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Extracellular vesicles set the stage for brain plasticity and recovery by multimodal neuroimmune signalling

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Abstract

Extracellular vesicles (EVs) are extremely versatile naturally occurring membrane particles that convey complex signals between cells. Several EVs are also capable of inducing therapeutic responses in disease models. Differently from pharmacological compounds that act by modulating defined signalling pathways, EV-based therapeutics possess multiple abilities via a variety of effectors, thus allowing the modulation of complex disease processes that may have very potent effects on brain tissue recovery. When applied to neurological disease models, EV-based therapeutics reveal striking effects on immune responses, cell metabolism and neuronal plasticity. The multimodal modulation of neuroimmune networks by EVs profoundly influences disease processes in a highly synergistic and context-dependent way. Ultimately, this EV-mediated restoration of cellular function may help to set the stage for neurological recovery. With this review we aim to outline the current understanding of the mechanisms of action of EVs, describing how EVs released from various cellular sources interact with target cells. Mechanisms applicable to key neurological conditions, such as stroke, multiple sclerosis, and neurodegenerative diseases, are presented. Pathways that deserve attention in specific disease contexts are discussed. By sketching a broad view of EV-orchestrated brain plasticity and recovery, we define their possible future clinical applications and propose necessary information that should be provided ahead of clinical trials. Our goal is to provide a steppingstone that can be used to critically discuss EVs as next generation therapeutics for brain diseases.

Keywords: Exosomes; immune modulation; cell metabolism; mitochondria; neuronal plasticity.

Therapeutic challenges and clinical promises

In diseases of the central nervous system (CNS), such as stroke, multiple sclerosis (MS), or neurodegenerative disorders, damage to neurons, axons, or synapses result in the disruption of neuronal networks [1-3]. Neuronal damage may be direct or indirect as a consequence of demyelination. Independent of the nature of neuronal damage, local and systemic inflammatory responses are activated, which further exacerbate neuronal injury and propagate degenerative processes to distant brain areas [1,4,5]. The resulting perpetuated degenerative process inhibits neuronal plasticity, myelin regeneration (remyelination) and rewiring [1,4,5], which in turn lead to the persistent neurological deficits associated with daily life impairments. The complexity of brain damage creates the need of large-scale brain tissue remodelling to compensate for lost functions. However, the endogenous capacity of the brain to cope with injury stressors is extremely limited, and integrated into a multiorgan network in which response abilities decline with ageing across life [6].

Over the past few years, significant progress has been made towards new therapeutic options in all three disease areas. In ischemic stroke, the advancement of thrombolysis and mechanical thrombectomy (i.e., strategies to reopen the occluded artery) has markedly reduced ischemic injuries and improved outcomes [7,8]. In MS, immunomodulatory treatments that dampen brain inflammatory responses reduce disease relapses, although modestly affecting disease progression [9,10]. In Alzheimer's disease, a particularly devastating neurodegenerative condition, an immune therapy targeting soluble β-amyloid (Aβ) protofibrils has recently been shown to slow the clinical decline [11], although to moderate extent. Despite the progress made, significant neurological deficits persist in the vast majority of stroke patients [12], while the deficits of most multiple sclerosis and neurodegenerative disease patients still continue to

progress in the long run [13,14]. Moreover, in none of these three disease areas, neurorestorative treatments have been made available, which could reverse existing injuries or efficiently shift the balance from neurodegeneration to brain repair.

Compared to medicinal chemistry-inspired approaches, advanced therapeutics - including gene delivery methodologies and cellular/ acellular therapeutics - hold the promise to provide unprecedented improvement to structural and functional brain plasticity and regeneration. This is achieved by a combination of suppression of neuroinflammation, preservation of host neuronal structures, and improvement of motor and cognitive functions [15-18]. Among advanced therapeutics, those based on extracellular vesicles (EVs) are particularly versatile agents. EVs are cell-derived, lipid membrane-enclosed vesicles carrying a broad spectrum of biologically active molecules, which play a crucial role in intercellular communication [19]. EVs traffic a plethora of signalling molecules, which are dependent on the tissue origin of the producer cells, and the molecular determinants of the recipient cells [20]. These signalling molecules, including proteins, RNAs and bioactive lipids [21], constrain inflammatory responses that would otherwise result in secondary neuronal injury [22-24]. Besides, EVs carry small molecules, critical enzymes, respiratory chain machineries, and even entire cell organelles that restore cell metabolism, thus enabling functional neurological recovery [25,26]. In contrast to pharmacological compounds, which act by modulating defined signalling pathways, EV therapeutics possess multiple abilities and a variety of effectors allowing the modulation of complex disease processes in a highly synergistic and context-dependent way [21,22].

When delivered therapeutically in animal disease models, stem/ progenitor cell-derived EVs exhibit striking plasticity-promoting restorative effects, leading to functional neurological improvements [27]. In the middle cerebral artery occlusion

(MCAO) model, intravenously administered mesenchymal stromal cell (MSC)-derived EVs enhance motor-coordination recovery, similarly to parental MSCs, by mechanisms involving long-term neuroprotection, angiogenesis, neurogenesis, axonal sprouting, remyelination, and increased synaptic plasticity [28,29]. In the myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), a model of MS, intracerebroventricularly delivered neural stem cell (NSC)-derived EVs improved clinical outcome in mice almost identical to NSCs, modulating adaptive and innate immune responses while promoting neuronal survival, remyelination, and white matter repair [26,30]. In transgenic mouse models of Alzheimer's disease, intranasally administered MSC-EVs reduce the progression of cognitive deficits via mechanisms involving the polarization of microglia to an anti-inflammatory phenotype and reduction of cerebral Aβ plaque load [31,32]. Hence, EVs have very potent effects on brain tissue recovery in multiple disease context.

With the demonstration of consistent therapeutic effects of EVs in clinically relevant brain disease models [26,29], including pilot studies in non-human primates [33], the EV field is moving fast towards clinical applications. This review aims to outline our current understanding of the mechanisms of action of EVs, describing how EVs from various cellular sources interact with brain cells to set the stage for functional recovery. Mechanisms applicable to different neurological diseases will be presented, focusing on pathways that deserve attention in specific disease contexts. By sketching a broader view of EV-orchestrated brain plasticity and recovery, we will further define their possible clinical applications for EV. Finally, necessary information and quality controls for EV-based therapeutics that should be provided ahead of clinical studies and first human studies will be summarized.

In preparation of this review, we performed a detailed literature search in Pubmed combining the keywords ("extracellular vesicle" or "exosome") with ("neuronal plasticity" or "axonal plasticity" or "synaptic plasticity" or "neurological recovery" or "clinical recovery" or "neuronal survival" or "neuroprotection"). Due to the eminent importance of immune modulation and metabolic recovery in the therapeutic effects of EVs, we also combined ("extracellular vesicle" or "exosome") with ("immunomodulation" or "immunomodulatory" or "anti-inflammation" or "anti-inflammatory" or "immune tolerance" or "metabolic" or "mitochondrial" or "energy metabolism") and ("brain" or "central nervous system" or "neuron"). Besides, literature searches combining the key words ("extracellular vesicle" or "exosome") and ("ischemic stroke" or "multiple sclerosis" or "experimental autoimmune encephalomyelitis" or "neurodegeneration" or "neurodegenerative" or "Alzheimer" or "Parkinson") were performed.

Cellular origins, composition, and target cell interactions

EVs are particles released by virtually all eukaryotic and prokaryotic cells, which are abundant in all body liquids and tissues including the blood, cerebrospinal fluid (CSF), and brain [22]. Based on their biogenesis in different cell compartments [21], EVs are classified into various categories, which strongly differ in their physiological function and size **(Box 1)**. Most EVs released from cells are rather small (diameter typically ≤150 nm). Among these EVs, exosomes are formed by inward budding of the limiting membrane in the late endosomal compartment [21,22], while nuclear EVs are generated by membrane budding at the inner nuclear membrane [34,35]. Conversely, larger EVs (diameter up to 1,000 nm or more) are frequently formed as bud-offs from the plasma membrane. Among these, ectosomes are microvesicles with a size larger than 100 nm

[22], while apoptotic bodies with a size typically larger than 500 nm are released as part of a cellular decomposition process [21]. Still, there are considerable size overlaps between EV categories.

The EV membrane consists of highly organized assemblies of lipids (including cholesterol and sphingolipids) and proteins, which constitute membrane microdomains **(Glossary)**. Different membrane microdomains are organized by different proteins, such as tetraspanins and flotillins [36,37] **(Box 2)**. Membrane microdomains enrich many signalling proteins, among them several ligands and receptors, forming ligand and receptor platforms that have unique mobility features and signalling properties [38,39]. The temporally restricted interaction of membrane microdomains represents a key principle underlying cellular communication, and the combination of surface molecules which include (e.g., integrins and adhesion molecules) defines the membrane microdomain tropism towards selected cells. [38,40]. Thus, EVs represent *mobile ligand platform carriers* interacting with specific receptor platforms on defined cells.

EVs may form transient contacts and activate receptor platforms on their target cells, while retaining their integrity and shape [41,42]. After protease-triggered resolution of cell-cell contacts, the activated receptors are endocytosed to transmit their signals to target cells [43,44]. In the meantime, EVs get separated from their target cells before activated receptor platforms are endocytosed. For this mechanism, the term *kiss-and-run signalling* has been coined **(Figure 1)**. One example of this process are phosphatidylserine $(PS)^+$ EVs that form contacts with T cells via MHC class I binding to CD8, which induces T cell receptor (TCR) activation associated with the nuclear translocation of the transcription factor nuclear factor (NFATc1) [45]. Alternatively, EVs forming kiss-and-run contacts have been suggested to transfer luminal contents to the target cell cytosol via transient nanometer-sized fusion pores [41]. The release of luminal

cargos across such fusion pores is well established for the exocytosis and ultrafast recycling of presynaptic vesicle contents [41,46]. The role of *kiss-and-run* signalling associated with EV signalling in the nervous system is far from being fully established. Future studies will have to find out whether fusion pore formation enables the cellular uptake of luminal EV cargos.

Kiss-and-run signalling must be discriminated from *cellular EV uptake* via large-scale plasma membrane fusion or endosomal endocytosis, which results in transfer of cargo **(Figure 1)**. Indeed, seminal studies on *Cre* mRNA and CRE protein transfer studies implied that luminal cargos can functionally be delivered into the cytosol of target cells, which also includes brain cells [47]. While membrane fusion enables the passage of luminal EV cargos into the cell cytosol, endosomal endocytosis still maintains a barrier for luminal EV cargos, which need to escape the endosomal compartment and reach the cytosol to exert their function. Previous studies on engineered EVs loaded with luminal cargo proteins suggest that luminal cargos are effectively delivered to the cytosol only in the presence of endosomal escape-facilitating mediators [48]. Interestingly, endosomal escape proteins have been recently identified in EVs in select conditions [49]. This mechanism appears to be similar to that of viruses that have evolved endosomal escape strategies to deliver nucleic acids into their host cytoplasm (either via fusogenic proteins or dissolution of the endosomal membranes) [50]. More research is required to identify how EVs transmit signals and how they overcome membrane barriers to deliver luminal cargos.

