They found that the exosome primarily uses endocytosis to enter the cytoplasm of the endothelial cells, accumulate in endosomes, and achieve transcellular trespassing by forming MVBs and exocytosis to brain parenchyma to ensure efficient cargo delivery to desired cells under stroke-like conditions [39]. They also observed that many exosomes approached the brain via the endothelial intercellular gap. In addition, during endocytosis and transcytosis, some of the exosomes were digested, and cargo was *de novo* uploaded and exocytosed from endothelial cells. Similarly, Banks et al. investigated the BBB crossing ability of 10 different exosome types isolated from different species (i.e., mouse vs. human) and various cell types (i.e., neoplastic vs. healthy) [40]. They observed that all exosomes had the BBB crossing ability; however, the traversing rate among various studied exosome types varied up to 10-fold. Lipopolysaccharide (LPS) could enhance the traversing of most exosome types across BBB. Meanwhile, the Wheatgerm agglutinin (WGA) could modulate the transport in most exosomes. LPS and WGA were independent of endothelial cell markers, i.e., ICAM-1, AVβ6, AVβ3, and CD46, and their origin (i.e., human vs. mouse or healthy vs. cancer cells). In differential cell investigation, the mannose 6-phosphate receptor was identified as a potential transport receptor for mouse macrophage cells (J774A.1) as their novel finding. Nevertheless, researchers test a series molecules with different molecular weight and found the size of cargo molecules higher than 1100 Da may compromise

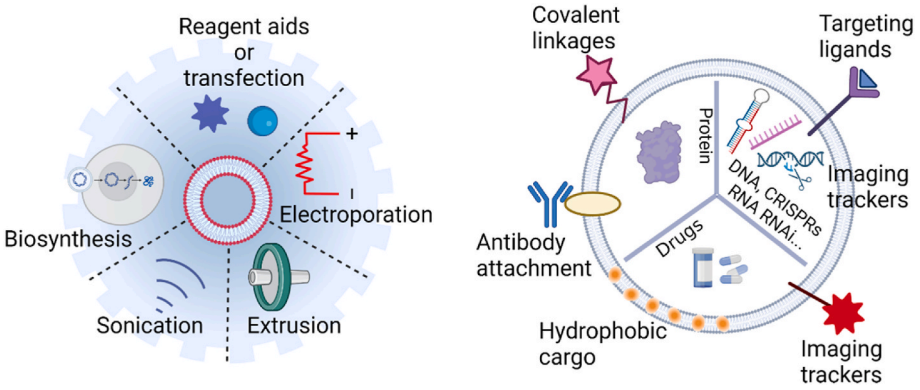

Fig. 3. The most common exosome cargo loading techniques currently employed (left) and cargo types for exosome loading (right).

Table 2
Exosome cargo loading techniques currently in vogue.

Technique	Procedure	Favored application	Advantages	Disadvantages	Ref.
Electroporation	Exosome (i.e. around 20 μg refer to protein weight) electroporation at around 400 V in a total 200 μL buffer adapting to specific nucleic acid transfection machine.	Highly favorable for small hydrophilic molecules, DNA/RNA and protein.	Relative higher efficiency, suitable for some macromolecules.	Compromised exosome membrane quality and formation of exosome or content aggregates.	[47,48, 49,50]
Sonication	Repeated sonication for a specific time (in seconds) with rest on ice.	Nanoparticles, drugs.	Low cost and general.	Limited loading for some drug and it may impact exosome membrane quality. Sonication condition may change even on same machine due to the vibrator durability.	[51]
Freeze and thaw	Rapid froze at < -80 °C and thawed for several cycles.	Protein and macromolecules.	Low cost and facile.	Exosome may aggregate and efficiency is relative low, structure may be destroyed.	[45, 49]
Biosynthesis	Preloading cargo to cells utilizing the exosomes can inherit the cargo from parent cells.	Transfected materials, Nanoprobes, drugs, nanoparticles.	Low cost, complete membrane, and can be highly efficient.	Loading efficient optimization and regulation need further confirm.	[52, 53]
Extrusion	A syringe extruder with filter delivers a mechanical force to reform the exosome membrane and cargo loading.	Membrane hybridization, nanoparticle coating.	Efficient and relatively facile. Can obtain more uniform size.	Mechanical force may damage the exosome membrane and loss content. Higher risk of contamination and waste of exosome.	[45]
Chemical permeabilization	Chemicals like saponins can generate pores on exosomes.	Hydrophobic drugs, DNA/RNA and protein.	Higher efficiency.	Risks in haemolysis and toxicity if not washing out.	[45, 49]
Transfection	Transfection reagent (i.e. lipofectamine 2000) assist the exosome fusion.	Favored for hydrophobic drugs, DNA/RNA and protein.	Higher efficiency, suitable for large cargo.	Chances of toxicity, less cost-effective, may jeopardize the cargo quality.	[54]

the exosomes' BBB traversing ability [41]. These data suggest that exosomes' higher BBB traversing ability depends on the cell origin and disease condition. A detailed exosome BBB traversing mechanism and fates have been reviewed in Ref. [42], and schematically presented in Fig. 2.

3. Exosome cargo loading techniques

The origin, payload type, and loading technique are crucial in ensuring exosome efficient therapeutic application. Exosomes comprise lipid bilayer mosaic with signature proteins and thus form a lipoprotein structure [43]. Understanding exosomal structure and biogenesis informs how the desired therapeutics may be 'uploaded' via active and passive modalities in exosomes. To promote passive upload, exosomes are incubated with a hydrophobic drug in a suitable medium, which allows the drug to diffuse through the exosomal membrane. Although passive cargo uploading is facile and convenient, the uploading efficacy is very low, compromising therapeutic efficacy in cell types that yield low numbers of exosomes. Examples of successful passive exosome upload include curcumin which was successfully loaded after a 5-min incubation at room temperature [44], or the catalase enzyme, which was successfully loaded after 18 h of incubation in PBS at room temperature and applied for the potential treatment of the Parkinson's disease (PD) [45]. Passive loading of exosomes is also possible in whole living cells; for example, mesenchymal stromal cells, when incubated with low concentrations of paclitaxel, produced exosomes with a paclitaxel payload that was highly efficient against pancreatic tumor cells [46]. One possible mechanism of whole-cell passive upload is that parent cells, when exposed to foreign and potentially toxic substances, try to 'quarantine' these in exosomes before elimination by secretion to surrounding microenvironment.

External activating source or substance aids are used in uploading cargo on exosomes (Fig. 3). These approaches compared with simply co-incubation are highly efficient. However, exosomal integrity may be compromised without proper experimental conditions optimization. The primary modalities employed for active exosomal cargo loading are also summarized in Table 2, case by case optimization should be applied for different cargo with different loading approaches and conditions. Current cargo loading technique is still facing changelings (discussed in Table 2 and Box 4), for example, loaded exosome ratio is hard to define, batch-to-batch control is difficult and the efficiency is still not satisfied for many drugs.

4. Function augmentation technologies for exosome

Although exosomes have considerable promise as brain drug delivery shuttles reflecting their vital roles in brain homeostasis and favorable physical properties such as reduced immunogenicity and prolonged blood plasma half-life, therapeutic potential of exosomes can still be augmented with various biotechnologies to enhance target recognition, biodistribution, and efficient BBB crossing. In addition, the challenge of attaining the necessary amount of exosomes to achieve clinical application can also be enhanced by relevant biotechnologies adaptation.

4.1. Genetic engineering

4.1.1. Exosomes generation control

Producing enough exosomes with desired targeting properties is a persistent challenge in developing new exosome-based therapeutics. Exosome production is cell type and environment-dependent but can be artificially enhanced, notably by induction of cell stress. Cells exposed to hypoxia, heat shock, oxidative stress, or neoplastic conditions; exosome number is higher relative to physiologically normal counterparts. Additionally, specific drug treatments increase exosome production; for example, the antimicrobial drug Monensin enhances the generation of

Table 3
Representative examples for peptide decoration and modalities for exosomes loading in brain diseases.

