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REVIEW

Transplantation of stem cell-derived astrocytes for the treatment of amyotrophic lateral sclerosis and spinal cord injury

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Abstract

Neglected for years, astrocytes are now recognized to fulfill and support many, if not all, homeostatic functions of the healthy central nervous system (CNS). During neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI), astrocytes in the vicinity of degenerating areas undergo both morphological and functional changes that might compromise their intrinsic properties. Evidence from human and animal studies show that deficient astrocyte functions or loss-of-astrocytes largely contribute to increased susceptibility to cell death for neurons, oligodendrocytes and axons during ALS and SCI disease progression. Despite exciting advances in experimental CNS repair, most of current approaches that are translated into clinical trials focus on the replacement or support of spinal neurons through stem cell transplantation, while none focus on the specific replacement of astroglial populations. Knowing the important functions carried out by astrocytes in the CNS, astrocyte replacement-based therapies might be a promising approach to alleviate overall astrocyte dysfunction, deliver neurotrophic support to degenerating spinal tissue and stimulate endogenous CNS repair abilities. Enclosed in this review, we gathered experimental evidence that argue in favor of astrocyte transplantation during ALS and SCI. Based on their intrinsic properties and according to the cell type transplanted, astrocyte precursors or stem cell-derived astrocytes promote axonal growth, support mechanisms and cells involved in myelination, are able to modulate the host immune response, deliver neurotrophic factors and provide protective molecules against oxidative or excitotoxic insults, amongst many possible benefits. Embryonic or adult stem cells can even be genetically engineered in order to deliver missing gene products and therefore maximize the chance of neuroprotection and functional recovery. However, before broad clinical translation, further preclinical data on safety, reliability and therapeutic efficiency should be collected. Although several technical challenges need to be overcome, we discuss the major hurdles that have already been met or solved by targeting the astrocyte population

in experimental ALS and SCI models and we discuss avenues for future directions based on latest molecular findings regarding astrocyte biology.

Key words: Neuroprotection; Stem cell; Cell therapy; Astrocyte; Transplantation; Amyotrophic lateral sclerosis; Spinal cord injury

Core tip: Amyotrophic lateral sclerosis (ALS) and spinal cord injury (SCI) result in incurable neurological dysfunction due to loss of spinal motor neurons and axonal degeneration, amongst other mechanisms. Astrocytes are increasingly recognized as being necessary for neuroprotection and regeneration in the central nervous system as they promote axonal growth and deliver essential neurotrophic factors under both physiological and pathophysiological conditions. Given the central role played by astrocytes, we gathered convincing results from ALS and SCI literature that argue in favor of stem cell-based astrocyte replacement therapies and stress the scientific community to investigate more deeply the molecular understanding of astrocyte biology.

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MULTIPLE FACETS OF ASTROCYTES IN THE CENTRAL NERVOUS SYSTEM

Astrocyte functions

Astrocytes are the most abundant cells in the central nervous system (CNS), outnumbering neuronal cells by several fold in some CNS regions. They have long been relegated to a secondary position, behind neurons or oligodendrocytes, as many thought that astrocytes were barely by-standers of the CNS. Accounting for a large fraction of the brain volume, their particular shape and tissue distribution are known to elaborate an extensive network of fine interconnected processes. Their star-shaped morphology projecting long branched processes provides a large coverage of CNS structures and the ensheathment of the brain or spinal cord in the pia matter. They draw the whole brain microanatomy by secreting astrocyte-derived extracellular matrix proteins (*e.g.,* chondroitin sulfate proteoglycans, hyaluronan, tenascin proteins family, thrombospondin), thereby providing structural support for nervous system cells. Beside their structural role, astrocytes are recognized to fulfill and support many, if not all, functions of the healthy CNS[1]. Astrocyte end-feet cover more than 90% of the CNS vasculature and come in contact with endothelial cells. Expressing key glucose transporter-1, astrocytes convoy glucose from blood vessels to nervous system cells, most of them being devoid of direct access to this high-end source of energy. Hence, astrocytes synthetize *via* specific metabolic pathways glycogen and lactate, main energy fuels for neurons or distant synapses. Through humoral factors released at the perivascular space, astrocytes control local cerebral blood flow and blood-brain barrier (BBB) integrity. Transforming growth factor-beta, glial-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2) and angiopoietin 1 (binding the endothelium-specific receptor TIE2), all secreted at the vascular end-feet, act on endothelial cells in order to induce or maintain an operational BBB[2,3]. Astrocytes-released growth factors [*e.g.,* brain-derived neurotrophic factor, BDNF; ciliary neurotrophic factor (CNTF)] exert beneficial effects far beyond the perivascular space and act as neurotrophic factors on all CNS cells[4,5]. The perivascular end-feet of astrocytes show other specialized features characteristic of this location, including a high density of orthogonal arrays containing the water channel aquaporin 4 (AQP4) and the Kir4.1 K^+ channel[6,7]. Those proteins closely interact with endothelial basal lamina and regulate ions/water fluxes through the BBB[7-10]. Astrocytes finely tune the dynamics of cerebral blood flow in order to increase the availability of oxygen and glucose locally to highly active brain areas; this process is mediated via secreted vasoactive compounds (*i.e.*, endothelin-1, arachidonic acid, prostaglandins, nitric oxide) and intercellular Ca^{2+} signaling[11-13]. As a member of the tripartite synapse, astrocytes are responsible for proper neurotransmission. Astrocyte end-feet express Kir4.1 channel, which is responsible for the rapid uptake of K^+ released in the interstitial CNS fluid and thus for spatial extracellular potassium siphoning *needed d*uring high-frequency firing of neurons[14-16]. Astrocytes also express membrane-bound transporters and receptors involved in the re-uptake and the response to neurotransmitters (*i.e.*, glutamate, GABA, glycine), whose synaptic concentrations must be tightly controlled in the synaptic cleft. In the case of glutamate homeostasis, two major CNS glutamate transporters, GLT1/EAAT2 and GLAST/EAAT1, are expressed almost exclusively by astrocytes in the adult mammals. GLT1 accounts for upward of 90% of glutamate uptake in most CNS regions[17]. By regulating extracellular glutamate homeostasis, GLT1 assures proper synaptic function and prevents excitotoxic insult to susceptible neurons, axons and oligodendrocytes. In addition, astrocytes form a gap junction-coupled network of very narrow overlapping domains throughout the CNS[18,19]. Each domain covers a CNS area encompassing from 20000 neuronal synapses in rodents up to 2000000 in humans and connects neighboring astrocytes by

junctional complexes made of connexin (Cx) family proteins. On a functional point-of-view, the astrocyte syncytium supports ATP-mediated long-distance propagation of Ca^{2+} waves[20,21], which is involved in the control of local cerebral blood flow and the fine tuning of electrical neuronal activity[22]. Cx channels allow the intercellular passage of monovalent ions (Na^+, K^+) or small molecules up to 1.5 kDa (*e.g.,* ATP, glutamate, d-serine), contributing to signaling, metabolic cooperation and ionic spatial buffering[23,24]. Astrocytes express Cx26, Cx30 and Cx43 that interact with homologous connexins in astrocyte-to-astrocyte (A/A) gap junctions and that can also bind in a heterologous fashion Cx29, Cx32 and Cx47 on oligodendrocytes partners[24-26]. In mammals at least, the presence of theses 6 connexins suggests a high level of complexity in coupling partners within the glial cell populations and reveals close cellular interactions between astrocytes and oligodendrocytes. Astrocyte-oligodendrocyte (A/O) coupling via connexins is necessary to sustain myelin formation and maintenance throughout life. In humans, mutations in *Cx29*, *Cx32* and *Cx47* genes lead to myelin formation abnormalities and are linked to Charcot-Marie-Tooth disease and several forms of leukodystrophies[27]. Beyond mutations targeting oligodendroglial connexins, the effect of loss-of-function of astrocyte connexins has been experimentally investigated using knockout mice. Double $Cx30^{-/-}$ and $Cx43^{-/-}$ knockout mice showed white matter pathology comprising vacuolated oligodendrocytes and intramyelinic edema[28]. Histopathological changes were accompanied by significant sensorimotor and cognitive deficits. Similar findings were shown when double-deleting Cx43 and Cx32 in mice[29]. All of these findings suggest an essential role of A/A and A/O coupling in maintaining overall CNS functions[30] and pave the way for developing integrated therapies targeting the astrocyte syncytium and its dysfunction(s) during neurodegenerative conditions.