Physiological and pathogenic roles of EVs

In the brain, EVs are abundantly released in microenvironments exhibiting vivid intercellular communication, such as cerebral endothelial cells, pericytes, astrocytic

end feet at the neurovascular unit [51,52], neurons at synaptic and astrocytic contact sites [53,54], oligodendrocytes [55,56], astrocytes along axonal surfaces[57,58], and neural stem/ precursor cells (NSCs) within stem cell niches [25,26]. In these environments, brain cells are arranged in proximity with each other, forming tight physical contacts. Herein, under physiological conditions, EVs mediate interneuronal and glial-neuron crosstalk, modulate synaptic plasticity and survival of neurons, regulate myelination, and manage immune and stress responses [59]. The underlying mechanisms will be outlined below, as they are the basis for current efforts to establish EVs as therapeutic concepts.

When considering brain-protective effects of EVs, it must be considered that under pathophysiological conditions, EVs can also transfer injury-exacerbating damage-associated molecular patterns (DAMPs) to surrounding cells, as shown in a variety of disease models. Moreover, EV trafficking at the BBB markedly differs from the brain parenchyma. The BBB forms an efficient barrier, which impedes the passage of brain-derived EVs into the blood and of blood-derived EVs into the brain [60]. The release of brain-derived EVs into the blood occurs mainly under pathophysiological conditions associated with neuronal injury and BBB breakdown. In response to intracerebral interleukin (IL)-1β injection, astrocytic EVs were shown to accumulate the blood and promote the transmigration of leukocytes into inflammatory brain lesions via mechanisms involving modulation of peripheral cytokine responses through inhibition of peroxisome proliferator-activated receptor-α (PPARα) [61]. In stroke, macrophage-derived EVs were shown to transfer the DAMPs IL1α, IL1β and Rantes to peri-infarct cells, inducing cellular dysfunction and injury [62]. In the MS-like lesion model of lysolecithin-induced axonal demyelination, the local injection of EVs collected from proinflammatory microglial cells, which were enriched in IL1α, IL1β and tumour necrosis factor-α (TNFα), prevented the remyelination of corpus callosum axons,

whereas that of EVs produced by microglia co-cultured with immunosuppressive MSCs promoted oligodendrocyte precursor cell recruitment and myelin regeneration [63].

In neurodegenerative diseases, EVs play a role in the propagation of misfolded proteins. EVs obtained from brain tissue of Alzheimer patients exhibited elevated levels of Aβ oligomers [64] and hyperphosphorylated tau protein [65], which were shown to act as vehicles for the neuron-to-neuron transfer of those toxic species *in vitro* and *in vivo*, respectively. Under conditions of Alzheimer's disease, microglia derived EVs were found to carry Aβ anterogradely along axonal surfaces, propagating long-term potentiation dysfunction from the entorhinal cortex to the dentate gyrus [66]. Under conditions of Parkinson's disease, microglia-derived EVs containing α-synuclein were shown to spread α-synuclein aggregates along axonal connections from the striatum to the substantia nigra [67,68]. EV α-synuclein internalization was initiated by α-synuclein binding to toll-like receptor (TLR)-2 of microglia [68]. These results indicate that EVs may act as seeds of protein aggregation to remote brain areas. The mechanisms of EV transport on axons are currently examined [66]. By propagating protein folding pathologies, EVs can contribute to neurological disease development. The careful selection of cellular sources thus is key in the implementation of successful EV-based therapeutics, and signalling mechanisms thoroughly need to be considered in clinically relevant settings.

Mechanisms of action mediating neurorestorative and recovery-promoting responses

Immunomodulatory target engagement at the plasma membrane

When therapeutically administered into the blood, EVs interact with adhesion molecules on inflamed endothelial cells enabling their passage into the injured tissue parenchyma [23,69]. This process involves EV interactions with extracellular matrix (ECM) proteoglycans in the corona of EVs, which expose signals and mediate their binding to cell membranes **(Box 2)**. Among the signals mediating therapeutic responses, cytokines such as transforming growth factor-β (TGFβ) play a decisive role **(Figure 1)**. Bound to EVs via the proteoglycan betaglycan, TGFβ interacts with TGFβ receptors (TGFβR) on cell membranes [70]. TGFβ signalling involves the endosomal uptake of activated TGFβ-TGFβR complexes [71]. Following MCAO, TGFβ localized on intravenously administered microglia-derived EVs was found to promote neuronal survival, angiogenesis and M2 microglia polarization by activating the small body size-mothers-against-decapentaplegic (SMAD)-2/3 pathway in ischemic brain tissue [72].

Besides cytokines, EVs can also directly transfer active cytokine receptors to target cells and modulate their biological responses in the nervous system. For example, under proinflammatory conditions, NSC-EVs were found to transfer functional interferon-γ receptor-1 (IFNγR1) to recipient cells, in which EV IFNγ-IFNγR1 complexes promoted STAT1 signalling [73]. The latter processes likely involved cytokines and cytokine receptors decorated on the EV surface. Whether cytokines, cytokine receptors, or associated signalling proteins encapsulated in the EV lumen can transmit signals to recipient cells, as proposed by some studies [74,75], needs further assessment. To the best of our knowledge, there still is no unequivocal evidence indicating functionally significant delivery of luminal cytokine or cytokine receptor cargos from EVs to target cells in the brain.

Chemokines, which are able to induce directional cell movements along concentration gradients, are also present on EVs and can attract cells to modulate their

biological responses to cell injury [76]. Among these, CC-chemokine ligand-2 (CCL2) is a chemokine, which in the brain is produced by astrocytes and decorates glycosaminoglycan sidechains of proteoglycans on the EV surface [77,78]. In models of breast, lung and prostate cancer, EV-bound CCL2 was found to induce cancer cell migration across a 3D BBB *in vitro* and promote brain metastasis *in vivo* via its receptor C-C-chemokine receptor-2 (CCR2) [77,78]. Upon brain injury, chemokines play a crucial role in the homing of inflammatory cells to the site of brain damage [76]. In spinal cord trauma, CCL2 on astrocytic EVs increased microglial activation and neuronal death via CCR2 in the acute injury phase [79], whereas CCR2 activation induced spinal motor circuit synapse pruning in the recovery phase [80].

In addition to cytokines and chemokines, EV can carry death receptor ligands, such as FAS ligand (FASL) or TNF-related apoptosis-inducing ligand (TRAIL) **(Figure 1)**, and checkpoint proteins, namely cytotoxic T lymphocyte antigen-4 (CTLA4) or programmed death-ligand-1 (PD-L1), on their surface, which can induce immune tolerance via corresponding receptor binding on T and NK cells [70]. Receptor binding of these ligands was shown to induce immune cell death, providing protection against autoimmune pathologies, e.g., under conditions of EAE [70]. When released by oligodendroglioma cells, EV-bound FASL and TRAIL cooperatively promoted cell death of astrocytes and neurons and prevented neurite growth [81].

Further, ECM proteoglycans and proteins on EVs can directly modify cellular signalling responses. For example, the laminin-binding protein fibulin-2, which is enriched on astrocyte-derived EVs, was shown to activate the TGFβR/ SMAD2 pathway in primary cortical neurons, enhancing spine and synapse formation [82]. Fibulin-2 knockdown abolished SMAD2-dependent spine and synapse growth. On the surface of EVs, several ECM proteases and glycosidases including membrane-type 1

matrix metalloproteinase (MT1-MMP), insulin-degrading enzyme, sialidase and heparanase, among others, have furthermore been localized [83]. These surface enzymes were shown to retain their activity and degrade their natural substrates present in the extracellular space. To date, ECM enzymes on EVs have been associated with the mobilization of growth factors, degradation of ECM macromolecules and destruction of Aβ plaques [83]. Their role in brain remodelling and plasticity still requires assessment. ECM proteins and proteoglycans play a decisive role in regulating neuronal survival and plasticity [84,85].

EV can also carry ectonucleotidases like CD39 and CD73 on their surface **(Figure 1)**, which restrain brain inflammatory responses by cleaving damage-associated adenosine triphosphate (ATP) to anti-inflammatory adenosine [86]. Via adenosine A2 receptor binding on target cells, adenosine was found to suppress $CD4^+$/ $FoxP3^+$ regulatory T cell [87] and $CD8^+$ effector T cell [86] activities. In glioblastoma, tumour EV-bound CD73 inhibited aerobic T cell glycolysis, reduced T cell proliferation and promoted tumour growth [88]. In EAE, CD39 and CD73 activation mediated activing-A-induced neurological improvements and axonal remyelination by inhibiting proinflammatory Th17 cells [89]. In Parkinson's disease, conversely, CD73-mediated adenosine formation sustained adenosine A2A receptor overactivation, resulting in the promotion of neuronal degeneration, motor, and cognitive impairments [90]. In MCAO, $CD73^{-/-}$ did not influence ischemic injury or neurological outcome [91]. Possibly, the role of CD39 and CD73 depends on pathophysiological contexts and cellular targets.

Some EVs display functional major histocompatibility (MHC) class-I and II complexes on their surfaces **(Figure 1)**, which present endogenous or exogenous antigens to T cells [92]. Dendritic cells may reveal antigens to T cells via EV-bound MHC complexes. This process, termed *crossdressing*, circumvents cellular antigen uptake

and processing that is otherwise required for antigen presentation [93]. EV-mediated antigen presentation may contribute to autoimmune brain pathology [22]. Upregulation of MHC complexes and integrins on EVs of IL1β-preconditioned astrocytes was made responsible for the inhibition of neurite outgrowth under neuroinflammatory conditions [94]. EV transfer is particularly intense in areas of immune cell contacts, where the transmitted signals, MHC complexes and costimulatory molecules coordinate interactions between cells [22]. By taking up MHC complexes, recipient cells can achieve new immunological features which fundamentally reprogram injury responses.

Nuclear signalling, transcriptional and posttranscriptional regulation

EVs can transport nuclear constituents and signals, among these proteins and RNAs, to the nucleus of target cells **(Figure 1)** [21]. Nuclear receptors carried via EVs bind DNA and modulate gene transcription. Using mutant receptor constructs, cancer cells were found to transport EV-bound epidermal growth factor receptor (EGFR, also called ErbB1) and androgen receptor (AR, also called nuclear receptor-3C4) to recipient cell nuclei, where they activated transcriptional responses [95]. EGFR is a tyrosine kinase which upon activation and dimerization phosphorylates a variety of transcription factors, whereas activated AR directly acts a DNA-binding transcription factor. Nuclear EGFR delivery was shown to confer chemotherapy resistance in cancer [96], while EGFR activation by EGF reduced neurological deficits and histopathological damage in EAE [97].

