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Stem cells and their extracellular vesicles as natural and bioinspired carriers for the treatment of neurological disorders

Anne des Rieux

Abstract

One of the most explored strategies to cure neurological disorders is the transplantation of stem cells and their derived products. Different stem cells, as well as their extracellular vesicles (EV), modified or not, have been administrated in a large array of preclinical neurological disorder models. EV represent the hope of a "cell-free" therapy that would combine the therapeutic potential of stem cells without their drawbacks. Stem cells and EV showed various degrees of efficiency but, overall, provided benefits and improvements. The administration route has a considerable impact on stem cell and EV safety and therapeutic effect. However, despite evidences of preclinical success, the different strategies developed based on stem cells to treat neurological disorders do not exactly recapitulate in clinical trials. Discrepancies between preclinical and clinical experimental conditions and settings, cell availability and difficulties to scale up and to produce cells and EV in a GMP environment limit translation.

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Stem cells, Extracellular vesicles, Secretome, Delivery, Central nervous system, Peripheral nervous system, Repair, Biomaterials, Clinical trials.

Introduction

Diseases of the nervous system are still unmet medical needs as, even if treatments are available to alleviate their symptoms, very few to none can cure those diseases. Indeed, neurological affections are very complex independently of their nature (traumatic event, neurodegenerative diseases, cancer) and affect organs that have limited regenerative potential. Currently, although the central nervous system (CNS) has a certain level of plasticity and the peripheral nerves can grow back over few millimeter gaps, little can be currently done for larger lesions. The impact of these diseases is quite considerable as in 2017, 307.9 million patients were affected by a neurological disease in the European Union (EU28) (The total population in the EU28 was 512.4 million), 74.5 million of which were newly diagnosed [1]. In the same year, the total number of deaths due to neurological pathologies was 1.1 million. In the EU, neurological disorders ranked third after cardiovascular diseases and cancers, representing 19.5% of total deaths; headache, stroke, Alzheimer and other dementias, and Parkinson's disease being the commonest. In addition, glioblastoma multiform is the most common primary brain cancer of glial origin and is almost universally fatal within 2 years of diagnosis despite maximal surgical and medical therapy [2].

Consequently, the research field dedicated to neurological pathologies is very broad and the community very active. Numerous strategies have been developed to find cures, or at least to stimulate the repair of the nervous system. Of note, whether the disease is degenerative or cancerous, the objective of the treatment is logically different.

In this review, we focused on therapies based on stem cells and their derived products, namely conditioned medium and extracellular vesicles (EV). Indeed, stem cell therapy has gained an increased interest as a treatment to manage uncurable disorders such as stroke, multiple sclerosis (MS), Parkinson's or Alzheimer's diseases, spinal cord injury and also cancer, even if stem cell benefit is more controversial for this application. Stem cells are believed to exert their therapeutic effect by the secretion of various neural trophic factors, immunomodulation, neuroprotection, angiogenesis, and may be even neural cell replacement.

It is quite a vast subject with a lot of approaches, using different stem cells and routes of administration, combining or not stem cells (or EV) with biomaterials, and addressing different pathologies (trauma versus diseases; central versus peripheral system; repair versus treatment [cancer]). It is the reason why, in this review,

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we focused mostly on the later developments (2018–2021), providing information on the different approaches and main current tendencies without pretending to be exhaustive. We first described the recent work that has been done on different types of stem cells (modified or not) and their derived products. Then we explored the solutions developed to deliver them to the nervous system and finally, we discussed their translation to the clinic and the market.

Stem cells and their derived products

Stem cells

When scientists realized that stem cells could be used as a therapeutic tool, every type, whether newly discovered or known for years, was tested as a treatment for a large variety of uncurable diseases. First embryonic, then adult and now induced pluripotent stem cells (iPSCs) have raised a lot of interest, hope and, inevitably, deceptions.

Different types and origins of stem cells have been tested in the quest for neurological disease treatment (Table 1) (Figure 1). Each has its own advantages and drawbacks, and the decision to use one type/origin over another depends on the study stage (early work, preclinical, clinical), the objective (cell replacement versus secretome-based effect), of the availability of the cell source (embryonic versus adult tissue for instance), and its translation value (amount of cell required and delay required to produce them, cell availability, safety).

Historically, **fetal cells** were the first to be considered to address unmet medical needs but their use has been strongly limited by ethical concerns and logistical challenges. Several articles reported the transplantation of neural stem cells (NSCs) derived from embryonic cells, but as research on these cells for regenerative medicine purposes has been restricted or banned (1990s in the USA, 2006 in Japan), little development has been recently done on that particular field. Preference has been given to physiologically available adult stem cells and iPSCs.

The development of techniques allowing the production of **human iPSC** [4] (exhibiting similar

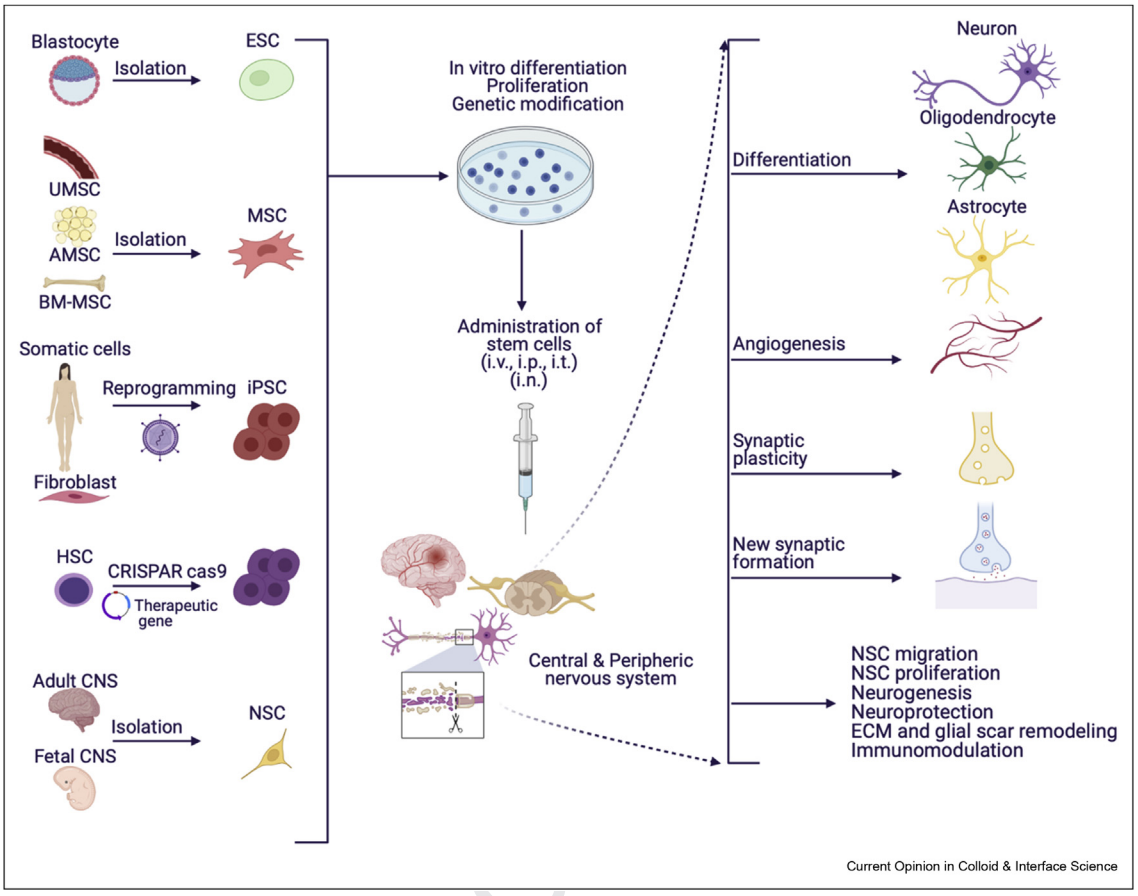
Table 1

Type and origin of stem cells used for the treatment of neurological disorders.

Type of stem cells	Origin	Advantages	Drawbacks
Embryonic	Human, mouse	• Limitless self-renewal • Theoretically differentiation in all three germ layers	• Ethical concerns • Heterogeneity • Immunological response • Limited availability
Mesenchymal (bone marrow, adipose and dental tissues, umbilical cord blood, Wharton's jelly)	Human, rodent, canine	• Adult tissues (no ethical issues) • Multipotent • Autologous use/allogenic for "off-the-shelf" application • Antitumor effect (controversial) • Pleiotropic effects • Migration to injured sites • Safe • Can cross epithelial barriers and the BBB (excepted Wharton's jelly-derived MSC) • Strong immunomodulatory potential	• Protumor effect (controversial) • Promising preclinical results do not always translate to efficiency during clinical trials. • Mode of action not always clearly understood. • Limited number of cells at the sampling site
Induced pluripotent stem cells	Human, mouse	• High proliferation rate • Multipotent • Autologous but ⚠ if elderly or sick patient • Low immune response if autologous • Very useful for human-relevant *in vitro* models • Unlimited source of autologous cells.	• Tumorigenicity • Long process (>2 months) • Lack of perspective
Neural stem cells	Human, mouse	• Easily differentiate into neural cells • Safe • Paracrine and cell replacement • Migration to injury site	• Ethical concerns • Limited availability • Low proliferation rate • Poor long-term *in vitro* expansion • Poor cell survival after administration

BBB, blood-brain barrier; MSC, mesenchymal stem cells.

Figure 1



Therapeutic effects of stem cells in neurological disorders. Adapted with permission from Ref. [3]. ESC, embryonic stem cells; MSC, mesenchymal stem cells; UMSC, umbilical stem cells; A/BM-MSC, adipose-derived/bone marrow MSC; iPSC, induced pluripotent stem cells; HSC, hematopoietic stem cells; CNS, central nervous system; NSC, neural stem cells; IV, intravenous; IP, intraperitoneal; IT, intrathecal; ECM, extracellular matrix. Created with BioRender.com. Q3

characteristics to embryonic stem cells) has allowed to overcome issues related to embryonic stem cells, but they still present a limited translation potential, as artificial induction methods can induce genetic and epigenetic abnormalities and potentially tumorigenicity after transplantation [5]. New methods using chemicals and microRNA or omitting the oncogenic factors c-MYC and KLF4 are being developed to produce iPSC aiming to limit the risk of tumor formation [6]. Nevertheless, preclinical studies showed that NSC obtained from mouse and human iPSC transplanted in models of spinal cord injury (rodents and primates) did not form tumors and supported functional recovery [5] (Table 2). The therapeutic potential of mouse and human-derived iPSC was also evaluated in preclinical models of MS and showed promising results [6]. One of the main advantages of using human-derived iPSC is that they can be obtained from the patient itself which would reduce or event remove the need for immunosuppressive therapy. However, iPSCs derived from patients affected by a disease, such as MS for instance, do not have the same therapeutic potential as cells obtained from heathy individuals. For instance, iPSC-derived NSCs obtained from primary progressive MS patients provided no protection against cuprizone-induced demyelination, while cells derived from healthy patients prevented the occurrence of the lesions [7]. In consequence, iPSC autograft might not be clinically relevant to treat patients affected by a neurological disease if healthy donors are required to obtain a therapeutic effect. An alternative is the constitution of iPSC databases with iPSC HLA signature that could provide quality-controlled cellular products and abate the risk of graft rejection ([8] and NCT03434808). Up to now, when searching the clinical data base (https://clinicaltrials.gov) for the keyword "induced pluripotent stem cells", only two interventional trials where iPSCs are actually injected in patients were reported: one aiming at autologous transplantation of iPSC-derived retinal pigment epithelium for age-related macular

Table 2

Examples of studies using iPSCs as a treatment against neurological diseases.