Wealth through diversity

A rapid look at the morphology of white matter astrocytes compared to gray matter astrocytes reveals the complexity and heterogeneity of this class of cells. Their different morphologies are most likely to be related with their wide range of functions, their neuroanatomical sites and the stem cells from which they derive (reviewed by[31]). During development, astrocytes mainly arise from radial glial cells located in the brain and spinal cord. During adulthood, astrocytes are still generated from differentiating progenitors in stem cell niches[32] or from dividing mature astrocytes in specific brain regions[33]. Historically, two classes of astrocytes were described: "type Ⅰ" fibrous astrocytes mostly found in white matter tracts and "type Ⅱ" protoplasmic astrocytes found in the grey matter[34,35]. Nowadays, the scientific community agrees that astrocyte complexity, in particular within the protoplasmic subfamily, has increased along with phylogenic evolution. As far as we know, this complexity culminates in the human CNS. Compared to rodents[19], human astrocytes have a greater size, a more complex morphology, a large pleiomorphism[36] and are able to propagate calcium waves five times more rapidly[31,37,38]. Although mice represent a useful tool to study astrocytes and their *in vivo* functions through genetic manipulation, one limitation of rodent-to-human extrapolations is the wider diversity of human counterparts. For instance, primate brain contains two types of astrocytes not found in rodent brain: interlaminar and varicose astrocytes, whose functions are as of yet undetermined but seem to be related to the higher complexity of neuronal layers inside the human cortex[37]. Despite this heterogeneity, astrocytes share similarities such as the expression of several common proteins. Intermediate filament proteins are very abundant in the cytoplasmic compartment among all astrocytes types: glial fibrillary acidic protein (GFAP), vimentin, desmin and synemin. Recently, the cell surface marker CD44, the receptor of extracellular matrix hyaluronan, has been described to distinguish, more accurately than GFAP, protoplasmic astrocytes from fibrous-like astrocytes[36]. Resting astrocytes also display specific immunoreactivity for aldehyde dehydrogenase 1 family member L1, AQP4, S100, GLT1, GLAST and glutamine synthetase. Under pathological conditions affecting CNS [*e.g.*, Alzheimer's disease, multiple sclerosis, stroke, amyotrophic laterals sclerosis (ALS), spinal cord injury (SCI)], astrocytes can switch to an activated phenotype. Activated astrocytes, which can in some case include a population of proliferating astrocytes, are commonly observed around focal CNS lesions, in areas of neuronal loss/damage, in demyelinating areas, during neuroinflammation or during CNS repair. Astrocyte hypertrophy and upregulation of GFAP, vimentin, nestin or S100 are hallmarks of activated astrocytes. For instance, new protein markers may also be expressed. Expression of S100A6, a calcium binding protein, is drastically increased in astrocytes from ALS spinal cord[39,40], after traumatic CNS injury[41] or in Alzheimer brain tissue[42]. Given that ALS and SCI affect either diffuse or discrete areas, respectively within the CNS (*i.e.,* in ALS from motor cortex to bulbar respiratory nuclei to the lumbar spinal motor neurons; in SCI, both gray and white matter astrocytes are lost over a distance of several millimeters), therapeutically-targeting astrocytes would undoubtedly take into account their diversity of shape and function, with respect to their location. Our knowledge about astrocyte heterogeneity now allows for generating *in vitro* subpopulations of stem cell-derived astrocytes endowed with specific fates or defined functional abilities. These cells, isolated and expanded *in vitro,* can be further used in transplantation paradigms aiming at providing neuroprotection or replacing astrocyte-deficient

functions observed during neurodegenerative and traumatic diseases of the spinal cord such as ALS or SCI.

SOURCES OF STEM CELL-DERIVED ASTROCYTES

Glial-restricted precursor cells

Use of lineage-restricted progenitors as a source for deriving astrocytes has gained wide popularity in recent years due to their ability to generate specific glial types following transplantation. Glial-restricted progenitors are present in the embryonic spinal cord and through their ability to self-renew and differentiate give rise to oligodendrocytes and astrocyte populations. The first type of glial restricted progenitor that was originally characterized was the $A2B5^+$ O-2A progenitor cell. This was isolated from optic nerve of embryonic and post-natal rats and through *in vitro* differentiation was shown to be able to differentiate into oligodendrocytes and a particular type of astrocyte called the type-2 astrocyte[43-45]. More recently, a different type of progenitor cell was characterized that is able to differentiate into oligodendrocytes and two different populations of astrocytes. This cell type was isolated from E13.5 rat spinal cord and is also positive for the A2B5 antigen. *In vitro* conditions that supported their above mentioned differentiation included culture medium supplemented with fetal calf serum or platelet-derived growth factor (PDGF) and T3 thyroid hormone. Clonal analysis confirmed that these individual GRPs are tripotent. Furthermore, these cells are capable of extensive self-renewal in the presence of PDGF and FGF2. These GRPs differed from the original $A2B5^+$ O-2A progenitor cell in several aspects such as absence of mitogen requirements, responsiveness to PDGF and morphologies[46].

Neural stem cells or radial glia

Using cell genesis and fate specifying processes, neural tube-derived neural stem cells give rise to 10^{11} neurons and at least 10^{12} glial cells of many different phenotypes in the adult brain[47-49]. Neural stem cells (NSCs) are an attractive source for generating astroglial cells through their multipotency and high potential of self-renewal. NSCs represent an endogenous population existing during brain development and to a lesser extent in adult brain niches. In adult CNS, they are found around the sub-ventricular zone, the sub-granular layer of the hippocampus and spinal ependymal canal. Their phenotype is characterized by an astrocyte-like morphology and GFAP expression[50,51] together with other markers of an undifferentiated state (*e.g.,* nestin, SOX2). The most common sources for NSC isolation include embryonic brain cortex and adult sub-ventricular zones. One of the basic features of NSCs is that they can easily divide *in vitro* either as spherical aggregates called "neurospheres" or adherent layers of cells when supplemented with epidermal growth factor and fibroblast growth factor 2. Culture conditions involving growth factor withdrawal and serum exposure elicit a differentiation program into either neurons, oligodendrocytes or $GFAP^+$ astrocytes, with various yields[52-54]. Transplantation of NSCs into the CNS results in the generation of multiple cell types, including astrocytes[53] and oligodendrocytes[55]. Another source of astrocytes in the adult CNS is radial glial cells[56,57]. Several lines of evidence support the existence of a small pool of such cells expressing classic radial glial markers (*i.e.,* NG2, RC1, 3CB2, brain lipid-binding protein)[58] and located in limited regions of ongoing neurogenesis in the adult CNS[59-61].

