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Extracellular Vesicles as an Emerging Frontier in Spinal Cord Injury Pathobiology and Therapy

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Abstract

Extracellular vesicles (EVs) are membrane-delimited particles secreted by nearly all cell types. EVs mediate critical physiological functions and pathophysiological processes in the central nervous system (CNS). As carriers of diverse bioactive cargo (e.g., proteins, lipids and nucleic acids) that can be modified in response to external stimuli, EVs have emerged as pathological mediators after neurotrauma, such as spinal cord injury (SCI). Here, we discuss the roles of endogenous EVs within the CNS as well as crosstalk with peripheral EVs in relation to neurotrauma, with particular focus on SCI. We then summarize the status of EV-based therapeutic advances in preclinical animal models for these conditions. Finally, we discuss new bioengineering strategies that are poised to enhance CNS-specific therapeutic capabilities of EVs.

Keywords

Exosomes; Neural Regeneration; Neurotrauma

Extracellular Vesicles in Spinal Cord Injury Pathobiology and Therapy

Traumatic injuries to the central nervous system (CNS), such as spinal cord injury (SCI) and traumatic brain injury (TBI), constitute a significant portion of the global injury burden, with 0.935 million new cases and an incidence rate of 17,730 new SCI cases each year according to the National SCI Statistical Center [1,2]. Historical advances in symptom management

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have reduced mortality rates, with an estimated 363,000 SCI survivors currently living in the United States. However, there is no effective treatment to counteract the long-term functional deficits following SCI. In part, this reflects an incomplete understanding of the complex pathophysiology after SCI as well as a lack of effective tools that safely regulate known therapeutic targets.

One emerging therapeutic option is the use of **extracellular vesicles** (EVs; see Glossary) that comprise the secretome of nearly all cell types, including those of the CNS. EVs can be detected in all body fluids including blood, urine, and cerebrospinal fluid (CSF) and play significant roles in regulation of neuro-glia communication as well as **neural plasticity** and the immune response during development, adulthood, and pathology based on their payload of diverse biological cargo [3–9]. Additionally, EVs have emerged as alternatives to cell-based therapies due to their potential for improved safety and therapeutic efficacy across diverse regenerative applications [10]. Yet, the pathophysiological roles of EVs in the CNS are still largely unknown, hampering rational design of novel EV-based treatment approaches.

This review summarizes current knowledge of the role of CNS EVs after neurotrauma, with an emphasis on SCI. It further discusses how this knowledge can be applied in combination with biotechnological advances to inform development of therapeutic EVs for SCI treatment. Additionally, the emerging role of EV crosstalk between the CNS and peripheral systems is considered throughout. It should be noted that the literature specific to EV-based interventions for SCI is relatively nascent compared to other treatment modalities, such as gene- or cell-therapy. As pathophysiological mechanisms of SCI considerably overlap with that of TBI, stroke, and some neurodegenerative diseases (e.g. inflammation, apoptosis, metabolic dysfunction, etc.), relevant studies from broader applications have also been included in this discussion.

CNS Injury Involves Multicellular EV-Mediated Crosstalk

Neurotrauma in the brain and spinal cord share many features that are often categorized into primary and secondary phases. Primary injury is defined by the acute damage to neural tissue resulting from mechanical forces that leads to largely irreversible cell loss [11]. The ensuing secondary injury phase is characterized by a delayed biochemical sequelae encompassing metabolic and inflammatory dysfunction that may contribute to further tissue damage over time [11]. Secondary injury mechanisms in particular represent critical therapeutic targets after SCI, with investigational strategies aimed to promote angiogenesis, axonal sparing, and regeneration/remyelination, while limiting **autophagy** dysfunction, inflammation, and **glial scarring** [12–14]. Recent studies suggest that EVs participate in the progression of secondary injury by transporting parent cell-specific signaling cargo (e.g. signal lipids, genetic information, cytokines, receptors, etc.) that alter the function of recipient cells within the CNS and beyond [15]. EV bioactivity and biological cargo are related to the phenotype of the EV parent cell and can vary depending on stimulus and the surrounding microenvironment.

Inflammation Alters EV Cargo, Thereby Affecting Neuro-Glia Communication

While current understanding of physiological EV communication in the CNS is limited (see also Box 1), emerging evidence indicates that EV-mediated functions are likely altered in association with many pathological features of neurotrauma. Modifications in EV cargo, such as microRNAs (miRNAs), may significantly disrupt homeostatic balance between neurons and surrounding glia, as indicated by *in vitro* studies (Figure 1). For example, upon ATP or proinflammatory (e.g. IL-1β, TNFα, IFNγ, or LPS) stimulation, microglia release EVs enriched with proinflammatory cytokines (e.g. IL-1β) and miRNAs (e.g. miR-146a-5p, miR-155) [3,16,17]. Transfer of miR-146a-5p from microglia to neurons leads to downregulation of key pre- and post-synaptic proteins (e.g., synaptotagmin1, neuroligin1), thus reducing synaptic density and strength [17]. EVs from LPS-stimulated microglia also carry the enzyme glutaminase that may contribute to neurotoxicity *in vitro* through excessive glutamate production [18]. Microglial EVs released under these stimuli may also contribute as mediators of inflammation by activating surrounding microglia and astrocytes [16,19].

In response to similar pro-inflammatory stimuli, astrocytes undergo a phenotypic transformation known as **reactive astrogliosis**, which includes an increased rate of EV release relative to baseline [20,21]. EVs derived from reactive astrocytes are enriched with small GTPases (e.g. profilin-1, fascin actin-bundling protein-1 and destrin), proteins (e.g. IL-1β, human immunodeficiency virus 1 protein Nef), and miRNAs that largely inhibit neuronal function by decreasing neurite outgrowth and spike firing rates and may also lead to neuronal apoptosis [20–23]. In response to TNFα or IL-1β stimulation, astrocyte EVs are enriched with miR-125a-5p and miR-16–5p that downregulate expression of the neurotrophin receptor NTRK3 in neurons, leading to reduced dendrite complexity and synaptic burst activity [23]. While current understanding of EV-mediated neuro-glia communication largely derives from *in vitro* systems, emerging evidence supports a role for EVs from activated glia in mediating secondary injury after SCI. Future work involving isolating cell-specific EVs *in vivo* will help define the complex and dynamic factors that dictate their cargo and associated bioactivity after SCI.

EVs Contribute to Axonal Regeneration and Myelination

Repair of white matter damage remains a major hurdle in SCI treatment. The limited capacity of axon regeneration in the adult mammalian CNS is well established and results from a low intrinsic capacity for neurons to regenerate and formation of the glial scar [24]. Recent studies have demonstrated that EVs can serve as both positive and negative cues for axonal regeneration [25–28]. For example, the C-terminal fragment of Nogo-A, a myelin-associated inhibitor (MAI) protein localized in the oligodendrocyte membrane, can be released into EVs after cleavage by BACE1 and inhibit axon growth by binding to the NgR1 receptor on neurons [25]. Moreover, EVs containing Nogo-A have greater potency for axonal inhibition than purified Nogo-A alone [25]. A diffusible means for modulation of axonal growth through EVs is particularly relevant after SCI, where normal contact-mediated interactions between neurons and oligodendrocytes are mechanically disrupted. Whether Nogo-A containing EVs may contribute to sustained axonal regrowth failure after SCI requires further investigation.