Target brain ailment	Exosome origin	Payload type	Loading mechanism	Targeting ligand	Ref.
GBM	Endothelial cells	Paclitaxel/Doxorubicin	Incubation (37 °C, 2 h)	–	[70]
GBM	RAW264.7	Curcumin/Iron Oxide nanoparticles	Electroporation	neuropilin-1 RGD peptide	[71]
GBM metastasis	BMSC	miR-146b	Electroporation	–	[72]
Downregulation of miR-21 expression in GBM, causing size regression	MSC	antisense miRNA oligonucleotide against miR-21	Electroporation (miR-21 loading), Lipofectamine 2000 (T7 peptide)	T7 peptide	[73]
AD	Dendritic cells	Anti BACE1 siRNA	electroporation	RVG	[47]
AD	Wharton's jelly isolated MSC	Catalase enzyme	–	–	[74]
AD	Macrophage (RAW264.7)	Curcumin	cells treated with curcumin @ 40 μg/mL and exosomes collected)	ICAM-1	[75]
AD	BMSC	–	DOPE-NHS linker conjugation	RVG peptide	[76]
PD	Microglia CD11b + cells	α-synuclein (α-Sny) oligomers	Lipofectamine 2000	–	[77]
PD	Macrophage/ monocytes	Catalase	Saponins	–	[45]
PD, AD, Autism, and stroke	MSC	Gold nanoparticles	Incubation (37 °C, 3 h)	–	[78]
PD	HEK293T	Catalase	Cell engineering (EXOtic)	RVG-Lamp2b	[60]
PD	Dendritic cells	α-Sny siRNA	Electroporation	RVG-Lamp2b	[79]
Oxidative Resistance 1 gene downregulation in PD	Serum isolated exosomes	miR-37	Transfection (Lipofectamine 2000)	–	[80]
PD	Dendritic cells	Short hairpin RNA (shRNA)	Electroporation	RVG	[81]
PD	HEK293T	DNA aptamer	Incubation (PBS, 30 mint), Transfection (myc-RVG-Lamp2b plasmid)	RVG-Lamp2b	[82]
Stroke	BMSC	Curcumin	Incubation (5 min, room temperature)	peptide (c (RGDyK))	[67]
Stroke	BMSC	Iron oxide nanoparticles	Extrusion	–	[83]
Neuro–trauma	BMSC	phosphatase and tensin homolog siRNA (PTEN)	Incubation (2–3 h at 37 °C)	–	[84]
Neuro–trauma	BMSC	miR-124	Plasmid transfection (pSUPER-mir-124 vector)	–	[85]
EAE (experimental autoimmune encephalomyelitis), LPS-induced inflammation, and GL26 glioblastoma	T cells	JSI-124 (anti STAT3 agent), Curcumin	Incubation at 22 °C for 5 min	–	[86]
Neuroinflammation	BMSC	miR-193b-3p	Electroporation	RVG-Lamp2b	[48]
REI-silencing transcription factor (REST) downregulation in Huntington's Disease	HEK293T	miR-124	pSUPER-mir-124 vector via Lipofectamine 2000	–	[87]
Status Epilepticus theranostics	BMSC	PKH26 (i.e., a red cell fluorescent dye)	Incubation following the standard protocol provided with the kit	–	[88]

exosomes in a target cell population, elevates the intracellular calcium ions (Ca^{2+}), and increases the generation of MVBs. Likewise, 1 μM of phorbol 12-myristate 13-acetate inoculation to cell culture media for 30 min can enhance exosome production in microglial cells (BV2) [55]. In contrast, GW4869, a neutral sphingomyelinase inhibitor, inhibits the ceramide-mediated inward budding of MVBs, limiting the generation of exosomes [56]. Another study showed that inhibiting mTORC1 (mechanistic target of rapamycin complex 1) by rapamycin or deprivation of nutrient and growth factors stimulates exosome release. In contrast, mTORC1 activation inhibited the release of exosomes both *in vitro* and *in vivo* [57]. Additionally, inhibition of Rho GTPase also suppresses the generation of exosomes [58].

An alternative approach of low electric current employment to augment exosome production was an exciting breakthrough in exosome research. Treatment of both neoplastic and non-neoplastic cells with a 0.34 mA cm^{-2} current can significantly enhance exosome generation [59]. Interestingly, the low current electric treatment mechanism was also attributed to Rho GTPase intracellular activity.

A landmark proof of concept study by Kojima et al. reported the development of an exosome booster to increase exosome production by 25 to 40-fold, depending on cell condition [60]. They identified the STEAP gene as a pivotal regulator of exosome biogenesis. They created their booster by adding syndecan-4 (SDC4) to support endosomal membrane budding and MVBs formation, as well as supplementation of L-aspartate oxidase (NadB). This citric acid cycle stimulator enhances cell metabolism. They further generated stable high-exosome-secreting cells by overexpressing STEAP through plasmid transfection (vector: pDB60; P_{hCMV}-STEAP-IRES-SDC4-IRES-nadB fragment-pA).

4.1.2. Payload and surface marker engineering

With extensive research in cell engineering, researchers have been able to program cells to upload the desired RNA as a payload on exosomes. Recently, leukemia T cells were programmed to express miR-335, usually not secreted by these cells. After isolation, the T cell exosomes contained miR-335 and transferred miR-335 RNA to surrounding cells that lacked miR-335 RNA [61]. RVG-Lamp2b as a surface targeting ligand that could facilitate exosome traverse the BBB and accumulate within the brain milieu was also engaged in many studies [62]. In addition, the peptide T7 has also been decorated on the surface of the exosomes to enhance glioblastoma targeting with brain milieu (details can be seen in the Brain Cancer therapeutics section). Likewise, Rufino-Ramos et al. designed a transfusible lentiviral construct (NoMi; Nanoluciferase outside and MCherry inside) to tag exosomal surface marker (CD63) with bioluminescence (luciferase) and fluorescent (mCherry) proteins within the cells implanted in the brain of murine models for the detection of exosomes in blood. These exosomes served as proof of concept for detecting brain exosomes in blood [63].

4.2. Surface decoration

The exosome membrane comprises multiple molecules that provide a scaffold for the bioconjugation of desired molecules to achieve therapeutic and diagnostic goals. Covalent bonding assists in directly attaching various molecules to the exosome surface. Additionally, "click chemistry" is ideal for the bioconjugation of molecules on the exosome membrane surface [64]. The alkyl and azide chemical groups react with triazole linkages more rapidly than traditional cross-linking and provide