Embryonic or Induced-pluripotent stem cells

Embryonic stem cells (ESCs) and induced-pluripotent stem cells (iPSCs) represent an ideal pluripotent source for deriving human astrocytes. Even though early reports demonstrated differentiation of astrocytes from human iPSCs or ESCs, there was a great diversity in terms of reported methods and the total yields of neural cells were not high. Furthermore, astrocytes generated by these protocols were not defined in terms of their subtype identity. On the other hand, specific protocols to generate astrocytes with regional or subtype identity were not reported[62-68]. This gap was fulfilled by a recently reported protocol, which allows directed differentiation of astrocytes *via* a prior differentiation of human stem cell derived neuro-epithelial cells into glial progenitor cells by repression of neurogenesis. Astrocytes generated by this protocol were characterized by specific gene expression profiles, neurotransmitter and ionic uptake, support of neuronal maturation, and importantly expression of specific sets of homeodomain transcription factors that specify functional identity[69]. On the basis of this, another protocol was recently reported for an efficient generation of immature astrocytes from human pluripotent stem cells (hPSCs)[70]. The protocol consists of three steps. In the first stage, hPSCs were differentiated into neuroepithelial cells either by co-culturing with mouse embryonic fibroblasts or by differentiation in feeder free conditions. These appeared in the form of columnar epithelia that organized into neural tube-like rosettes at about day 10-15 of differentiation. Addition of morphogens at days 8 to 15 allows differentiation of specific astrocyte subtypes. In the second stage, floating neural progenitor aggregates were expanded with epidermal growth factor, FGF2 and mitogenic factors. Neuronal differentiation was repressed and potential for gliogenic progenitor was enhanced by weekly mechanical trituration. By around 90 d, most of the progenitors expressed markers for astrocyte progenitors or astrocytes such as CD44 and S100. Thus at this stage, they are referred to as "astrospheres". In the third and

final stage, astroglial progenitors were differentiated into functional astrocytes by removal of mitogens. Addition of CNTF for 6 d stimulated gliogenic gene expression. If neuroepithelial cells were patterned in stage 1, terminally differentiated astrocytes expressed specific markers such as Hoxb4 for posterior identity or Nkx2.1 for ventral identity.

In vivo or in vitro transdifferentiation

Some cases of *in vivo* transdifferentiation into astrocytes have been described in the literature following transplantation of bone marrow-derived stem cells or mesenchymal stem cells into rodent CNS. It is not yet clear whether transdifferentiation normally occurs in healthy CNS and, if so, whether it requires a de-differentiation step. Several criteria must be filled to truly establish transdifferentiation into astrocytes: original cells must lose their committed-lineage differentiation markers; they have to adopt a astrocyte-like morphology and express astrocyte markers (*e.g.,* GFAP, S100). Several reports showed that GFP reporter-expressing bone marrow stem cells transplanted into adult rodent CNS gave rise to GFP^+ $GFAP^+$ astrocytes[71-73]. Similarly, mesenchymal stem cells (MSCs) isolated from bone marrow, when grafted into neonatal mouse brain, have been shown to migrate extensively and differentiate into olfactory bulb granule cells and astrocytes[74]. In an original experimental approach, Boucherie and colleagues intrathecally grafted MSCs in ALS-affected rodent spinal cord and observed that MSCs were able to extensively migrate while differentiating mostly into astrocytes at the sites of neurodegeneration[75]. In recent years, *in vitro* transdifferentiation, starting from single skin fibroblasts, has also made possible the modeling of CNS diseases in a dish, bypassing invasive neurosurgical procedures to get human diseased tissue. Recent cell reprogramming protocols describe the direct conversion of human fibroblasts to "induced" neural stem cells, able to further differentiate into the three CNS lineages (neurons, astrocytes and oligodendrocytes) following appropriate culture conditions. Artificial and temporal expression of specific transcription factors such as Oct-3/4, Sox2, Klf4, Brn4 and c-myc seems to govern the cell conversion towards a neural fate[76-78]. Molecular pathways and key transcription factors making transdifferentiation possible are currently under investigation for different cell types[79].

RATIONALE FOR ASTROCYTE REPLACEMENT IN ALS AND SCI

What is amyotrophic lateral sclerosis?

Amyotrophic lateral sclerosis (ALS) is a common adult-onset neurodegenerative disease of the motor system, with a prevalence of 2-5/100000 people. It is characterized by a rapidly progressing neurodegeneration selectively affecting cortical, brainstem and spinal motor neurons. ALS remains an incurable disease, leading to fatal respiratory failure usually within 5 years following diagnosis[80]. Pathophysiology of ALS is poorly understood and likely multifactorial. Proposed starting points for this complex disease targeting the motor neuron population include mitochondrial dysfunction, intracellular protein aggregation, disturbances of RNA metabolism, extracellular toxic environment, impairment at the level of axonal transport and at the neuromuscular synapse (reviewed by[81]), together with extrinsic events: blood-brain barrier breakdown, glial cell reaction/dysfunction and neuroinflammation[82-88]. It is known that dying motor neurons influence surrounding cells, of which astrocytes are most commonly investigated. Astrocytes shift from an anti-inflammatory and neuroprotective role to one that is pro-inflammatory and neurotoxic, thus adding to complexity of this fatal cascade[80]. In 5%-10% of patients, ALS is inherited (familial ALS). In those cases, every fifth patient carries a detected mutation in superoxide dismutase 1 (SOD1), which is at present the most reliable and most widely used genetic animal model for ALS[89]. Among others, the most commonly described mutations include genes encoding the DNA/RNA-binding proteins FUS, TDP43[90], the ubiquitin-like protein Ubiquilin 2[91] and optineurin[92]. On the other hand, an intronic hexanucleotide repeat in the *C9ORF72* gene was discovered in both familial and sporadic cases of ALS[93].

What are spinal cord injuries?

SCI are devastating and diverse set of conditions that result from damage to spinal cord grey matter and white matter, as well as corresponding spinal nerves. The National Statistics for SCI estimates approximately 12000 new cases each year in the United States alone, most of which result from preventable causes (*e.g.,* motor vehicle accidents, falls, sports or violence). Some common outcomes of SCI include dysfunctions in the musculoskeletal, respiratory, uro-genital and gastrointestinal systems. The diversity of this condition results from differences in the location, type and severity of trauma, as well as on the consequent types and degree of functional impairment. Despite this diversity, all forms of SCI are linked to specific phenotypic changes in populations of spinal cord astrocytes. Some examples of these changes are: (1) acquisition of specific protective functions such as glial scar formation to constrain the secondary expansion of the lesion; (2) loss of certain crucial homeostatic functions such as the astrocyte glutamate transporter system that are key to normal CNS physiology; and (3) gain of toxic functions such as the generation of pro-inflammatory signaling molecules that contribute to degeneration, neuronal hyperexcitability and other detrimental effects. The response of astrocytes is a gradated response such that there is diversity in these changes that vary with the type and severity of trauma and with proximity

to the lesion. Furthermore, even within a population of astrocytes within the spinal cord, the response of individual astrocytes to the same injury can vary, likely reflecting the normal heterogeneity amongst astrocytes that is now becoming increasingly appreciated.