In contrast, endogenous EV release may also promote axonal regeneration. For example, systemic treatment with an agonist for retinoic acid receptor β (RARβ) was shown to improve axonal regeneration through EV release in a rat SCI model (Figure 1) [26–28]. Following treatment, neuronal EVs transferred PTEN into astrocytes, which had a dual benefit: extrusion of PTEN removed inhibition of the **PI3K/Akt/mTOR** pathway within neurons and thus improved intrinsic growth capacity, while the transfer of PTEN into astrocytes reduced their proliferation and led to a reorganization of the glial scar into a more permissive environment for axonal growth [26]. RARβ agonist treatment also promotes neuronal interaction with **oligodendrocyte precursor cells** (OPCs), leading to upregulation of retinoic acid (RA) synthesis in the latter [27]. OPCs release RA in association with EVs, which can be taken up by nearby axons and serve as a positive signal for axonal outgrowth [27]. When OPCs transition into a mature myelinating oligodendrocyte (OL) phenotype, EV secretion is reduced and may coincide with myelination of regenerated axons [28]. Interestingly, bone marrow derived macrophages (BMDMs) also stimulate intrinsic axonal growth capacity mechanistically through modulation of PTEN. After peripheral nerve transection, BMDMs recruited to the injury site release EVs containing NADPH oxidase-2 (NOX2), which are taken up by the remaining axon and retrogradely transported back to the soma [29]. Reactive oxygen species produced by NOX2 oxidizes PTEN, leading to its inactivation and promotion of PI3K/Akt/mTOR signaling [29]. While BMDMs are present in the CNS after SCI, it is unknown whether a similar mechanism occurs endogenously to promote axonal regeneration within the CNS and, if so, whether there is a limitation on this capacity compared to peripheral nerves.

Bidirectional Blood-Borne EV Crosstalk between CNS and Peripheral Systems

In addition to local communication, EVs transported in circulation play a role in long-distance signaling and may contribute to overall SCI progression. While the relative contribution of CNS-specific EVs in the bloodstream is unknown, plasma EVs bearing neuronal markers (L1CAM, Glur2), as well as astrocytic (GLT-1, GLAST) and microglial (CD45, CD11b, P2yr12) markers are a focus of recent clinical studies that examine specific populations for diagnostic and prognostic monitoring after neurotrauma [30–32]. Beyond biomarker potential, emerging preclinical data suggest that circulating EVs play an active role in CNS injury progression (Figure 1). It is well-established that CNS inflammation and injury robustly activate the systemic immune system, leading to peripheral leukocyte infiltration. This effect is largely coordinated by cytokine/chemokine production in the liver, but the factors that modulate the hepatic **acute phase response (APR)** were largely unknown until recent evidence implicated EV release from the CNS [8][21]. In a GFAP-GFP transgenic mouse model, astrocyte EVs rapidly mobilized to the liver, lung, and spleen within two hours after striatal IL-1β injection. The associated cytokine responses in these peripheral organs were attenuated by local co-administration of a **sphingomyelinase** inhibitor to prevent CNS EV release and could be recovered by intravenous (IV) injection of EVs from IL-1β stimulated astrocytes [21]. Bioinformatics analysis based on the miRNA and protein content in stimulated astrocyte EVs identified specific modulation of the PPARα/NFκB pathway in recipient organs, which was further validated *in vivo* [21]. In addition to astrocytes, CNS endothelial cells may also contribute to circulating EVs that regulate the APR [33,34]. IV administration of EVs from LPS-stimulated endothelial cells

after acute mouse TBI enhanced the magnitude of the liver APR, which subsequently expanded lesion volume size [34]. Thus far, no studies (to our knowledge) have characterized the role of EVs during APR specifically after SCI. Future work in this area will allow rational design of EV-based therapeutics that tune APR to limit its detrimental effects.

EVs have also been implicated in the development of various systemic inflammatory complications after neurotrauma, including pulmonary dysfunction [35,36]. Proteins related to the **inflammasome** complex increase locally after neurotrauma and are present in EVs isolated from CSF from human SCI patients and from blood in human TBI patients [35–37]. EVs released from the injured CNS may also carry pro-coagulant molecules (e.g. tissue factor, phosphatidylserine) and can associate with other members of the coagulation cascade [38–40]. Together, these effects can disrupt endothelial cell barrier function leading to vascular edema and fibrin deposition in the lung [38,40].

Just as important but less well examined are the effects of circulating EVs directly on CNS cells *in vivo*. CNS cells receive functional RNA via EVs from hematopoietic cell sources, which is further enhanced under LPS-induced peripheral inflammation [41]. Additional studies using this same model suggest that microglia are an important recipient cell type for plasma EVs (specifically from hematopoietic cells) and further highlight the importance of immune-related crosstalk between the CNS and peripheral systems [42,43]. Interestingly, neuronal activity also drives the uptake of hematopoietic cell-derived EVs into neurons under various paradigms including kainite injection (to induce seizure-like activity), optogenetic stimulation, and behavioral exploration of novel objects [43]. These studies are among the earliest to demonstrate the functional transfer of EV cargo from blood cells to the CNS and suggest that circulating EVs may impact brain function under a variety of normal physiological situations. The functional significance of this blood-to-brain signaling in SCI needs to be elucidated further.

Spinal Cord Injury Alters Circulating EV Count and miRNA Cargo

While a few studies have analyzed alterations in circulating EVs after rodent TBI [34,35], little data is currently available from animal models of SCI. Two studies published in 2020 presented RNA-sequencing data of EV-associated miRNAs in serum at 1 and 7 days post-injury in female rats [44,45]. Adding to these findings, our group recently described dynamic and multifaceted changes in plasma EVs after thoracic contusion SCI in adult male mice (Figure 2) [46]. Using a combination of techniques to measure EV count, size, and cargo, we reported a significant increase in plasma **tetraspanin** CD81+ EVs after SCI in conjunction with an overall reduction in total plasma EV count [46]. At the injury site, there was a robust decrease in CD81 surface expression on astrocytes specifically, suggesting these cells may shed CD81+ EVs into circulation. Furthermore, SCI modified CNS-related miRNA content in the total plasma EV population, where changes in miRNA profiles were similar to those previously described in astrocyte EVs after IL-1β or TNFα stimulation *in vitro* (Figure 2) [23,46].