EVs can also deliver mRNAs to target cells, where these are translated into proteins **(Box 2, Figure 1)**. For example, EV-encapsulated mRNAs from human endothelial progenitor cells were found to promote endothelial survival, proliferation, and tube formation [98]. The successful transfer and translation of mRNA in endothelial cells was

shown by EV-encapsulated *Gfp* mRNA transduction, and the biological relevance by the angiogenic effect of EV-mRNA extract delivered by lipofectamine [98]. In MCAO mice, mRNAs enriched in brain-derived EVs were most often of microglial and oligodendroglial origin [99]. They were involved in immune signalling, cell differentiation, adhesion, and motility, indicating brain-reparative roles.

Several studies reported EV-encapsulated non-coding RNA (ncRNA) transfer to target cells under conditions of cerebral hypoxia-ischemia. The current literature on EV-associated ncRNAs has been reviewed recently [100]. We therefore focus the following paragraphs on implications for neuronal survival, neuroplasticity and neurological recovery. miRNAs are short single-strand non-coding RNAs, which typically are 21-23 nucleotides in size. Released from the nucleus as pre-miRNA hairpins, they are processed in the cytosol to mature miRNAs [101]. As part of the RNA-induced silencing complex (RISC) [102], miRNAs interact with complementary gene sequences of target mRNA, repressing gene expression by mRNA cleavage or interference with mRNA-ribosome interactions [103-105]. The human genome contains >600 genes with robust evidence of miRNA functions [106], which target >60% of all genes [107]. Thus, miRNAs have potent biological effects when transferred via EVs, which modify disease recovery.

Following MCAO, miR-133b has been found to mediate effects of MSC-EVs on axonal plasticity and neurological recovery in rats via mechanisms involving downregulation of the miR-133b targets connective tissue growth factor and Ras-homolog gene-family member-A [108]. Besides, miR-17-92, which was enriched in MSC-EVs, stimulated oligodendrogenesis, neurogenesis and axon-myelin remodelling following MCAO by downregulating the miR-17-92 target phosphatase-and-tensin homolog (PTEN) [109]. Also following MCAO, MSC-EV miR-25-3p decreased neuronal

autophagic flux and injury and enhanced neurological recovery in mice by downregulating the miR-25-3p target p53 and BNIP3 [110]. In type-2 diabetic mice exposed to cortical photothrombotic stroke, endothelial cell-derived EV miR-126 promoted axonal plasticity, myelin remodeling and neurological recovery by mechanisms involving M2 macrophage polarization and enhanced angiogenesis [111].

In contrast to miRNAs, long ncRNAs (lncRNAs) are transcripts with more than 200 nucleotides [112], which control gene expression in multiple ways, acting as transcription regulators, regulators of epigenetic modifications, assistants of DNA repair and regulators of mRNA processing [113,114]. Due to their circular structure, circRNAs have high exonuclease resistance [115]. They act as miRNA sponges and scaffolds for chromatin-modification, transcription regulation and mRNA splicing [116,117].

Primary astrocyte EV-associated circSHOC2 has been shown to increase neuronal survival and inhibited autophagy in mice exposed to MCAO via miR-7670-3p sponging that resulted in the elevation of the miR-7670-3p target SIRT1 [118]. Under conditions of oxidative stress, MSC-EV lncRNA MALAT1 increased HT22 neuronal survival and proliferation via mechanisms involving serine and arginine rich splicing factor (SRSF)-2 recruitment, alternative protein kinase (PK)-CδII splicing and B-cell lymphoma protein (BCL)-2 elevation [119]. Following photothrombotic stroke, circSCMH1 enriched in EVs of genetically engineered HEK293T cells increased dendritic and synaptic plasticity, reduced microglial activation, reduced proinflammatory cytokine (IL1β, TNFα and IL6) formation and improved neurological recovery in mice and rhesus monkeys through repression of transcription factor methyl-CpG binding protein (MeCP)-2, a nuclear transcription factor directly binding methylated DNA [120]. By MeCP2 binding, MeCP2 target gene transcription repression was released.

A prerequisite for biological actions is that EV-encapsulated RNAs reach their targets in the cell, specifically in the nucleus and endoplasmic reticulum. Importantly, not all EV-contained RNAs are involved in intercellular communication. Several RNAs are released for cellular waste disposal [100]. Some RNAs may also represent artifacts, since RNAs tend to precipitate with EVs [100]. Further research is needed on the mechanisms responsible for RNA packaging into EVs, and the mechanisms enabling the delivery of EV-loaded RNAs to their subcellular targets in recipient cells.

Metabolic and mitochondrial reprogramming

Most evidence supporting a role for EVs in regulating cell metabolism comes from non-neural cells. Upon glucose deprivation, cardiomyocytes increase the synthesis and secretion of EVs, which are loaded with functional glucose transporters and glycolytic enzymes that increase glucose uptake, glycolysis, and pyruvate production in recipient endothelial cells [121]. Similarly, EVs produced by prostate cells (exosome-like prostasomes) contain glycolytic enzymes and enzymes involved in ATP turnover (e.g., adenylate kinase, ATPase, 5'-nucleotidase), which contribute to ATP formation when supplied with substrates [122].

This intrinsic metabolic activity of EVs plays an important role in cancer, where energy metabolism is targeted to block tumour progression [123]. Indeed, up to one quarter of proteins enriched in cancer derived large EVs (i.e., oncosomes) are enzymes involved in glucose, glutamine, and amino acid metabolism [124], processes relevant to cancer progression. Via EV-bound amino acids and tricarboxylic cycle intermediates, tumours induce a metabolic switch of their microenvironment from oxidative phosphorylation to glycolysis [125]. The resulting lactate is utilized by cancer cells to promote tumour growth. Oxidative phosphorylation/ glycolysis balance

decisively controls neuronal survival and synaptic plasticity in the injured CNS through astrocytes [126].

Recent data obtained from CNS cells have shown that NSC-EVs harbour a specific L-asparaginase activity due to the presence of the asparaginase-like protein-1 (ASRGL1) **(Figure 1)**, a key enzyme specific for asparagine that is devoid of glutaminase activity [25]. Thereby, EVs act as independent, metabolically active units capable of perturbing the extracellular milieu by influencing metabolic substrate levels.

In the brain, axons are critical sites at which energy metabolism is stabilized by oligodendrocyte-derived [55,56] and astrocyte-derived [58,127] EVs. An important mechanism is the transcellular delivery of the NAD-dependent deacetylase SIRT2 **(Figures 1, 2)**, which is produced in oligodendrocytes and transferred to neurons via EVs [56]. EVs obtained from wildtype, but not *Sirt2*$^{-/-}$ oligodendrocytes induced mitochondrial adenine nucleotide translocases-1/2 (ANT1/2) deacetylation, elevated ATP level and rescued mitochondrial integrity in *Sirt2*$^{-/-}$ mouse spinal cords [56].

Deficient oligodendrocytic metabolic support was made responsible for progressive axonal degeneration in proteolipid protein (*Plp*)$^{-/-}$ and 2',3'-cyclic-nucleotide-3'-phosphodiesterase (*Cnp*)$^{-/-}$ mice characterized by deficient retrograde and anterograde axonal transport and axonal swelling [55]. EV release of oligodendrocytes was reduced in both mice, indicating roles of PLP and CNP in EV biogenesis. Notably, EVs of *Plp*$^{-/-}$ and *Cnp*$^{-/-}$ oligodendrocytes revealed reduced SIRT2 and heat shock protein-72 contents compared with wildtype oligodendrocyte EVs [55]. Progressive axonal degeneration and transport in both mice were reversed by wildtype oligodendrocyte EVs.

The mechanisms via which oligodendroglial EVs sustain axonal structure and function have recently been thoroughly reviewed [128]. The latter review specifically

pointed out the cooperation between exosome-dependent and metabolic support mechanisms in the maintenance of axonal integrity.

Oxidative stress in mitochondria closely accompanies delayed neuronal loss, brain atrophy and cognitive impairment in rodent traumatic brain injury models [127]. Astrocytic EVs can reduce neuronal loss, brain atrophy and mitochondrial oxidative stress, as shown post traumatic brain injury by activating nuclear factor erythroid-2-related factor-2 (NRF2)/ heme oxygenase-1 signalling and increasing antioxidant superoxide dismutase (SOD) and catalase activity [127]. The neuroprotective effects of astrocytic EVs were abrogated in brain-specific *Nrf2*$^{-/-}$ mice.

EVs also contain mitochondrial proteins, mitochondrial DNA, and even entire mitochondria [129,130]. EVs may help to unload injured mitochondria from stressed cells in a process termed *transmitophagy*, as demonstrated for retinal ganglion cell axons releasing acidified mitochondria associated with lysosomes, which were taken up by neighbouring astrocytes for degradation [131]. Lysosomal uptake protects the cells against inflammatory responses elicited by oxidized mitochondrial proteins [132]. The Parkinson's disease-associated protein parkin recognizes damaged mitochondrial proteins and membrane fractions and directs them to the lysosomes [132]. Less severely injured mitochondria may be reutilized by recipient cells. Thus, depolarized mitochondria released from MSCs via EVs were engulfed and restored by macrophages and regained bioenergetic function [129].

Upon ischemia, astrocytes can release functionally intact mitochondria by a calcium-dependent mechanism involving CD38/ cyclic ADP-ribose signalling, which are transferred to adjacent neurons [130]. When administered to MCAO mice, the mitochondrial transfer increased cellular ATP level, neuronal survival, and dendritic growth [130]. CD38 knockdown reduced cellular mitochondrial transfer and worsened

neurological outcome. Endothelial precursor cells similarly can release viable mitochondria, which are taken up by brain endothelial cells, promoting intracellular ATP level, microvascular integrity and angiogenesis [133].

Structurally and functionally intact (free and EV-encapsulated) mitochondria can finally be released by NSCs [26]. These MitoEVs can rescue the mitochondrial dysfunction of mitochondrial DNA-deficient L929 Rho^0 cells, and integrate into mitochondria of inflammatory macrophages, modifying their metabolic profile and pro-inflammatory gene expression *in vitro* and *in vivo* in rodents with chronic EAE [26]. These effects are relevant for persistent neuroinflammation [134]. Further research is required on the mechanisms underlying mitochondrial packaging, release, and cellular uptake.

