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Review

Advanced engineering strategies of extracellular vesicles for enhanced central nervous system regeneration and disease modulation

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THE BIGGER PICTURE Central nervous system (CNS) diseases and injuries are leading causes of disability and death worldwide, largely due to the CNS's limited capacity for self-repair. Stem cell therapy has emerged as a promising and effective treatment option. However, clinical administration is hindered by technical challenges in preparing patient-derived cells and biological limitations during administration. Extracellular vesicles (EVs) have become an appealing allogenic alternative, as they can deliver paracrine signaling factors from the parent cells. In this review, we explore methods to enhance the effectiveness of EVs through various engineering techniques. These methods involve loading specific therapeutic agents into the EVs for targeted therapeutic repair. We discuss different engineering approaches and examine their applications for specific CNS injuries and diseases. Additionally, the review highlights the limitations and emerging strategies in EV engineering aimed at developing effective treatments for CNS injuries and diseases.

SUMMARY

The limited regenerative capacity of the central nervous system (CNS) following injury or disease requires the development of novel therapeutic strategies to promote endogenous repair. Extracellular vesicles (EVs) have emerged as a promising cell-free therapeutic that naturally encapsulates bioactive paracrine factors responsible for tissue repair. Achieving optimal therapeutic outcomes with EVs requires rational engineering to improve their delivery, targeting, and efficacy. Here, we present a comprehensive overview of the recent advacements in EV engineering. To improve their capacity to bypass biological barriers and enhance therapeutic effectiveness, key strategies such as surface ligand functionalization, therapeutic drug loading, and cellular preconditioning are used. We then explore the application of these engineered EVs in treating major CNS pathologies, including traumatic brain and spinal cord injuries (TBIs and SCIs), glioma, stroke, and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Finally, we discuss current challenges in accelerating the clinical translation of engineered EVs as next-generation therapeutics for CNS disorders.

INTRODUCTION

Neurological disorders affect over one billion people globally, representing the leading cause of disability and the second leading cause of mortality worldwide.[1] Central nervous system (CNS) pathologies, including traumatic injuries, neurodegenerative diseases, and glioblastoma (GBM), arise from complex and multifactorial mechanisms that are increasingly difficult to target. These challenges are compounded by the CNS's inherently limited regenerative capacity, which restricts spontaneous repair following injury or disease.[2,3] The complexity of CNS disorders, combined with the brain's limited self-healing ability, makes it challenging to develop effective treatment. Despite advances in clinical management, current therapeutic interventions remain largely palliative, offering symptomatic relief without meaningfully modifying the underlying pathological mechanisms driving disease progression. This critical gap underscores an urgent and unmet need for the development of novel therapeutic strategies that directly target core pathogenic processes. One promising therapeutic approach to address these challenges involves harnessing the regenerative potential of stem cells. Stem cell-based therapies can modulate recovery following neurological injury and disease through paracrine signaling, releasing bioactive factors that restore dysregulated pathways driving disease progression.[4] Clinical studies have demonstrated the therapeutic efficacy of stem cell interventions in restoring neurological function across multiple CNS disorders (Figure 1A).[5–7] However, significant translational barriers remain, including the substantial

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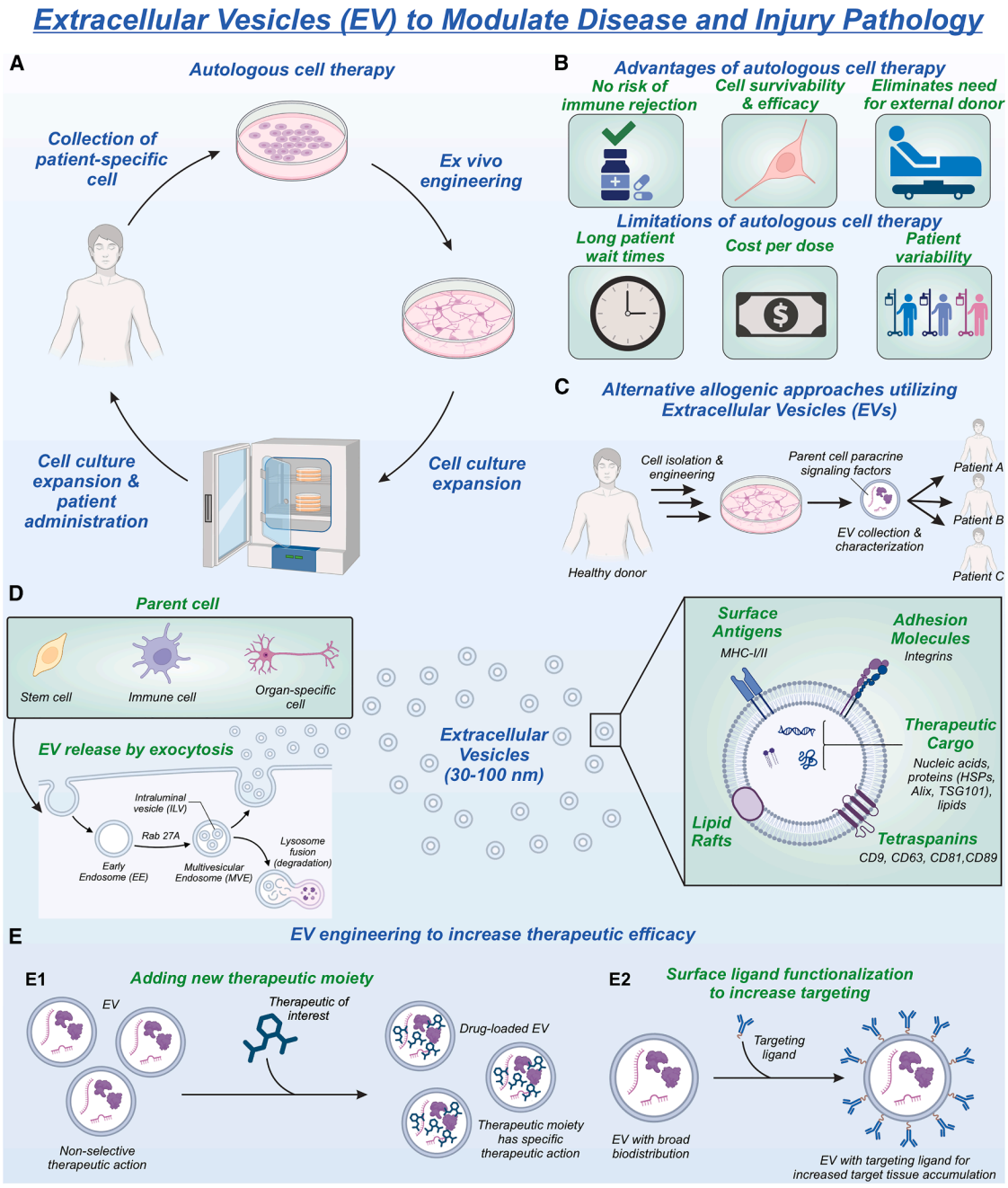

Figure 1. EVs can address the limitations of autologous cell therapy by delivering paracrine signaling factors from the parent cell for cell-free therapy

While autologous stem cell therapy has shown promise in the clinic, several technical and financial limitations prevent its widespread administration. Extracellular vesicles (EVs) are released by the parent cell to be ~30–100 nm and consist of vital therapeutic content. EVs have emerged in recent years as an alternative allogenic approach. The EVs can be engineered to maximize therapeutic efficacy by adding a new therapeutic moiety or conjugating a targeting ligand to the EV surface.

time and cost associated with generating patient-specific autologous stem cells,[8,9] as well as poor engraftment and survival post-transplantation due to the hostile inflammatory microenvironment of the injured CNS.[10,11] These limitations have impeded clinical translation of stem cell therapies, creating an urgent need for alternative approaches that retain regenerative efficacy while addressing these practical and biological constraints (Figure 1B).

In response to these translational bottlenecks, the field has pivoted toward allogeneic, cell-free approaches using extracellular vesicles (EVs).[12,13] EVs are nanosized vesicles that transfer

therapeutic proteins, lipids, and nucleic acids between cells, effectively acting as the primary mediators of stem cell paracrine activity. This unique function allows them to recapitulate the therapeutic efficacy of stem cells without the challenges of whole-cell therapy, such as low survival and complex manufacturing (Figure 1C).[14–17] The immense interest in this platform is reflected in its rapidly growing market, projected to exceed $700 million by 2030.[18,19] While native EVs represent a significant advance, their therapeutic potential can be further amplified through bioengineering. Recent studies have therefore focused on integrating the innate biological advantages of EVs with the tunable properties of synthetic nanocarriers.[20] These engineered systems can be precisely modified to improve cargo stability, control drug-release kinetics, and enhance target-cell uptake, ultimately promising greater therapeutic efficacy and fewer off-target effects.

Despite this promise, the clinical translation of EV-based therapies faces critical hurdles that must be overcome.[21] Natural EVs, for instance, exhibit low intrinsic loading efficiency for therapeutic cargo and lack inherent specificity for target tissues, which can limit therapeutic efficacy and lead to off-target effects (Figure 1D).[22,23] To unlock the full potential of this platform, a new generation of engineering strategies has been developed to enhance payload enrichment and improve tissue-specific accumulation, particularly for CNS applications. In this review, we provide a state-of-the-art, critical evaluation of recent bioengineering advances for the design of EV-based therapies. We highlight their application in neurological disorders and discuss their broader relevance for other diseases (Figure 1E).

EVs

Crucial mediators of intercellular communication, EVs are membrane-bound lipid bilayer vesicles released by cells into the extracellular space.[24] Their membranes are characteristically enriched in cholesterol, sphingomyelin, and ceramides. They are present in nearly all cell types and can carry proteins, nucleic acids, and small molecules. EVs can be broadly classified into three main subtypes—EV, microvesicles, and apoptotic bodies (ABs)—based on their biogenesis and size. Among these, EVs are the most extensively studied, owing to their consistent composition, stability, and established isolation protocols, which make them particularly suitable for brain-targeted delivery applications. Exosomes are highly enriched in signaling molecules such as tetraspanins, while microvesicles display more variable content with less selective cargo loading. Small GTPase ADP-ribosylation factor 6 (ARF6) has also been implicated in mediating the release of microvesicles into the environment to regulate membrane dynamics and remodeling.[25–27] By contrast, ABs often contain nuclear fragments, making them less desirable for therapeutic applications. To distinguish these vesicles from other extracellular populations, studies frequently employ a combination of markers such as Annexin V, which binds to exposed phosphatidylserine on apoptotic membranes, and activated caspases, whose cleavage product can confirm the origin of these vesicles.[28–30] The distinct molecular and compositional profiles of these vesicles underpin their specific biological roles and potential in therapeutic delivery.

Initially dismissed as cellular debris, EVs gained recognition in the 1980s following the discovery that reticulocytes secrete small vesicles to eliminate transferrin receptors during erythrocyte maturation.[31–33] This pivotal finding transformed the understanding of EVs from passive byproducts to dynamic mediators of intercellular communication.[34] Since then, EVs have gained considerable attention as natural, cell-derived nanocarriers capable of crossing biological barriers, transporting bioactive cargos, and modulating diverse physiological and pathological processes. At the molecular level, EV biogenesis involves several distinct yet interconnected mechanisms. The best-characterized is the endosomal sorting complex required for transport (ESCRT) machinery, which orchestrates intraluminal vesicle (ILV) formation within multivesicular bodies (MVBs). Besides the canonical pathway, cells can also produce EVs through ESCRT-independent, lipid-driven mechanisms, such as the ceramide pathway and the Ras-related protein (RAB31)-flotillin(FLOT) complex. These pathways collectively determine EV formation, cargo composition, and secretion of EVs.

The ESCRT machinery plays a central role in the formation of ILVs and MVBs that give rise to EVs. This highly conserved system comprises approximately thirty proteins organized into ESCRT-0, -I, -II, and -III complexes, together with VPS4-VTA1 adapters and associated cofactors.[35,36] ESCRT-0 components, including the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and signal-transducing adapter molecule (STAM), contain ubiquitin-binding domains that are recognized by ESCRT-I and -II, promoting cargo clustering and endosomal membrane deformation. Subsequent recruitment of ESCRT-III subsequently mediates membrane scission, driving ILV budding and EV formation, while VPS4-VTA1 facilitates final membrane fission and ESCRT recycling. Evidence shows that core ESCRT components regulate both EV biogenesis and selective cargo loading.[37] A broad short hairpin RNA (shRNA) screen in HeLa-CIITA cells identified HRS, signal-transducing adapter molecule 1 (STAM1), tumor susceptibility gene 101 (TSG101), and vacuolar protein sorting-associated protein 4B (VPS4B) as essential for EV output and composition, with gene-specific effects on vesicle size and marker content (CD63 and MHC-II), highlighting the mechanistic diversity even among ESCRT-positive vesicles.[38]

Beyond the classical ESCRT machinery, EVs can also form through lipid-driven, ESCRT-independent processes. A central example is the ceramide pathway, in which neutral sphingomyelinase 2 (nSMase2) produces ceramide, promoting the clustering of lipid microdomains and inducing negative membrane curvature that drives ILV budding.[39] This biogenesis pathway preferentially incorporates cargos that partition into lipid raft microdomains, including Glycosylphosphatidylinositol (GPI)-anchored proteins, palmitoylated proteins, and cholesterol-associated molecules.[40] Importantly, pharmacological inhibition of nSMase2 using agents like GW4869, DDL-112, or PDDC reduces the exosomal spread of neurotoxic proteins, including amyloid-β (Aβ), tau, and α-synuclein, in models of Alzheimer's disease (AD) and Parkinson's disease (PD).[41–43] These findings underscore the therapeutic potential of modulating ceramide-dependent cargo selection to mitigate neurodegenerative pathology. Another ESCRT-independent route involves the RAB31-FLOT complex, which marks a lipid

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raft-dependent pathway of EV formation. Upon activation, RAB31 engages flotillins in ceramide- and cholesterol-rich microdomains while simultaneously inactivating RAB7, preventing lysosomal degradation and redirecting cargos such as receptor tyrosine kinases into exosomal secretion.[44] In this way, lipid raft microdomains act not only as structural scaffolds for vesicle budding but also as selective filters that determine whether cargos are degraded or secreted.