Defective astrocyte glutamate handling: one rationale among others

ALS and SCI are both spinal cord disorders involving all CNS cells: neurons, oligodendrocytes, microglial cells, endothelial cells and astrocytes. In both cases, there is significant motor, as well as sensory, dysfunction, although sensory involvement is still a matter of debate in ALS. To some extent, they share histopathological features in the spinal cord tissue: death of spinal motor neurons, axonal damage in white matter tracts, anterograde axonal degeneration, BBB dysfunction, impaired astrocyte function and neuroinflammation. In the case where neurodegeneration reaches the cervical level of the spinal cord, the loss of phrenic motor neurons that control contraction of the diaphragm leads to severe respiratory deficits and represents a life-threatening condition. ALS, historically characterized by the loss of upper and lower motor neurons, is more and more recognized as endowed with a non-cell-autonomous component, in which microglia and astrocytes act as significant contributors to overall spinal cord dysfunction. It has been shown that microglia carrying ALS-linked mutant SOD1 drive ALS disease progression by fostering neuroinflammation and a toxic environment for spinal motor neurons[81,94]. With respect to astrocytes in ALS, independent groups demonstrated that astrocytes bearing mutant SOD1 are key determinants of disease progression[95-97]. In the absence of any pathology, normal astrocytes uptake glutamate, released at the synaptic cleft, through their glutamate transporters (GLT1/EAAT2 and GLAST/EAAT1). Furthermore, normal astrocytes protect motor neurons from glutamate excitotoxicity by stimulating the neuronal expression of AMPA-GluR2, which is less permeable to calcium making motor neurons less sensitive to excitotoxicity[98]. During ALS disease course, it is known from human and animal model samples that astrocyte-based glutamate uptake is compromised due to an early loss of astrocyte GLT1. Drastic GLT1 downregulation is most likely to be linked with deleterious extrasynaptic glutamate accumulation, which gave rise to the theory of glutamate-induced excitotoxic motor neuron death and to the unique FDA-approved drug that slows ALS: Riluzole, based on its anti-glutamatergic action.

Following SCI, the entire architecture of the spinal cord is disrupted at the lesion site; the trauma produces both immediate and delayed cell death affecting all nervous system populations: neurons, astrocytes and oligodendrocytes. Shortly after injury, it has been demonstrated that glutamate starts to accumulate in the extracellular space[99], and can persist for over a week, depending on injury severity[100]. Overload of CNS extracellular glutamate may cause excitotoxic damage to neurons, axons and oligodendrocytes *via* overactivation of both AMPA and NMDA receptors[101,102]. Failure of long-term extrasynaptic glutamate clearance is suspected to be one major cause of secondary cell loss following SCI. Noteworthy, we and others demonstrated that astrocyte GLT1 was chronically lost at the injury epicenter following SCI but also downregulated in spinal cord regions distant from the lesion core[103-106]. Furthermore, experimental data showed that the newly-generated astrocytes arising during the SCI repair phase lacked GLT1 expression, possibly compromising long-term astrocyte glutamate homeostasis[107]. Other consequences of astrocyte dysfunction or loss should be considered in ALS and SCI: shortage of neurotrophic factors important for neuronal survival, overwhelming of anti-oxidative defenses, lack of support to maintain endothelial BBB integrity, impaired water, ionic and metabolic transport, release of harmful proinflammatory cytokines and synthesis of glial scar-related ECM proteins that block axonal regrowth[1]. Regardless of whether astrocyte dysfunction is a cause of disease or a consequence of neuronal loss, altered physiology of pathologic astrocytes likely results in further susceptibility to CNS tissue loss, justifying the rationale for transplantation-based astrocyte replacement[108]. Numerous studies indicate that transplantation of stem cell-derived astrocytes has exhibited beneficial effects on histological/functional outcomes in ALS and SCI animal models. In this review, we summarize the data from recent literature regarding transplantation of stem cell-derived astrocytes targeting the replacement of deficient or lost astrocytes during ALS and SCI.

METHODOLOGICAL CONSIDERATIONS

Feasibility of astrocyte transplantation in healthy spinal cord

Pilot studies were done on spinal GRPs isolated and characterized from the human aborted embryos[46,109,110]. Upon transplantation into neonatal and adult rodent brain, human GRPs survived, migrated and differentiated into (im)mature oligodendrocytes and into GFAP-expressing astrocytes[111]. These results were later confirmed by another group who analyzed the fate of GRPs harvested from alkaline phosphatase-expressing transgenic rats injected into intact and injured spinal cord. In the first set of experiments, GRPs survived at least 6 wk[112] and in the second experiment up to 15 mo post-transplantation[113], demonstrating their long-term integration. Cells showed morphological maturation and differentiated along astrocyte and oligodendrocyte lineages and not into neuronal lineages. Interestingly, cells grafted into the intact spinal cord showed a particular tropism for white matter tracts and robust migration capacities

since they were found more than 15 mm away from the injection sites[112,113]. Transplantation of GRP-derived astrocytes seems to be a safe procedure, at least experimentally, since no tumor formation or pronounced immune response were observed at the graft sites. Isolated human GRPs express *in vitro* functional glutamate transporters EAAT1, EAAT3 and EAAT4 but not GLT1/EAAT2[114]. According to the nature of *in vitro* pre-differentiation signals, GRPs do not give rise to homogenous astrocyte populations upon transplantation[115,116]. Following bone morphogenic protein 4 (BMP4) driven differentiation, GRPs start to express EAAT2/GLT1 together with AQP4, AKAP12, Cx43 as markers of mature astrocytes[116]. On the contrary, *in vitro* GRP exposure to CNTF give rise to mature astrocytes expressing FGF receptor-3 and several axon growth inhibitory proteoglycans, such as neurocan, brevican and phosphacan, suggestive of a phenotype of reactive astrocytes.

Routes of administration

Considering the route of administration for a stem cell therapy product is a significant factor when targeting the CNS and the spinal cord in particular. Stem cell transplantation into the spinal cord was successfully reported, in terms of homing and long-term engraftment, using different cell populations and different route of administration. Intraparenchymal injections of MSCs and NSCs into the thoracolumbar region[117-123] and of GRPs into the cervical region[104,124] have been successfully reported in animal models of ALS. Although direct targeting of the spinal cord is technically challenging and an invasive application, this route promises to be the most versatile and accurate method of targeted CNS therapeutic delivery, and therefore has been chosen for clinical translation of stem cell-based therapies in ALS patients. As both ALS and SCI affect long segments of spinal cord (several millimeters long and sometimes independent regions within the cord), a need for multiple injections adds to the complexity of the procedure. Aware of this issue, neurosurgeons are developing new surgical techniques or tools (*e.g.,* radially-branched cannulae) able to deliver therapeutic agents to large CNS areas in a single shot[125]. Other attempts to find the optimal delivery route used intranasal[126], intrathecal[127-129], intraperitoneal[130], intramuscular[131], intravenous[132-135] and intra-bone marrow cell[136] transplantation. Major shortcomings are poor CNS homing of the therapeutic product compared to the initial dose given (dilution of cells in unwanted organs/sites) and adverse effects such as blockade of intravenously-injected stem cells in lung capillaries or spleen (reviewed by[137]). In many cases, researchers reported beneficial effects of peripherally-delivered stem cells associated with improved histological and functional outcomes in ALS animal models. While various routes of stem cell delivery have been investigated in ALS therapy (Table 1), experimental SCI pathology was mainly targeted by focal stem cell injections in and around the lesion core, regardless of the spinal level affected (Table 2). Although the most efficient delivery method is still a matter of debate, the dose of transplanted cells, the timing, the location, the type of cells for particular needs, and their migratory abilities are additional parameters to take into account in designing CNS stem cell-based therapies. Numerous preclinical studies using animal transplantation paradigms will still be required to assess biodistribution, viability, integration into host tissue, differentiation into functional cells, lack of tumorigenicity and safety of delivery before broad clinical application[138-140].

LESSONS FROM TRANSPLANTATION PARADIGMS IN ALS MODELS

Taking into account that neurons represent only a portion of the various nervous system cell types, it is not surprising that during onset and especially during progression of disease non-neuronal cells contribute significantly to neuronal dysfunction and death. For example, it has been shown that wild-type motor neurons in close proximity to mutant SOD1-containing non-neuronal cells became affected by pathological chain reactions specific for ALS and eventually die with many features of this disease[141]. In the same way, wild type non-neuronal cells extend survival of SOD1 mutant motor neurons[142]. It has been clearly shown that reduction of mutant SOD1 selectively in astrocytes results in a prolongation of disease duration, but has no effects on disease onset. At least a part of this process is modulated by dysfunction and decrease in levels of the primary astrocyte glutamate transporter, GLT1, in areas of motor neuron loss[143]. Taken together, these results suggest a particular role for astrocytes in later progression of disease[81,97]. Main studies that have assessed replacement of astrocytes based on transplantation in ALS animal models have been gathered in Table 1.