Cells	Model	Cell fate	Impact	Ref
iPSC–NPC (mouse embryonic fibroblasts)	Subacute phase SCI in rats	• Differentiation into 3 neuronal lineages • No tumor formation	• Differentiated oligodendrocytes enhanced remyelination • Neurite regrowth of 5-HT + serotonergic fibers around the lesion area. • Locomotor functional recovery	[10]
Human fibroblast-derived iPSC	MCAo stroke model in rats	Transfer to injury zone	Partial sensorimotor function recovery	[11]
GMP-grade HLA-homozygous hiPSC line into neural precursor cells	Subacute stage of ischemic stroke in rats (ie at 7 days after the induction of MCAo).	• High proportion of transplanted cells survived • Differentiation into MAP2+ mature neurons, GABAergic neurons and DARPP32+ medium spiny neurons. • Transplanted cells formed neuronal connections with striatal neurons • Enhanced endogenous repair processes	Significant behavioral improvements (12 weeks after transplantation)	[12]
Dopaminergic neurons from healthy and Parkinsonian fibroblasts	Parkinson monkey (MPTP)	Long-term survival of dopaminergic cells	Increase in spontaneous movements	[13]
Human-induced pluripotent stem cells into OPC	Intracerebrally injected hiPSC-derived OPCs in a nonhuman primate (marmoset) model for progressive MS	• Grafted OPCs migrated toward the MS-like lesions in the corpus callosum • Myelination of denuded axons		[14]
Mouse iPSC-derived NPCs	Intrathecally transplanted after disease onset- EAE	Secretion of leukemia inhibitory factor.	• Ameliorate clinical and pathological features • Neuroprotective effect through the secretion LIF • Promoted survival, differentiation and remyelination capacity of both endogenous OPC and	[15]

		mature oligodendrocytes. • Early preservation of tissue integrity limits BBB damage and CNS infiltration of encephalitogenic leukocytes.	
iPSC-derived NSC obtained from primary progressive MS patients (blood samples)	Cuprizone-induced demyelination	No information	• No protection against demyelination while cells derived from healthy patients prevented the occurrence of the lesions. • No support of OPC differentiation *in vitro*. • Reduced response to pro-myelinating experimental compounds.	[7]

iPSC–NPC, induced pluripotent stem cell–derived neural progenitors; SCI, spinal cord injury; 5-HT, 5-hydrotryptamine (serotonin); MCAo, middle cerebral artery occlusion model; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; OPC, oligodendrocyte progenitor cells; EAE, experimental autoimmune encephalomyelitis; BBB, blood–brain barrier; MS, multiple sclerosis.

degeneration (NCT04339764, phase 1/2a started in September 2020) and another one using allogeneic iPSC-derived cardiomyocyte sheet for ischemic cardiomyopathy (NCT04696328, phase 1 started in January 2021). All in all, even if a considerable number of studies are performed with iPSC, these cells are currently mostly used for the development of relevant human models [9] (i.e. NCT04476225 and NCT03867526).

NSCs are a logical choice when addressing neurological disorders and as such, they have been extensively used to try to find cures for these diseases. They are derived from any part of the nervous system and differentiate into cells expressing neural markers (i.e. astrocytes, oligodendrocytes and neurons) *in vitro* [16]. NSCs are primely located in the subgranular and subventricular zones of the brain but can also be found in the spinal cord [17]. Their therapeutic potential has been vastly explored in the last 20 years, applying two main strategies: administration of exogenous cells and *in situ* differentiation of endogenous cells [18]. *In vivo* differentiation of exogenous NSCs after transplantation is also an option. Exogenous NSCs can migrate to the lesion, exert neuroprotective and proangiogenic effects and stimulate the proliferation and differentiation of endogenous NSCs, leading to functional recovery [19]. However, as they are isolated from embryonic, fetal or brain tissues, their use is limited by donor availability (ethical and safety concerns), low proliferation rate, poor long-term *in vitro* expansion and the need for a large number of cells per patient combined with poor cell survival after administration (i.e. due to immune reaction or low engraftment). As mentioned previously, one alternative is the use of iPSC-derived NSCs but for now, their translational value is still low. Another option is to directly stimulate *in situ* differentiation of endogenous NSCs [18]. The administration of active compounds (i.e. small molecules, siRNA, virus) is the most common approach to enhance neurological functions in neurodegenerative disease animal models, but electrical stimulation and oxygen supply modulation have been also used with that objective [18]. However, bioactive molecules, if administered in solution by intravenous injection, present usually a low therapeutic index because of short half-life, low bioavailability and/or poor solubility. In our laboratory, we have developed nanomedicines to improve the therapeutic efficiency of these bioactive cues. We tested polymeric nanoparticles loaded with stromal cell–derived factor-1 (SDF-1) to increase the migration of endogenous precursors [20] and lipid nanocapsules loaded with retinoic acid specifically targeting brain NSCs [21] to stimulate their *in situ* differentiation into myelin basic protein–mature oligodendrocytes [22]. While administration of exogenous cells and *in situ* differentiation of endogenous cells are used in preclinical studies, the

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large majority of NSC-based therapies evaluated in clinical trials rely on the grafting of exogenous NSCs [18]. When looking for NSC-related clinical trials (https://clinicaltrials.gov), 60 are listed as interventional with the keyword "neural stem cells", but if filtered based on "With results", only 2 studies appear and they are related to NSC protection during radiation for glioblastoma treatment. Only 2 studies are aiming at endogenous NSC differentiation (NCT00397423 and NCT04121468). No results are available for the first one and the second one ("*A Phase I Double Blind Study of Metformin Acting on Endogenous Neural Progenitor Cells in Children With Multiple Sclerosis*") started one year ago and is still recruiting. If looking at active and recruiting trials (Table 3), a few studies are going on, mostly consisting of safety Phase 1 trials for NSC injection. This illustrates the severe bottleneck between preclinical exciting results and their translation to actual clinical efficiency.

Adult stem cells, or **mesenchymal stem cells (MSCs)**, have been seen as the solution to circumscribe limitations and issues related to the use of embryonic stem cells and NSCs. MSCs are adult stem cells that can self-renew and are multipotent (differentiate into mesodermal, i.e. adipocytes, chondrocytes, osteoblasts, and nonmesodermal, i.e. glial cells, neuronal cells, hepatocytes cell types). They were originally found in the bone marrow but have been since identified in other tissues [23]. Owing to numerous advantages, MSCs have become the most popular source for cell and regenerative therapy. They can be quite easily isolated from adult tissue and expanded for clinical use and have a high therapeutic potential. However, while the use of autologous cells was the paramount a few years ago, the elongated time frame required to reach an adequate number of cells from the patient's own tissue as well as the impact of the patient's age and health state has been tipping the scale toward allografts. Several excellent reviews provide abundant details regarding the therapeutic potential of MSCs and their secretome (more than 190 in 2021−2020) [24−27]. Briefly, the main advantages of MSC are that they can migrate to the site of injury (i.e. thanks to the CXCR4-CXCL12 axis), modulate the immune response, promote cell survival and induce angiogenesis [23] (Figure 2). MSCs can exert their effect not only via direct contact, fusion, secretome, mitochondria transfer and cell replacement but also by activation of mechanisms in the host niche, such as proliferation and differentiation of local precursors [23]. MSCs have been reported to be hypoimmunogenic or "immune privileged". But more than being hypoimmunogenic, MSCs present a high immunosuppressive potential which allows them to delay or evade allo-rejection [28]. However, MSC that do not tip the balance toward immunosuppression are detected and destroyed as any other foreign cell [29]. Rejection of allogenic MSCs happens more slowly than other allogeneic cell types, but MSCs cannot be considered truly immune-privileged [28]. Incidentally, MSCs have been used as local immunosuppressors to lengthen the survival of co-transplanted allogenic cells in the nervous system (i.e. NSCs of iPSC-derived cells) [30]. Most popular sources of MSCs are bone-marrow, adipose and, more recently, dental tissues but also umbilical cord blood and Wharton's jelly.

Regarding their use for neural diseases, MSCs have been mostly used for CNS affections and less for peripheral diseases (i.e. 531 references for "MSC/CNS" keywords versus 61 references for "MSC/peripheral nervous system" in PubMed), but have been equally used for acute injuries and for chronic neurodegenerative diseases (Table 4). One of the most common acute injuries is ischemic brain injury, and traumatic injury for the brain, spinal cord and peripheral nerves. Ischemic brain injury (mostly ischemic stroke but also perinatal hypoxic-ischemic encephalopathy and cerebral ischemia secondary to cardiac arrest [19]) is the second leading cause of death worldwide and a major cause of adult long-term disability and mortality [31]. Administration of bone marrow and adipose tissue−derived MSCs has been widely explored for this pathology, and encouraging results have been obtained in rodent stroke models.

MSCs derived from umbilical cord blood can also be beneficial, but interestingly, contrary to other MSCs, they cannot cross the BBB [32]. MSCs have also been successful in improving the outcome of animals subjected to traumatic brain and spinal cord injury, including the reduction in brain edema, apoptosis, neuroinflammation, microglia accumulation, ischemia, and the enhancement in angiogenesis, neurogenesis and cell survival [33−36]. Although less explored, MSCs have also been used to stimulate peripheral nerve regeneration, either as MSCs [37] or as Schwan cell−like cells [38,39]. If MSCs are injected at the nerve lesion site, enhanced survival and neuronal growth cone elongation have been observed, as well as improved restoration of motor and sensory functions, mostly due to paracrine factor secretion (ciliary neurotrophic factor, glial cell−derived neurotrophic factor [GDNF], basic fibroblast growth factor [bFGF]) [40]. However, most of the work done on peripheral nerve regeneration associated stem cells with a physical support of sorts, similar to a conduit or a tube [38]. It makes sense considering that in non−self-regenerating lesions (typically a gap longer than 3 mm) a physical bridge is required to connect the two stumps and see any repair.