Collectively, these diverse biogenesis pathways highlight the intricate interplay between protein sorting machinery and lipid microdomains in determining EV cargo composition. Leveraging these endogenous mechanisms enables the rational design of EVs with enhanced therapeutic cargo-loading, tissue-specific targeting, and controlled release for CNS applications. However, little is known about the ESCRT pathways in the context of EV engineering for disease and injury treatment. More research into this field is required to fully understand the connection.

EV ENGINEERING FOR TARGETING THE CNS AND ACHIEVING CELL-SELECTIVE DELIVERY

The field of precision nanomedicine requires that the nanomaterial platform be capable of carrying and delivering therapeutic agents across biological barriers, targeting specific organs, and internalizing within selected cell types while maintaining a constant concentration over a defined timeframe.[45] To be considered a viable platform for precision nanomedicine, EVs must satisfy these criteria by efficiently delivering their bioactive cargo to target cells in the CNS (Figure 2). However, systemically administered EVs exhibit limited organ specificity, accumulating predominantly in the liver, spleen, and lungs, with minimal CNS penetration.[46] Additionally, rapid clearance kinetics necessitate repeated dosing regimens to maintain therapeutic efficacy.[47] Given the formidable challenge of CNS delivery, this section examines strategies to enhance EV targeting to neural tissues and specific cell populations (Table 1). EVs can traverse the blood-brain barrier (BBB), albeit with limited efficiency.[48] The precise mechanism underlying BBB penetration remains unclear, but preliminary studies suggest that transport occurs via transcytosis.[48–50] EVs derived from different cell sources display considerable variability in BBB permeability.[51] To address this, several strategies have been developed to functionalize the EV surface with targeting ligands to achieve cell-selective delivery.[21] Many of these strategies are analogous to those developed for liposomes, a similarity driven by their shared phospholipid bilayer structure. For example, Sabini et al. engineered parent cells to express glycosylphosphatidylinositol-anchored avidin, which binds to biotinylated ligands through the strong non-covalent avidin-biotin interaction.[52]

Another common approach is the post-insertion of ligand-conjugated polyethylene glycol (PEG)-lipids, in which a lipid-PEG conjugate is incorporated into the EV membrane at moderately elevated temperatures. PEG insertion has been shown to increase circulation time by preventing immune cell uptake. This method is performed under mild conditions, preserves membrane integrity, and allows coupling of various targeting ligands, including antibodies, peptides, and proteins.[53–58] Several conjugation strategies exist for linking PEG to targeting ligands, including thiol-maleimide click chemistry, azide-alkyne click chemistry, and NHS/EDC coupling.[54,59] Novel enzymatic ligation strategies have also been developed to attach targeting ligands directly to the EV surface. These approaches have been validated using several cancer-targeting ligands and may hold promise for CNS applications.[60] Many targeting ligands have been reported to enable precise delivery. Although the surface functionalization of EVs for brain-specific targeting has not yet been extensively explored, studies using liposomes suggest several promising ligands that could be adapted for EVs. Two widely used ligands for brain delivery target the transferrin receptor and the nicotinic acetylcholine receptor (nAChR).[61–66] The OX26 antibody crosses the BBB through receptor-mediated transcytosis by binding to transferrin receptors on endothelial cells.[67,68] Specific OX26 variants have demonstrated high binding affinity (approximately 76–108 nM).[69] When conjugated to nanoparticles, the antibody facilitates BBB penetration. Another commonly used ligand to enhance neural tissue localization is the rabies virus glycoprotein (RVG) peptide, which binds to the nAChR to induce receptor-mediated endocytosis.[70] RVG peptides have been successfully used to shuttle nanocarriers across the BBB[71] (Figure 3A). Other ligands also exhibit moderate efficacy, and an expanded list can be found in previous publications.[72,73] Aside from intravenous administration, intranasal delivery of EVs has also been shown to be a feasible route for CNS targeting.[74,75]

Beyond systemic delivery, the ability to selectively target discrete tissues and cell populations within the CNS represents a critical frontier in the development of precision therapeutic strategies for neurological disorders. Terashima et al. reported the development of a 10-amino acid peptide that mediated efficient plasmid DNA delivery to cells within the spinal cord.[77] While this peptide targeted microglia and astrocytes, uptake was highest in neurons. In an earlier study, the same group identified a distinct peptide that selectively targeted spinal cord microglia.[78] Sellers et al. developed a targeted "ANS to CNS uptake ligand" (TACL) capable of delivering therapeutic cargos into the spinal cord. Among the tested candidates, one peptide showed statistically higher uptake in the spinal cord compared with other organs[76] (Figure 3B). These targeting ligands may increase EV accumulation in the affected organ while reducing accumulation in healthy tissue.

Although EVs represent a promising and biogenic nanoplatform for CNS repair, they still face significant challenges *in vivo* and in clinical translation. Future research should focus on improving these targeting strategies. EVs exhibit rapid clearance and poor retention at target sites.[79] When administered intraperitoneally or intravenously, EVs display short circulation half-lives and rapidly release their cargos upon internalization by the recipient cells.[80] In recent years, biomaterials have emerged as promising tools to improve EV efficacy by prolonging release, enhancing localization, and maintaining structural stability.[81] Because the physical interaction between the EV bilayer and the encapsulated matrix is identical regardless of whether the EVs are engineered or native, a robust body of literature currently exists to further understand this topic.[82–84]

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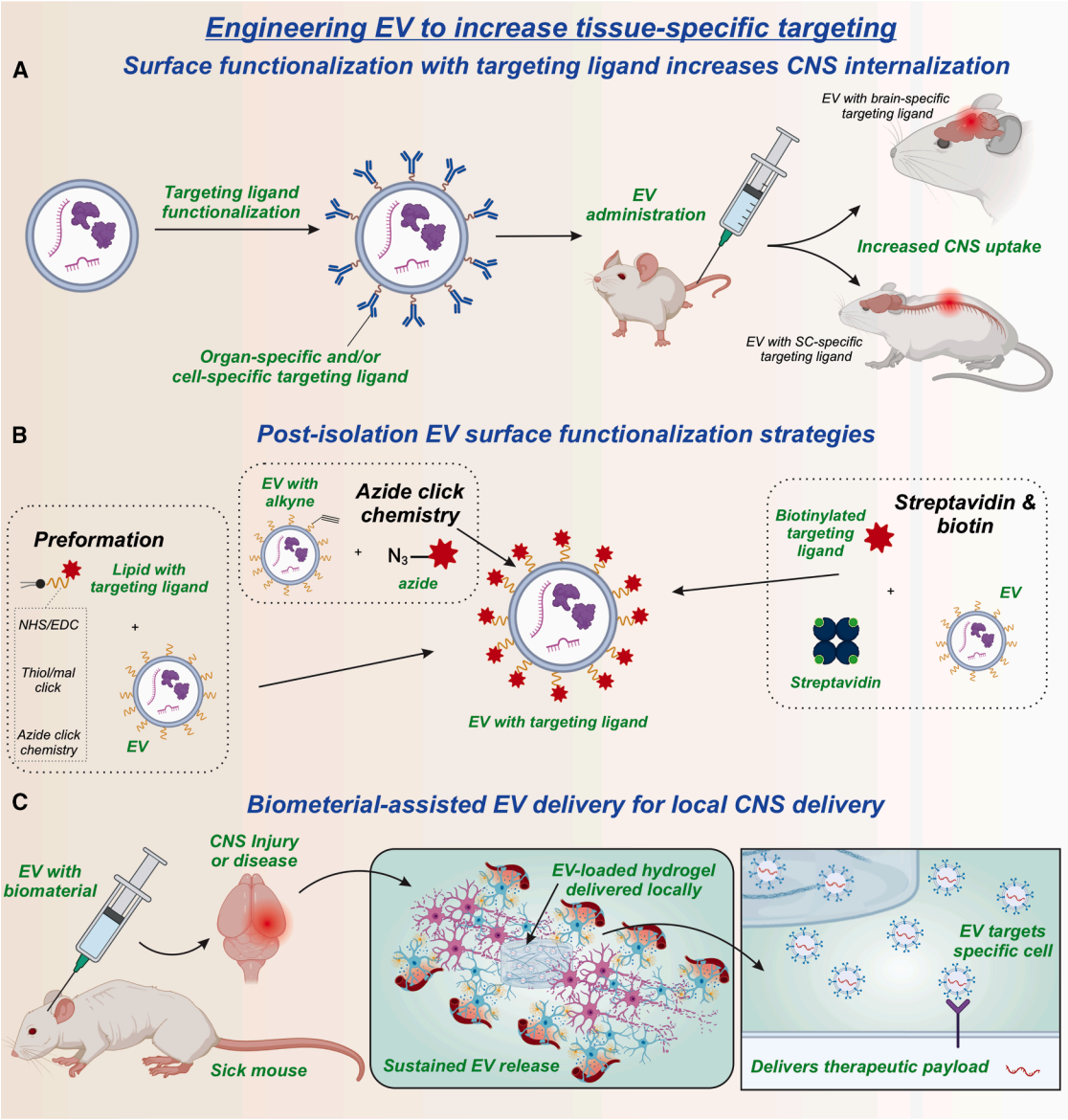

Figure 2. EV engineering strategies to increase accumulation in the target tissue

Targeting ligands can be conjugated to the surface for increased CNS uptake when delivered systemically. Chemical strategies exist to functionalize the EV surface with targeting ligands, including azide click chemistry and streptavidin/biotin binding. Preformation is another strategy to reduce off-target conjugation. Biomaterials can deliver the EV for sustained local delivery following traumatic CNS injury.

METHODS FOR EV ENGINEERING

Beyond systemic delivery, the ability to selectively target discrete tissues and cell populations within the CNS represents a critical frontier in the development of precision therapeutic strategies for neurological disorders. These strategies aim to either introduce novel therapeutic moieties—such as drugs or nucleic acids—or enrich the concentration of beneficial endogenous molecules already present. Fundamentally, these modification techniques are classified into two major categories: endogenous approaches, which manipulate the EV-producing cell (termed the "parent cell"), and exogenous approaches, which modify the EVs post-isolation (Figure 4). Endogenous engineering typically involves preconditioning with small molecules, stress-inducing stimuli, or genetic material (e.g., DNA plasmids) to produce cells that alter the levels of RNAs, proteins, or other bioactive molecules. Since a cell's biological contents are naturally packaged into secreted vesicles, transcriptomic or proteomic changes induced at this stage will ultimately modify the therapeutic cargo of the resulting EVs. Exogenous loading involves incorporating therapeutic agents into EVs after they are synthesized and isolated. In many cases, techniques that

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Table 1. Targeting ligands for increased accumulation in the CNS

Targeting ligand	Target/receptor	Proposed mechanism	Outcomes	Reference(s)
Rabies virus glycoprotein (RVG)	nicotinic acetylcholine receptor (nAChR)	receptor-mediated endocytosis	RVG nanoparticles showed 2 to 3 times higher crossing than the control	Dos Santos Rodrigues et al.[63] and Friden et al.[243]
OX-26	transferrin	–	approximately five times more labeled EVs in the brain parenchyma than in the capillaries	Rodrigues et al.[64]
Spinal cord homing peptides SP1 and SP2	unidentified	unidentified	nanoparticle with targeting ligands showed 100–1,000× increased accumulation in the spinal cord	Terashima et al.[77]
Spinal cord homing peptides AS1, MG1, and MG2	unidentified	unidentified	cell-specific targeting in the spinal cord was observed; AS1 targeted astrocytes, MG1 targeted pro-inflammatory microglia, and MG2 targeted anti-inflammatory microglia	Terashima et al.[78]
Targeted ANS-to-CNS uptake ligands (TCL)	unidentified	retrograde axonal transport	a 2 to 5 fold increase in the delivery of active enzyme to the brain and spinal cord	Seller et al.[76]

temporarily disrupt the lipid membrane are required, including extrusion, sonication, electroporation, and freeze-thaw cycles.[85,86] However, disrupting the lipid membrane has been shown to compromise EV integrity and reduce their biological functionality.[87]

Exogenous engineering

Exogenous engineering enables loading of therapeutics that are impractical to produce endogenously, including small molecules and peptides difficult to overexpress in parent cells. Available methods include electroporation, passive incubation, freeze-thaw cycles, saponin-assisted sonication, extrusion, and pH-gradient-driven loading.[88–90] Osmotic shock demonstrates superior loading efficiency for hydrophilic small molecules compared with conventional approaches.[91] Method selection depends on cargo properties (molecular weight, charge, and hydrophobicity), desired loading efficiency, manufacturing scale, and application.