Transplantation of wild-type astrocytes or their precursors into CNS tissue affected by ALS represents a promising experimental approach. With the aim to rescue motor neurons responsible for breathing, which is the primary cause of death in human ALS, GRPs were transplanted into the cervical spinal cord of the SOD1^{G93A} rat model. GRPs survived in diseased tissue (32.2% ± 4.6% of transplanted cells survived at least 80 d post-transplantation), differentiated efficiently into astrocytes, and reduced microgliosis in the cervical spinal cord. Most notably, GRPs extended survival and disease duration, attenuated motor neuron loss, and slowed declines in forelimb motor and respiratory function. Since GRPs that did not express the glutamate transporter GLT1 did not have similar effects on behavioral measures or

Table 1 Transplantation-based astrocyte replacement in amyotrophic lateral sclerosis animal models

Animal	Type of cells	Delivery	Effect on disease	Main outcomes	Ref.
$SOD1^{G93A}$ ALS rats (80 d-old)	GRPs from rat E13.5 spinal cord, wild-type and overexpressing GLT1	Injections into C4-C6 cervical spinal cord, 6 sites, bilateral 1.5×10^5 cells/site	Delayed decline in motor function and survival extension	Differentiation into functional astrocytes. Prevented motor neurons loss independently from growth factors secretion, sustained GLT1 levels, alleviated microgliosis	[104]
$SOD1^{G93A}$ ALS mice (75 d-old)	Human neural precursors (hNPs) overexpressing BDNF, IGF-1, VEGF, NT-3, or GDNF	Injection in cisterna magna and cerebral ventricles	No effect on motor function or animal survival	Differentiation in $GFAP^+$ $GLT1^-$ expressing and growth factors-secreting astrocytes. Prevented motor neurons loss	[147]
$SOD1^{G93A}$ ALS rats (90 d-old)	Rat adult MSCs	Intrathecal delivery in lumbar cisterna magna, 1.95×10^6 cells	Preserved motor function and survival extension	Differentiation into astroglial cells. Decreased neuroinflammation	[75]
$SOD1^{G93A}$ ALS mice (24-26 wk-old)	Human umbilical cord blood cells overexpressing VEGF and FGF2	Intravenous delivery, 1×10^6 cells	Not investigated	Differentiation in $S100^+$ astrocytes	[146]
$SOD1^{G93A}$ ALS rats (14-26 wk-old)	NSCs from rat E16 brain cortex	Intravenous delivery, 1×10^7 cells	Not investigated	Preferential homing to late symptomatic ALS brain and spinal cord. Differentiation into neurons and astrocytes	[135]
$SOD1^{G93A}$ ALS mice (50-60 d-old)	hGRPs from fetal cadaver brain tissue (week 17-24 of gestational)	Injections into C4-C5 cervical spinal cord, 4 sites, bilateral 1.2×10^5 cells/site	No effect on histological or functional outcomes	Poor cell survival	[124]

ALS: Amyotrophic lateral sclerosis; BDNF: Brain-derived neurotrophic factor; C: Cervical; E: Embryonic; FGF2: Fibroblast growth factor 2; GDNF: Glial cell-derived neurotrophic factor; GFAP: Glial fibrillary acidic protein; GLT1: Glutamate transporter 1; GRP: Glial-restricted precursor; IGF-1: Insulin-like growth factor 1; MSC: Mesenchymal stem cell; NP: Neural precursor; NSC: Neural stem cell; NT-3: Neutrophin-3; SOD1: Superoxide dismutase 1; VEGF: Vascular endothelial growth factor.

animal survival, this highly suggested that glutamate-relevant pathways contribute to the cascade of events leading to cell death in this model and that the focal beneficial effects of GRP transplantation could be explained, at least in part, by increases in glutamate transporter expression[104].

With the aim to translate astrocyte-based transplantation towards treatment of patients, human GRPs have been transplanted into $SOD1^{G9A3}$ mice. At disease end-stage, 10% of initial hGRP transplants survived and distributed throughout of the cervical spinal cord and up to 80% of all human derived cells co-expressed the astrocyte marker GFAP. Unlike the results obtained with rodent-derived GRPs, this xenograft transplantation paradigm using human GRPs did not provide a phenotypic preservation of motor function[124].

In another study, Boucherie and colleagues tested a different approach: they injected MSCs into the cerebrospinal fluid of symptomatic $SOD1^{G93A}$ rats. They successfully penetrated the CNS parenchyma and accumulated at sites of motor neuron degeneration. They differentiated into astrocytes and, most notably, decreased motor neuron loss in the lumbar spinal cord, preserving hindlimb motor function and extending the survival of $SOD1^{G93A}$ rats. This neuroprotection correlated with decreased inflammation, as shown by a decrease in the proliferation of microglial cells and reduced expression of inflammatory-related genes, *COX-2* and *NOX-2*[75]. Recent publications confirmed that transplantation of stem cells indeed reduces neuroinflammation and suggests that alleviating astrocytosis or microgliosis is an important parameter leading to both histological and functional improvement in ALS animal models[144,145].

Another approach was tested by Mitrecić *et al*[135]. They transplanted NSCs into the rat model of ALS using the intravascular route. It was clearly shown that in animals affected by disease cells did cross the blood-brain barrier and accumulated in the regions affected by disease (motor cortex, ventral horns of the spinal cord) more than in healthy non-diseased animals. In the diseased CNS tissue, although only 6% of the initial stem cell dose was found at 7 d post-infusion, the transplanted cells differentiated into neurons and astrocytes[135]. Transplantation of genetically-engineered cells is another strategy to provide additional support or growth factors to ALS-affected cells and could also unexpectedly influence (stem) cell differentiation. This is what happened in a study from Rizvanov *et al*[146] who showed that wild-type human umbilical cord blood cells differentiated into endothelial and microglial lineages after transplantation into $SOD1^{G93A}$ mice, while the same cells genetically modified to overexpress vascular endothelial growth factor (VEGF) and FGF2 exhibited preferentially an astrocytic differentiation[146]. In the same vein, human neural precursors were also genetically engineered to express insulin-like growth factor-1, neurotrophin-3, BDNF, VEGF or GDNF. These cells were then transplanted into the spinal cord or brain of $SOD1^{G93A}$ mice where they migrated and differentiated into neurons, oligodendrocytes or GLT1-expressing astrocytes. Unfortunately, although cells of interest

Table 2 Transplantation-based astrocyte replacement in spinal cord injury animal models

