Published in final edited form as:

Stroke. 2018 May ; 49(5): 1083–1090. doi:10.1161/STROKEAHA.117.018292.

Exosome Therapy for Stroke

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Keywords

Exosome; Stroke; MicroRNA; Mesenchymal Stromal Cells

Introduction

Nearly all cells generate and eject vesicles, and these vesicles constitute major vehicles for intercellular communication. Exosomes, as nanosized vesicles (~30–100 nm in diameter[1]), target cell function by delivering proteins, lipids, and nucleic acids. Exosomes are emerging as a valuable source for disease stage-specific information and as fingerprints of disease progression, and as potential biomarkers in different pathophysiological states[2–5]. However, since exosomes provide a major medium of intercellular communication[6], they likely also impact the treatment of diseases[7, 8]. Recent reports have highlighted the critical application of exosomes as personalized targeted drug delivery vehicles[6, 9]. Exosomes harvested from multipotent mesenchymal stromal cells (MSCs) mediate the restorative therapeutic effects of MSCs for stroke[10]. Here, we review the biogenesis of exosomes, their molecular composition and role as messengers of intercellular communication, and describe using exosomes for treatment of stroke. We also focus on therapeutic effects and underlying mechanisms of action of exosomes as therapeutic vectors for stroke[11], but do not discuss the role of exosomes as disease or injury biomarkers. Capitalizing on the function of exosomes as vehicles for intercellular communication in physiological and patho-physiological conditions such as stroke, provides a paradigm shift and enormous potential for safe and effective therapeutic approaches for stroke and for other diseases/injury.

Exosome biogenesis and content

Exosomes are highly conserved among most eukaryotic organisms, from microorganisms up to mammals[12]. Exosomes originate from the endocytic route, and are formed by the inward

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Disclosures: None

budding of the plasma membrane. The membrane of late endosomes invaginates and forms small vesicles that are pinched off into the endosomal space. The internal intralumenal vesicles with their cargo secreted into the extracellular space are exosomes [13].

Exosomes contain conserved proteins such as CD81, CD63 (membrane associated proteins like LAMP-3), and CD9, Alix and tumor susceptibility gene 101 protein[9], as well as tissue/cell type specific proteins that reflect their cellular source[14]. The exosome membranes are enriched with cholesterol, sphingomyelin, and ceramide[15]. Exosomes contain many biologically active molecules, such as proteins, ribonucleic acids (RNAs), deoxyribonucleic acids, lipids and microRNAs (miRs)[16]. These bioactive molecules mediate exosomal intercellular communication and may target specific cell types, and thereby modify their target cell function by delivering proteins, lipids, and nucleic acids[17]. Most proteins within exosomes derive from parent cell membranes, the cytosol, and Golgi, but rarely from endoplasmic reticulum or mitochondria[12]. Cytosolic proteins remain within the exosomes, and those derived from the plasma membrane are retained in the vesicle membrane, maintaining the same topology of the cell, with potential roles in sequestering soluble ligands[18]. Exosome proteins participate in antigen presentation, cell adhesion, cell structure and motility, and are stress regulators, involved in transcription and protein synthesis, and in trafficking and membrane fusion[19]. Many functional effects of exosomes may be attributed to the transfer of their RNA and miR content[17]. RNAs and miRs are the most relevant cargo in exosomes in terms of the ability of a small number of molecules to influence several proteins/enzymes within one or more cellular pathways in target cells [20].