The therapeutic potential of MSCs can also be harvested for chronic neurodegenerative diseases such as MS, multiple system atrophy, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease and autism [41−43]. Vaes et al. recently showed that nasal administration of bone marrow−derived MSCs to a

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Table 3

Recent clinical trials (either recruiting or active) related to NSC.

NCT Number	Title	Status	Pathology	Interventions	Phases	First posted	Last update posted
Injection of NSCs							
NCT04631406	A Safety and Tolerability Study of Neural Stem Cells (NR1) in Subjects With Chronic Ischemic Subcortical Stroke (ISS)	Recruiting	Ischemic stroke	Biological: NSCs	Phase 1|Phase 2	2020	2021
NCT01772810	Safety Study of Human Spinal Cord–derived Neural Stem Cell Transplantation for the Treatment of Chronic SCI	Recruiting	Spinal cord injury (SCI)	Drug: human spinal cord stem cells.	Phase 1	2013	2017
NCT04284293	CNS10-NPC for the Treatment of RP	Recruiting	Retinitis pigmentosa	Biological: CNS10-NPC implantation	Phase 1	Febr. 2020	Febr. 2020
NCT03629275	Investigation of Neural Stem Cells in Ischemic Stroke	Active, not recruiting	Ischemic stroke	Combination product: CTX0E03 cells and delivery device Drug: placebo	Phase 2	2018	2020
NCT01151124	Pilot Investigation of Stem Cells in Stroke	Active, not recruiting	Stroke	Biological: CTX0E03 NSCs	Phase 1	2010	2019
NCT02452723	A Study to Evaluate the Safety of Neural Stem Cells in Patients With Parkinson's Disease	Active, not recruiting	Parkinson's disease	Biological: ISC-hpNSC	Phase 1	2015	2019
Genetically modified NSC							
NCT03072134	Neural Stem Cell–Based Virotherapy of Newly Diagnosed Malignant Glioma	Active, not recruiting	Glioma	Biological: NSCs loaded with an oncolytic adenovirus	Phase 1	2017	2020
Other cell type differentiated into NSC							
NCT03684122	Use of MSC Differentiated Into NSC in People With Parkinson's disease (PD).	Recruiting	Parkinson's disease	Biological: injection of umbilical cord–derived MSC	Phase 1|Phase 2	2018	2020
NCT03355365	Intrathecal Administration of Autologous MSC-derived Neural Progenitors (MSC-NP) in Progressive Multiple Sclerosis	Active, not recruiting	Multiple sclerosis	Biological: intrathecal MSC-NP injection Other: intrathecal saline injection	Phase 2	2017	2020
NSC + Scaffold							
NCT02688049	NeuroRegen Scaffold™ Combined With Stem	Enrolling by invitation	SCI	Biological: NeuroRegen scaffold/ MSC transplantationBiological:	Phase 1|Phase 2	2016	2020

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Table 3. (*continued*)

NCT Number	Title	Status	Pathology	Interventions	Phases	First posted	Last update posted
	Cells for Chronic Spinal Cord Injury Repair			NeuroRegen scaffold/NSC transplantation			
Endogenous modification of NSC							
NCT04121468	A Phase I Double Blind Study of Metformin Acting on Endogenous Neural Progenitor Cells in Children With Multiple Sclerosis	Recruiting	Multiple sclerosis (MS)	Drug: Metformin Other: Placebo	Phase 1|Phase 2	2019	2020

NSC, neural stem cells; MSC, mesenchymal stem cells.

diffuse white matter injury mouse model improved myelination and functional deficits, decreased microglia and astrocyte activation and stimulated oligodendrocyte maturation [44]. Treating parkinsonian rats with MSCs isolated from Wharton's jelly significantly restored the level of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) and improved their cognitive abilities [45].

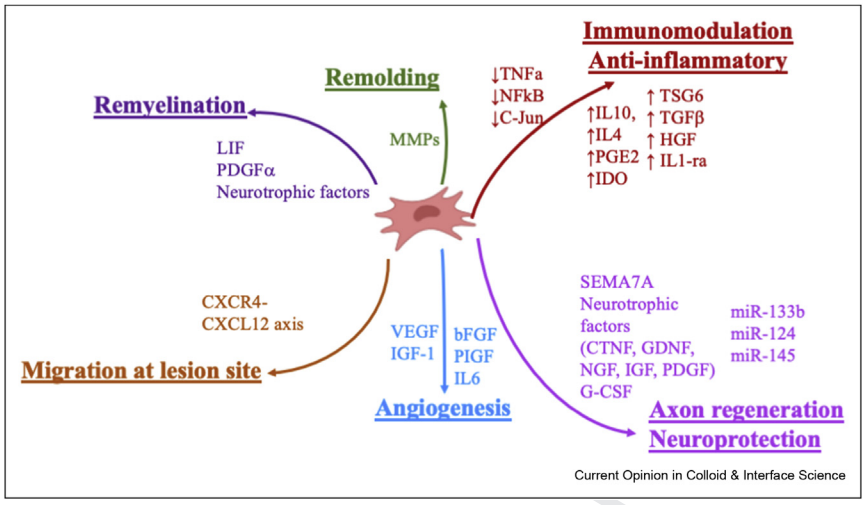
The mechanisms by which MSCs can reduce deficits and promote recovery are not fully elucidated but three main leads have been proposed regardless of the pathology: i) endogenous repair stimulated by MSC secretome (i.e. neurotrophic factors such as BDNF, vascular endothelial growth factor [VEGF], hepatic growth factor [HGF]); ii) endogenous neurogenesis by recruitment of progenitors (NSCs from the subventricular zone and subgranular zones); iii) direct replacement of dead or non-functional cells by differentiated MSCs [31,33]. Lalu et al. performed a meta-analysis on the outcome of MSC administration after a stroke [46]. They collected evidence showing that MSC injection was safe (in clinical and preclinical settings), but unfortunately, so far, promising results obtained in preclinical models did not translate in patients. The same observation has been made for other pathologies such as traumatic brain injury [33]. Mostly, it is due to translational discrepancy between the laboratory and clinical protocols. Efficacy was reached in the laboratory by following precise dose and timing of transplantation that are not strictly applied during clinical trials [47].

Besides regenerative medicine, MSCs have also been used in cancer therapy. However, the role of MSCs in cancer progression is still controversial. MSCs are involved in the regulation of tumor growth and metastasis through cell fusion [52]. They can enhance malignant characteristics, promoting tumor growth and metastasis by fusing with malignant cells, while other conflicting reports showed that MSC can reduce tumorigenicity on fusion with malignant cells [52]. As such, MSCs could potentially be used as a therapeutic tool to treat cancers of the nervous system, but MSC administration, in this scope, could lead to the promotion of tumor growth and metastasis. In consequence, this kind of approach should be handled with outmost precautions.

In conclusion, there are a large number of studies that demonstrate the feasibility, safety and efficiency of stem cells as a therapeutic tool in the arsenal against neurological disorders. However, as potent as they might be, stem cell efficiency can be further improved by various modifications to, for instance, increase the secretion of a specific molecule, enhance their migratory abilities, and control their fate by incorporation of a suicide gene.

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Figure 2



Important aspects of MSC secretome for neurological diseases. MSC, mesenchymal stem cells; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; TNFα, tumor necrosis factor alpha; PGE2, prostaglandine E2; IDO, indoleamine-pyrrole 2,3-dioxygenase; TSG6, tumor necrosis factor-inducible gene 6 protein; TGFβ, transforming growth factor beta; HGF, hepatic growth factor; GDNF, glial cell–derived neurotrophic factor; NGF, nerve growth factor.

Modified stem cells

They are different strategies to produce "enhanced" stem cells whose choice depends on the objective and the inherent cell properties [53]. The main reasons for stem cell modification are i) to increase the production of a therapeutic/bioactive molecule (i.e. neurotrophic and angiogenic factors), ii) to track cells (i.e. GFP), iii) to confer a higher migratory ability to the cells (i.e. CXCL4), iv) to improve cell homing and survival and v) to better control fate of implanted cells (i.e. suicide gene [54]) (Figure 3). Mostly, these alterations are performed by genetic manipulations using viruses, even though more and more nonviral vectors are being developed and used. Alternatively, cells can be preconditioned before administration but the effect is likely to be more transient.

Viral vectors are the most common and efficient vectors for the genetic modification of cells as they can easily infect them, cross barriers, and deliver genetic material into the host cell nucleus [55]. Different types of viruses can be used to modify stem cells, including lentivirus, adenovirus, retrovirus, adenovirus-associated virus and Sendai virus [56]. Viruses can be integrative (retrovirus and lentivirus) or nonintegrative (adeno-associated virus, adenovirus and Sendai virus), meaning that they can integrate the transgene in the cell genome (long-term expression) or not (short-term expression), respectively. Although viral vectors are really efficient, they raise safety concerns that limit their clinical translation. They could induce insertional mutagenesis, proto-oncogene activation and immune response activation [55,56]. Some strategies like common insertion site analysis to predict the risk of insertional mutagenesis can be applied to reduce these issues [56]. The establishment of a benefit-to-risk ratio is thus essential. To address these safety issues, numerous nonviral vectors have been developed, ranging from transfection agents (i.e. lipofectamine) to synthetic viruses and nanomedicines (i.e. liposomes). Several good reviews have been published on this subject [57–60]. Of note, compared with direct injection of gene therapy vectors, *ex vivo* stem cell modification is safer as it can be more easily controlled and limited to a mean of one or two vector per cell [55].

The large majority of studies reporting the production and use of genetically modified cells for neural disorders aims at the production of different molecules: i) a specific growth factor like BDNF [61,62], GDNF or NGF [63] to stimulate neurogenesis or VEGF [64] for angiogenesis, ii) cytokines (i.e. interleukin 12 [IL12] and IL17 [65], CX3CL1 [66], CCL2 [67], IL10 [68]) to increase cell immunomodulatory properties [25], iii) a receptor or regulator to enhance cell homing abilities (i.e. CXCR4 [69], SDF-1 [70], FGF2 [71], polysialylating enzyme sialyltransferase [6]). Genetic modifications can also be used to improve the safety of cell therapy by introducing a gene coding for a tracking molecule (i.e. GFP [72]) or a suicide gene [73].