Promotion of neuronal plasticity

Via their immunomodulatory, transcriptional/ post-transcriptional and metabolic effects, therapeutically administered EVs help creating a microenvironment favourable for neuronal plasticity and neurological recovery. Axons and dendrites in the vicinity and at distance to brain lesions sprout accompanied by myelin remodelling enabling functional neuronal network rewiring in rodents [108,109]. This plasticity-promoting action was recently also demonstrated in the perilesional cortex of rhesus monkeys exposed to motor cortical cold injury, in which intravenously delivered MSC-EVs increased dendritic branching and synaptic spine density [33]. In this rhesus monkey study, the plasticity-promoting structural effects went along with functional fine motor improvements [33]. The authors of this studies found that microglial immunomodulatory responses were crucially involved in the plasticity-promoting actions of MSC-EVs [135]. Mechanistically, a number of nervous system-intrinsic effects of EVs also exist that

specifically contribute to axonal growth, axon-myelin interaction, astrocytic function and synaptic plasticity. These effects ore outlined in the following.

EV delivery profoundly regulates axonal signalling. This process involves communication with the perikaryon and nucleus. For example, macrophage EVs were found to transfer functional NADPH oxidase-2 (NOX2) to injured mouse axons, in which NOX2 was taken up by endocytosis [136]. In axonal endosomes, active NOX2 was retrogradely transported to the soma through an importin-β1-dynein-dependent mechanism **(Figure 2)**. Endosomal NOX2 oxidized PTEN, leading to its inactivation, stimulating phosphatidylinositol-3-kinase (PI3K)/ Akt signalling and regenerative axon growth [136]. Besides, internalized EVs obtained from ischemic cerebral endothelial cells can specifically transfer miRNAs to the nucleus via retrograde transport, which have the ability of downregulating axonal growth inhibitors in distal axons via gene expression repression [137]. Blockage of axonal transport suppressed cerebral endothelial EV miRNA and protein responses in somata but not in distal axons.

Neurons and astrocytes mutually support each other following brain injury via EV-bound signals. Astrocytes play decisive roles in the maintenance neuronal energy metabolism, most notably via lactate shuttling [126]. They besides have important trophic functions, controlling neuronal survival and plasticity [126]. The oligomannose-mimicking peptide synapsin-I is a neurite growth stimulant released from mouse astrocytes via EVs **(Figure 2)**. When transferred to neurons, astrocytic synapsin-I increased neurite outgrowth and promoted neuronal survival after hydrogen peroxide treatment or oxygen-glucose deprivation [57]. Coculture experiments using wild-type neurons and wild-type, or synapsin-deficient glial cells showed enhanced neurite outgrowth when synapsin was expressed by glial cells. Synapsin-induced neurite outgrowth was

dependent on oligomannose on synapsin I and neural cell adhesion molecule (NCAM) at the neuronal cell surface [57].

Perisynaptic astrocytes express glutamate transporters, namely glutamate transporter-1 (GLT1), which control extracellular glutamate levels at tripartite synapses and modulate synaptic activation and plasticity. EVs released from mouse neurons were found to contain abundant microRNAs and other small RNAs [54]. When internalized into astrocytes, these EVs increased GLT1 protein levels via mechanisms involving miR124a transfer **(Figure 2)** [54]. Intrastriatal injection of antisense RNA against miR-124a into adult mice dramatically reduced GLT1 protein expression and glutamate uptake in the striatum, yet without reducing *Glt1* mRNA levels [54]. miR-124a was reduced in spinal cords of endstage SOD1 G93A mice, an amyotrophic lateral sclerosis model. Exogenous miR-124a delivery prevented the loss of GLT1 protein in spinal cord astrocytes of SOD1 G93A mice [54].

Synaptic contacts are sites of activity-dependent plasticity [138]. In the regulation of activity-dependent plasticity, EVs possess a central role. EVs are constantly released at the presynaptic membrane in an activity-dependent way [53]. The activity-dependent EV release involves syntaxin-1A (SYX1A), a protein otherwise involved in synaptic vesicle secretion, as shown in *Drosophila* [53]. EVs released via SYX1A were found to contain the Wingless-binding protein Evenness-interrupted (EVI)/ WNTless that binds to Frizzled-2 (FRZ2) at the pre- and postsynaptic membrane **(Figure 2)**, inducing coordinated synaptic growth at both membranes occurring in a glycogen synthase kinase-3β (GSK3β)/ β-catenin-dependent way [53].

The cytoskeleton-associated protein ARC regulates activity-dependent synaptic plasticity. ARC protein was demonstrated to self-assemble into capsid-like structures in with a size of 20-60 nm that encapsulate RNA [49]. In mouse hippocampal neurons,

ARC protein capsids released via EVs were shown to transfer mRNA into recipient neurons, in which this mRNA was successfully translated **(Figure 2)** [49]. Structurally, ARC resembles retroviral GAG retrotransposons which may have been repurposed phylogenetically for synaptic communication [139]. The retrotransposon ARC might provide an endosomal packaging and escape mechanism, via which mRNA and miRNA can be exchanged between cells.

Activity-dependent EV release at synapses is controlled by neurotrophic growth factors. In a model of electrophysiological stimulus-induced EV release in primary rat hippocampal neurons, basic fibroblast growth factor (bFGF) was found to increase the activity-dependent release of EVs by late endosomes **(Figure 2)** [140]. Proteome analysis showed that EVs released by bFGF were rich in vesicle-associated membrane protein-3 (VAMP3) [140]. VAMP3 was indispensable for bFGF-induced EV secretion. VAMP3 knockdown attenuated the bFGF-induced EV release.

Brain-derived neurotrophic factor (BDNF) coordinates the sorting and release of miRNAs via neuronal EVs, which promote synaptic plasticity **(Figure 2)**. In mouse cortical neurons, miR-132-5p, miR-218-5p, and miR-690 were packaged into small EVs upon BDNF-induced TrkB activation [141]. EV formation occurred in a neutral sphingomyelinase and ceramide-dependent way. When added to mouse hippocampal neurons, BDNF-induced EVs increased excitatory synapse formation by elevating a broad set of developmental and synaptogenesis-related genes (such as *Sema4a*, *-6c*, and *-7a*, *Wnt7a/b*, *NeuroD2*), which depended on EV-associated miRNA transfer [141]. BDNF-induced EVs furthermore amplified synaptic vesicle clustering, thereby increasing synaptic transmission and synchronous neuronal activity [141].

The presynaptic endosomal system maintains a stock of release-competent EVs and EV cargos, which supports activity-dependent plasticity. The formation of this stock relies on the functionality of endocytic proteins, namely nervous wreck (NWK),

564 shibire/ dynamin and AP-2 adaptor complex [142]. In *Drosophila*, the deficiency of these
565 proteins locally depleted EV cargos from presynaptic terminals. As such, *Nwk* mutants
566 exhibited synaptic plasticity defects phenocopying those associated with deficiency of
567 synaptotagmin-4 (SYT4), a known EV cargo [142]. Mechanistically, NWK assisted in the
568 loading of cargos into EVs. Activity-dependent synaptic EV signalling has not been
569 modulated therapeutically in the injured brain. Stimulating or mimicking synaptic EV
570 responses might allow enhancing use-dependent plasticity, e.g., under conditions of
571 neurorehabilitation.
572

573 ## Tissue accumulation and functionalization of EV-based therapeutics

574 ### *Unmodified EVs*

575 In order to reach potential targets in the CNS, systemically administered EVs need to
576 pass the BBB, a tight barrier preventing the diffusion of macromolecules. In direction
577 to the brain, the penetration of blood-injected unmodified EVs is scarce under
578 physiological conditions in rodents [47,143-145] and in macaque monkeys [146].
579 Pharmacokinetics is disappointingly rapid, and circulation time is short [47,143-146]. In
580 macaques, the achieved brain EV concentrations after intravenous EV administration
581 were 100-1000 times lower than concentrations in the liver and spleen and 10-50 times
582 lower than concentrations in the lungs and heart [146]. EV accumulation in the brain was
583 markedly increased under inflammatory conditions, e.g., upon peripheral
584 lipopolysaccharide administration or in cancer, when EVs derived from hematopoietic
585 lineage cells expressing CRE recombinase displayed more widespread recombination
586 and reporter gene expression in neuronal populations of the cortex, hippocampus,
587 substantia nigra and cerebellum [47,147]. EV uptake by neurons was augmented by
588 neuronal activity, as shown in pharmacological and optogenetics studies [147]. In

rodents, intranasal EV administration significantly more efficiently delivered EVs to the ischemic brain that intravenous delivery [148,149]. Unfortunately, the enhanced brain accumulation following intranasal delivery could not be replicated in macaque monkeys. In macaques, brain concentrations of EVs were even lower after intranasal than after intravenous delivery [146]. EV biodistribution will carefully have to be considered in the preparation of human proof-of-concept studies. It yet remains to be determined in which disease settings systemically administered EVs achieve sufficient concentrations in the brain that allow to modify disease processes.

In view to the limited brain parenchymal uptake, considerable scepticism has been raised about the systemic intravenous delivery of EVs in neurological disease contexts. Yet, it may not be mandatory that EVs accumulate in the CNS parenchyma in order to exert their neurorestorative and recovery-promoting actions. In fact, EVs rapidly accumulate within minutes at high concentrations in peripheral blood leukocytes, specifically in monocytes, granulocytes and B cells, following intravenous delivery both in rodents or macaque monkeys [146]. Blood-derived leukocytes massively invade the injured brain parenchyma in all major neurological disorders [23,150]. Hence, leukocytes might mediate the neuroprotective and neurorestorative activities of systemically administered EVs even in the absence of EV BBB passage. In line with this notion, the protective effects of MSC-EVs on neurological deficits and brain injury following ischemic stroke induced by MCAO depended on their anti-inflammatory actions, namely the prevention of polymorphonuclear neutrophil, monocyte and lymphocyte entry in the ischemic brain tissue [23,24]. Neutrophil depletion by delivery of an antibody against the neutrophil-specific antigen Ly6G mimicked the effects of intravenously administered MSC-EVs on neurological deficits, brain injury and brain monocyte/ macrophage and lymphocyte infiltrates [23]. Yet, in neutrophil-depleted mice, MSC-EVs did not have any further effect on neurological deficits and brain injury, and brain

monocyte/ macrophage and lymphocyte infiltrates were not reduced by MSC-EVs [23]. Notably, the role of peripheral blood leukocytes in mediating post-ischemic actions of MSC-EVs is not limited to the acute stroke phase. When administered in the post-acute stroke phase, from 24 hours to 5 days post-MCAO, EVs obtained from hypoxic MSCs were found to promote peri-infarct angiogenesis [151]. The angiogenic effects of the MSC-EVs were abolished in neutrophil-depleted mice [151]. Apparently, polymorphonuclear neutrophils are early invaders of the brain after MCAO, which promote brain monocyte/ macrophage and lymphocyte entry and exacerbate ischemic damage in the early injury phase [152,153], but support brain tissue remodelling and recovery in the post-acute stroke phase in response to MSC-EV treatment. The modulation of peripheral immune responses might represent a potent mode of action via which disease processes can be modified even under conditions in which brain EV uptake is low.