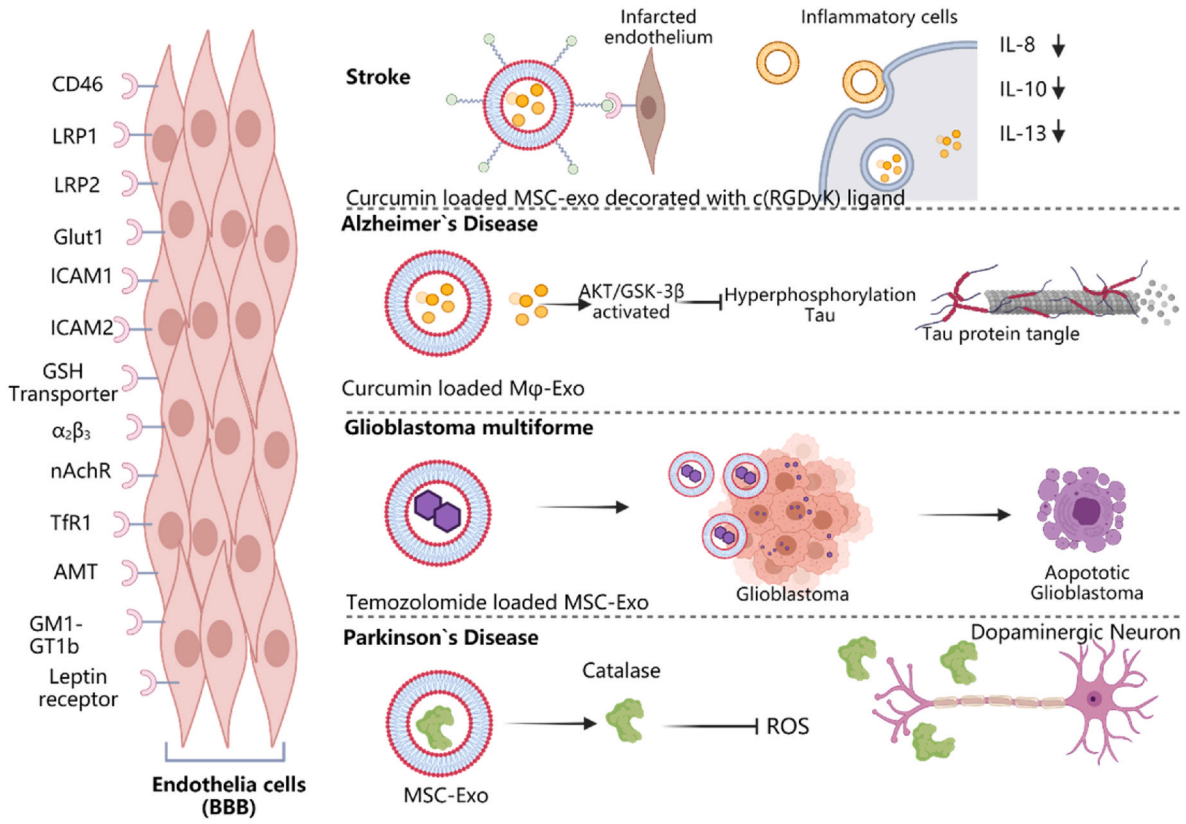

Fig. 4. Selected examples of exosome role as therapeutic vehicles in neurodegenerative diseases and glioblastoma, highlighting BBB traversal and major defined transportation receptors. The illustration data details can be found in the following references; Stroke [67], Alzheimer's Disease [75], Glioblastoma [103], and Parkinson's Disease [45].

better control over conjugation reaction at the desired site [65,66]. Likewise, another study with BMSC isolated exosomes incorporated reactive dibenzylcyclootyne (DBCO) groups in amine-containing molecules on the exosome surface using a heterobifunctional crosslinker. The c (RGDyK) peptide was then successfully decorated on the surface of exosomes by conjugation to azide groups incorporated in the peptide. These functionalized exosomes were then able to target ischemic brain lesions and decreased the inflammatory response and apoptosis when loaded with curcumin [67]. As described above, exosomes reflect their parent cells' physiology and present the same types of membrane proteins. Hence, ligation of cell-specific antibodies may also be an isolating marker of the exosome from a mixed population. Recently, research endeavors have been made to sort and analyze fluorescence vesicles from the whole cells via antibody conjugation to the epidermal growth factor receptor (EGFR) of human colorectal cells (DiFi) isolated exosomes [68]. Table 3 lists current developments in peptide decoration and modalities for loading desired cargoes in exosomes for neurodegenerative disease therapy across the BBB. Another feasible option for exosome surface decoration is incorporating moiety conjugated lipid polymers or amphiphilic lipids (ie. PEG-lipid, PEG-DSPE) to the exosome membrane [69], which take advantage of the similar amphiphilic property of the exosome membrane.

4.3. *Exosome membrane hybridization*

Membrane hybridization has recently attained attention for employment in the drug delivery system. This innovative approach camouflages nanocarrier systems with compatible cell membranes and can target desired tissues, particularly cancer, owing to their cell tropism properties [89]. Exosome membrane hybridization also offers an avenue for specific tissue targeting compared to naïve exosomes, as demonstrated by Piffoux et al. [90]. These authors hybridized the exosome membrane with liposomes using polyethylene glycol (PEG). This method enriched the exosomes with hydrophobic or lipophilic compounds without jeopardizing their intrinsic contents or biological properties. The ability of the hybrid exosomes to deliver chemotherapeutics was 3–4 times higher than free drug or unhybridized counterparts, i.e., naïve exosome and liposome. Likewise, Sato et al. used a freeze-thaw method to hybridize the exosome membrane with liposome to develop an efficient biomimetic nano-drug delivery system [91].

5. Brain cancer therapeutics

Gliomas are the most lethal cancers that primarily affect the central nervous system. Glioblastoma multiforme (GBM) comprises 50% of all diagnosed gliomas without effective treatment. Annually, 2–3 new cases per 100,000 individuals worldwide are reported with a mean post-diagnosis survival rate of 12–15 months [92]. Only a few people survive up to five years after diagnosis. The standard treatment for GBM is surgical resection, followed by adjuvant radio and chemotherapies. A particular challenge presented by GBM is that tumor cell diffuses into the healthy brain parenchyma that, hinders complete resection of GBM and valuable BBB penetrating agents are very rare to be seen which leads to high recurrence and drug resistant. To date, temozolomide (TMZ) is one of the most commonly employed FDA-approved chemotherapeutic agents for GBM that is administered orally or via intravenous infusion [93,94]. In addition, GLIADEL wafers are another FDA-approved agent implanted in the patient after surgical removal of GBM. Up to eight wafers can be placed at a time, depending on the size and type of cavity. These wafers then degrade and release carmustine drug into the surrounding tissue [95]. All these chemotherapeutic agents modestly prolong GBM patient survival time, not complete tumor resection.

Unfortunately, the chemotherapeutics' suboptimal bioavailability in brain tissue and frequent resistance development has hampered its

efficacy against GBM. Therefore, nanotechnology-based endeavors for improving GBM targeting and treatment are eagerly sought. Biocompatible nanoscale materials such as exosomes promise to translate bench-side research to bedside [67,96].

5.1. Chemical drugs as exosome payloads

BBB penetrating capacity of exosome enable many BBB reject drug to be delivered into brain and glioma site. Recent study engaged paclitaxel and doxorubicin loaded exosomes isolated from brain endothelial cells to treat GBM [70]. The systemic injections demonstrated GBM growth harnessing ability and found that the drug was highly concentrated in brain capillaries rather than parenchyma. Likewise, owing to the inflammation-driven nature of neutrophils and easy BBB crossing ability, their exosomes were employed to deliver doxorubicin for GBM ablation in C6 glioma-bearing mice and zebra fish models [97]. These neutrophil exosomes could chemotactically respond to inflammatory stimuli and efficiently target the infiltrating GBM cells within the brain milieu. These exosomes were internalized by glioma and endothelial cells via clathrin endocytosis as a common route, whereas the endothelial cells also had additional pathways involved for exosome internalization.

Combining TMZ-loaded exosome therapy with radiotherapy may provide a new avenue in radio-chemo-adjuvant treatment. In this vein, Erel-Akbaba et al. used radiation to trigger BBB opening to target GBM [98]. The nanomedicine was also ligated with a particular peptide to target the EGFR on the cell surface and it was observed that irradiation significantly enhanced nanomedicine accumulation in the neoplasm [99]. Recently Zhu et al. used exosomes derived from embryonic stem cells to deliver paclitaxel as the therapeutic agent to the GBM. They modified the exosome surface with Cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide (RGDyK), which has a strong affinity to $\alpha_v\beta_3$ integrin present on the surface of the proliferating GBM and endothelium [100]. Likewise, Jia et al. decorated the exosome surface with the neuropilin-1 RGD peptide and loaded with curcumin and superparamagnetic iron oxide nanoparticles [71], which allowed theranostics of GBM by imaging and hyperthermia via magnetic radiation and curcumin as chemotherapeutic agents.

Our research group has recently demonstrated that BMSC isolated exosomes, when decorated with heme oxygenase 1 (HMOX1) selective short peptide (HSSP), could target the TMZ-resistant GBM in orthotopic mice models. The BMSC isolated exosomes exhibited higher BBB traversing ability than somatic cells isolated exosomes. Significantly higher HMOX1 expression was found in TMZ-resistant GBM could than chemosensitive GBM and normal glia cells, which enables the HSSP targeting strategy. These engineered exosomes were loaded with TMZ and STAT3 siRNA (siSTAT3) to restore the TMZ sensitivity of the GBM [101]. Likewise, suicide gene (yCD:UPRT) engineered human MSCs isolated exosomes were reported with gene-directed enzyme prodrug therapy for GBM. The isolated exosome in the presence of 5-fluorocytosine could induce an anticancer effect on the C6 glioblastoma cell line and human primary glioblastoma cells [102]. Selected exosome-based endeavors for cancer and other brain diseases that provide novel ideas for drug delivery are depicted in Fig. 4.