Cell preconditioning is another strategy that can be used to enhance MSC therapeutic properties, mostly immunomodulation, to improve their chances of survival when transplanted into a harsh environment, and migration. Priming MSCs with immunomodulatory factors such as

interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) activates the synthesis of indoleamine-pyrrole 2,3-dioxygenase, HGF and transforming growth factor beta (TGFβ) [25], which leads to a significant increase of MSC T-cell suppressive function. MSC conditioning could as well increase their therapeutic action. For instance, Beigi Boroujeni et al. showed a strong benefit of bone marrow–derived MSC preconditioning with SDF-1 on remyelination in a cuprizone model [74]. Conditioning of MSCs can also be used as an MSC-based vaccination against cancer, like when the conditioning of MSC with small heat-shock proteins 27 and 20 as well as with E7 oncoprotein significantly enhanced E7-specific T-cell response and suppressed tumor growth in mice [75].

Culture in hypoxic condition before implantation activates the hypoxia-induced factor 1 alpha pathway and consequently downregulated the NFκB pathways, leading to the secretion of immunosuppressive/antioxydative and pro-regenerative molecules [76,77]. It also increases cell migrating and homing abilities via an upregulation of CXCR4 [70].

MSC conditioned medium can also be used to prestimulate NSCs before transplantation [78]. Preconditioning increased NSC survival after transplantation in an adult rat spinal cord, oligodendroglial differentiation and ability to myelinate axons *in vivo*.

Thus, genetic modification or preconditioning significantly enhances stem cell therapeutic efficiency. However, the feasibility of large-scale GMP production and, ultimately the translational value of this approach, are questionable as they increase associated risks and complexify the process and quality control. In regard to the challenges raised by GMP production of a sufficient number of cells, safety concerns and lack of an undisputable efficiency during clinical trials, the use of stem cell–derived products instead has appeared as a credible alternative. The postulate is that by harnessing stem cell secretome, it is possible to benefit from the therapeutic potential of stem cells in a much easier to translate cell-free product.

Stem cell–derived products

Over the last years, new insights regarding the mechanisms of action of transplanted cells have emerged, focusing on their paracrine action rather than cell replacement or cell–cell interactions. It seems that whatever the cell type or source, at least some of their effects are linked to their secretome. Consequently, the administration of stem cell secretome as a conditioned medium would provide "cell-free" therapies by harnessing stem cell potential while limiting the drawbacks of cell transplantation (i.e. ethics, safety, cell survival, allogenic immune response).

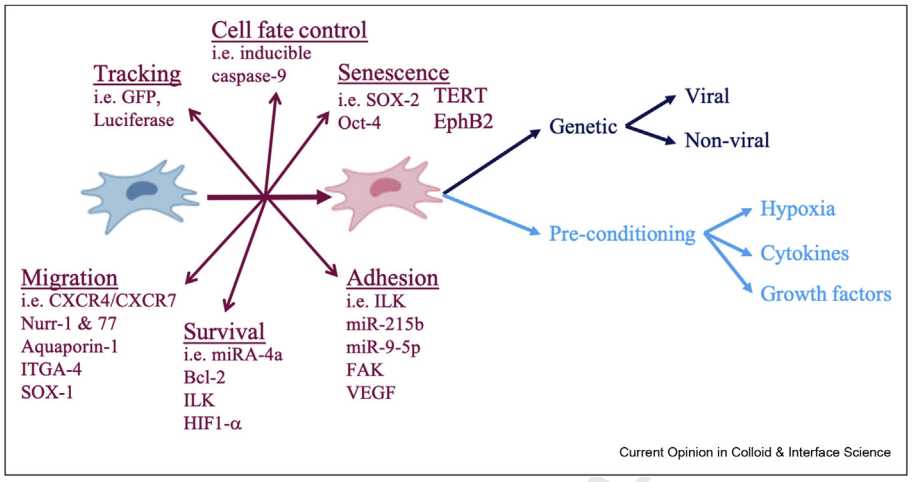
Cell-derived products such as growth factors, lipids, micro-RNA, mitochondria and EV have been identified as the most potent elements explaining stem cell therapeutic effects [47,79]. For instance, Matsubara et al. demonstrated that the significant improvement of rat locomotor function after dental stem cell injection in a spinal cord model was largely due to their secretome and more specifically by the production of inducers of anti-inflammatory M2-like macrophages, among which monocyte chemoattractant protein-1 and the secreted ectodomain of sialic acid–binding Ig-like lectin-9 (ED-Siglec-9) [80]. Similarly, the therapeutic effect of human NSCs observed in several neurological diseases has been vastly linked to their secretome, mostly neurotrophic factors (i.e. BDNF, NGF, GDNF) [81]. As an example, the injection of human NSC conditioned medium in a 6-OHDA rodent model of Parkinson's Q10 disease resulted in a significant increase of dopaminergic neuron survival [82]. More and more articles are being published reporting the crucial role of stem cell secretome in their therapeutic effect on CNS diseases [83], mostly about MSC secretome but not restricted to this type of cells [81].

Conditioned medium

Conditioned medium is typically obtained from cells in culture whose medium has been replaced by serum-free medium for 24h–72h. This medium is then collected, cleared of debris and frozen until further use. It can be used as such or concentrated (usually by ultrafiltration on a 3 kDa filter). Cell secretome contains bioactive factors (soluble proteins, nucleic acids, lipids) secreted to the extracellular space, either as soluble molecules or associated to EV. In the last decades, stem cell preconditioning has also been used to increase or modify cell secretome properties. MSCs have been primed using different stimuli like hypoxia and inflammatory, molecular or mechanical stimuli [25,83]. This resulted in a higher content of pro-angiogenic molecules (i.e. angiogenin, matrix metalloproteinase-9 [MMP-9], VEGF-A, and VEGF-C), and immunomodulatory cytokines (i.e. IL6, IL8, IFNγ, IL10, prostaglandine E2) in their secretome with improved effects on wound healing, angiogenesis, neuroprotection, neurogenesis, and even neuro-regeneration [83]. However, depending on the strategy, priming does not always result in improved properties, so it has to be chosen, optimized and characterize carefully [84]. As mentioned previously, cell-derived secretome presents the advantage to recapitulate the effects of stem cells without most of their side effects. Stem cell–derived secretome would also be easier to manage, as it could be immediately available for the treatment of acute conditions, would not require the invasive collection of donor cells and would be considered as a pharmaceutical agent for regulatory purposes.

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Figure 3



Strategies and objectives to enhance stem cell properties. HIF-1α, hypoxia-induced factor 1 alpha; VEGF, vascular endothelial growth factor. Q9

The therapeutic efficiency of conditioned medium has often been studied as a follow-up after the efficiency of the donor cells has been proved. Alternatively, conditioned medium was compared within the same study to donor cells. In the scope of neurological diseases, conditioned media have been obtained from NSCs and MSCs mainly (Table 5), from native or primed cells. Media have been tested in various models, where their therapeutic effect has been linked to their content in neurotrophic factors (BDNF, GDNF, NGF), cytokines (IL10) and growth factors (TIMP-1, VEGF, FGF, TGF-β). As far as we saw when writing this review, published work always reported a therapeutic effect for conditioned medium, sometimes but not always superior to donor cells. One might wonder whether this reflects the reality, as researchers might have refrained from publishing what would be considered negative results considering that the main trend tends toward a beneficial effect of conditioned medium. Q11

One limitation of conditioned medium is the difficulty to precisely control its content. Indeed, its composition can be influenced by cell passage, batch, culture conditions, and operator. Cell source has to be carefully selected as different cell types have different transcriptional signatures [85]. One approach would be then to perform a throughout analysis of the content of a therapeutically effective conditioned medium and to recreate it artificially. However, the cost would have to be taken into consideration and balanced with the benefit of this approach. Another limitation is the short-lasting effect of a conditioned medium injection compared with the continuous secretion of implanted cells. This means that multiple administrations would be required for conditioned medium where only one cell injection would suffice. While some studies reported a significant therapeutic effect in preclinical models after only one administration, others reported a more sustained effect with chronic administration of conditioned medium [86].

When looking at clinical trials, surprisingly, only one trial currently aims at evaluating the therapeutic efficiency of conditioned medium for a neurological disease (umbilical stem cell conditioned medium), and it is tested in combination with umbilical stem cells (versus cells alone), not alone (NCT04314687) (Table 5). Clearly, the choice has been made by actors of the field to rather invest on EV/exosomes where cell-derived products are concerned.

Extracellular vesicles

EV used to be considered as a way for the cells to eliminate wastes, but these last years, they received more and more attention as researchers realized that they could be used not only as diagnostic tools but also as therapeutics, either on their own or as vehicles for bioactive molecules (nanomedicines). Consequently, they are the focus of numerous recent publications on stem cell therapy. As an example, the search terms "extracellular vesicles" or "exosomes" associated with "stem cells" identified 2396 articles in PubMed for 2020 alone. EV encompass exosomes, microvesicles and apoptotic bodies, three broad categories used to classify EV according to their mode of genesis, size and composition. The diameter of most EV is around 100 nm (exosomes and microvesicles) with apoptotic bodies being much larger (in the range of microns) [92]. Practically, most studies are focused on EV that measure 1 μm or less: exosomes and microvesicles. As they are quite difficult to separate, the consensus is to use the term EV for the heterogenous population composed of

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Table 4

Examples of studies using MSCs as a treatment against neurological diseases.