Cognitive impairment and affective symptoms are reported clinically in nearly half of SCI patients [47,48], but the mechanisms underlying this brain dysfunction have not been well

explored. Studies from our group [49,50] and others [51] report a link between neurodegeneration and neuroinflammation in the brain after rodent SCI, leading to cognitive and depressive-like behavioral deficits. Based on prior evidence of astrocyte EVs regulating the APR [21], the possibility that circulating EVs after SCI could promote inflammation in recipient target organs – specifically the brain – was assessed by intracerebroventricular (ICV) injection of plasma EVs from either SCI or control (uninjured) mice into healthy mice (Figure 2) [46]. At 24 hours post-injection, increased expression of several key inflammatory genes, including markers related to astrocyte reactivity [52], was observed in associated with injection of SCI plasma EVs [46]. Furthermore, flow cytometry analysis demonstrated increased intracellular IL-1β and IL-1α levels in brain astrocytes with this same paradigm [46]. These data, in conjunction with prior studies using neuroinflammation models, suggest a critical role for astrocytes in both the production of and response to plasma EVs after SCI.

Potential for EV-based Therapeutics for SCI

The emerging evidence for roles of EVs in regulating SCI biology can inform not only a better understanding of pathophysiology but also the development of a variety of new treatment strategies and in particular, provides impetus for investigation of CNS cell-derived EVs as potential SCI therapeutics. Further, many of the reparative and functional benefits of stem cell transplantation in various CNS disease models can now be linked to the combined autocrine/paracrine bioactivity of its secretome [53]. As a key component of the cellular secretome, EVs have emerged as a viable candidate for cell-free therapies due to their potential for therapeutic bioactivity, inherent biocompatibility, and potential for cell targeting while mitigating concerns related to immunogenicity and uncontrolled proliferation/differentiation of cellular transplants [54].

Cell Source is a Critical Determinant of EV Bioactivity

EV producer cell phenotype partly determines the array of cytosolic proteins (i.e. molecular chaperones, metabolic enzymes, ribosomal proteins, etc.), cell-surface proteins, and nucleic acids available to be packaged within EVs during their biogenesis (Figure 3) [55]. Although the multifunctional properties of EVs and their mechanisms of action relative to producer cell types are not well understood, the diversity of EV-associated cargo suggests that pleiotropic, complementary, and/or synergistic effects could be critical to their therapeutic potential [10]. In particular, small ncRNAs (<200 nucleotides) have attracted substantial attention, as these molecules represent a significant proportion of EV cargo and EV-mediated horizontal transfer of ncRNA appears to be an important mechanism for regulating CNS development, homeostasis, aging, and pathology [56,57]. The EV content of one critical class of small ncRNAs, miRNAs, varies widely with producer cell type (Box 2). This highlights the possibility of cell source as an important design criterion when developing EV-based therapeutic strategies for SCI.

The role of cell source in EV therapeutics has also been examined via direct comparison. Mesenchymal stem/stromal cell (MSC) transplants have anti-inflammatory and pro-angiogenic effects in a wide array of regenerative applications [58–60] and thus have been extensively studied as a potential source for therapeutic EVs for SCI and other types of

neurotrauma [61–64]. However, EVs from MSCs and other non-CNS types to date have generally been unable to induce key neuroregenerative processes relevant to SCI therapy, including neurogenesis, adaptive neural plasticity, and remyelination and regeneration of injured axons over long-distances. Notably, EVs from isogenic human pluripotent stem cell (hPSC)-derived NPSCs and MSCs were assessed in a rodent model of ischemic stroke, with the NPSC-derived EVs inducing improved cognitive and behavioral outcomes with significantly reduced lesion volumes relative to MSC EVs [65].

Further, EVs isolated from CNS cell types have shown the potential to recapitulate the regenerative properties of their parent cells in several animal models of CNS pathology. In a rodent model of radiation-induced brain injury, neuroprotective effects after administration of EVs from human **neural progenitor/stem cells** (NPSCs) were comparable to cranial transplantation of the cells themselves [66]. Additional studies also showed improved functional recovery and neuroprotection after transplants of both exogenous NPSCs [67] and NPSC-derived EVs in experimental SCI and TBI [68–70]. Other studies include a rodent model of focal TBI in which IV administration of NPSC EVs led to decreased glial scarring and cortical lesion volume that were correlated with motor function recovery [68]. IV administration of NPSC EVs also showed efficacy in a clinically relevant porcine model of ischemic stroke by decreasing cerebral lesion volume, edema, and recovery time while improving fine-motor coordination [71]. Schwann cell (SC)-derived EVs also improved therapeutic outcome in a rodent model of peripheral neuropathy by increasing axonal sprouting and remyelination of sciatic nerves [72].

Only a limited number of studies have characterized administration of EVs derived from resident CNS cell types in animal models of SCI (Table 1). The available data indicates that intrathecal or IV administration of NPSC-derived EVs after SCI can modulate autophagy, neuroprotection, angiogenesis, and inflammation, and that some of these changes were correlated with functional improvements [70,73,74]. NPSC-derived EVs are linked with suppressing the formation of NLRP3 inflammasome complexes, which corroborates a separate study that shows NPSC-EVs are likely enriched with 14–3-3 family of proteins, known to regulate autophagy and inflammasome activation [73,74]. Another report suggests that NPSC EV-mediated angiogenesis after SCI in mice likely occurs through transfer of EV-associated proteins from the vascular endothelial growth factor (VEGF) family to spinal cord microvascular endothelial cells (SMEC) [75]. Further, EVs derived from primary cortical neurons reduced neuroinflammation after SCI in mice by suppressing activation of pro-inflammatory microglia and neurotoxic astrocytes via transfer of EV-associated miR-124–3p [76]. Although these initial studies indicate that EVs from CNS-resident cells can improve functional deficits after SCI, overall it is still unclear how their therapeutic potency and mechanism of action in SCI contrasts with EVs from non-CNS cells (with MSCs being the most widely studied thus far).

Enhanced EV Potency Through Engineering Producer Cell Microenvironments

In addition to elucidating the relationship between cellular phenotype and EV therapeutic potential in SCI, optimal cell culture parameters and microenvironment on tuning the bioactivity of EVs is another important topic for study (Figure 3). Various culture parameters

such as cell seeding density, cell age/passage, and EV collection frequency can lead to significant changes in EV bioactivity and their production rates across multiple cell types [77]. For example, EVs isolated from microglia differentially regulated myelination under proinflammatory or pro-regenerative conditions in an *in vivo* model of lysolecithin-induced demyelinated lesions [78]. Further, oxygen- and glucose-deprived astrocytes secreted EVs that exerted neuroprotection *in vitro,* likely due to increased EV-associated miR-92b-3p content compared to unstressed astrocytic EVs [79,80]. Similarly, NPSCs primed with insulin-like growth factor-1 produced EVs enriched with miR-219a-2–3p, associated with OPC maturation, which was linked with attenuated neuroinflammation, apoptosis, and improved axonal regeneration after SCI [81]. NPSCs primed with ethanol increased EV-associated miR-140–3p and miR-140–5p content, which alters NPSC proliferation and differentiation dynamics *in vitro* [82]. Under proinflammatory conditions, RNA and protein content of NPSC-derived EVs can change significantly, with one study showing that enrichment of EV membrane-bound IFN-γ and interferon gamma receptor 1 (Ifngr1) complexes can induce Ifngr1 and Stat1 signal transduction (key regulators of cell proliferation and neuroinflammation) in target cells [83].