5.2. Nucleotide drugs as exosome payloads

Nucleotide based gene therapy showed great potential in clinic. Exosomes as nanocarrier is capable for mRNA, siRNA, antisense oligonucleotide (ASO) delivery. For example, recent study suggested that BMSC exosomes loaded with miR-146b could reduce tumor metastasis by up to 60% [72]. Another study showed anti-miR-9 cargo-loaded exosomes could restore the GBM sensitivity chemotherapeutics due to miR-9 is involved in chemo-resistance of GBM by inducing higher P-glycoprotein expression, a drug efflux transporter on the cell surface [104]. Likewise, antisense oligonucleotide against miR-21 (AMO-21) was loaded into exosomes decorated with T7 peptide and tested for GBM treatment [73]. Another study investigated the natural tropism to hypoxic tumors of the neural stem cells (NSC) and their exosomes. Antisense oligonucleotide of STAT3 was loaded by programming NSC derived exosomes, and it was found can stimulate macrophages and dendric cells and immune response in GL261 glioma orthotopic models [105].

5.3. Exploitation of inherited exosome potential

Parent cell or cell origin is critical for the therapeutic potential of exosomes. For instance, embryonic stem cells environment have been reported to exert tumor suppression through epigenetic regulation [106,107]. On the other hand, some exosome was reported has a strong affinity to endothelial cells and promotes angiogenesis on demand [108]. The exosome potential to reverse the chemo-sensitivity has also been investigated in chemotherapy resistant GBM models. A study reported that the exosomes isolated from LPS/INFγ activated microglial cells could phenotypically switch tumor-associated myeloid cells by upregulating inflammation-related genes and suppress glioma growth [109]. The chimeric antigen receptors (CAR) based immunotherapeutic technologies utilize genetically modified T lymphocytes to achieve the dual function of tumor targeting and immune activation, and has been well applied in blood tumor therapy. These cells continuously induce cytotoxicity in neoplastic cells, thus providing a rapid and durable anticancer effect. However, acute toxicities as well as insufficient solid tumor and tissue penetration have limited their general clinical applications beyond blood system [110]. Nevertheless, isolated exosomes from the CAR T-lymphocytes, while still containing chimeric parent-cell antigens that mediate antineoplastic effects and can reduce the toxicity via surface engineering [111]. Continued adaptation of CAR exosome may lead to new, highly efficient GBM treatments.

Some researchers consider exosome based immunotherapy and vaccines can be developed into the most effective approaches for GBM and neurodegenerative disease [112,113]. Evidences showed dendritic cell-based immunization containing total glioblastoma lysates has been promising in experimental and clinical trials against GBM [114,115]. A recent study reported that GBM-bearing rats vaccinated with exosomes derived from tumor cells with α-galactosylceramide on pulsed dendritic cells elicited a potent-immunotherapeutic response [116]. This vaccine significantly mimicked and activated the tumor-specific cytotoxic T lymphocytes to re-establish immunosurveillance. The mechanisms of dendritic cell exosome uptake have been investigated by Dusoswa et al. [117]. They found that the glycocalyx component of exosomes could target the dendritic cells for internalization. The modification of the exosome surface with high-affinity ligand ($Lewis^Y$) increased the exosome internalization four-fold via dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (CD209). Ultrasound technologies are also an FDA-approved approach and are favored to open BBB and deliver therapeutic agents to brain parenchyma. Recently, Bai et al. employed focused ultrasound to assist exosomes traverse the BBB. In a glioblastoma model, these authors employed focused ultrasound to facilitate exosomes isolated from macrophages and whole blood serum for GBM treatment and achieved 4.45 times more efficient at crossing the BBB after focused ultrasound *in vivo* [118].

6. Exosome-based therapeutics for central nervous system disease and traumatic brain injury

The BBB penetrating ability of exosomes enables its applications in multiple brain diseases. Recent study found exosomes can also target to the diseases region in multiple models of neurodegenerative disease, for example PD (Parkinson diseases), AD (Alzheimers' diseaseas), Autism, and stroke. It reported that MSC-derived exosomes loaded with gold nanoparticles could specifically target pathological regions characterized by inflammation in brain tissue [78]. In this study researchers investigated the uptake of exosomes by fluorescence imaging and

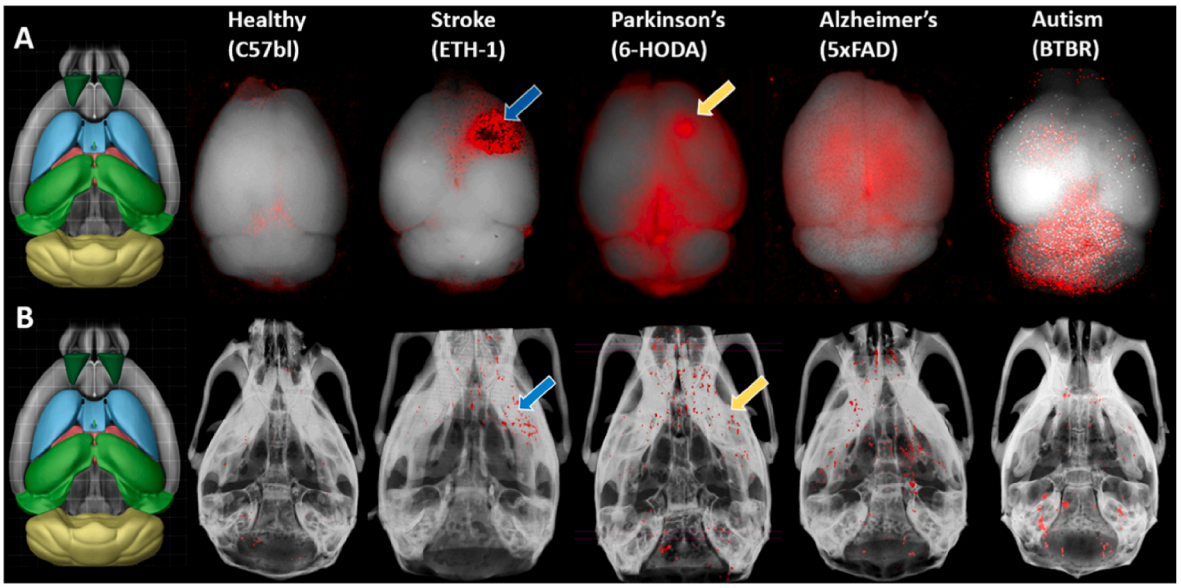

Fig. 5. Distribution of exosomes in various brain associated diseases, including stroke, Parkinson's disease, Alzheimer's disease, and autism mice models. (A) Ex-vivo imaging for accumulation of PKH-26 (a red cell fluorescent dye) labelled MSC exosomes and (B) quantitative CT imaging for accumulation of gold nanoparticle loaded MSC exosomes in various neurodegenerative diseases after 24 h post intranasal administration. These MSC isolated exosomes exhibited distinct brain homing and migration patterns under different brain pathologies. In ischemic stroke, the exosomes could migrate to the injection site (striatal region), whereas in Parkinson's disease, the striatal region, including the midbrain and cerebellum, was occupied with exosomes. In Alzheimer's disease, exosome accumulation was found in the hippocampus, whereas in autism, the cerebellum and cortex were the main exosome migration aims. The reported exosome migration sites are also considered ast the primary pathological lesions in related diseases. The blue arrow noted the injection site for ETH-1 (ischemic stroke model inducer), orange arrow noted the 6-OHDA injection site (Parkinson diseases model inducer). C57bl is wild type mice, 5xFAD is Alzheimer's diseases model, BTBR is autistic-like behavior mouse model for autism. The brain region was indicated by color, the olfactory bulb is dark green; the striatum is blue; the thalamus is red; the hippocampus is green; and the cerebellum is yellow-colored. Adapted from Ref. [78], copyright 2019, American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

quantitative X-ray computed tomography (CT) images, data revealed that neurons took up exosomes but not in glial cells and murine neurodegenerative models accumulated exosomes in the pathological lesion up to 96 h post-administration. In a normal mouse brain, a diffuse uptake pattern was observed for 24 h only. Interestingly, exosome accumulation was driven by the nature of the disease. In a PD mice model, exosomes attained higher concentrations in the striatal region, midbrain, and cerebellum. In contrast, AD mice exhibited more significant exosome accumulation in the hippocampus, whereas, in the autism model, this occurred in the cerebellum. Likewise, the striatal region had a higher exosome concentration in ischemic stroke. Different uptake pattern for these diseases was demonstrated by fluorescence imaging and CT images and cited in Fig. 5. Some representative exosome applications for neurodegenerative diseases are discussed below.