Cells	Model	Cell fate	Impact	Ref
hAMSC	Cardiac arrest–induced brain injury Intravenous 1 hour after cardiac arrest at 1, 3 and 7 days	Expression of neuroprotective IL-6 and BDNF	• Neuroprotection • Improved neurological function	[48]
Mouse (C57BL/6) bone marrow-derived MSC	• Encephalopathy of prematurity • Intranasal application of mesenchymal stem cells	Migrated dispersedly into the injured brain	• Improved myelination and functional outcome • Dampened cerebral inflammation and oligodendrocyte maturation • IGF1, EGF, LIF, and IL11	[44]
Schwann-like cells deriving from human adipose–derived MSC	• Loaded into collagen sponge biomaterials • Implanted around transected sciatic nerves with a 10-mm gap	No information	Significantly enhanced axon regrowth, myelination and functional recovery after sciatic nerve injury.	[49]
hAMSC in fibrin glue	Sciatic nerve injury model	Enter the nerve and migrate mainly retrogradely after transplantation	• Neuroprotective effect on DRG L5 sensory neurons • Stimulated axon growth and myelination. • Nerve angiogenesis and motor function recovery.	[50]
hWJ-MSC	Intrathecally transplanted in the 3 consecutive weeks after the induction of a balloon compression lesion SCI	No information	• Significant functional improvement • Higher amount of spared gray and white matter • Enhanced expression of genes related to axonal growth. • WJ-MSCs enhanced the expression of inflammatory and chemotactic markers in plasma.	[51]
hWJ-MSC	Rat model of Parkinson Intravenous injection twice on the 14th and		• Significantly restored the levels of NGF and BDNF	[45]

	28th days after PD induction	• Improved cognition and hippocampal long-term potentiation (hLTP)

MSC, mesenchymal stem cells; hAMSC, human adipose derived MSC; DRG, dorsal root ganglia; hWJ-MSC, human Wharton's jelly-derived; IL, interleukin; BDNF, brain-derived neurotrophic factor; SCI, spinal cord injury; NGF, nerve growth factorMSC.

both types of vesicles. EV can transport molecules involved in biological process (i.e. proteins [enzymes, signaling proteins, immunomodulatory, and growth factors], lipids, genetic materials [DNA, miRNA, mRNA] and mitochondrial components in their lumen and membrane but also associated with their surface) [93,94]. EV in general and EV as diagnostic tools are not the focus of this review, so we refer the readers to other publications where more information regarding these matters can be found [95–98]. Similar to conditioned medium, EV could be used as cell-free treatments as they bypass many limitations of cell-based therapies as mentioned previously. EV also present the advantage of crossing the BBB [99] and eliciting a limited allogenic immune reaction while having intrinsic properties. In the scope of neurological affections, EV are mostly isolated from MSC, but several studies reported also the use of EV isolated from NSCs, induced NSCs, and iPSCs [100].

EV obtained from NSCs have strong neuroprotective, neuroregenerative, neuroproliferating, and anti-aging properties [81,100]. An intravenous injection of human NSC-derived EV decreased the lesion volume and swelling, preserved white matter and improved functional behavior of animals in a stroke model [101]. EV derived from induced NSCs have been used to treat stroke [102]. They promoted a proresolutive phenotype of the macrophages, increased the number of Tregs, reduced lesion volume and improved behavior in stroke models. However, like for cell therapy, the access to NSCs can be limited and problematic, and their expansion for industrial production is delicate.

This is the reason why most of the current studies and clinical trials focus now on **MSC-derived EV**. In addition to the advantages of MSCs versus other stem cell source (as described previously), MSCs are prolific regarding EV production [103], and their EV have been attributed numerous beneficial activities. As done to increase MSC potential, pre-conditioning using 3D culture, chemicals (azoles, cytochalasin B), hypoxia, gene editing (phospholipase D2) and cytokines (TNFα, IFNγ, IL-1β) increases the production of EV and their therapeutic efficiency [104]. EV have been tested in all the most prominent neurological diseases and showed benefits in every one of them, although, like for conditioned medium, bias can be created by researcher auto-censure.

MSC-derived EV have the same effects as their donor cells: They would modulate immune response, be neuroprotective, be proangiogenic, would support neurogenesis, and in general, stimulate the repair of the nervous system, peripheric or central, resulting in an improved function. MSC-derived EV effects can be plethoric, and their reported mechanism(s) of action depend(s) on numerous factors, such as the preclinical

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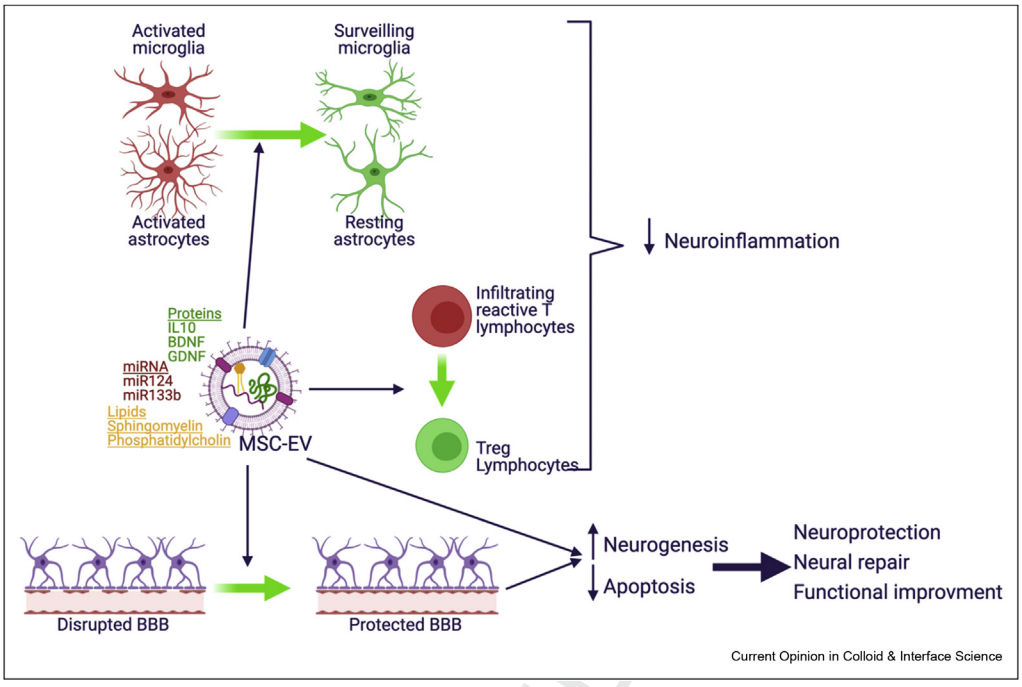
Table 5

Examples of studies using cell conditioned medium as a treatment against neurological diseases.

Cell source	Pathology (model)	Administration	Impact	Active molecules	Ref.
Preclinical studies					
hWJ-MSC	*In vitro*/*in vivo* NSC	Inj. in dental gyrus	↑NSC differentiation, proliferation in dental gyrus	↑ NGF and FGF	[87]
hAMSC	Human astrocytes (*in vitro* scratch assay)	Incubation with cells	↑ Cell viability ↓ ROS Preserve mitochondria	Neuroglobin	[88]
OL-derived from hWJ-MSC	Multiple sclerosis (EAE)	Intranasal adm. 1 dose/12 h 100× conc.	↑Mouse weight and motor function, remyelination ↓Inflammation	BDNF, GDNF, CNTF	[89]
hSHED	Parkinson's disease (Rotenone rat model)	Intravenous inj. (100 µg prot., one-shot)	↓ Symptoms ↑Tyrosine hydroxylase ↓Alpha-synuclein in striatum ↓ Iba-1 and CD4	IGFBP-6, TIM-2, TIMP-1, TGFβ-1	[90]
hWJ-MSC	SCI (rat)	Intrathecal lumbar puncture 3 first weeks post-SCI 20× conc.	↑ Spared tissue ↑Genes related to axonal growth No inflammation markers in serum for conditioned medium (vs donor cells)	HGF, MCP1, VEGFA, IL6, sICAM1, SDF1α, IDO, FGF2, sVCAM1, BDNF, NGF	[51]
hDPSC Leukocyte- and platelet-rich fibrin priming	Primary cortical neurons	Incubation with cells	↑ Neurogenesis Chemoattractant on NSC No effect of priming	↑ BDNF by priming	[84]
Rat BM-MSC	SCI (rat)	Continuous inj. 2 for weeks via lateral ventricle (ALZET osmotic pump)	↑Locomotion and axonal growth ↓ Astrocytes	No information	[91]
hNSC	Parkinson's disease (6-OHDA)	Substantia nigra and 4 striatum inj.	↑Recovery of dopaminergic neurons ↑Motor function ↑Tyrosin hydroxylase (>donor cells inj.)	595 proteins identified	[82]
hAMSC Activated with IFNγ and TNFα	Alcool and nicotine dependence (rat)	Intranasal adm. 1×/week for 5 weeks 30× conc.	↑ Oligogenesis Myelin sheath generation	Proteins associated with oligodendroglial lineage regulation (i.e. TIMP-1)	[78]
hBM-MSC Exposed to brain homogenate	Alzheimer's disease (Transgenic APP/PS1 mouse)	Intravenous (one-shot) and intranasal (1/week) Concentrated	Memory recovery ↑ Neuronal density and mouse life span ↓Plaques, lower density of beta-amyloid oligomers, gliosis	No information	[86]
Clinical study					
hUMSC Conditioned medium + cells Allogenic	Cerebral palsy in children	Intrathecal inj.	Phase 1/2 Started in March 2020	No information	NCT04314687

hWJ-MSC, human Wharton Jelly umbilical MSC; hAMSC, human adipose derived MSC; OL, oligodendrocyte; hDPSC, human dental pulp stem cells; ROS, reactive oxygen species; EAE, experimental autoimmune encephalomyelitis; conc., concentrated; hSHED, human exfoliated deciduous teeth; SCI, spinal cord injury; hBM-MSC, human bone derived MSC; NSC, neural stem cells; MSC, mesenchymal stem cells; NGF, nerve growth factor; FGF, fibroblast growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell–derived neurotrophic factor; CNTF, ciliary neurotrophic factor; TGFβ-1, transforming growth factor beta; HGF, hepatic growth factor; MCP-1, monocyte chemoattractant protein-1; IL, interleukin; SDF-1, stromal cell–derived factor-1 alpha; IDO, indoleamine-pyrrole 2,3-dioxygenase; BM-MSC, bone marrow MSC; IFNγ, interferon gamma.

Figure 4



Impact of EV on nervous system repair. IL, interleukin; BDNF, brain-derived neurotrophic factor; GDNF, glial cell–derived neurotrophic factor; BBB, blood-brain barrier; EV, extracellular vesicles. Created with BioRender.com.

