Engineered extracellular vesicles as brain therapeutics

Miguel M. Lino[1], Susana Simões[1], Francesca Tomatis[1], Inês Albino[1], Angela Barrera[1], Denis Vivien[2,3], Tomas Sobrino[4] and Lino Ferreira[1,5]

[1] *CNC-Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal*

[2] *Normandie Univ, UNICAEN, INSERM U1237, Etablissement Français du Sang (EFS), Physiopathology and Imaging of Neurological Disorders (PhIND), Cyceron, Institut Blood and Brain @ Caen-Normandie (BB@C), 14000 Caen, France.*

[3] *Department of clinical research, Caen-Normandie University Hospital, CHU, Avenue de la côte de Nacre, Caen, France.*

[4] *Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain.*

[5] *Faculty of Medicine, University of Coimbra, Coimbra, Portugal*

Abstract

Extracellular vesicles (EVs) are communication channels between different cell types in the brain, between the brain and the periphery and *vice-versa*, playing a fundamental role in physiology and pathology. The evidence that EVs might be able to cross the blood-brain barrier (BBB) make them very promising candidates as nanocarriers to treat brain pathologies. EVs contain a cocktail of bioactive factors, yet their content and surface can be further engineered to enhance their biological activity, stability and targeting ability. Native and engineered EVs have been reported for the treatment of different brain pathologies, although issues related to their modest accumulation and limited local therapeutic effect in the brain still need to be addressed. In this review, we cover the therapeutic applications of native and bioengineered EVs for brain diseases. We also review recent data about the interaction between EVs and the BBB and discuss the challenges and opportunities in clinical translation of EVs as brain therapeutics.

1- Introduction

Brain pathologies such as stroke, Alzheimer´s (AD), Parkinson´s disease (PD), traumatic brain injury are leading causes of disability[1] in the increasingly aged worldwide population which requires the development of new treatments. In the past two decades, one of the advanced treatments investigated in pre-clinical and clinical tests for brain pathologies was based on cell therapies, however, with limited efficacy due to poor cell survival and engraftment[2]. The demonstration that many of the functional benefits achieved with cell therapies were a result of a paracrine effect mediated by extracellular vesicles (EVs) released from transplanted cells[3-6] has led to an increased interest in EVs as an alternative cell-free therapy. The advantages of EVs are related to their low immunogenicity, low cost and longer shelf-live. Moreover, the growing evidence that EVs mediate the cross-talk within the different cell types of the central nervous system (CNS)[7-9] and between the CNS and the periphery[10-12] and that they have the ability to cross the BBB[13, 14] is motivating several studies either with native or engineered EVs to address brain pathologies.

EVs are naturally released lipidic vesicles that carry a cocktail of bioactive molecules (microRNAs, mRNAs, proteins, lipids) from the parental cell and mediate cell-to-cell communication[15-18]. EVs are a heterogeneous population of vesicles and depending on their biogenesis they can be classified in three different categories: i) exosomes; ii) microvesicles and iii) apoptotic bodies. Exosomes, formed by the inward budding of endosomal membrane during maturation of multivesicular endosomes, are the smallest class of EVs with diameters between 40 and 100 nm and a cup shape morphology according to previous studies using electron microscopy[18]. Microvesicles, are the second largest vesicle type between 100 and 1000 nm in diameter, which are formed by the outward budding and fission of the plasma membrane. Apoptotic bodies are the largest vesicle population, with a diameter ranging from 1 to 5 µm and have a heterogeneous morphology. Apoptotic bodies are released when cells undergo apoptosis and

56 therefore they contain various components from their parental cells often including organelles and
57 DNA fragments[15-18].

58 EVs are important players in the intricate communication between neurons, glia and vascular
59 cells, with an important role in the modulation of homeostasis and also in the progression and outcome
60 of pathology. EVs are involved in physiological processes such as neuronal maintenance and
61 repair[19], synaptic activity[9], neurovascular integrity[20] and maintenance of myelination[21] and
62 also in the onset and progression of different brain pathologies, namely neurodegenerative
63 diseases[22, 23] and stroke[24]. Over the last years, several studies have shown the potential of EVs
64 as nanotherapeutics in the context of brain pathologies[6, 25, 26]. Indeed, this field has witnessed a
65 considerable interest by the academic community with a significant number of studies showing the
66 neuroprotective and regenerative effects achieved with native EVs from different sources[25-29].
67 Because of the limited bioactivity and targeting efficacy of the native EVs, several approaches have
68 been investigated in the last 5 years to engineer their payload and surface for enhanced bioactivity
69 and targeting, respectively[30-36]. Awareness to the potential of EVs as nanotherapeutics has also
70 risen within the biotechnological community, with more than 10 companies working on the
71 translation of EV-based therapies[37]. First clinical trials using EV-based therapies are expected in
72 the coming years for stroke and amyotrophic lateral sclerosis[37].

73 In this review, we propose that engineered EVs will leverage the therapeutic efficacy of EV-
74 based therapies for the brain. We cover the therapeutic potential of native EVs for brain applications
75 followed by a discussion about critical parameters for their therapeutic efficacy. We also describe
76 recent progresses related to the regulation of the BBB by EVs and their migration across the BBB.
77 Finally, we make an overview of the different engineering methodologies developed for the
78 modulation of the content and surface of EVs, particularly focused on the type of strategies adopted
79 for different therapeutic/targeting agents as well as for theranostic purposes. Although recent studies

80 have reviewed the potential of EVs for brain diseases[38-40] the focus of those reviews was not in
81 engineered EVs.

82

83 **2. Native EVs as brain therapeutics**

84 The importance of EV signaling in the context of the brain has been documented for the first
85 time in early 1950s based on electron microscopy studies[41-43]. Since then, the role of EVs secreted
86 by neural cells such as microglia and astrocytes in immune signaling[44, 45], in synaptic plasticity[9],
87 in the specificity of neural cell communication[46], in the spreading of certain neurological conditions
88 including neurodegenerative diseases and brain tumors, has been unveiled (reviewed in ref.[47]). In
89 addition, the therapeutic effect of EVs in the context of the brain, such as stroke[3, 26], traumatic
90 brain injury[48], AD [14] autism [49] and schizophrenia [50], has been reported since 2011. The last
91 decade witnessed a transition from cell-based therapies into EV therapeutics, with a multitude of pre-
92 clinical studies showing the protective and regenerative potential of EVs in different therapeutic
93 applications in the brain (**Table 1**). Because of the number of studies and social impact, the
94 application of EVs in the context of stroke and AD will be highlighted in the sections below.

95

96 **2.1- EV source**

97 Although there is evidence that the source of EVs is determinant to their biodistribution after
98 systemic administration[51], this parameter is yet to be fully explored when developing EVs as brain
99 therapeutics. One might consider taking advantage of an innate brain tropism to leverage the efficacy
100 of an EV-based therapeutic (**Figure 1**), however, to the best of our knowledge no study has
101 specifically compared the brain tropism of EVs from different sources. So far, the studies on native
102 EVs for brain pathologies have not been particularly instigated by an evident brain targeting capacity
103 of these EVs but mainly by their therapeutic effect. The majority of them have used EVs isolated
104 from mesenchymal stem cells (MSCs), either for the treatment of stroke, traumatic brain injury or

AD (**Table 1**), recapitulating the effects already achieved in previous studies focused on cell therapies. Experimental data suggests that the homing mechanism of EVs isolated from MSCs towards injured regions in the brain could be driven by inflammation[52]. Other studies have used EVs secreted by mouse neural stem cells (NSCs) isolated from the subventricular zone[5, 27] or by human NSCs[25, 53] obtained after the differentiation of induced pluripotent stem cells (iPSCs). In this last case, the patient may benefit from his own cells after the generation of iPSCs[25, 53]. It is also important to note that NSCs have been described as having an exquisite tropism for brain with the ability to migrate to the injury site[54]. Preliminary studies using mouse NSC-EVs showed that these EVs accumulated preferentially in the liver and lung as compared to brain when they were administered by intravenous or retro-orbital routes[5].

EVs innate targeting ability seems to be mediated by their surface molecules[55, 56]. For example, the study of metastatic progression revealed that the brain tropism of EVs from breast cells was dictated by the expression of integrin β_3[56]. Further studies on surface molecules mediating targeting to the brain could provide clues for the choice of cell sources or engineering strategies for enhanced brain tropism.