Exosome isolation and storage

Many methods for exosome isolation have been described[21, 22]. They include: 1) differential centrifugation coupled with ultracentrifugation (UC)[15, 23]; 2) using anti-EpCAM (epithelial cell adhesion molecule) coated magnetic beads immunoaffinity pull-down[24, 25]; 3) density gradient separation[25]; 4) sequential centrifugal ultrafiltration by tangential flow filtration[26]; 5) using ExoQuick-TC[27]; 6) rapid isolation of exosome by alternating current electrokinetic microarray chip device [28]; 7) using a commercially available size exclusion chromatography column (SEC) for rapid vesicle purification[29]. Ultracentrifugation isolated exosomes have the highest protein purity, but the lowest recovery of particles.[30] Specific g-force/k factor usage during differential ultracentrifugation also influences the purity and yield of exosomes[31]. Baranyai et al compared the purity of exosomes using differential ultracentrifugation and SEC. They found that using ultracentrifugation isolation, the diameter of the majority of isolated particles fell into the size range of exosomes; however, albumin was also present in the preparations, when 1h of ultracentrifugation at 4°C was applied[32]. SEC isolation showed good reproducibility, and rapid vesicle purification (less than 10 minutes); however, post-column exosome concentration steps resulted in some protein loss and also leads to low exosome recovery and reduced purity (assessed by the particle-to-protein ratio)[29]. Van Deun et al, [33] compared four methods of exosome extraction: differential ultracentrifugation, OptiPrep density gradient centrifugation (ODG), ExoQuick™ precipitation and Total Exosome Isolation precipitation. They found that ultracentrifugation and ODG showed better clean exosome preparations measured by CD63-immuno-TEM than ExoQuick™ and Total Exosome Isolation precipitation. ODG showed

the purest exosome preparations [33]. However, Taylor et al, indicated that circulating exosomes isolated by ExoQuick precipitation produces exosomal RNA and protein with greater purity and quantity than chromatography, ultracentrifugation, and DynaBeads.[34] Therefore, to-date there is no gold-standard method for exosome isolation, which complicates inter-laboratory comparisons of data [21].

Exosome storage conditions (such as temperature and duration) influence the therapeutic utility of exosomes and their stability. Protein and RNA content of exosomes decrease at 10 days of room temperature storage compared with storage at −70°C and 4°C[35]. Exosomes are stable at 4°C for short term (within 7 days) and when stored at −80°C for at least 90 days[25]. Storage at below −70°C is the most favorable condition for long term preservation of fresh exosomes for clinical application and basic research[35].

Interaction of exosomes with target cells

Exosomes are taken up by target cells by several mechanisms, most of which are mediated by the endocytosis route, such as clathrin mediated endocytosis[36], phagocytosis[37], lipid raft mediated internalization[38], and macropinocytosis[39] as well as by direct fusion with the plasma membrane[40]. Exosomes bind to target cells *via* ligand-receptor interactions, such as integrins, tetraspanins and intercellular adhesion molecules. Tetraspanins as a functionally important component of exosomes, also have specific effects on distinct cell fission and fusion machineries[41]. After binding, exosomal contents are internalized by recipient cells *via* fusion with the plasma membrane of recipient cells and direct release of contents into the cytoplasm, or by exosome internalization by endocytosis into recipient cells. The exosomal tetraspanin web regulates target cell selection, as well as facilitates tailoring exosomes for drug delivery[42]. Human brain endothelial cell derived exosomes (hBEC-Exos) contain several receptors to carry macromolecules across the BBB, including transferrin receptor, insulin receptor, low density lipoprotein (LDL), LDL receptor-related protein, and TMEM30A (a putative antigen for the single-domain antibody), and hBEC-Exos act as cell communication vesicles with both brain astrocytes and cortical neurons[43]. Therefore, exosomes are promising tools to target drugs or biological material to specific cells across different biological barriers, and exosomes mediate cell-to-cell interaction[6, 8].

Exosome effects on immunoresponse

Exosomes communicate with cells, participate in the cascade of antigen presentation, and are implicated in various essential immunological processes such as immune surveillance[44]. Exosomes derived from dendritic cells are antigen-presenting, and have been used for treatment of brain tumor in phase I and II clinical trials[45, 46]. Exosomes can cross the BBB and transfer brain antigens to the periphery, and regulate the peripheral immune system[47, 48]. Exosomes secreted by resident brain cells in response to pathogenic stimuli also influence bystander cells by the transfer of dysregulated miRs that suppress the expression of essential genes in the recipient cells[49]. Stroke and central nervous system (CNS) neuroinflammatory diseases, such as multiple sclerosis, regulate peripheral immune response via exosomes[47, 50]. Microglial-Exos and astrocyte-Exos store and release the inflammatory cytokine IL-1β[51, 52].