Engineered EVs

Although extremely versatile in nature, unmodified EV therapeutics suffer from their very low accumulation and fast clearance in target tissues. Therefore, genetic engineering methods are being employed to allow the modification of EVs to make them longer lasting in the blood, more selective towards their brain target tissue, and more potent **(Figure 3)**. To increase circulation time in the blood and improve their delivery to target tissues, one promising approach is the decoration of EVs with the polyether polyethylene glycol (PEG), called PEGylation. PEGylation is expected to delay EV degradation and increase circulation time, as described for lipid nanoparticles showing 10-15-fold increased blood half-life, compared to unmodified lipid nanoparticles [155].

The prevention of EV phagocytosis by host innate immune cells using *do-not-eat* signalling molecules is another mean to prolong the circulation time of EVs in the blood. As such, EV decoration with the *do-not-eat* signalling protein CD47 was found to reduce EV endocytosis by macrophages, augment circulation time and increase EV accumulation in tumours following systemic injection in rat cancer models [156]. The strategy induced a more than 2-fold concentration increase in tumours compared to conventional EVs.

The functionalization of PEG derivatives on EVs with nanobodies aims to increase the target tissue specificity of EVs. In a proof-of-concept study, nanobodies directed against EGFR were conjugated to phospholipid-PEG derivatives [157]. This process did not affect EV morphology, size distribution, or composition. After introduction of PEG-conjugated anti-EGFR nanobodies to EVs, cellular binding to EGFR-expressing cancer cells was increased compared with PEG-conjugated control antibody [157]. Whereas unmodified EVs were rapidly cleared from the circulation within 10 minutes after intravenous injection in mice, EVs modified with nanobody-PEG-phospholipids were still detectable in plasma for more than 60 minutes [157].

To increase brain parenchymal targeting, click chemistry is a particularly versatile method for the conjugation of ligands to the EV surface [158]. Specifically, click chemistry-based expression of the neuropilin-1 receptor peptide RGERPPR has been shown to increase BBB passage and promote the therapeutic efficacy of systemically administered EVs in a rodent glioma model [159]. Combined with hyperthermic therapy, RGERPPR-engineered EVs revealed a synergistic anti-tumour effect [159].

For the functionalization of EVs, two alternative modalities approaches have become available, each of which with its own pros and cons. These approaches imply the loading of the EV producer cell line with defined cargoes, or the direct loading of

EVs with cargoes using a variety of loading strategies. One of the most exciting strategies is the EV functionalisation with key components of the genome editing machinery through EV-Cas9 ribonucleoprotein (RNP) complexes [160]. While EV-Cas9 RNP therapeutics have been validated in acute liver injury, chronic liver fibrosis, and hepatocellular carcinoma mouse models, the applicability of these new principles of tissue specific therapeutic gene editing for brain disease is yet to be established.

Modified EVs are not the main focus of this work. Technologies used for EV engineering have been reviewed in depth recently [154]. This earlier review addresses both target tissue delivery and functionality aspects of EVs.

Therapeutic potential and clinical translation

As pointed out in this review, EVs have multimodal actions when obtained from the right cell sources that promote neurological recovery by modulating gene expression, immune responses, cell metabolism, at the same time stimulating neuron-glia interactions, neuronal survival and plasticity **(Figure 3)**. Whereas each of these actions may have beneficial actions in defined disease states, we have to assume that the majority of EVs has a plethora of signalling mechanisms, which act in synergy to set the stage for functional neurological recovery. The combination of actions explains the potent effects of EVs. Having a clear therapeutic potential in a variety of disease contexts, supported by a large number of experimental studies, clinical translation is promising. First clinical proof-of-concept studies are on the way. We need to rule out that critical mistakes are made at this stage.

Therapeutic effects of EVs besides cell sources critically depend on culturing conditions and EV isolation protocols. MSC-EVs, for example, may have immune tolerance-promoting or cytotoxic actions depending on the MSC culturing conditions

even when a defined MSC donor is used [23,24]. Preconditioning in the right setting by physiological or chemical stimuli may augment the restorative effects of EVs, whereas inappropriate preconditioning and loading with proinflammatory signals (e.g., DAMPs) or pathogenic proteins (e.g., Aβ) may abolish brain protective effects or even confer detrimental activities. When applied in ischemic stroke models, for example, hypoxic preconditioning enhanced the neurovascular, angiogenic and long-term neuroprotective effects of MSC-EVs by modifying a large number of EV proteins [23,151]. When administered in brain tumours, the same hypoxic stimulus was found to increase tumour malignancy and growth [161-163]. Solid pathophysiological concepts are needed with in depth knowledge about cell sources and culturing conditions to ensure that EV preparations are used that successfully stimulate neurological recovery. In order to retain restorative properties, cell sources, culturing conditions and EV isolation protocols should be standardized in clinical studies and precisely mirror those in experimental studies **(Box 3)**. Since the biological activity of EVs differs from preparation to preparation even when the same cell source is used, the biological activity of each EV preparation should be evaluated with potency assays before administered to human patients **(Box 3)**.

In the preparation of clinical studies, an important question relates to the selection of cellular EV sources. Certain mechanisms of action, namely mitochondrial stabilization and neuronal plasticity promotion, have genuinely been linked to EVs derived from neural cell sources, namely NSCs, oligodendrocytes or astrocytes [26,56,57]. In contrast, potent immunomodulatory actions have been reported following the delivery of MSC-EVs [23,24]. Hence, the selection of the optimal cell sources will depend on disease contexts. Studies targeting inflammatory responses may prefer MSC-EVs [23], while studies primarily modulating neuronal plasticity might prefer brain-derived, namely NSC-EVs [26]. Another key question is the mode of EV delivery. Potent immune

modulation can be achieved by systemic (namely intravenous) EV delivery [23,24], whereas mitochondrial stabilization may require more local, i.e., intracerebroventricular EV administration [26]. In the choice of EV delivery strategies, potential benefits of a certain mode of administration need to be weighed carefully against associated efforts and risks. Due to the risk of peri-procedural bleedings and infections, the intracerebral delivery of EVs, for example, via a trephination of the skull is not feasible in a large number of disease contexts. Repeated EV doses will be needed in several disease settings.

Mitochondrial disturbances are a joint hallmark of various neurodegenerative and neuroinflammatory conditions. Thus, EVs with mitochondria-stabilizing action may have broad application not only in contexts, in which they have hitherto been evaluated (e.g., stroke, multiple sclerosis, Parkinson's disease) but also beyond, e.g., in rare hereditary neurodegenerative diseases, in which they should be able of restoring cellular energy state. Gene therapies are currently making great progress in the treatment of metabolic disturbances in rare hereditary neurodegenerative diseases [164,165]. For enhancing their biological properties, EVs may genetically be loaded with defined genes or proteins **(Figure 3)**. As outlined above, genetic engineering strategies may also be used for increasing the EV circulation time in the blood or enhancing EV brain tissue targeting **(Figure 3)**. Interestingly, compelling evidence exists in support of a multi-cargo biological anti-ageing signature of genetically non-modified small EVs, which can be used therapeutically to delay the degenerative processes associated with aging and frailty [166].

An important requirement for clinical studies is that the proof-of-concept for a given mode of action has unequivocally been documented in experimental disease models. This implies that the assumed mediator (i.e., a protein or RNA) reaches its target on the surface or inside recipient cells. Considering that cargos encapsulated in the EV

lumen must escape endosomal confinements, proofs-of-concept built on luminal EV signals may pose greater challenges than proofs-of-concept built on membrane-bound signals. We urgently need to learn more about the target cell uptake of EVs, specifically about how EV cargos reach their site of action in recipient cells.

Concluding remarks and outlook

Envisaging the clinical translation of EV therapeutics, several tasks remain to be resolved at this moment. For ensuring therapeutic efficacy, EV production should be standardized, and EV activity should be evaluated in well-selected potency assays. Potency testing raises important challenges **(Box 3)**. Depending on disease contexts, sets of assays may have to be screened. The clinical implementation will require stringent proof-of-concept studies which closely mimic experimental studies regarding cell sources, EV isolation strategies and delivery protocols. Often, information on EV sources and isolation strategies is critically missing in ongoing interventional clinical trials **(Table 1)**. Future clinical phase-1/2a studies should vividly examine surrogate markers (e.g., immune responses in blood or CSF), which ideally match readouts in experimental studies and potency assays. These surrogate markers may provide the proof-of-concept that a presumed mode of action (e.g., anti-inflammation) can successfully be modified in human patients ahead of phase-2b/3 efficacy studies. These principles are pivotal for the success of clinical trials. The scientific community should not risk the clinical implementation of EVs by premature studies neglecting them.

Declaration of interests

DMH and BG hold patents for the application of extracellular vesicles for the treatment of inflammatory conditions (EP2687219A1; US9877989B2). BG is founding director of Exosla Ltd., scientific advisory board member of Innovex Therapeutics SL, Mursla Ltd., PL Bioscience GmbH and ReNeuron Plc., and consultant of Fujifilm. SP is founder, chief scientific officer, and shareholder (>5%) of CITC Ltd. and chair of the scientific advisory board of ReNeuron Plc.

Additional elements:

Box 1: EV categories defined by biogenesis

EVs can be classified into the following categories:

1. Exosomes are formed by inward budding of the limiting membrane of late endosomes. The resulting intraluminal vesicles are released into the extracellular space by endosomal plasma membrane fusion [21,22]. Exosomes are small EVs that typically have diameters of 60-150 nm and have important roles in cellular nutrition and intercellular communication.

2. Exosomes are also formed by inward limiting membrane budding followed by the extracellular release of autophagosomes/lysosomes [21]. The size of autophagosomal/ lysosomal exosomes overlaps with late endosomal exosomes, but larger EVs that may include organelles or organelle fragments, including those of mitochondria, can also be secreted [26]. The release of autophagosomes/lysosomes and their exosomes represents a cellular waste excretion mechanism, when autophagy activity is overchallenged or inhibited [167]. Many contents are not involved in intercellular communication.

3. At the endoplasmic reticulum, EVs are formed by budding at specific membrane contact sites [168]. EVs formed at these contact sites are rich in RNAs including miRNAs. Via direct endoplasmic reticulum-endosomal or endoplasmic reticulum-autophagosomal contacts, newly-formed proteins are transferred to late endosomes/lysosomes/autophagosomes [169], from where they are further processed or released.

4. Nuclear EVs are generated by membrane budding at the inner nuclear membrane [34,35]. They are passaged across the cytosol and released into the extracellular space. Nuclear EVs are rich in pre-miRNAs. Pre-miRNAs need to be processed to miRNAs to exert biological roles.