2.2-Administration route

The administration route is an important parameter in the study of the biodistribution of a drug and this is also relevant for EVs (**Figure 1**). EVs have been administered in different animal models by intracerebral[57-60], intravenous[6, 25-29, 48, 53, 61], intranasal[62, 63], intra-arterial, intraperitoneal[34] and retro-orbital[5, 64] routes (**Table 1**). Few studies have compared the amount of EVs accumulated in the brain using different administration routes[63] and using different EV sources[51]. Usually, EVs are cleared by organs such as the liver, kidneys, lungs and spleen[51, 65-67].

129 The intracerebral injection assures that the majority of the EVs will be taken up by the cells of
130 interest, even if a certain level of diffusion to other parts of the brain is observed. Indeed, two studies
131 have reported intracerebral administration of EVs harvested from plasma[57] and HEK293 cell
132 line[58] in the hippocampus of AD mice[57] and non-diseased mice[58] and showed the capacity of
133 the vesicles to diffuse from the place where they were injected. Another way of administration that
134 was exploited to target the brain with EVs, and in particular the traumatic brain injury, was the retro-
135 orbital route[64]. However, both the stereotactic and the retro-orbital injections are invasive
136 procedures requiring a clinical intervention and thus they are not desirable approaches for human
137 patients. Intraperitoneal administration is not a desirable route in humans and results in mice indicate
138 that this route does not enhance the accumulation of EVs in the brain as compared to other organs
139 such as the liver, spleen, lungs and kidneys[34]. Experimental results indicate that intranasal delivery
140 may be an interesting possibility to deliver EVs in the brain. For example, intranasal administration
141 of EVs labeled with gold nanoparticles led to increased concentration of gold in the brain 1 h and 24
142 h post administration[63]. In particular, the amount of gold found in the brain 1 h after the intranasal
143 injection was twice the gold recovered at the same time point after the intravenous administration.
144 The difference in the accumulation became even more evident 1 day after the administrations,
145 probably due to the faster clearance of the vesicles from the brain when injected intravenously. The
146 main problem of intranasal administration is precisely the requirement for low volume for liquids and
147 low mass for powders as well as the presence of enzymes in the nasal cavity that can affect the
148 stability of EVs [68].

149 Most studies using EVs for brain pathologies have done their administration by intravenous route
150 (**Table 1**). The intravenous administration is less invasive than intracerebral administration, but also
151 characterized by fast clearance in the bloodstream and liver (both mediated by macrophages[69]) and
152 low accumulation in the brain. Using a highly sensitive PET/MRI imaging system to monitor *in vivo*

153 EVs isolated from human mononuclear cells and administered by intravenous route, most of the EVs
154 accumulated in the liver while 0.5% of the injected dose was found in the brain[66].
155 The intra-arterial administration of EVs may be more effective than the intravenous for brain
156 targeting because the EVs are delivered in the proximity of the brain while reducing the clearance by
157 the other organs. EVs derived from human bone marrow mesenchymal stem cells (BM-MSCs) were
158 intra-arterially injected in rat models with focal brain injuries simulating the conditions of ischemic
159 stroke[70]. The presence of macrophages was reduced in the damaged zone after treatment with EVs
160 compared to the controls, together with the fact that the astrocytes activation and pro-inflammatory
161 cytokines expression were reduced.
162
163 **2.3- EV dose: single *versus* multiple administrations**
164 Besides the delivery route and cell source, the dose of EVs and the administration regimen are
165 very important parameters for the efficacy of the treatment (**Figure 1**), already demonstrated in a
166 different context[71]. Both for the treatment of stroke and AD with native EVs, different doses and
167 administration regimens have been used (**Table 1**). However, a comparison of the effect of distinct
168 dose schemes for brain pathologies within the same study has not been reported yet. One additional
169 challenge to a more consistent comparison between studies is related to the units in which the
170 administered dose is reported, either in protein content, particle number or initial number of secreting
171 cells. As a matter of fact, the amount of protein in EV samples may be variable depending on the
172 source as well as the isolation and purification methods[72].
173 In the case of AD, reduction of Aβ oligomers in rats[60] and in a transgenic mouse model[59]
174 has been achieved by single local administration of native EVs with doses ranging from 4 to 22.4 µg
175 respectively. For stroke therapy, doses of 10-100 µg and 30-100 µg were tested in mice[27, 29] and
176 rats[28, 73-75], respectively. While some studies report a single administration of EVs, others use
177 multiple administrations motivated by the rapid clearance of EVs from the infarct site observed 24 h

178 after the first intravenous administration[53] and with the purpose of achieving prolonged
179 accumulation in the brain. The time of administration was variable, ranging from 2 h[27] up to 48
180 h[74] after ischemia in single dose treatments[28, 74] and from 2 h up to 5 days in multiple dose
181 regimens[25, 53]. It has been suggested that starting the treatment in the acute phase, as early as 2 h
182 after stroke, promotes a downregulation of the systemic inflammatory response in the blood, with an
183 increase in M2-type macrophages and Treg populations and a concurrent decrease in Th17
184 lymphocytes, thus establishing an appropriate external milieu for successful brain remodeling[53].
185 Importantly, high doses of EVs are not necessarily better from a therapeutic point of view. Increased
186 neuronal densities were observed in stroke mice treated with medium dose of NPC-EVs or MSC-EVs
187 but not low or high doses[5].

188 Further preclinical studies will be needed to define optimal doses and administration times of
189 therapeutic EVs as they are crucial aspects for the success of the therapy. For example, for the
190 treatment of stroke three time windows for therapeutic intervention are defined and the benefits that
191 can be attained in each one are distinct in terms of neuroprotection and brain remodeling[76].
192 Additionally, multiple administration regimens might be needed to achieve effective treatments for
193 neurodegenerative diseases.

194

195 **2.4- Are EVs able to cross the blood brain barrier?**

196 It has been recently shown that EVs are able to regulate the integrity of the brain vasculature
197 through specific microRNAs[20, 77, 78]. For example, neurons secrete exosomes enriched in miR-
198 132, capable of being translocated to endothelial cells and regulate the expression of vascular
199 endothelial cadherin. Impairment of neuronal exosome secretion or knockdown of miR-132 in
200 zebrafish larvae caused intracranial hemorrhage[20]. In addition, there are experimental evidences
201 that EVs may mediate *in vivo* the transport of proteins through barriers. For example, it has been
202 reported that EVs secreted by red blood cells from PD patients can transfer α-synuclein across the

203 BBB *via* adsorption mediated transcytosis (AMT)[23]. Using wheat germ agglutinin (WGA), an
204 inducer of AMT, the amount of radiolabeled EVs increased significantly in the brain after intravenous
205 injection in the jugular vein of mice[23].

206 Although there is data suggesting the bidirectional transport of EVs across the BBB[11, 12,
207 23], studies about the detailed mechanisms involved in BBB crossing are still very scarce.
208 Experimental data indicate that EVs derived from HEK293T cells are taken up by caveolae and
209 clathrin-dependent endocytosis by a monolayer of mouse brain microvascular endothelial cells but
210 are not able to cross it by transcytosis[79]. Yet, if the cell monolayer is treated for 6h with TNF-α and
211 then cultured for additional 12h without the inflammatory stimulus, 10% of the initial EVs are able
212 to cross the barrier via transcellular route[79]. It is also evident that certain type of EVs (e.g. cancer
213 cell-derived) are able to modulate the endocytic pathway in brain endothelial cells to facilitate their
214 transcellular transport[80]. For example, EVs are able to decrease the expression of the late
215 endosomal marker Rab7 in brain endothelial cells, and thus accumulate preferentially in endosomes
216 labeled for early endosome marker (EEA1) and with rab11, a marker of recycling endosomes[80].

217

218 **2.5- Mechanism of action**

219 Functional benefits of vesicles secreted from MSCs[6, 28, 74, 75, 81] and NSCs[25, 27, 53] have
220 been observed in mice[6, 27], rats [28, 74, 75] and pigs[25] with cerebral ischemia induced by the
221 occlusion of the middle cerebral artery. MSC-derived EVs have been reported to reduce infarct
222 volume, improve functional recovery and to increase angiogenesis and neovascularization[6, 28],
223 reduce astrocyte activation[53, 74] and modulate peripheral immune responses, and these effects were
224 comparable to the ones described with MSC transplantation[3, 26]. Likewise, EVs from NSCs have
225 a therapeutic effect by altering the systemic immune response[53].