Small molecule loading is a common strategy, particularly for targeting neuroinflammation. Naturally occurring small hydrophobic molecules have shown the most promising results. For example, encapsulating Bryostatin-1 by simple incubation into EVs derived from neural stem cells (NSCs) reduced immune cell infiltration, decreased astrogliosis, and preserved BBB integrity in experimental autoimmune encephalomyelitis (EAE) mice.[92] Loading quercetin via sonication resulted in a nearly 10-fold increase in drug concentration within mesenchymal stem cell (MSC)-derived EVs. Quercetin has poor water solubility, high lipophilicity, and a short systemic half-life, but EV loading increased drug accumulation in the spinal cord and significantly reversed disease pathology. The EVs exerted anti-inflammatory effects by suppressing upregulated Toll-like receptor 4 (TLR4) expression in microglia. Functional recovery was confirmed using the Basso, Beattie, and Bresnahan locomotor test.[93] In another study, berberine was loaded into MSC-derived EVs via ultra-sonication. The EVs were then incubated at 37°C for 2 h to restore membrane integrity. Anti-inflammatory effects, axonal regeneration, and recovery of motor function were observed.[94] Exogenous drug loading using simple EV engineering strategies proved highly effective for lipophilic small molecules. Further investigation is needed to optimize these methods for hydrophilic small molecules, which often exhibit low bioavailability and rapid systemic clearance.

Other unique approaches have also been introduced, such as a covalent modification of therapeutic moieties (cholesterol) to increase lipophilicity and improve EV encapsulation. Attaching the stem cell recruiting factor (stromal cell-derived factor-1 [SDF-1]) and vascular targeting peptide (i.e., the d-peptide DA7R) induced neuronal differentiation in NSCs.[95] The approach is less optimal for small molecules due to the possibility of conjugation impacting the drug's therapeutic efficacy.[96] Studies have shown that drug loading capacity also depends on the amount of naturally occurring biological material already present within the EVs, as reducing these endogenous cargos can increase drug loading capacity.[97] Therefore, drug-loading approaches should be evaluated across multiple parent cell types to obtain a more comprehensive understanding of loading efficiency and therapeutic performance.

Therapeutic cargos can also be loaded into EVs through fusion with lipid nanoparticles or liposomes. Transferring proteomic cargo to the EV is best represented by encapsulated protein nanocages, which are self-assembling structures with similarity to viruses in their mechanism to mediate membrane fusion.[98,99] Fusion can also be increased by increasing the liposome's PEG concentration to 30%. Verification is conducted by fluorescence resonance energy transfer (FRET) imaging. Specifically, Nitrobenzofurazan (NBD)- and rhodamine-labeled lipids were doped into liposomes that are FRET-active when in close proximity.[100]

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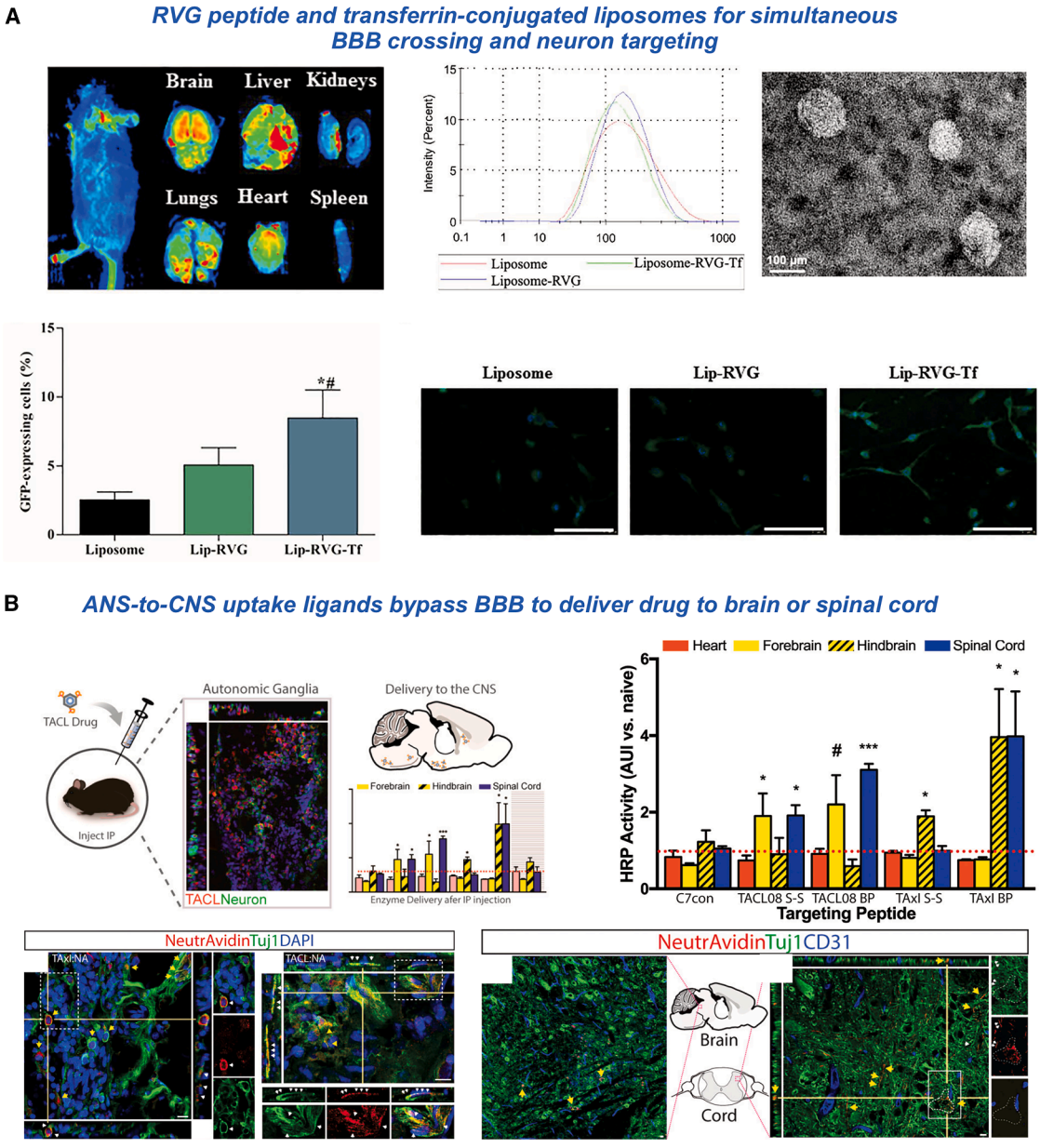

Figure 3. Lipid-based engineering strategies for CNS targeting

(A) Liposomes conjugated with RVG and transferring cross the BBB and target neurons. Reprinted with permission from Dos Santos Rodrigues et al.[63] Elsevier Copyright 2020.

(B) Autonomic nervous system (ANS) to central nervous system (CNS) uptake ligands (TACL) were reported to selectively target the brain and spinal cord. Reprinted Adapted with permission from Seller et al.[76] Copyright 2019 American Chemical Society.

Loading siRNA therapeutics into EVs combines the natural advantages of EVs with the sequence-specific gene-silencing capabilities of siRNA. Current engineering strategies offer several options for loading siRNA into EVs. For instance, fusion assays between EVs and siRNA-loaded liposomes have been developed to increase siRNA encapsulation efficiency. siRNA liposomes were first synthesized using a thin-film hydration method and then combined with cardiac progenitor cell (CPC)-derived EVs. The resulting hybrid nanoparticles exhibited successful endothelial signaling and migration.[101]

Inorganic nanomaterials can also aid siRNA loading into EVs. D-cysteine-derived chiral graphene quantum dots were functionalized with Pygo2 siRNA via van der Waals interactions, yielding a loading efficiency of approximately 60%.[102] Modifying RNA with hydrophobic linkers also facilitated passive incubation without altering EV physical properties.[103,104] In other cases, inorganic nanoparticles can impart new functionality to EVs.

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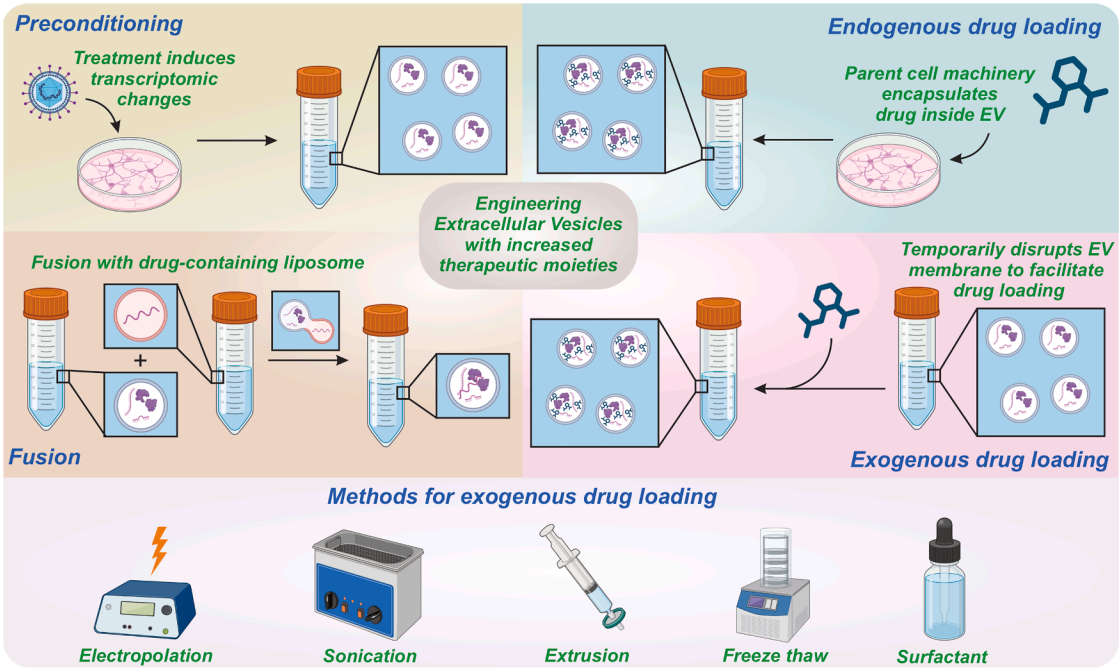

Figure 4. EVs can be engineered to increase their therapeutic efficacy

Preconditioning induces specific transcriptomic changes to the parent cell that are reflected in the EV's therapeutic contents. Drugs can be encapsulated either endogenously (before EV synthesis) or exogenously (after EV synthesis). Several methods for exogenous drug loading are available. Fusion assay includes fusing the EV with a liposome encapsulating the therapeutic of interest.

For example, iron-oxide nanoparticles have been encapsulated for their photodynamic, MRI, and hyperthermia properties. Simple incubation enabled nanoparticle loading, as confirmed by imaging flow cytometry (IFC).[105]

A major limitation of exogenous loading methods is their propensity to disrupt EV lipid membrane integrity, thereby compromising vesicle stability, altering biodistribution, and ultimately diminishing therapeutic efficacy in the context of CNS injury and disease. As summarized in Table 2, varying electroporation settings showed that medium voltage levels of 750 V and a large pulse number of 10 yielded 30% transfection yield with little morphological damage to the EV membrane, as shown by transmission electron microscopy (TEM).[106] Electroporation in 400 mM sucrose buffer increased voltage settings to 950 V and subsequently increased drug loading by 2%. Little EV membrane damage was stated, which the authors evaluated by measuring the EV's therapeutic efficacy after electroporation.[85] Despite these promising results, electroporation irreversibly increased EV size from 176 to 425.7 nm, despite lowering the voltage exposure to 500 V.[107]

Conversely, little change in size was found when applying sonication to produce drug-loaded EVs. Nanoparticle characterization using TEM found the EVs to be unchanged and spherical, with a slightly smaller dynamic light scattering (DLS) reading, approximately 60–80 nm.[108]

A comprehensive evaluation of the impact of exogenous engineering protocols on EV membranes remains challenging. Current studies often conclude no change in EV morphology by TEM, but these analytical techniques are limited and require higher resolution to fully understand changes in the lipid membrane. In addition, these studies are also limited in their comparability due to the difference in loading conditions and substrates employed in each study. Therefore, more studies are needed that adequately compare each protocol. Conducting single-particle assessments using nano-flow cytometry (nFCM) appears to be a leading technique to analyze the EV membrane.[109,110]

Endogenous engineering

Preconditioning is a robust and versatile strategy for EV engineering. In this approach, bioactive stimuli (e.g., small molecules, cytokines, and hypoxia) are applied to the parent cells to induce defined transcriptional and post-transcriptional programs, leading to preferential packaging of the newly expressed or modulated therapeutic molecules into EVs via the cell's native sorting machinery. Compared with active post-isolation loading, preconditioning minimally perturbs the EV lipid membrane and often yields more consistent payload incorporation, reducing batch-to-batch variability. Delivering genetic material to parent cells is a powerful approach to induce transcriptomic modulation. For example, mRNA-mediated expression of the neurogenic transcription factors Ascl1, Brn2, and Myt1l drives reprogramming of mouse embryonic fibroblasts into induced neurons. The resulting EVs were decorated with glutamate-targeting ligands to enhance intracranial accumulation and were shown to modulate neuronal responses, including electrophysiological activity and pro-neuronal differentiation.[111] Similarly, mouse fibroblasts were transduced with three neurotrophic factors (Brn1, Sox2, and Foxg1) via retroviral delivery to reprogram