5. Under conditions of inflammation, structurally and functionally intact free and EV-encapsulated mitochondria and mitochondria fractions are released by stem/precursor cells, namely NSCs [26]. These structures can restore mitochondrial and metabolic dysfunction of inflammatory macrophages [26].

6. Microvesicles are formed by outward budding of the plasma membrane into the extracellular space [21,22]. Microvesicles typically have a diameter of 100-1000 nm. They possess important roles in intercellular communication, particularly under conditions of inflammation and injury. Under inflammatory conditions, microvesicles can traffic damage-associated molecular patterns (DAMPs), including IL1α, IL1β and Rantes, to adjacent cells, which induces cellular dysfunction and injury [62,63]. Under conditions of neurodegenerative diseases, microglia-derived microvesicles can carry misfolded proteins, namely Aβ or α-synuclein, along axonal surfaces, propagating synaptic dysfunction across the brain [66-68].

7. Apoptotic bodies with diameters typically larger than 500 nm are released by the outward budding of larger plasma membrane fractions as part of a cellular decomposition process in apoptotic cell death [21]. Of note, apoptotic cells in addition may also release small EVs within the size range of exosomes that confer pro-inflammatory signals to myeloid leukocytes [170].

8. EV formation can also result from cell migration, during which raptured filopodia may condense to vesicles called migrasomes [22]. Follicular dendritic cells release immune complex-loaded vesicles called iccosomes [22].

Box 2: Composition and signalling properties of EVs

The composition of EVs closely defines their biological roles:

1. EVs abundantly contain membrane-organising proteins including tetraspanins and flotillins. Tetraspanins are a family of 34 transmembrane proteins in mammals which contain four transmembrane domains and two extracellular loops, among which are the classical exosomal markers CD9, CD63 and CD81 [36]. Although each tetraspanin exhibits different tissue and subcellular distributions, they are detected in nearly all cell-types as components of plasma membranes, endosomes, and exosomes. Forming homodimers or heterodimers, tetraspanins are able to assemble to tetraspanin-enriched microdomains (TEMs) or '*tetraspanin webs*'. Unlike lipid-rafts organised by the inner membrane proteins flotillins-1 and -2, which are constituents of caveolae and have been described to be insoluble in the non-ionic detergent Triton X-100 [171], TEMs are Triton X-100-soluble [37]. Tetraspanins arrange the spatial juxtaposition of associated transmembrane proteins and receptors. Clustering with transmembrane integrins, selectins, cell adhesion molecules, cadherins and receptor proteins, tetraspanins regulate biological processes including cell adhesion, motility, proliferation and immune cell activation.

2. Associated with glycosylphosphatidylinositol (GPI)-anchored proteins and binding proteins on the outer membrane leaflet, EVs carry various protein cargos. These proteins include cytokines, cytokine receptors, enzymes, enzyme inhibitors, ephrins, ephrin receptors, death receptor ligands and MHC proteins/complexes [70,172,173]. These cargos have immunomodulatory properties and control cell proliferation, migration and guidance, as well as axonal growth.

3. EVs may contain RNAs, namely miRNAs, pre-miRNAs, lncRNAs and mRNAs [100], as well as DNA, including mitochondrial DNA [26] in their lumen and on their surface. According to recent findings, RNAs might be more abundant in larger EVs than small

EVs in the exosome size, at least when stringent isolation techniques are used [174]. Indeed, bead-capturing experiments using MSC-EVs revealed that EVs recovered by choleratoxin b, a GM1 ganglioside ligand and membrane microdomain marker, contained many exosome markers but hardly any RNAs [175]. Conversely, EVs captured by the globotriaosylceramide ligand shigatoxin b were abundant in nuclear markers and contained large RNA amounts [175].

4. Important functions of EVs have been attributed to lipids, namely phosphatidic acid, phosphatidylserine, and sphingolipids [21]. Phosphatidylserine is highly abundant in the inner membrane leaflet, but serves as signal for phagocyte removal when exposed on outer membrane leaflets derived from apoptotic cells [176]. The sphingolipids sphingomyelin, ceramide and sphingosine-1-phosphate (S1P) crucially control EV budding and release and modulate cell migration and differentiation upon target cell binding [52,177].

Box 3: Tasks for the successful clinical implementation of EVs

The following steps, procedures and principles will have to enable the successful clinical implementation of EVs:

- Cell sources, culturing conditions and EV isolation protocols should be standardized and precisely mimic those in experimental studies. Protocols should not be modified for large scale production of EVs without again confirming therapeutic actions in experimental model systems.
- The biological activity of each EV preparation should be evaluated with well-selected potency assays before EVs are administered to human patients. The biological activity of EVs differs from preparation to preparation, even when the same cell source is used. Accordingly, the biological activity should be monitored in subsequent EV preparations. The activities evaluated should measure biological responses relevant for the presumed modes of action. Depending on the disease context, sets of assays may have to be screened.
- Clinical study protocols shuld closely mirror experimental conditions in animal studies, including disease severities, temporal disease progression, age profile and comorbidities. EV delivery routes should be identical to experimental studies. Treatment dosing and timing should match each other.
- Early clinical (phase-1/2a) studies should vividly examine biological actions of EVs by surrogate markers. In case of systemic EV delivery, surrogate markers in the blood or CSF may prove that a given mode of action (e.g., anti-inflammation) can successfully be targeted in human patients. The surrogate markers ideally reflect readouts of experimental studies and potency assays.
- The subsequent clinical implementation will require randomized, double-blind, placebo-controlled phase-2b/3 studies. These studies will have to evaluate therapeutic responses with endpoints able to detect clinical improvements relevant for daily life.

Figures

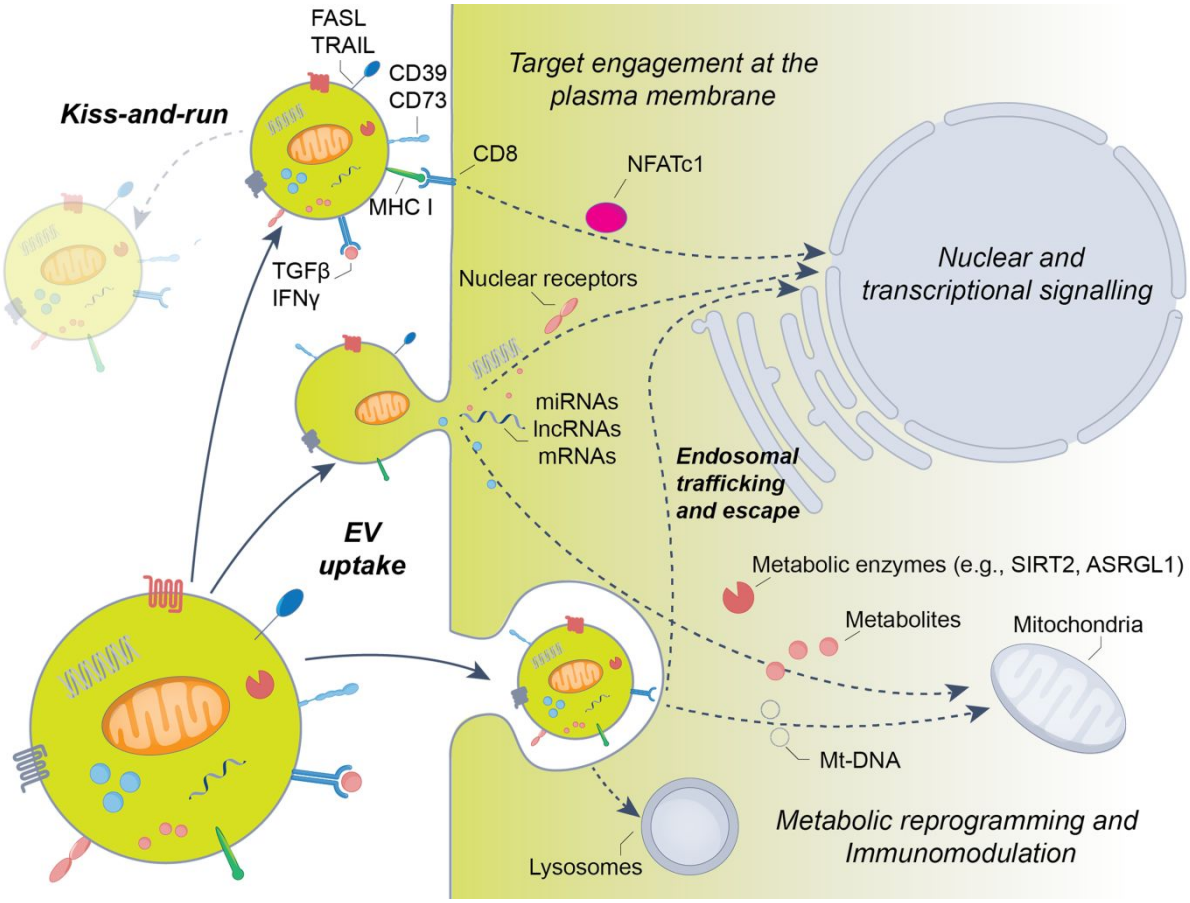


Figure 1. Mechanisms of EV interaction with brain cells. EVs interact with brain cells as mobile ligand carriers binding corresponding receptors on the plasma membrane. As receptor ligands, immunomodulatory cytokines/ chemokines (e.g., TGFβ, IFNγ) play important roles. According to the *kiss-and-run* hypothesis, EVs conferring a signal get separated from their target cell and fade off before activated receptor platforms are endocytosed. Following target engagement, receptor activation is transmitted to the cytosol and nucleus via a variety of signals that include activated receptor platforms (e.g., nuclear EGFR) and signalling proteins (e.g., SMAD2-4, STAT1). In addition to cytokines/ chemokines, endonucleotidases (namely CD73), death receptor ligands (such as FASL and TRAIL), and MHC class-I/II molecules transmit immunomodulatory signals to brain cells. Importantly, *kiss-and-run* signalling does not enable the cellular uptake of luminal EV cargos. The latter process requires plasma membrane fusion or endocytotic EV uptake. Luminal EV contents transferred to brain cells include metabolic enzymes, metabolites, RNA (including mRNAs, miRNAs and lncRNAs), DNA (specifically mt-DNA), mitochondrial membrane fragments and intact mitochondria. Importantly, not all contents transmitted between cells via EVs are involved in intercellular communication. Some contents are transferred for cellular degradation in the lysosome.