6.1. *Alzheimer's disease*

Alzheimer's disease (AD) is a major neurodegenerative disorder leading to progressive memory loss in patients [119]. Extracellular vesicles' role is crucial in AD pathogenesis [120,121]. Understanding the extracellular vesicle pathways may not only spotlight the AD progression mechanism but also provide opportunities for treatment development. As part of normal physiological processes occurring inside the brain and other parts of the body, the exosomes' role in cargo delivery across BBB becomes crucial. This cargo may be either inherited from the parent engineered cells or drug-loaded with active or passive mechanism.

6.1.1. *Exosome delivery system for Alzheimer's disease*

A decade ago, pioneering work by Alvarez et al. opened a new avenue in brain disease therapy by employing exosomes isolated from engineered dendritic cells loaded with *BACE1* siRNA to inhibit the production of Aβ (β-amyroid) [47]. Similarly, exosomes from MSC loaded with active catalase enzyme were reported can mediate a protective effect against Aβ associated oxidative stress and synaptic damage in hippocampal neurons [122,74]. Study also showed curcumin-loaded exosomes isolated from macrophages (RAW264.7) were able to improve the cognitive function in AD mice with tau pathology, crossing BBB via exosome signature receptor ICAM-1 and parent cell inherited LFA-1 receptors [75]. Likewise, the potential of peptide decorated exosome has been proved, researchers employed DOPE-NHS linker conjugated RVG peptide for exosomes isolated from BMSC and successfully restored the memory defective in APP/PS1 AD murine model [76]. These strategies concluded exosome based delivery is capable in AD treatments, but more clinical relevant evaluations shall be further investigated.

6.1.2. *Inherited potential of exosomes from parent cells*

The exosome can inherit surface interact molecules and inner content from the parent cells. Exosomes isolated from neuronal cells are abundant in glycosphingolipids and were reported to sequester Aβ in the brain milieu, leading to AD amelioration in primates and murine animal models [123]. Previous study reported that neuronal exosomes induce conformational changes of Aβ and transforming to nontoxic fibrils, which can be quickly taken up by microglia and scavenged through lysosomal degradation pathway [124]. Moreover, phosphatidylserine on the surface of the neuronal exosomes was responsible for their microglial internalization and the sphingolipid-metabolizing enzymes, i.e., neural sphingomyelinase 2 (nSMase2) and sphingomyelin synthase 2 (SMS2), which can modulate the secretion of the neuronal exosomes and promoted Aβ microglial scavenging. In another study, glioblastoma-derived exosomes were injected into an AD mice model and demonstrated significantly higher Aβ scavenging by microglial cells within the brain milieu [125]. Likewise, exosomes derived from adipose progenitor cells are abundant in neprilysin, an essential protein for Aβ proteolysis. In AD, lowered neprilysin levels promote Aβ oligomerization and contribute to AD pathology [126]. Another evidence also

showed exosomes isolated from BMSC could lower Aβ plaques formation and dystrophic neurite burden [127]. Adipose tissue-derived stem cells (ADSC) was also been proved can reduce toxic Aβ induced apoptosis in TG2576 AD mice isolated brain stem cells [128]. The exosomes of hippocampus neuronal stem cells (NSC) were also reported to exert ameliorating effects on AD-related pathophysiology. When NSC exosomes loaded with miRNAs (miR-322, miR-17, and miR-485), it reversed Aβ oligomer-induced suppression of long-term hippocampal potentiation (LTP) and memory deficits in mice and induced phosphorylation of synaptic CaMKII receptors, preventing the toxic binding of Aβ oligomers to the synapse [129].

Overall, MSC derived exosomes is promising in AD treatment due to the therapeutic effect of exosome itself and the therapeutic agent delivery potentials. Drugs direct intervene AD pathology are very limited, the controversial Aβ targeting monoantibody aducanumab may be combined with exosome therapy by decoration or loading approaches for better BBB penetrating and at-site targeting. AD is one of the most burdensome neurodegenerative diseases with very complex pathology, beyond the classic Aβ, Tau, APOE, neuro inflammation hypothesis, exosome mediated cell communication may play an unexpected role and need to be further investigated.

6.2. Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease [130]. Clinically, it is characterized by the progressive degradation of dopaminergic neurons and dopamine loss. The appearance of Lewy bodies composed mainly of α-synuclein (α-Syn) is one pathological hallmark of PD. PD pathology is also associated with neuro-inflammation, increased microglial infiltration, and redox imbalance in the brain milieu [131]. Current PD therapy relay on surgery and very limited drugs, deep brain stimulation, stem cells-derived dopaminergic neurons transplantation and gene therapy was supposed to be the cure [132,133]. The future treatments of PD is pursuing non-invasive and personalized treatment, where the advantages of exosomes can be utilized. Like other neurodegenerative diseases, the exosome therapeutic application in PD was supposed due to its excellent BBB crossing ability and target recognition for payload delivery [134].

6.2.1. Optional cargoes for exosome delivery in PD

Exosomes are excellent cargo vehicles that can traverse the BBB easily. Therefore, their application in PD therapeutics has been of immense interest. A recent study confirmed that the microglial $CD11b^{+}$ exosomes contained a payload of α-Syn oligomers that induced protein aggregation in neurons. Inhibition of microglial synthesis decreases α-Syn in neurons [77]. Therefore, modulating the expression of microglial exosomes or using exosomes to deliver therapeutic cargoes could be a valuable therapeutic strategy in PD. A recent study demonstrated that exosomes isolated from macrophage/monocytes loaded with catalase could reduce oxidative stress and exhibit neuroprotective effects in PD mouse models by intranasal administration [45]. To supply the reduced dopamine in PD model, Qu et al. loaded dopamine on blood derived exosomes by incubation in a saturated dopamine solution [135]. Exploiting transferrin-transferrin receptor-mediated transcytosis, these authors found 15-fold higher dopamine accumulation in the brain (primarily in striatum and substantia nigra) and therapeutic effect after exosome treatment. To reduce the key PD hallmark α-syn expression, exosome delivering for α-syn targeting antisense oligonucleotides (ASO) was supposed. Yang et al. employed MSC isolated exosomes to deliver ASO (sequence: GCTCCCTCCACTGTCT) to the brain of the A53T transgenic PD mice model [136]. These ASO loaded exosomes could efficiently attenuate the α-Syn aggregates and ameliorate the dopaminergic neuron degradation, resulting in improved locomotor functionality in murine models. Similarly, exosomes derived from dendritic cells containing short hairpin RNA (shRNA) against α-Syn were employed to ameliorate PD [81]. DNA aptamers was also tested for exosome delivery, study employed RVG-Lamp2b decorated exosomes from HEK293T cells loaded with α-Syn targeting DNA aptamers and decreased α-Syn aggregates [82]. Exosome isolated from serum loaded with MicroRNA miR-137 were reported can reduce oxidative stress in neurons and leading to physiological improvements in PD *in vivo* model [80]. All these at-site delivery for therapeutic nucleic acid based cargos may enable the direct intervention on α-Syn and reduced the chemical drug development process for PD.