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Table 2. Evaluating exogenous loading methods on EV integrity

Loading method	Specific parameters	Loading efficiency/yield	Impact on membrane integrity and size	Reference(s)
Electropolation	750 V, 10 pulses	30% transfection yield	little morphological damage (TEM)	Piffoux et al.[100]
Electropolation	950 V, 400 mM sucrose	increased drug loading by 2%	minimal damage (assessed via therapeutic efficacy)	Seller et al.[76]
Electropolation	500 V	not specified	irreversible size increase (176 to 425.7 nm)	Evers et al.[101]
Sonication	not specified	not specified	unchanged/spherical (TEM) slight size decrease (60–80 nm vis DLS)	Zhang et al.[102]

them into neural progenitor cells (NPCs). The resulting EVs activated the Mitogen-activated protein kinase/ Extracellular signal-regulated kinase (MEK/ERK) signaling pathway to promote proliferation, with activity comparable to that of wild-type NPCs.[112] NSC-derived EVs were also functionalized with platelet-derived growth factor receptor α (PDGFRα) for selective targeting of oligodendrocyte progenitor cells (OPCs) by delivering a lentivirus encoding PDGFRα. When loaded with the anti-inflammatory drug montelukast, these EVs significantly increased myelinated axon formation.[113]

Similar approaches have been applied to immune cells. Casella et al. transfected BV2 microglia to overexpress the "eat-me" signal lactadherin (Mfg-8) and loaded an anti-inflammatory cytokine into the resulting EVs. The interleukin (IL)-4-loaded, Mfg-8-functionalized EVs selectively targeted phagocytes in an EAE mouse model, inducing anti-inflammatory effects in recipient cells and reducing tissue damage.[114] Delivering genetic material to modulate anti-inflammatory responses has also been demonstrated using EVs derived from HepG2 cells overexpressing serpinB3, which markedly reduced oxidative stress *in vitro*.[115] Lentiviral delivery has also been used to overexpress specific microRNAs. Viera et al. transduced mesenchymal stromal cells with miR-135b and miR-210. Human umbilical vein endothelial cells (HUVECs) treated with the resulting EVs exhibited a significant enhancement in tubular structure formation, attributed to increased expression of angiogenic proteins (placental growth factor (PGF), endothelin-1, and artemin) and genes (vascular endothelial growth factor [VEGF], activin A, and insulin-like growth factor binding protein (IGFBP1)) following lentiviral treatment.[116] While these EVs display an angiogenic profile, their *in vivo* validation remains to be established.

Small molecules can also modulate the transcriptome of parent cells and alter the therapeutic cargo of their EVs. Interestingly, several clinically approved small molecules have been shown to induce transcriptomic changes in parent cells. For instance, curcumin-treated cells secreted EVs that restored vascular junction protein cadherin and increased the late-stage neuronal marker NeuN.[117] Metformin treatment enhanced EV production through SNAP29 phosphorylation and increased the loading of inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), promoting anti-senescence effects.[118] Inhibition of cholesterol synthesis has also been shown to increase EV yield without affecting EV size, protein concentration, or glycan composition.[119] Exposure to specific ions has been shown to trigger substantial changes in cellular transcriptomic profiles. Treating oxygen-deprived neurons with lithium-conditioned MSC-derived EVs elevated levels of miR-1906, which in turn reduced inflammatory mediators, including TLR4, nuclear factor κB (NF-κB), and nitric oxide synthase. *In vivo* validation using a mouse stroke model revealed improved axonal density and significant neurological recovery, as evidenced by balance beam and rotarod tests.[120] Modulating calcium influx has also been shown to regulate immunostimulatory activity in adaptive immune cells. Sako et al. identified a structure-activity relationship (SAR) by targeting store-operated Ca^{2+} entry (SOCE) in bone-marrow-derived dendritic cells (BMDCs).[121]

Hypoxic preconditioning enhances the production of EVs with substantial neuroprotective properties. Culturing NSCs under hypoxic conditions (~8% oxygen) induced a distinct proteomic profile, upregulating more than 22 neuroprotective proteins and downregulating 20 proteins associated with neurodegenerative diseases. Among the upregulated proteins were 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), CYFIP1, calcium/calmodulin-dependent serine protein kinase (CASK), and tubulin beta-5 (TUBB5)—molecules implicated in neuronal function.[122] This hypoxia-induced signature appears to be cell-type specific, as bone marrow MSC-derived EVs (BMSC-EVs) exhibited altered expression of 74 unique proteins, including several redox enzymes (e.g., catalase and superoxide dismutase).[123] Upregulation of these proteins, along with specific microRNAs, has been linked to enhanced angiogenesis.[124] Induced pluripotent stem cells (iPSCs) cultured at 1% oxygen using tangential flow filtration (TFF) showed elevated levels of angiogenic factors. Numerous reports have demonstrated that EVs derived from hypoxia-conditioned cells effectively reverse stroke pathology.[125] The oxygen concentration used for conditioning is a critical parameter influencing EV content. Angeles de Pedro et al. compared EVs generated under different oxygen levels of 21%, 1%–2%, and <1% O_2. EVs collected at 21% O_2 contained proteins associated with innate anti-inflammatory responses, whereas those produced under 1%–2% and <1% O_2 were enriched with immunomodulatory proteins that target the adaptive immune system.[126] Cyclic hypoxia-reoxygenation, inspired by remote ischemic conditioning (RIC), involves alternating periods of hypoxia and reoxygenation to induce distinct miRNA (notably miR-181-5p, which protects dopaminergic neurons) and protein (GTP-binding protein Rheb, which regulates cell growth and the cell cycle) signatures in EVs. The resulting EVs improved cell viability, reduced inflammation, and induced angiogenesis. Additionally, they exhibited preferential accumulation in the ischemic hemisphere after transient middle cerebral artery occlusion (tMCAO) in mice.[127]

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Many small molecules can mimic hypoxia by stabilizing hypoxia-inducible factor-1α (HIF-1α). Deferoxamine mesylate (DFO), a well-known hypoxia mimetic, modulates EV cargo by upregulating 12 mitochondrial genes related to oxidative phosphorylation, enhancing ATP production. It also upregulated 11 pro-angiogenic and downregulated 7 anti-angiogenic miRNAs.[128] Several studies have employed DFO-conditioned EVs for diverse biomedical applications.[129–131] Emerging approaches include isolating EVs from conditioned plasma. Plasma from animals treated with Buyang Huanwu decoction (BHD) exhibited upregulation of more than 18 genes to promote NSC differentiation, including Sox9, SCP1, BAF53a, BAF45a, RE1-silencing transcription factor (REST), tailless homolog (TLX), forkhead box G1 (FOXG1), Her5, and Her9, as shown by RNA sequencing. *In vitro* validation demonstrated increased microtuble-asociated protein (MAP2) expression, while *in vivo* studies revealed improved cognitive performance after ischemic stroke and enhanced neurovascular repair. These findings suggest that traditional Chinese medicine can profoundly influence modern therapeutic strategies.[132] In other cases, exposure to pro-inflammatory stimuli has surprisingly produced therapeutically efficacious EVs. MSCs conditioned with activated microglial cell supernatant and hydrogen peroxide displayed enhanced anti-inflammatory and antioxidant activity, as indicated by proteomic and phosphoproteomic analyses. This activity was associated with serine/threonine kinase phosphorylation and inhibition of insulin-like growth factor-1 receptor (IGF1R). Similarly, menstrual stem cells incubated with tumor necrosis factor α (TNF-α) and interferon (IFN)-γ released EVs with immunomodulatory properties targeting the innate immune system.[133] MSCs treated with the same cytokines produced EVs upregulating pro-angiogenic proteins IL-8, MCP-1, and CXCL16, which increased migration, proliferation, and tube formation in HUVECs.[134]

Using growth factors as preconditioning stimuli also enhances EV bioactivity. A recent study incubated nerve growth factor (NGF) with NPCs and validated the resulting EVs in a rat nerve crush injury model. NGF-conditioned EVs increased PC12 cell proliferation, reduced IL-6 expression, and promoted neurite extension to a greater extent than oligodendrocyte-derived EVs.[135] *In vivo* analysis confirmed greater myelinated axon formation, showing that preconditioning with neurotrophic factors enhances the neuroregenerative potential of EVs. Mechanical stimulation is another emerging approach in EV engineering. Mechanical cues act on the cell membrane to activate signaling pathways that induce transcriptomic reprogramming. EVs released from dental pulp stem cells under mechanical stimulation significantly increased axonal sprouting. Cells were seeded either into a bioreactor to induce flow or onto a 3D stretchable scaffold to apply cyclic stretch. Both conditions activated Yes-associated protein (YAP) signaling and upregulated associated transcription factors. The amount of EVs released under mechanical stimulation was markedly greater than in static conditions, while their mean size remained unchanged. These EVs promoted axonal outgrowth in rat dorsal root ganglion (DRG) neurons, as shown by increases in axon length, volume, branching complexity, and depth. Mechanical stimulation has also been shown to modulate inflammatory mediators in MSCs: wall shear stress upregulated the NF-κB cyclooxygenase-2(COX2)- prostaglandin E2 (PGE2) axis, which subsequently suppressed TNF-α production.[136] In rats with traumatic brain injury (TBI), treatment with mechanically conditioned EVs reduced apoptosis and suppressed microglial activation, demonstrating their capacity to promote neural repair.

Beyond stimulation with soluble bioactive agents, the preconditioning landscape has expanded to include a range of physicochemical and genetic strategies. Yet, many preconditioning strategies have not yet been investigated in the field of CNS regeneration. Parent cells can also be modulated using various forms of biophysical stimulation, such as electrical fields, electromagnetic waves, ultrasound, heat shock, or alterations in environmental pH, to elicit desired changes in EV cargo.[137] At a more targeted level, permanent transcriptomic modifications can be achieved through precise gene-editing technologies such as CRISPR-Cas9, enabling stable, long-term production of engineered EVs.[138] Future investigations that strategically integrate these diverse preconditioning modalities will be pivotal for developing next-generation EV therapies for neuroregeneration.

As usual with large bodies of biomedical academic literature, a lack of standardization and defined nomenclature still plagues the field. As a consequence, the lack of standardized protocols for parent cell culture conditions, EV isolation methodologies, and EV engineering approaches across laboratories has introduced substantial variability into the literature, significantly complicating efforts to reproduce and compare findings across studies. More attention needs to be given to standardization as outlined by the minimal information for studies of extracellular vesicles (MISEVs) and the critical quality attributes (CQAs).[139–141]

TBI

EVs have shown considerable therapeutic efficacy in models of CNS injury and neurodegenerative diseases. While native EVs possess intrinsic activity, engineered EVs substantially outperform unmodified EVs in therapeutic potency, specificity, and clinical efficacy by enhancing cargo delivery, improving cellular uptake, optimizing neural targeting, and augmenting neuroprotective/neuroregenerative function. EV engineering has therefore emerged as a critical strategy for advancing viable EV-based therapeutics. This section examines current engineering approaches, highlights successful CNS applications, identifies translational challenges, and discusses optimization strategies. The clinical significance of engineered EV approaches becomes clear in TBI, where a direct mechanical insult to the brain causes immediate neuronal death and irreversible damage. The injury triggers a systemic inflammatory response that diminishes BBB integrity and disrupts natural biochemical gradients essential for CNS protection.[142] Conventional therapeutic strategies primarily address clinical symptoms but are ineffective at modulating the detrimental neuroinflammatory environment or stimulating endogenous neural repair mechanism.[143] Many patients with moderate-to-severe injury experience permanent cognitive deficits. EVs have shown great promise in treating TBI by promoting neuroregeneration.

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Endogenous loading is widely employed to engineer EVs for TBI therapy because it is versatile for loading large biomolecules, mainly proteins and nucleic acids. Introducing genetic material into the parent cell can correspondingly alter miRNA levels in the resulting EVs. Transfection of miR-124-3p into BV2 microglia exerted potent neuroprotective effects: miR-124-3p bound FAK family kinase-interacting protein of 200 kDa (FIP200) mRNA and reduced autophagy in HT22 cells, while EVs decreased oxidative stress and increased neurite outgrowth. *In vivo*, a substantial reduction in behavioral scores was observed compared with EVs lacking miR-124-3p.[144] This strategy can also be applied to the immune system. Transfecting MSCs with miR-17-92 inhibited microglial activation and TLR4 expression. In a rat model, the EVs strongly downregulated inflammation, increased hippocampal neuronal density, and reduced activated macrophages and astrocytes.[145] Lentiviral systems are a common approach for transfecting parent cells. Overexpressing miR-424-5p in MSCs using a lentiviral system produced EVs that significantly improved visual acuity and contrast after blast injury; however, the engineered EVs did not show a significant improvement in contrast with non-engineered EVs.[146] Lentiviral approaches were also used to transfect human adipose-derived MSCs, and the resulting EVs suppressed the NF-κB pathway and inflammatory markers (Iba-1, inducible nitric oxide synthase [iNOS], and IL-1β) while upregulating Arg-1[147]·Figure 5. Antisense oligonucleotides were also used for gene downregulation: depleting MALAT1 from human adipose-derived stem cells removed the corresponding biomolecule from their EVs.[148]

Astrocyte-derived engineered EVs can strongly modulate inflammation following TBI. Transfection of long non-coding RNAs corresponding to miR-195 and NLRX1 into astrocytes upregulated NK-κB interacting lcRNA (NKILA) in injured neurons, and reduced neuronal apoptosis was observed.[150] EVs derived from astrocytes conditioned with rat brain extract also showed substantial recovery benefits. miR-148a-3p was overexpressed, polarizing microglia to the pro-regenerative M2 phenotype. *In vivo*, the EVs' anti-inflammatory properties were evident from decreased iNOS and Iba-1.[151] Cellular stress (a form of preconditioning) has also been shown to produce EVs with substantial efficacy for TBI. EVs harvested from microglia under hypothermic conditions increased the expression of miR-20b-5p, promoting the number and length of neuronal dendritic branches by activating the PTEN/PI3K/AKT pathway in neurons. In a murine controlled cortical impact (CCI) model, significant improvements in behavioral testing were observed[149] (Figure 5B). In another study, hypoxia upregulated miR-212/132 in microvascular endothelial cell-derived EVs.[152] Mechanical cues have also been investigated: microglia-derived EVs isolated following stretch-induced injury using a BioFlex plate contained elevated miR-5121 and promoted neurite outgrowth and synapse recovery *in vivo*.[153] These studies indicate that inducing cellular stress in the parent cell can yield therapeutically effective EVs for TBI treatment.