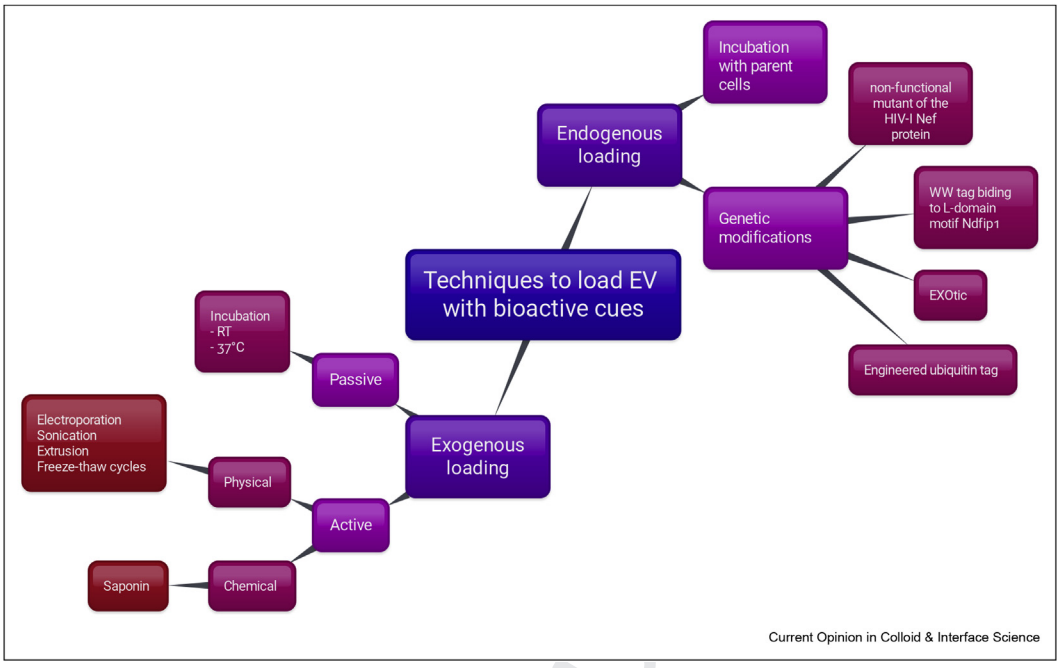
model, the source of EV, the administration route, the dose of EV used and also the researchers "hypothesis and working angle." It is then quite difficult to link them to a generic mode of action. It is generally accepted that these EV can turn the lesion micro-environment into a more favorable, pro-regenerative one, thanks to their anti-inflammatory and pro-angiogenic effects [79]. They can modulate the immune response via different mechanisms (i.e. regulation of macrophage polarization [105,106], increase of Treg lymphocytes [107]) (Figure 4). Several studies provide evidences that MSC-derived EV could support traumatic and brain injury via miR-124 and miR-133b, IL10/NFκB-p65 or BDNF/GDNF/IL10 pathways [79]. Intravenous injection of MSC-derived EV improved neurogenesis and axonal growth in rodent models of ischemic brain injury, resulting in improved functional outcome [108]. Several articles report that MSC-derived EV promoted the repair of spinal cord injury in different animal models, whether administrated locally (direct injection in the spinal cord parenchyma or intrathecally [109,110]), systemically (intravenous [111]), or intranasally [112]. They induced axonal growth and neuroprotection, reduced neuroinflammation, glial scar formation and astroglial activation, supported angiogenesis and protected the blood-spinal barrier [100]. The positive effect of MSCs on peripheral nerve repair has also been linked to their EV. Adipose tissue–derived MSC-EV promoted *in vitro* Schwann cell proliferation and survival by upregulation of Bcl-2 and downregulation of Bax gene expression [113]. Intrathecal infusion of human umbilical cord MSC-derived EV reduced pain in a rat with sciatic nerve ligation injury by modulating neuroinflammation and neuroregeneration [114,115]. Like for traumatic affections, EV isolated from MSCs have also improved the outcome of neurodegenerative diseases, for instance, MS or epilepsy. They reduced clinical score, demyelination and neuroinflammation and increased Tregs in experimental autoimmune encephalomyelitis mouse models after intravenous injection [105,116]. Of note, in the work of Riazifar et al., MSCs were primed with IFNγ (10 ng/ml 48h before EV isolation) [116]. Intranasal administration of human bone marrow MSC-derived EV in a rat model of status epilepticus significantly reduced the loss of glutamatergic and GABAergic neurons and reduced inflammation in the hippocampus [117]. EV were visible in the hippocampus 6h after administration.

Extracellular vesicles as nanomedicines

Recently, in addition to their endogenous role, EV have been more and more used as drug delivery vehicles. They have the natural ability to cross barriers [118], have strong homing capacities toward targeted tissues and can be tuned for site-specific drug delivery without eliciting immune responses, hence qualifying as an ideal

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Figure 5



Different techniques used to load extracellular vesicles (EV) with a bioactive cue.

delivery vehicle [119,120]. Proteins, small molecules, and miRNA are the most popular among the exogenous molecules encapsulated in EV for therapeutic purposes [121,122]. In addition, EV surface can also be modified either after isolation [123,124] or by genetic engineering of the producing cell to increase their specific targeting abilities and, consequently, their drug delivery efficiency [122,125]. Different techniques can be used to encapsulate active molecules in EV, depending mostly on the nature of the molecule to be encapsulated (i.e. nucleic acid, lipophile or hydrophile molecules) (Figure 5).

EV drug loading is very variable and depends on the molecule and the method used [126]. EV can be enriched with a given cue by exogenous or endogenous loading. Exogenous loading is performed on EV after their isolation and two approaches can be used: passive and active loading [127]. Passive loading consists incubation of the EV with the therapeutic cargo so that it is passively loaded into the EV. Active loading uses a physical (i.e. sonication) or chemical agent (i.e. surfactant) that disrupts the EV lipid membrane. Endogenous loading refers to the addition of the therapeutic molecule before EV shedding, either by simple incubation of the molecule with the EV-producing cells or by genetic modification (transient or permanent). Complete and good reviews summarize the main techniques used to load EV with therapeutic molecules [121,126,127].

In the scope of neurological diseases, EV have been loaded with miRNA (i.e. miR-17-92, miR-186, miR-124), siRNA (i.e. BACE1, a-Syn), small molecules (i.e. paclitaxel or curcumin), and proteins (i.e. catalase, tripeptidyl peptidase-1 [TPP-1]). Catalase, a potent anti-oxidant, has been encapsulated in mouse bone marrow macrophage-derived EV via four methods [128]. Sonication was selected as the best compromise and catalase-loaded EV were administrated to a Parkinson's disease 6-OHDA mouse model. Catalase-EV significantly reduced oxidative stress and increased neuronal survival. The same group loaded TPP-1 in EV for the treatment of a lysosomal disease, CLN2 Batten disease (BD), by transfection of EV parent cells or active loading in EV after isolation [129]. They compared four routes of administration for TPP-1-loaded EV in a mouse BD model and observed that administration through intrathecal and intranasal routes resulted in high TPP1 accumulation in the brain, decreased neurodegeneration and neuroinflammation, and reduced aggregation of lysosomal storage material in BD mouse model [130]. They also observed that the combination of intrathecal and intraperitoneal injections of TPP-1 loaded EV significantly prolonged the lifespan in BD mice. Curcumin, an efficient but very hydrophobic anti-inflammatory molecule, has been encapsulated in macrophage-derived EV. Incorporation in EV significantly improved curcumin solubility and bioavailability and increased drug penetration across the BBB, prevented the death of neurons *in vitro* and *in vivo* and

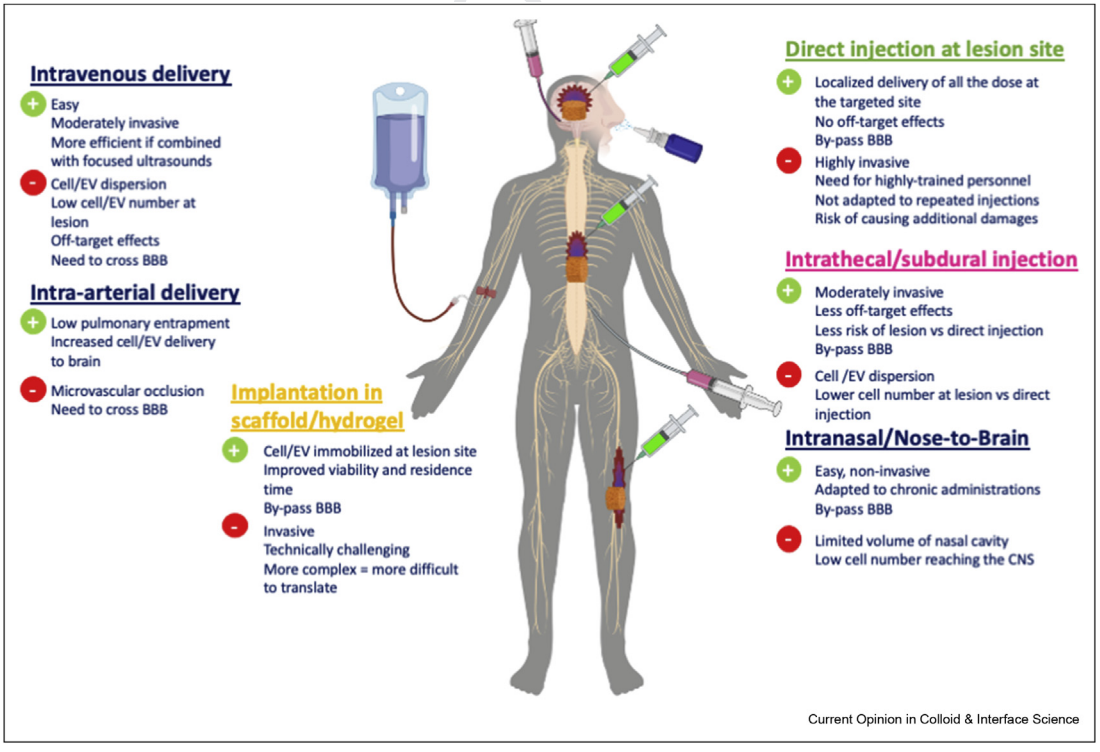
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inhibited the phosphorylation of the Tau protein in an Alzheimer's disease model [131]. In another model, they alleviated cerebral injury by inhibiting reactive oxygen species (ROS)-mediated mitochondrial apoptosis in a transient cerebral ischemia rat model [132].

Drug-loaded EV have also been used as treatment against brain cancer [133]. Atorvastatin has been loaded in EV derived from human endometrial stem cells as a treatment against glioblastoma. These EV were able to reduce the viability and proliferation of glioblastoma cell spheroids [134]. A Stat3 inhibitor JSI-124 encapsulated in EL-4 cell EV (lymphoma cell line) reduced the growth of GL-26 (glioblastoma cells) tumor growth [135]. And MSCs transduced with a lentivirus coding for miR-124a increased by 50% the survival of GSC267-tumor bearing mice [136].

Besides proteins and small molecules, nucleic acids such as miRNA have also been delivered to the nervous system using EV. The therapeutic potential of miRNA has recently raised a lot of interest. MiRNAs are a family of noncoding RNAs ranging between 20 and 25 bases in length that are able to affect protein levels by post-transcriptional regulation of mRNA. They complement the 3′ UTR of mRNA and are able to shut down the translation of the mRNA transcript as a part of the RNA-induced silencing complex [94]. MiRNA exert fundamental effects on neural tissue regeneration and strongly take part in neural progenitor cell proliferation and differentiation, regulatory activities of glial cells and synaptic plasticity [137]. As EV are natural transporters for miRNA, a few studies so far have been aiming to enrich EV with exogenous miRNA, either by direct loading or gene transfection of parent cells [119]. MSC-EV loaded with miR-124 have been seen to promote neurogenesis after stroke in mice [138]. EV loaded with miR-133b or miR-126 promoted axon regeneration in a spinal cord injury model via the Stat-3 and Erk1/2 pathways [139,140]. As another example, Wharton's jelly MSCs transfected to express miR-124 enhanced the U87 glioblastoma cell line chemosensitivity to temozolomide and decreased their migration *in vitro* [141]. Kojima et al. even developed a set of plasmids allowing an efficient, customizable, increased production of EV as well as a higher loading of miRNA (EXOtic) [142]. They incorporated mRNA coding for catalase in EV and observed a reduced neurotoxicity and neuroinflammation in *in vitro* and *in vivo* models of Parkinson's disease. This system seems really efficient and elegant but quite complicated for an easy translation.