6.2.2. MSCs derived exosomes boosts PD therapy

MSC transplantation has a promising role in neuroprotection. However, *in vitro* preparation for large amount and uncontrolled cell proliferation are major drawbacks. Chen et al. employed human umbilical cord isolated MSCs derived exosomes for dopaminergic neuron protection in PD and rescued the PD phenotypes in neurotoxin 6-hydroxydopamine (6-OHDA) and apomorphine induced PD model [137]. Another study assessed the effects of BMSC or BMSC secretome in the 6-OHDA mouse PD model and found the BMSC secretome was highly efficient in protecting dopaminergic neurons is competitive to whole-cell treatment, where the exosomes play a crucial role [138]. Compared with cell-therapy, exosome based therapy may be more feasible and non-invasive, future investigation may also combine exosome based therapy to non-invasive deep-brain stimulation and local microenvironment regulation.

6.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive muscular paralysis due to motor cortex brainstem and spinal cord motor neurons degradation [139–141]. In terms of novel treatment strategies, several exosome-based approaches have been explored for ALS. Exosomes isolated from ADSC were found to exert neuroprotective effects in an *in vitro* model of ALS. The administration of exosomes significantly increased the survival of motor neuron-like NSC-34 cells by overexpressing human SOD1 and apoptosis resistance signaling [142]. Further studies by the same group identified a total of 189 exosome proteins that were involved in cell adhesion and prevention of apoptosis by downregulation of the proapoptotic gene (Bax) and upregulation of the anti-apoptotic gene (Bcl-2) [143]. Several studies have suggested that exosomes can restore the mitochondria defects in ALS. A study reported that ADSC-derived exosomes restored the mitochondria function in a SOD1 (G93A) ALS *in vitro* and lowered mutant SOD1 aggregation in neuronal stem cells [144]. Likewise, Calabria et al. reported that mitochondrial coupling efficiency, complex I activity, and mitochondrial membrane potential were restored in SOD1 (GA93A) mutant NSC-34 cells after treatment with ADSC exosomes *in vitro* [145]. ALS is one fatal disease but the animal models and disease pathology is still not fully established, current evidences showed exosomes may be a valuable treatment but the therapeutic targets are still need further investigation.

6.4. Huntington's disease

Huntington's disease (HD) is a neurodegenerative disease caused by the expansion of CAG repeats encoding for the huntingtin gene, characterized by neuropsychiatric symptoms, cognitive impairment, and choreiform movements [146]. Although the genetic mutation is clear, the pathology of HD is largely unknown, endeavors have been made to restore the REI-silencing transcription factor (REST), which may ameliorate the disease [147]. In this case, Lee et al. used miR-124 as exosome delivery cargo for R6/2 HD mouse treatment. MiR-124 containing exosomes were initially isolated from HEK293T cells transfected by the pSUPER-mir-124 vector. These exosomes successfully downregulated the expression of REST, but did not result in behavioral improvement [87]. The genetic correction by gene editing tools such as prime editors are the most promising approach, but the at-site delivery,

Box 2
Representative stem cell derived exosomes' role in stroke amelioration.

- Promotion of neurite branching and neuronal elongation in stroke models.
- Inflammation suppression and promotion of white matter repair makers at injury site [158].
- Otero-Ortega et al. reported the fiber tract recovery and axonal sprouting in stroke-bearing murine models [159].
- In porcine models, the neural stem cells could improve sensorimotor functions, hemorrhagic tissue lesions, and inflammation. In addition, the white matter integrity was improved with increased corpus callosum fractional anisotropy [160].
- The paracrine effect of stem cell exosomes promotes angiogenesis and increased neuronal functional recovery [161].

sufficient gene editing, less miss-target and cost is still challenging.

6.5. Epilepsy

Epilepsy is a neurological disorder characterized by abnormal brain function, seizures or sensations, and loss of awareness and has a close relationship to many neurodegenerative disorders, epilepsy also has a poor prognosis with very limited drug therapy [148]. Many epilepsy subtypes are caused by genetic mutations, therefore the gene therapy materials can be delivered for therapy. Exosomes was found may have an at-site delivery capacity for epilepsy, that Kodali et al. traced the BMSC derived-exosomes with PKH26 (a red cell fluorescent dye) in the kainate-induced status epilepticus mouse model, [88]. The mice were intranasally dosed with two billion exosomes. After 6 h, a significantly higher number of exosomes accumulated in the neurons at the hippocampal CAI subfield and the entorhinal cortex region of the brain, known to be susceptible to neurodegeneration after status epilepticus. Moreover, exosomes were selectively taken up by microglia compared to brain parenchyma. These results suggest the suitability of exosomes as a potential drug carrier for epilepsy therapy.

6.5.1. Naïve exosomes with inherited amelioration properties for epilepsy

MSC transplantation therapy for epilepticus has improved the therapy development [149–151]. However, transplantation has certain limitations, as discussed before. In this regard, MSC exosomes may circumvent these bottlenecks to improve therapeutic efficacy by reflecting their inherent properties and incorporating therapeutic cargoes such as RNAi [152]. Recently, a study using the pilocarpine-mediated epilepsy mouse model showed the intranasal administration of exosomes from MSCs accumulated in the hippocampus within 6 h of administration and reduced the neuronal loss and increased neurogenesis in the hippocampus, thus improving status epilepticus and brain function [153]. Considering naïve exosomes may ameliorate epilepsy and can act as delivery tools, exosomes are responsible for the gene editing tools delivery of future epilepsy causing genetic mutation correction.

6.6. Stroke

Stroke is a medical condition attributed to compromised blood flow to the brain, resulting in severe disability or death of the individual. Around 700 drugs have failed to achieve clinical approval to ameliorate stroke, while exosomes were supposed to improve tissue and functional recovery after stroke [154].

6.6.1. Exosomes with payload

To improve drug therapies for ischemic stroke treatment, exosomes have been assessed due to their neuroprotective and angiogenesis-promoting properties [155]. Study showed exosomes isolated from BMSC with surface decoration of peptide c (RGDyK) have ischemic brain tissue targeting capacity. Additionally, these exosomes were loaded with curcumin, which strongly suppressed inflammatory lesions and apoptosis in the ischemic brain [67]. Passive in-cell exosome cargo packaging is an exciting strategy for various disease theranostics. A recent study showed that magnetic nanoparticles (iron oxide) packaged in exosomes in cells significantly accumulated at stroke-affected brain tissue [83], showing a 5.1-fold higher accumulation at the injury site. Owing to the anti-inflammatory, anti-apoptotic and angiogenic properties of BMSC exosomes, this strategy is potentially highly useful in stroke patient recovery but requires further preclinical evaluation.

6.6.2. Inherited exosome potential

The inherited properties of exosomes without exotic payload are also plausible in stroke amelioration (see Box 2). Isolated exosomes from MSC can promote functional recovery of the primary motor cortex in the rhesus monkey (*M. mulatta*) stroke model [156]. The authors demonstrated that MSC exosomes reduce reduced injury-induced hyperstability, restored the inhibitory balance and significantly correlated to behavioral recovery in the primate model. Another exosome-based approach borrowed from the inflammatory cascade follows ischemic brain injury, researchers hypothesized that converting microglial cells from the proinflammatory M1 phenotype to the M2 anti-inflammatory phenotype might aid in ameliorating ischemic stroke and proved lipopolysaccharide-activated macrophages derived exosomes conversed the microglial cells from the M1 to the M2 phenotype, ameliorated brain inflammation [157]. These regulation role of naïve exosomes makes it perfect in delivering nutrient and anti-inflammatory agents for the recovery.

6.7. CNS trauma and approaches to subside neuroinflammation

CNS trauma following an accident is also one of the significant causes of disability and death. The sensory recovery after prognosis is often prolonged. Recently, exosome-based therapies have been employed to alleviate traumatic brain injury (TBI) in experimental models [162]. Exosomes isolated from BMSC significantly improved sensorimotor recovery, enhanced neuroblast and mature neuron formation, endogenous angiogenesis, and lowered neuro-inflammation in a rat TBI model. It was also shown that sciatic nerve injury could be improved by employing the BMSC isolated exosomes that facilitated axonal regeneration in a rat model of nerve damage [163].