EVs produced under 2D or 3D culture conditions are of interest because they can better mimic *in vivo* environments. These systems more effectively model the extracellular matrix (ECM) and activate mechanotransduction pathways that may influence EV cargo. EVs isolated under 3D culture conditions from human MSCs reduced glial fibrillary acidic protein (GFAP) expression while increasing neuronal nuclear antigen (NeuN) expression. However, no significant differences were observed in brain lesion volume or performance in several behavioral tests compared with controls, indicating a need for further development of this approach.[154]

While endogenous engineering is a valuable strategy, it presents notable limitations, including an incomplete understanding of the parent cell's transcriptomic response to stimuli and, critically, a lack of precise control over the final cargo dosage. Exogenous loading methods, therefore, are often superior for applications requiring the delivery of a specific biomolecule at a defined concentration. For instance, in a study aimed at targeting pathological tau following TBI-induced AD, an anti-p-tau antibody was successfully loaded into MSC-derived EVs using several exogenous techniques. Among these, saponin-based permeabilization proved most effective, achieving an encapsulation efficiency of 82.3%, whereas simple room-temperature incubation was least efficient (62.3%). Interestingly, despite these significant differences in loading efficiency, EVs produced by all methods yielded comparable improvements in TBI-related behavioral outcomes, suggesting that even the lower encapsulation rate was sufficient to achieve a therapeutic effect.[155] Electroporation is another widely used method. During miR-26a-5p loading, EV aggregation was minimal, and the engineered vesicles performed therapeutically comparably to non-engineered EVs *in vivo*.[156] Electroporation was also used to load miR-328a-3p into EVs from rat plasma, and the engineered EVs showed a much greater ability to promote osteogenesis following TBI.[157] Commercially available kits have been used to load nucleic acids into EVs, as with Bcl-2-associated X-protein shRNA.[158] However, methodological details are often not publicly available, and the stability of engineered EVs is unknown. Further characterization is warranted.

EVs modified with targeting ligands have shown great efficacy in treating TBI. To achieve CNS-specific targeting, the RVG29 peptide was covalently attached to the EV surface through a bio-orthogonal click chemistry reaction. Higher brain localization was observed *in vivo*, with signal decreasing after 48 h, and reduced accumulation was observed in the liver and spleen[159] (Figure 5C). Click chemistry was also used to conjugate DA7R and SDF-1 to the EV surface, yielding higher therapeutic efficacy for downregulating microglial inflammation *in vitro* and increased brain fluorescence *in vivo*.[95] Local delivery and sustained release of EVs can maximize therapeutic efficacy while reducing off-target effects.

Hydrogels have emerged as an essential platform for encapsulating EVs and releasing them over several days. Hydrogels can also restore aspects of the native ECM and promote anti-inflammatory effects by tuning their stiffness to match that of brain tissue. 3D-printed hydrogels offer synthetic versatility by controlling scaffold size, porosity, and morphology, and they provide favorable water-absorption characteristics for cell growth and tissue regeneration. For CNS injuries and diseases, 3D-printed scaffolds can closely mimic CNS tissue for repair.

3D-printed collagen/chitosan scaffolds can meet these requirements. A hybrid scaffold delivering IFN-γ-preconditioned NSC-derived EVs synergistically reduced inflammation and

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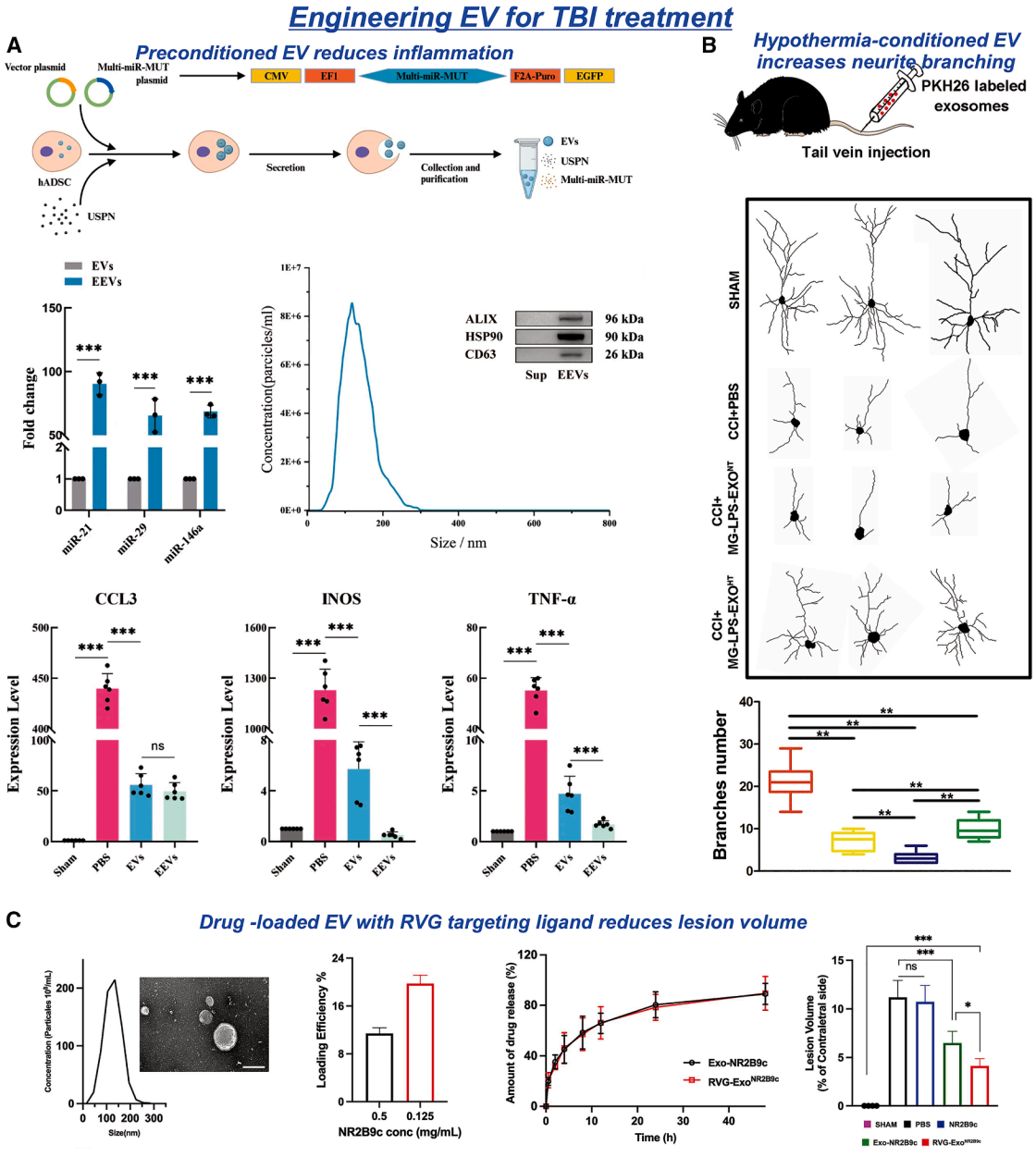

Figure 5. EV engineering strategies for treating TBI

(A) Human ADSCs transduced with lentivirus to increase miRNA levels. The EVs were found to reduce inflammatory markers. Licensed from the *Journal of Nanobiotechnology*[146] under CC BY-NC-ND 4.0.

(B) Hypothermia-conditioned EVs from microglia increased neurite branching *in vivo*. Reprinted with permission from Neurobiology of Disease[149] with Permission from Elsevier Copyright 2023.

(C) Microglia EVs were loaded with NR2B9 and the RVG peptide and showed significant protective effects. Reprinted from *International Journal of Pharmaceutics*[147] copyright (2024), with permission from Elsevier.

encouraged neurogenesis, with long-term EV release at the injury site.[160] Similar results were obtained using 3D-printed implants delivering hypoxia-derived MSC-EVs over 2 weeks.[161] A 3D-printed collagen/chitosan scaffold loaded with IGF-1-primed EVs downregulated NF-κB following TBI. Significant upregulation of late neuronal markers, including MAP2 and NeuN, as well as myelin basic protein, was observed, alongside improved spatial memory.[162] 3D-printed collagen/chitosan scaffolds were also used to deliver brain-derived neurotrophic factor (BDNF)-derived human umbilical cord MSC (BComSc) EVs. Chitosan binding slowed collagen degradation, enabling sustained EV release to the injury site.[163] Other materials include hyaluronan-based hydrogels, which degraded over three days following injury. Hyaluronan is a natural ECM-mimetic polymer

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that can upregulate neurogenesis (NeuN marker) and angiogenesis (CD31 marker).[164] In some cases, synthetically derived polymers have enabled sustained release of engineered EVs. Thioketal-based polymers have emerged for the treatment of CNS injuries due to their reactive oxygen species (ROS)-scavenging ability to alleviate oxidative stress after injury. Most EVs were released within 24–48 h, with sufficient reversal of pathology at the injury site being observed.[165] Electrospun nanofibers have also been shown to provide 28-day sustained release of EVs, enabling prolonged suppression of inflammatory pathways.[162]

SCI

In recent years, the engineering of EVs as therapeutic agents for spinal cord injury (SCI) has garnered significant attention. Preconditioning has emerged as a simple yet powerful strategy to enhance the bioactivity of EVs. For instance, hypoxia has been shown to prime neural or MSC-derived EVs to activate regenerative cascades via the HIF-1α/RAB17 pathway in NSCs and enhance angiogenesis in HUVECs.[166,167] Furthermore, such preconditioned EVs were demonstrated to promote ECM regeneration by stimulating nucleus pulposus cell proliferation and collagen synthesis.[168] Other preconditioning cues can entirely reshape EV cargo composition. For example, melatonin pretreatment in MSCs has been shown to upregulate USP29, giving rise to an anti-inflammatory EV phenotype.[169] Another study took this a step further by treating M2-microglia-derived EVs with melatonin and Angiopep-2, generating EVs with site-specific delivery that not only reduced inflammation but also promoted axonal growth and remyelination.[170] Together, these studies underscore how modifying the cellular microenvironment can reprogram vesicular output toward neuroprotection.

Genetic manipulation of parent cells provides a more targeted route to augment EV potency. Lentiviral transduction of MSCs has been widely used to enrich EVs with specific therapeutic molecules such as CD73, miR-146a-5p, or Sonic Hedgehog, each of which confers potent anti-inflammatory and regenerative benefits.[171–173] Similarly, transfection of circular or modified RNAs like circZFHX3 and netrin-1 modRNA has been shown to load EVs with neuroprotective transcripts.[174,175] Increasing neurotrophic factors is also achieved by overexpressing NGF or BDNF. BDNF introduced via plasmid electroporation led to improved neuronal differentiation and axonal outgrowth. Other studies have shown that EVs enriched in non-coding RNAs like miR-26a, miR-544, and long non-coding RNA TCN2 (lncRNA-TCTN2) to promote neurogenesis and motor recovery post-SCI.[176–179] In a related effort a TAR-RNA/TAT-peptide-based system was also designed to efficiently load miRNAs into iPSC-derived EVs.[180] Collectively, these works highlight how precise genetic engineering can convert EVs into customizable gene and RNA-delivery vehicles.