Exosomes also transfer pro-inflammatory messages from the periphery to recipient brain cells. Balusu et al. found that the choroid plexus epithelium (CPE) cells sense and transmit peripheral inflammatory signals to the brain via the release of exosomes.[53] These CPE-Exos enter brain parenchyma and are taken up by astrocytes and microglia, inducing miR target repression and inflammatory gene up-regulation[53]. Microglia-derived extracellular vesicles can stimulate neuronal activity and participate in the propagation of inflammatory signals[54]. Exosomes isolated from circulating immune cells from conditions of environmental enrichment increase oligodendrocyte progenitor cell differentiation into myelinating cells in cultured hippocampal slices and promote myelination in vivo when intranasally administered to naïve rats[55]. Thus, exosomes as mediators of neuroinflammation may impact stroke outcome[49].

Exosome effects on thrombosis

Circulating exosomes participate in the coagulation cascade by providing a surface for the assembly of clotting factors [56]. In intracerebral hemorrhage (ICH), CSF and plasma pro-coagulant microvesicle (MV)/Exo levels are significantly increased, and may contribute to stroke pathogenesis[57]. Platelet-MVs/Exos have 50–100 fold higher specific pro-coagulant activity than activated platelets[58]. Circulating MVs/Exos derived from endothelial cells and blood cells may promote procoagulant activity and thrombin generation[59]. However, compared with MVs and apoptotic vesicles, exosomes have reduced coagulation and immunogenic effects[60]. Manipulating thrombosis and the coagulation cascade via exosomes as an intervention for ischemic stroke, warrants investigation.

Therapeutic effects of exosomes on stroke

Exosomes transport cell type-specific molecular cargo extracellularly and over large distances. In addition, the same exosomes may evoke differential response in different cells. Neural released exosomes not only regulate the onset and progression of neurodegenerative and neuroinflammatory diseases, but also may play a role in the regeneration and remodeling of the nervous system after stroke[61]. Neural secreted exosomes contribute to local synaptic plasticity, and also influence neuronal networks by long-range communication within the CNS. Therefore, by inhibiting their release from diseased cells and/or by manipulating their cargo to enable shuttling of secretory RNA, miR or molecules such as cytokines, chemokines, and growth factors[62], exosomes may function as therapeutic agents[56].

Advantages of using stem cell-derived exosomes for stroke therapy

Cell-based therapies for stroke improve neurological outcome[63–66]. The mechanisms of cell-based therapy induced therapeutic effects after stroke are not mediated via cell-replacement or transplanted cell differentiation into brain cells[14, 66]. Secreted paracrine factors from stem cells are the principal mechanism underlying their therapeutic action in stroke[14]. Using stem cell-secreted paracrine factors and cell–free therapy are likely safer alternatives in promoting brain plasticity after stroke, and in neurodegenerative disease. Recently, a variety of cell types have been shown to secrete paracrine factors that are contained within membrane

vesicles, such as exosomes, microvesicles, ectosomes, membrane particles, and apoptotic bodies[18]. Extracellular vesicles have emerged as important mediators of intercellular communication, being involved in the transmission of biological signals between cells[56]. Treatment of stroke and neural injury with extracellular vesicles, i.e. exosomes, harvested from MSCs, rather than the exosome parent MSCs supplants the therapeutic benefits of administration of the parent MSCs[8, 10]. Exosomes are specifically internalized by recipient cells, which avoids a multiplicity of potential concerns associated with administration of living cells, and exosomes provide therapeutic benefit, at least the equivalent of their cellular source. Compared to cell-therapy, the advantages of exosome-based therapy include[9, 14]: 1) low immunogenicity[56]; 2) no vascular obstructive effect, and reduced risk of secondary microvascular thrombosis[14]; 3) systemically injected exosomes are able to cross the BBB and enter the brain parenchyma[67, 68]; 4) the potential to develop large scale cellular factories of engineered therapeutic vesicles[17]; 5) exosomes have higher surface/volume ratio and amplify ligand gated signaling pathways and the transfer of biomolecules from stem cells to target tissues; 6) ability to readily modify exosome microRNA (miR) content. Here, we review the possible application of exosomes for the treatment of stroke, to provide a safe and effective alternative to cell-based therapy[7, 69]. In the following section, we discuss exosome therapy for stroke, which is summarized in Figure 1.