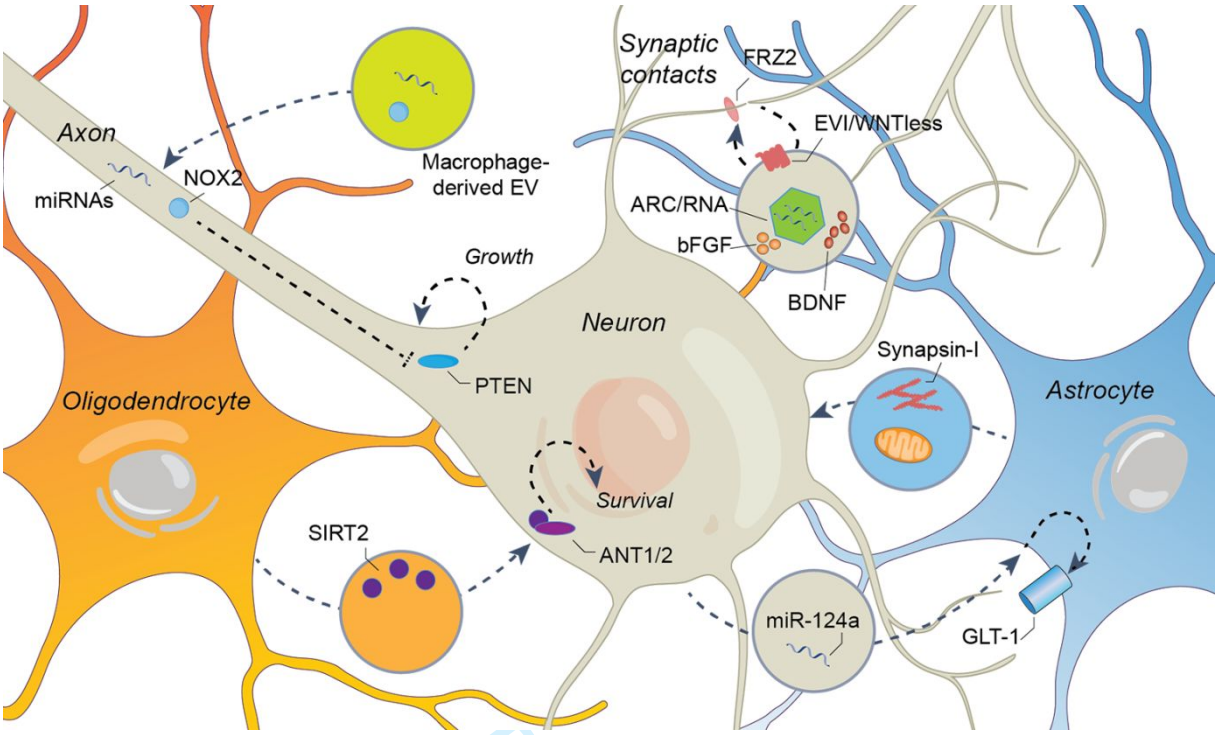
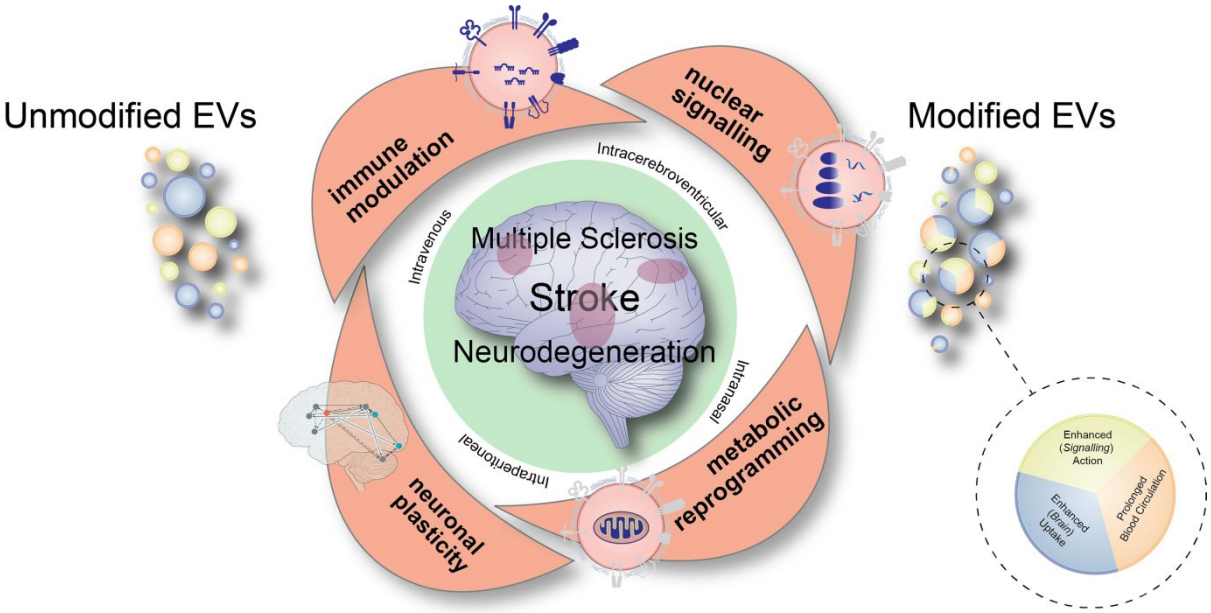


Figure 2. Molecular mechanisms and signals via which EVs induce neuronal plasticity and functional recovery. In the injured brain, EVs carrying a large variety of proregenerative signals are released by neurons, oligodendrocytes and astrocytes. EVs derived from oligodendrocytes (in orange) can transfer NAD-dependent deacetylase SIRT2 to neurons (in grey), which helps to stabilize cellular energy state and prevents axonal degeneration via ANT1/2 deacetylation. Under conditions of ischemia, astrocytes (in blue) can shuttle synapsin-I and functional mitochondria to neurons via EVs, promoting cell survival and neurite growth. In amyotrophic lateral sclerosis, neurons can traffic EV-encapsulated miR-124a to astrocytes, elevating the glutamate transporter GLT1 by transcriptional regulation, which reduces extracellular glutamate levels and reverses synaptic over-activation that otherwise threatens neuronal survival. In the inflamed brain, EVs are furthermore released by macrophages, which can transport functional NADPH oxidase NOX2 and miRNAs to neuronal axons, from which they are retrogradely carried to the perikaryon, inducing axonal regeneration via PTEN deactivation. A unique, recently discovered mechanism is the activity-dependent EV release at the presynaptic membrane at synaptic contact sites. By trafficking the FRZ2 ligand EVI/ WNTless and RNA-loaded capsid-like structures formed by the retrotransposon ARC to the postsynaptic membrane, these EVs can coordinate pre- and postsynaptic growth. The growth factors bFGF and BDNF are major modulators of EV release at synapses.



931

932 **Figure 3. Cartoon summarizing major modes of actions of EVs that are**
933 **therapeutically administered via different routes in diverse disease conditions**
934 **including stroke, multiple sclerosis or neurodegenerative diseases.** The different
935 modes of action, which comprise immune modulation, nuclear signalling, metabolic
936 reprogramming and promotion of neuronal plasticity, synergistically contribute to the
937 recovery-promoting effects of EVs. For therapeutic purposes, unmodified EVs are
938 currently evaluated, as well as EVs that have genetically been modified enabling
939 prolonged EV circulation in the blood, enhanced brain uptake or enhanced signalling
940 action, respectively.

941 ## Table

942 **Table 1.** List of interventional clinical trials on Clinicaltrials.gov investigating the
943 delivery of EVs for the treatment of brain disorders.

944

NCT Number	Source	Conditions	Phase	Country	Status
NCT03384433	Allogenic MSC-derived EVs transfected with miR-124	Ischemic stroke	1/2	Iran	Passed completion date
NCT04202770	Amniotic fluid EVs	Dementia, depression, anxiety	1	USA	Suspended (pending COVID-19 pandemic)
NCT04202783	EVs (not further specified)	Craniofacial neuralgia	1	USA	Suspended (pending COVID-19 pandemic)
NCT04388982	Allogenic adipose MSC-derived EVs	Alzheimer's disease	1/2	China	Passed completion date
NCT05490173	MSC-derived EVs	Neurodevelopmental disorders of prematurity	1	Russia	Not yet recruiting

945

946 Keywords used in this search: exosome, EV, and extracellular vesicle.

947

Glossary

Activity-regulated cytoskeleton-associated protein (ARC): Master-regulator controlling synaptic plasticity, which was suggested to act as phylogenetically repurposed retrotransposon packaging/unpackaging RNA in EVs. ARC might represent an endosomal escape mechanism allowing EV-encapsulated RNA transfer into the cytosol.

Axonal demyelination: Loss of axonal myelin-sheaths associated with oligodendrocyte death during brain injury/inflammation.

Axonal remyelination: Reconstruction of myelin-sheaths by surviving or new-formed oligodendrocytes during brain repair.

Endosome: Organelle involved cellular nutrition, sorting, transport and waste disposal.

Endosomal escape: Ability of luminal endosomal contents (including EVs) to pass the endosomal limiting membrane to accumulate in the cytosol.

Exosome: Small EV (diameter typically 60-150 nm) formed by inward budding of late endosomes or autophagosomes/lysosomes. The vesicle is released into the extracellular space by endosomal plasma membrane fusion.

Experimental autoimmune encephalomyelitis (EAE): Model of multiple sclerosis induced by CNS antigen immunization.

Extracellular vesicle (EV): Heterogeneous class of vesicles released from different cell compartments, which strongly differ in their function and size.

EV isolation: EV enrichment in supernatants/fluids using physicochemical (e.g., differential ultracentrifugation, precipitation, affinity-selection) techniques.

Immune modulation: Shifting immune balance towards cytotoxicity or immune tolerance.

Kiss-and-run signalling: Temporally restricted EV-cell interaction associated with receptor activation followed by protease-triggered EV-cell contact resolution, upon

974 which activated receptors are endocytosed to transmit their signals to the cell. This
975 mechanism does not require cytosolic ligand uptake.
976 **Long non-coding (lnc)RNA:** RNA containing >200 nucleotides which is not translated
977 into proteins with roles in transcriptional/post-transcriptional regulation.
978 **Membrane microdomain:** Assembly of lipids (including cholesterol, sphingolipids)
979 and proteins within membranes that forms the basis for ligand/receptor platforms.
980 **Mesenchymal stromal cell (MSC):** Multipotent cell with capacity to differentiate into
981 osteogenic, chrondrogenic, myogenic and adipogenic cell lineages
982 **MicroRNA:** Non-coding RNA containing 21-23 nucleotides with roles in
983 transcriptional/post-transcriptional regulation.
984 **Microvesicle:** EV formed by outward budding of the plasma membrane (diameter 100-
985 1000 nm).
986 **Middle cerebral artery occlusion (MCAO):** Blockage of a major artery that supplies
987 large parts of the striatum and overlying cerebral cortex.
988 **Mitochondrial transfer:** Cell-to-cell exchange of injured or healthy mitochondria.
989 **Neural stem/precursor cell (NSC):** Multipotent cell with capacity to differentiate into
990 neurons, astrocytes and oligodendrocytes.
991 **Neurological recovery:** Recovery of sensorimotor, cognitive, or language
992 impairments following brain injury/inflammation.
993 **Neuronal plasticity:** Capacity of neurons, axons, dendrites and synapses to change
994 through growth and reorganisation.
995 **Phase-1/2a study:** Early clinical study with focus on surrogate markers and side-
996 effects.
997 **Phase-2b/3 study:** Pivotal clinical study evaluating therapeutic efficacy using
998 predefined endpoints.
999 **Potency assay:** Assay able to predict therapeutic effects of EVs.