Because native EVs are rapidly cleared *in vivo*, biomaterial scaffolds have been developed to ensure localized and sustained delivery to the spinal cord. A range of hydrogel formulations such as those constructed from hyaluronic acid, fibrin, or electroconductive composites using tannic acid and polypyrrole, or methyl acrylate-gelatin nanomaterial hybrids, have been investigated for their ability to encapsulate and release EVs in a controlled manner.[94,181–187] For example, a poly-L-lysine hydrogel was shown to allow rapid aminoguanidine release and prolonged EV delivery simultaneously.[188,189] Roh's group used TNF-α/IFN-γ-primed MSCs combined with decellularized matrices and polydeoxyribonucleotide (PDRN) molecules within hydrogels to potentiate anti-inflammatory and regenerative outcomes.[190] Hydrogels comprising sodium alginate-gelatin have been shown to repair both local and distal spinal tissues, achieving dual-organ recovery.[191] In another study, Cao and team fabricated 3D exosomes derived from MSC spheroids and subsequently sonicated them to load dexamethasone, dramatically improving angiogenic and neurotrophic outcomes by facilitating ROS-responsive release of EVs.[192] Similar synergy was observed when scaffolds derived from NSCs were used to mimic spinal architecture, promoting axonal regrowth and vascularization.[181,193] Temperature-sensitive triblock copolymers and collagen-based matrices have further expanded the EV-delivery toolkit, further bridging the gap between bioengineering and regenerative medicine.[183,194]

EVs are natural nanocarriers for therapeutic payloads, with engineered EVs demonstrating clinically significant synergistic efficacy enhancing drug bioavailability, target specificity, and combinatorial effects beyond additive benefits. Various small molecules, including dexamethasone, curcumin, berberine, cerebrolysin, and isoliquiritin, have all been successfully encapsulated through sonication, freeze-thaw, or incubation methods, achieving loading efficiencies between 13% and 32%.[94,192,195–201] These drug-loaded vesicles consistently showed reduced inflammation, enhanced neuronal survival, and improved functional recovery. Interestingly, platelet-rich plasma-derived EVs and milk EVs coated with platelet membranes have also proven feasible for translation-friendly formulations.[195,198]

Targeting injured tissue remains critical for therapeutic success. Peptide modification and surface functionalization have therefore been employed to direct EVs specifically to lesion sites, including glial scars and neurons. RGD peptides expressed on macrophage or MSC-derived EVs via lentiviral transduction enhanced endothelial uptake and improved barrier integrity.[202,203] Other targeting strategies employed Lamp2b fusion constructs or peptides such as the injury-homing peptide CAQK and viral macrophage inflammatory protein-II (vMIP-II) to facilitate lesion homing.[191,204]

Novel approaches continue to emerge. Ran et al. developed autologous plasma EVs functionalized with neuron-targeting peptides that facilitated axonal regrowth across astrocyte borders, whereas Zheng et al. used simple sulfo-NHS chemistry to conjugate IKVAV peptides onto M2-macrophage EVs for boosting neuronal regeneration.[205,206] Loading superparamagnetic nanoparticles to EVs has provided magnetically guided axon regeneration within the glial scars.[186] Meanwhile, electroconductive hydrogels built from gelatin-methacrylate crosslinked with polypyrrole and tannic acid allowed synchronized EV release and local electrical stimulation.[207] Collectively, this demonstrates that SCI interventions can exploit engineered EVs whose tunability and targetability can render them uniquely multifunctional.

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GLIOMA

One of the central challenges in GBM therapy is the effective delivery of therapeutics across the BBB. Although EVs show an inherent capacity to cross the BBB through mechanisms such as transcytosis, the efficiency is generally low, necessitating engineered approaches. For example, EVs derived from canine GBM demonstrate an innate ability to cross the BBB and exhibit a natural tropism for brain tissue, rendering them an effective platform for targeted imaging applications.[208] Brain endothelial cell-derived EVs loaded with a mitochondria-targeting photosensitizer hijacked the BBB, leveraging intrinsic endothelial mechanisms to deliver photodynamic therapy.[209] In addition, functionalization with ligands such as cyclic arginylglycylaspartic acid (RGD) for GBM-targeted drug delivery and T7 peptide-decorated EVs for Galectin-9 siRNA delivery has been reported to enhance receptor-mediated uptake and selective tumor accumulation.[210] Other groups have explored thermo-responsive EV membranes for controlled release and microfluidic-based scalable EV platforms for intranasal delivery as noninvasive strategies for BBB penetration[211,212] (Figure 6A). Collectively, these approaches highlight the promise of BBB-directed EV engineering for GBM therapy. Despite their biological advantages, native EVs face limitations in yield and consistency, which has motivated the development of EV-mimetic and hybrid platforms. Multifunctional EV mimetics designed to modulate the protein corona achieved extended circulation times and improved tumor homing *in vivo*.[213] Hybrid EV-liposome nanomedicines for NIR-II imaging and photothermal therapy combine the stability and loading capacity of synthetic liposomes with the biological interface of EVs.[214] Other innovations include fruit-derived EV-engineered droplet drugs for chemotherapy and EVs enveloped by nanolipid particles for signal transducer and activator of transcription 3 (STAT3) siRNA delivery.[215,216] These hybrid designs address limitations of scale-up and drug encapsulation, offering versatile theranostic opportunities.

EV-mediated delivery of RNA therapeutics, including siRNA, miRNA, and mRNA, is among the most actively investigated approaches for GBM treatment. EVs have been engineered to deliver siRNAs against STAT3, cytosolic phospholipase (cPLA2) (in combination with metformin), and cholesterol-modified Yin Yang 1 (YY1), resulting in the suppression of oncogenic signaling and improved sensitivity to chemoradiotherapy.[216,219,220] T7 peptide-decorated EVs delivering Galectin-9 siRNA demonstrated potent immune modulation by reprogramming macrophages.[221] Beyond siRNAs, tumor-specific polycistronic miRNA delivery by engineered EVs simultaneously downregulated multiple oncogenic pathways. Genome editing is another promising strategy: engineered EVs delivering CRISPR-Cas9 for radiotherapy sensitization and CRISPR-Cas9 library screening via EV delivery to address temozolomide (TMZ) resistance showed strong potential in overcoming therapeutic resistance[217] (Figure 6B). These studies demonstrate the versatility of EVs as RNA carriers. EVs have also been investigated as carriers for conventional chemotherapeutics, aiming to improve drug accumulation in GBM while reducing systemic toxicity. Doxorubicin-loaded nanoparticles coated with endothelial cell-derived EVs elicited strong immunogenic chemotherapy effects.[222] These findings highlight how EV platforms can extend the therapeutic window of chemotherapies.

The immunosuppressive microenvironment of GBM remains a major barrier to treatment. EV engineering provides innovative strategies to reprogram immune responses. For example, engineered brain-targeting EVs designed to modulate the immunosuppressive tumor microenvironment (TME) and Galectin-9 siRNA-loaded EVs promoting macrophage repolarization both enhanced antitumor immunity (brain-targeting immuno-EVs; T7-Gal9 EVs). EVs carrying immune checkpoint inhibitors or TRAIL (TNF-related apoptosis-inducing ligand), derived from induced NSCs (iNSCs), further amplified immune responses (checkpoint EVs; TRAIL-iNSC-EVs). In addition, trypsinized EVs carrying tumor-suppressive miRNAs simultaneously targeted both tumor cells and tumor-associated macrophages (trypsinized EVs). These approaches position EVs as powerful tools to overcome GBM-associated immunosuppression.

Beyond therapeutic applications, several studies have provided key mechanistic insights into EV biology in GBM. For example, under hypoxic conditions, glioma cells were shown to adapt to stress by increasing their uptake of EVs via proteoglycan-dependent endocytosis, which in turn fuels a lipid-droplet phenotype (termed "hypoxia-uptake EVs"). In another study, microglia-derived EVs carrying miR-124 were found to reduce GBM growth by altering tumor metabolism and enhancing glutamate clearance ("microglia-miR-124 EVs"). Similarly, EVs from M1-macrophages demonstrated effective tumor accumulation and highlighted the intrinsic antitumor role of immune-derived vesicles ("M1-mac EVs"). Together, such mechanistic discoveries are crucial for informing the rational design of next-generation therapeutic EVs.

STROKE

Ischemic stroke results from an acute interruption of cerebral blood flow, leading to rapid neuronal death within the infarct core and progressive damage to the surrounding ischemic penumbra. The primary pathological consequences include severe neurological deficits, expansion of the infarct size, and widespread neuronal apoptosis. Current treatment strategies, such as intravenous thrombolysis and endovascular thrombectomy, are restricted by narrow therapeutic windows and provide only partial restoration of neurological function. Recent work reported that although endovascular thrombectomy improves acute reperfusion in patients with large-vessel occlusion, a substantial proportion of patients did not achieve functional independence beyond 90 days, underscoring the limited long-term efficacy of existing interventions.[222] Consequently, novel therapeutic approaches are required to directly address infarct progression, neuronal loss, and secondary complications, including BBB disruption, neuroinflammation, oxidative stress, and impaired angiogenesis. In this context, EVs derived from stem cells and engineered sources have emerged as promising candidates for ischemic stroke therapy.

MSC-derived EVs have demonstrated the ability to enhance angiogenesis, modulate inflammation, and promote neuroregeneration. While NSC-derived EVs generally demonstrate superior efficacy in reducing infarct core volume, engineering strategies

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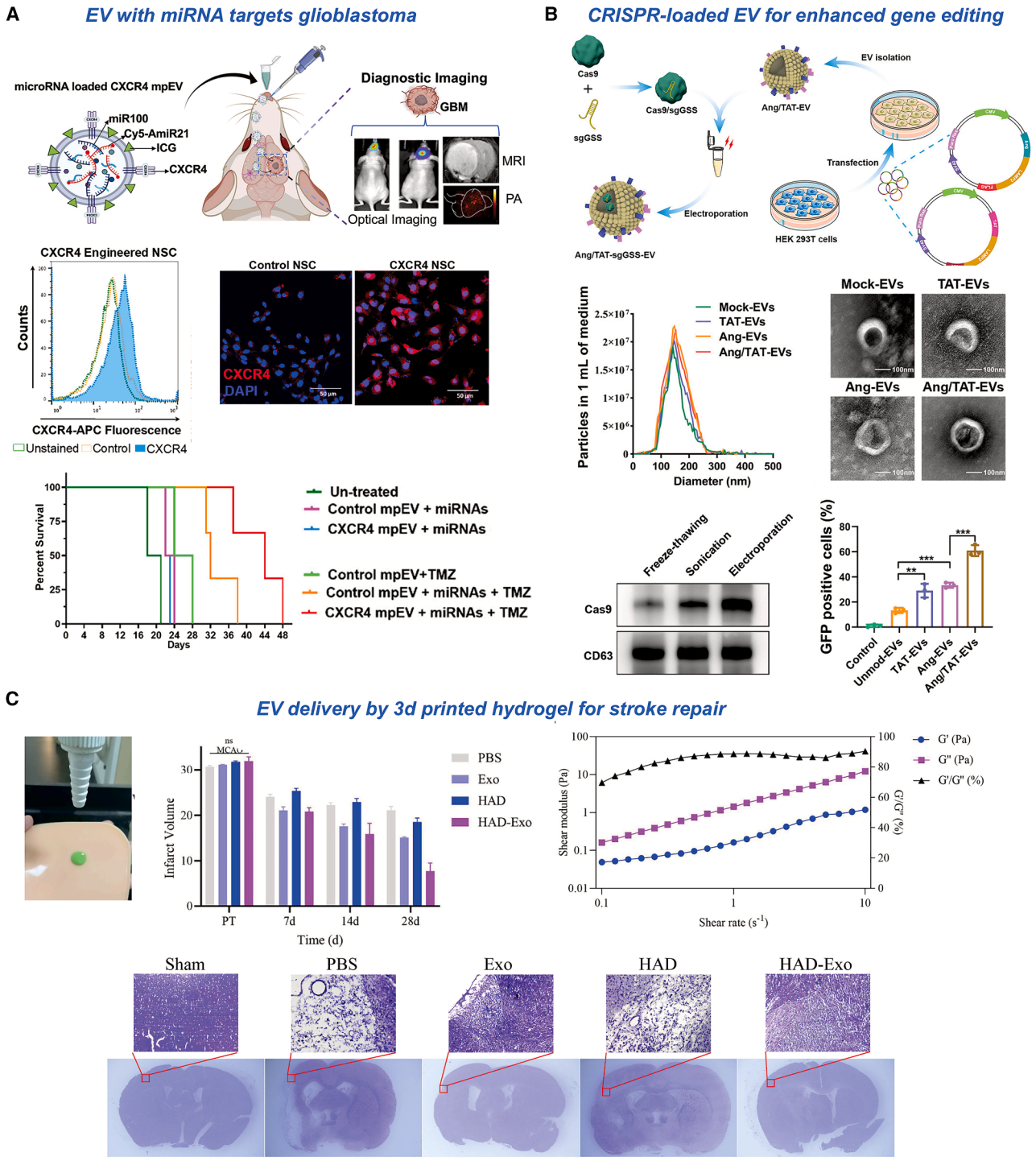

Figure 6. EVs engineering strategies for treating glioblastoma and stroke

(A) EVs were engineered to express miRNA-100 and to increase CXCR4 expression for increased CNS accumulation. Reduced tumor progression was observed. Reprinted with permission, Copyright (2021) American Chemical Society.[212]

(B) The CRISPR plasmid was loaded into the EV, and high gene editing efficiency was observed. Figure reproduced from *ACS Nano*[217] licensed under Creative Commons Attribution 4.0 International License (CC BY 4.0).

(C) 3D printed catechol-grafted hyaluronic acid hydrogel facilitated sustained EV release and decreased infarct volume. Reprinted from *Experimental Neurology*[218] Copyright (2023), with permission from Elsevier.