Ongoing biomaterials-based development of artificial extracellular microenvironments as *in vitro* models for neurotrauma, neurodegenerative diseases, and neurodevelopmental disorders may inform future engineering of optimal EV production environments for SCI [84]. To this end, three-dimensional (3D) bioprinting provides increased control over spatial distribution of materials (e.g. bioactive components, cells, drug depots, etc.) using “bioinks” (e.g. designer hydrogels and biocompatible plastics) to construct spatially complex architectures at milli/micro-meter resolution with high reproducibility [85]. Fully recapturing *in vivo* EV production microenvironments in artificial constructs is currently not feasible, but implementing existing knowledge from biomolecular frameworks into biomaterials design may aid in generating therapeutic EVs with potent CNS-specific regenerative capacity. For example, one study reported that EVs derived from dental pulp stem cells cultured in 3D had neuroprotective effects, while EVs from the same cell type in two-dimensional (2D) culture did not [86]. Although EVs derived from CNS cell types have scarcely been scrutinized with respect to one or more key biophysical cues, studies using other cell types have shown that, in principal, it is possible to improve therapeutic potency and biomanufacturing yield of collected EVs using 3D bioprinted perfusion bioreactors [87,88]. A potential design limitation to note about 3D cultures is the difficulty in isolating EVs secreted within hydrogel-based scaffolds due to potential EV-hydrogel interactions [87]. It remains to be seen whether rational leveraging of EV producer cell phenotype along with EV production microenvironment can: 1) yield EV populations with potent CNS-specific therapeutic capacity and, 2) enable identification of EV-associated bioactive components with relevance for SCI.

Enhanced EV Potency Through Loading of Therapeutic Cargo

The endogenous roles of EVs include protection of nucleic acids and other bioactive components from degradation while ensuring delivery to target recipient cell cytosol without eliciting an immunogenic response. These properties make EVs a promising delivery vehicle for RNA-based therapeutics such as antisense oligonucleotides, small interfering RNAs

(siRNAs), miRNAs, mRNAs, and others [89]. However, average endogenous ncRNA loading can be as low as one bioactive copy of a given miRNA per EV, which may not potently modulate gene expression in target cells [90,91]. Thus, even after identifying populations of therapeutic EV populations and their bioactive components, clinical translation may still be hampered by low potency that necessitates high or repeated EV doses to achieve a therapeutic effect.

Several strategies to manipulate EV parent cells have been proposed to control cargo loading into EVs during biogenesis (Figure 3A–D) [89,92]. In the context of neurotrauma, EVs derived from gene-modified MSCs that overexpress miRNAs associated with neurogenesis and neurite outgrowth (e.g. miR-124 & miR-133b) led to functional improvements in animal models of SCI and TBI [93,94]. Similarly, a separate study generated EVs enriched with miR-219a-5p (specific to OL-lineage cells that regulate OPC proliferation and maturation) using a gene-modified HEK293T cell line [95]. These studies highlight the possibility of loading CNS-specific miRNA cargo into non-resident CNS cell types using genetic modification and subsequent overexpression [93]. As for gene-modified CNS cell types, neurons overexpressing miR-21–5p induced neuroprotective effects *in vitro* [96] while EVs from microglia overexpressing miR-124–3p exerted neuroprotection and improved functional outcome in a rodent model of TBI [97]. However, several challenges remain for optimizing strategies for producer cell loading of EVs with desired cargo. Simple cytosolic overexpression of ncRNA via gene modification does not always improve endogenous bioactive cargo loading in EVs, and plasmid modifications, such as inclusion of targeting moieties, may be necessary to direct ncRNA cargo loading to EVs specifically [98]. Even after successful enrichment of desired cargo into EVs, the process of stable gene overexpression itself can produce changes in parent cell phenotype that may alter the overall cargo/bioactivity of secreted EV populations, potentially causing undesired off-target effects. Additionally, endogenous enrichment of individual ncRNA cargo within EVs may not provide control over other factors in intercellular EV transfer, such as uptake and subsequent processing by acceptor cells [89,99].

Several studies have also explored means for modifying EV cargo after biogenesis and isolation (Figure 3F–J). A straightforward method albeit with limited efficacy for small molecule drugs is passive loading via co-incubation of EVs with desired cargo, where the presence of detergents may facilitate EV encapsulation [100]. Lipid- or cholesterol functionalization of desired cargo to target association with EV membranes is another option that has been effective in delivering functional siRNA [101]. Several studies have also exploited the high surface concentration of phosphatidylserine (PS) on EVs to decorate bifunctional fusion proteins that contain the C1C2 domain of lactadherin that binds to PS with high affinity [102]. However, it is important to note that surface modification is unlikely to shield EV membrane-associated functional cargo from degradation by endogenous factors relative to cargo encapsulated inside the EV itself. Other strategies for active internal cargo loading use techniques that transiently permeabilize EV membranes to allow encapsulation of desired cargo. Electroporation can successfully load functionally active small ncRNA into EVs [103]. Sonication is another demonstrated method for active EV loading of various types of functional protein cargo including siRNA [104]. Some potential disadvantages for the electroporation and sonication-based loading strategies include loss, aggregation or

degradation of EV-associated cargo due to the energy added and disruption of EV membrane integrity. More recently, our group has used a pH gradient across EV membranes as an effective means for loading functional miRNA, siRNA, and single-stranded DNA without compromising EV cargo/bioactivity [105]. However, all post-isolation EV modification strategies have scalability concerns that could potentially limit the large-scale biomanufacturing that would be necessary to create a translational therapy for SCI patients.

Concluding Remarks and Future Perspectives

How CNS-specific regenerative processes after SCI are regulated by EV transfer between specific producer and acceptor cells is not well understood. Furthermore, the extent to which crosstalk between circulating EVs and CNS resident cells contributes to pathophysiological mechanisms after SCI and other forms of neurotrauma still remains to be established. A significant technical hurdle is the difficulty in identifying cell-specific EVs from *in vivo* tissue samples within the CNS, where neurons, astrocytes, microglia, and other resident cells contribute to a heterogeneous pool of EVs. EV-associated cargo can include cell-specific biomarkers, but the relative packaging of these molecules into EVs has not been well examined (see Outstanding Questions) [106,107]. Emerging strategies that fluorescently tag EV subtypes *in situ* during biogenesis in a cell-type-specific manner represent an important step towards addressing some of these unanswered questions [108].