226 Most of the EV therapeutic effects reported in the context of stroke are likely indirect
227 (extracranial organs), i.e., EVs seem to mediate a downregulation of the systemic inflammatory

228 response after stroke which in turn may lead to a reduction in the infiltration of leukocytes in the brain
229 and finally a reduction in blood brain permeability and neurologic inflammation[5, 53] (**Figure 2**).
230 Indeed, EVs from different sources (MSCs[5, 53], NSCs[5]) applied by different administration
231 routes (intravenous or retro-orbital injections[5]) accumulate preferentially in the liver and lungs, as
232 determined by imaging platforms. NSC-EVs have been found to promote macrophage polarization
233 toward an anti-inflammatory M2 phenotype while increasing the regulatory T cell population and
234 decreasing proinflammatory T helper 17 cells[53]. In fact, both local and systemic inflammatory
235 response to the disruption of tissue homeostasis dictate the extent of brain lesion after stroke[82] and
236 may be linked to peripheral organ dysfunction[83]. For instance, lymphocyte recruitment has been
237 associated with the progression of cerebral ischemia-reperfusion injury. Specifically, infiltrating γδT
238 lymphocytes producing interleukin-17 have an important role in the evolution of brain infarction in
239 delayed ischemia-reperfusion injury[84], when apoptotic neuronal death occurs in the penumbra
240 region. Thus, the immunomodulatory effect of EVs might contribute to establish a milieu more
241 favorable for brain remodeling.

242 The molecular mechanisms involved in the therapeutic effect of EVs in the context of stroke
243 have been attributed, in most cases, to miRNAs within EVs (**Figure 2**). The direct effect of EVs in
244 brain cells was evaluated, in most cases, by *in vitro* assays. For example, miR-133b-containing EVs
245 secreted by MSCs enhanced neurite outgrowth by the suppression of RhoA in neurons and inhibited
246 connective tissue growth factor in astrocytes[3]. In addition, miR-124-containing EVs secreted by
247 M2-microglia cells induced *in vivo* neuronal survival by regulating its downstream target ubiquitin-
248 specific protease 14; however, a direct correlation between transfected cells and downregulation of
249 ubiquitin-specific protease 14 has not been provided and thus is not clear whether the effect is a direct
250 or systemic effect[29].

255 The therapeutic effect of native EVs was also reported in the context of neurodegenerative
256 pathologies (**Figure 2**; **Table 1**). Amyloid beta peptide (Aβ) aggregation is known as part of the

257 pathophysiology of AD and its clearance has been proposed as a therapeutic approach [85, 86]. Both
258 MSC-[87-89] and cancer cell lines[60, 90]-derived EVs have been evaluated in the context of AD as
259 therapeutic strategies. EVs from mouse neuroblastoma cells can reduce the synaptic-plasticity
260 disrupting activities of Aβ by sequestration of Aβ oligomers *via* exosomal surface proteins such as
261 the prion protein[60]. Glycosphingolipids, a group of membrane glycolipids and highly abundant in
262 the EVs from mouse neuroblastoma cells also play a role in the sequestration of Aβ oligomers, acting
263 as scavengers of Aβ, which are then incorporated into microglia for degradation[90]. The therapeutic
264 effect of mouse[88] and human[87, 89] MSC-EVs in AD was also studied using *in vitro*[87] and *in*
265 *vivo*[88] models. The results show that these EVs induce neuroprotection from oxidative stress
266 induced by Aβ oligomers and decreased activation of microglia.[87, 88] The effects of MSC-EVs
267 were found to be partially mediated by active enzymes packaged within these EVs, namely catalase,
268 which confers anti-oxidant properties acting as reactive oxygen species scavenger[87], and
269 neprilysin, a type II membrane-associated metalloendopeptidase involved in the proteolysis of
270 Aβ[89].

271

272 **3- Engineered EVs as brain therapeutics**

273 Despite the progresses done in the last 10 years in the pre-clinical use of native EVs as brain
274 therapeutics, further improvements are needed to maximize their therapeutic effect and facilitate their
275 clinical translation. The first is related with EV bioactivity. Native EVs are heterogeneous, even when
276 harvested from the same cell source, and thus the enrichment of EV content in a single therapeutic
277 entity, with the highest brain activity, may potentiate their therapeutic effect. The second is related
278 with EV targeting. Only a small percentage (typically below 5%)[66, 67, 91] of EVs accumulate in
279 the brain after systemic administration. Advances in engineering the surface of EVs to increase
280 travelling distance and targeting specific cell surface epitopes are needed to maximize their
281 local/direct effect in the brain. By other hand, the evaluation of the *in vivo* targeting and therapeutic

282 processes of EVs requires the development of very sensitive and high-resolution analytical and
283 imaging platforms, respectively. Therefore, engineering approaches to tailor EV bioactivity[27, 35,
284 92-102], targeting[14, 32, 103-111] and tracking[35, 63, 104] have been developed to address the
285 previous challenges. These approaches can be performed in EVs after their isolation (post-isolation
286 method) or in the EV-producing cells (either by genetic engineering[35], metabolic and residue-
287 specific protein labelling[112] or by incubating cells with exogenous molecules[30] or
288 nanoparticles[113]) (**Figures 3 and 4**; **Tables 2 and 3**). Although many methods to rapidly and
289 efficiently engineer EVs with functional groups, nucleic acids and bioactive proteins and peptides
290 exist[14, 98, 114, 115], developing methods to engineer EVs without negatively impacting their
291 function remains challenging. In this section, we will discuss how techniques such as genetic
292 engineering, exogenous delivery and chemically-inspired methods have been explored for the
293 modification of the surface and content of EVs for brain drug delivery.
294

295 ### 3.1- Content modulation

296 In the last 5 years, there was a blast of reported methods for loading of functional molecules into
297 EVs[27, 93, 94, 97, 99, 116, 117] (**Table 2**). The majority of these methods used genetic manipulation
298 (by plasmid transfection) of the EV-secreting cells to efficiently control the content of EVs[27, 94,
299 97, 100, 116] (**Figure 3**). Plasmid transfection may be achieved by electroporation[100] or by
300 incubation with transfection reagents[35, 98]. Hence, functional proteins, mRNAs[35], microRNAs
301 (miRNAs)[27, 92, 97, 100] and other short noncoding RNAs[94, 116] were introduced in EVs to
302 regulate gene expression in *in vitro* and *in vivo* human disease models. Direct modulation of isolated
303 EVs has been adopted for the loading of small drugs[95, 99, 104, 118-120] and also for the packaging
304 of proteins[93, 121] and small non-coding RNAs[30] in EVs (**Figure 3**). In all these strategies, some
305 aspects should be considered. First, the cargo that can be loaded into the EV depends in the size of
306 the molecules. A large number of small molecules like miRNAs or small drugs can be encapsulated

in the EV; however, for large molecules like mRNA or proteins, the capacity of the EV is limited. Second, a larger quantity of cargo does not necessarily imply increased biological relevance. Each EV population is very heterogeneous and some sub-populations may have higher cargo levels than others. In addition, the impact of the modulated EVs in the recipient cell will depend ultimately in the internalization efficiency, intracellular trafficking and pathways modulated by the EV-based biomolecules.

3.1.1- Proteins

The selection of the strategy to load therapeutic proteins into EVs will ultimately depend on their target application. Transfection of EV-secreting cells with plasmids has been adopted for the generation of EVs loaded with enzymes, namely catalase[121], Cre-recombinase[98] and lysosomal enzyme tripeptidylpeptidase-1 (TPP-1)[34]. Elegant approaches took advantage of plasmids coding fusion cargo proteins with tags or proteins that enable the loading of the cargo protein in EVs with higher efficiency. For example, a protein-protein interaction module activated by blue light was developed by fusing a cargo protein with photoreceptor cryptochrome 2 and conjugating CRY-interacting basic-helix-loophelix 1 with a representative marker of exosomes, CD9 protein[101]. Optogenetics allowed to control the loading of the protein in EVs and its detachment from the EV membrane into the intraluminal space, with an efficiency 4 times higher compared to a commercialized method for protein loading. The system was validated for the intracellular delivery of mCherry, Bax, super-repressor IkB protein and Cre recombinase enzyme as functional proteins into the target cells *in vitro*[101]. Post-isolated EVs have also been used to load therapeutic enzymes for the treatment of neurodegenerative diseases with loading efficiencies up to 26%[121]. The post-isolation methods seem to compare favorably to the genetic modulation of EV-secreting cells in terms of loading magnitude. For example, the (i) transfection of EV-producing macrophages with a plasmid for the expression of TPP1 protein or (ii) direct loading of TPP1 into native EVs isolated from

332 macrophages using sonication or permeabilization with saponin showed in both cases EVs with
333 enzymatic activity; however, the post-isolation strategy yielded EVs with 5 to 7 times higher amount
334 of TPP1 compared to EVs obtained from the genetic manipulation of EV-secreting cells [93]. TPP1-
335 loaded EVs were able to passively target and accumulate in the lysosomal compartments of neural
336 cells both *in vitro* and *in vivo*. Intraperitoneal administration of EV-TPP1 reduced neuroinflammation
337 and astrocytosis, and at the same time has increased the life span of LINCL mice[93].