Figure 6



Different EV and cell administration routes to the nervous system. Created with BioRender.com.

It seems then that by using EV it is possible to recapitulate at least some therapeutic effects of stem cell transplantation, effects that can be significantly enhanced by EV modification, either by loading of a therapeutic cue and/or specific targeting [122]. On the bright side, EV are quite stable in biological fluids, immuno-compatible, can cross the BBB, have intrinsic homing capacity and are safe to use as demonstrated by recent clinical trials. However, numerous challenges remain to be addressed before EV can be fully exploited as a potential "cell-free" therapy [143,144]. Indeed, large-scale production is difficult, yields are often low, there is no optimal purification method, low drug loading efficiency, lack of *in vivo* tracking, and a lack of clinical study for delivery of therapeutic molecules to the CNS. In addition, the composition and effect of EV are quite dependent on the parent cell source, method and medium of culture and isolation technique.

Administration of stem cells and derived products to the nervous system

The most efficient strategy to deliver stem cells and their derived products remains unclear. It is a balance between the number of cells (or EV) that reaches the desired site of action, their engraftment, their immediate survival and medium- to long-term residency, with, of course, their therapeutic efficiency being the ultimate endpoint (Figure 6). Cell delivery is a compromise between invasiveness, ease of use and efficacy [19]. For instance, direct injection in the parenchyma (brain or spinal cord) or in the peripheral nerve lesion presents the most direct route of administration that ensures that most of the cells reach the intended site of action. However, it is extremely invasive and requires highly trained personnel and up-to-date installations, presenting a considerable risk of infection or additional lesions due to the injection process. By comparison, intravenous injection is the easiest to perform with less risk of morbidity associated with the administration, but the percentage of cells that reaches the nervous system is quite limited, and the risk that cells take up residence in nontargeted organs is high. Another parameter to take into account is the timing of the injection. In traumatic events, such as ischemic stroke or spinal cord injury, it significantly impacts the efficiency of cell therapy and outcome, the earliest the administration, the highest the chances of a therapeutic effect.

Local injection

The most direct administration technique to deliver cells and therapeutics to the nervous system is the local injection at the desired site of action. It allows bypassing the BBB and a delivery of all the intended dose at once (cells/conditioned medium EV) and thus the best control on the cell or EV number administrated to the patient. However, it is highly invasive, could cause additional lesions and the delivery of a massive number of cells at once is not always in favor of their survival. It is not ideal for repeated injections and thus for chronic diseases such as neurodegenerative pathologies. Nevertheless, this method of administration has been widely used for NSC and MSC injection in the brain and spinal cord, mostly via a microneedle fixed on a stereotaxic frame and pump [47,145]. The main reason is that it allows crossing off any issue with the whole dose reaching the lesion as a possible cause for inefficiency. It seems that lateral ventricle injection allows a higher cell survival rate than injection in the parenchyma [145]. In the case of stroke for instance, local injection of cells to the ischemic area was more effective than intravenous administration [3]. Specific instruments, such as the intracerebral microinjection instrument, have been developed for the safe and minimally invasive delivery of stem cells in the CNS [146]. It has been successfully tested in stroke patients.

Alternatively, cells and their derived products can also be injected via catheter (subdural or intrathecal injections) and osmotic pumps for cell-derived products. This is a slightly less invasive alternative where cells are infused under the meninges, so still bypassing the BBB, but not directly injected at the lesion site. It is more acceptable when performing multiple injections than direct administration but there is also more risk of cell/EV loss or dilution. Intrathecal injection of bone marrow and adipose tissue–derived MSCs has been used to administrate stem cells for spinal cord injury in humans and is considered to be safe [147]. It has also been used to deliver EV [130] in a BD model and MSCs to the brain [148]. An Alzet osmotic pump connected to the fourth ventricle was also used immediately after spinal cord contusion in rats to deliver the conditioned medium of bone marrow MSCs through the cerebrospinal fluid for 2 weeks [91]. The authors observed beneficial effects on locomotor function and tissue repair, including axonal regeneration. More recently, human MSCs have been injected in rats via the nose by trans-septal transplantation [149].

Systemic administration

At the other end of the spectrum of administration strategies, intravenous and intra-arterial injections are probably the most used to deliver cells and EV [150]. Indeed, as mentioned previously, it is considered less invasive and easier to deliver treatments. It has been extensively used to deliver NSCs, MSCs, and EV to treat neurological affections [47,79,151], based, at least partially, on the ability of stem cells and EV to cross the BBB. Indeed, one of the main advantages of EV compared with synthetic nanomedicines is their ability to cross epithelial barriers including the BBB. EV can cross intercellular junctions between endothelial cells and enter the CNS. MiRNA in EV could also reduce the

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tightness of junctions (miR-105 downregulates expression of Zonula occludens-1, and miR181c impacts the polymerization of actine) [152], which would increase BBB vascular permeability. But it absolutely needs to be transient as a permeabilized BBB would induce or contribute to the pathology, as in many neurological pathologies the integrity of the BBB is compromised (i.e. MS, glioblastoma, Parkinson's disease). Of note, pathological permeability of BBB can be an advantage for the passage of bigger entities such as cells and EV. Intra-arterial delivery of NSCs seemed to allow a more diffuse distribution of cells in the brain parenchyma and reduced trapping in the liver and spleen as the first-pass effect is less pronounced [145]. It is also possible to combine intravenous and subarachnoid injections. For instance, Vaquero et al. conducted a clinical trial on spinal cord−injured patients where bone marrow MSCs were first administrated by intravenous injection, and then later on, a second subarachnoid injection was performed [153]. However, although easy to set, systemic delivery has been limited by unpredictable cell destination, limited accumulation in the CNS and engraftment. Indeed, even if EV and MSCs can cross the BBB [154], they are quickly removed from the bloodstream and end up in the liver and lungs [144].

Alternative routes of administration

Alternative routes of administration have been sought, such as the intraperitoneal or intranasal routes, the use of BBB permeation enhancement techniques (i.e. focused ultrasounds [155], mannitol injection [32]) or magnetic targeting [156]. EV intraperitoneal injection resulted in a lower accumulation in peripheric organs and allowed the delivery of larger doses [144] while intravenous delivery of MSCs combined with focused ultrasounds increased accumulation of cells in the brain [155]. The intranasal route is becoming more and more popular as it is truly noninvasive, thus more adapted for chronic administration. More and more studies report the use of the nasal route to administrate cells and EV [144]. Evidences accumulate regarding the ability of MSCs and EV to cross the nasal epithelium, notably to reach the CNS via the nose-to-brain route. SDF-1 preconditioned bone marrow MSCs administrated by the nasal route were able to reach the brain of cuprizone treated mice and to stimulate remyelination [74]. EV can also reach the brain or the spinal cord after nasal administration and exert their therapeutic action (anti-inflammatory in a model of Alzheimer's disease [157] and delivery of siRNA in a spinal cord model [112], respectively). Haney et al. recently compared four administrations routes for the delivery of TPP-1-loaded EV: intravenous, intraperitoneal, intrathecal and intranasal [130]. The biodistribution of EV was logically impacted by the administration route. The highest accumulation in the brain was observed for the intrathecal administration and in peripheric organs (liver and spleen) for the intraperitoneal and intravenous administrations. The therapeutic effects were more pronounced for the intranasal and intrathecal routes. The combined administration of TPP-1 EV by intraperitoneal and intrathecal injections significantly prolonged the BD mouse survival.

Biomaterial assisted delivery

The efficacy of cell therapy has been limited by the low rate of cell engraftment and survival after transplantation. Mostly, the hostile microenvironment (proinflammatory cytokines, activated immune cells, ROS), immune rejection and the lack of support, notably from the often-disturbed extracellular matrix precipitate cell death [158]. EV are quickly eliminated from the circulation, diluted in the CSF or cleared from the tissue that are intended to be treated.

To address these issues, several strategies relying on the use of biomaterials, either as hydrogels, scaffolds or microcarriers, have been developed to provide the cells with an anchorage at the site of injection and a more permissive environment that would soften and ease the transition from the plastic of cell culture flasks to the body. This strategy is logically intended for local delivery.

Various natural or synthetic biomaterials have been used including alginate, hyaluronic acid, fibrin, chitosan, polyesters and polyethylene glycol. They have to be biocompatible, biodegradable and ideally injectable followed by gelification *in situ* to limit the invasiveness of their administration. Cells can be incorporated alone in scaffolds/hydrogels or co-encapsulated with EV or bioactive cues (in nanomedicines or in solution). Stem cells can also be seeded on microcarriers before injection, allowing their administration without any detachment procedure, which would preserve their integrins and thus their engraftment potential. In addition, especially in the case of peripheral nerve and spinal cord repair, biomaterials can also be used to provide directional support and bridging to transplanted cells and endogenous tissue.

A multitude of examples can be found in the literature regarding the use and development of biomaterials for neurodegenerative disorders [159−164]. Scaffolds can be used alone but are mostly combined with cells and bioactive or electrical cues, as the consensus is that neurological affections are so complex that multifaceted approaches have more changes to be efficient.

Porous collagen-chitosan scaffolds have been used to transplant MSCs in a rat traumatic brain injury model [165]. The scaffold was well tolerated and the neuroprotective and neuroregenerative effects were higher than the cells or the scaffold alone. An aligned collagen

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Table 6

Current on-going clinical trials using EV for neurological disorders.