Along with an improved understanding of EV-mediated signaling after SCI, we believe that selection of biologically-relevant EV parent cell(s) and production microenvironments (incorporating key biophysical and biochemical cues) will aid in the discovery of EV populations that target CNS-specific regenerative processes after SCI (e.g. axonal sparing/regeneration, remyelination, autophagy, glial scar formation, etc.). Profiling of EV-associated cargo under these circumstances can be used to identify and validate any critical bioactive components that may mediate therapeutic benefit. Finally, emerging technologies such as 3D bioprinting and new approaches to therapeutic cargo loading into EVs may enable the reproducible and scalable biomanufacturing of EVs with high therapeutic potency required for clinical translation (see Outstanding Questions).

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Glossary

Extracellular vesicles (EVs)
A general term for any non-nucleated particle released from cells with a double-leaflet membrane. EVs originate via various biogenesis pathways, including budding of the plasma membrane or release from multivesicular bodies

Tetraspanin
Membrane proteins (e.g. CD9, CD63, CD81) with four transmembrane domains that are highly enriched in certain EV subtypes

Neural plasticity
Innate capacity of the developing and adult CNS to undergo functional and biochemical reorganization within individual neurons (e.g. synaptic plasticity) as well as across large-scale neural networks in response to experience, injury, and disease

Autophagy
Greek for “self-eating,” autophagy is a regulated process for removal and degradation of damaged cytosolic components, misfolded/aggregated proteins, and invasive microbes during physiological and pathological conditions

Glial scarring
Reactive astrocytes and microglia encapsulate the lesion site after neurotrauma, creating a lesion core enriched with extracellular matrix proteins that are inhibitory for axonal growth and remyelination. The glial scar may also be neuroprotective by further confining inflammatory pathology to the lesion core

Neural progenitor/stem cells (NPSCs)
Multipotent progenitor cells of the CNS that retain the ability to differentiate and generate most, if not all, glial or neuronal cell types. These cells can be found anatomically in specific neurogenic niches in the brain and spinal cord

Inflammasome
Multiprotein molecular complex involved in innate immune defense that leads to caspase-1 activation and downstream production of pro-inflammatory cytokines such as IL-1β and IL-18. Activation can also result in pyroptosis, a caspase-1 dependent form of cell death

Acute phase response (APR)
Systemic inflammatory and metabolic response rapidly (minutes to hours) initiated to enhance body defenses to injury, trauma, or infection. If not resolved, long-term activation of the APR may lead to further secondary tissue damage

Glutamate homeostasis
Primarily glial cell-mediated control over non-synaptic extracellular glutamate through direct uptake and cystine/glutamate exchange to regulate synaptic efficiency and plasticity

Oligodendrocyte precursor cell (OPC)
Progenitor cells that are abundant in the adult CNS and have the capacity to generate mature oligodendrocytes, contributing to adaptive myelination and remyelination following injury/disease

Macropinocytosis
Non-specific, endocytic sampling of the external environment through projections of the cellular membrane that engulf and internalize extracellular fluid and particles

Sphingomyelinase

Cellular enzyme that hydrolyzes sphingomyelin to ceramide, which contributes to the biogenesis of certain EV subtypes

PI3K/Akt/mTOR pathway

A critical intracellular signaling pathway that regulates cell growth, metabolism, and survival. Negatively regulated by the phosphatase and tensin homolog (PTEN) protein

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Box 1:

EVs play roles in regulating physiological processes in the CNS

During physiological conditions, neuronal depolarization and glutamate release stimulate EV transfer from multiple cell types mediating neuro-glia communication [3,109]. Neuronal EVs act as vehicles for anterograde/retrograde transfer of biomolecules such as L1 cell adhesion molecule and AMPA-type receptor subunits, which indicates their potential contribution to regulating synaptic transmission and plasticity mechanisms [109]. Neuronal EVs enriched with miR-124a are trafficked selectively to astrocytes, upregulating excitatory amino acid transporter 2 (EAAT2/GLT-1) expression as a means for maintaining **glutamate homeostasis** [108,110]. In fact, selective transfer of EVs between specific producer/acceptor cells appears to be an important feature in CNS physiology. EVs containing proteolipid protein (PLP) isolated from a mouse oligodendrocyte precursor cell (OPC) line *in vitro* selectively accumulated in unstimulated (MHC-class-II-negative) microglia via **macropinocytosis**, and not in astrocytes or neurons [111]. In the same study, activation of microglia through interferon gamma (IFNγ) or lipopolysaccharide (LPS) stimulation significantly decreased macropinocytosis, indicating a potential role of unstimulated microglia in internalizing and degrading OPC-derived EVs during physiological conditions [111].

Astrocyte-derived EVs under physiological conditions can carry synapsin I, molecular chaperones and matrix metalloproteinases (involved in extracellular matrix remodeling) that regulate neuronal survival and neurite outgrowth [22]. Microglial EVs modulate synaptic activity and neurotransmission by inducing sphingolipid metabolism [3]. Oligodendrocyte (OL)-derived EVs can carry catalase, superoxide dismutase (SOD), heat shock proteins, glycolytic enzymes, and various myelin-related proteins (e.g. proteolipid protein, myelin oligodendrocyte glycoprotein, etc.) that regulate myelination, neuronal activity (e.g. action potential firing rate and gene expression) and exert neuroprotection from oxygen/glucose deprivation [109,112]. NPSCs reside in neurogenic niches throughout the brain (e.g. subgranular zone, dentate gyrus and subventricular zone; SVZ) as well as in the spinal cord central canal (CC). EVs from SVZ-derived **neural progenitor/stem cells (**NPSCs) have been reported to carry miRNAs (e.g. miR-9, Let-7, miR-26, and miR-181), mRNA, proteins (e.g. IFNγ) and active enzymes (e.g. asparaginase-like protein 1; Asrgl1) related to NPSC function such as proliferation, migration and differentiation [113,114].

Box 2:

CNS EV-associated miRNA cargo is specific to cell type and microenvironment

Cellular content of miRNAs varies based on cell type, and the CNS is no exception. For example, miR-383 is highly enriched in neurons relative to glial cells [115] whereas miR-219 and miR-338 (crucial in regulating myelination) are thought to be specific to oligodendrocyte-lineage cells under physiological conditions [116]. Additionally, miRNA content for the same cell-type may depend on its location in the CNS. In particular, miR-326 is strongly expressed in microglia within the brainstem yet nearly absent in spinal microglia [115]. Further, heterogeneity in primary adult NPSCs populations from distinct stem cell niches within the CNS is well documented and may also be a key feature in determining the miRNA cargo function of their EVs [117]. Critically, pathological progression after neurotrauma appears to be a factor that differentially affects spatiotemporal miRNA expression across CNS cell types [115]. Furthermore, cargo loading into EVs comprises both active (selective) and passive mechanisms that are yet to be fully elucidated [118], and there is strong evidence that suggests the cellular transcriptome/proteome does not always correlate directly with EV cargo makeup [89]. Uncovering the complex relationships and interplay that determine EV bioactivity secreted by CNS cell types as a function of pathological progression after neurotrauma remains largely in its infancy. Only a limited number of studies have gone beyond identifying predicted mRNA targets for CNS cell-specific miRNAs towards characterizing/validating their bioactivity and potential means of intercellular trafficking.