338

339 **3.1.2- Non-coding RNAs**

340 Two different strategies may be used to load non-coding RNAs in EVs: (i) chemical (e.g.
341 transfection with chemical agents)[122, 123] or physical (e.g. electroporation)[14, 30] strategies after
342 the isolation of EVs and (ii) transfection of EV-secreting cells with plasmid-encoding noncoding
343 RNAs or, directly, with non-coding RNAs[92, 94, 100, 122]. In the first strategy, EVs have been
344 loaded with siRNAs[14, 122] and miRNAs[27, 30, 92, 100] for the treatment of morphine- or cocaine-
345 mediated disorders[122, 123], LPS-induced microglial proliferation[94], AD[14] and stroke[30, 92,
346 100]. The loading of EVs with non-coding RNAs by electroporation had efficiencies between 1%[30]
347 and 25%[14]. The enrichment of the non-coding RNA within the EV can be 3,000 higher than the
348 one found in native EVs[30]. In the second strategy, EV-secreting cells have been transfected with
349 plasmid-encoding miRNAs[27, 100] via electroporation or with siRNAs[94, 124] and miRNAs[92]
350 via incubation with transfection reagents[92, 94, 124]. From the 2 strategies, the most popular one is
351 by the regulation of the EV-secreting cell since the genetic manipulation of cells is an established
352 method in biology. Yet, this strategy has some limitations including the fact the levels of the
353 biomolecule of interest may not reach the desired concentration within the EV and the fact that the
354 genetic manipulation of the cell may alter the content of the EV.

355 A popular source of EVs for miRNA modulation is the one obtained from MSCs. This is part
356 due to the potential of MSCs for the treatment of several brain pathologies[2]. EVs have been

modulated with miR-17-92 cluster[100], miR-126[92] and miR-124[30], which are found enriched in the CNS and play important roles in neuronal cell function. EVs enriched for these miRNAs have shown enhanced neurogenesis and vasculogenesis after stroke. For example, delivery of EVs enriched with miR-126 in a rat model of ischemic stroke was able to increase by a factor of 2 both neurogenesis and vasculogenesis and decrease neuronal apoptosis more than 4 times compared to non-modulated EVs[92].

3.1.3- Small molecules

The motivation for the development of EV-based delivery vehicles of small molecules for brain pathologies is explained by the expectations of increased bioavailability and stability of the drug as well as increased accumulation of it in the brain. Loading of small molecules such as anti-inflammatory/anti-oxidant compounds (e.g. curcumin) [62, 99], neurotransmitters (e.g. dopamine)[95], anti-cancer drugs (e.g. paclitaxel, doxorubicin)[120] into EVs have been attempted to develop new therapies for neurodegenerative (e.g. AD[99, 118] and PD[95]) and brain cancer diseases[119, 120]. The loading of the drugs in EVs was performed *via* incubation of secreting cells or post-isolated EVs with the drugs of interest[95, 99]. These methods reported variable encapsulation efficiencies, from 15%[95] to 84.8%[99]. Dopamine replacement is a known therapy for the early stage treatment of PD[125], which has an important role in preventing neurological impairment. The use of blood-derived EVs has been reported as an efficient carrier of dopamine to the brain. Dopamine-loaded EVs showed much better therapeutic efficiency, increasing more than 15-fold the accumulation in the brain in a PD mouse model with lower systemic toxicity after intravenous administration than a free dopamine therapy[95]. Curcumin, a small molecule with effect on the regulation of Tau phosphorylation[126] and oxidative damage of beta-amyloid in AD, was loaded in EVs secreted by lymphocytes, taking advantage of the specific active targeting inherited by the lymphocyte function-associated antigen 1 (LFA-1) and endothelial intercellular adhesion molecule 1

382 (ICAM-1) present in brain endothelium[99]. The results suggested that EVs crossed the BBB *via*
383 receptor-mediated transcytosis to access brain tissue and inhibit Tau phosphorylation through the
384 AKT/GSK-3β pathway.

385

386 **3.1.4- Nanoparticles**

387 EVs have been used to encapsulate therapeutic nanoparticles (in general nanoparticles containing
388 a therapeutic agent) with diameters between 10 nm[104] and 150 nm[127] to facilitate their transport
389 through the BBB. The loading of EVs occurred by two processes: (i) after their isolation followed by
390 an electroporation procedure with nanoparticles[104] or (ii) after transfection of EV-secreting cells
391 with nanoparticles[127]. In process (i), EVs were transfected with an anti-inflammatory drug
392 (curcumin) and superparamagnetic iron oxide nanoparticles (SPION) that could be remotely guided
393 by an external magnetic field. The hybrid EVs were delivered to the brain, more specifically to the
394 tumor and the synergistic effect of curcumin and the hyperthermia induced by the magnetic field in
395 the nanoparticles exhibited a strong antitumor effect on glioma cells[104]. In other cases, EVs were
396 transfected with SPION to guide their transport to the brain, while allowing MRI detection (the
397 nanoparticles acted as contrast agent)[113]. In process (ii), the transfected cells showed an increase
398 in autophagy which in turn lead to an increase in nanoparticle exocytosis. Approximately 60 µg of
399 EVs were secreted from 10^7 cells while 2 µg of EVs were secreted from the same number of cells
400 without nanoparticle transfection[127]. The hybrid EVs showed efficient cellular uptake and strong
401 cytotoxicity against *in vitro* bulk cancer cells, as well as cancer stem cells, and showed higher
402 accumulation in tumors (more than 3 times) following intravenous administration in animal models.
403 Hence, EVs biomimetic nanoparticles are emerging as new candidates for *in vivo* enrichment of
404 therapeutic EVs.

405

406 **3.2- Surface modification/functionalization**

EV surface modification has been reported to allow/enhance their (i) stability in the bloodstream, (ii) targeting a specific organ/tissue and (iii) *in vivo* monitoring (see section 3.3) (**Figure 4**; **Table 3**). To enhance EV stability in the bloodstream (i.e. to prolong their circulation times), the surface of EVs has been conjugated with anti-fouling agents such as poly(ethylene glycol)[128, 129]. This has increased 6-fold the lifetime of EVs in the blood[128]. To enhance EV targeting properties, the surface of EVs has been modified with peptides, including RVG[33], cyclo(RGDyK)[103, 111, 119] and neuropilin-1 peptides[104], for brain targeting. The use of RVG peptide has been inspired by the mechanism of neurotropic viruses, which have the ability to cross the BBB and infect brain cells[33]. Indeed, RVG peptide is known to interact with acetylcholine receptor and enable viral entry into neuronal cells. The use of cyclo(RGDyK) was motivated by the fact that this peptide targets integrin $\alpha_v\beta_3$ in reactive cerebral vascular endothelial cells after ischemia[103]. It has been estimated that between 52[104] and 263[103] peptides can be immobilized per EV. The modification of the EVs with peptides increased their brain targeting in almost 3-fold[33] or 10-fold in the ischemic hemisphere in relation to the non-ischemic hemisphere[103]. Yet, it should be noted that most studies do not report the absolute quantification of EVs in the brain.

The EV surface can be altered by two strategies: (i) by physico-chemical procedures after the isolation of EVs[33, 66, 103, 104, 111, 112, 119] or (ii) by genetic modification of the EV-secreting cells[14, 30, 32, 35, 79, 114]. Regarding the first strategy (post-isolation strategy), EV surface modulation can be achieved by two means: (i) by initially conjugating the molecule (peptide, protein) to a phospholipid, forming micelles and mixing them with EVs to allow a temperature-dependent transfer of the peptide/protein to the EVs[33, 119] or (ii) *via* direct reaction of bioactive molecules or linkers with functional groups naturally present on EV membrane (namely amines[130], carboxylic acids[131] and thiol[66] groups; the concentration of thiols in EVs has been estimated to be 1500±200 per EV[66]) or artificially introduced in EVs, such as azide groups, via residue-specific labeling of proteins (e.g. an exogenous amino acid L-azidohomoalanine replaced methionine in newly