MSC-Exosome (MSC-Exo) for treatment of stroke

Compared to other cell types, the MSC is a prolific exosome producer[70]. Using proteomic analysis, Otero-Ortega identified more than 2000 proteins in MSC-Exo, many of which may be implicated in brain repair[67]. MSCs induce neurological recovery post stroke and neural injury, primarily by paracrine effects via MSC secreted exosomes, which mediate restorative actions of MSCs[10, 71–73]. MSC-Exos taken up by endothelial cells, dose-dependently increase endothelial cell proliferation, migration, and capillary tube formation, as well as impair T-cell function by inhibiting T cell proliferation in vitro[74]. Systemically injecting bone marrow derived MSC-Exo at one day after ischemic stroke or traumatic brain injury (TBI) significantly improves functional outcome, as well as enhances angiogenesis, neurogenesis and neurite remodeling in rats[10, 75–78]. Similarly, hMSC-Exo treatment of stroke increases long-term neuroprotection, promotes neuroregeneration, enhances neurological recovery, and modulates peripheral post-stroke immune responses, but does not affect cerebral immune cell infiltration in mice[79]. MSC-Exos significantly improve functional outcome and reduce structural injury, and show promise in treating global hypoxic-ischemic injury of the fetal brain[80]. hMSC-Exo treatment dose-dependently reduces brain neuroinflammation after TBI in mice[81], and significantly ameliorates inflammation-induced neuronal cellular degeneration, reduces microgliosis and prevents reactive astrogliosis, as well as improves functional recovery in TBI animals[82, 83]. Adipose-derived MSC-Exo treatment initiated at 3h after ischemic stroke was safe, decreasing lesion volume, increasing angiogenesis, demonstrating anti-inflammatory and immunomodulatory capacity, as well as improving neurological function in rats[84]. Intravenous administration of MSC-Exos to rats subjected to intracerebral hemorrhage (ICH) significantly promotes white matter/axonal remodeling identified by fiber tract integrity, white matter repair and axonal

sprouting, as well as decreases neurological functional deficits compared with the control group at 28 days after ICH[67].

Multicellular sources of microparticles or exosomes as a treatment of stroke

In addition to MSC-Exo, exosomes derived from other cell types also induce neuroprotective and neurorestorative effects after stroke. Exosomes derived from human adipose-derived stem cells increase expression of protein-kinaseCδII in immortalized mouse hippocampal cell line and increase neuronal survival and proliferation[85]. Platelet derived microparticles dose-dependently increase endogenous neural stem cell proliferation, neurogenesis and angiogenesis in the ischemic brain and significantly improve neurological functional outcome after ischemic stroke in rats[86]. Microparticles derived from activated platelets contain a variety of growth factors augmenting endogenous neural progenitor cell proliferation and neurovascular remodeling, which may be utilized for stroke therapy[86, 87]. Altmann et al. showed that secretomes derived from rat and human apoptotic mononuclear cells also induce neuroprotective effects identified by decreased lesion volume and improved functional neurological outcome[88]. Therefore, exosomes and microvesicles derived from a variety of cells induce angiogenesis, suppress apoptosis and stimulate cell proliferation, promote neurogenesis and synaptic plasticity, deliver immunomodulatory signals, as well as recruit and/or reprogram cells, restorative events, that in-concert improve functional recovery after stroke.

Exosome treatment effects by transfer of microRNA (miR)

Exosomes contain miRs which play important roles in cell function, disease, and immunomodulation[7, 8, 89]. Systemic administration of MSC-Exos improve neurological functional outcome in animal models of stroke, impacting post transcriptional gene expression and ensuing protein expression in their target cells via transfer of miRs[76]. MiRs are short sequences of non-coding RNA that function in RNA silencing and post-transcriptional regulation of gene expression[90]. Among their myriad of functional properties, miRs also regulate neurovascular remodeling, inflammation and stem cell biology[91]. We also note, that MSC harvested exosomes also stimulate endogenous brain cells to subsequently release miRs, in a “chain-reaction” like manner, ultimately promoting brain plasticity after stroke[92]. In addition, MSCs inhibit macrophage activation by shedding miR-containing exosomes[89]. In vitro data show that exosomes derived from environmental enrichment serum promote oligodendrocyte precursor cell differentiation into myelinating cells and reduce oxidative stress[55]. MSC-Exos promote axonal growth, and inhibition of argonaut 2 protein (a primary miR machinery protein) abolishes MSC-Exos induced axonal growth[75]. MSCs communicate with brain parenchymal cells and regulate neurite outgrowth by transfer of miRs, such as, miR-133b to neural cells via exosomes[76]. Collectively, these data demonstrate that exosomes mediate their functional benefit in stroke at least partially by the transfer of miRs to parenchymal cells.