Retrotransposon: Gene product copying and pasting RNA into different genomic locations, which includes RNA transport in capsid-like complexes. Within synapses, Arc was found to package/unpackage RNA-loaded capsid-like structures in EVs presumably as a phylogenetically repurposed retrotransposon. Arc might represent an endosomal escape mechanism for EV-RNA.

Transmitophagy: Cell-to-cell exchange of injured mitochondria for remote degradation.

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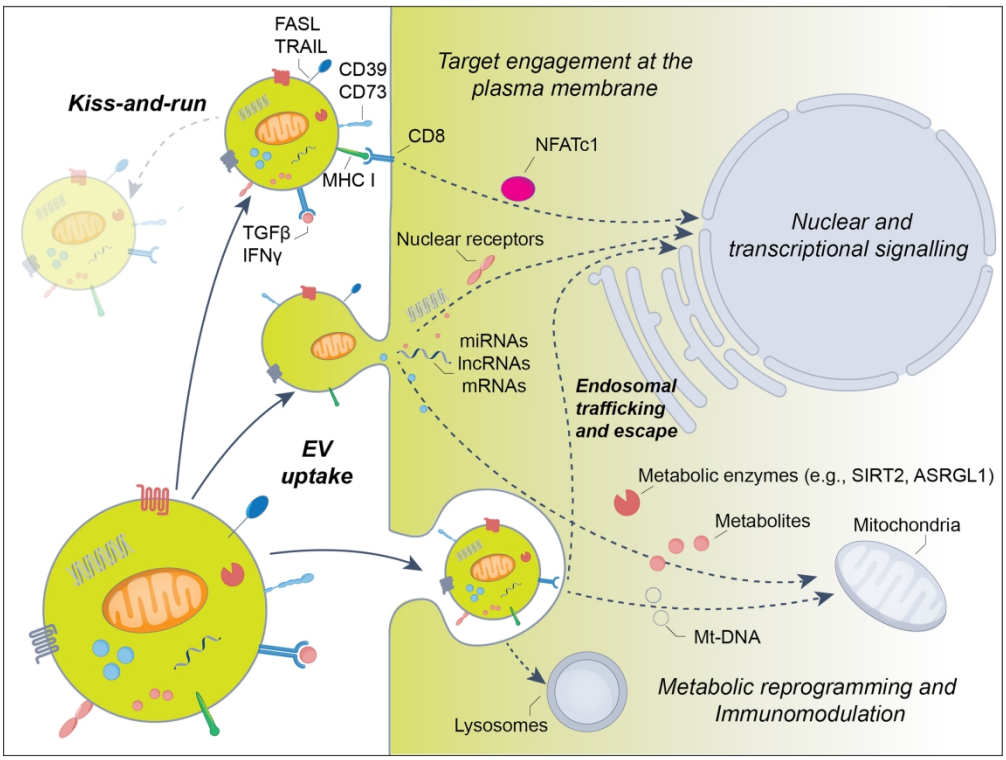

Hermann et al. Figure 1

Figure 1. Mechanisms of EV interaction with brain cells. EVs interact with brain cells as mobile ligand carriers binding corresponding receptors on the plasma membrane. As receptor ligands, immunomodulatory cytokines/ chemokines (e.g., TGFβ, IFNγ) play important roles. According to the kiss-and-run hypothesis, EVs conferring a signal get separated from their target cell and fade off before activated receptor platforms are endocytosed. Following target engagement, receptor activation is transmitted to the cytosol and nucleus via a variety of signals that include activated receptor platforms (e.g., nuclear EGFR) and signalling proteins (e.g., SMAD2-4, STAT1). In addition to cytokines/ chemokines, endonucleotidases (namely CD73), death receptor ligands (such as FASL and TRAIL), and MHC class-I/II molecules transmit immunomodulatory signals to brain cells. Importantly, kiss-and-run signalling does not enable the cellular uptake of luminal EV cargos. The latter process requires plasma membrane fusion or endocytotic EV uptake. Luminal EV contents transferred to brain cells include metabolic enzymes, metabolites, RNA (including mRNAs, miRNAs and lncRNAs), DNA (specifically mt-DNA), mitochondrial membrane fragments and intact mitochondria. Importantly, not all contents transmitted between cells via EVs are involved in intercellular communication. Some contents are transferred for cellular degradation in the lysosome.

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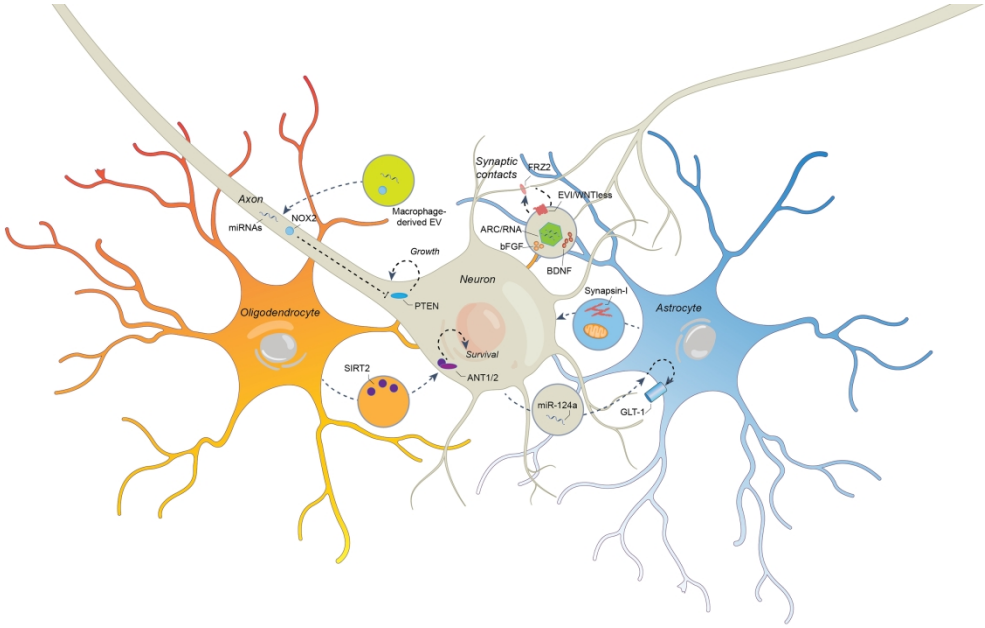

Hermann et al. Figure 2

Figure 2. Molecular mechanisms and signals via which EVs induce neuronal plasticity and functional recovery. In the injured brain, EVs carrying a large variety of proregenerative signals are released by neurons, oligodendrocytes and astrocytes. EVs derived from oligodendrocytes (in orange) can transfer NAD-dependent deacetylase SIRT2 to neurons (in grey), which helps to stabilize cellular energy state and prevents axonal degeneration via ANT1/2 deacetylation. Under conditions of ischemia, astrocytes (in blue) can shuttle synapsin-I and functional mitochondria to neurons via EVs, promoting cell survival and neurite growth. In amyotrophic lateral sclerosis, neurons can traffic EV-encapsulated miR-124a to astrocytes, elevating the glutamate transporter GLT1 by transcriptional regulation, which reduces extracellular glutamate levels and reverses synaptic over-activation that otherwise threatens neuronal survival. In the inflamed brain, EVs are furthermore released by macrophages, which can transport functional NADPH oxidase NOX2 and miRNAs to neuronal axons, from which they are retrogradely carried to the perikaryon, inducing axonal regeneration via PTEN deactivation. A unique, recently discovered mechanism is the activity-dependent EV release at the presynaptic membrane at synaptic contact sites. By trafficking the FRZ2 ligand EVI/ WNTless and RNA-loaded capsid-like structures formed by the retrotransposon ARC to the postsynaptic membrane, these EVs can coordinate pre- and postsynaptic growth. The growth factors bFGF and BDNF are major modulators of EV release at synapses.

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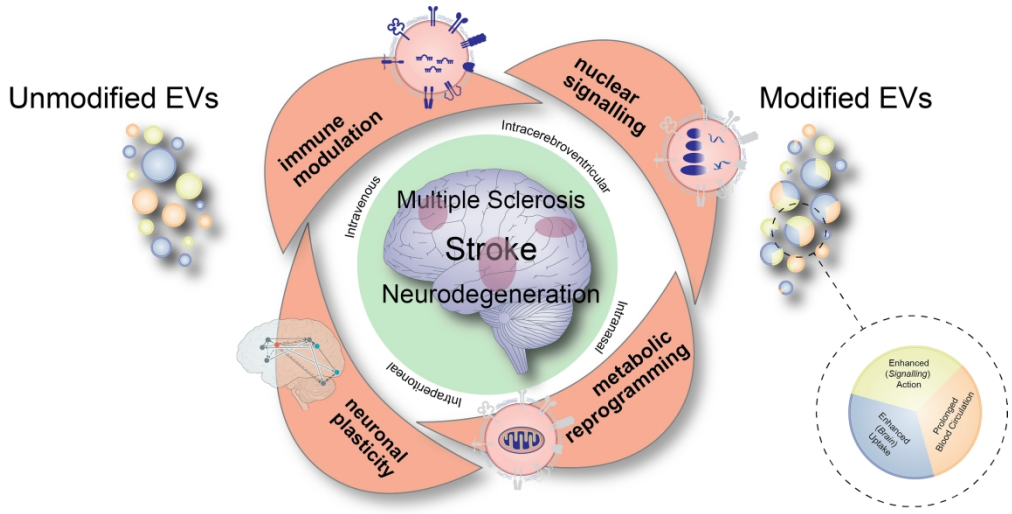

Hermann et al. Figure 3

Figure 3. Cartoon summarizing major modes of actions of EVs that are therapeutically administered via different routes in diverse disease conditions including stroke, multiple sclerosis or neurodegenerative diseases. The different modes of action, which comprise immune modulation, nuclear signalling, metabolic reprogramming and promotion of neuronal plasticity, synergistically contribute to the recovery-promoting effects of EVs. For therapeutic purposes, unmodified EVs are currently evaluated, as well as EVs that have genetically been modified enabling prolonged EV circulation in the blood, enhanced brain uptake or enhanced signalling action, respectively.

381x234mm (200 x 200 DPI)