Outstanding Questions

- Much of our understanding of local EV cell-to-cell communication is inferred from *in vitro* work as technical challenges remain to visualize and monitor EV trafficking *in vivo*. Can cell-specific EVs and their cargo be identified from this complex environment? How do these parameters change over time after SCI? And how might they be similar to or differ from other forms of neurotrauma, such as TBI?
- How do EVs in blood circulation impact the progression of SCI? Are there specific peripheral cell types (e.g. BMDMs, red blood cells) that communicate preferentially with CNS cells after injury? What are the critical cargoes, including proteins, lipids, and nucleic acids, that contribute to this type of signaling?
- Do cells with direct functional involvement in CNS repair (e.g. neurogenesis, axonal sprouting, remyelination, etc.) secrete EVs that specifically enhance CNS-specific regenerative responses after SCI? For example, EVs isolated from NPSCs appear to regulate neuroinflammation and mediate neuroprotection after SCI. What are the putative EV-associated cargo(s) that mediate these CNS-specific regenerative processes?
- Can emerging technologies such as 3D bioprinting and control over cell-cell communication (e.g. co-cultures) be used to generate biomimetic EV production environments that endow EVs with enhanced CNS-specific regenerative potency? Can these technologies also assist in moving towards scalable biomanufacturing of therapeutic EVs for SCI treatment?
- Even after EV populations with therapeutic effects are identified, a low therapeutic potency requiring high/repeated EV dosages may be a critical hurdle, as demonstrated across other applications. Once EV-associated cargo linked to CNS repair are identified, can these mediators be efficiently loaded in manufactured EVs (during or after EV biogenesis) to further improve their therapeutic potency?

Highlights

- EVs have functional roles in mediating neuro-glia and CNS-periphery communication after neurotrauma by regulating neuronal function, axonal regeneration and remyelination, metabolic activity, and the inflammatory milieu.
- Understanding of EV signaling after SCI is limited, but recent studies have identified differences in circulating EV count and miR cargo that may contribute to remote inflammatory changes.
- The therapeutic potential of EVs for SCI treatment depends on source cell phenotype and is linked to the repertoire of EV-associated cargo.
- New bioengineering approaches such as 3D printing demonstrate improved control over the EV production microenvironment and cellular phenotype that can accelerate the development of therapeutic EVs with high potency for applications in neurotrauma, including SCI, in a reproducible and scalable fashion.

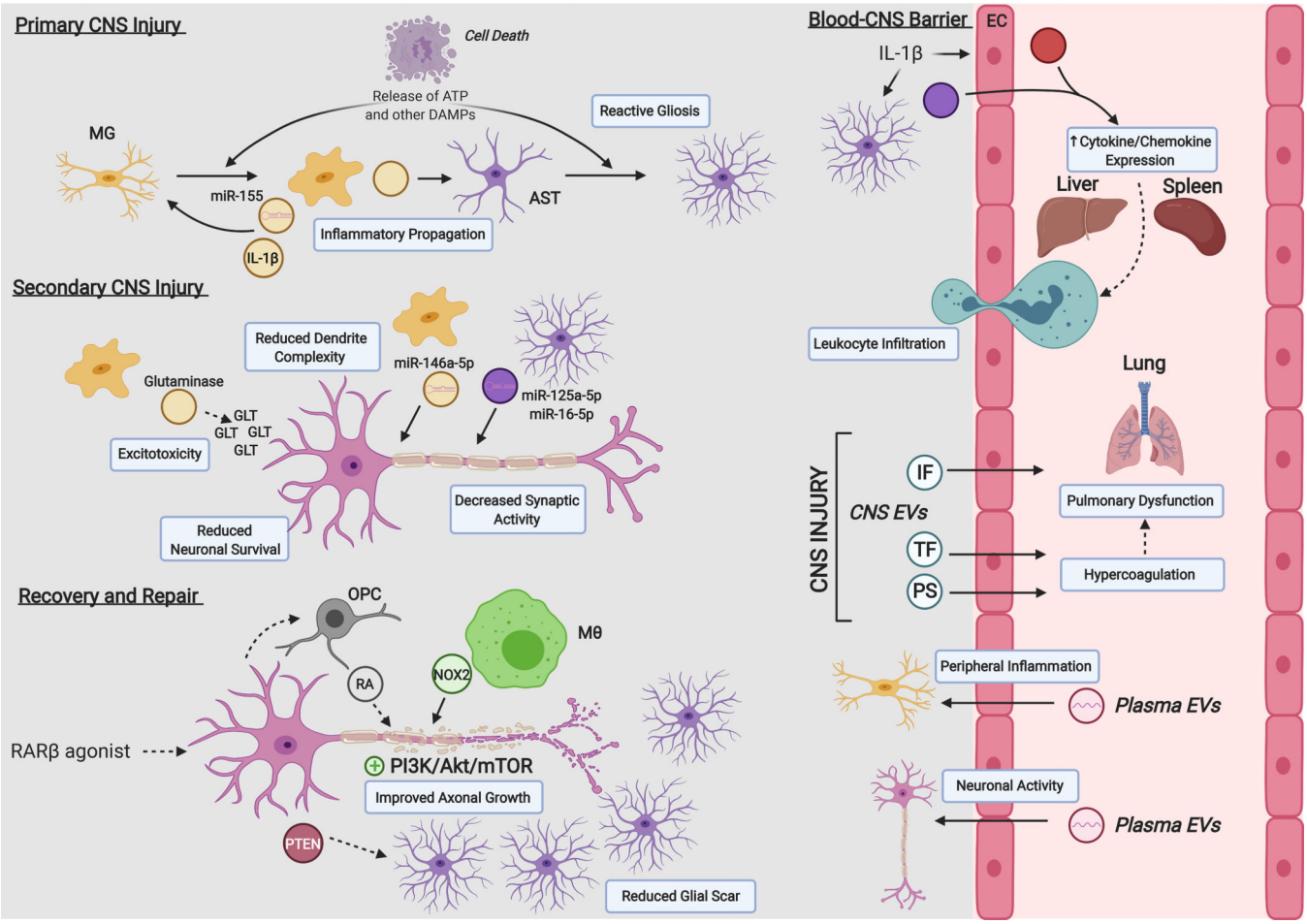

Figure 1: EVs contribute to local and systemic crosstalk after neurotrauma, including SCI.