432 synthesized proteins) in parental cells, which are then incorporated in the exosomal membrane[112]
433 **(Figure 4)**. In this last approach, the chemical modification of EV surface with molecules has been
434 mainly achieved by click chemistry. This chemistry is rapid, high specific, and compatible in aqueous
435 buffers and thus an attractive strategy to modify the surface of EVs[103, 104, 111]. Regarding the
436 second strategy, EV-secreting cells can be genetically manipulated in order to overexpress a certain
437 protein (such as Lamp2b fused to RVG peptide[14, 35, 116] or T7 peptide[114]) and then EVs are
438 harvested from these cells. The benefit of this strategy is the isolation of large number of EVs after
439 the generation of the genetically modified cell while the post-isolation strategy (the first one) requires
440 laborious modification steps each time that EVs are isolated. Yet, this strategy has also limitations.
441 For example, it does not allow the incorporation of non-natural molecules and it is relatively difficult
442 to control the density of the targeting molecule in the surface of the EVs. Therefore, it is likely that
443 the post-isolation strategy allows a better control of the type and density of the targeting epitope in
444 the surface of the EVs.
445

446 **3.3- Theranostic strategies**

447 Besides modification with targeting peptides, the membrane of EVs has been modified with
448 different reporter systems for *in vitro* and *in vivo* tracking of EVs using direct methodologies such as
449 insertion of lipophilic dyes or chemical modification of the membrane[66] and indirect methodologies
450 (i.e. by modification of the EV-secreting cell) for the generation of membrane-bound
451 bioluminescence reporter systems[35, 79]. In this context, several imaging modalities have been
452 described to track the labeled EVs such as optical imaging (fluorescence and bioluminescence
453 imaging), single-photon emission computed tomography (SPECT)/position emission tomography
454 (PET) and magnetic resonance imaging (MRI). Fluorescence imaging has been explored to track EVs
455 in brain cell communication[132], brain cell tropism[104, 111] intracellular trafficking[79], and the
456 advantages and limitations of this type of imaging has been recently reviewed[133] These studies

have shown that engineered EVs could accumulate within microglia, neurons and astrocytes in the lesion area after stroke[134] and they are internalized by clathrin- and caveolae-dependent routes at least in brain endothelial cells[79]. In many of these studies, EVs were labeled with lipophilic dyes such as PKH67[79, 134], PKH26[70, 79], DiI[30, 33, 57, 104, 114], DiR[51] or CFSE[135] to be tracked by florescence imaging. Bioluminescence imaging (BLI) allows the *in vivo* tracking of EVs labeled with Gaussia luciferase[65, 67]. Unfortunately, the sensitivity of bioluminescence technique to track *in vivo* small populations of EVs is modest. Thus, other noninvasive imaging methods have been developed based on MRI (by labeling the EVs with SPIONs[113]) and mainly SPCET/PET imaging modalities. In this last case, several strategies have been used to label EVs with radioligands such as ^{125}I[136], ^{99}mTc[91], $^{64}Cu^{2+}$[66, 129] and ^{111}In[137]. In some cases, the surface of the EV is conjugated with a chelator followed by its complexation with a radioligand[66]. The labeling procedure had no impact in the surface receptor proteins or internal miR content of EVs[66]. In other cases, cells have been engineered to express streptavidin in the membrane of EVs which was then complexed with ^{125}I-tagged biotin[136].

Results obtained from different imaging modalities indicate that EVs injected from different cell sources (e.g. mouse breast cancer cells[129], mouse mammary carcinoma[138], mouse melanoma cells[136], mouse melanoma B16-BL6 cells[65], human mammary adenocarcinoma[138], human prostate adenocarcinoma[138], human embryonic kidney 293T[67], human MSCs, human umbilical-derived mononuclear cells[66]) by intravenous route had an accumulation below 5% of the initial dose in the brain. Most of administered EVs are captured in the liver and spleen or in the lungs[65-67]. Yet, as discussed in section 3.2, the modification of EVs with specific peptides increased their brain targeting[33].

482 **4- Conclusions and future perspectives**

483

484 EVs isolated from different cell sources have shown a therapeutic effect in the context of several
485 brain diseases. In the last 10 years, advances have been made in: (i) showing the therapeutic effect of
486 certain populations of EVs in pre-clinical models of brain diseases at cognitive and locomotor levels
487 (**Table 1**); (ii) unraveling EV brain regenerative mechanisms either by systemic[53] and local[73, 75]
488 effects; (iii) demonstrating the impact of EVs in neurogenesis[30], neuroprotection, angiogenesis and
489 brain remodeling and (iv) in characterizing the transport mechanism of EVs through the BBB[23,
490 80]. Despite these progresses, many questions need to be addressed to benefit of the full potential of
491 EVs in the context of the brain. For example, whether an increase in the accumulation of EVs in the
492 disease area is of benefit for regenerative effect or what is the most effective EV composition to
493 attenuate the effect of stroke or lead to brain remodeling in the context of AD remains to be
494 determined.

495 EV accumulation in the brain needs to be improved to further explore its therapeutic effect at the
496 disease area. Direct administration in the brain is a very invasive procedure and after systemic
497 administration, EVs have a modest accumulation in the brain[66]. In fact, part of the results obtained
498 in the treatment of ischemic stroke with native EVs were mainly caused by modulation of the immune
499 response and not by a local effect in the brain[53] yet, an increased accumulation in the lesion area
500 might lead to a higher neuroprotective and pro-angiogenic effect. A deeper understanding of the
501 mechanisms underlying interaction and transport of tumor-derived EVs in the brain may inspire the
502 development of new approaches to increase brain accumulation of engineered EVs for therapeutic
503 purposes. In addition, a topic that deserves further investigation is the identification of the best avidity
504 of EVs to the BBB. Because the density of targeting moieties in nanomaterials is a critical factor for
505 the interaction with the BBB[139], the investigation of the best avidity of EVs to the BBB is
506 fundamental for both targeting and transport of EVs through the BBB.

507 Unravelling the molecular mechanisms involved in neuroprotection and brain regeneration are
508 of utmost importance for the fine-tuning of the biological effect of EVs by bioengineering
509 methodologies. Although a considerable number of studies have shown the therapeutic effect of EVs
510 in several brain diseases, further studies are needed to understand better their bioactivity at cellular
511 and molecular levels. Some of the molecular mechanisms mediated by EVs, particularly miR133-b,
512 have been identified in neurons and involved the decrease in the expression of connective tissue
513 growth factor and RhoA[3, 26], while in other cases (e.g. EVs enriched for miR124[30]), it is not
514 known whether the identified putative molecular targets (Gli3 and Stat3) are the result of local or
515 systemic effect of EVs. In addition, a better understanding of the EV mechanism at cellular level is
516 necessary and may require the use of transgenic animal models, in which EVs carrying a gene editing
517 protein (e.g. Cre recombinase) may be administered in the brains of transgenic animals (e.g. floxed
518 mice) with a brain disease[7, 98].

519 Although the therapeutic potential of native EVs for brain pathologies has been shown at pre-
520 clinical stage, clinical translation of these therapies has not been fulfilled yet. For this, critical
521 parameters must be taken into consideration for the maximization of the efficacy of EV-based
522 therapies, such as the optimization of dose regimens, route of administration and EV source as well
523 as the definition of potency assays for the evaluation of efficacy. Clinical translation of EVs also
524 carries technical challenges related to the considerable effort required for the collection of large
525 amounts of EVs (although it is still unclear, effective therapeutic doses are estimated to be in the
526 order of 10^{14} EVs/Kg [140]). Thus, it is essential to further develop efficient isolation and purification
527 processes under GMP conditions, compliant with strict regulatory hurdles, to ensure high purity and
528 homogeneity between batches of EVs as this could have an impact on their biological activity[141].

529 In summary, EVs hold great potential for therapeutic purposes. It is worth noting that
530 methodologies following the same engineering principles have also been applied to other purposes
531 besides brain pathologies, for instance for cancer treatment [142], for which increased activity by

532 cargo loading with different molecules (e.g. miRNAs and small molecules) and enhanced targeting
533 by surface modification have also been pursued. In both cases (brain and cancer pathologies), the
534 enrichment of the EVs with single or multiple biomolecules (e.g., small molecules or non-coding
535 RNAs) able to act at multiples stages of the disease, and likely at multiple cell types, is required. In
536 addition, both EV interventions share similitudes in EVs able to interact with the vasculature at the
537 regions where they are required. Yet, there may be significant differences in the transport
538 requirements of EVs through the vasculature for brain and cancer pathologies. In the brain, EVs may
539 cross the BBB by transcytosis (caveolae seems to have an important role in transcellular transport
540 [143]) and therefore the type of ligand and their density is critical for this transport process, as it was
541 reported for receptor mediated transport of biomolecules [144]. In cancer pathologies, it is possible
542 that EVs may cross the vasculature through endothelial cells pathways as found recently for synthetic
543 nanoparticles [145]; however, caveolae –mediated transport has no significant impact in the
544 accumulation of the nanoparticles. Therefore, it is possible that differences in the intracellular
545 transport at the BBB and in the cancer vasculature should exist and should be taken in consideration
546 for EV interventions. Another important difference between both pathologies, is the effect of EVs
547 after crossing the vasculature. In the case of the brain and cancer pathologies, EVs may encounter
548 microglia cells (immunocompetent cells of the brain) or macrophages, respectively, and the impact
549 of the EVs may be different taking in account the phagocytic capacity of both type of cells as well as
550 differences in their intracellular pathways.