Taking these promising results, studies also combined the gene therapy with BMSC derived exosomes. Intranasal delivery of BMSC isolated exosomes with phosphatase and tensin homolog siRNA (PTEN) payload was proved can home in spinal cord lesions and attenuated the spinal cord injury [84]. Moreover, exosomes isolated from microglia with a payload of miR-124 could successfully deliver the miRNA-124 cargo to TBI trauma sites in a rat model, promoting M2 polarization in microglial cells, improving hippocampal neurogenesis and overall brain functionality [85]. Collectively, these results may represent a solution for TBI and spinal cord injury, which currently lack of effective therapies.

Neuroinflammation plays a cruel role in CNS diseases development, it is important signals and can be beneficial, but usually hinders the therapy outcomes [164]. There is growing evidence suggesting that exosomes can inherit useful properties from parent cells for ameliorating

Table 4
Representative exosome payload inherited from parent cells to alleviate brain inflammation.

Exosome cell origin	Inherited Payload	Mode of action	Inflammation type	Ref.
MSC	miR-133b	Enhanced neurite branching and neuronal elongation	Stroke	[158]
Microglia M2	miRNA-137	Neuroprotective effect via Notch1 gene	Ischemic Brain injury	[167]
Adipose derived MSC	miR-22–3p	Inhibiting KDM6B mediated effects on BMP2/BMF axis	Ischemic Brain injury	[168]
Urinary tract derived MSC	miR-206	Promote neurogenesis by enhancing proliferation and differentiation of neural stem cells to reduce infarction	Ischemic stroke	[169]
MSC	miR-542–3p	Lower the TLR4 expression that resulted in the prevention of ischemia-induced glial cells inflammatory response	Ischemic Stroke	[170]
BMSCs	–	Neuroprotective effect by reducing total number and duration of seizures, preserving baroreceptor reflex sensitivity, prevention of hypomyelination	Neonatal brain hypoxia	[171]
MSC	–	Reduced inflammation resulting in neuronal protection, restoration of short-term myelination defects, and long-term white matter microstructural abnormalities	Ischemic injury	[172]
BMSCs	–	Decrease levels of complement factors, i.e., complement C4 and C4 binding protein alpha, C5, C6, and complement factor H, reduced spinal trauma by reversing activation of nuclear factor kappa-B (NF-kB)	Spinal cord injury	[173]
Microglia	miR-124–3p	Increased neurite outgrowth characterized by increased neurite length and branching, lowering the expression of neurodegenerative proteins p-Tau and Aβ-peptide, inhibiting mTOR signaling	Traumatic Brian Injury	[174]
MSC	Proinflammatory cytokines	Attenuated neuroinflammation as judged by reduced reactive astrocytes and microglia at the injury site	Spinal cord injury	[175]
BMSC	–	Increased the number of regulatory T cells (Treg) cells that promoted the remyelination of neurons and reduced inflammation	Experimental autoimmune encephalitis	[176]
Adipose derived MSC	–	inhibited NF-kB and MAPK to reduce neuroinflammation mediated by microglial cells	Neural Injury	[177]
BMSC	miRNA-181c	Inhibition of PTEN and suppression of NF-kB signal, resulting in inflammation and apoptosis	Spinal cord injury	[178]

neuroinflammation by reducing the numbers of activated microglial cells and supporting oligodendrocytes (as shown in Table 4) [165]. Moreover, the stem cells' natural inflammation ameliorating properties are also inherited to exosomes which can be efficiently employed in brain inflammatory disease treatment, for instant experimental autoimmune encephalitis [166], shown in Fig. 6.

Intranasal administrated exosomes isolated from T cells loaded with curcumin and JSI-124 (anti STAT3 agent) were showed can protected mice from LPS-induced brain inflammation and slowed the progression of an experimental autoimmune encephalomyelitis. In mice brain tumor model, it can also significantly delayed tumor growth by delivering drug encapsulated exosome to the brain [86]. Recently, exosomes from microglia were also examined in HIV-related neuroinflammation in *trans*-well *in vitro* models [179]. It was reported that siRNA for tetraspanin 2 (Tspan2) downregulated IL-13 and IL-10 and increased the Fc gamma receptor 2A (FCGR2A) and TNF-α microglial cells, thereby modulating microglia cytokines and phenotype. Similarly, the potential of exosome-delivered miR-193b-3p to reduce neuroinflammation in a mouse model of post-subarachnoid hemorrhage has been explored and BMSC exosomes expressing RVG-Lamp2b targeting ligand successfully delivered miR-193-3b to injury sites and ameliorated neuroinflammation by upregulating the acetylation of NF-kB p65 [48]. Recently, Yuan et al. employed macrophage isolated exosomes to traverse the BBB and navigate to the inflamed brain without surface modification [180]. The macrophage-derived exosomes crossed the BBB by interacting with the brain capillary endothelium molecules: ICAM 1 and 2, carbohydrate-binding C-type lectin receptor, and lymphocyte function-associated antigen (LFA-1) receptors. These exosomes were loaded with brain-derived neurotrophic factor (BDNF) protein cargo and promoted the growth and differentiation of new neurons. Overall, as natural intercellular communicators, exosomes showed very promising inflammation regulation and inflammation site homing capacity, this enables it act as anti-inflammation agent perfectly and can be very useful in many CNS diseases treatments.

7. Outlook and conclusion

Currently, ~40 companies are worldwide registered to offer exosome-based therapeutics and exponentially growing to substitute stem cell-based therapies (https://bioinformant.com/companies-developing-exosome-technologies/). The accumulated knowledge of exosomes has also been systematically assigned in many exosome databases