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have been shown to enhance the therapeutic potential of MSC-EVs.[213] For instance, Zhou et al. showed that intranasal delivery of BDNF loaded into MSC-EVs significantly improved outcomes compared with unmodified MSC-EVs, with greater reductions in infarct volume, attenuation of synaptic loss, and promotion of axonal regeneration. These benefits were mediated by activation of the BDNF/TrkB pathway, which in turn upregulated Akt, Erk, and PLCγ1 signaling.[223] Song and colleagues also found that microglia polarized to an M2 phenotype following stroke, releasing small EVs (sEVs) that were highly enriched for miR-124. The administration of these miR-124-loaded sEVs subsequently reduced infarct volume and promoted functional neurological recovery. Mechanistically, these vesicles promoted NSC differentiation and proliferation by activating the AP2-associated protein kinase 1 (AAK1)/Notch1 signaling pathway. In another study, IL-4-treated microglia-derived EVs supported remyelination and BBB repair by increasing the expression of tight-junction proteins ZO-1 and Claudin-5.[224,225] iPSC-derived EVs have also been reported to rejuvenate the BBB by activating the endothelial nitric oxide synthase (eNOS)-Sirt1 axis, thereby reducing neuronal apoptosis and inflammation in aged mouse ischemia models.[226,227]

Recent advances highlight the potential of engineering EVs for targeted delivery and enhanced efficacy. Haroon et al. developed a neuron-specific EV cocktail using bio-orthogonal chemistry to conjugate therapeutic peptides such as NR2B9c, achieving efficient BBB penetration and reduction of oxidative stress.[228] Likewise, functionalized MSC-derived EVs with RGD-modified peptides were developed and loaded with curcumin, yielding synergistic suppression of inflammation and apoptosis.[229] In another approach, Tian and colleagues generated fusion proteins combining RGD-4C peptides with lactadherin (C1C2) domains to increase EV targeting to microglial cells, leading to marked inhibition of MAPK-driven inflammation.[230] Hydrogel-based sustained-release platforms have also been explored. For example, Gu et al. demonstrated that encapsulating NSC-derived EVs in hydrogels improved release kinetics, motor recovery, and infarct reduction in rodent models[218] (Figure 6C).

Beyond classical EVs, ABs are emerging as a controllable and highly scalable platform for therapeutic delivery. To this end, You and team engineered ABs loaded with the cargo α-mangostin and surface-functionalized them with matrix metalloproteinase (MMP)-activatable peptides. This targeted system exerted potent, localized effects, providing both anti-inflammatory/antioxidant activity and pro-angiogenic/neuroprotective support.[231] Endothelial-derived EVs enriched with miR-27a have been shown to enhance axonal rewiring and neuronal circuit recovery.[232] Furthermore, adipose stem cell-derived EVs with enhanced microglial targeting were engineered to mitigate ferroptosis in ischemic models.[233] The feasibility of EV-mediated RNA therapeutics is increasingly evident, where one study showed that EVs loaded with circSCMH1 improved post-stroke recovery by promoting synaptic plasticity and reducing immune infiltration. Crucially, the study's preclinical validation in both murine and nonhuman primate models demonstrated a favorable safety profile and significant translational potential.[234]

AD

AD is the most common cause of dementia, characterized by progressive memory loss, Aβ plaque deposition, tau hyperphosphorylation, synaptic dysfunction, and chronic neuroinflammation. Despite extensive efforts, current FDA-approved drugs mainly offer symptomatic relief and cannot halt or reverse disease progression. Recently, EVs, including exosomes and microvesicles, have emerged as promising therapeutic candidates due to their intrinsic ability to cross the BBB, carry bioactive cargos, and modulate cellular pathways implicated in neurodegeneration.[225] MSC-derived EVs have been extensively studied for their neuroprotective potential in AD. Katsuda et al. first demonstrated that adipose-derived MSC-EVs deliver neprilysin, an enzyme that degrades Aβ, leading to a reduced amyloid burden both *in vitro* and in transgenic mice.[226] Subsequent studies confirmed that MSC-EVs attenuate oxidative stress and synaptic loss in hippocampal neurons exposed to Aβ oligomers, mediated in part by EV-associated catalase and cytokine secretion.[227]

In vivo, administration of MSC-EVs to amyloid precursor protein (APP)/ presenilin-1 (PS1) mice rescued synaptic plasticity and memory deficits while downregulating iNOS expression, a critical mediator of neurotoxicity.[228] Hypoxia-preconditioned MSC-EVs further enhanced therapeutic efficacy by enriching miR-21, which restored synaptic function and suppressed STAT3/NF-κB-driven neuroinflammation.[229] More recently, bone-marrow-derived MSC-EVs have been shown to suppress APP expression and reduce plaque formation in rat models of AD, improving cognitive performance.[230] Collectively, MSC-EVs exert their effects through multifaceted mechanisms, including Aβ clearance, anti-inflammatory modulation, and reduction of oxidative stress. NSC-derived EVs (NSC-EVs) appear to possess unique regenerative advantages compared with MSC-EVs. Li et al. demonstrated that NSC-EVs restore mitochondrial function, increase Sirtuin 1 (SIRT1) expression, and rescue synaptic integrity in APP/PS1 mice, though without significantly altering Aβ plaque burden.[218] Apodaca et al. extended these findings by administering human NSC-derived EVs administered intravenously, which reduced dense-core Aβ plaques, attenuated microglial activation, and improved memory and anxiety-related behaviors in 5xFAD mice.[231]

A recent study demonstrated that iNSC-EVs exhibited therapeutic efficacy comparable to or exceeding that of primary NSC-EVs. Both EV populations significantly reduced Aβ deposition, p-Tau propagation, and neuroinflammation, while promoting dendritic spine density and neurogenesis in AD mice.[232] These studies suggest that NSC-EVs may be particularly effective in restoring synaptic plasticity and mitochondrial health, which are crucial to AD pathology. Beyond naturally secreted vesicles, engineering approaches aim to augment therapeutic delivery. Yang et al. developed macrophage-derived EVs loaded with curcumin and methylene blue, demonstrating efficient BBB penetration and synergistic inhibition of tau hyperphosphorylation and apoptosis in an OA-induced AD mouse model.[233] Such bioengineered EVs offer a flexible platform for co-delivery of neuroprotective drugs and may overcome pharmacokinetic limitations of free compounds.

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Despite encouraging preclinical results, several challenges remain before clinical translation. Current studies are confined to rodent models, with no human trials yet reported. EV isolation and characterization methods vary widely, limiting reproducibility. Large-scale, GMP-compliant production remains another bottleneck. Additionally, the heterogeneity of EV cargo poses safety concerns, as unwanted oncogenic or inflammatory molecules could be co-delivered. Advancing the clinical translation of EVs necessitates several key future directions. These include establishing standardized protocols for EV biomanufacturing, conducting comprehensive comparative studies across diverse EV sources (for example, MSCs, NSCs, and engineered variants), and optimizing drug/EV hybrid systems. Furthermore, iPSC-derived EVs or iNSC-EVs present a promising avenue, offering scalable and ethically favorable alternatives to primary stem cell sources. Integrating EVs with nanotechnology, gene-editing tools (such as CRISPR-Cas9 delivery), or targeted ligands could further enhance their specificity and therapeutic efficacy.

EVs are a highly versatile and promising modality for AD therapy. EVs derived from MSCs and NSCs exhibit neuroprotective, anti-inflammatory, and regenerative effects across multiple preclinical models. Engineered EVs that carry therapeutic payloads further broaden the intervention landscape, overcoming limitations of conventional small molecules. Although clinical translation will require rigorous standardization and safety evaluation, EVs hold strong promise as a next-generation platform to combat AD.[234]

PD

PD, the second most common neurodegenerative disorder, is marked by progressive loss of dopaminergic neurons in the substantia nigra, accumulation of misfolded α-synuclein, oxidative stress, and chronic neuroinflammation. Existing pharmacological interventions, such as levodopa and dopamine agonists, offer symptomatic relief but cannot halt disease progression. Consequently, EVs are now recognized as highly promising therapeutic agents, primarily due to their intrinsic capacity to cross the BBB, deliver functional biomolecules, and modulate both neuroinflammation and oxidative stress.[235]

EVs secreted by MSCs have been extensively investigated for PD therapy due to their anti-inflammatory and neuroprotective cargo. Umbilical cord MSC-derived EVs improved motor function and reduced neuroinflammation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) rodent models, partly by suppressing microglial activation and oxidative stress.[236] Similarly, bone-marrow-derived MSC-EVs rescued cognitive and motor deficits in A53T α-synuclein transgenic mice by modulating cholesterol metabolism and inflammatory signaling.[237]

Preconditioning strategies enhance their efficacy. For example, hypoxia or growth factor stimulation increases the abundance of neuroprotective miRNAs and antioxidant enzymes in MSC-EVs, strengthening their ability to protect dopaminergic neurons.[238] Collectively, MSC-EVs exert their effects through anti-inflammatory signaling, reduction of oxidative stress, and trophic support, making them leading candidates for translational PD therapy. NSC-derived EVs may provide distinct benefits by promoting neuronal survival and synaptic integrity. Human NSC-EVs reduced oxidative stress, apoptosis, and inflammatory cytokines in neuronal and microglial cultures, while preserving dopaminergic neurons in 6-OHDA PD mice.[235] In another study, NSC-EVs delivered antioxidant enzymes, such as catalase, which improved dopaminergic neuron survival in PD models.[239]

These results highlight that NSC-EVs target both neuronal and glial dysfunction, with beneficial cargo including neurogenic and anti-inflammatory miRNAs (miR-182, miR-183, miR-9, and let-7). Their ability to simultaneously reduce oxidative stress and promote neuronal regeneration positions NSC-EVs as a powerful platform for disease modification in PD.

Engineering EVs expands their therapeutic potential. Yang et al. designed MSC-derived EVs loaded with curcumin (PR-EXO/PP@Cur) and administered intranasally, which alleviated motor dysfunction and improved neuronal survival in PD mice.[240] Another approach used EVs as carriers for BDNF, achieving enhanced dopaminergic neuroprotection and behavioral recovery in toxin-induced PD models. Beyond stem cell sources, platelet-derived EVs administered intranasally protected dopaminergic neurons and improved outcomes in PD and TBI models, suggesting that blood cell-derived vesicles may offer a clinically feasible alternative.[241] Likewise, early work proposed blood-derived EVs as flexible drug delivery systems for PD, underscoring the versatility of EV-based platforms.[242]

In short, EVs offer a versatile therapeutic platform for PD by combining intrinsic neuroprotective properties with the capacity to deliver engineered cargo. MSC-EVs primarily act through anti-inflammatory and antioxidant mechanisms, whereas NSC-EVs emphasize neuronal regeneration and synaptic protection. Engineered EVs carrying therapeutic drugs or trophic factors further broaden the scope of intervention. While translation to the clinic requires standardization and safety validation, EV-based therapeutics represent a promising frontier for disease-modifying therapy in PD.

DISCUSSION AND CONCLUSIONS

EV engineering has witnessed remarkable advancements over the past decade, driven by the imperative to enhance therapeutic efficacy through improved cellular internalization and the diversification of bioactive cargo formulations. These efforts have solidified EVs as a highly promising modality for addressing complex neurological disorders and injuries (Table 3). However, despite this rapid progress, several fundamental limitations and technical hurdles persist, which collectively hinder the full realization of EVs' therapeutic potential and their successful translation into clinical applications (Figure 7). Addressing these challenges systematically is paramount for advancing the field.

A primary concern in EV biomanufacturing revolves around the culture systems used for EV production. Most studies exploring EV engineering for CNS injury and repair have historically relied on two-dimensional (2D) cell culture systems. While convenient, these systems inherently suffer from significant drawbacks.[244] They typically yield lower EV quantities, critically impacting the scalability required for clinical doses. More importantly, 2D cultures fail to accurately recapitulate the intricate physiological conditions of the *in vivo* environment, lacking crucial cues such as three-dimensional cell-to-cell interactions, ECM components,

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Table 3. EVs for modulating CNS injury and disease

Diseases	EV sources	Platforms	Outcomes	Reference(s)
TBI	MSCs	preconditioned EVs developed using suspension bioreactors	improved method to scale-up for clinical translation and promote angiogenesis	Phelps et al.[250]
TBI	MSCs	hypoxia-preconditioned EVs loaded in 3D-collagen/fibroin scaffold	noticeable neuronal regeneration, angiogenesis, anti-inflammation, and reduced cell apoptosis, promoted motor function recovery in dogs	Liu et al.[161]
TBI	MSCs	exosomes derived from hMSCs cultured in a 3D-collagen scaffold	improved spatial learning, neurogenesis, reduced inflammation, and motor function recovery	Zhang et al.[154]
TBI	NSCs	3D-printed collagen/chitosan scaffold with IGF1/IFN-γ-pretreated EVs	promotes anti-inflammation, neuronal remyelination, and regeneration; enhanced differentiation and maturation; motor and cognitive functions *in vivo* rats	Chen et al.[160] and Liu et al.[251]
TBI	NSCs/MSCs	polydopamine-conjugated-nanofibers for effective loading of exosomes	sustained delivery of dual exosomes, enhanced loading of the exosomes, anti-inflammation and neuronal repair, improved functional recovery in mice, and reduced reactive astrocytes and glial activity	Li et al.[162]
TBI	hASC	lyophilized small EVs	improved efficiency and stability of the exosomes for over 2 months, promoted wound healing, anti-inflammatory benefits, and reduced oxidative stress	Jones et al.[252]
TBI	iPSC neurons	decellularized extracellular matrix-based adhesive hydrogel to load exosomes	anti-apoptotic, created a regenerative microenvironment, axonal regeneration, and remyelination along with functional recovery in rats	Wang et al.[253]
SCI	ADSC	silk fibrin-based hydrogel	enhanced neuro- and oligodendrogenesis and motor function in rats	He et al.[184]
SCI	bovine milk	curcumin/platelet-membrane fused	enhanced targeting of lesion site, axonal regeneration, and motor function recovery in mice with anti-inflammation with reduced cellular senescence and reduced blood-spinal cord barrier (BSCS) leakage	He et al.[198]
SCI	HEK293T	collagen-based miR-21 enriched with CBD-Lamp2b functionalization	improved tissue wound repair, anti-apoptosis, and promoted recovery and regeneration	Liu et al.[194]