ClinicalTrials.gov Identifier	Title	Status	Phase	Pathology	Treatment	Timing	Administration route	Nber of patients	Sponsor
NCT03384433	Allogenic MSC-derived EV in Patients With Acute Ischemic Stroke	Recruiting	Phase1/2	CVA patients with disability	Allogenic MSC-generated EV transfected by miR-124	One month after attack	Stereotaxis/ intraparanchymal	5	Isfahan University of Medical Sciences (Iran)
NCT04202770	Focused Ultrasound and Exosomes to Treat Depression, Anxiety, and Dementias	Enrolling by invitation	NA	Refractory depression/ anxiety disorders/ neurodegenerative diseases	EV derived from amniotic fluid i.v. (21.10 + 6 equiv. stem cells)	NA	Transcranial focused ultrasound + i.v.	300	Neurological Associates of West Los Angeles (USA)
NCT04388982	The Safety and the Efficacy Evaluation of Allogenic Adipose MSC-Exos in Patients With Alzheimer's Disease	Recruiting	Phase1/2 Open-label	Alzheimer's disease	EV derived from allogenic adipose mesenchymal stem cells transfected by miR-124	Twice a week for 12 weeks	Nasal drip	9	Ruijin Hospital (China)
NCT04202783	Focused ultrasound delivery of intravenously-infused exosomes	Enrolling by invitation	NA Open-label	Craniofacial neuralgia	EV-derived from neonatal stem cell	NA	Transcranial focused ultrasound + i.v.	100	Neurological Associates of West Los Angeles (USA)

MSC, mesenchymal stem cells; EV, extracellular vesicles.

sponge scaffold was used to deliver human fetal brain and spinal cord NSCs to a complete spinal cord lesion in rats [166]. Aligned biomaterial scaffolds provide a support and guidance for cell growth and differentiation, and a guide for axial axonal extension. The implantation of NSC-loaded aligned scaffolds promoted the long-term cell survival and neuronal differentiation of cells. It decreased inflammation and reduced glial scar formation, which led to a functional improvement. Biomaterials have also been used to produce nerve conduits for peripheral nerve repair. Luo et al. built a bioactive system where a cellulose/soy protein isolate composite membrane hollow nerve conduit was filled with a gelatin methacryloyl hydrogel, human bFGF and dental-MSC [167]. It was implanted in a 15-mm-long gap in the sciatic nerve defect, and was able to improve the recovery of the nerve and led to a significantly increased sciatic function. Another study tested the impact of a construct where amine functionalized multiwalled carbon nanotubes were incorporated with polycaprolactone and gelatin to fabricate aligned conductive nanofibers [168]. Bone marrow-MSC trans-differentiation into Schwann cells was increased when cultured in the construct with aligned conductive nanofibers and their implantation in a 10 mm-gap sciatic nerve model stimulated axon elongation. Complex systems have also been developed that evolve with the microenvironment and cell behavior, like the "intelligent" double layer alginate hydrogel that contains an inner layer modified with RGD moieties and metalloprotease peptides and an outer layer of alginate grafted with Cripto-1 antibodies to transplant NSCs [158]. The cells would proliferate in the inner layer and doing so, they secrete MMP enzymes that would break the inner materials and allow their migration toward the outer layer once the "inflammatory storm" is passed. Cripto-1 antibodies would then stimulate their differentiation.

An alternative to cell encapsulation in a scaffold/hydrogel is to seed them on biocompatible and biodegradable microcarriers that would allow their proliferation and subsequent injection. Somville et al. developed green and tunable animal free microcarriers that allow the proliferation of MSCs and could eventually be used for cell transplantation [169]. Montero-Menei et al developed a platform of pharmacologically active microcarriers (PAM) that present a bioactive surface, are loaded with a growth factor and covered with stem cells before their injection at the site of action. This technology was used in a Huntington's disease model (combination of BDNF and GABAergic progenitors/neuronal-like cells derived from MSCs) [170], in an ischemic stroke model (combination of VEGF and MSCs) [171] or incorporated in an hydrogel (combination of BDNF, human marrow–isolated adult multilineage-inducible stem cells and an injectable nontoxic silanized-hydroxypropyl methylcellulose hydrogel [172]), showing each time significant positive effects. Recently, Kandalam et al. injected dental stem cell/BDNF-loaded PAM in a rat spinal cord contusion model and observed a significant improvement of the rat gait [173]. Culture on PAM induced an increased gene expression of immunomodulatory markers (i.e. tumor necrosis factor-inducible gene 6 protein and IL10). Finally, the 3D bioprinting technology has been recently used to produce cell-laden hydrogels for peripheral nerve injury repair [174] and spinal cord injury [175].

While numerous biomaterial-based supports have been developed to deliver stem cells, not so many systems have been proposed yet to encapsulate EV; the ones that do mostly use hydrogels. EV incorporation in hydrogels would physically maintain EV at the site of action and allow the loading of a relatively low amount of EV, while still having the desired therapeutic effect thanks to limited clearance and a slow sustained release [176]. Recent studies report the incorporation of human placenta–derived MSCs in a thermosensitive chitosan hydrogel or the encapsulation of adipose tissue–derived MSCs in a polypeptide hydrogel made of Pluronic F127, oxidative hyaluronic acid, and poly-ε-L-lysine [177]. Li et al. loaded EV in a peptide-modified hyaluronic acid hydrogel that was locally injected in a spinal cord lesion [176]. The combination induced a significant nerve recovery and urinary tissue preservation by effective modulation of the inflammation and oxidative responses. EV have also been incorporated in alginate and chitosan-based hydrogels. All studies reported a slowed down release and improved therapeutic efficiency.

Clinical considerations

Stem cells

There are thousands of scientific articles related to the use of stem cells for neurological pathologies. When looking at the clinical trials database (https://www.clinicaltrials.gov), the keyword "stem cells" identified 8179 clinical trials among which 3985 were completed or terminated with only 1/3 with results (371 suspended or withdrawn). Regarding the neurological affections specifically, 133 studies (excluding withdrawn and suspended studies) were listed for "stem cells" and "neurodegenerative disease", 51 for "stem cells" and "spinal cord injury" and 78 for "stem cells" and "stroke". This has to be looked at against the number of cell therapy products that are currently on the market. The Alliance for Regenerative Medicine (https://alliancerm.org) has established a nonexhaustive list of marketed products based on stem cells. They listed 55 products so far, and only two for neurological diseases: 1) NeuroNata-R by Corestem, a korean company, for ALS and 2) Stemirac by Nipro, a Japanese company, for spinal cord injury. NeuroNata-R consists in the intrathecal injection (2 injection 4 weeks apart) of autologous bone marrow MSCs (1.10^{+6}/kg). Stemirac is based on the intravenous

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injection of autologous bone marrow MSCs but has been the subject of a large controversy.

They are indeed several reasons that seriously limit the translation to the clinic, and then to the market, of stem cell therapy products in general, and for neurological affections in particular.

One of the first bottlenecks is the translation of pre-clinical positive results to clinical efficiency. Indeed, while clinical trials demonstrated the safety of stem cell administration in the nervous system, conclusions on efficiency in patients are hindered by the limited number of patients enrolled in the study, the open-labeled status of some of those studies and the discrepancy between conditions used for the pre-clinical studies and the clinical one. Borlongan et al. very clearly demonstrated how the number of cells injected in patients with stroke was considerably lower in humans than that in rodents: At least 10 times less cells/kg were transplanted in humans than the dose used in preclinical trials [47]. Only patients who received the equivalent number of cells displayed clinical improvements.

Other parameters, such as administration route or timing are not always concordant either. Only when the conditions of the pre-clinical and clinical studies were equivalent, clinical improvement was observed. In addition, the comparison between different clinical trials is almost impossible as the cell origin, dose, transplantation technique, and administration route, as well as therapeutic window vary. Another factor that limits the translation of efficiency from preclinical to clinical is the lack of relevant and reliable animal models. Indeed, especially for neurodegenerative diseases, it is very difficult to recapitulate all the aspects of the pathology in one model. To test the efficiency or new treatments against MS for instance, different models need to be used in combination to really recapitulate all the aspects of the disease [178]. It is the same for Parkinson's disease where none of the animal models recapitulates all the biochemical characteristics of the pathology [179].

Then, the source of cells (autologous versus allogenic), availability and thus timing of injection are important considerations. One might consider that the best combination would be an "as early as possible" injection of autologous cells but this is not that simple. Indeed, to act early, enough cells have to be ready, and millions are required, which takes time to produce. In addition, if the patient is not in good health or too old, the proregenerative properties of his/her stem cells might be diminished. Allogenic cells can be expanded and stored to be available on-demand. However, they elicit an immune reaction and are quickly cleared from the body. Another limitation is sometimes the lack of sufficiently well-defined markers and limited understanding of stem cell mechanisms of action that complicate their strict characterization during and at the end of the production process, ensuring the homogeneity and consistency of the different batches. Finally, one of the main sources of variability, notably regarding stem cell properties, is the translation from research-grade to the GMP clinical-grade cell production. Indeed, the constrains of replacing animal-derived reagents, the scale and the time of the extension process, the support on which the cells are grown (cell stacks, microcarriers) and setting (static versus dynamic culture) may generate cells with different properties, notably immunomodulatory, compared with those observed in the labratory [47].

Derived products

Although more recent and thus less explored, the field of conditioned medium and EV faces the same challenges as the stem cells regarding translation to the clinic and market. Being cell-free products, EV and conditioned medium avoid the safety concerns that might be associated with stem cell transplantation, but they are still subjected to the same variations in properties and efficiency. Indeed, cell source and production settings (reagents, cell support, 2D versus 3D, static versus dynamic) but also dose, administration route and timing affect the therapeutic potential of stem cell-derived products. In addition, there is still no consensus on the best protocol to isolate EV at a large-scale and to cryopreserve them while preserving their physical integrity and inherent properties, although work has been published on that aspect [144]. EV characterization also lacks standardized protocols even if the International Society for Extracellular Vesicles is trying to establish guidelines [180,181]. Hence, to promote the use of EV as therapeutics, it is essential to develop and validate GMP conditions at which EV production will be reliable, sustained, robust, safe, and consistent [144]. They are currently a few clinical trials looking at the therapeutic potential of conditioned medium and exosomes/EV in the scope of neurological diseases (Table 6). Several companies are currently developing and investing in EV as therapeutics: Codiak Bioscience, Evox Therapeutics, Exopharm or Aruna Bio to site a few [122].

Conclusion

Stem cell therapy and EV have raised a lot of hopes regarding the treatment of neurological diseases, and enormous progresses have been made in this field. We have now a better understanding of what is happening when stem cells and EV are transplanted in the nervous systems, the safety of such treatments and what kind of benefits can be obtained. For now, allogenic human-derived adult MSCs have been identified as the best

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compromise between safety, efficiency and feasibility but as we will gain more information and perspective on iPSC, this might change. EV represent the hope of a "cell-free" therapy that would be as efficient and safer as stem cells, but their production, characterization, and understanding of mechanisms of actions are still far from being optimized and defined.

In conclusion, in a field where efficient and curative therapeutic options are lacking, stem cells and their derived products represent a serious hope, but the efforts of scientists need to be pursued to plainly fulfill their potential.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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