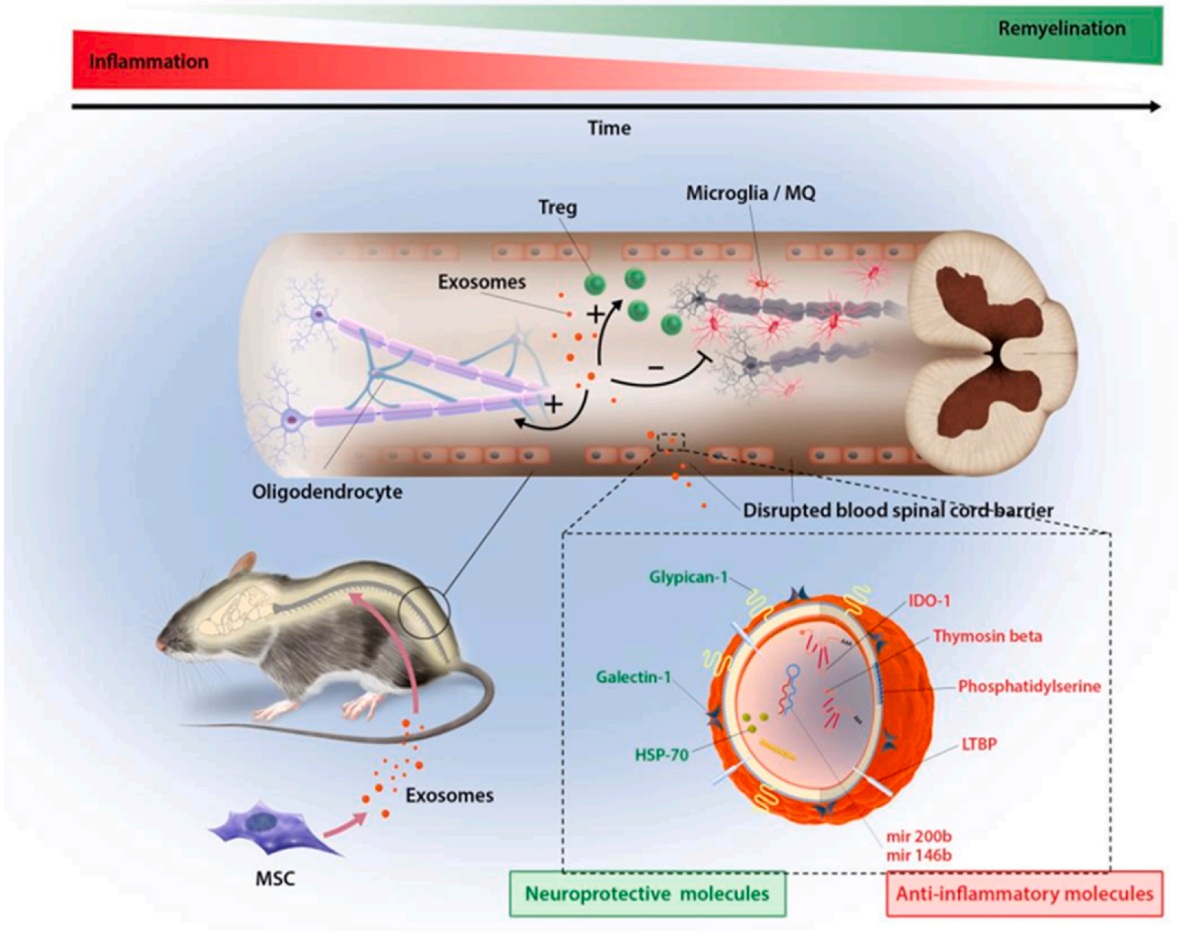

Fig. 6. Application of exosomes derived from MSC in the experimental autoimmune encephalitis model. These exosomes restored myelination and reduced inflammation in a time-dependent manner. Adapted from Ref. [166], copyright 2019, American Chemical Society.

Box 3
Representative exosome online databases.

- **Vesiclepedia.** It offers open access standalone tool for bioinformatics analysis of extracellular vesicle data set and manually curated compendium of molecular data. http://www.microvesicles.org/
- **ExoCarta.** A manually curated database of exosomal proteins, RNA and lipids. www.exocarta.org/
- **Exosomes. gene-quantification.info.** The RNA content and the physiological functions for EVs. https://www.gene-quantification.de/exosomes.html
- **GOA.** Exosome Gene Ontology Annotation Initiative. https://www.ebi.ac.uk/GOA/EXOSOME
- **ExRNA.** The exRNA Atlas is the data repository of the Extracellular RNA Communication Consortium (ERCC), which includes small RNA sequencing and RT-qPCR-derived exRNA profiles from human and mouse biofluids. http://exrna-atlas.org/
- **ExoRBase.** Collecting about 1000 RNA-seq data of EVs from four types of human body fluids. www.exrobase.org.

(Box 3). Given the merit of biogenic origin and already pivotal role in intercellular communications that pave the way for cell proliferation, angiogenesis, and the traversal of physiological barriers such as the BBB, exosomes are regarded as promising candidates for brain disease therapeutics and diagnosis. Although the clinical translation of exosomes is likely short, drug loading efficiency, quality control, efficient means of production and purification, and optimized engineering protocols are significant challenges to be addressed (Box 4). Many exosome-based strategies currently entered clinic trial evaluating stages (Table 1), while the thorniest obstacles to final clinical use remain in the purity and batch-to-batch variation control in manufacturing. Moreover, highly efficient exosome generation strategies must be explored to achieve mass production. A new generation of the exosome production system, such as automated manufacturing from raw material to product may be helpful. The exosome-based delivery platform is also involved in the concept of personal interventions and precise medicine. The exosome donor cells can be the somatic stem cells or induced pluripotent stem cells derived from patients, which may avoid the virus or allergens in exogenic exosome sources. Exosomes compared with synthesized nanoparticles or cell therapy showed a shorten path to clinic evaluation (Fig. 7). Compared with synthesized nanoparticles, exosomes can be patient derived, therefore the immunogenicity and cytotoxic can be reduced greatly, in addition exosomes itself usually has BBB penetrating lesion navigating role, and the inherited content may also act as

Box 4
Exosome clinical application challenges.

- Large-scale production of exosomes for clinical application.
- Lower yield from cell cultures (1 μg/mL) vs. applications in animal models is 10–100 μg reported.
- Batch to batch quality control.
- Lack of standard manufacturing methods, storage conditions and QC.
- Variation given by source cell type, source cell physiological condition, genetic background in patients.
- Surface marker, biophysical property and content heterogeneity within one batch.
- Purity and physicochemical properties are dependent upon the isolation method employed.
- Unexpected virus infection and pathogen.
- Insufficient cargo loading given by physiochemical properties of cargo and loading modality.
- The ratio of drug loaded to exosome is hard to quantify. And the completed removal of unloaded drug usually greatly reduce the yield of exosomes when loading exogenesis drug.

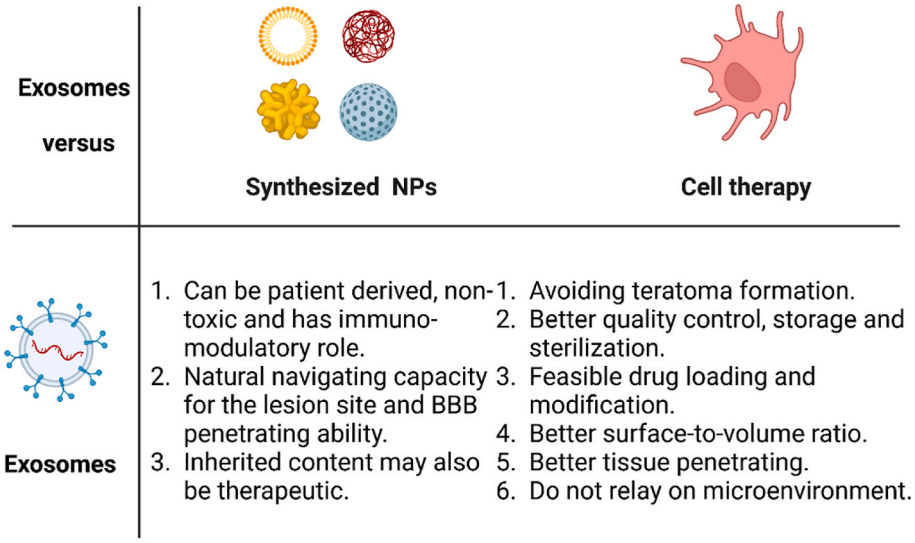

Fig. 7. Comparison of exosomes with synthesized nanoparticles and cell therapy.

therapeutic agent. Compared with cell therapy, exosomes are lacking proliferation ability which can avoid the teratoma formation concern of stem cell transplantation, and the nanoscale size can avoid embolism as well as offer more robust tissue penetrating together with the exosome surface moiety. Exosomes also offer feasible quality control, sterilization and storage condition compared with whole cell therapy. In addition, transplanted cells some time can be impact greatly by the microenvironment, for example, stem cells need specific niche for function, the diseases environment may impact the performance of transplanted cells, while exosome itself has immuno- and microenvironment modulating role. These advantages boosted the translation of exosomes. Moreover, gene editing approaches may be helpful to program the exosome parent cells, such as gain of therapeutic content or enhancing secretory pathways, thus increasing exosome production. Identifying the exact composition of exosomes is also a vital issue to address. Disclosing the ligands and content of exosomes can promote the *de novo* synthesis of exosome-like nanoparticles such as membrane-based NPs. Synthesis of exosome-like nanoparticles may also have a higher yield, stability, and purity.

The immune complementarity and lower adverse effects give exosomes an upper hand over the conventional drug delivery system. Accordingly, the best-suited exosomes for brain disease clinical use are autologous patient-derived MSC or neural cells. Moreover, it is reasonable to expect that developments in surface modification technologies will continue to emerge, enhancing BBB permeability and specific tissue targeting by exosomes. The prospects of achieving the goal of effective exosome-based therapy for one or more brain diseases seem high, given current progress in the field.

Author statement

Y. Liu and F.U. Rehman contributed equally to this work. M. Zheng, B. Shi, F.U. Rehman and Y. Liu generated the idea and wrote the manuscript. F.U. Rehman, Y. Liu and M. Zheng wrote the draft. Y. Liu generated the new figures and discussions. Y. Liu, M. Zheng and B. Shi revised the manuscript. M. Zheng and B. Shi supervised the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC 32071388, 52073079), the National Key Technologies R&D Program of China (2018YFA0209800), China Postdoctoral

Program and China Postdoctoral foundation batch 64 (2018M642740). Figures were created with Biorender.com.

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