Intercellular interactions and EV-associated cargos contribute to the injury response within the CNS and beyond. Primary injury leads to the release of intracellular ATP and other danger associated molecular patterns (DAMPs) that activate surrounding microglia (MG) and astrocytes (AST). Reactive MG independently activate other MG and AST through the transfer of pro-inflammatory molecules within EVs. Both reactive MG and AST can transfer EV-associated microRNAs to surrounding neurons that impact their overall synaptic function and survival in the secondary injury phase. Glutaminase-carrying EVs from MG may also contribute to glutamate (GLT) excitotoxic neuronal cell death. AST and endothelial cells (EC) under pro-inflammatory conditions (e.g. IL-1β stimulation) release EVs into blood circulation that can trigger peripheral organ cytokine/chemokine release regulating leukocyte infiltration into the CNS. CNS-derived EVs of unknown cellular origin may carry inflammasome (IF) components and pro-coagulant factors (e.g. TF, PS) after injury that can contribute to secondary pulmonary dysfunction. Under conditions of peripheral inflammation or neuronal activity, MG and neurons can receive functional RNA from hematopoietic cell-derived plasma EVs. Local EV release may also contribute to recovery and repair mechanisms relevant to SCI. Neuronal RARβ stimulation leads to the release of PTEN in neuronal EVs that promote intrinsic axonal growth (increased PI3K/Akt/mTOR signaling) and reduced AST proliferation; RA-containing EVs from oligodendrocyte

precursor cells (OPCs) also contribute to axonal growth under this paradigm. NOX2-containing EVs from macrophages (MΘ) taken up at injured axons and transferred to the neuronal soma promote axon growth as well. Abbreviations: IL-1β, interleukin-1β; NOX2, NAPDH-oxidase 2; PS, phosphatidylserine; PTEN, phosphatase and tensin homolog; RA, retinoic acid; RARβ, retinoic acid receptor β; TF, tissue factor.

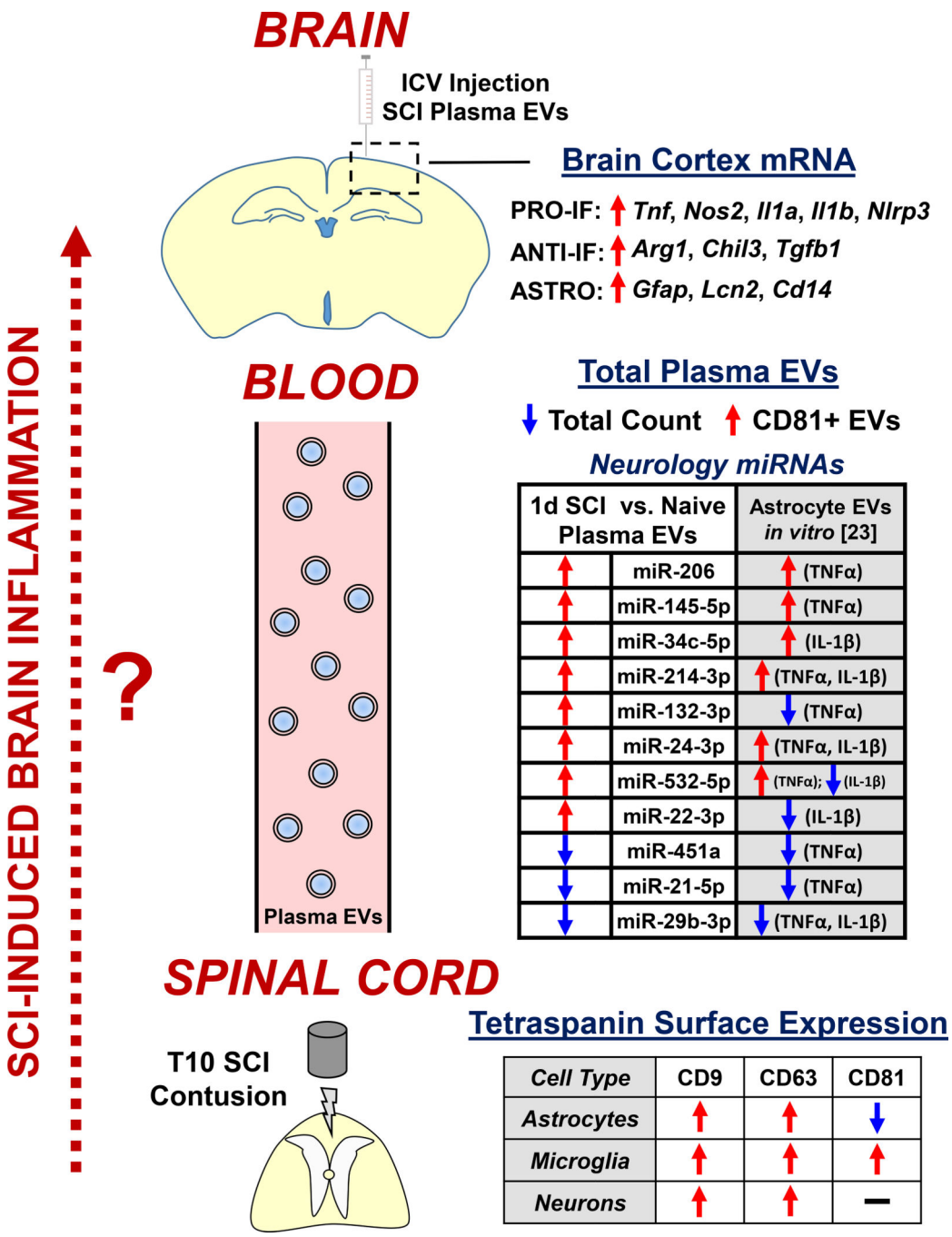

Figure 2: Alterations in circulating EVs and miRNAs after SCI may contribute to remote brain inflammation.

Schematic diagram that summarizes key findings from [46] analyzing SCI-induced changes in circulating plasma EVs. Thoracic contusion SCI in male mice resulted in multifaceted changes in total plasma EVs at 1d post-injury including a decrease in the overall count, an increase in tetraspanin CD81+ EVs, and modifications in CNS-related miRNA cargo associated with proinflammatory stimulation of astrocytes (comparison with *in vitro* data from [23]). Tetraspanin protein expression primarily increased in cells at the injury site except for a unique decrease in surface CD81 on astrocytes, which may be the source of

increased plasma CD81+ EVs. ICV injection of total plasma EVs from 1d SCI animals induced robust inflammatory gene expression in the brain cortex, including increased reactive astrocyte genes. Future investigation (dotted red arrow, left) is required to further test the hypothesis that EVs released by cells directly from the injury site travel through the blood circulation to seed brain inflammation, which may contribute to long-term neurodegeneration and associated cognitive deficits and depressive-like behavior after SCI. Abbreviations: ANTI-IF, anti-inflammatory; ASTRO, astrocyte; ICV, intracerebroventricular; PRO-IF, pro-inflammatory; miRNA, microRNA; T10, thoracic segment 10; TNFα; tumor necrosis factor α.