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Table 3. ***Continued***

Diseases	EV sources	Platforms	Outcomes	Reference(s)
SCI	MSC/OECs	miR-26a/146a-5p modified through magnetic superparamagnetic iron oxide nanoparticle (SPIONs) or lentiviral transduction	promoted neurofilament generation, axonal growth, and functional recovery, inhibited reactive astrogliosis and autophagy	Lai et al.[172], Chen et al.[176], and Gao et al.[177]
SCI	MSCs	pH-responsive aminoguanidine (AG)/ hydrogel with short and sustained release	targeted release of AG only in an acidic environment, anti-inflammatory, reduces oxidative stress and formation of scar tissue, and improves axonal regeneration	Wang et al.[189]
SCI	MSCs	thermal-responsive triblock-polymer-based hydrogel loaded with miR-138 modified exosomes	reduced oxidative stress and inflammation with improved axonal regeneration and motor function recovery	Xiao et al.[183]
SCI	MSCs	RDG-CD146/CD271 specific through flow cytometry extraction	specific targeting of neovascular endothelial cells, increased BSCB stabilization and junction repair, improved neurogenesis and function recovery in mice	Xie et al.[203]
SCI	MSCs	3D spheroid-derived EVs	ROS-responsive release of EVs, anti-inflammatory, enhanced angiogenesis and neurotrophic effects, anti-oxidative, reduced secondary injury, and promoted functional and tissue repair in rats	Cao et al.[192]
SCI	MSCs	magneto-electric Fe_3O_4@$BaTiO_3$ based hybrid-hydrogel	anti-inflammation promoted, improved neurogenesis, axonal regeneration, and functional recovery in SCI rats	Liu et al.[186]
SCI	microglia	M2-induced pretreated electroconductive hydrogel	promoted M2-like polarization in BV2 cells, improved neuronal and axonal regeneration and remyelination, motor function recovery, and enhanced neurogenesis	Guan et al.[182]
Glioblastoma	endothelial cells	doxorubicin-loaded exosomes	higher tumor accumulation; strong immunogenic chemotherapy; reduced systemic toxicity	Zhang et al.[254]
Glioblastoma	GL261	RNA and Dox loaded coated with acid-cleavable transferrin	reprogrammed immunosuppressive TME; improved survival/antitumor immunity	Yang et al.[255]
Glioblastoma	HEK293T	siRNA loaded through electroporation and functionalized with T7 peptides	enhanced BBB crossing and tumor accumulation; macrophage repolarization; tumor growth inhibition	Li et al.[210]

(*Continued on next page*)

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Table 3. ***Continued***

Diseases	EV sources	Platforms	Outcomes	Reference(s)
Glioblastoma	NSCs	loaded with the TRAIL transmembrane protein	dual targeting of GBM cells and tumor-associated macrophages; apoptosis induction; prolonged survival *in vivo*	Zhang et al.[256]
Glioblastoma	RAW264.7 macrophages	exosome-liposome hybrid vesicles through lipid film hydration and extrusion	NIR imaging guidance; potent photothermal tumor ablation; improved stability/ loading	Liu et al.[257]
Glioblastoma	U87 MG	hypoxia-preconditioned	higher EV uptake: lipid-droplet phenotype linked to stress adaptation	Cerezo-Magana et al.[258]
Glioblastoma	bEnd3 cells	TPP-Chlorin e6 for targeted delivery to mitochondria	improved BBB traversal; effective photodynamic therapy; increased intratumoral delivery	Nguyen Cao et al.[209]
Glioblastoma	HEK293T	CRISPR-Cas9 delivery with angiopep2TAT dual functionalization	gene editing of resistance pathways; radiosensitization; reduced recurrence	Hazrati et al.[137]
Ischemic stroke	ADSCs	Lamp2b-M2pep functionalized	anti-ferroptosis, anti-inflammation, improved microglia targeting	Wang et al.[253]
Ischemic stroke	MSCs	magnetic nanovesicles derived from iron-oxide nanoparticle preconditioning	improved targeting to the lesion site, anti-apoptosis, inflammation, and angiogenesis	Kim et al.[259]
Ischemic stroke	MSCs	BDNF-loaded EVs	improved functional behavior, neurogenesis, angiogenesis, synaptic plasticity, fiber preservation, anti-inflammation, and reduced infarct volume	Huang et al.[260]
Ischemic stroke	microglia	RVG29 functionalized via click chemistry, loaded with NR2B9c	functional recovery, decreased neuronal apoptosis, reduced oxidative stress, targeted neuron delivery	Haroon et al.[159]
Ischemic stroke	NSCs	loaded to hyaluronic acid hydrogel	improved neurological functions, angiogenesis, anti-inflammation, and reduced infarct volume	Gu et al.[218]
Alzheimer's disease	MSCs	hypoxia-preconditioned exosomes	enhanced neurogenesis, cognitive recovery, and attenuated inflammation	Cui et al.[261]
Parkinson's disease	blood	dopamine-loaded exosomes	delivered dopamine directly to the brain, improved motor and behavioral symptoms	Qu et al.[262]
Parkinson's disease	MSCs	BDNF-loaded exosomes	promoted dopaminergic neuron regeneration and improved behavioral recovery	Wang et al.[263]

and physiological shear stress. Consequently, EVs derived from such systems often exhibit reduced therapeutic potency and altered biodistribution profiles compared with those produced under more physiological conditions. Therefore, future efforts must prioritize the development and adoption of advanced production platforms, such as 3D bioreactors, microfluidic systems, or

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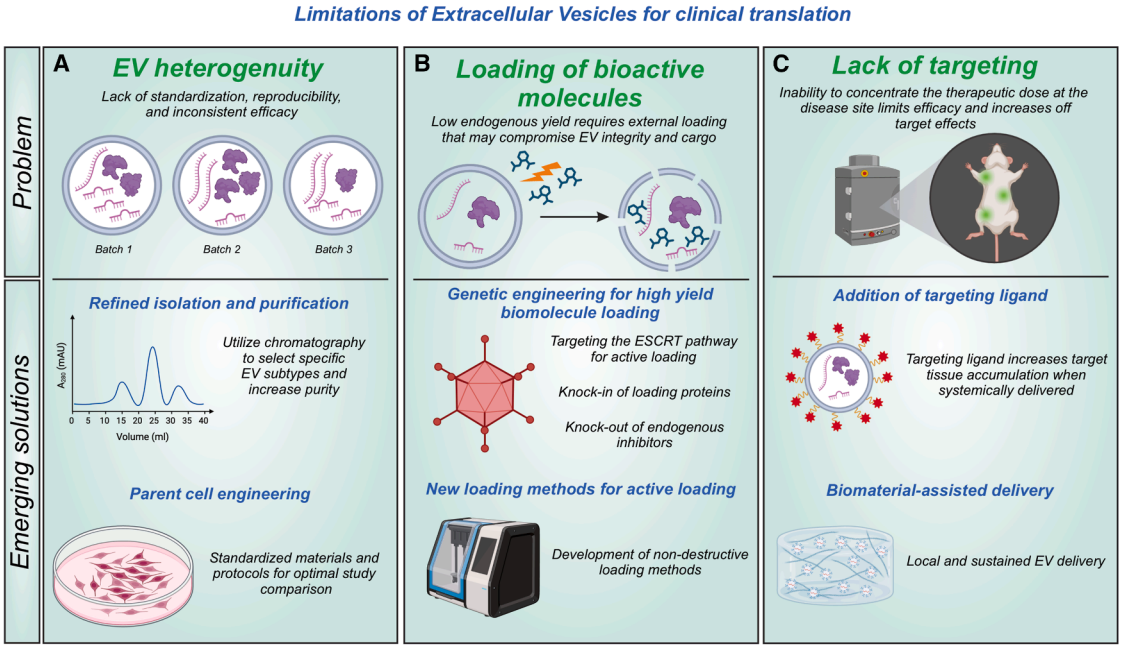

Figure 7. Limitations of EVs preventing their clinical translation

Three main limitations plaguing EV therapy development are heterogeneity, an inaccurate bioactive moiety-loading procedure, and a lack of targeting to the disease microenvironment. To address limitations associated with heterogeneity, more accurate isolation and purification procedures need to be developed, as well as standardized cells and materials. Low biomolecule loading can be addressed by genetic engineering of the parent cell and by developing new non-destructive loading methods. The third limitation, targeting, can be addressed by adding specific targeting ligands to the EV surface or delivering the EV with a biomaterial.

scaffold-based cultures, that more closely mimic *in vivo* biological environments to generate clinically relevant quantities of highly potent and reproducible EVs.

Concurrently, a more comprehensive and robust molecular characterization of EV composition is indispensable. The precise molecular composition and bioactive contents of EVs remain incompletely understood, hindering our ability to decipher their exact mechanisms of action and ensure consistent product quality. High-throughput multi-omics approaches, including transcriptomic, proteomic, metabolomic, and lipidomic profiling, must systematically define the intricate landscape of EV cargo. While advancements in these analytical technologies are emerging, further refinement is still needed to overcome challenges such as the low abundance and heterogeneity of EV contents. This detailed characterization is particularly crucial because EVs are known to carry a vast array of metabolites, beyond nucleic acids and proteins, that can promote critical processes like neurogenesis and angiogenesis. Untargeted metabolomic platforms, such as MetaboAnalyst, for instance, offer invaluable tools for identifying these potentially novel bioactive metabolites and biomarkers, paving the way for targeted engineering and quality control.[245]

Regarding EV loading strategies, maintaining EV membrane integrity is a critical consideration. Endogenous loading strategies, which involve genetically engineering parent cells to produce EVs containing specific therapeutic cargo, remain highly attractive as they circumvent the need for external manipulation and thus avoid disrupting the inherent structure and functionality of the EV membrane. However, when exogenous loading is necessary to incorporate specific molecules not naturally expressed by the parent cell, careful selection of the loading method is crucial. Saponin-assisted treatment represents a preferable loading strategy compared with more disruptive methods like electroporation or sonication. By inducing only minimal membrane perturbation, the saponin-based approach better preserves the inherent stability and biological activity of the EVs. Continued research into novel, gentle, and highly efficient exogenous loading methods is vital for expanding the therapeutic repertoire of EVs. The therapeutic promise of EVs for chronic conditions, such as neurodegenerative diseases or sustained neuronal injury, depends on achieving long-term bioactivity. Therefore, establishing methods for sustained and targeted delivery is critical to maximizing their clinical efficacy. The rapid clearance of intravenously administered EVs and the inherent challenges in traversing biological barriers, most notably the BBB or blood-spinal cord barrier (BSCB), remain significant hurdles. Current biomaterial-based delivery systems that can mimic brain and spinal cord tissue, enabling controlled and prolonged EV release over a defined time frame, are still under active development. To achieve sustained delivery, biocompatible hydrogels such as chitosan- and collagen-based formulations have emerged as promising platforms, particularly when fabricated via 3D bioprinting for precise spatial control and tailored release kinetics. These innovative platforms have demonstrated high efficacy in both prolonging EV release kinetics and enhancing regenerative outcomes in preclinical models. Future work must intensify efforts on optimizing EV surface modifications with

specific targeting ligands or functional peptides, as well as designing sophisticated bio-responsive scaffolds, to improve CNS targeting accuracy, reduce off-target effects, and ensure therapeutic concentrations reach the intended site over extended periods.

Manufacturing still remains a challenge moving forward. TFF and chromatography appear to remain the best approach for large-scale isolation of EVs.[246–249] Engineered EVs will suffer from additional limitations due to the addition of therapeutic cargo or targeting ligands. CRISPR engineering of the parent cell shows initial promise for inducing endogenous engineering, but many more studies are required to fully address limitations that still remain. No optimal method appears in the literature for drug loading or targeting ligand addition to the EV at scale.

In summary, EV-based therapeutics represent a rapidly developing and immensely promising approach for promoting neuroregeneration and mitigating pathology following CNS injury or disease. The field is poised at an exciting juncture where foundational scientific understanding must converge with advanced engineering principles. However, realizing their full clinical potential hinges upon continued and concerted advancements in several key areas: establishing robust, scalable EV engineering and large-scale manufacturing protocols, undertaking comprehensive multi-omics characterization for quality control and mechanistic insights, developing gentle and efficient loading strategies, and designing sophisticated biomaterial-based delivery systems for sustained and targeted CNS administration. Overcoming these limitations will undoubtedly pave the way for a new generation of highly effective, EV-based neurotherapeutics.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.S. and K.-B.L.; data curation, J.S., M.A., and H.C.; writing—original draft, J.S., M.A., and H.C.; writing—review and editing, J.S., M.A., H.C., and K.-B.L.; visualization, J.S.; project administration, J.S. and K.-B.L.; supervision, K.-B.L.; funding acquisition, K.-B.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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