551

552

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Competing Interests

The authors declare no competing interests

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Table 1- Native EVs for the treatment of brain pathologies.

Disease	Model	EV source	Route	Dose (µg or particles)	Outcome	Ref.
AD	*In vivo* (rat)	Mouse neuroblastoma cells Human CSF	ICV	4 µg	↓Aβ oligomers	[60]
	In vivo (mouse)	Mouse MSCs	IC	22.4 µg (~1×10^{9} particles)	↓Aβ oligomers; ↓Dystrophic neurites	[59]
	In vitro	Mouse MSCs	N.A.	2.4×10^{8} particles	↑Neuronal protection	[146]
	In vitro	Human MSCs	N.A.	6×10^{8} particles	↑Neuronal protection	[87]
Stroke	MCAO in mouse	Human MSCs	IV	N.A. (multiple administrations)	↑Neurogenesis and angiogenesis Modulation of immune response	[6]
		M2 microglia	IV	100 µg (multiple administrations)	↑Neuronal protection ↓Infarct volume ↓Behavioral deficits	[147]
		Mouse NSCs	IV	10 µg	↑Astrocytes viability ↓Infarct volume	[27]
		Mouse NSCs and MSCs	RO	1-100 µg (multiple administrations)	↓Motor coordination impairment ↑Neuroregeneration Modulation of immune response	[5]
	TE-MCAO in mouse	Human NSCs	IV	2.7×10^{11} EVs/kg (multiple administrations)	↓Post-stroke cerebral atrophy ↑Functional recovery	[53]
	MCAO in rat	Porcine MSCs	IV	100 µg	↑Angiogenesis; ↓Infarct volume ↑Functional recovery	[28]
		Human MSCs	IA	200 µg/kg	↑Neurogenesis; ↓Infarct volume; ↑Functional recovery	[74]

		Rat NSCs	ICV	30 μg	↑Neuronal protection; ↓Microgliosis; ↓Infarct volume ↓Behavioral deficits	[148]
		Rat MSCs	ICV	100 μg	↓Infarct volume ↑Functional recovery	[75]
		Rat MSCs	IV	100 μg	↑Neurite remodeling ↑Neurogenesis and angiogenesis ↑Functional recovery.	[149]
	MCAO in pigs	Human NSCs	IV	N.A. (multiple administrations)	Preserve cellular integrity ↑Functional recovery	[25]
TBI	TBI in mouse	Human MSCs		30 μg (15×10^9 particles)	↓ Neuroinflammation ↑Spatial learning	[150]
	TBI in rats	Human MSCs	IV	100 μg	↑Neurogenesis and angiogenesis ↓Neuroinflammation	[61]
		Human MSCs	IV	100 μg	↑Cell proliferation ↑Neurogenesis and angiogenesis ↑Functional recovery	[48]
	TBI in pig	Human MSCs	IV	1×10^{13} (multiple administrations)	↓Neurologic injury ↑ Functional recovery	[151]
ICH	ICH in rats	Rat MSCs	IV	N.A.	↑Odor- based recognition ↑Neurological function ↑Neurogenesis and angiogenesis	[152]
		Rat MSCs	IV	100 μg	↑Functional recovery; ↑Axonal sprouting; ↓Lesion size; ↑White repair markers	[153]

AD – Alzheimer's disease; CSF- cerebrospinal fluid; IA - Intra-arterial; IC-Intracerebral; ICH- Intracerebral hemorrhage; ICV- Intracerebroventricularly; IV- Intravenous injection; MCAO- Middle cerebral artery occlusion; MSCs – Mesenchymal stem cells; NSCs- Neural stem cells; RO- Retroorbital; TE-MCAO – thromboembolic Middle cerebral artery occlusion; TBI- Traumatic brain injury; N.A.-Not applicable.

Table 2 – Strategies for EV cargo modulation.

EV source	Method	Model	Outcome	Ref.
Modulation of EV-secreting cells				
Mouse macrophages	Tripeptidyl peptidase-1 (TPP1) enzyme overexpression	Batten disease mouse model	↑TPP1 accumulation in lysosomes; ↑ Lifespan of Batten disease mouse model	[34]
Mouse embryonic fibroblasts	Cre recombinase enzyme overexpression	Transgenic mouse model	Delivery of active proteins to the brain by intranasal route	[98]
Human HEK-293T	Catalase enzyme overexpression	PD mouse model	↓Neuroinflammation	[35]
Rat MSCs	miR-17-92 cluster overexpression	MCAO rat model	↑Neurological function; ↑Oligodendrogenesis; ↑Neurogenesis	[100]
Rat MSCs	miR-133b overexpression	ICH rat model	↑Neuroprotection	[97]
Human ADSCs	miR-126 overexpression	Rat MCAO	↑Neurogenesis; ↑Vasculogenesis; ↓ Inflammation	[92]
Mouse EPCs	miR-126 overexpression	Mouse MCAO	↑Neurogenesis; ↑Vasculogenesis; ↓Infarct size	[154]
Mouse astrocytes	Transfection with lincRNA-Cox2-siRNA	*In vitro/in vivo* lincRNA-Cox2 knockout model Intranasal	↓Expression of lincRNA-Cox2; LPS-induced microglial proliferation	[94]

Human astrocytes	Transfection with lincRNA-Cox2-siRNA	*In vitro/in vivo* lincRNA-Cox2 knockout model Intranasal	Restored microglial phagocytic activity	[122]
Mouse macrophages	Transfection with curcumin	Rat AD model	↑Neuron survival; ↓Tau phosphorylation	[99]
Post-isolation methods				
Mouse macrophages	EV loading with TPP1 protein: saponin or sonication	Batten disease mouse model	↑TPP1 accumulation in lysosomes; ↑ Lifespan of Batten disease mouse model	[34]
Mouse macrophages	EV loading with catalase: sonication, extrusion or saponin	Mouse PD model	↓Oxidative stress ↑Neuron survival	[155]
Mouse MSCs	EV loading with miR-124: electroporation	Photothrombosis mouse model	↑Neurogenesis ↑Neuronal differentiation	[156]
Mouse dendritic cells	EV loading with BACE1 siRNA: electroporation	Wild-type mouse	Knockdown of BACE1	[14]
Mouse MSCs	EV loading with curcumin: diffusion	Mouse MCAO model	↓Inflammation ↓Brain cell apoptosis	[103]
Human ESCs	EV loading with paclitaxel: diffusion	Orthotopic mouse xenografts	↑Accumulation in glioma site ↑Mouse survival	[157]
Mouse BECs	EV loading with paclitaxel or doxorubicin: diffusion	Xenotransplanted brain cancer zebrafish model	↑Brain cancer cell elimination	[158]
Mouse blood serum	EV loading with dopamine: diffusion	Mouse PD model	↑Dopaminergic neurogenesis ↑Symptomatic performance	[95]
Mouse macrophages	EV loading with curcumin: electroporation	Glioma mouse model	↑ Brain cancer cell elimination ↑Mouse survival	[104]

AD- Alzheimer's Disease; ADSCs- adipose derived stem cells; BECs- brain endothelial cells; EPCs- Endothelial progenitor cells; ESCs- Embryonic stem cells; EV- extracellular vesicle; ICH- Intracerebral hemorrhage; MSCs – Mesenchymal stem cells; MCAO- Middle cerebral artery occlusion; PD – Parkinson's Disease; TBI – Traumatic brain injury.

Table 3 – Strategies for EV targeting.