Animal and type of SCI	Type of cells	Delivery	Effects on disease	Main outcomes	Ref.
Rat, aspiration of C3 fasciculus gracilis	E14 rat spinal cord astrocytes	Intraparenchymal graft at lesion site	Worsened hindlimb function compared to controls	Migration of grafted GFAP+ astrocytes toward the nucleus gracilis of the host medulla	[184]
Rat, L3 hemisection	Neonatal rat cortical astrocytes	Intraparenchymal injection at lesion site, 2.5×10^5 cells, in suspension or in gelfoam	Not investigated	Migration more than 4 mm away from the injection site, reduced glial scarring	[185]
Rat, photochemically-induced infarction of dorsal funiculus	Neonatal rat mixed glial cells (close to type-1 astrocytes) Neonatal kitten mixed glial cells (giving rise to type-2 astrocytes) CG4-mixed glial cells (differentiated into type-2 astrocytes)	Intraparenchymal injection at lesion site	Not investigated	Produced dense clusters of astrocytes surrounded by meningeal cells within the cyst Produced cells that filled the cyst with a loose network devoid of meningeal cell infiltration at the lesion Filled the cyst with a loose network and increased the density of blood vessels in the lesion core	[186]
Rat, T9/10 contusion	GRPs from rat E13.5 spinal cord	Intraparenchymal injection at lesion site, 5×10^5 cells	Not investigated	Differentiated into oligodendrocytes and astrocytes. Reducing glial scar and proteoglycans synthesis. Supported axonal regrowth in the lesion but not on long-distance	[155]
Rat, T8 dorsal hemisection	P3 rat neonatal cortical astrocytes (mainly type I astrocytes)	Intraparenchymal injection at lesion site, 2.5×10^5 astrocytes in a collagen I scaffold	Modest temporary improvements of locomotor function	No migration of astroglial cells out of the implant. Significant increase in the number of ingrowing axonal fibres	[152]
Rat, T8/9 contusion	Mixed NRPs and GRPs (ratio 1:3) from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 3 sites, 1×10^6 cells	Improvement of bladder, sensory and motor functions	Differentiation into neurons and glia. Volume of spinal cord spared was increased and local lumbosacral circuitry was modified	[187]
Rat, T8 complete transection	Adult rat cortical astrocytes	Intraparenchymal injection below lesion site (T11), 1.5×10^5 cells	Not investigated	Massive rostral migration (8 mm)	[188]
Rat, C1/2 or C3/4 dorsal hemisection	$GDAs^{BMP4}$ from rat E13.5 spinal cord	Intraparenchymal injections at and around lesion site, 6 sites, 2-3 $\times 10^4$ cells/site	Functional locomotor recovery	Significant axonal regrowth, decreased synthesis of inhibitory proteoglycans, suppression of axotomized neurons atrophy	[156]
Rat, C1/2 or C3/4 dorsal hemisection	$GDAs^{BMP4}$ from rat E13.5 spinal cord $GDAs^{CNTF}$ from rat E13.5 spinal cord GRPs	Intraparenchymal injections at and around lesion site, 6 sites, 3×10^4 cells/site	Same for $GDAs^{BMP4}$ as in[156], GRPs and $GDAs^{CNTF}$ caused mechanical allodynia and thermal hyperalgesia	Same results for $GDAs^{BMP4}$ as in[156], GRPs and $GDAs^{CNTF}$ failed to support axonal regrowth	[115]
Rat, C3/4 dorsal hemisection	$GDAs^{BMP4}$ and $GDAs^{CNTF}$ from human embryonic spinal cord tissue (week 9 of gestation)	Intraparenchymal injections at and around lesion site, 6 sites, 3×10^4 cells/site	$GDAs^{BMP4}$ promoted locomotor recovery	$GDAs^{BMP4}$ supported axonal regrowth, neuronal survival more efficiently than $GDAs^{CNTF}$	[116]
Mouse, T9/10 contusion	Mouse iPS-derived astrocytes	Intraparenchymal injection at lesion site, 1 site, 1×10^5 cells	No improvement of locomotor or sensory functions	No tumor formation. Long $GFAP^+$ processes from transplanted cells. No interaction with host cells.	[189]
Athymic rats, T10 contusion	hGRPs from fetal cadaver brain tissue (week 18-24 of gestational) $GDAs^{BMP4}$ derived from hGRPs	Intraparenchymal injections at and around lesion site, 3 sites, 1×10^6 cells	No significant improvements in motor function recovery. hGRP grafts attenuated hyperactive bladder reflexes	Differentiation for 80% of grafted cells into $GFAP^+$ astrocytes	[159]
Athymic rat, C4/5 dorsal hemisection	GRPs, $GDAs^{BMP4}$ and $GDAs^{CNTF}$ from human and rat embryonic tissue	Intraparenchymal injection at lesion site, 1 site, 6×10^5 cells	Not investigated	In all 3 groups differentiation into astrocytes generating a permissive environment for axonal regrowth, but not out of the lesion	[157]
Rat, T9 contusion	$GDAs^{BMP4}$ from rat E14 spinal cord and overexpressing D15A	Intraparenchymal injections around lesion site, 4 sites, 4×10^5 cells	Improved locomotor function. No changes in neuropathic pain	Differentiation into GFAP+ astrocytes not secreting CSPG and allowing robust axonal regeneration. Increased spared white matter and decreased injury size compared to controls	[181]
Athymic rat, C4/5 dorsal hemisection	GRPs, $GDAs^{BMP4}$ and $GDAs^{CNTF}$ from fetal cadaver brain tissue (week 20-21 of gestation)	Intraparenchymal injection at lesion site, 1 site, 6×10^5 cells	Not investigated	Differentiated astrocytes from all 3 groups generated a permissive environment for axonal regrowth	[160]
Rat, T8 contusion	$GDAs^{BMP4}$ from rat E13.5 spinal cord	Intraparenchymal injection at and around lesion site, 12 sites, 1.5×10^4 cells/site	Improved hindlimb motor function	Promoted axonal regrowth, reduced glial scarring, inhibited neuroinflammation	[190]

BMP4: Bone morphogenic protein 4; CNTF: Ciliary neurotrophic factor; E: Embryonic; CSPG: Chondroitin sulfate proteoglycans; GDA: GRP-derived astrocyte; GFAP: Glial fibrillary acidic protein; GRP: Glial-restricted precursor; h: Human; iPS: Induced-pluripotent stem cells; L: Lumbar; NRP: Neural-restricted precursor; P: Post-natal; SCI: Spinal cord injury; T: Thoracic.

were generated and produced substantial amount of growth factors, they were unable to modify ALS disease onset or progression[147].

In the last few years, we have been witnessing translation of the basic research into clinical trials for ALS. Maybe too prematurely, clinical trials have been launched around the world, without consensus on the cell types used, the delivery methods, or targeted outcome measurements. The majority of the these studies have focused on safety measurements, and all of them have reported transplantation of stem cells as a safe procedure (reviewed by[148]). As we are approaching the stage when some of these trials are moving from phase 1/2 to phase 2/3, the field is eagerly awaiting detailed reports of the clinical benefits of these cellular interventions. Currently, there are approximately 15 ongoing clinical trials in the EU and United States. Most of them are focused on intraspinal delivery of fetal/mesenchymal/neural stem cells (United States, Italy, Spain) and some used intrathecal delivery of autologous bone marrow-derived cells; so far, none of them is specifically addressing the replacement of ALS-affected astrocytes.

LESSONS FROM TRANSPLANTATION PARADIGMS IN SCI MODELS

Some of the major experimental studies addressing the replacement of astrocytes based on transplantation paradigm in SCI animal models are summarized in Table 2. Historical studies conducted in the laboratories of George Smith and Jerry Silver tested the therapeutic potential of rodent neonatal astrocytes by transplantation following CNS insults. In this paradigm, cerebral midline was lesioned causing severing of callosal axons. A nitrocellulose bridge was introduced at the site of injury. When conducted at postnatal day 8 or younger ages, no necrosis was observed within 24 h. Furthermore, glial cells migrated and integrated into the graft, which provided a substrate for axon growth. However, when conducted later, there was extensive tissue degeneration and the implant was covered with a scar like mixture of fibroblasts and astrocytes. This glial scar failed to promote axon regeneration. Interestingly, when this paradigm was modified such that glial cells from younger pups were grafted into lesions of the older pups, they retained their ability to promote axon outgrowth. These pioneering studies demonstrated an age dependent decrease in the ability of endogenous and transplanted astrocytes to promote axon regeneration[149].