Modification of exosomes

Exosomes have low toxicity, high stability in the circulation, and high efficiency of transport to donor cells. Modified exosomes have been used as vehicles to transport exogenous chemical compounds to recipient cells. Exosomes are valuable for the delivery of RNA interference and miR regulatory molecules, in addition to other single-stranded oligonucleotides[93]. Intravenous administration of neuronal-targeted exosomes loaded with specific siRNA knocked down their target genes in neurons[94]. Curcumin, an anti-inflammatory agent, can be encapsulated in exosomes[95]. Intranasal administration of curcumin-encapsulated exosomes in ischemic stroke mice significantly reduced astrogliosis and increased the expression of NeuN as well as vascular endothelial tight junction proteins expression when compared to non-treated stroke mice[95].

Exosomes also contain distinct subsets of miRs depending upon the cell type source[96]. Modulation of miRs within stem cells and thereby within exosomes derived from the parent cell, may enhance exosome induced therapeutic efficacy. MSC-Exo can be enriched with specific miRs to enhance recovery of injured tissues[8, 14]. MSCs release functional small RNAs via their exosomes[10, 71–73]. In vitro, the miR-17-92 cluster promotes oligodendrogenesis, neurogenesis, and axonal outgrowth[97]. Treatment of stroke in rat with MSC-Exo and miR-17-92 enriched MSC-Exo both significantly improved neurological functional recovery, but miR-17-92 cluster enriched exosome treatment induced significantly more improvement of functional outcome and enhancement of neurogenesis, oligodendrogenesis and neuronal dendrite plasticity in the ischemic brain than the control MSC-Exo treatment[97]. Tailored exosomes derived from MSCs further enhance neurite growth via the phosphatase and tensin homologue/mammalian target of rapamycin signals by increasing the miR-17-92 cluster[98]. Exosomes derived from miR-133b-overexpressed MSCs as well as miR-17-92 cluster enriched exosomes significantly increase brain plasticity and neurological functional recovery after stroke compared to MSC-Exo treated stroke rats[97, 99]. In vitro data also show that exosomes harvested from astrocytes subjected to oxygen glucose deptrivation (OGD) treated with miR-133b-overexpressing MSC-Exo significantly increased neurite outgrowth in cultured primary cortical neurons compared with the exosomes derived from OGD astrocytes subjected to MSC-Exo-Control[99]. Thus, in vivo and in vitro data suggest that modulating miR content of exosome may be an effective means to amplify the therapeutic effects of exosomes for the treatment of stroke and neurological injury, as well as degenerative diseases[8].

Caveats and future studies

Although exosomes exhibit very promising therapeutic effects, exosome-based therapy for stroke has just recently emerged, and many additional studies are warranted in order to move this therapy to the clinic. Among the considerations and studies to be performed are the following: 1) Scaling production of exosomes for human clinical trials will be required. Recent publications suggest that these production and scaling methods are actively being pursued[21, 22]. In addition, cell culture conditions and storage methods may have a major impact on the exosome content and their functionality. Appropriate exosome isolation methods, storage, and functional read-out systems need to be standardized. 2) Exosome