EV source	Targeting peptide, construct or nanoparticle	Model	Outcome	Ref.
Chemical modification of EV membrane with peptides, proteins or magnetic nanoparticles				
Mouse MSCs	RGD	MCAO mouse model	Enhanced accumulation in the lesion site as evaluated by IVIS imaging	[103]
Mouse MSCs	RVG	AD mouse model	Enhanced accumulation in cortex and hippocampus by fluorescence imaging	[33]
Mouse MSCs	c(RGDyK)	MCAO mouse model	Enhanced accumulation in the lesion site as evaluated by IVIS imaging	[111]
Human ESCs	c(RGDyK)	Orthotopic glioma mouse model	Enhanced accumulation in the glioma site as evaluated by IVIS imaging	[119]
Mouse melanoma cells	Horseradish peroxidase	N.A.	*In vitro* targeting	[112]
Mouse macrophages	Neuropilin-1-targeted peptide	Orthotopic glioma mouse model	Enhanced accumulation in glioma site as evaluated by fluorescence imaging	[104]
	Magnetic nanoparticle	Subcutaneous cancer mouse model	Enhanced accumulation in tumor site by IVIS imaging	[109]

Genetic of EV-secreting cell to express a peptide or protein				
Mouse MSCs	Lamp2b-RVG	Photothrombosis rat ischemic model	Enhanced accumulation in the ischemic area as evaluated by fluorescence imaging	[156]
Human HEK-293T	Lamp2b-T7	Glioblastoma rat model	Enhanced accumulation in the brain as evaluated by IVIS imaging	[114]
Human HEK-293T	Lamp2b-RVG	Mouse PD model	Enhanced accumulation as evaluated by luminescence analyses	[35]
Mouse dendritic cells	Lamp2b-RVG	Wild-type mouse	Enhanced accumulation in the brain as evaluated by fluorescence microscopy	[14]
Mouse dendritic cells	Lamp2b-RVG	Wild-type mouse	Enhanced accumulation in acetylcholine receptor-rich tissues by IVIS imaging	[51]
Mouse MSCs	Lamp2b-RVG	Photothrombosis mouse ischemic model	Enhanced accumulation in the lesion site as evaluated by fluorescence microscopy	[30]
Human HEK-293T	Lamp2b-RVG	Morphine relapse mouse model	Enhanced accumulation in the brain as evaluated by fluorescence microscopy	[32]

AD- Alzheimer's Disease; ESCs- embryonic stem cells; HEK- human embryonic kidney; LAMP2B-lysosome- associated membrane protein 2; MCAO- Middle cerebral artery occlusion; MSCs – Mesenchymal stem cells; PD – Parkinson's Disease; N.A.- not applicable.

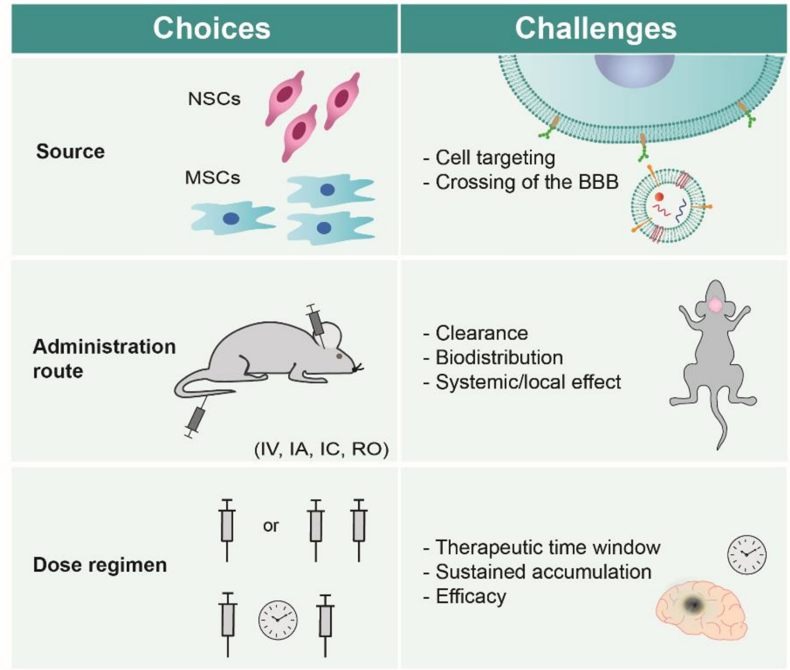

Figure 1. Choices and challenges when using EVs. EVs from different cell sources have been used for the treatment of brain pathologies. Although not fully evaluated yet, each cell type might be associated with different properties of EVs that can lead to a higher or lower tropism for brain vasculature or for neuronal cells, resulting in a better or worse targeting to the brain. An important challenge related to the chosen source is also the ability to cross the BBB. The administration route not only has an effect on the biodistribution of the EVs and on their clearance, but it may also have an impact in the type of effect, *i.e.,* local and/or a systemic effect. Finally, there are challenges created by the dose regimen: single and multiple administrations are possible, with different impact on the accumulation and efficacy. In pathologies such as stroke, the time between the event and the treatment represents an important variable in saving or repairing as much brain tissue as possible.

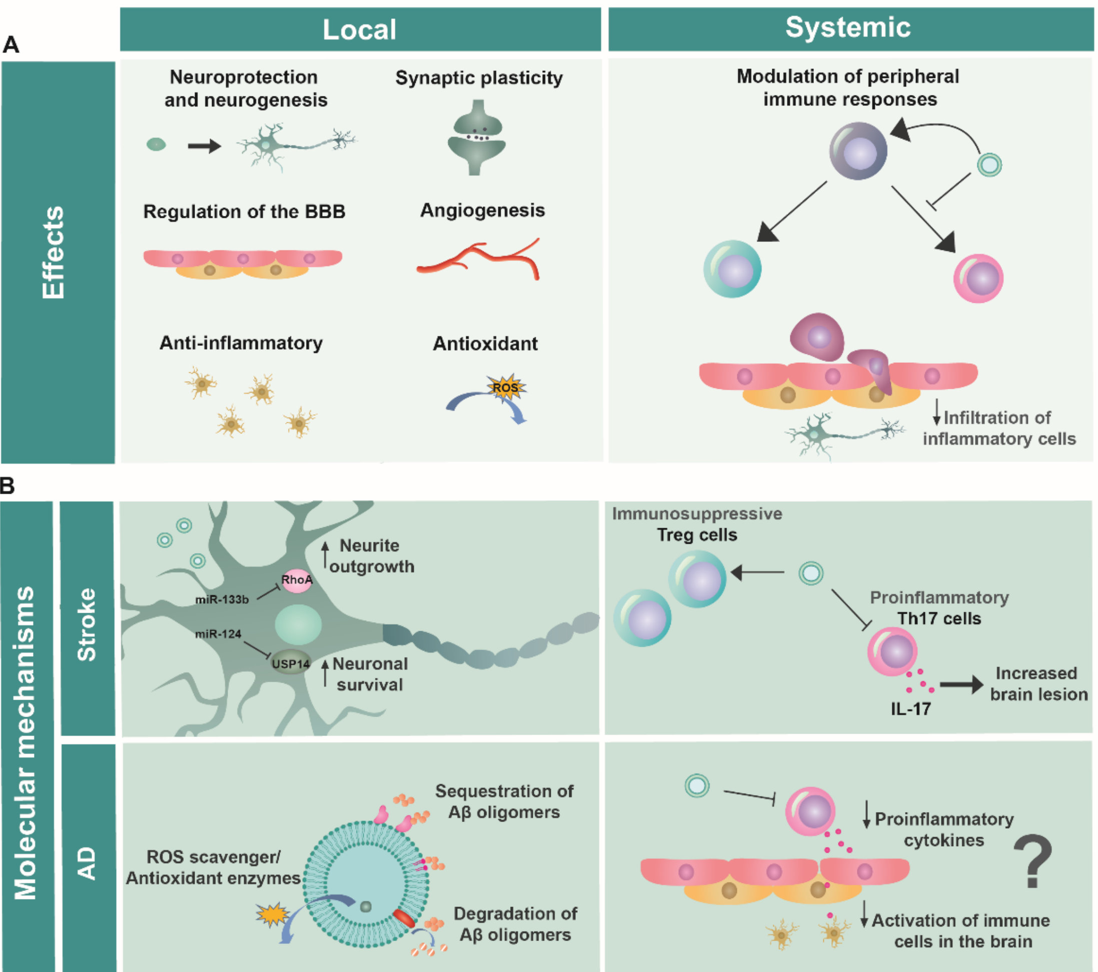

Figure 2. Biological effects and mechanisms of action of native EVs in the context of brain pathologies. (A) Benefits in the treatment of brain pathologies have been achieved not only by direct local effects (neuroprotection, neurogenesis, angiogenesis, anti-inflammatory and antioxidant properties, etc.) in the brain and but also by systemic effects through the modulation of peripheral immune response that might lead to the establishment of a favorable milieu for brain regeneration. **(B)** Molecular mechanisms mediated by native EVs in the treatment of stroke and AD. MiR-133b carried by EVs from MSCs is able to promote neurite outgrowth by targeting the transforming protein RhoA and miR-124 has been associated with increased neuronal survival by targeting USP-14 (ubiquitin-specific protease 14). At systemic level, EVs from NSC were shown to decrease proinflammatory Th17 cells while increasing immunosuppressive Treg cells. In the case of AD, local effects are related to the sequestration and degradation of Aβ oligomers by exosomal surface molecules or to antioxidant effects mediated by enzymes. No systemic effect has been described yet in the context of AD, although modulation of systemic inflammation might have an indirect local effect in inflammatory markers in the brain.