Successive *in vitro* studies conducted by these researchers confirmed age dependent changes in intrinsic properties and molecular basis for supporting axon growth in astrocytes. The rate and extent of chick, as well as rodent, axon outgrowth was consistently greater over the surface of immature compared to mature astrocytes. In terms of molecular properties, NCAM and G4/L1 antibodies reduced the rate of outgrowth over immature but not mature astrocytes, whereas integrin B1 receptor antibody reduced outgrowth on immature, and to a lesser extent, over mature astrocytes[150].

Furthermore, there are differences in the motile properties of the two cell populations such that following transplantation into adult brain immature astrocytes demonstrate extensive migration and interaction with host blood vessels, unlike mature astrocytes. This ability may be linked to their potential to limit glial scar formation and support axon outgrowth[151]. Transplantation of neonatal astrocytes at the site of thoracic SCI resulted in outgrowth of axons along the transplants, as well as some motor recovery[152].

Even though these early studies produced promising results, because of the difficulty in harvesting these beneficial immature astrocytes from human tissues, further studies explored more practically relevant cell types such as multipotent NSCs as a viable source to generate transplantable astrocyte populations. In studies conducted by the Whittemore lab, NSCs isolated from E14 rat cortex were transplanted into intact and injured spinal cord, without prior differentiation. In the intact spinal cord, over a 2-mo period, a 50% of multipotent stem cells differentiated into GFAP positive astrocytes and a small percentage differentiated into oligodendrocytes and neurons. Following injury to the spinal cord, astrocyte differentiation dropped to 35% and neuronal differentiation was not observed at all[153], demonstrating an effect of the micro-environment on the differentiation pattern of the transplanted stem cells. To overcome this, in a separate study, NSCs were transfected with Ngn-2, followed by transplantation into injured spinal cord. This resulted in a significant reduction in astrocyte differentiation and an increase in differentiation into oligodendrocytes and neurons. Furthermore, Ngn-2-NSC treated rats showed improved motor function. These studies also showed that transplantation of control NSCs enhanced neuropathic pain associated with SCI, whereas this unexpected side effect was not observed in Ngn-2-NSC transplanted rats[154].

While NSCs provide the benefit of being able to give rise to neurons as well as glia, the use of GRPs harvested from fetal CNS has gained wide popularity in recent years for targeting astrocytes. Early studies documented extensive migration of transplanted GRPs in injured and intact spinal cord and differentiation into cells of the glial lineage, predominantly into astrocytes[112]. Transplantation of these cells into thoracic SCI reduced scar formation and expression of growth-inhibiting proteoglycans; however, no axon outgrowth was observed[155]. This obstacle was overcome by pre-differentiating GRPs into astrocytes (GDAs) by treatment with BMP-4. These GDAs were able to promote axon outgrowth when transplanted into lesioned spinal cord without causing neuropathic

pain[156]. These investigators also differentiated GRPs into astrocytes by treatment with a different estrogenic signaling molecule, CNTF. This type of GDA however failed to promote axon regeneration and promoted thermal hyperalgesia and mechanical allodynia following transplantation into lesioned spinal cord[115]. These studies highlighted that heterogeneous astrocyte populations may have distinct therapeutic potentials. Contrasting results from the Fischer lab indicated that, although heterogeneous, both BMP-4 and CNTF GDAs promoted axon outgrowth to a similar extent[157]. Although it is not possible to compare in detail the precursor populations used by the two labs, the properties of specific glial precursor populations, differences in the culture conditions, and different transplantation and tracing protocols may have led to these contrasting results. Advantages derived from the graft are manifold. For example, a recent report from the Proschel lab illustrated an underlying mechanism through which BMP4-GDAs promote axon regeneration involving secretion of periostin[158].

While the above-mentioned studies involving rodent GRPs provided an understanding of their properties and therapeutic potential, further studies involving more clinically relevant human GRPs were designed based on these previous studies. hGRPs harvested from fetal human brain were pre-differentiated into astrocytes (hGDAs) by treatment with BMP-4 and transplanted into injured spinal cords of athymic rats. Graft expansion was seen over an 8 wk time period, and in both hGRP and hGDA transplant groups, cyst and glial scar formation was reduced. Although there was no significant motor recovery, lack of neuropathic pain and permissive properties of these cells make them promising therapeutic candidates after SCI[159]. Based on their rodent GRP work, the Fischer group generated GDAs by treatment with either CNTF or BMP-4 and showed that these cells have similar therapeutic potential in terms of promotion of axon generation[160], whereas the Davies and Proschel groups again reported contrasting results[116]. These differences may be related to the age, region and technique of isolation of hGRPs, as well as differences in the culture conditions used by the two labs.

In recent years, the discovery of induced pluripotent stem cells (iPSC) cells has generated great excitement as large quantities of clinically relevant mature cell types can be generated from these pluripotent cells, without facing the ethical concerns presented by pluripotent embryonic stem cells (ESCs). However, this field is in its infancy, especially in the context of astrocytes. Recently, the Zhang laboratory developed a protocol for producing large quantities of regionally specific astrocytes from ES and iPSC cells[69]. A shortcoming of this study however is that it required 6 mo of culturing. Shorter duration protocols were subsequently reported, but these produced mainly populations of immature astrocytes[161,162]. Astrocyte maturation takes place in two distinct phases and marker expression for these phases has been characterized, which led to the development of protocols that generate astrocytes with mature phenotypes. While the treatment of immature astrocytes with FGF1 or FGF2 promoted up-regulation of maturation markers such as glutamate transporters and downregulation of GFAP, treatment with tumor necrosis factor- pushed cells toward a reactive astrocyte phenotype. These cells can be used to model human pathological processes *in vitro* and *in vivo* as well as for therapeutic transplantation[163]. More recently the Zhang laboratory reported another shorter duration protocol for generating astrocytes from iPSCs by differentiation of these cells into neuroepithelial cells, followed by removal of mitogen and treatment with CNTF to induce differentiation into astrocytes[70]. Recently, the Maragakis lab conducted transplantation of iPSC- and ESC-derived immature astrocytes into rodent spinal cord. Although limited, the grafts showed long-term survival, with less than 5% of the transplanted cells surviving at 12 wk post-transplantation. Furthermore, graft derived astrocytes expressed markers of mature, quiescent astrocytes such as AQP4, EAAT1, EAAT2, as assessed by immunostaining analysis and gene expression profiling, suggesting that these cells are a promising resource for transplantation therapies[164]. Additional reports suggest that it is important to evaluate the *in vivo* safety of these cells, especially in terms of their tumor formation tendency, prior to their use for clinical transplantation[165,166]. Recent work from the Lepore lab, conducted as an extension of their work related to rodent GRPs, generated glial progenitors from human iPSCs, followed by further differentiation into astrocytes. Following transplantation into injured spinal cord, these cells showed long-term survival, no tumor formation, and efficient differentiation into GFAP expressing cells. These cells could even be engineered to express GLT1, suggesting that hiPSCs could be used as a safe source for transplantation therapies for targeted replacement of astrocytes in SCI and other CNS diseases (unpublished data).

Until recently, the SCI field primarily focused on the role of astrocytes with regard to scar formation and their inhibitory effects on axon re-growth. Recent studies however have elucidated their heterogeneity and the changes exhibited by these cells following SCI, thereby underscoring the need to preserve their normal physiological functions. Studies mentioned above, as well as other studies that are not detailed here, are indicative of the growing appreciation of cell transplantation therapies for therapeutically targeting astrocytes in SCI.