content, function and activity depend on the generating cells of origin. Therefore, exosome cell source, including age, gender, comorbidities and other factors associated with the exosome generating cells should be optimized. 3) Although initial preclinical studies have shown that a single dose of exosomes administered post stroke is highly efficacious in promoting neurological recovery, we cannot exclude the possibility that multiple dosing, particularly for different types of stroke may further improve neurological outcomes. 4) Dose-response and therapeutic window studies are required. Given the reported very extended therapeutic window for treatment of stroke with cell based therapies, exosomes may likewise provide an extended therapeutic window. Previous studies have shown that exosome treatment initiated at 24h after brain hemorrhage or ischemic stroke, significantly improves functional outcome, decreases lesion volume and promotes axonal/WM remodeling for both hemorrhage and stroke[10, 67, 76, 79, 97, 99]. Adipose-derived mesenchymal stem cell (ADMSC) derived exosome treatment of stroke initiated at 50 mins or 3h after stroke reduced lesion volume and enhanced neurological recovery in rat[84]. Thus, exosome treatment of stroke not only induces neurorestoration, but also promotes neuroprotection. However, some therapeutic interventions provide therapeutic benefit in the acute phase of stroke, but impair regeneration in the chronic phase, and vice versa[100]. Therefore, investigation of the optimal timing of exosome therapy, which may be affected by the parental cell source, is required. 5) Of primary importance, is the performance of safety studies for stroke using exosomes. These studies should include safety in patients with co-morbidities. Particularly, studies should be performed to ensure that the restorative exosomes are not oncogenic, and further promote tumor growth. Enhancing tissue regeneration after stroke in the CNS may increase the risk to activate cancer[100]. Induction of neurovascular remodeling is itself associated with angiogenic and cell proliferative events [101]. In addition, exosomes contain many microRNAs, such as miR-9, miR-223 and miR-126 which not only induce neural repair or axonal growth responses, but are also closely linked with oncogenesis[102–105]. Potential oncogenic features of extracellular vesicles or exosomes are being addressed in the literature [106]. Further studies, however, are warranted to evaluate the oncogenic potential of restorative therapy for stroke. Therefore, safety, time window, dose-response, multiple dosing, and oncogenic potential studies, among others, are required for effective and safe translation of this very promising restorative and neuroprotective therapeutic approach for the treatment of stroke.

Conclusion

Exosomes have multifaceted roles in the regulation of physiological and pathological processes, and importantly may also function as therapeutic agents. Exosomes derived from different cells can induce neuroprotection and neurorestorative effects by modulating gene, protein and miR expression in their target cells and tissues. Exosomes from specific cells, such as MSCs, and likely other cells, reduce inflammation, and increase angiogenesis, neurogenesis and white matter remodeling after stroke. Modified exosomes can be used as vehicles to transport exogenous genes, proteins and chemical compounds to recipient cells. Modulation of genes and miRs in exosome parent cells enhances exosome induced therapeutic efficacy[72, 76, 97]. Therefore, exosomes may potentially be used as personalized targeted drug delivery vehicles[6, 9]. However, we should cautiously and carefully develop this

promising therapy. Safety, time window and dose-response studies are just a part of the range of investigations that will be performed prior to the translation of exosomes into the clinic for the treatment of stroke, and likely other forms of neurological injury and degenerative diseases.

Acknowledgments

This work was supported by National Institute of Neurological Disorders and Stroke R01NS083078 (JC), R01NS099030 (JC), R01NS097747 (JC) and R01NS088656 (MC).

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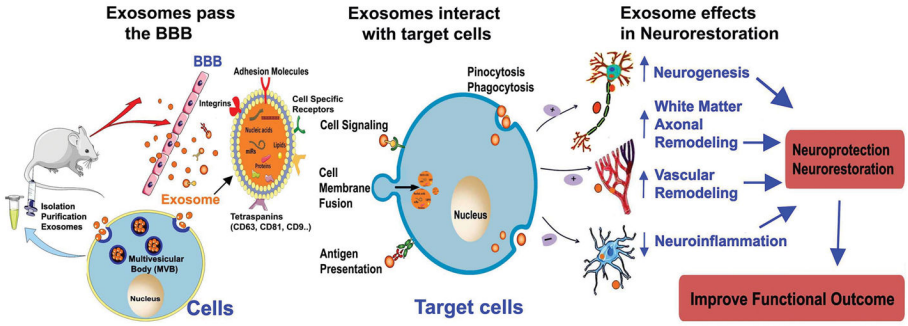

Figure 1. Summary the therapeutic effects of exosome in stroke

1) Intravenous administration of exosomes can pass the BBB and are taken up by endogenous brain cells. **2)** Exosomes contain miRs, mRNA and proteins (such as CD81, CD63, CD9, Alix et al, as well as cell type specific antigens); **3)** Exosomes interact with target cells and transfer their RNA, miR and protein content by: **A:** the endocytosis route (pinocytosis and phagocytosis); **B:** direct fusion with the plasma membrane; **C:** binding to target cell *via* ligand-receptor interactions (such as integrins, tetraspanins and intercellular adhesion molecules). **4)** Exosomes communicate with endogenous brain cells and induce neurogenesis, white matter/axonal and vascular remodeling, as well as inhibit neuroinflammation, and thereby promote neuroprotective or neurorestorative effects, as well as improve functional outcome after stroke.