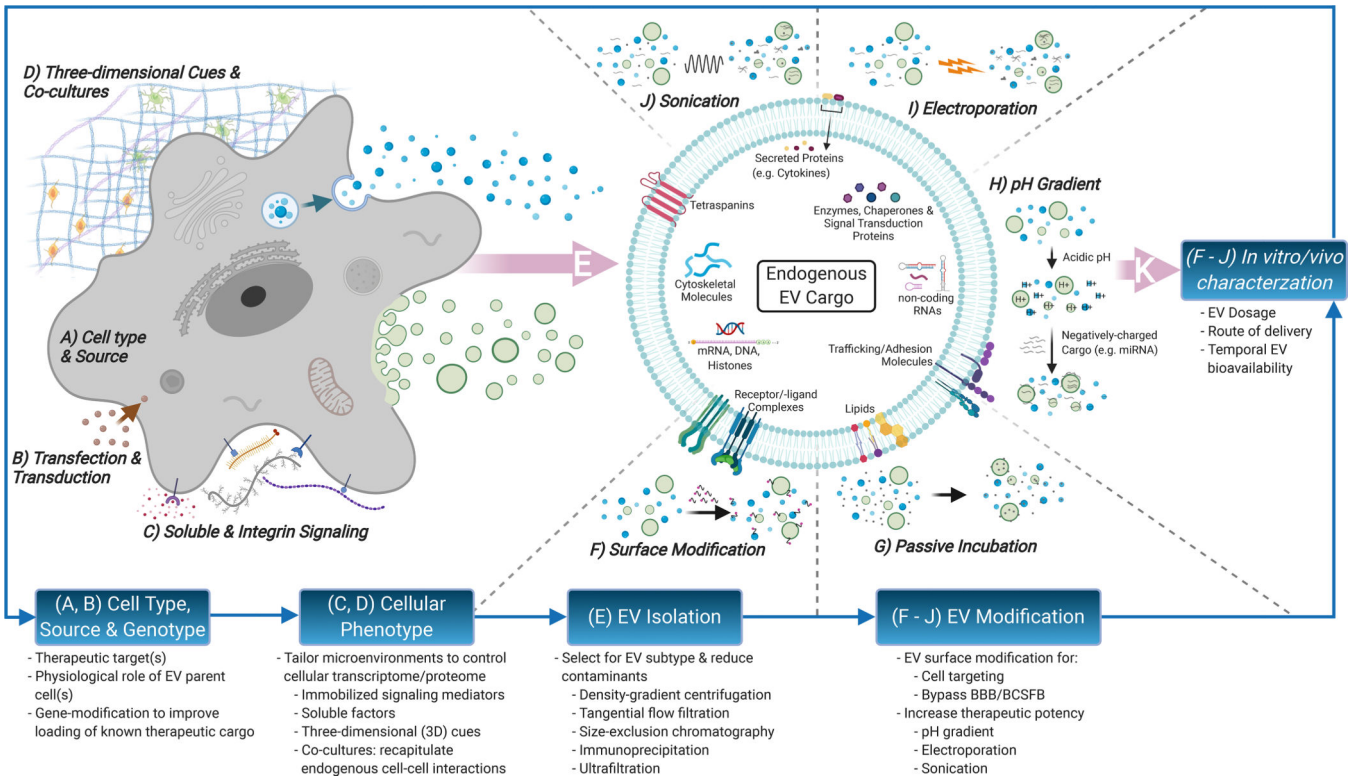

Figure 3: Strategies for enhancing CNS-specific regenerative potential of EV-based SCI therapeutics.

Engineering approaches to probe for populations of therapeutic EVs begin with selection of therapeutic target(s) for SCI (e.g. neurogenesis, axonal sprouting, remyelination, etc.) matched with appropriate EV producer cell types hypothesized to play direct/indirect physiological roles (A). EV producer cells may be genetically modified to further enhance EV loading of known biological mediators (B). Control over cellular phenotype through immobilized signaling mediators (e.g. extracellular proteins) or soluble factors (e.g. cytokines, hormones, etc.) can modify cargo and bioactivity of secreted EV populations (C). Additionally, biophysical cues such as three-dimensional culture and incorporation of specific cell-cell interactions using co-cultures (e.g. neuronal/oligodendroglial co-cultures to target remyelination) can further tune loading of EV-associated cargo (D). EVs are highly heterogeneous in size, membrane composition, cargo, and bioactivity. Many of these parameters are closely linked to the EVs' biogenesis pathways, which include: i) release of intraluminal vesicles within multivesicular bodies (MVBs) upon fusion with the plasma membrane, ii) direct budding of the plasma membrane and, iii) formation of apoptotic bodies. A significant hurdle in current EV isolation techniques is the inability to robustly differentiate between these EV subtypes. Thus, isolated EV populations will inevitably contain particles originating from multiple biogenesis pathways (E). Overall functional cargo makeup on EV surface (F) and/or EV interior (G-J) can also be modified to varying degrees after EV biogenesis and isolation. Lastly, *in vitro/vivo* characterization of biomanufactured EVs can inform further modifications to optimize for therapeutic efficacy and potency (K). Principal considerations for *in vitro* characterization include cell-based assays that probe for relevant EV bioactivity (e.g. neuroprotection, neuroinflammation).

Considerations for *in vivo* characterization include selection of SCI injury model, EV dosage, route of delivery to the CNS and temporal bioavailability of delivered EVs over an appropriate therapeutic time window.

Table 1.

Therapeutic outcomes and key bioactive components identified in EVs derived from CNS cell types in animal models of SCI

Cell Source	EV Parent Cell Micro-Environment	Route of Delivery	EV Dosage	Effects	Highlighted EV-associated cargo	Refs
Primary rodent neural stem cells		Tail-vein injection	200μg (PC)	• ↑ motor function • ↓ lesion volume and neuroinflammation • ↑ autophagy and neuroprotection	14-3-3t family of proteins	[70,74]
Primary rodent neurons		Tail-vein injection	200μg (PC)	• ↑ motor function • ↓ lesion volume • ↓ activation of proinflammatory microglia and neurotoxic astrocytes	miR-124-3p	[76]
Primary rodent neural stem cells		Tail-vein injection	5 doses of 200μg (PC) each over 12 days post-SCI	• ↑ motor function • ↓ lesion volume • ↑ angiogenesis	VEGF-A	[75]
Primary rodent neural stem cells		Intrathecal	10μg (PC)	• ↑ motor function • ↓ lesion volume • ↑ neuroprotection • ↓ expression of inflammasome proteins in the CNS		[73]
Primary rodent neural stem cells	+ Insulin-like growth factor-1	Tail-vein injection	100μg (PC)	• ↑ motor function • ↓ neuroinflammation • ↑ neuroprotection and axonal connectivity of neural fasciculus	miR-219a-2-3p	[81]

Key: PC – EV dosing determined via analysis of total protein content; ↑ – increased/upregulated; ↓ – decreased/downregulated