With a special focus on chronic SCI conditions, many clinical trials based on the transplantation of unmodified mesenchymal, neural or bone-marrow stem cells have been launched around the world[167-171]. Among phase 1 studies, most of them have focused on safety measurements and graft survival according to different routes of administration (mostly intrathecal

or focal intraparenchymal delivery). All of these studies have reported transplantation of stem cells as a risk-free procedure. To our best knowledge, none of the current human trials are specifically addressing the replacement of lost astrocytes following SCI.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

We must admit that, despite numerous preclinical animal studies demonstrating improvement in a variety of functional outcomes following astrocyte transplantation, the variability and the poor reproducibility of the results still hinder full clinical translation. The variability might be partly explained by the limited survival of transplanted cells in diseased spinal cord tissue. In published preclinical ALS and SCI studies, cell graft survival was found to be usually between 5% and 32% of the initial dose and was often assessed at early time points post-transplantation. Inter-laboratory discrepancies might also be due to the source or type of astrocytes (or precursors) used in the transplantation paradigms. Indeed, astrocytes, in rodents and even more in humans, are a broad class of CNS cells endowed with specific functions according to their location, their morphology, their degree of maturity and their activation state. Finally, lack of standardization, different type of cells, different animal species and single targeting strategies of multifactorial diseases may also be part of the failures. Therefore, future protocols should aim at standardizing the culture conditions, verifying the astrocyte population homogeneity, the cell purity and, above all, at enhancing cell survival after *in vivo* transplantation. Researchers are also encouraged to provide preclinical data about long-term follow-up of engrafted cells, including survival time, differentiation pattern and CNS integration several months post-transplantation.

During this last decade, detailed molecular dissection of astroglial populations has shown that astrocytes are active players in CNS homeostasis and far more than basic fibroblast-like cells of the CNS. Understanding the molecular mechanisms underlying the normal biology of astrocytes as well as their behavior during pathological conditions will provide us with undoubtedly novel astrocyte-specific therapeutic targets. Especially regarding ALS and SCI that share several common pathophysiology mechanisms, targeting astrocytes, whose dysfunction is now well established from animal and human studies, might be a promising therapeutic strategy. As far as we know, oxidative stress, neuroinflammation and glutamate excitotoxicity are all pathological events in which astrocytes are actively involved. Therapeutics that address each separately or all-in-one these specific pathological events are of great relevance to ALS and SCI.

Stem cell based therapy for ALS and SCI includes three main aims: (1) replacement of lost nervous cells for restoring homeostatic functions of the CNS; (2) neuroprotection of unaffected surrounding tissue *i.e.,* by secretion of neurotrophic factors or anti-inflammatory molecules; and (3) enhancement of the endogenous repair process. Based on the biology and the known functions of astrocytes, here are a few examples of neuroprotection-based mechanisms that are under investigation and might deserve particular attention in future transplantation paradigms targeting astrocytes.

Increased oxidative stress has been implicated in the pathogenesis of ALS and SCI, leading to the testing of different antioxidants in affected patients. Several studies have demonstrated that astrocyte-secreted glutathione greatly contributes to neuro-protection against oxidative stress. One of the main regulators of glutathione production and release is the transcription factor Nrf2. ALS mice with astrocyte driven Nrf2 overexpression showed increased lifespan, better functional outcomes and lower glial reactivity in spinal cord tissue[172]. Accordingly, it would be interesting to investigate the effect of transplantation of Nrf2-overexpressing astrocytes. Stat3 is another transcription factor regulating GSH synthesis[173], as well as a "master-switch" that mediates transition from quiescent to reactive astrocyte. Following SCI, transgenic mice ablated for Stat3 in the astroglial population showed less reactive astrocytes in the spinal cord, resulting in more widespread neuroinflammation, motor neuron loss and axonal damage and thus worsened motor deficits compared to wild-type counterparts. This study and others demonstrates that Stat3 is a key transcription factor controlling astrocyte reactivity during neurodegenerative processes, providing a potential target for intervention[174,175].

Far from being science fiction, researchers can genetically modify (stem) cells to make them produce more growth factors or proteins of interest, as Trojan horses to deliver molecules to specific CNS locations. For instance, NSCs engineered to overexpress CNTF exert neuroprotection by *in situ* differentiation into supportive astrocytes in a mouse model of photoreceptor degeneration[176]. Similarly, NSCs overexpressing nerve growth factor showed beneficial effects on cognitive functions after CNS transplantation and astrocyte differentiation in a learning deficit mouse model[177]. Another example is illustrated in a recent report from Gowing[178]. This group engineered GDNF-secreting human neural progenitors that were able to survive up to 7.5 mo after intraspinal transplantation, differentiate mainly into astrocytes and still maintain locally high levels of GDNF. More specifically, intranigral transplantation of astrocytes transduced with a lentiviral vector expressing GDNF resulted in sustained local production of growth factor and provided neuroprotection in a rat model of Parkinson[179]. Lastly, genetically-modified astrocytes designed to secrete BDNF promoted retinal ganglion cell survival[180], and transplantation of neurotrophin-releasing GRPs improved functional and histological outcomes after

SCI[181]. Growth factors released from transplanted cells act in a paracrine manner on surrounding host tissue but are likely to also act in an autocrine manner on grafted cells to promote survival and/or a specific differentiation pattern. All aforementioned examples should encourage us to investigate more deeply the therapeutic potential of grafted exogenous astrocytes or stem cell-derived astrocytes in neurodegenerative and traumatic CNS diseases, as well as to think about novel genetically-modified cells that specifically target pathogenic events occurring during ALS or SCI.

Inspired from encouraging results obtained following transplantation of GLT1-overexpressing GRPs in the rat model of ALS[104], the Lepore lab is studying their applicability and evaluating their efficacy in clinically-relevant SCI models[182,183]. The rationale behind such an approach is based on the findings that, following SCI, astrocyte GLT1 expression and function are compromised, resulting in excitotoxicity-induced cell death during the delayed secondary injury phase[105]. Unpublished data from our group show that transplantation of astrocytes engineered to overexpress GLT1 can prevent excitotoxicity and protect respiratory phrenic motor neurons following cervical SCI. While unmodified transplants showed robust survival, they expressed relatively low levels of GLT1 in injured spinal cord. Excitingly, GLT1 overexpressing transplant-derived astrocytes continued to express high levels of GLT1 protein following transplantation into the injured spinal cord and promoted survival of these important phrenic motor neurons and preservation of diaphragm function (unpublished work).

Finally, although ALS and SCI share many similarities including the death of spinal motor neurons, it must be emphasized that important differences between two diseases exist. ALS is a progressive neurodegenerative condition starting with discrete and asymptomatic loss of motor neurons in remote specific regions of the CNS (upper and lower motor neurons), whose symptomatology becomes apparent at approximately 50% of motor neuron loss and fatally progresses when disease affects bulbar and/or spinal respiratory centers. Therefore, therapeutic strategies in ALS, once diagnosed, should focus on astrocyte-mediated neuroprotection that prevent further motor neuron loss, worsening of functional outcomes and delayed fatal respiratory failure. Unlike ALS, SCI is characterized by different temporal sequence of events. First, acute and sub-acute phases following the initial trauma in the first hours-by-days post-injury result in a hostile environment in the spinal cord around the lesion epicenter, including inflammation, excitotoxicity and oxidative stress that lead to more cell loss and thus extension of initial lesion. This narrow time window would be appropriate for interventional strategies targeting astrocytes that favor neuroprotection or compensate for lost astrocyte-specific functions. At later time points, during the chronic stage of SCI, characterized by the resolution of subacute events, glial scarring and reorganization of spinal tissue architecture, one can imagine designing astrocyte transplants to make scar tissue permeable to axonal regrowth, to promote invasion by remyelinating oligodendroglial precursors, or to facilitate overall rewiring of ascending/descending tracts in the spinal cord.

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