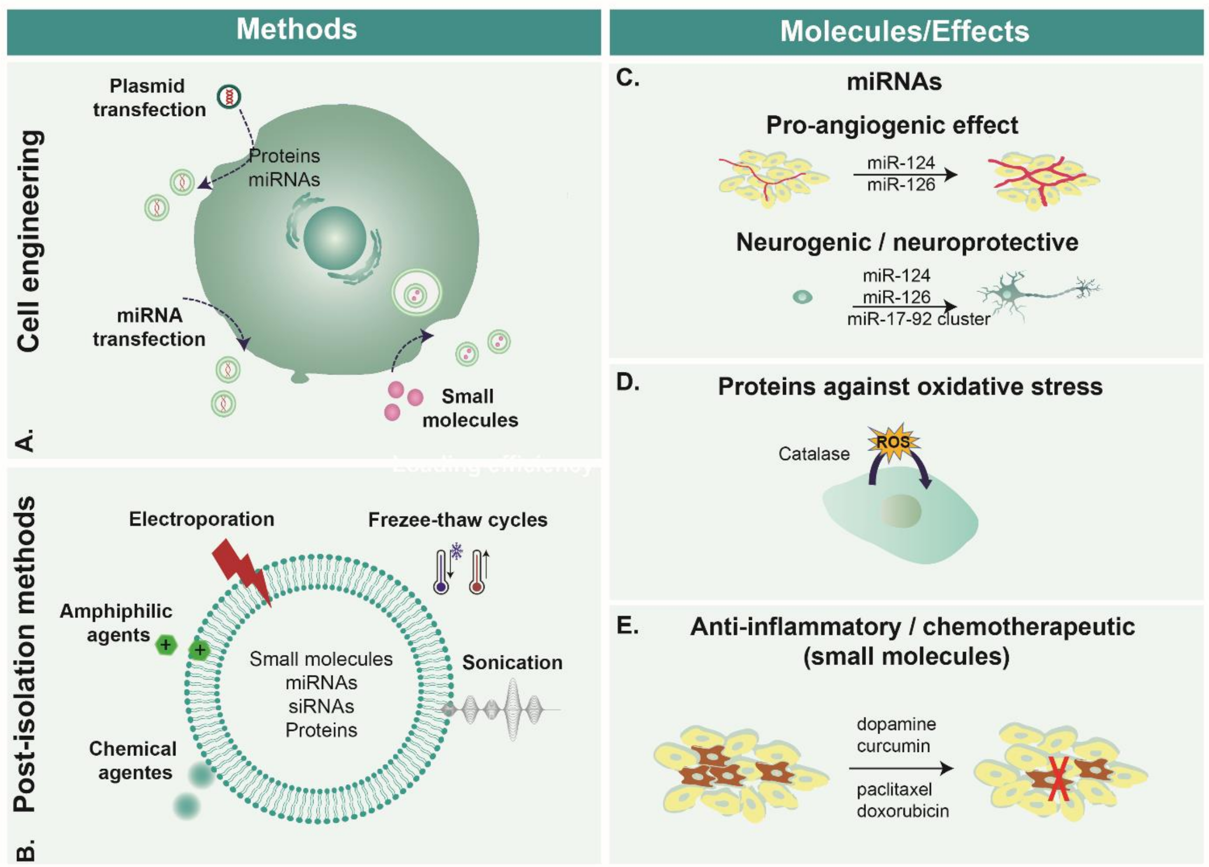

Figure 3. Engineering methods for modulation of EV content. EVs can be modulated by cell engineering (by manipulating progenitor cells) or by direct loading using different post-isolation methods. **(A)** Cell engineering is an indirect loading method via genetic manipulation of a parent cell by plasmid transfection, or by the enrichment of cells with miRNAs or small molecules. **(B)** Post-isolation methods represent a direct modulation of isolated EVs thought active methods such electroporation, sonication, freeze-thaw cycles, detergents, and chemical agents. The best method for EV modulation depends on the therapeutic molecules and on their loading efficiency. Effects of EVs cargo modulation in the treatment of brain pathologies can be achieved by the action of the type of cargo, such miRNAs **(C)**, proteins **(D)** and small molecules **(D)**.

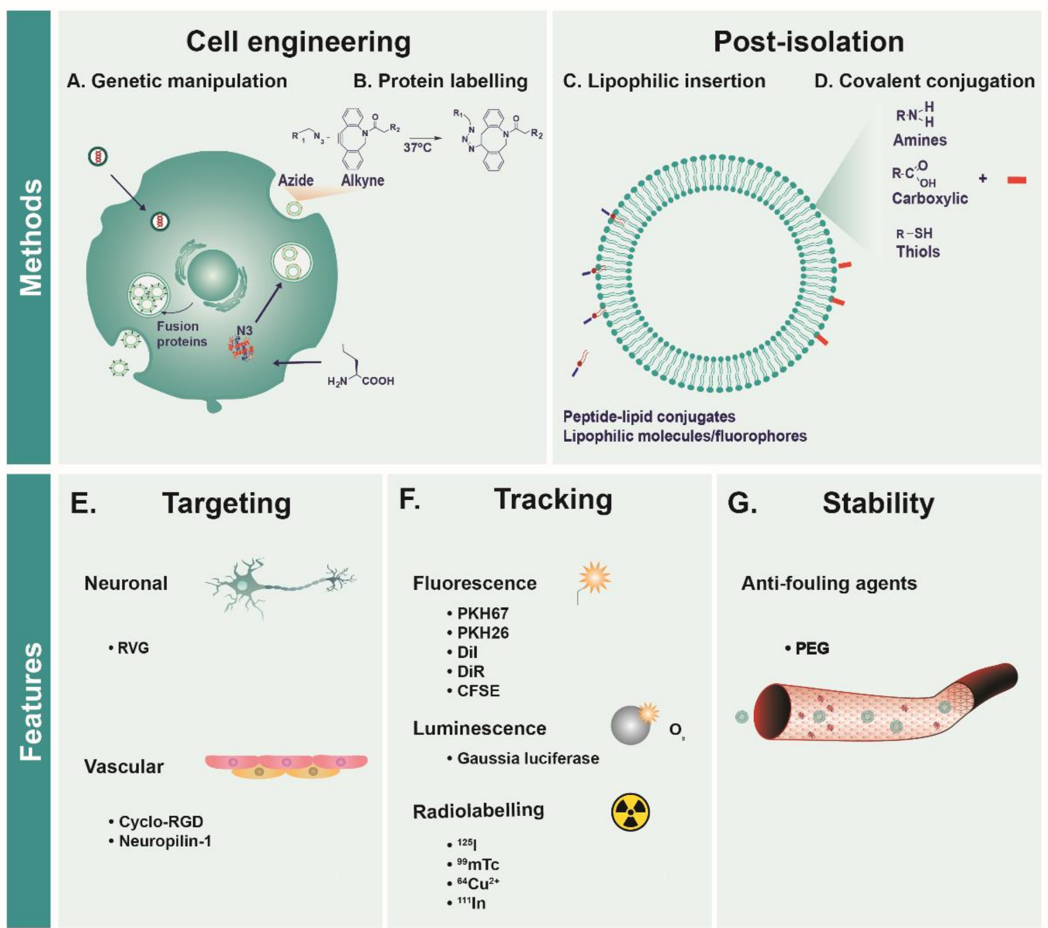

Figure 4. EVs surface engineering improves stability, targeting ability and EVs tracking. EVs surface modulation can be achieved indirectly by genetic modification of the EV-secreting cells. In this strategy, transfection with **(A)** protein plasmids or **(B)** protein-residues introduces exogenous groups such as, azides, alkynes, methacryloyls and thiols on the EVs surface. On the other hand, EVs can be directly conjugated with **(C)** lipids that are subsequently incorporated on the membrane of EVs, or reacted with **(D)** functional groups present on the EV surface via bio-orthogonal chemistry. The EVs membrane also allows tailoring of its surface properties with **(E)** targeting peptides, including RVG, cyclo(RGDyK) and neuropilin-1 peptides for brain targeting, **(F)** lipophilic probes and radiolabeling for *in vivo* monitoring of EVs that can also acts as contrast agents for diagnostic purposes of neurological disorders; and **(G)** amphiphilic polymers, such as PEG that prolong their circulation time in the bloodstream. Taken together, these agents make them versatile drug delivery systems.